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Research Article

ANATOMICAL CONSIDERATIONS ON THE ANNUAL SHOOT IN SOME WILD ROSES

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Abstract: The observations aim a comparative study of the anatomical structure of the stem and leaf, during anthesis, in some *Rosa* species and varieties (sports), in order to complete the data from the specialized literature because the morphological information is more numerous than the anatomical ones. Moreover, sometimes they are even inconsistent, as in the case of the glands/glandular trichomes on the leaves and stem.

A series of similarities are highlighted, which can be considered conservative characteristics of the *Rosa* genus, as for instance: the rapid transition to the secondary structure, the disposition of the vascular bundles (of collateral type) on a circle, the absence of the phelloderm's activity in the first year, the presence of the crystal idioblasts and the bifacial structure of the leaf. Also, a series of characteristics of each species is highlighted: disposition of the secondary xylem vessels, quantity of libriform fibres, the nature of the walls of the cells from the medullar rays, the thickness of the walls of the sclerenchyma fibres and the presence of the crystal idioblasts in various tissues.

Keywords: anatomy, annual shoot, leaf, *Rosa* species, stem.

Introduction

The *Rosa* L. genus is a vast one, with over 200 spontaneous species in the Holarctic region, spread between 20 and 70 degrees Northern latitude [KRÜSSMANN, 1986].

Arctic and tropical areas are excluded from this region. This genus is heterogeneous, with its species adapted to various environmental conditions. Wild roses grow on all continents in the northern hemisphere, being widespread from the plains to the mountain ranges (even 4000 m – *R. morrisonensis* Hayata in Asia). A few species are only found on plains (*R. setigera* Michx., *R. foetida* Herrm., *R. sempervirens* L.) or flood prone areas (*R. clinophylla* Thory, *R. palustris* Marshall), and only *R. persica* Michx ex Juss. grows in cold desert. They present morphological, anatomical and physiological adjustments [ADUMITRESEI, 2011] related with their diverse ecology.

Ten wild species and two sports were included in the study. Five of these are native in Romania (*R. agrestis*, *R. gallica*, *R. glauca*, *R. rubiginosa* and *R. spinosissima*), while the others originate from Asia Minor (*R. damascena* and *R. foetida*) and East Asia (*R. multibracteata*, *R. multiflora* and *R. rugosa*). The two sports are included in collections due to their significance: *Rosa canina* 'Inermis' is an important rootstock, while *Rosa chinensis* 'Viridiflora' is remarkable for its flowers, which consist solely of bracts.

The reasons for including them in the study stem from the need to update and expand the information regarding this genus, especially considering that scientific morpho-anatomical data in classical literature is fragmented and rather brief, compared to the diversity and

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variability that characterize this genus. It mainly concerns point-based anatomical examples of the main vegetative or reproductive organs or morpho-anatomical aspects as a support for physiological processes. Thus, the studies on the anatomy of the shoot are better represented and refer to: the primary and the secondary structure of the annual shoot [PARMENTIER, 1898; METCALFE & CHALK, 1988; NIKITIN & PANKOVA, 1982; TOMA & al. 1997; TOMA & RUGINĂ, 1998; ADUMITRESEI & TĂNĂSESCU, 2005; ADUMITRESEI & al. 2006; SHAMSO & al. 2019; PASHINA, 2021], prickles' ontogenesis and their peculiarities [UPHOF & HUMMEL, 1962; FAHN, 1968; NAPP-ZINN, 1984], similarities between prickles and glands [KAUSSMANN & SCHIEWER, 1989], anatomical aspects and their taxonomic implication [PARMENTIER, 1898; LOTOVA & TIMONIN, 1999; FATEMI & al. 2008; NIJSSE & al. 2007; ADUMITRESEI & GOSTIN, 2016], anatomical aspects as a support of some physiological processes [HASHIDOKO & al. 2001; CAISSARD & al. 2006; ZAMFIRACHE & al. 2006; ADUMITRESEI & al. 2009; BURZO & TOMA, 2012; MENG & FAN, 2022; TORRE & al. 2023].

Material and methods

The observations aim to provide a comparative study of the anatomical structure [TOMA & GOSTIN, 2000] of the stem and leaf, during anthesis, in *Rosa agrestis* Savi, *Rosa canina* L. 'Inermis', *Rosa chinensis* Jacq. 'Viridiflora', *Rosa damascena* Mill., *Rosa foetida* Herrm., *Rosa gallica* L., *Rosa glauca* Pourr., *Rosa multibracteata* Hemsl. et Wils., *Rosa multiflora* Thunb., *Rosa rubiginosa* L., *Rosa rugosa* Thunb. and *Rosa spinosissima* L., collected from the "Anastasie Fătu" Botanical Garden of Iași [OSTACIUC, 1994; CAIRNS, 2000].

Shoot fragments were collected during the anthesis period and preserved in ethanol 70%. Sample sections were made with handling microtome using a razor blade. Their coloring was imparted using iodine green and alum carmine or ruthenium red based on a classical method from histo-anatomical studies of plants. Sections were fixed with glycerol gelatin.

Sections were made on the following levels: top of shoot (the middle part of stem, under vegetative cone) and leaves (through petiole at the stipule level, through rachis, on middle zone, through petiole on the first pair of leaflets, through lamina, at the median vein between secondary veins). Furthermore, some superficial sections had been used for epidermis description.

Microphotographs of anatomical sections were taken using Olympus, respectively Olympus BX 41 Microscope and a BH-2 JAPAN camera and an Olympus E 330 digital camera.

Results and discussion

The stem generally has a circular contour in cross-section (e.g. *R. canina* 'Inermis', *R. glauca* and *R. rubiginosa*). The contour could be circular irregular, modified by prickles sometimes (as *R. spinosissima*, *R. rugosa* – Figures 6, 7), or elliptical (as *R. agrestis* and *R. chinensis* 'Viridiflora' – Figure 2).

At some taxa non glandular trichomes and glandular trichomes appear [WANG & al. 2019; ZHOU & al. 2021]. Non glandular trichomes appear only in juvenile stages at some species. Small glandular trichomes are present at *R. spinosissima*, even bigger at *R. agrestis*, *R. damascena*, *R. rugosa* and *R. rubiginosa*, they always have multicellular pedicel and it is pluriseriate, which consists of 4-6 or sometimes even 8 cell series. The glandular trichomes have a terminal clavate part which is formed of polygonal cells with cellulosic walls; their number is variable, but always in the tens of cells.

In all cases, epidermal cells are covered by cuticle. It can be very thick with intercellular cuticle blades very deeply inside (for *R. multibracteata*, *R. rubiginosa* and *R. rugosa*), thick (*R. agrestis*, *R. chinensis* 'Viridiflora', *R. foetida*, *R. glauca*, *R. gallica* and *R. spinosissima*) or relatively thin (*R. canina* ‘Inermis’) (Figure 4).

Epidermal cells are either isodiametric (e. g. *R. chinensis* 'Viridiflora', *R. damascena*, *R. glauca*, *R. rubiginosa* and *R. rugosa*) or tangentially elongated (*R. foetida*, *R. gallica*, *R. spinosissima* and *R. multiflora*) (Figure 8). Regarding epidermal cells size, it is usually constant, an exception being the prickle area where epidermal cells are slightly radially elongated. The external wall of epidermal cells is thicker than the walls of other walls of the same cells. The external walls are very thick for *R. canina* 'Inermis', *R. glauca*, *R. multibracteata*, *R. multiflora* and *R. rugosa* (Figures 4, 5 and 6).

Stomata are situated either at level of epidermal cells in *R. canina* 'Inermis', or slightly above the level of epidermal cells at *R. multibracteata*. An exception is *R. glauca*, in which stomata can be observed both at the level of the epidermal cells or at a nearby height (either above or below that level) [ADUMITRESEI & TĂNĂSESCU, 2005]. The substomatic chamber can be reduced in *R. glauca*, with a medium size (in *R. canina* 'Inermis') [ADUMITRESEI & al. 2006], the suprastomatic chamber, relatively tall, can be observed in *R. damascena* as a result of the presence of tall annex cells.

Prickles are always present on the stem surface in variable numbers, more frequently in *R. spinosissima* and *R. rugosa*. They have different sizes and shapes depending on the species. In section, it is observed that the shape of the cells changes from isodiametric cortical cells (at the base) to radially elongated parenchymal cells on the flanks and towards the tip of the prickles. The older prickles get, do they become sclerified and lignified, becoming harder, and the epidermis on their flanks exfoliated (Figures 19, 20).

Fully developed prickles are based on a multilayered suberous tissue to which exterior the parenchyma is moderately sclerified and lignified, which in time leads to exfoliation.

In *Rosa rugosa*, in most of the stem circumference, in a hypodermic position, the phellogen differentiated, producing 6-8 layers of suber outwards and several layers of phelloderm inwards. These anatomical features prove the fact that it is the most resistant species to low temperatures among the ones selected by this study.

The primary cortex is differentiated in an external collenchymatous area and an internal area cortical parenchyma of meatic type.

The hypodermal collenchyma is of the tangential type and is presented either as a continuous area with different thickness (in *R. agrestis*, *R. chinensis* 'Viridiflora', *R. foetida* and *R. rugosa*) or as collenchyma cords (larger or smaller) separated by some assimilating parenchyma islands (in *R. canina* 'Inermis', *R. damascena*, *R. gallica*, *R. multibracteata* and *R. multiflora*) or in an angular type as in *R. spinosissima* (Figure 7), which allows the shoot to grow in thickness. In *R. glauca* and *R. rubiginosa*, the entire hypodermic layer is collenchymatous [SHAMSO & al. 2019].

The number of cell layers included in the collenchyma structure is variable depending on taxa, but it is well represented in most cases. The cortex generally consists of 14-16 cell layers, from which 4-6 layers of external cells, in most species (*R. chinensis* 'Viridiflora'), are represented by collenchyma. The internal area of cortical parenchyma of the meatic type is composed of cells whose sizes are growing from beneath epidermis up to the central cylinder

(Figures 3-6, 15, 18). In most taxa, cells are isodiametric, but tangentially elongated cells can be found, for instance, in *R. multibracteata* (Figure 15).

Cortex thickness is variable in each taxon, being thin in *R. rubiginosa* and very thick in *R. canina* 'Inermis', *R. damascena*, *R. foetida*, *R. glauca* and *R. spinosissima*.

Central cylinder consists of a variable number of collateral vascular bundles, with a circular arrangement, and pith. The central cylinder is usually relatively thick, situated on the upper limit in species such as *R. multibracteata*, *R. canina* 'Inermis', *R. glauca*, *R. rubiginosa* and *R. rugosa*.

The number of vascular bundles is variable, yet constant for each taxon: many (over 60) at *R. canina* 'Inermis', fewer (about 35-40) for *R. rubiginosa* and *R. glauca*; relatively few (about 25-30) in *R. agrestis*, *R. chinensis* 'Viridiflora' and *R. rugosa*, even less (about 20-22) in *R. multibracteata* and *R. chinensis* 'Viridiflora' (Figures 1-6).

The vascular bundles are arranged in a perfect circle (in *R. canina* 'Inermis', *R. damascena*, *R. gallica*, *R. multibracteata*, *R. multiflora*, *R. rugosa* and *R. rubiginosa*) (Figures 1, 5, 11, 17, 19), or they can be organized in a tortuous circle (e.g. *R. agrestis*, *R. glauca*, *R. rubiginosa* and *R. spinosissima*) (Figures 4, 6, 18). The general architecture of the central cylinder in *R. chinensis* 'Viridiflora' is rather interesting, for in this case the vascular bundles are no longer strictly arranged on a circle, but on several arcs that converge, so that the xylem is always oriented towards the pith. Each of the arcs presents two types of bundles, collateral ones and other formed by elements of phloem only. Between the bundles of the same arch, medullary rays are extremely narrow (one or two series of cells), whereas between arches of bundles, medullary rays are quite large (5 or 8 series of cells) (Figure 2). This atypical arrangement of the vascular bundles has also been observed in several horticultural varieties of *Rosa*, such as 'Laminuette', 'Luchian', 'Rose Gaujard', and 'Hurdal' [ADUMITRESEI & al. 2005; ADUMITRESEI & al. 2012; TOMA & TOMA, 2005; MONDER & al. 2021].

Generally, bundles' sizes vary within the same taxon; however, in the case of *R. agrestis*, *R. damascena*, *R. foetida*, *R. multibracteata*, and *R. multiflora* they can be approximately equal (Figures 15, 18, 19, 21, 23).

Concerning the structure of vascular bundles in the annual shoot, we highlight the following observations for investigated taxa: in the most analysed species cambium generates less phloem outward and more xylem inward (Figures 18, 19, 23, 25). The presence of cork cambium and its differentiation into 4-6 layers of phelloderm on the outer layer, with just a few layers of cork cambium towards the inner part, is noticeable only in *R. rugosa*, which is the most resistant to low temperatures among all the species included in the study (Figure 16).

The position of secondary xylem vessels is sometimes in radial and parallel rows (as in *R. canina* 'Inermis', *R. glauca*, *R. multiflora* and *R. rugosa*), in other cases it is irregular within the libriform mass (as in *R. agrestis*, *R. chinensis* 'Viridiflora', *R. gallica*, *R. rubiginosa*, *R. multibracteata* and *R. spinosissima*).

The amount of libriform in the secondary xylem is as follows: abundant (in *R. agrestis*, *R. damascena*, *R. gallica*, *R. multibracteata* and *R. spinosissima*) and reduced (in *R. canina* 'Inermis').

Medullary rays are relatively thin, often uniseriate, and generally very thin (*R. canina* 'Inermis', *R. damascena*, *R. foetida*, *R. multibracteata* and *R. rugosa*), with some bundles which form a continuous ring inside of the xylem, because here pith rays are slightly sclerified and lignified (in *R. agrestis*, *R. gallica* and *R. rubiginosa*) (Figures 21, 22). In most cases, medullary rays consist of 2-3 cell rows. As mentioned before, *R. chinensis* 'Viridiflora' has extremely large rays between arches (8 rows of cells), making it an exception within the *Rosaceae* family.

Regarding the composition of cell walls in medullary rays: at the phloem level, they are parenchymatic, cellulosic, while at the xylem level they are moderately sclerified and lignified (in *R. glauca*, *R. damascena*, *R. multibracteata*, *R. multiflora* and *R. rugosa*); in some taxa, the cell walls of medullary rays from the xylem are slightly lignified and sclerified (in *R. agrestis*, *R. foetida* and *R. rubiginosa*) (Figures 18, 21, 22).

Perivascular sclerenchyma bundles are generally well represented at stem level. The differences are related to walls of sclerenchyma fibres, which can be: very thick and fully lignified with lumen cells that are almost punctiform (*R. agrestis*, *R. gallica*, *R. multibracteata*, *R. rubiginosa* and *R. rugosa*), strongly thickened, but partially lignified (*R. multiflora*), moderately thickened, but completely lignified, with a large lumen (*R. damascena*) or strongly thickened and partially lignified (*R. canina* 'Inermis' and *R. glauca*) (Figures 21, 22, 23, 25). In the perimedullary area, cells have lignified walls. Similar structures are mentioned by DELINSCHI (FLORIA) & al. (2009).

The configuration of the pith is similar in *Rosa* species and varieties. It is formed by very small cells or islands of cells, with moderately thickened and lignified walls, surrounded by extremely large cells with very thin, cellulose walls, often arranged in a rosette with respect to the small cells, suggesting a pattern (Figure 26).

Crystal idioblasts are found in various tissues, depending on cultivar or hybrids. Generally, they are relatively common, as in other *Rosaceae* species [METCALFE & CHALK, 1988; TOMA & al. 1983; FLORIA & DERID, 1998; FLORIA, 1998; LERSTEN & HORNER, 2006]. Some taxa predominantly contain druses while others are more likely to have prismatic crystals (Figures 16, 17, 22, 23, 24). Both forms can coexist in certain taxa, though one type may predominate in different tissues. For example, crystal idioblasts are present in hypodermic collenchyma layer contain either druses (*R. chinensis* 'Viridiflora', *R. glauca*, *R. rugosa*, *R. spinosissima* sometimes grouped together, *R. rubiginosa* relatively rare) or prismatic crystals (*R. foetida*).

Within of the cortical parenchyma, crystal idioblasts containing druses are present in *R. glauca* and *R. multiflora*. Some species, such as *R. chinensis* 'Viridiflora', *R. damascena*, *R. glauca* and *R. rubiginosa*, have only a few crystal idioblasts at the cortex level.

In the phloem parenchyma, we identified cells with druses in *R. glauca* and *R. multiflora* or both druses and fewer prismatic crystals in *R. rugosa*. *R. rubiginosa* contains fewer druses and more prismatic crystals. Species such as *R. damascena* and *R. multibracteata* are relatively poor in crystal idioblast cells at bark level.

In the perimedullary area and at the level of the medullary rays, crystal idioblast cells are more frequent in *R. damascena* (containing druses), *R. multibracteata* (containing druses and prismatic crystals).

Inside the pith there also are cristal idioblasts (containing druses) at *R. multibracteata*, *R. rugosa* and *R. glauca* (also appearing prismatic crystals). Crystal idioblasts are missing from the pith from *R. damascena* and *R. multiflora*.

"Hydrocites" are present both in the pith (in *R. damascena*, *R. glauca*, *R. rubiginosa*, *R. multibracteata*) and in the perimedullary area (in *R. agrestis*, *R. damascena* and *R. gallica*). Sometimes, they are present on the periphery of periphloemic sclerenchyma (Figure 18). These structures, characterized by thickened and lignified walls with visible punctations, have been assimilated to "hydrocytes" [ANDRONACHE & al. 2006; ABDINAZAROV & al. 2017]. The term "hydrocytes" was described especially in Russian botanical literature as part of a system that ensures the flow of nutritive and biologically active substances in areas of active growing processes [CHURIKOVA & BARYKINA, 2005; CHURIKOVA & BARYKINA, 2015].

In both wild roses and cultivated hybrids, the growth process is very fast; in spontaneous species and climbing roses, shoots go through the phenophases from bud to flowering in almost 3 weeks, reaching lengths of approximately 15-40 cm, and cultivated hybrids go through these phenophases in a month, reaching lengths ranging between 20 and 80 cm, sometimes even 160 cm. Also, in roses, the annual shoot has an intermediate "behavior" between herbaceous and woody plants in terms of growth, a fact supported both anatomically and physiologically. It is likely that this type of growth is correlated with the hydrocytic system that is present in the stem and the leaf, respectively in several types of tissues in both organs.

The petiole, at the level of the stipule, typically has a semicircular contour in cross-section, although it is rarely semielliptical. This shape is modified by two adaxial wings, represented by the stipule, which has an adaxial surface with a groove that can vary in width. Sometimes, the adaxial surface is almost flat (Figures 27, 29).

Epidermal cells are either isodiametric (*R. canina* 'Inermis', *R. glauca*, *R. damascena* and *R. multiflora*) or slightly tangentially elongated (*R. multibracteata*).

The cuticle can be thick (*R. chinensis* 'Viridiflora', *R. damascena*, *R. multiflora*, *R. multibracteata*, *R. rubiginosa* and *R. rugosa*) or thin (*R. canina* 'Inermis' and *R. glauca*).

Non glandular trichomes are present on both the petiole and the rachis surfaces in *R. agrestis*, *R. foetida* and *R. rugosa* species (some of them very long, others are shorter, fairly uniformly distributed). In *R. damascena*, non-glandular trichomes are of various sizes, primarily located on the adaxial face, though they are relatively rare. In *R. multiflora*, non-glandular trichomes are few, small, and mostly found in the adaxial groove (Figures 27, 29, 30, 31-33, 36). Generally, trichomes are most abundant on the bases of young leaves and gradually decrease in density toward the top. In all cases, non-glandular trichomes have very thick walls and an almost filiform lumen.

Glandular trichomes are present in all species, particularly on the stipule extremities, and are most frequent in *R. agrestis*, *R. damascena*, *R. foetida*, *R. multiflora*, *R. multibracteata*, *R. rubiginosa* and *R. rugosa*. In species such as *R. canina* 'Inermis', *R. glauca* and *R. gallica*, secretory glands are primarily located on the edge of the stipule and they tend to be scarce. Some taxa have glands both on the edge of stipules and on the rachis, the lower epidermis of leaflets or stipules (*R. agrestis*, *R. spinosissima*, *R. rubiginosa*, *R. rugosa*, more rare at *R. damascena*) (Figure 29). These glandular trichomes have a multicellular pluriseriate foot and clavate terminal head. In cross-section, the terminal head features a pluricellular core composed of isodiametric cells, surrounded by an external layer of elongated cells, the structure of which varies depending on the species (Figures 27-30, 31-33, 44).

In *R. rugosa* sepals there were observed [SULBORSKA & WERYSZKO-CHMIELEWSKA, 2014] similar structure called multicellular glandular trichomes, but it is specified that they have similar structures with colleters, already mentioned by FAHN (1968) and DALVI & al. (2024) in the *Rosa* genus. We found this type of emergence in *Rosa agrestis* on the rachis of the leaf (Figure 27).

Hypodermic collenchyma, of tangential type, is typically composed of 3-4 cell layers in most species, but is thinner in *R. damascena*. We observed angular collenchyma in *R. multibracteata* and *R. spinosissima* (only on the abaxial face of the petiole) which differs from the one found in the stem (Figures 35 and 43). In some cases, the collenchyma layers contain cells with druses (*R. spinosissima*), prismatic crystals (*R. rubiginosa* and *R. rugosa*) or both types (*R. glauca* and *R. multibracteata*). In *R. glauca* and *R. rubiginosa*, all the hypodermic layers are colenchymatous.

Cells with prismatic crystals are more frequent in *R. rugosa* and *R. multiflora*, and the ones with prismatic crystals are more common in *R. damascena* (Figure 38).

The number of vascular bundles varies from 3, in *R. chinensis* 'Viridiflora', *R. multiflora*, *R. gallica*, *R. rugosa* and *R. spinosissima* to 5-6 in *R. damascena*, *R. glauca*, *R. foetida*, *R. canina* 'Inermis' and *R. rubiginosa*. Sometimes, periphloemic sclerenchyma bundles are interrupted by medullary rays, which can be narrower (*R. multiflora* and *R. rubiginosa*) or larger (*R. damascena*). This suggests that multiple smaller bundles may merge to form a larger one.

The size of the bundles typically decreases, from the centre toward the periphery. In some cases, several bundles from the central area are approximately equal in size (*R. canina* 'Inermis').

The secondary structure at the level of vascular bundles is present in *R. multibracteata*. Primary and secondary structures at this level are found in *R. chinensis* 'Viridiflora', *R. damascena*, *R. foetida*, *R. rubiginosa* and *R. rugosa*, while only the primary structure appears in *R. canina* ‘Inermis’, *R. glauca* and *R. spinosissima*.

In all cases, vascular bundles have sclerenchyma at the periphery of the phloem forming thick sheaths (*R. canina* 'Inermis' and *R. glauca*), which can be interrupted by medullary rays more or less narrow (*R. multibracteata*, *R. multiflora* and *R. rubiginosa*).

Periphloemic sclerenchyma contains cells with very thick lignified walls, resulting in a reduced lumen, in most cases. However, in *R. multiflora*, cells with a larger lumen can be found, although they are completely lignified. In other cases, such as in *R. rubiginosa*, the cells have partially lignified walls but are still thick.

Stipules vary in width, being either wider or narrower, and are relatively thin. Structurally, the mesophyll consists of homogeneous parenchyma, composed of 2-4 layers of more or less elongated cells, with small air spaces in species with narrow stipules (*R. glauca*, *R. multibracteata*, *R. multiflora* and *R. spinosissima*). In species with wider stipules (*R. canina* 'Inermis' and *R. rugosa*), the mesophyll is differentiated into palisade and spongy tissue throughout the length of the stipule (*R. chinensis* 'Viridiflora') or only along the edge of the stipules (*R. agrestis*, *R. damascena*, *R. foetida* and *R. gallica*). In some cases, the homogeneous parenchyma is replaced by collenchyma, as observed in *R. canina* 'Inermis' and *R. multiflora*.

The non-glandular trichomes are present on the surface of the stipules in *R. agrestis*, *R. foetida*, *R. multiflora*, *R. rubiginosa*, *R. rugosa* and *R. spinosissima*. Glandular trichomes are found either only on the edges of the stipules in *R. agrestis*, *R. canina* 'Inermis', *R. chinensis* 'Viridiflora', *R. multibracteata*, *R. spinosissima*, *R. rubiginosa* and *R. rugosa* (Figure 28 and 30), or across their abaxial surface (*R. multiflora*).

The rachis, in its median position, has the same structure plan as the petiole. The cross-section contour is almost circular but modified by latero-adaxial crests, sometimes almost parallel, sometimes slightly divergent, which delimits a more or less wide groove, but usually shallow. The number of vascular bundles is maintained as in the petiole, though the central bundles may be grouped together to form a larger one. At this level, the structure is mainly of primary origin with some secondary development. (Figures 31, 34, 35, 39). Also, we can notice the presence of “hydrocytes”, at both poles of the xylem and phloem. Hypodermic collenchyma is almost continuous in all investigated cases (Figure 41). The sclerenchyma is not continuous in periphloemic position; it is interrupted at intervals by groups of parenchymatic cells, sometimes containing “hydrocytes” between them, which are more frequent in *R. gallica* (Figures 40-42).

Both the petiole at the stipule level and the rachis, including the midrib of the last leaflet display spines on the inner face. These spines are composed of rows of cells with strongly lignified, thick walls, especially in the outer layer. In species such as *R. canina* 'Inermis', *R. glauca*, and *R. gallica*, the spines are smaller and fewer in number.

The **petiole of the leaflets** has a similar structure to that of the midrib of the leaflet lamina with a notable presence of mechanical tissue surrounding the vascular bundles. In these cases, only collenchyma elements are present within the periphloemic area, alternating with sclerenchyma cells that have lignified walls. In species where sclerenchyma is present in the periphloemic areas, lignified parenchyma cells are found intermittently. For example, *R. chinensis* 'Viridiflora' and *R. foetida* exhibit sclerenchyma cells with thinner, more lignified walls. Both on the rachis and, especially, on the petiole of the leaflets, non-glandular trichomes and/or glandular trichomes are present, similar to those on stem or other leaf parts, often being more frequent at this level.

At the level of the leaflet lamina the midrib has a structure similar to that of the rachis and petiole, with the distinction that on this level conductive tissues form a single collateral bundle, which usually has a primary structure.

The periphloemic sclerenchyma fibres are absent in many species (*R. damascena*, *R. foetida*, *R. rubiginosa*), or they are present as a continuous ring, with cells having cellulosic walls in *R. multibracteata*, well developed in *R. spinosissima* and *R. multiflora*, or discontinuous in *R. rugosa*.

The upper epidermis has its external wall thickened and cutinized. The cuticle is very thick at *R. multibracteata* species. Notably, air space cavities with irregular contours are present on both flanks of the midrib in *R. rugosa*.

The palisade represents more than half of the mesophyll's surface in species such as *R. agrestis*, *R. gallica*, *R. glauca*, *R. multibracteata*, and *R. multiflora*.

The **leaflet's lamina** has a bifacial structure at all investigated taxa, the mesophyll is differentiated in palisade parenchyma under adaxial surface and spongy parenchyma under abaxial surface.

Palisade parenchyma is generally bistratified (Figures 45, 46, 54) consisting of tall cells. In some species, such as *R. agrestis*, *R. chinensis* 'Viridiflora', *R. gallica*, and *R. foetida*, it appears tristratified; in these cases, the inner layer is composed of much shorter cells (Figures 48, 49, 53, 58).

Spongy parenchyma consists of 3-5 layers of cells in most cases; the variation limits are from 2-3 cell layers (*R. chinensis* 'Viridiflora', *R. rugosa*) up to 3-4 layers of cells. Based on size and frequency of air spaces of spongy tissue, we identified two following situations: very lax (*R. chinensis* 'Viridiflora', *R. damascena* and *R. multibracteata*) (Figure 46) and relatively lax (*R. agrestis* and *R. multiflora*) (Figures 48, 49, 52).

Regarding the shape of spongy parenchyma cells, several patterns can be distinguished: *R. foetida* shows both round and elongated cells in the multilayered spongy tissue, *R. glauca* has small, tangentially elongated cells, *R. spinosissima* features a compact spongy parenchyma where the cells are almost vertically oriented, creating the appearance that the palisade tissue is continuous from one epidermis to another, and *R. glauca* which has narrow cells arranged into a horizontal disposition. The epidermal cells are larger in the superior epidermis than the inferior one. Larger cells were found in *R. chinensis* 'Viridiflora', *R. gallica*, *R. glauca*, yet the specie *R. gallica* shows considerably larger cells in the lower epidermis than in many other species.

The contour of epidermal cells is different from one taxon to another and, in some cases, from upper epidermis to lower epidermis, even at the same taxon: polygonal cells with straight walls were identified inside of upper and lower epidermis (in *R. multiflora*, *R. rubiginosa* and *R. rugosa*); cells with an irregular shape with slightly wavy walls in both epidermis (*R. glauca* and *R. multibracteata*); polygonal cells with slightly wavy walls in upper epidermis, and slightly wavy in the lower epidermis with lateral walls almost sinuous (*R. canina* 'Inermis' and *R. damascena*). These results are in concordance with those observed in similar taxa originated from areas with different climate conditions [AL-DOSKEY, 2023]. Papilliform cells occur in the inferior epidermis in *R. rugosa*, as METCALFE & CHALK (1988) mentioned (Figure 56).

Notably, in *R. chinensis* 'Viridiflora', the upper epidermis, when viewed from above, consists of cells with flat lateral walls. Among these, there are isolated, slightly circular cells surrounded by the polygonal ones, forming a rosette-like pattern. The size of polygonal cells is variable, while the circular cells are uniform in size.

A particular feature occurs in *R. rugosa*, where a layer of isodiametric, colourless cells acts as a hypoderm between the palisade parenchyma and the upper epidermis. This layer results from the division of cells through the tangential walls of the initial epidermis. A similar structure is found on the lower surface of the lamina, many spongy cells are idioblasts, predominantly containing druses.

Additionally, tangential wall divisions are observed in some cells of the upper epidermis of *R. canina* 'Inermis', where two layers of unequally sized cells are formed in scattered spots.

The cells of the upper epidermis have thickened (*R. rubiginosa*) and cutinized (*R. chinensis* 'Viridiflora' and *R. multiflora*) external walls, with the exception of *R. canina* 'Inermis' and *R. glauca*, which have thinner, less cutinized walls. The two latter are more sensitive to attacks by phytopathogenic agents than other taxa that have thicker cuticles.

Stomata are located in the lower epidermis. They are by anomocitic type, a characteristic of the *Rosaceae* family. Stomata are numerous in *R. canina* 'Inermis', *R. damascena*, *R. multiflora* and *R. multibracteata* (Figures 59). The phenomenon of stomatal clustering within the axils of veins, characteristic for hybrids, occurs in species such as *R. damascena*, *R. multibracteata* and *R. multiflora*. Also, there is sometimes a tendency for stomatal cells to be positioned close together, separated by just a single epidermal cell (*R. canina* 'Inermis' and *R. damascena*). Typically, stomata are located at the same level of epidermal cells. In *R. damascena*, we also observed a suprastomatic chamber, a feature shared with *R. gallica*, where stomata are placed above the epidermal cells and form a tall substomatic chamber.

The second wall of division in epidermal cells, a characteristic feature of the *Rosaceae* family, sometimes appears at the level of the upper epidermis (*R. canina* 'Inermis', *R. damascena* and *R. rugosa*) or at level of the lower epidermis (*R. multibracteata* and *R. rugosa*).

Crystal idioblast, which are relatively frequent in the foliar lamina, as in other *Rosaceae* species, are present in various tissues. In the palisade parenchyma they occur as prismatic crystals and druses (*R. canina* 'Inermis', *R. damascena*, *R. gallica*, *R. multibracteata*, *R. multiflora* and *R. spinosissima*), or only as druses (*R. rugosa*). In spongy parenchyma they appear as prismatic crystals and druses (*R. canina* 'Inermis', *R. damascena* and *R. spinosissima*); in the phloem they are found in form of prismatic crystals and druses (*R. damascena*, *R. multibracteata*, *R. multiflora* and *R. spinosissima*); along to the main veins or their lateral veins many crystal idioblast cells with druses are present (*R. canina* 'Inermis' – under lower epidermis, *R. multiflora* – in both epidermis) or prismatic crystal (*R. damascena* and *R. multiflora*) (Figures 45, 47, 52, 54, 56, 57, 60).

Conclusions

The general structural plan is conservative. Anatomical investigations reveal rapid development of secondary structure as characteristic of the taxa, with the primary structure maintained only beneath the shoot apical meristem. The transition to secondary structure is primarily due to intense cambium activity in most cases. Phellogen activity during anthesis is observed only in *R. rugosa*, where phelloderm and suber is produced in a hypodermic position.

The hypodermic collenchyma is most often of the tangential type. *R. spinosissima* is distinguished by angular collenchyma arranged in a continuous layer.

Vascular bundles are commonly arranged in a circular pattern, with exception of *R. chinensis* 'Viridiflora'. Secondary xylem is sometimes arranged in radial, parallel strings. Libriform fibres from the secondary xylem are more abundant in some species, but found in smaller quantities in most. Sheaths of periphloemic sclerenchyma are well-distributed, with varying wall thicknesses and lower lignification in some cases.

The leaf is bifacial, typically with bistratified palisade tissue, sometimes tristratified with a smaller inner layer. Spongy tissue consists of 3-5 layers, often thin. Stomata are numerous and often grouped in the axils of the veins, characteristic of hybrids. Crystal idioblast, including druses and prismatic crystals, are present in various tissues.

The pith consists of large, thin-walled, cellulosic cells surrounded by smaller, lignified cells in a network. Non-glandular trichomes are found on the petiole and rachis, covering the external layer or adaxial groove.

Glandular trichomes are more abundant at the edges of stipules.

The second division wall, typical of the Rosaceae family, is present at the epidermal cell level, occasionally in the upper or lower epidermis.

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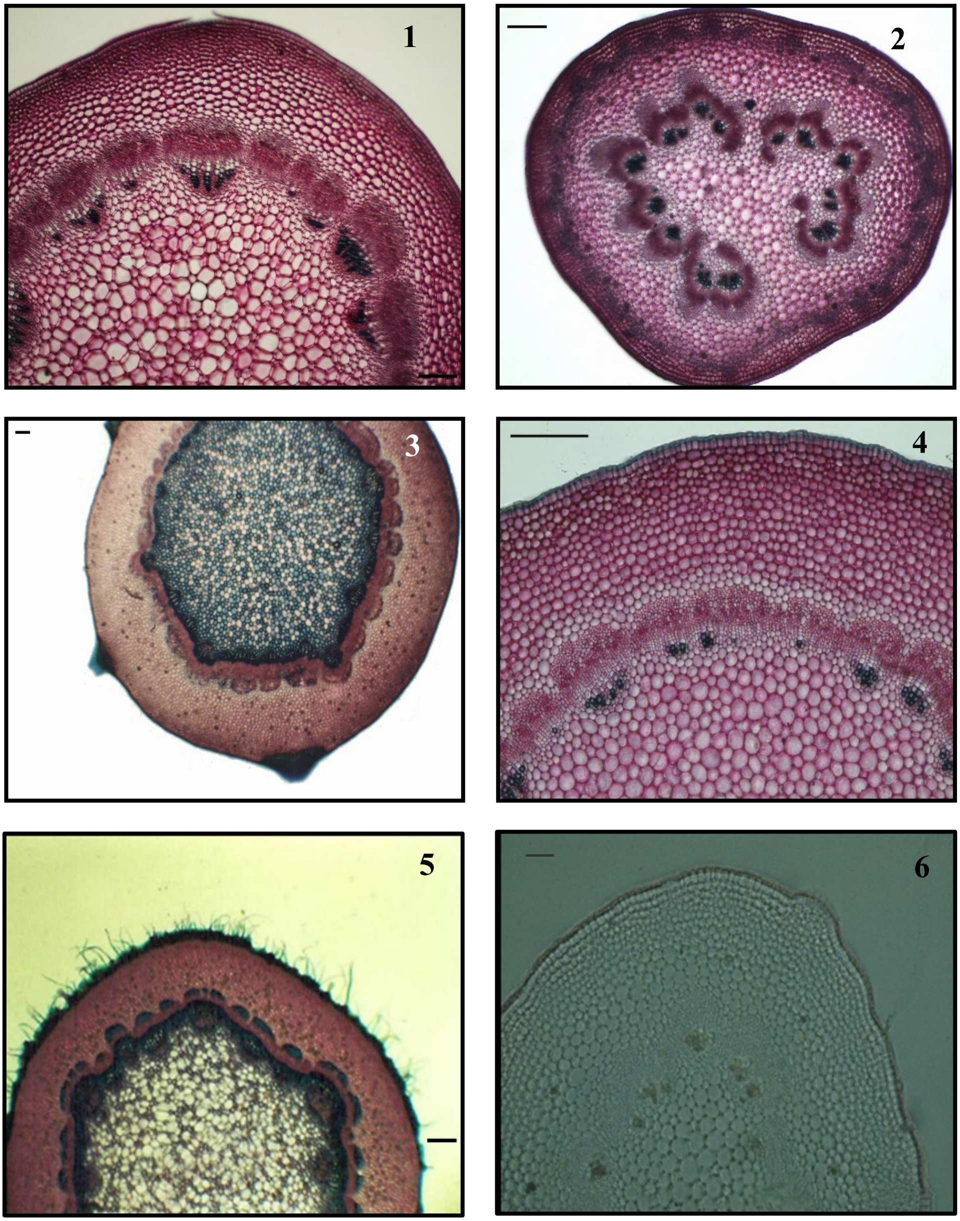
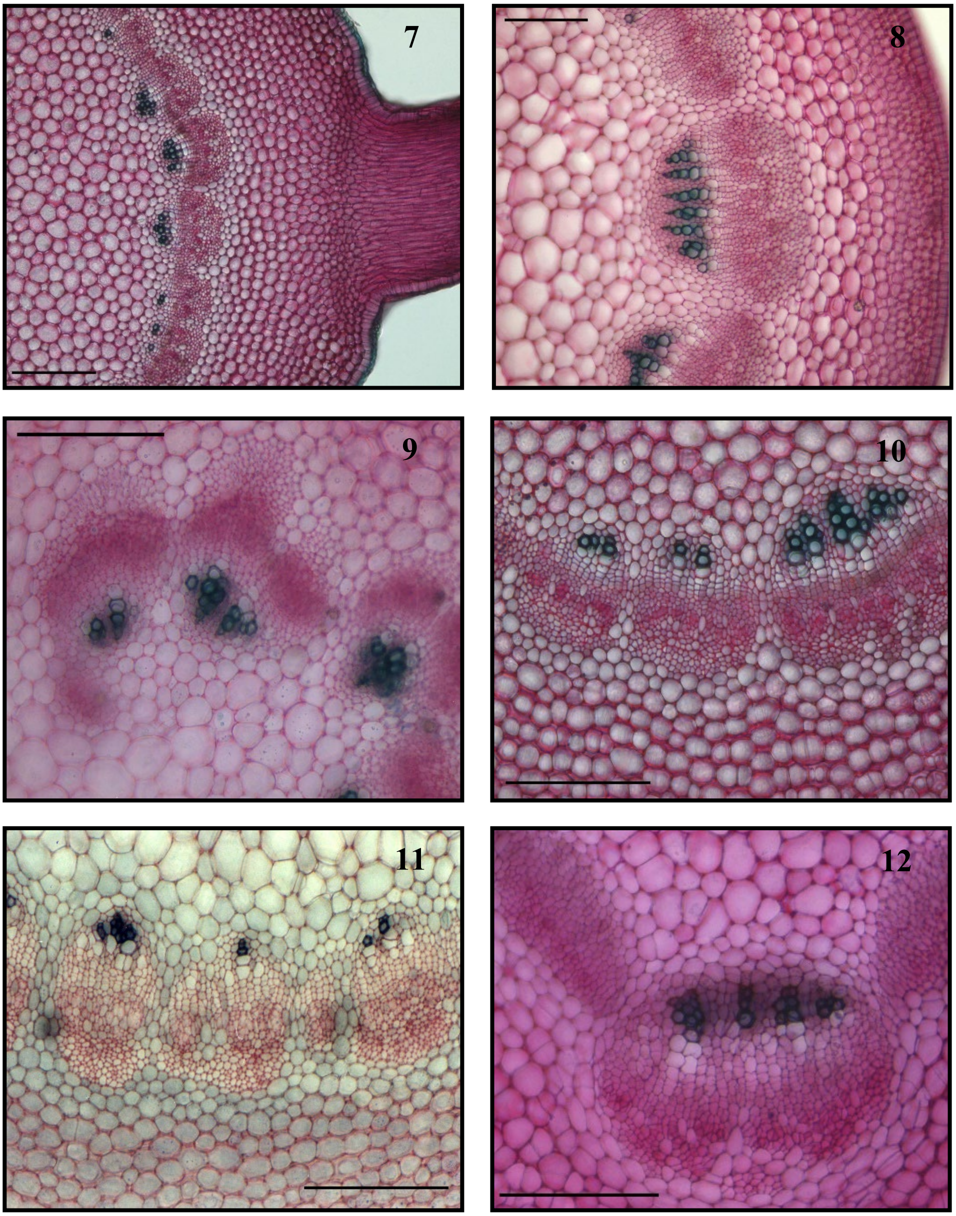
Plate 1



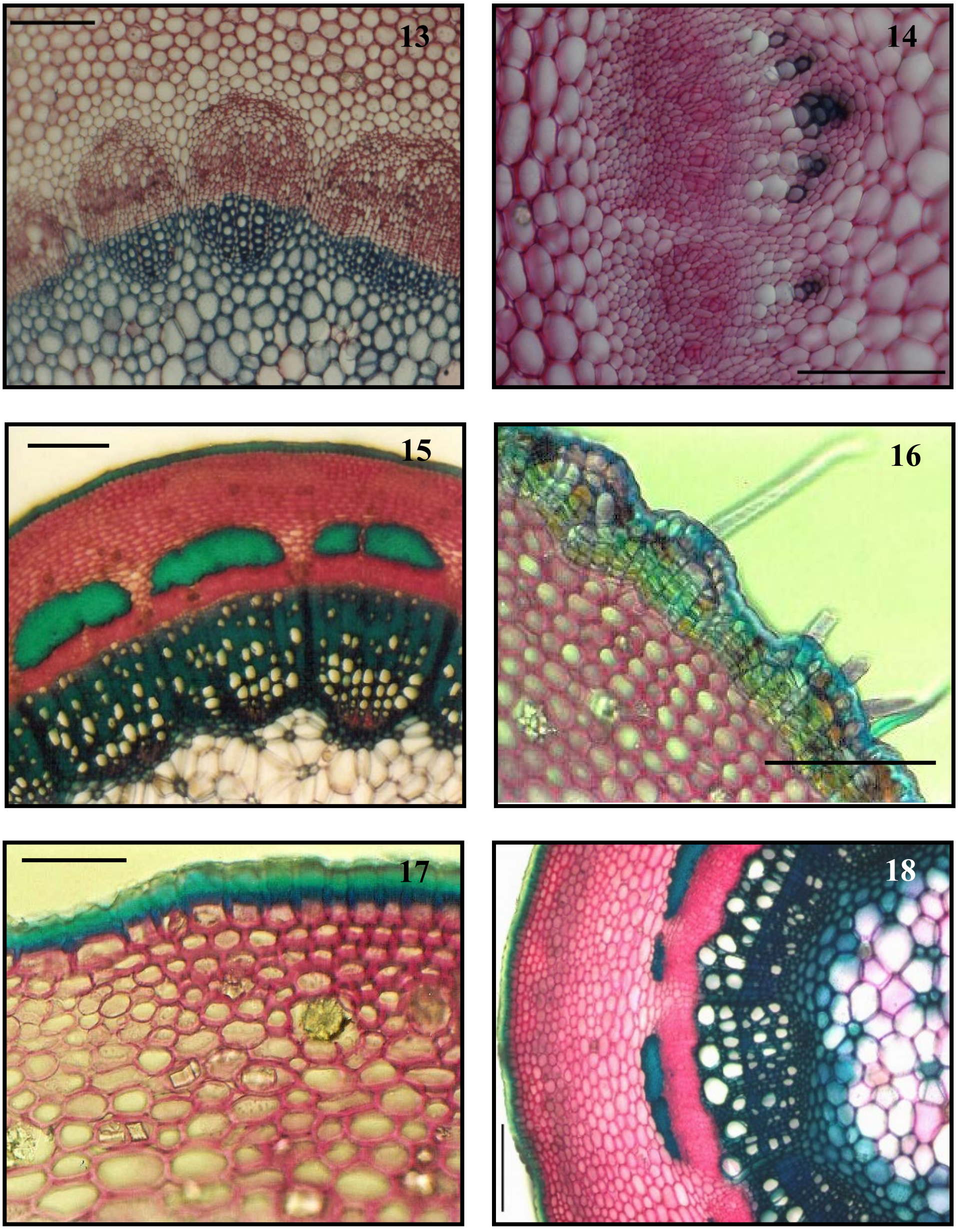
Figures 1-6. Anatomical aspects - upper third of the stem (scale = 100 µm): 1. Cross section through the stem of *Rosa damascena*; **2.** Cross section through the stem of de *Rosa chinensis* 'Viridiflora' (overview); **3.** Cross section through the stem of *Rosa rubiginosa* (overview); **4.** Cross section through the stem (overview); **5.** Cross section through the stem of *Rosa rugosa* (overview); **6.** *Rosa spinosissima* (overview).

Plate 2



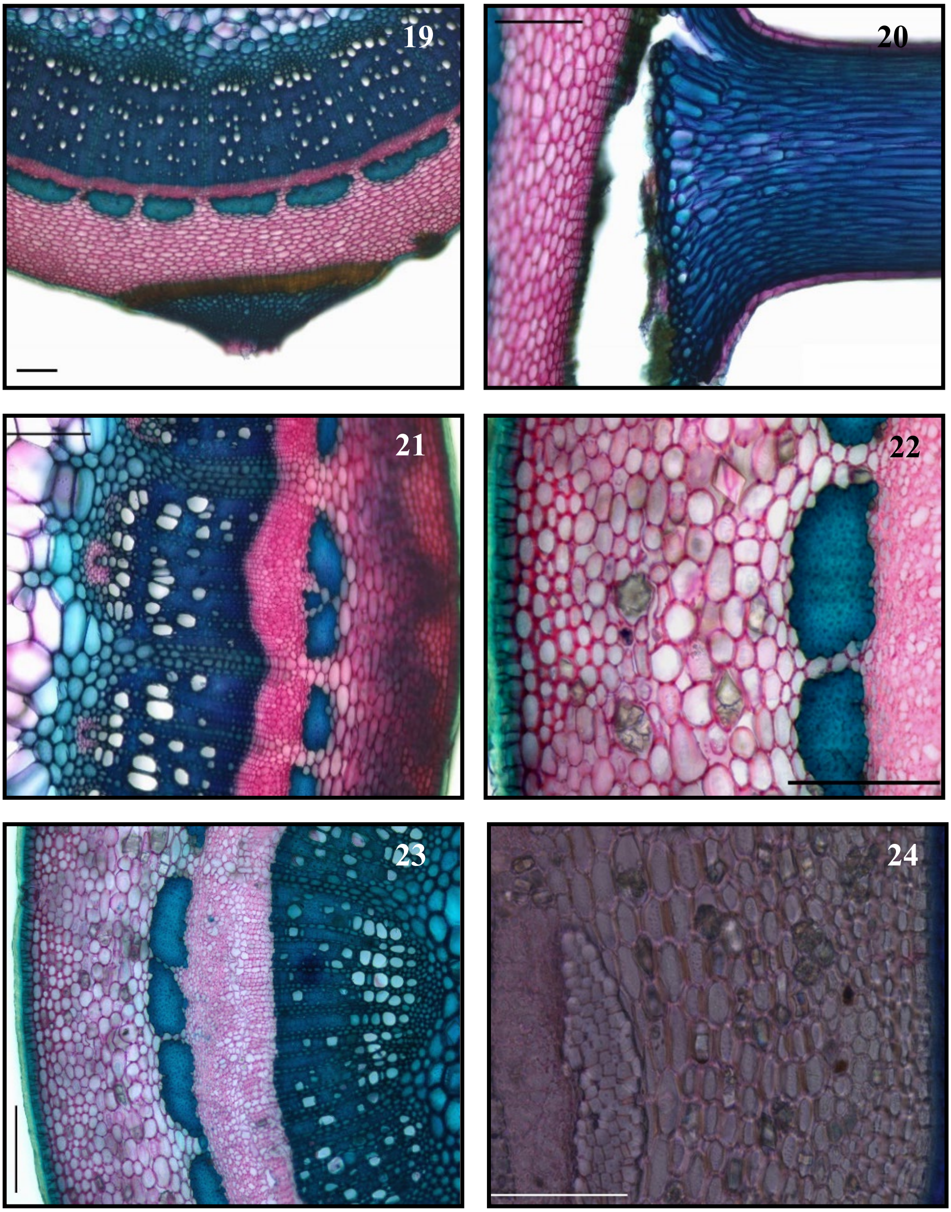
Figures 7-12. Anatomical aspects - upper third of the stem (scale = 100 µm): 7. Cross section through the stem of *Rosa foetida*; **8.** Cross section through the stem of *Rosa damascena* – bark and central cylinder detail; **9.** primary vascular bundles details at *Rosa chinensis* 'Viridiflora'; **10.** primary vascular bundles details at *Rosa foetida*; **11.** Cross section through the stem of *Rosa canina* 'Inermis' (cortex and central cylinder details); **12.** Cross section through the stem of *Rosa agrestis* (cortex and central cylinder details).

Plate 3



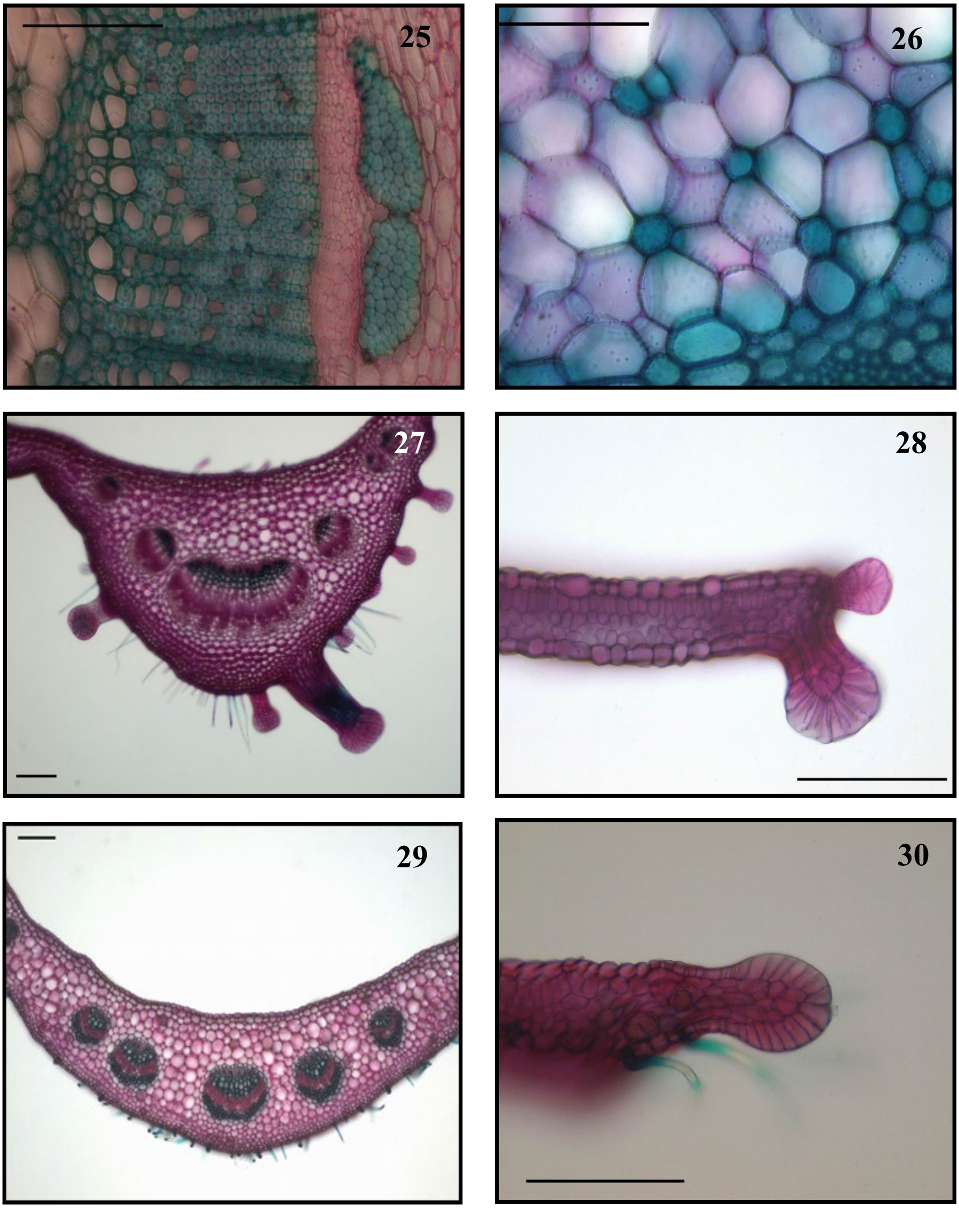
Figures 13-18. Anatomical aspects - upper third of the stem (scale = 100 µm): 13. the primary structure of the stem at *Rosa glauca*; **14.** primary vascular bundles details at *Rosa damascena*; **15.** *Rosa multibracteata* (overview); **16.** *Rosa rugosa* (bark detail and cork cambium, one of few species where the phelloderm is active since the first year giving birth to cork cambium); **17.** *Rosa rubiginosa* (detail of crystal idioblasts found in bark); **18.** *Rosa agrestis* (details of bark, central cylinder and pith).

Plate 4



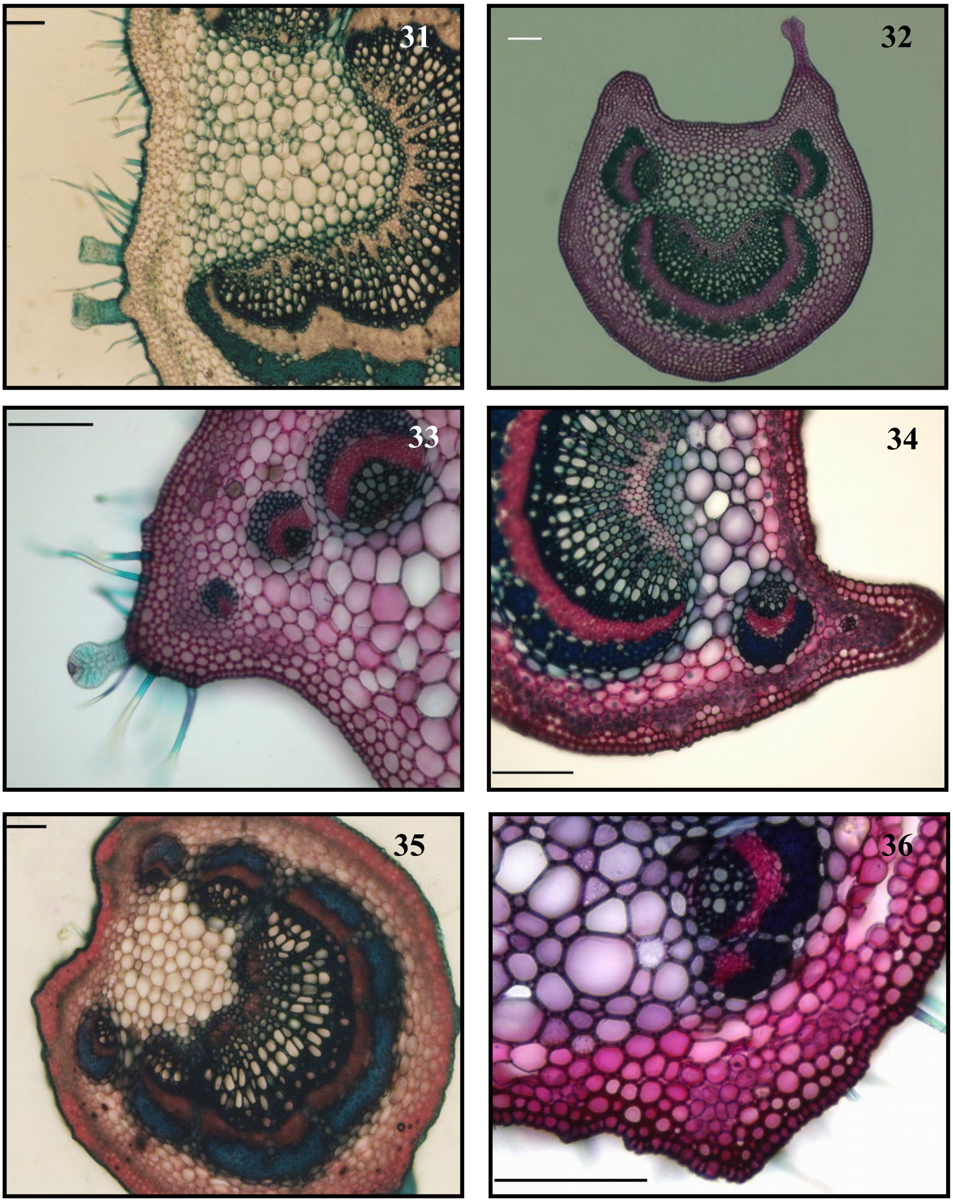
Figures 19-24. Anatomical aspects - middle/upper third of the stem (scale = 100 µm): 19-20. *Rosa foetida* (cortex and central cylinder details, spine that will detach from the stem, forming suber at its base); **21.** *Rosa chinensis* 'Viridiflora' (cortex and central cylinder details); **22.** *Rosa damascena* (cortex details); **23.** *Rosa damascena* (cortex and central cylinder details); **24.** *Rosa rubiginosa* (cortex detail with frequent crystal in cells).

Plate 5



Figures 25-30. Anatomical aspects in the stem and leaf of *Rosa* L. – Cross section through the middle third of the stem (scale = 100 µm): **25.** *Rosa gallica* (cortex and central cylinder details); **26**. *Rosa foetida* (pith pattern); **27-28.** *Rosa agrestis* (tector trichomes and secretory glands on petiole and stipules); **29-30.** *Rosa damascena* (tector trichomes and secretory glands on petiole and stipules).

Plate 6

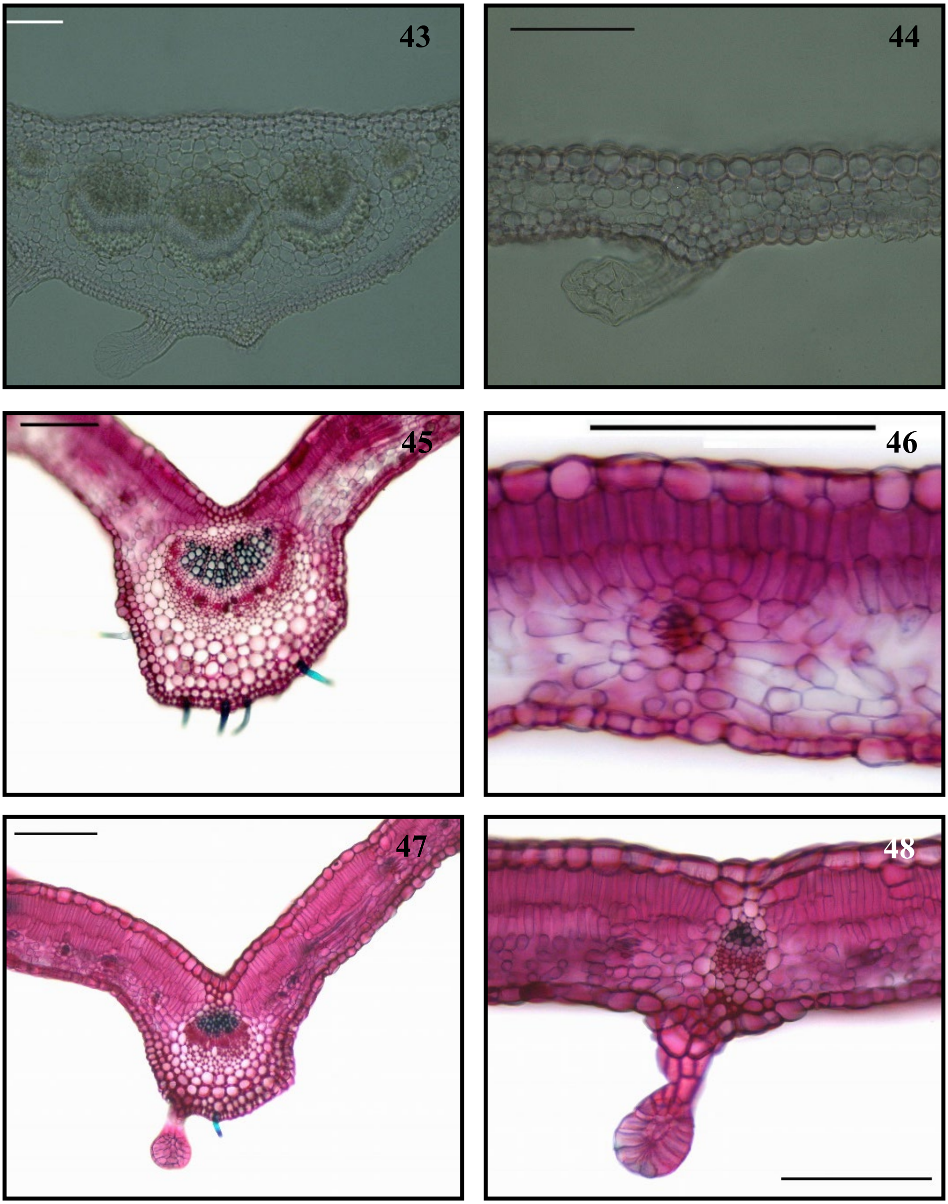


Figures 31-36. Anatomical aspects of the leaf of *Rosa* L. – Cross section through leaf's rachis (scale = 100 µm): 31-33. *Rosa damascena* (tector trichomes and secretory glands on rachis); **32.** *Rosa gallica*; **34.** *Rosa chinensis* 'Viridiflora'; **35.** *Rosa multibracteata*; **36.** *Rosa gallica*.

Plate 7

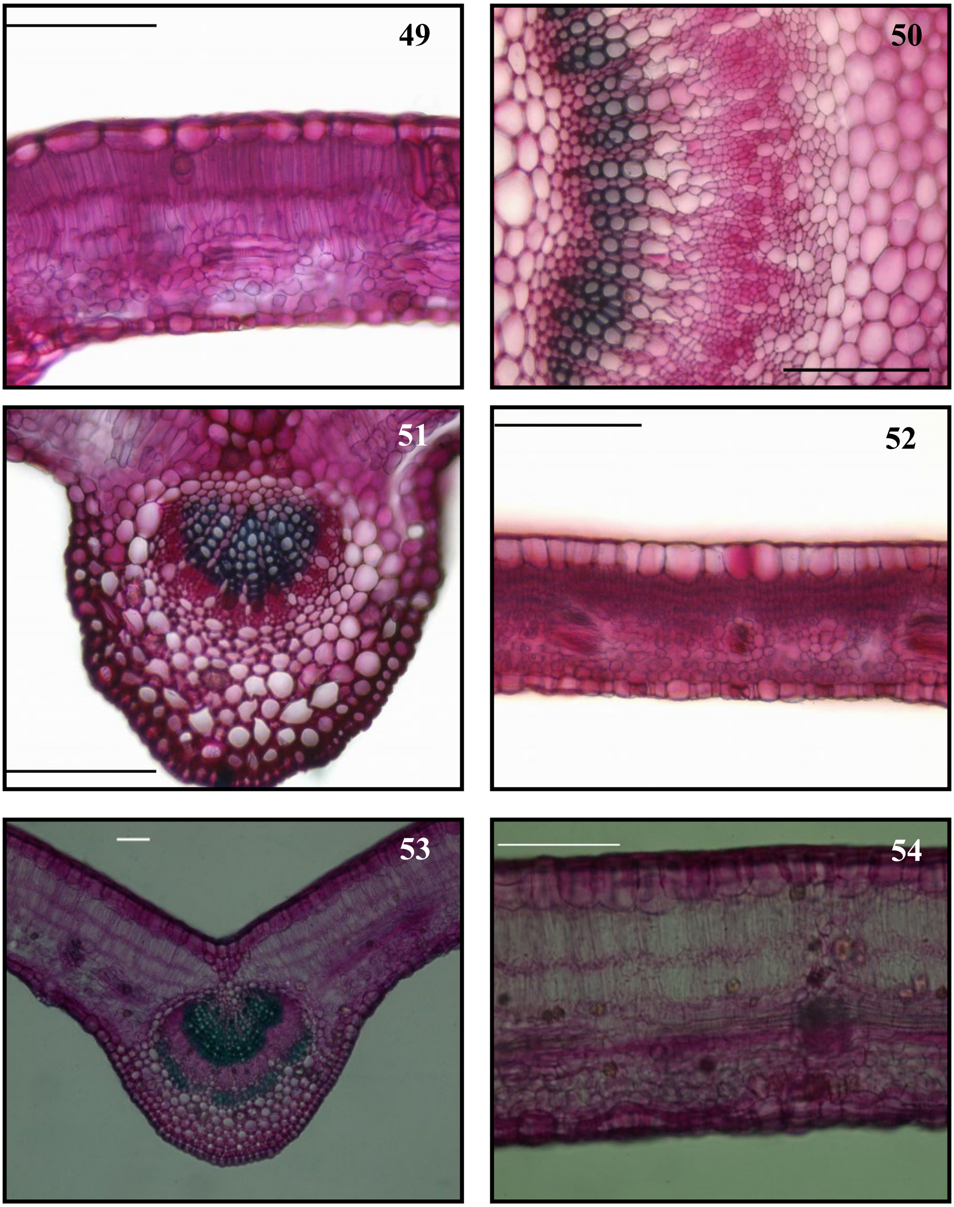
Figures 37-42. Anatomical aspects of the leaf of *Rosa* L. – Cross section through leaf's rachis (scale = 100 µm): 37-38. *Rosa rugosa*; **39-40.** *Rosa foetida*; **53-54)** *Rosa gallica*.

Plate 8



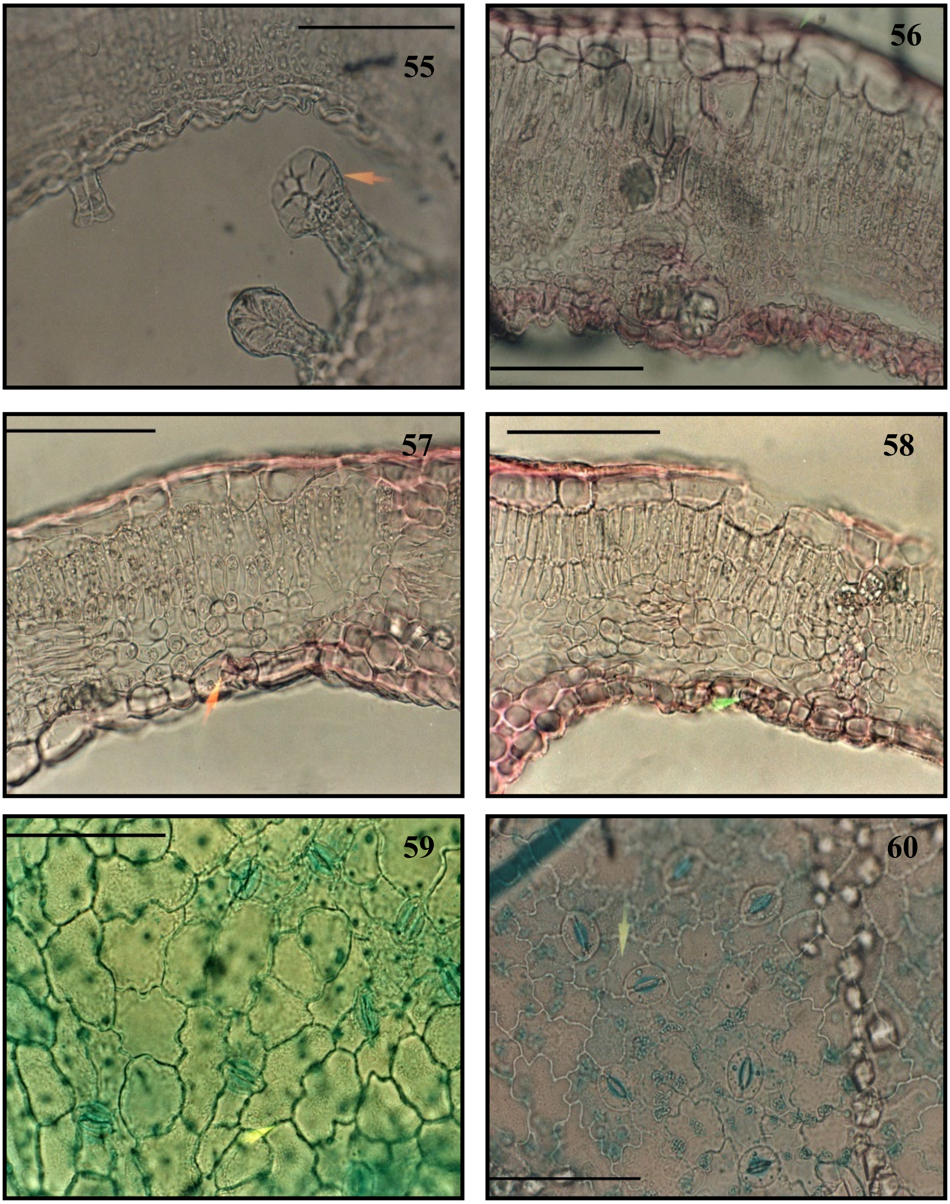
Figures 43-48. Anatomical aspects of the leaf of *Rosa* L. – Cross section through leaflet (scale = 100 µm): 43-44. *Rosa spinosissima*; **45-46.** *Rosa damascena* (midrib and leaflet lamina); **47-48.** *Rosa foetida* (leaflet's lamina).

Plate 9



Figures. 49-54. Anatomical aspects of the leaf of *Rosa* L. – Cross section through leaflet (scale = 100 µm): 49-50. *Rosa agrestis*; **51-52.** *Rosa chinensis* ‘Viridiflora’; **53-54.** *Rosa gallica*.

Plate 10



Figures 55-60. Anatomical aspects of the leaf of *Rosa* L. – Cross section through leaflet (scale = 100 µm): 55-56. *Rosa rugosa* (secretory glands on the abaxial face of the lamina and papilliform adaxial epidermis); **57.** *Rosa canina* 'Inermis'; **58.** *Rosa multibracteata* (detail of the midrib); **surface view of abaxial epidermis: 59.** *Rosa multibracteata*; **60.** *Rosa damascena*.

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Research Article

ASSEMBLAGES OF WOOD-INHABITING MACROFUNGI SPECIES IN BEECH FORESTS FROM EASTERN ROMANIA

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Abstract: Species composition and diversity of lignicolous fungi occurring in representative *Fagus* sp. forests have been analyzed along an altitude gradient, in 14 locations from the eastern region of Romania, including mountain forests (Borca, Goşman, Nemţisor, Tarcău), as well as hill forests (Arsura, Bârnova, Dălhăuţi, Dragomirna, Gâdinţi, Homiţa, Humosu, Măgura Ocnei, Runc, Valea Fagilor). The investigations were carried out over three successive years (2020-2022) in a total of 40 study plots with a size of 1000 square meters each. Identification of the main fungal groups was realized using a hierarchical agglomerative clustering procedure, and the differential species were determined based on their indicator value, while the relationship between fungal composition and environmental variables was assessed with detrended correspondence analysis. A total of 89 species of wood-inhabiting fungi has been identified, with approximately 2500 records. These species were grouped in three different clusters: one specific to the mixed broadleaved-coniferous forest in (sub) mountain areas, one specific to hornbeam - beech communities in hilly areas, and one specific to more thermophilic forest communities with *Fagus taurica*. The main ecological factors influencing the fungal composition were altitude, average annual precipitations and average annual temperatures, which also influence the trees species composition of the analyzed forests.

Keywords: assemblages, beech forests, climatic factors, lignicolous fungi, tree species.

Introduction

Beech forests occupy large areas in Europe (approximately 910,000 km^2) [LEUSCHNER & ELLENBERG, 2017], in low altitude areas, from NW and Central Europe to southern Sweden. They have been subjected to great anthropogenic pressure throughout history as they have been used as resources for human populations (e.g. timber, accessory products, etc.) [JAHN, 1991; ELLENBERG & LEUSCHNER, 1996]. Representative areas with natural ancient *Fagus* sp. forests are quite few and strictly protected [PETERKEN, 1996; DIACI, 1999; PARVIAINEN & al. 2014].

In Romania, the beech forests occupy the largest areas of the forestry fund (about 31%), covering about 2 million ha [MILESCU & al. 1967; ŞOFLETEA & CURTU, 2007]. In scientific literature have been described 15 types of beech forest ecosystems, depending on phytosociological associations, forest types, soil types and climatic characteristics [PAUCĂ-COMĂNESCU & al. 1989; DONIŢĂ & al. 1990; DONIŢĂ & al. 2005]. A part of these forest types is also found in the Ukrainian and Polish Carpathians, as well as in the plateaus of the Republic of Moldova reaching the eastern limit of species' distribution in Europe [SZAFER, 1932; STOYKO, 1992; STOYKO, 2005; NEDEALCOV & DONICA, 2019]. *Fagus sylvatica*

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edifies pure or mixed forests in (sub) mountain and hilly areas of the country, and in the case of thermal inversions, it replaces the spruce at higher altitudes. *Fagus taurica*, with Balkan and Crimean areal, is a more thermophilic taxon, sporadically encountered in Romania, that form in Valea Fagilor (Tulcea County) a particular forest habitat [OPREA & al. 2011].

Figure 1. Map presenting the distribution of investigates beech forests in Eastern Romania (adapted from www.geotutorials.ro)

These species present a shade temperament [MILESCU & al. 1967], and have a significant impact on the modification of forest ground light regime, on soil humidity and on buffering temperature and humidity changes. These properties favorably affect biological activity, and contribute at maintaining an increased biodiversity [SOLON, 2002]. Adult specimens are resistant to diseases and pests. Still, they are vulnerable to the attack of wood-inhabiting fungi, through mechanical wounds mainly generated by forestry operations, which are successful entry points for infections [IGMÁNDY, 1964]. A great asset of the forest ecosystems in Romania is their naturalness, depending, among other, on diversity and structure of trees layer, ground vegetation, the quantity of dead wood and management type. There have been preoccupations about habitat restoration by increasing the volume of dead wood, thus leading to increased biodiversity throughout the ecological chain. In protected areas and ancient forests, dead wood exists in different forms, from dead trees on the ground, to large pieces (fallen logs) and small wood fragments [CHRISTENSEN & al. 2005].

The different types of dead wood (roots, branches and logs) in different stages of decomposition offer a wide range of ecological niches and favor a high diversity of wood-inhabiting fungi. It is estimated that approximately half of the macrofungi species in forests are wood decomposers, lignicolous fungi playing an important role in the functioning of forest ecosystems [KÜFFER & SENN-IRLET, 2005; MÜLLER & al. 2007; STOKLAND & al. 2021].

Although fungi are components of natural communities, they were rarely used to describe them. Because of their specificity to particular microhabitats and environmental conditions, some fungal taxa could be better suited as indicator species than plants. Despite of the ecological importance of lignicolous macrofungi species in forest ecosystems, this group of organisms was generally neglected in the biodiversity studies carried out in Romania. Although some studies tried to document their diversity at the species level, there are very rare cases when plot-based sampling in combination with multivariate analyses were used [BÎRSAN & al. 2014; COPOŢ & al. 2020] in order to distinguish some macro-fungal communities.

The current study was focused on identifying the main assemblages of wood-inhabiting fungi specific to the *Fagus* forests from the eastern part of Romania, on identification of differential species for these assemblages and on the main abiotic drivers shaping their species composition.

Material and methods

The present study was carried out in 14 forests (13 edified by *Fagus sylvatica* and one by *Fagus taurica*) located in the eastern part of Romania (Figure 1). The sampling plots were established along an altitude gradient, from low elevation areas, of approximately 150 meters altitude (Valea Fagilor), to mid-altitudes ranging from 361 meters to 540 meters (Arsura, Bârnova, Dălhăuţi, Dragomirna, Gâdinţi, Homiţa, Humosu, Măgura Ocnei, Runc), up to relative high altitudes of about 900 m in the mountain area (Borca). The silvicultural history differs between the study areas. Some forests were completely logged in the past and now have a uniform age structure with dominant tree ages ranging from 100 to 250 years. The selected sample areas cover the main types of beech forests and are characterized by various degrees of human interventions.

The mycological investigations were carried out in 40 plots, each with a size of 1000 square meters, over 3 successive years (2020-2022), starting from April to November. The forests massifs are subject to different types of management, a part of them characterized by various degrees of intervention (e.g. Bârnova, Gâdinţi, Homiţa, Măgura Ocnei, Borca, Tarcău), while other sites were declared natural reserves (e.g. Arsura, Dragomirna, Dălhăuţi, Goşman, Nemţisor, Humosu, Runc, Valea Fagilor). In all the investigated sample areas, beech is the dominant species, but other tree species that form the respective forest stands also have a major importance, as host species for various lignicolous fungi. For each sample, the geographical coordinates, altitude, slope and aspect using a geographic positioning device (GPS II Plus Garmin Ltd.) were recorded (Table 1). The species of lignicolous fungi collected were identified at the species level based on macroscopic and microscopic characters, according to identification keys and reference guides [SĂLĂGEANU & SĂLĂGEANU, 1985; BREITENBACH & KRÄNZLIN, 1986; GERHARDT, 1999; BERNICCHIA, 2005; TĂNASE & al. 2009; COURTECUISSE & DUHEM, 2013]. Nomenclature follows the Index Fungorum database [http://www.indexfungorum.org/Names/Names.asp].

Table 1. Geographical features of sampling plots investigated

Acr.	Sampling plots	Localities / County	Latitude	Longitude	Altitude (m a.s.l.)	Aspect	Slope
A 1	Arsura 1	Gârleni BC	46˚38'20.27"	26˚47'17.68"	361	NNV	small
A 2	Arsura 2	Gârleni BC	46˚37'48.37"	26˚48'40.34"	364	NV	medium
A 3	Arsura 3	Gârleni BC	46˚37'44.88"	26˚48'34.72"	343	NE	small
B 1	Bârnova 1	Bârnova IS	47˚00'28.75"	27˚35'10.10"	404	ENE	small
B 2	Bârnova 2	Bârnova IS	47˚00'34.54"	27˚35'12.58"	394	E	medium
Bo 1	Borca 1	Borca NT	47˚14'44.36"	25˚52'30.66"	868	VNV	high
Bo 2	Borca 2	Borca NT	47˚14'33.93"	25˚52'26.19"	877	V	small
Bo 3	Borca 3	Borca NT	47˚14'31.40"	25˚52'30.07"	895	V	high
D 1	Dălhăuți 1	Dălhăuţi VN	45˚41'47.21"	27˚00'50.46"	413	E	medium
D 2	Dălhăuți 2	Dălhăuţi VN	45˚41'34.91"	27˚00'41.43"	470	NE	small
D 3	Dălhăuți 3	Dălhăuţi VN	45˚41'52.22"	27˚00'51.92"	401	N	medium
Dr 1	Dragomirna 1	Dragomirna SV	47˚46'43.50"	26˚13'09.89"	460	ESE	small
Dr 2	Dragomirna 2	Dragomirna SV	47˚46'42.89"	26˚13'05.17"	460	V	medium
Dr 3	Dragomirna 3	Dragomirna SV	47˚46'45.55"	26˚13'02.85"	469	V	small
Dr 4	Dragomirna 4	Dragomirna SV	47˚46'42.48"	26˚13'00.11"	466	ESE	high
G 1	Gâdinți 1	Roman NT	46˚55'15.56"	27˚04'11.41"	418	NE	medium
G 2	Gâdinți 2	Roman NT	46˚55'05.53"	27˚04'13.76"	394	NE	medium
G 3	Gâdinți 3	Roman NT	46˚55'21.06"	27˚04'06.16"	382	NNV	high
Go 1	Goşman 1	Brateş NT	46˚42'50.79"	26˚12'12.08"	823	NV	high
Go 2	Goşman 2	Brateş NT	46˚42'49.62"	26˚12'08,31"	803	ENE	medium
H 1	Homița 1	Cristeşti IS	47˚16'39.37"	26˚36'21.20"	412	NNE	medium
H 2	Homița 2	Cristeşti IS	47˚16'37.48"	26˚36'31.24"	413	NNE	medium

H 3	Homița 3	Cristeşti IS	47˚18'16.71"	26˚36'19.90"	401	ESE	small
Hu 1	Humosu 1	Pârcovaci IS	47˚29'43.64"	26˚43'29.23"	439	ESE	small
Hu 2	Humosu 2	Pârcovaci IS	47˚29'49.50"	26˚43'24.97"	458	E	small
Hu 3	Humosu 3	Pârcovaci IS	47˚29'55.70"	26˚43'17.23"	470	ENE	small
Hu 4	Humosu 4	Pârcovaci IS	47˚30'05.19"	26˚43'15.15"	475	ENE	small
MO 1	Măgura Ocnei 1	Târgu Ocna BC	46˚15'56.62"	26˚35'34.47"	540	ESE	medium
MO 2	Măgura Ocnei 2	Târgu Ocna BC	46˚16'13.61"	26˚35'18.77"	500	E	medium
N 1	Vânători Neamţ 1	Mrea Neamţ NT	47˚16'27.84"	26˚07'47.88"	656	V	high
N 2	Vânători Neamţ 2	Mrea Neamţ NT	47˚16'28.60"	26˚07'38.20"	652	ESE	medium
N 3	Vânători Neamţ 3	Mrea Neamţ NT	47˚16'45.00"	26˚06'10.16"	692	S	high
R 1	Runc 1	Buhuşi BC	46˚45'11.74"	26˚44'48.34"	461	S	small
R 2	Runc 2	Buhuşi BC	46˚45'18.67"	26˚45'23.37"	454	VNV	small
R 3	Runc 3	Buhuşi BC	46˚45'22.51"	26˚45'29.81"	480	VNV	small
T 1	Tarcău 1	Tarcău NT	46˚53'09.53"	26˚07'30.79"	530	NV	high
T 2	Tarcău 2	Tarcău NT	46˚53'08.00"	26˚07'35.39"	525	SE	high
VF 1	Valea Fagilor 1	Luncaviţa TL	45˚12'58.33"	28˚18'24.98"	130	NE	medium
VF 2	Valea Fagilor 2	Luncaviţa TL	45˚12'55.15"	28˚18'25.44"	153	ENE	medium
VF 3	Valea Fagilor 3	Luncaviţa TL	45˚12'52.61"	28˚18'32.82"	163	E	high

Identification of the main groups of wood inhabiting fungi was realized in a hierarchical agglomerative clustering method, using the Sorensen index (on presence-absence data) and the UPGMA algorithm. The main gradients in fungal composition and the environment (altitudes, mean annual temperatures, mean annual precipitations, etc.) influence on fungal assemblages were assessed using detrended correspondence analysis (DCA). Climatic variables were extracted from WorldClim database [HIJMANS & al. 2005]. Differential species were assessed using the indicator value index [DUFRÊNE & LEGENDRE, 1997] and a permutation test allowed the selection of those species statistically significant associated to the clusters [DE CÁCERES & LEGENDRE, 2009]. The clustering procedure has been realized using the GINKGO software [DE CÁCERES & al. 2003; BOUXIN, 2005]. DCA have been realized in CANOCO 5 programme [TER BRAAK & ŠMILAUER, 2012].

Results and discussions

Field mycological investigations had as result the identification of 89 lignicolous fungi species, with approximately 2500 records. The most species rich plot included 47 species, while the poorest species plot had only 10 species. Among the most frequent species, inventoried in more than 40% of all records, were: *Xylaria polymorpha*, *Trametes versicolor*, *Stereum hirsutum*, *Schizophyllum commune*, *Fomes fomentarius*, *Apioperdon pyriforme*. In addition, in the investigated sample areas were found more species less frequent such as *Amylostereum areolatum*, *Coniophora puteana*, *Dasyscyphella nivea*, *Gloephyllum odoratum*, *Gymnopus erythropus*, *Mycena aetites*, *Mycena haematopus*, *Pluteus granulatus*, *Sarcoscypha austriaca*, *Trametes cinnabarina*.

The areas with the greatest fungal diversity were those from Humosu, Valea Fagilor and Dragomirna, some of the most important natural reserves for conservation of *Fagus sylvatica* in our country. In contrast with areas where the forest management is strongly focused on conservation, in the areas with intensive silvicultural management, focused on economic activities, and do not benefit of protection status, lignicolous fungal diversity is lowest, for example Tarcău, Măgura Ocnei and Gâdinţi with 13, 10 and 11 species (Figure 2).

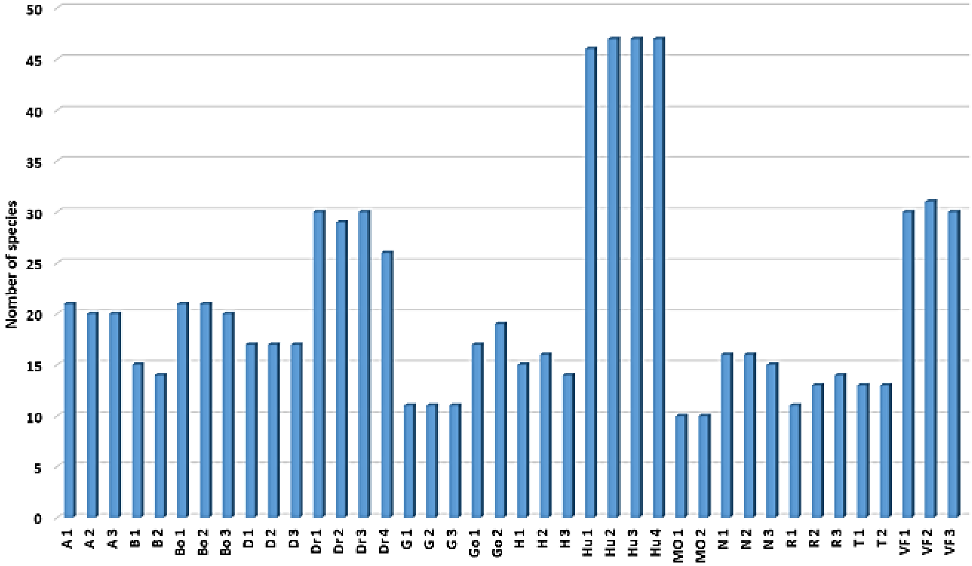

Figure 2. Species richness of wood inhabiting fungi in the investigated plots.

This aspect is related to the quantity and the different types of dead wood, with different decomposition stages, offering a wide range of ecological niches for wood-inhabiting fungi. In all investigated beech natural reserves were identified dead trees which represent true treasures for biodiversity. As the amount of dead wood decreased, a decline in the number of fungi species was observed due to the lack of support for their development. From the point of view of forest management and administration, dead wood has been perceived negatively, supposedly indicating lack of management, negligence and waste. Articles have also been published on the functions of dead wood, thus improving people's perception functions of this type of substrate [MERGANIČOVA & al. 2012]. Nowadays, the amount of dead wood is increasing not only due to ecologists but also due to foresters and scientific research. The amount of dead wood is increasing after its component and importance in the functioning of forest ecosystems have been demonstrated [BÄSSLER & al. 2011; GAO & al. 2015]. Dead wood has become an indicator of sustainable forest management, being of major importance for biodiversity. Fungi are the main saproxylic taxa, first colonizing wood and starting its decomposition. The presence of large amounts of dead wood in forest ecosystems is often connected to low anthropogenic influences, and in regions where human activities are reduced, the degree of naturalness is expected to increase over time and space [LOMBARDI & al. 2008]. In conclusion, the greater the volume of dead wood, the greater the number of lignicolous species were encountered, some of which were rare.

In the dendrogram generated by hierarchical clustering (Figure 3), a first separation was highlighted between fungal assemblages in mountain mixed deciduous - coniferous forests (cluster 1) and fungal assemblages in pure deciduous forest stands. Further, the assemblages in deciduous forests differentiated into communities from more mesothermic and mesophilic forests typical to hilly areas (cluster 3) and more thermophilic and more xeric forests in lower areas (cluster 2). This separation highlights besides the importance of climate in fungal species distribution, the fundamental role of the host tree species.

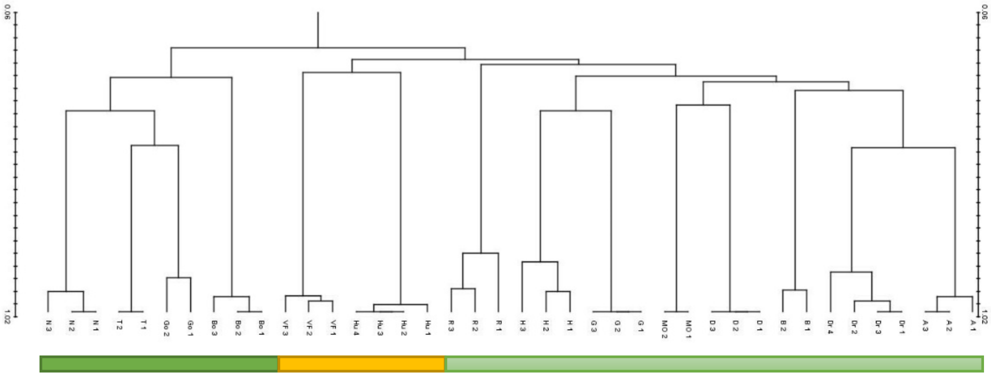

Figure 3. Dendrogram highlighting the three clusters resulted from hierarchical clustering of the 40 sampled plots (cluster 1 – dark green, cluster 2 – yellow, cluster 3 – light green)

Cluster 1 – groups the plots from the mountain and submountain areas, in Borca, Goşman, Tarcău, and Nemţisor areas. The forests stands are edified by *Fagus sylvatica* mixed with *Abies alba*, *Picea abies*, *Betula pendula*, etc., and are characterized by low means of annual temperatures, larger temperature amplitudes, short vegetation seasons, and high values of precipitations. Most of the diagnostic species were identified on logs and woody debris from conifers, in various degrees of decomposition: *Hypholoma capnoides*, *Pseudohydnum gelatinosum*, *Plicaturopsis crispa*, *Flammulina velutipes*, *Pholiota squarrosa*, *Meripilus giganteus*, *Amylostereum areolatum*, *Gloeophyllum odoratum*, *Mycena polygramma*, *Trametes pubescens*.

Cluster 2 – included the plots in more thermophilic forest stands from Valea Fagilor as well as plots investigated in Humosu forest reserve. These areas share a great number of common fungal species (among them) and are characterized by a continental, dry climate, a suboptimal precipitation level. The forests are edified by *Fagus taurica* (in Dobrogea) and *Fagus sylvatica* (in Moldova) with *Carpinus betulus*, *Tilia tomentosa*, *Tilia platyphyllos*, *Fraxinus excelsior*, *Acer campestre*, *Acer platanoides*, *Ulmus glabra*, etc. in the tree layer. The fungal species which differentiate from the other clusters are: *Chondrostereum purpureum*, *Ganoderma lucidum*, *Auricularia auricula-judae*, *Cerioporus varius*, *Hericium coralloides*, *Trametes gibbosa*, *Armillaria cepistipes*, *Mucidula mucida*, *Phlebia tremellosa*, *Pluteus cervinus*, *Auricularia mesenterica*, *Bulgaria inquinans*, *Cerioporus squamosus*, *Collybiopsis* *ramealis*, *Coprinellus micaceus*, *Daldinia concentrica*, *Gymnopus fusipes*, *Ischnoderma resinosum*, *Phlebia rufa*, *Mycena galericulata*, *Lycogala epidendrum*, *Calycina citrina*, *Daedaleopsis confragosa*, *Xerula pudens*, *Schizophyllum commune*, *Hymenochaete rubiginosa*, *Pleurotus ostreatus*, *Volvariella bombycina*, *Coniophora puteana*, *Dasyscyphella nivea*, *Pluteus ephebeus*, *Pluteus granulatus*, *Pluteus leoninus*, *Sarcoscypha austriaca*, *Trametes cinnabarina*, *Panellus stipticus*, *Ganoderma applanatum*, *Daedaleopsis tricolor*, *Hypoxylon fragiforme*, *Sarcoscypha coccinea*.

Cluster 3 – included the plots analyzed in mesophilic *Fagus sylvatica* forests from the nemoral zone (e.g. Bârnova, Dragomirna, Gâdinţi, Homiţa, etc.). The tree layer includes other species, such as *Carpinus betulus*, *Cerasus avium*, *Acer platanoides*, *Tilia cordata*, and were subjected to a high degree of human intervention. As a consequence, these are species poor stands, and are characterized by only one diagnostic species, namely *Hymenopellis radicata*.

The influence of host tree species in determining fungal assemblages is well known in the literature [BERNICCHIA & al. 2007; KEBLI & al. 2011]. It was found that areas dominated by deciduous species present different communities of lignicolous fungi compared to those dominated by conifers [BLASER & al. 2013]. The ecological profile of the component species in the sampled areas indicates a variety of diagnostic species in the assembly of wood-inhabiting fungal communities, closely related to the composition of host tree species, achieving a clear distinction between conifers and deciduous trees, which was also demonstrated in other studies [KÜFFER & al. 2008; O'HANLON & HARRINGTON, 2012]. Although in the current study the most common deciduous species was *Fagus sylvatica*, the presence of other tree species in the investigated forest stands determined the agglomeration of fungal species in different types of assemblages.

In DCA, the most important factors influencing the distribution of lignicolous macrofungi species, as well as the composition of the groups of wood inhabiting fungi, in the studied forests, were the average annual precipitations and mean annual temperatures, which depend, at larger scales, of terrain elevation (Figure 4). Thus, the mountainous and sub-montane regions are characterized by a higher amount of annual precipitation and lower temperatures that maintain moisture for longer periods of time. As demonstrated in other studies [BODDY, 1993; HEILMANN-CLAUSEN & CHRISTENSEN, 2003; KARADELEV & al. 2008], water (along with temperature) is the main limiting factor in the appearance of sporocarps and for the distribution of lignicolous species. In addition, the floristic composition of trees and shrub layers separate fungal assemblages from higher altitudes from those in medium and low hilly areas. The slope aspect of the sampled areas was also important for the distribution of lignified species, because those that are exposed to high brightness (especially southern aspect) lose moisture faster than those that have shade for longer periods of time. The edges of the massif, with greater fluctuations in humidity and solar radiation are poorer in species than those located under the canopy of secular beech forests, with a high degree of shading at ground level [BÄSSLER & al. 2010; ABREGO & SALCEDO, 2014].

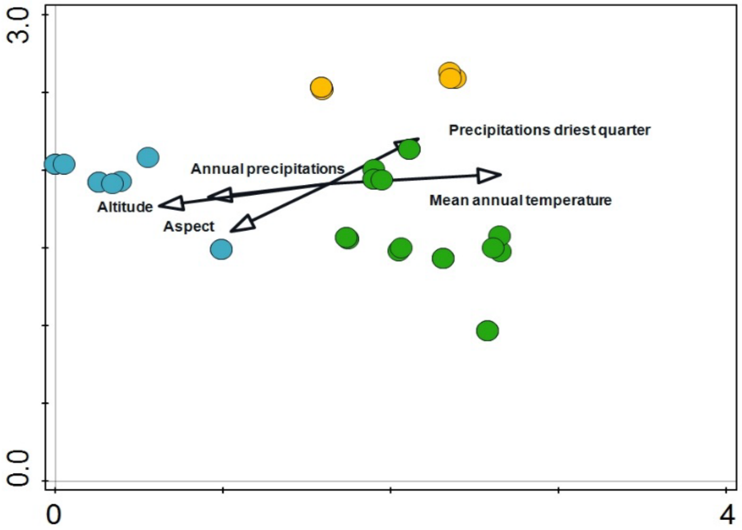

Figure 4. DCA ordination diagram of the 40 investigated mycological plots (only first two axes are presented). Explained variation: 44.1%. Eigenvalues: Axis 1 – 0.412, Axis 2 – 0.223. Fungal assemblages resulted from hierarchical clustering: cluster 1 – blue circles, cluster 2 – yellow circles, cluster 3 – green circles.

Conclusions

The communities of wood-inhabiting fungi were separated into three main groups: one of mixed broad-leaved - coniferous forests in higher areas, a group typical to mesothermic and mesophilous forests from hilly to sub-montane areas and another group specific to more thermophilic *Fagus* sp. forests in lower areas. Average annual precipitations and mean annual temperatures (an underlying effect of altitude) were the main factors influencing their composition. These climatic characteristics induce regional differences between sample plots having as a result different tree composition of forests and consequently, different fungal assemblages. Different tree species in the forest stands provide various quantities of dead wood, with different physical and chemical properties, which are colonized by different fungal species. A greater fungal diversity was recorded in protected areas (forest reserves) than in those with strong anthropogenic influences, mainly due to the amount of the available dead wood.

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Research Article

EVALUATION OF ANTIOXIDANT ACTIVITY IN OAK LEAVES FROM THE REPUBLIC OF MOLDOVA EXPOSED TO HEAT STRESS

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Abstract: The total activity of antioxidant substances, oxidases, and catalases was evaluated in the leaves of three oak species – pedunculate oak (*Quercus robur* L.), sessile oak (*Q. petraea* Liebl.), and downy oak (*Q. pubescens* Willd.) from the Republic of Moldova, subjected to thermal shock at 50 °C for durations of 20, 40, and 60 minutes. The study aimed to identify variations as well as common and divergent responses of these species according to ecological zone, thermal shock duration, and recovery period, in order to assess their thermotolerance and specific adaptations to environmental conditions. The results showed that during the recovery period, leaves of pedunculate oak and downy oak from the center and southern regions, as well as sessile oak from the center region, exhibited high total antioxidant activity. Additionally, pedunculate oak and sessile oak from the center and southern regions displayed increased oxidase activity, while sessile oak from the center region demonstrated high catalase activity, indicating the activation of adaptive processes to thermal shock. Conversely, pedunculate oak and sessile oak from the northern region exhibited a decrease in total antioxidant and oxidase activity during the recovery period, suggesting an increase in leaf thermotolerance. Moreover, the downy oak from the northern region demonstrated an increase in thermotolerance, reflected in the activity of all types of antioxidant substances after extended recovery periods. The study demonstrated that the investigated oak species enhance their thermotolerance in the northern region by developing specific adaptive strategies to thermal shock. These oak species adjust their antioxidant and enzymatic activity in accordance with the specific environmental conditions of the ecological zone in which they grow.

Keywords: *Quercus robur* L., *Q. petraea* Lielb., *Q. pubescens* Willd., leaves, thermal shock, recovery, adaptation, thermotolerance.

Introduction

Currently, extreme temperatures during the summer, along with prolonged heat waves, have expanded their impact to approximately 10% of the Earth's surface, compared to just 1% recorded in the 1960s [HANSEN & al. 2012]. Estimates suggest an increased probability of both the frequency and intensity of heat waves rising in the 21st century [YAO & al. 2013], which has already led to a 0.5 °C increase in the global average temperature [COUMOU & ROBINSON, 2013; COUMOU & al. 2013]. This trend aligns with forecasts indicating that global warming will be partially driven by increasingly frequent and intense high-temperature conditions [COUMOU & ROBINSON, 2013; DULIÈRE & al. 2013].

Moreover, climatic conditions, particularly temperature and photoperiod, significantly influence plant species distribution through direct physiological constraints related to growth and reproduction, as well as indirectly through ecological factors such as competition for resources. Climate change, including temperature variations over the past century, has resulted in significant adverse effects on the geographic distribution, abundance, phenology, and

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physiological state of numerous species [CHEN & HILL, 2011]. Simulation models indicate that climate change can induce extensive modifications in terms of the geographic distribution and survival of species. This underscores the urgent need for comprehensive strategies to mitigate climate change effects on both plant species and broader ecosystems.

Species capable of rapid migration and adaptation to higher altitudes gain survival and competitive advantages, and under specific conditions, they may experience geographic expansion [THOMAS & al. 2004; MENENDEZ & al. 2006]. However, such shifts in species distribution can jeopardize their abundance by fragmenting migration corridors and reducing dispersal distances due to barriers arising from the multifunctional land use. These constraints ultimately contribute to a decline in population numbers [GASTON, 1994].

High temperatures associated with drought can induce metabolic dysfunctions in organisms distributed in a particular area, such as most plants, a phenomenon that can affect multiple physiological processes and trigger stress conditions. This combination of factors can trigger heat stress in plants, initiating the synthesis of antioxidant enzymes and related metabolic pathways to reduce the excessive accumulation of unwanted and harmful reactive oxygen species. Among the most common reactive oxygen species are singlet oxygen ($1O_2$), superoxide radical ($O_2{}^{*-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^*), which are responsible for oxidative stress [ASADA, 2006]. Uncontrolled increase in the concentration of reactive oxygen species can cause cellular damage to plants, including lipid peroxidation, protein damage, and disruption of cellular membranes. These processes can lead to physiological dysfunctions and weaken the viability of plants.

The reaction centers of photosystem I and II in chloroplasts represent primary sources of reactive oxygen species generation, although these can also be generated in other organelles such as peroxisomes and mitochondria [SOLIMAN & al. 2011]. Scientific data indicate that thermal damage to the photosystems under the influence of high temperatures results in reduced photon absorption [HALLIWELL, 2006]. Under stress conditions, when the photon intensity absorbed by photosystem I and II is excessive, this surplus energy is considered as an excess of electrons, thus serving as a source for reactive oxygen species [HALLIWELL, 2006].

Under stress conditions, characterized by high temperatures and drought, plants activate antioxidant mechanisms to mitigate the harmful effects of thermal stress and maintain metabolic homeostasis. These defense mechanisms against oxidative stress are achieved through molecules, including both enzymatic and non-enzymatic components. Key components of this system include enzymes such as superoxide dismutase, peroxidase, catalase, polyphenol oxidase, and alternative oxidase essential in metabolic pathways for the elimination of reactive oxygen species and the control of lipid peroxidation [BOECKX & al. 2015; SAHA & al. 2016]. Increasing the concentration of these enzymes represents an efficient strategy for cell protection against the harmful effects of oxidative stress, thereby ensuring the prevention of excessive accumulation of reactive oxygen species.

By predominantly localizing in mitochondria, the function of alternative oxidase is closely linked to the mitochondrial respiratory chain, playing an essential role in energy generation within cells and providing an alternative pathway for electron transfer in the respiratory chain. Thus, alternative oxidase limits the generation of reactive oxygen species, such as superoxide ($O_2{}^{*-}$) and hydrogen peroxide (H_2O_2). This mechanism contributes to maintaining a redox balance within the cell and protecting it against potential damage caused by reactive oxygen species, thereby regulating oxidative stress in cells [SAHA & al. 2016].

In the forests of the Republic of Moldova, indigenous oak species such as *Quercus robur*, *Q. petraea*, and *Q. pubescens* exhibit sensitivity to stressors generated by high temperatures and heat waves. Their vulnerability is largely due to the fact that, over an extended period, the regeneration of forest stands has been predominantly through shoots, a process that has led to pronounced negative selection. This selection has involved the elimination of valuable trees and stands from populations, both in terms of dendrometric and qualitative aspects. Additionally, these species face excessive habitat fragmentation [CUZA, 2001; DASCALIUC & al. 2005]. Although oak species fulfill multiple essential ecological functions, significantly impacting environmental protection, they also hold considerable economic importance. They provide timber used in furniture production, flooring, wine barrels, and more. However, threats such as prolonged droughts and increasingly frequent heat waves can lead to succession phenomena or alterations in the distribution of indigenous oak species.

Given the current context, studies on the thermotolerance of oak species become highly relevant to anticipate and sustainably manage forests in the future. In this regard, conducting in-depth research to assess the adaptive capacity of oak species to climate change and to develop effective strategies for conservation and sustainable forest management is essential. Additionally, it is important to mention that in the Republic of Moldova, there is an ongoing campaign to expand the forest area through afforestation. In this context, the proper use of species, considering the habitat's specificity, is a particular concern within the efforts for the conservation and sustainable development of forest resources.

Material and methods

Leaf samples for laboratory experiments were collected in July, with fifteen specimens taken from the lower and southern sections of the tree crowns for each species and provenance. The selected species included pedunculate oak (*Quercus robur*), sessile oak (*Q. petraea*), and downy oak (*Q. pubescens*). These trees are located within the Filimon Carcea Forestry District (northern region), Cociulia Forestry District (southern region), and the “Plaiul Fagului” Scientific Reserve (central region).

In the laboratory, leaves from each species and location were subjected to heat shock at 50 °C for intervals of 20, 40, and 60 minutes. Following the heat shock treatment, the leaves were transferred to desiccators, where they were maintained under controlled conditions: a temperature of 25 °C, relative humidity of 85%, illumination of 20 lux, and a photoperiod of 16 hours of light and 8 hours of darkness.

The antioxidant activity in the leaves was assessed using the method described by DASCALIUC & al. (2018), as detailed below. On days 1, 3, and 5 after the heat shock treatment, leaf samples were taken from the desiccators for biochemical analysis to determine total oxidase activity. Leaf samples, weighing 0.1 g each, were cold-macerated, and the resulting material was extracted for 30 minutes at 25 °C in a 0.2 M Tris buffer solution (pH 7). The extract was then centrifuged for 15 minutes at 4000 g.

To determine the total capacity for reducing free oxygen, reflecting both the direct reduction potential of antioxidant substances and enzymatic activity, 40 µl of the supernatant obtained after centrifugation was added to 1.6 ml of buffer solution to ensure a stable testing environment. The mixture was then incubated at 25 °C.

In all experiments, the dynamics of oxygen content reduction in the experimental solutions were measured using a YSI oximeter (USA), with a control solution (containing only 1.6 ml of buffer solution) used for comparison. After 15 minutes of incubation at 25 °C, the oxygen content in the experimental solution reached a stationary phase, at which point the rate of oxygen consumption equaled the rate of its diffusion into the solution.

The difference in oxygen percentage between the control solution (without extract) and the experimental solution (with extract) was determined based on the capacity of antioxidants and oxidases to bind oxygen. Consequently, the activity of antioxidants and oxidases led to a reduction in oxygen content in the solution during the stationary phase, compared to the control variant.

To determine catalase activity, 40 µl of the solution was added to 1.46 ml of buffer solution, along with 60 µl of 0.05% H_2O_2, followed by incubation at 25 °C. The dynamics of oxygen content change were influenced by the activity of oxygen removal through the degradation of hydrogen peroxide by catalases, as well as the activity of oxygen binding by oxidases. The combined outcome of these processes was evaluated based on the oxygen percentage in the solution at the stationary phase. Thus, using the oximetry method, the activities of oxidases and catalases were determined separately, along with the combined effect of these enzymatic activities.

To illustrate the specificity of processes within certain antioxidant components, the graphs in Figures 1-3 depict the oxygen utilization activity by antioxidants and oxidases as negative values, while the oxygen removal activity by catalases is represented by positive values. The studies were conducted in three replicates, with the mean value and standard deviation of the mean determined [GIURGIU, 1972].

Results

Variability in the activity of antioxidant substances, oxidases, and catalases in the leaves of oak species

1. Activity of antioxidant substances, oxidases, and catalases in pedunculate oak (*Quercus robur*) leaves

The data presented in Figures 1a, 1b, and 1c indicate that, over a 5-day recovery period following heat shock, the oxygen-binding processes in the extracts from pedunculate oak leaves exhibited distinct processes. Specifically, the activity of antioxidant substances and oxidases differed from that of catalases in terms of oxygen removal. These variations highlight the differential responses of these biochemical components to heat stress.

In the northern region, the overall activity of antioxidant substances and oxidases was comparable and elevated on the first day following thermal shock, with an increase observed as the treatment period extended. This activity was significantly higher than that in the control sample, indicating a strong response of the oak trees to thermal stress. Additionally, catalase activity in the leaf extracts was high, surpassing that of antioxidant substances and oxidases. However, despite the duration of thermal shock exposure, catalase activity remained lower than in the control sample. This suggests that recovery processes are more advanced for catalases, while antioxidant substances and oxidases continue to exhibit increased activity compared to the control.

After three and five days of thermal shock, a trend towards reduced activity of antioxidant substances and oxidases was observed, with levels reaching those comparable to the control sample and lower than on the first day post-shock. In contrast, catalase activity increased somewhat after 20 and 40 minutes of shock, and after five days, it was similar to that of the control sample.

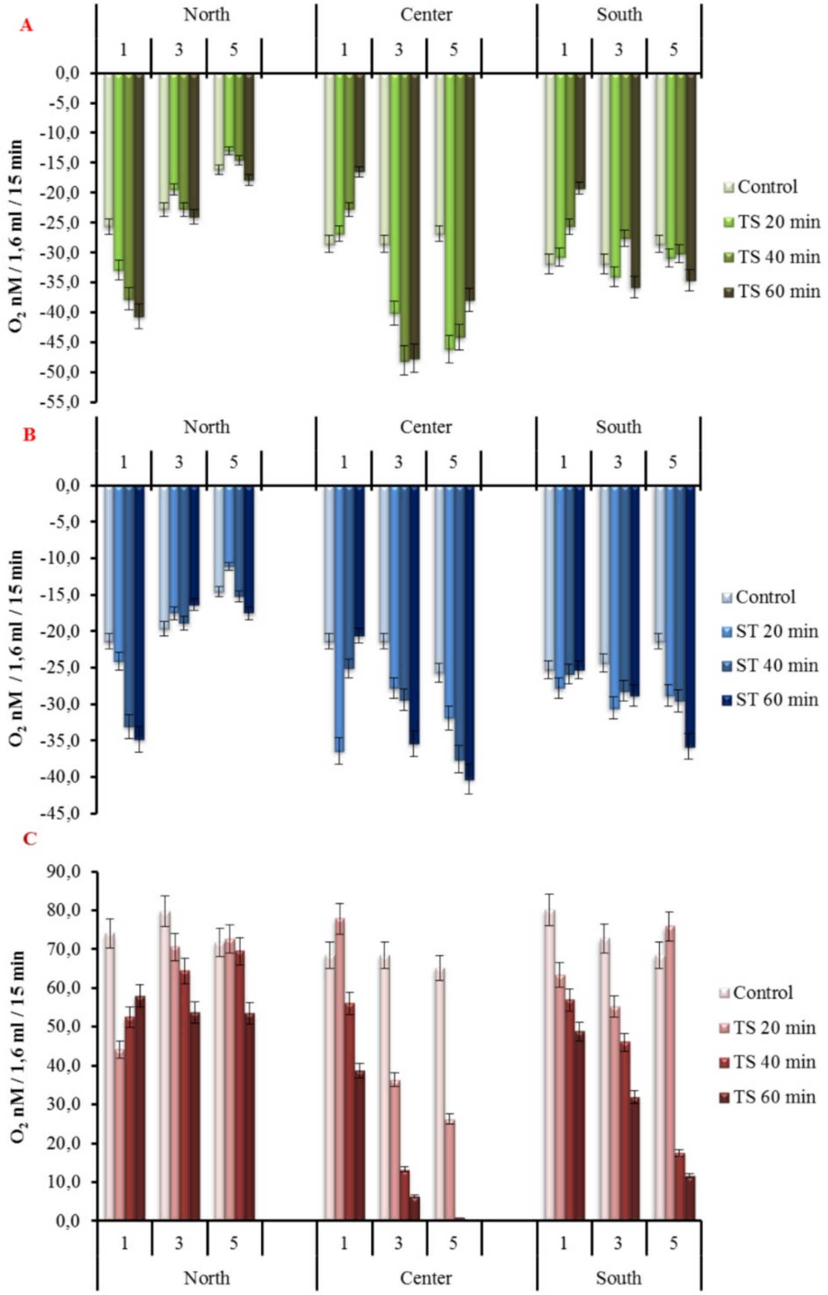

Figure 1. Dynamics of changes in the activity of antioxidant substances (A), oxidases (B), and catalases (C) in extracts from the leaves of pedunculate oak, exposed to thermal shock at 50 °C for 20, 40, and 60 minutes. 1, 3, 5 – recovery of antioxidant components' activity on the first, third, and fifth day after the application of thermal shock

In the center region, the analysis of the overall activity of antioxidant substances, oxidases, and catalases in leaf extracts of pedunculate oak trees reveals a clear trend of reduction following the first day of thermal shock. This trend is observed in conjunction with an increase in the duration of thermal shock exposure, with activity levels becoming lower than those in the control sample as the thermal shock duration extends. These results indicate that exposure to thermal shock induces recovery processes among antioxidant substances. On the third and fifth days post-exposure, there was an increase in the overall activity of antioxidant substances and oxidases, while the control sample activity was significantly lower. This suggests adaptive processes in the trees, activating antioxidant defense mechanisms to mitigate the effects of thermal shock. In contrast, catalase activity showed a different trend, significantly decreasing throughout the post-shock period with prolonged exposure to thermal shock. These changes suggest that the enzymatic system of pedunculate oak trees undergoes complex alterations in response to thermal stress, with catalase activity potentially being affected differently compared to other antioxidant enzymes.

In the southern region, a high and relatively constant activity of antioxidant substances and oxidases was observed, indicating that the antioxidant mechanisms of oak trees in this area are active and effective in managing thermal stress. Additionally, the analysis of overall antioxidant activity and oxidase levels during the recovery period, specifically after three and five days, revealed an increase compared to the control leaf extracts. This suggests that exposure to thermal shock stimulates the activity of these antioxidant substances in response to the stress, demonstrating the trees' ability to mobilize their defense mechanisms to mitigate the negative effects of thermal stress. In contrast, catalase activity decreased with the extension of the recovery period following exposure to thermal shock, indicating a specific regulation of these enzymes in the trees' response to thermal stress in the southern region. Pedunculate oak trees in this area appear to be well-adapted to thermal stress, possessing efficient antioxidant mechanisms to cope with it, along with a specific regulation of catalase activity during the recovery period.

2. The activity of antioxidant substances, oxidases and catalases in the leaves of the sessile oak (*Quercus petraea*)

Figures 2a, 2b, and 2c provide a detailed illustration of changes in the activity of antioxidant substances, oxidases, and catalases in the leaves of sessile oak trees under thermal shock in three distinct regions: north, center, and south.

In the northern region, the overall activity of antioxidant substances and oxidases extracted from leaves one day after thermal shock is moderate. However, a significant increase is observed with extended thermal exposure. This increase clearly surpasses the corresponding activity levels in the control leaf extracts (Figures 2a and 2b). These processes suggest that the response of sessile oak leaves to thermal shock initiates the trees' protective mechanisms to alleviate the generated oxidative stress. After three and five days of recovery post-thermal shock, the overall activity of antioxidant substances and oxidases decreased significantly compared to the first day post-shock. Simultaneously, their activity remained at levels comparable to those of the control samples. These observations indicate that the antioxidant system of the trees may experience a significant reduction in efficiency as the recovery time progresses. Nevertheless, the maintenance of activity at the level of the control samples could also suggest the plants' capacity to activate compensatory mechanisms to mitigate the negative effects of thermal stress and maintain internal homeostasis.

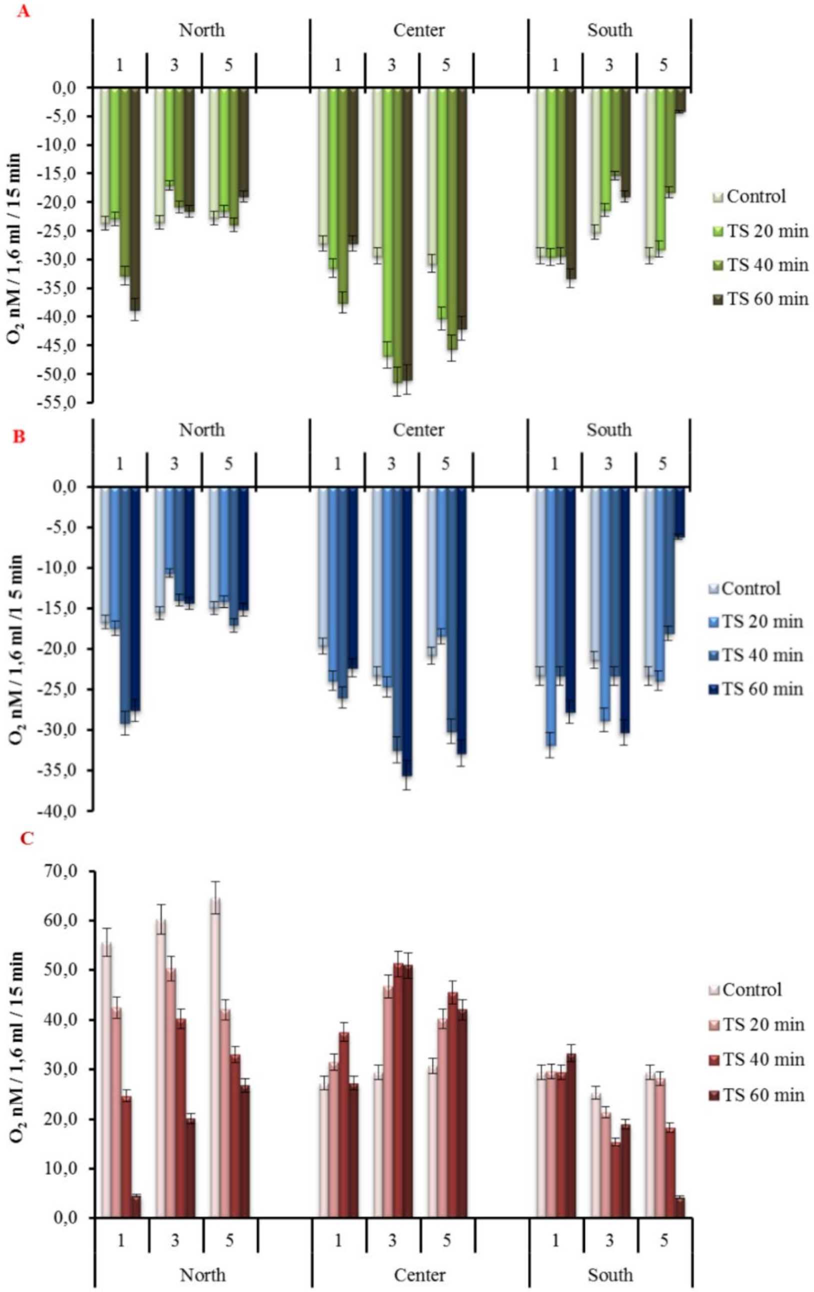

Figure 2. Dynamics of changes in the activity of antioxidant substances (A), oxidases (B), and catalases (C) in extracts from the leaves of sessile oak, exposed to thermal shock at 50 °C for 20, 40, and 60 minutes. 1, 3, 5 – recovery of antioxidant components' activity on the first, third, and fifth day following thermal shock application

In contrast, catalase activity exhibits an opposing trend over the five-day recovery period, showing a progressive decrease as the duration of thermal shock exposure increases, remaining significantly lower than in the control sample. This suggests that catalase activity may have adverse consequences as the post-shock recovery time advances.

In the center region, antioxidant substances and oxidases show moderate activity on the first day after thermal shock exposure, with antioxidant substances exhibiting a slightly higher level. Figures 2a and 2b clearly illustrate that, in both cases, the activity of these antioxidant components exceeds the levels observed in the control leaf extracts, indicating that their activity is directed towards strengthening protective mechanisms and controlling reactive oxygen species.

However, the overall activity of antioxidant substances and oxidases notably increases after three and five days of thermal shock exposure, significantly surpassing the control sample. The same trend is evident for catalase activity. The observed phenomenon demonstrates that, during an extended recovery period, sessile oak trees exhibit an adaptive response, highlighting protective mechanisms that include metabolic and enzymatic processes of antioxidation and detoxification, which intervene to mitigate the negative effects of thermal stress.

In the southern region, on the first day post-shock, the activity of antioxidant substances, oxidases, and catalases remains at a moderate level. However, the overall activity of antioxidant substances and catalases generally shows levels similar to those in the control sample, while the activity of oxidases in the thermal shock-exposed leaf extracts is noticeably higher compared to the control. As the recovery period and thermal dose increase, all antioxidant compounds show a trend towards decreased activity, with levels being lower five days after the shock compared to the control sample. This suggests either a “fatigue” of the antioxidant system or a reduction in the processes generating reactive oxygen species, indicating an improvement in the physiological state of the sessile oak trees.

The results indicate significant variations in the enzymatic and non-enzymatic responses of sessile oak leaves to thermal shock, suggesting that the impact of this thermal perturbation depends on geographic location.

3. The activity of antioxidant substances, oxidases and catalases in the leaves of downy oak (*Quercus pubescens*)

The data presented in Figures 3a, 3b, and 3c reflect the dynamics of changes in the overall activity of antioxidant substances, oxidases, and catalases in the leaves of downy oak across the three ecological regions, five days after exposure to thermal shock.

In the northern region, there is a significant increase in the overall activity of antioxidant substances and oxidases on the first day following thermal shock exposure compared to the control sample. As the duration of thermal shock exposure increases, the activity of these antioxidant substances appears to be positively influenced, continuing to rise with extended exposure of the leaves. This reaction indicates a strong response of downy oak trees to thermal shock.

In contrast, catalases activity decreases as the duration of thermal shock extends, reaching minimal levels after five days of recovery, particularly following exposure periods of 40 and 60 minutes. This phenomenon suggests a specific adaptation of downy oak trees to thermal stress, where the sensitivity of catalases plays a significant role.

The analysis of histograms presented in Figures 3a and 3b demonstrates that the overall activity of antioxidant substances and oxidases during a recovery period of three and five days shows a significant decrease compared to the first day post-thermal shock. These changes are influenced by both the duration of thermal shock and the recovery period of the plants, reflecting their regulatory capacity in response to thermal stress. This phenomenon highlights the ability of downy oak, classified as a heliophyte and thermophile species, to adjust the activity levels of antioxidant substances and oxidases during the post-stress recovery process in accordance with the ecological requirements of the trees.

In the center region, the overall activity of antioxidant substances and oxidases remained consistently high on the first and third days post-thermal shock, including in the control sample. A noticeable decrease began after five days of exposure to extended doses, particularly between 40 and 60 minutes. The specific activity of these antioxidant substances, maintained at high and relatively stable levels in the initial days post-stress, indicates their adjustment through protective mechanisms to control reactive oxygen species. Furthermore, the reduction in antioxidant activity after five days of recovery from prolonged thermal doses suggests a metabolic and biochemical stabilization of downy oak trees, indicating their adaptation to thermal shock conditions.

In contrast, catalase activity exhibited a significant reduction on the first, third, and fifth days of recovery, showing a decreasing trend from short thermal doses (20 minutes) to extended thermal doses (60 minutes). Throughout the recovery period, catalase activity remained at lower levels compared to the control sample (Figure 3b).

The significant decrease in catalase activity during the recovery period, especially after exposure to prolonged thermal doses, suggests a specific adaptation of the plants to thermal stress. This adaptation may involve the regulation of gene expression and metabolism to mitigate the negative effects of thermal shock on cellular function and to maintain homeostasis under post-shock conditions.

In the southern region, the overall activity of antioxidant substances remained high during the first three days of recovery post-shock, with values exceeding those of the control sample. This indicates a strong response of these components in the leaf extracts to thermal shock. On the fifth day, a decline in the overall activity of antioxidant substances was observed, with this decrease becoming more pronounced with the extension of the shock duration. Regarding oxidase activity, it was elevated on the first day but somewhat diminished by the third day of recovery. However, on the fifth day post-shock, a marked decrease in activity was noted, comparable to the control sample (Figure 3c).

Regarding catalases, activity demonstrated significant increases after the first day of shock with extended exposure durations, reaching high levels. However, by the third and fifth days of recovery, there was a notable decrease in catalase activity, particularly after prolonged thermal doses.

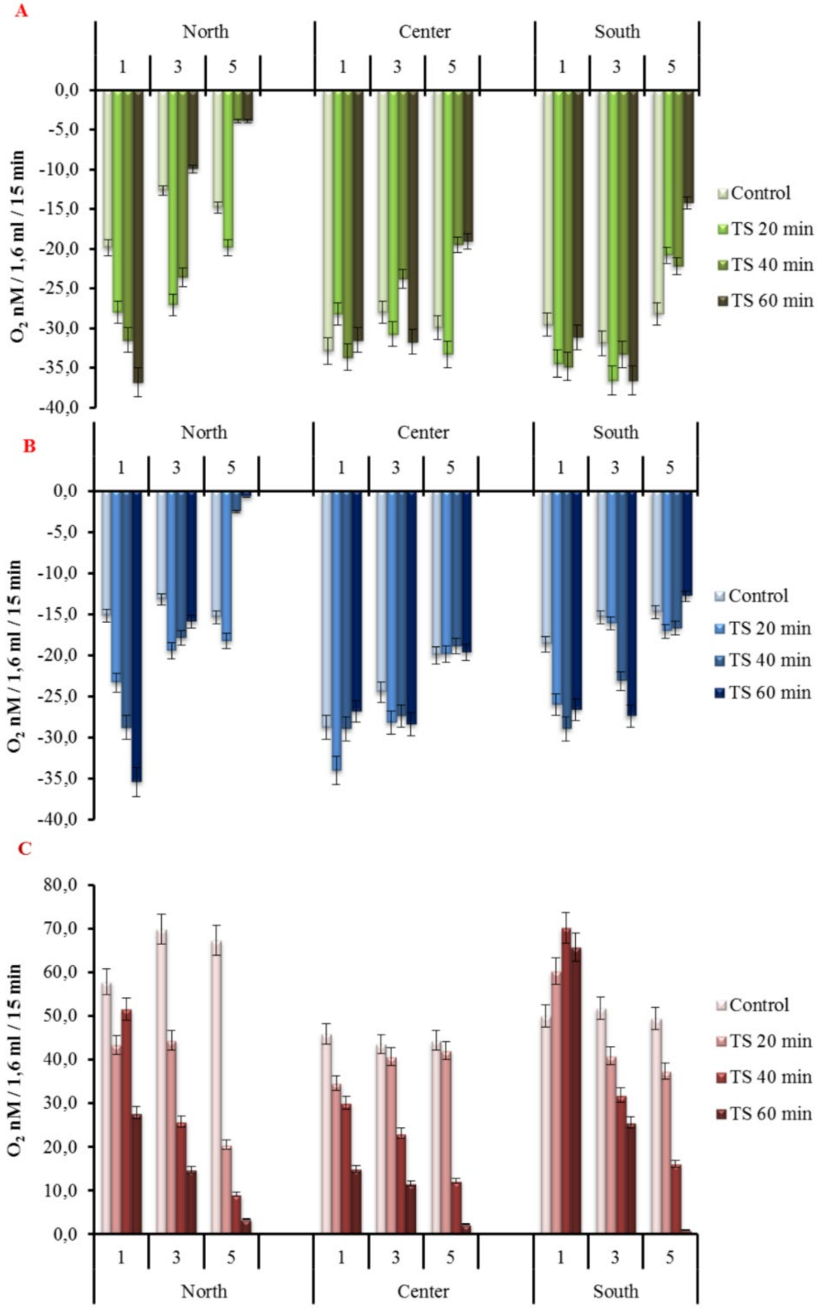

Figure 3. Dynamics of changes in the activity of antioxidant substances (A), oxidases (B), and catalases (C) in extracts from the leaves of downy oak, exposed to thermal shock at 50 °C for durations of 20, 40, and 60 minutes. Days 1, 3, and 5 – recovery of antioxidant components' activity on the first, third, and fifth day after thermal shock application

Discussions

In recent decades, the health and survival of oak species have been alarmingly threatened, with oak dieback observed in forests worldwide [ALLEN & al. 2010; GENTILESCA & al. 2017]. Researchers believe that the decline and dieback of oak forests are caused by a range of factors, with climate change, prolonged droughts, and defoliating insects significantly contributing to this phenomenon [ROMAGNOLI & al. 2018]. Some studies suggest that tree species, including oaks, growing in regions with high precipitation are less resilient to high temperatures compared to those in arid regions characterized by prolonged droughts [HODGSON & al. 2015].

In our study, we compared three indigenous oak species growing in different ecological zones of Moldova, each characterized by distinct temperature and precipitation regimes. Oak leaves were subjected to thermal shock for varying durations at a constant temperature to elucidate their physiological responses, based on the overall activity of antioxidant substances, oxidases, and catalases. Measurements were conducted on leaf extracts at different recovery time points. We hypothesized that populations of these species develop optimal adaptations in specific habitats, where changing environmental factors positively influence their genotypes.

Our results highlight a robust response of pedunculate oak trees from the northern region to thermal shock, as evidenced by a significant increase in the overall activity of antioxidants and oxidases in leaf extracts on the first day post-shock. Interestingly, elevated levels of these substances are also observed in the leaves of oak trees from the center and southern regions, indicating a similar adaptation to thermal stress. These findings align with previous research demonstrating that plants can respond to prolonged thermal stress by increasing antioxidant activity [PINTÓ-MARIJUAN & al. 2013; ONO & al. 2021; NETSHIMBUPFE & al. 2023].

Additionally, we observed enhanced catalase activity on the first day after thermal shock in the leaf extracts of pedunculate oaks. However, following prolonged exposure to thermal shock, there is a significant decrease in catalase activity in oaks from the center and southern ecological zones of the republic. This observation suggests either a direct inhibition of catalase activity by high temperatures over an extended period or a reallocation of cellular resources toward alternative hydrogen peroxide detoxification pathways that may be more effective under prolonged thermal stress.

In a broader context, research on the plant *Arabidopsis thaliana* has revealed that prolonged thermal stress can lead to a significant accumulation of hydrogen peroxide in the plant leaves, with catalase activity being induced as a response to this stress. However, this adaptation does not appear to be sufficient to significantly enhance plant heat tolerance under the specific conditions of the study [ONO & al. 2021].

It is evident that the overall activity of oxidases in pedunculate oak leaf extracts, following thermal shock, is higher under conditions of effective recovery. This observation is supported by previous studies indicating that oxidase activity provides essential energy sources for cellular functions and helps to regulate reactive oxygen species within the mitochondrial electron transport chain during stress [FINNEGAN & al. 2004]. Thus, our study's results suggest a relationship between enzymatic activity and the efficiency of cellular recovery processes, providing a coherent explanation for the observed phenomena.

Analysis of the response of sessile oak to thermal stress reveals significant variations in the overall activity of antioxidant substances and oxidases in leaf extracts, influenced by specific ecological zones. In the center and southern regions, the activity of oxidases in sessile oak leaf extracts was elevated, surpassing the levels observed in control samples, and showed similar trends to those observed in pedunculate oak. This may reflect common adaptive responses of these oak species to increased oxidative stress levels.

In the southern region, a trend towards decreased overall activity of antioxidant substances and oxidases was observed in sessile oak leaf extracts, particularly after five days of recovery following extended thermal shock exposure. Catalase activity demonstrated a clear decline in the northern and southern regions following prolonged thermal exposure, whereas in the control samples, activity remained high, highlighting the recovery processes. The enhancement of these recovery processes in catalase activity appears to contribute to increased thermotolerance of leaves over time in these regions.

It is well-established that a plant's resistance to a stress factor depends on its ability to activate tolerance mechanisms and the presence of adaptive traits for avoidance, which are directly related to its habitat [LARCHER, 2004]. Specialized studies indicate that high temperatures can induce harmful effects on plants, including cellular membrane damage, protein inactivation, excessive production of reactive oxygen species, and disruption of key metabolic functions [RUELLAND & ZACHOWSKI, 2010]. Despite these investigations, recent research, such as that by ONO & al. (2021), has emphasized the importance of studying the specific plant responses to various thermal stress conditions, including the impact of thermal shock duration, whether short or prolonged.

In our studies on sessile oak, a species of both ecological and economic interest, we observed that prolonged exposure to thermal shock leads to a decrease in the activity of antioxidant substances and catalases. This variation in the metabolic and enzymatic response of sessile oak under different environmental conditions suggests the presence of complex and adaptive stress protection systems that are differentially activated depending on the duration of thermal shock and the recovery period.

In this context, the research conducted by WANG & al. (2023) on *Brassica campestris* is particularly relevant. Their study demonstrated that after exposure to thermal stress, there is a transiently induced expression of the BcWRKY22 gene, leading to the activation and significant increase in catalase activity. Concurrently, under thermal stress conditions, the expression of this gene showed the capability to reduce hydrogen peroxide accumulation. These findings highlight the crucial role of BcWRKY22 in modulating the response to thermal stress, positively impacting catalase activity and mitigating H_2O_2 accumulation through gene regulation. These results support our observations and suggest that the antioxidant protection mechanisms in sessile oak may be similar to those in other species, such as *Brassica campestris*, indicating a common adaptive strategy to thermal stress.

Another study conducted by SAHA & al. (2016) demonstrated that stress tolerance, through the attenuation and neutralization of reactive oxygen species, is mediated by the activity of the antioxidant enzyme alternative oxidase. This enzyme plays a crucial role in protecting leaves from oxidative damage through specific metabolic pathways, thereby contributing to the adaptation and survival of plants under abiotic stress conditions.

Our results highlight significant variability in the total activity of antioxidant substances, oxidases, and catalases, influenced by the trees' location, the duration of leaf exposure to thermal shock, and the recovery period. These findings underscore the complexity of protective mechanisms against thermal stress in sessile oak. Our observations are supported by similar studies conducted on other species [ONO & al. 2021; WANG & al. 2023], suggesting the existence of common adaptive processes in plant responses to thermal stress.

The dynamic analysis of the total activity of antioxidant substances and oxidases in the leaves of downy oak reveals significant aspects related to thermal stress adaptation across different ecological zones. In the northern zone, there is a notable increase in enzymatic activity on the first day of recovery, correlated with the intensity of the thermal shock. However, after three and five days of recovery, the activity of antioxidant substances progressively decreased. Conversely, in the center zone, there was an initially high activity of antioxidant substances and oxidases, but catalase activity diminished with prolonged exposure to thermal shock. In the southern zone, the activity of antioxidant substances, oxidases, and catalases declined after five days of recovery. These variations in enzymatic activity suggest that adaptive processes to thermal stress differ depending on the ecological zone and the duration of the recovery period.

The study conducted by DINAKAR & al. (2016) on *Pisum sativum* highlighted the importance of the alternative oxidase pathway in optimizing the photosynthetic process under thermal stress conditions in the mesophyll protoplasts of leaves. The capacity of the alternative oxidase pathway significantly increased with elevated intracellular levels of reactive oxygen species under suboptimal temperature and saturating light conditions. The authors demonstrated the crucial role of the alternative oxidase pathway in regulating reactive oxygen species and the antioxidant system's response to thermal stress.

When comparing our results with those from the *Pisum sativum* study, we observe that both emphasize the significance of oxidase activity, including the alternative oxidase pathway, in responding to thermal stress. While our study focused on how thermotolerance in downy oak leaves varies depending on the recovery period following exposure to thermal stress, the research on pea plants evaluated the role of the alternative oxidase pathway in optimizing photosynthetic activity. This difference in approach underscores the distinct responses of the two species to thermal stress, highlighting the enhancement of thermotolerance in downy oak and the optimization of photosynthetic activity in pea plants.

The findings presented here illustrate that the overall activity of antioxidant substances, oxidases, and catalases varies specifically depending on the oak species studied, taking into account the origin of the trees, the intensity of thermal shock, and the recovery period. The specific variation in the activity of oxidases and catalases in these oak species can be used as an indicator of recovery processes, reflecting the metabolic transformations involving these enzymes. An increase in the activity of these enzymes in the cells of the oak species studied suggests an efficient adaptive response to thermal stress and the species' ability to detoxify reactive oxygen species. Conversely, a decrease in activity might indicate either a reduction in thermal stress or a diminished need for an antioxidant response, or a deficiency in the antioxidant capacity, signaling potential oxidative damage at the cellular level. Thus, our study contributes to the understanding of adaptive mechanisms to thermal stress under various ecological conditions, highlighting the role of both antioxidant substances and oxidases and catalases in these adaptive processes.

When evaluating plant resistance, studies have reported that the activity of antioxidant enzymes increases under thermal stress to neutralize the reactive oxygen species generated (DAS & al. 2014; SEWELAM & al. 2016). These findings suggest that adaptation to thermal stress involves finely tuned regulation of both enzymatic and non-enzymatic activities to mitigate the negative effects of reactive oxygen species and maintain cellular homeostasis. Additionally, it has been observed that low activity of these enzymes may indicate two distinct scenarios:

1. **Low Stress levels:** If the thermal stress is reduced or the plant has effectively adapted and stabilized the reactive oxygen species, the antioxidant enzymatic activity decreases as the demand for neutralization diminishes.
2. **Inability to respond:** When enzymatic activity remains low despite the presence of thermal stress, it could indicate an insufficiency or “fatigue” in the plant's antioxidant response.

Building on previous discussions, it has been observed that pedunculate oak trees from center and southern regions exhibit an enhanced adaptive response to thermal stress, mirroring the reactions noted in sessile oak trees from the same areas. The comprehensive assessment of all antioxidant substances in downy oak revealed a decline in their activity in the leaves of trees from northern regions following a prolonged recovery period, particularly after five days. A similar trend was noted for catalase activity in trees from the central region. These findings suggest that cellular homeostasis in downy oak is reestablished after extended recovery periods following thermal stress. Therefore, both enzymatic and non-enzymatic adaptations allow oak species to maintain a balance between the production of reactive oxygen species and their detoxification capacity, thereby optimizing their function and survival under stress conditions.

Conclusions

This study assessed the total activity of antioxidant substances, oxidases, and catalases in the leaves of three oak species (*Quercus robur*, *Q. petraea*, and *Q. pubescens*) subjected to thermal shock, followed by a five-day recovery period. The findings revealed distinct variations as well as common responses in antioxidant activity, influenced by the ecological origin of the oak stands in the Republic of Moldova.

Leaves of pedunculate oak and downy oak from center and southern regions, as well as sessile oak from the center region, exhibited consistently high total antioxidant activity throughout the recovery period. Additionally, increased oxidase activity was observed in pedunculate oak and sessile oak from the center and southern regions, and in downy oak from the center region. Moreover, high catalase activity was evident in the leaves of sessile oak from the central region. These specific antioxidant responses in particular ecological zones suggest an enhanced adaptive capacity of these trees to thermal shock.

Conversely, pedunculate oak and sessile oak trees from the northern region showed a decrease in total antioxidant and oxidase activity during the recovery period, indicating an increase in leaf thermotolerance. Similar responses were observed across all types of antioxidant substances in pubescent oak from the northern region, indicating an enhancement of leaf thermotolerance after extended recovery periods.

Overall, the results indicate that leaf thermotolerance in the investigated oak species varies and exhibits specific changes depending on environmental conditions. These adaptations reflect distinct responses of oak stands to thermal stress, highlighting regional variations in the physiological responses of oak species.

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Research Article

SHADE-INDUCED PHENOTYPIC PLASTICITY AND ITS IMPACT ON THE ECONOMIC TRAITS OF PATCHOULI, *POGOSTEMON CABLIN* (BLANCO) BENTH., A HIGH-VALUE AROMATIC CROP

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Abstract: The study was conducted to assess the patchouli crop responses to varying light intensities: full-sunlight (open-condition), 30% shade, and 50% shade. It showed notable variations in growth, development, essential oil yield, and chemical composition between full-sunlight and partial-shade conditions. Plants grown in 50% shade exhibited significant increases in height (113.83 cm), inter-nodal length (8.81 cm), leaf area (59.17 cm^2), petiole length (3.78 cm), and width (3.44 mm) compared to open conditions. However, reductions in branches/plant (14.26), fresh weight (152.95 g), dry weight (29.64 g), leaf thickness (117.57 µm), number of trichomes (13.14 no./mm^2), number of oil glands (10.14 no./mm^2), and oil content (0.98%) were observed in 50% shade plants compared to open conditions. Anatomical modifications like bundle sheath extensions (BSEs) were present in 30% and 50% of shade-grown plants but absent in open-condition-grown plants.

Keywords: bundle sheath extensions (BSEs), oil glands, patchouli, phenotypic plasticity, shade stress.

Introduction

Shade affects a variety of developmental, physiological, and phenological traits in many plants. Increased shoot length via inter-node elongation, petiole growth, leaf position alteration, increased apical dominance, decreased tillering or branching, earlier flowering and a higher leaf area to biomass ratio [EVANS & POORTER, 2001]. The shade avoidance syndrome is defined by these characteristic alterations taken together. The physiological and developmental mechanisms behind this syndrome, which involve photoreceptors and phytohormones, are thoroughly described by [BALLARÉ & PIERIK, 2017]. A common feature of sun–shade adaptation changes in leaf anatomy. Sun leaves are smaller, have more leaf mass per area, are thicker, and have a larger palisade/spongy parenchyma ratio than shade leaves, and also have a higher stomatal density [TERASHIMA & al. 2006]. On the other hand, their stomata are noticeably smaller than those of shade leaves [BRESINSKY & al. 2008]. Besides these morpho-anatomical differences, sun and shade leaves have significant physiological characteristics. The photosynthetic light saturation rate is much higher in sun leaves than in shade leaves, as is the light compensation point, light saturation irradiance, and chlorophyll a/b ratio [MENDES & al. 2001]. Shade leaves, on the other hand, have more chlorophyll content per leaf dry mass and area, as well as a larger nitrogen allocation to light-harvesting complexes [NIINEMETS, 2010]. Furthermore, photoprotection based on non-photochemical quenching

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(NPQ), which relies on the violaxanthin cycle and the plastidic protein PsbS, is significantly enhanced in sun leaves [NIYOGI & TRUONG, 2013]. Sun and shade leaves differ in size, shape, and number of chloroplasts, in addition to structural morpho-anatomical and physiological characteristics. The size of the chloroplasts in sun leaves is smaller, and the thylakoid/grana ratio is lower than in shade leaves [BRESINSKY & al. 2008]. On the other hand, bundle sheath extensions (BSEs) confer plasticity in leaf structure and function in response to low irradiance levels and may act as a hub connecting leaf structure, photosynthetic performance, and water supply and demand [BARBOSA & al. 2018].

Patchouli (*Pogostemon cablin* (Blanco) Benth.) is an aromatic herbaceous plant that belongs to the Lamiaceae family. Because of its strong fixative characteristics, its oil is employed as a “base” substance in the fragrance industry. It is utilized as a flavoring agent in a variety of foods and also has a variety of medical benefits. It is an effective under-crop in areca nut and coconut orchards. It could also be accomplished by planting appropriate shade trees. The plant prefers partial shade but can also be grown in full-sun conditions. In horticultural crops, phenotypic plasticity can be used to boost productivity [LANDE, 2009], and it is an important concern for breeders grappling with the genetic association between important morphological attributes [WAITT & VLEVIN, 1998]. In this context, the present detailed investigation was undertaken into shade-induced morphological and anatomical plasticity in patchouli concerning economic traits.

Material and methods

Study area, methodology, and plant material

The present study was conducted at the CSIR-CIMAP Research Centre in Bengaluru with three different light intensity treatments: full sunlight (50000 lux), 30% shade (35000 lux), and 50% shade (25000 lux). The sunlight intensity was measured under the canopy of the Jack fruit tree via a handheld Lux meter (LX-102); the respective PAR values are 925 µmol $s^{-1}m^{-2}$, 647.5 µmol $s^{-1}m^{-2}$, and 462.5 µmol $s^{-1}m^{-2}$, respectively. Before plantations, the selected experimental area was confirmed for constant light intensity under the trees. The patchouli cultivar CIM-Shrestha was selected for the present study and was grown under field conditions at an optimum temperature of 32±3 °C. The treatments were replicated eight times in a randomized complete block design (RCBD), and recommended doses of fertilizer and cultural practices were adopted.

Morphological, growth, and yield parameters

Plant growth and yield parameters were taken at the harvest time (after five months of plantation). The leaf and stem color of the plants were recorded by visual observations. Plant height (cm), inter-nodal length (cm), and petiole length (cm) were measured using a graduated ruler. Petiole diameter (mm) was measured using a digital caliper rule. The number of branches and leaves was counted. The leaf area was determined by using a leaf area meter (LI-COR 3100C, Nebraska, USA). Chlorophyll content was recorded by the SPAD-502 meter at the time of harvest, and mean values for each treatment were calculated. The infestation of leaf rollers was observed and recorded as the number of larvae per plant.

Anatomical observations

The leaf samples for the anatomical studies were collected fully expanded in the third node from the apical bud. Leaves were cross-sectioned freehand with a razor aid in a manual microtome and mounted with 5% glycerin for leaf thickness measurement. The thickness of the leaf blade and palisade parenchyma was measured in µm at the midrib and then at the lamina, 1 mm to its left and right. Stomatal density (mm^2) and chloroplast number in the guard cells were counted on the abaxial side using the nail polish impression method. Trichomes (non-glandular) and oil glands (glandular) were counted by staining the decolorized leaf segment with 1% Methylene blue for one minute. All the observations were taken under the light microscope (Olympus CX 31) at 40 X magnification. In all the cases, ten readings were taken five times, and the mean was calculated.

Phenotypic plasticity analysis

The phenotypic plasticity index (PI) was calculated separately for the measured plant traits such as growth and yield, leaf anatomy, chlorophyll content, and chemical compounds [GREWELL & al. 2016]. The difference between the maximum and minimum values was divided by the maximum value to produce the index. Higher PI values, which are closer to one, imply that the variable is more plastic [CHEPLICK, 1995]. The mean PI was derived by averaging the plasticity index of individual groups such as growth and yield, leaf anatomy, chlorophyll content, and chemical compounds.

Oil extraction and analysis

To evaluate the essential oil contents, dried samples of patchouli leaves were subjected to hydro distillation (2000 mL capacity flask) for 5-6 hours using a Clevenger-type apparatus. The percentage yield of oil (g/100 g) in each of the samples was calculated concerning the dry weight of the leaves. The extracted oil samples were further subjected to the identification of compounds with GC and GC-MS.

Statistical analysis

The experimental data of all observations were subjected to one-way ANOVA by SPSS (19.0) and the values of least significant differences (LSD) were set at 1% and 5%. The simple correlation coefficient between 11 characters was carried out as per the procedure suggested by SINGH & CHAUDHARY (1995). The principal component analysis (PCA) method explained by HARMAN (1976) was followed in the extraction of the components. PCA and biplot graphical display was performed using R-studio software. A PCA-based selection index was constructed using computed principal components wherein data was normalized and weights were assigned based on the PCs having an Eigenvalue greater than one.

Results

Plants ability to grow effectively is severely hampered by shading stress. For the development of aromatic crops, it is critical to understand how the patchouli plant's morpho-anatomical adaptations are formed in response to reduced light conditions. The effects of shade stress on the growth, development, and yield of the patchouli cultivar CIM-Shrestha were investigated and analyzed. The data are shown in Table 1.

Morphological, growth, and yield traits

Patchouli plants exhibited significant variability in morphological traits under different light intensity regimes that were observed and recorded. The leaf color was dark green and the young shoot was green in color in 30% (Figure 1b) and 50% (Figure 1c) shade grown patchouli, whereas it was green-yellowish in leaf color and purple in young shoots in plants that were grown under open conditions (Figure 1a). Significant increases in shoot length (113.83 cm), inter-node (3.78 cm), and petiole (3.78 cm), as well as leaf area (59.17 cm^2), while a drastic reduction in the number of branches (14.26), leaves (96.62), total fresh weight (152.95 g), dry weight (29.64 g), oil content (0.98%) and oil yield (0.30 g) per plant were recorded in the 50%

shade grown plants compared to open-condition grown patchouli plants. However, there is no significant variation noticed in the patchouli plants between the 30% shade grown and open–condition-grown plants in terms of oil content (1.97% and 2.04%, respectively) and oil yield (1.67 g and 1.89 g, respectively).

Leaf anatomical traits and SPAD chlorophyll content

Significant leaf anatomical plasticity was detected in the plants grown under different light environmental conditions. The leaf thickness was drastically reduced with increasing shade conditions. It was observed in the 50% shade (Figure 1f) grown plants (117.57 μm) followed by 30% shade (Figure 1e) grown plants (216.00 μm) compared to open condition grown (Figure 1d) plants (239.14 μm), while the thickness of palisade parenchyma tissue was also decreased in the patchouli plants grown under 50% shade (26.28 μm) followed by 30% shade (46.21 μm) compared to open condition grown (Figure 1d) plants (56.86 μm). Furthermore, the number of stomata (12.43 no./mm^2), trichomes (13.14 no./mm^2), and oil glands (10.14 no./mm^2) was found to be very low in 50% shade (Figure 1l) grown patchouli plants compared to 30% shade (Figure 1k) grown and open condition (Figure 1j) grown plants, whereas the number of chloroplasts per stomatal guard cell was found to be highest in 50% shade (13.71 no./stomata). Interestingly, major anatomical modifications such as bundle sheath extensions (BSEs) were detected in the leaf petiole of 50% (Figure 1i) and 30% of shade-grown plants (Figure 1h). Moreover, the BSEs were not found in the patchouli plants grown in open condition (Figure 1g). Furthermore, the chlorophyll content was highest in patchouli plants grown in 50% shade (46.96), 30% shade (43.03), and open conditions (40.43).

Chemical composition of patchouli essential oil

The chemical composition was analyzed, and the major chemical constituents of the oil, such as patchouli alcohol (patchoulol), α-Bulnesene (δ-guaiene), and β-Caryophyllene were considered. Of these, the compound β-Caryophyllene was recorded at the highest (3.54%) in 50% shade grown plants, followed by open-condition grown plants (3.13%), compared to 30% shade grown (2.94%) patchouli plants. However, the patchouli alcohol percentage was found to be the lowest (42.44%) in 50% shade-grown plants when compared to open-condition (47.72%) and 30% shade-grown plants (45.21%). There was no significant variation in α-Bulnesene between treatments, such as 12.28%, 11.59%, and 11.41% in open-condition, 30%, and 50% shade-grown plants, respectively.

Phenotypic plasticity index

The plasticity index showed variable values between the four analyzed groups, viz., growth and yield, leaf anatomy, chlorophyll content, and chemical compounds of the oil under two different shade levels. Box plots (Figure 2) comprising these groups exhibited the highest PI for growth and yield traits (0.56) followed by leaf anatomical traits (0.51), and the lowest for the chemical component traits (0.11) and chlorophyll content (0.14) in the 50% shade-grown plants. In the 30% shade, plants also exhibited phenotypic plasticity, but they had the lowest PI of all the analyzed traits, such as 0.20 for leaf anatomical traits, 0.18 for growth and yield traits, 0.06 for chlorophyll content, and 0.05 for chemical components.

Correlation studies

The oil yield per plant in patchouli had a positive correlation with the plant height (r=0.109), inter-nodal length (r=0.141), the number of branches per plant (r=0.067), number of leaves per plant (r=0.202), leaf area (r=0.201), oil glands (r=0.315), trichomes (r=0.013), fresh weight (r =0.227), and dry weight (r=0.669), while the leaf thickness (r=-0.041) was negatively correlated with the oil yield. Concomitantly, the oil glands had a strong association with leaf area (r=0.566), leaf thickness (r=0.974), as well as trichomes (r=0.306). However, the dry weight per plant had a highly significant positive correlation with the plant height (r=0.483), the number of branches per plant (r=0.672), the number of leaves per plant (r=0.573), leaf area (r=0.562), leaf thickness, and fresh weight (r=0.606), while the inter-nodal length (r=-0.447), oil glands (r=-0.407), and trichomes (r=-0.501), were negatively correlated with the dry weight in the patchouli (Table 2).

Principal component analysis (PCA)

To analyze the pattern of relationships between traits, PCA was carried out, considering the selected 11 quantitative traits simultaneously (Table 3). Out of eleven PCs formed, only one PCs exhibited more than one Eigenvalue (3.040) and showed about 84.07% variability. Eigenvalues above one generates components with significant quantities of information about the original variations. The PC I accounted for the maximum proportion of total variability in the set of all variables studied, and the remaining components accounted for progressively less variation. PC I accounts for approximately 84.07% of the variation and is primarily contributed by leaf area (0.312), internode length (0.300), and plant height (0.292), whereas PC II (5.51) is more related to plant growth and oil yield contributing traits such as oil glands (0.354), fresh weight (0.341), plant height (0.327), internode length (0.309), leaf area (0.307), dry weight (0.303), oil yield (0.275), trichomes In addition, biplot analysis of eleven traits formed by the components PC I and PC II retained 89.58% of the original variance in the patchouli plant grown under three different conditions, *viz.*, open, 30% shade, and 50% shade. The length of arrows showed the proportion of contribution, and the direction of the arrows indicated whether the proportion was positive or negative, as shown in Figure 3.

Pest infestation

During the study period, leaf roller (*Pachyzacia stultalis*) infestation exclusively noted in 50% shade-grown patchouli, with two to five larvae per plant, resulting in the browning and drying of the affected areas.

Discussions

Plants can change their physiology and morphology in response to environmental challenges, which is reflected in their phenotypic variation. Plants that grow in low-light environments frequently undergo such changes [MARKESTEIJN & al. 2007]. The present findings showed that there are strong morpho-anatomical variations between sun and shade-grown leaves in patchouli, which match the situation in “traditional” angiosperm sun and shade leaves [GRATANI & al. 2006]. The morphological characteristics of patchouli plants grown in 50% shade showed a significant increase in leaf color, plant height, inter-nodal length, and leaf area. The improved leaf color was correlated with increased foliar chloroplast number as well as chlorophyll concentration in response to shading. A comparison of the relative levels of photosynthetic pigments in shade-growing patchouli plants revealed that chlorophyll b and carotenoids accumulate preferentially over chlorophyll a [MISRA, 1995]. To sustain the crop's normal life under shade conditions, increasing chlorophyll content is a mechanism to boost light interception. The increased chlorophyll content is to improve the effectiveness of photosynthetically active radiation (PAR) absorption, showing crop adaptability to reduced light intensity [CARTECHINI & PALLIOTTI, 1995].

Plants usually develop a higher leaf area when grown under low-light conditions. In shade-grown patchouli plants, leaf area increased while leaf number decreased as a result of a 50% increase in the shade. Several studies have shown that increasing leaf area increases net photosynthesis [SULANDJARI & al. 2005]. The plastic response of leaf area increase enables plants to maintain high performance under shading and has to constitute adaptive plasticity [EVANS & POORTER, 2001]. On the other hand, increasing the shade level, which decreases the leaf size by reducing palisade parenchyma tissue, was observed in shade-grown (30% and 50%) patchouli plants. The shade reduces the size of the plant leaf by controlling cell proliferation and expansion while also reducing the quantity and size of cells. It causes thinner leaves with thinner palisade tissues [EVANS & POORTER, 2001], resulting in a structure that hinders CO_2 dissolution and transport. Many plant species have smaller and thinner leaves as a reaction to shade stress. Changes in stomatal densities on the adaxial and abaxial surfaces reveal the anatomical plasticity of patchouli as a consequence of the cultural environment [TERASHIMA & al. 2001].

The development of bundle sheath tissue in the leaf petiole of patchouli plants has been shown to be adaptive anatomical plasticity under shade conditions. Bundle sheaths may act as flux sensors or “control centers” for leaf water transport [LEEGOOD, 2008] and bundle sheath extensions (BSEs), which connect the vascular bundle to the epidermis and so lower the resistance in the water path between the supply structures (veins) and the water vapor exits, improving hydraulic integration of the lamina. The BSEs are most typically found in minor veins, but depending on the species, they can occur in veins of any order [WYLIE, 1952]. As a result, such leaves, and thus the species that have them, are referred to as “heterobaric”, as opposed to “homobaric” plants that do not have BSEs. It was reported that BSEs could play an ecological role in the leaf by influencing mechanical and physiological characteristics [TERASHIMA, 1992] and more experimental support, implying that BSEs could be adaptive traits. Concomitantly, BSEs increased in the leaves, which improved the light transmission within the leaf blade [NIKOLOPOULOS & al. 2002].

The oil yield in patchouli is mainly dependent on the number of oil glands and trichomes, leaf area, plant height, the number of branches and leaves, as well as fresh weight and dry weight. This phenotypic variation is highly influenced by the shade (50%) condition. As a result, a 50% reduction in the oil yield has been recorded in patchouli plants which were grown under the 50% shade conditions. When patchouli is grown in a plantation with more than 30% shade, the infestation of leaf roller (*Pachyzacia stultalis*) is severe, causing significant crop yield damage. During the months of October and December, the leaves are completely infested [GAHUKAR, 2018]. However, patchouli has strong insecticidal activity against lepidopteran pests, and this oil has sufficient efficacy to be considered as a component of an essential oil-based insecticide that targets lepidopteran pests [MACHIAL & al. 2010]. Trichomes are a type of morphological adaptation that protects plants by secreting chemicals to protect the leaves from insect pest [BARBOSA & al. 2018]. This clearly indicated that decreases in the number of glandular and non-glandular trichomes and oil content in the 50% shade-grown plants make them most susceptible to leaf roller infestation due to the decreases in defense mechanisms.

Table 1. Comparison of growth, yield, leaf anatomy, chlorophyll content, and oil chemical compounds in full-sunlight and shade (30% and 50%) grown patchouli plants

Parameters	Full Sunlight	30% Shade	50% Shade	CV (%)	Phenotypic Plasticity Index (PI)	
					(30% Shade)	(50% Shade)
Growth and yield traits						
Plant height (cm)	80.71^c	97.50^b	113.83^a	10.06	0.20	0.29
Number of branches plant^{-1}	30.51^a	24.62^b	14.26^c	15.87	0.19	0.53
Number of leaves plant^{-1}	412.35^a	338.46^b	96.62^c	5.02	0.17	0.77
Leaf area (cm^2)	25.83^c	38.45^b	59.17^a	12.73	0.48	0.56
Petiole length (cm)	1.52^b	1.77^b	3.78^a	13.31	0.14	0.6
Petiole width (mm)	1.59^c	2.31^b	3.44^a	5.88	0.31	0.54
Internode length (cm)	4.95^c	6.56^b	8.81^a	11.02	0.24	0.44
Fresh weight plant^{-1} (g)	266.35^a	271.32^a	152.95^b	6.66	0.01	0.44
Dry weight plant^{-1} (g)	92.99^a	85.01^b	29.64^c	8.40	0.08	0.68
Oil content plant^{-1}(%)	2.04^a	1.97^a	0.98^b	13.54	0.03	0.52
Oil yield plant^{-1}(g)	1.89^a	1.67^a	0.30^b	14.79	0.11	0.84
Leaf anatomical traits						
Leaf thickness (µm)	239.14^a	216.00^b	117.57^c	9.49	0.09	0.51
Cuticle thickness(µm)	6.84^a	5.88^b	3.7^c	13.17	0.14	0.46
Thickness of palisade parenchyma (µm)	56.86^a	46.21^b	26.28^c	6.77	0.18	0.53
stomatal density (number mm^{-2})	20.43^a	15.43^b	12.43^b	23.33	0.24	0.39
Chloroplasts number stomata^{-1}	8.43^c	11.71^b	13.71^a	9.67	0.38	0.38
Oil glands (number mm^{-2})	22.71^a	18.57^a	10.14^b	24.16	0.18	0.55
Trichomes (number mm^{-2})	68.29^a	51.00^b	13.14^c	14.35	0.25	0.80
SPAD Chlorophyll content						
Chlorophyll content	40.43^c	43.03^b	46.96^a	4.93	0.06	0.14
Chemical compounds of oil (%)						
Patchouli alcohol	47.72^a	45.21^{ab}	42.44^b	6.49	0.05	0.11
α-Bulnesene (= δ-guaiene)	12.28^a	11.59^a	11.41^a	10.05	0.05	0.07
β-Caryophyllene	3.13^b	2.94^b	3.54^a	5.13	0.06	0.17

Table 2. Correlation matrix for oil yield in patchouli

	Oil yield	**Plant height**	**Inter-node length**	**No. of branches**	**No. of leaves**	**Leaf area**	**Leaf thickness**	**Oil gland**	**Trichomes**	**Fresh wt.**	**Dry wt.**
Oil yield	1										
Plant height	0.109	1									
Inter-node length	0.141	0.994**	1								
No. of branches	0.067	0.398**	-0.388**	1							
No. of leaves	0.202	0.302**	0.305**	0.766**	1						
Leaf area	0.201	-0.329**	0.321**	-0.718**	-0.995**	1					
Leaf thickness	-0.041	-0.072	-0.131	0.559**	0.468**	0.397**	1				
Oil gland	0.315**	-0.062	-0.125	-0.623**	-0.637**	0.566**	0.974**	1			
Trichomes	0.013	-0.851**	-0.849**	-0.644**	-0.349**	0.334**	0.107	0.306**	1		
Fresh wt.	0.227*	0.952**	-0.942**	0.534**	0.342**	0.321**	0.356**	-0.064	-0.928**	1	
Dry wt.	0.669**	0.483**	-0.447**	0.672**	0.573**	0.562**	0.343**	-0.407**	-0.501**	0.606**	1

Highly significant ($p<0.001$) = **; Significant ($p<0.05$) = *

Table 3. The Eigen values, per cent variance and per cent cumulative variance for three principal components (PCs) and factor loading between PCs and traits studied in patchouli plant

Principle component	**PC I**	**PC II**	**PC III**
Eigen values	3.040	0.778	0.668
Proportion of variation	84.07	5.51	4.05
Cumulative proportion	84.07	89.58	93.63
Oil yield/plant	-0.315	-0.048	0.275
Plant height	0.292	-0.423	0.327
Inter-node length	0.300	-0.095	0.309
No. of branches/plant	-0.284	-0.132	-0.509
No. of leaves/plant	-0.323	-0.107	0.105
Leaf area	0.312	-0.102	0.307
Leaf thickness	-0.290	-0.468	-0.072
Oil glands	-0.261	0.672	0.354
Trichomes	-0.310	-0.164	0.125
Fresh wt.	-0.305	-0.266	0.341
Dry wt.	-0.312	-0.013	0.303

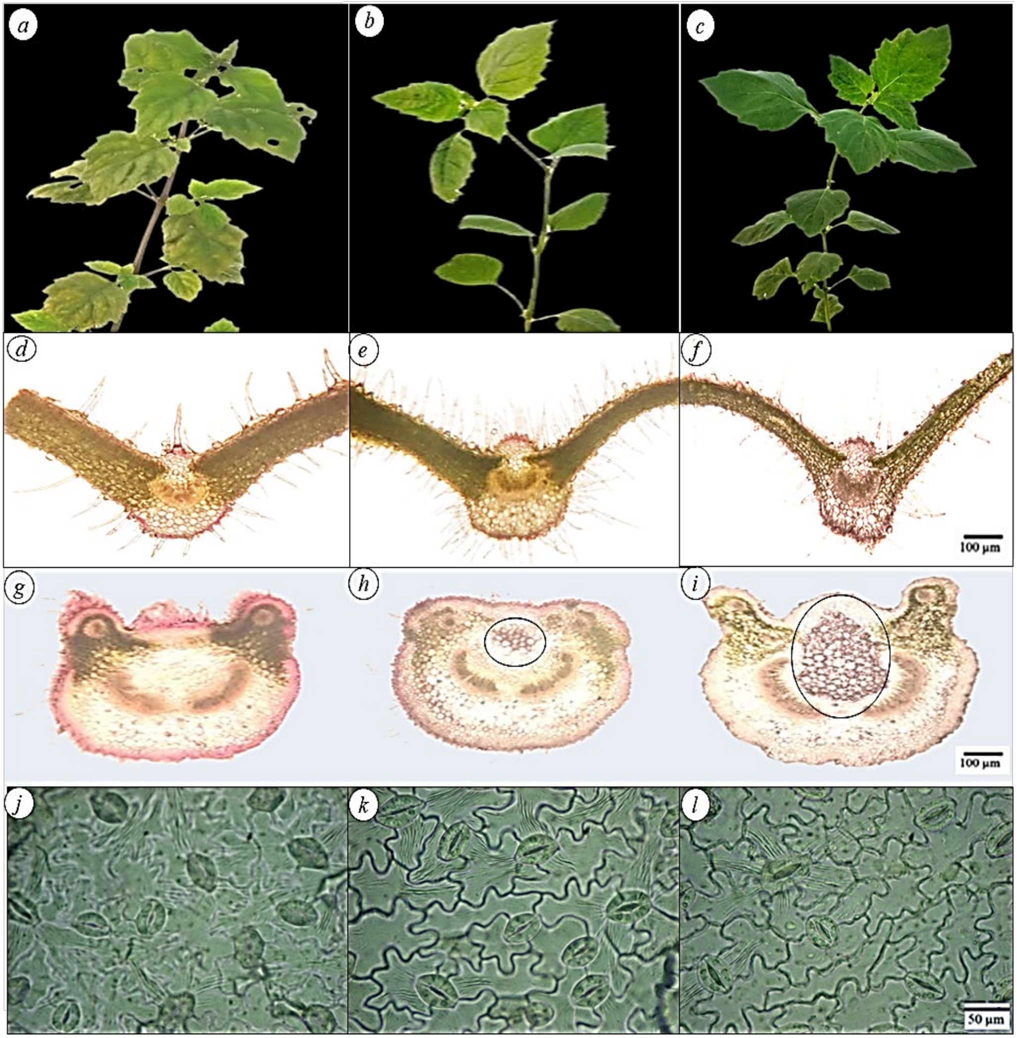

Figure 1. Plant morphology: (a) full-sunlight grown plant, (b) 30% shade grown plant and (c) 50% shade grown plant; Cross section of the leaf blade: (d) full-sunlight, (e) 30% shade, and (f) 50% shade grown plant; Cross section of the petiole: (g) full-sunlight, (h) 30% shade, and (i) 50% shade grown plant; Leaf stomatal density: (j) full-sunlight, (k) 30% shade, and (l) 50% shade grown plant. (* Circle marks show the bundle sheath extensions region in the cross section of petiole)

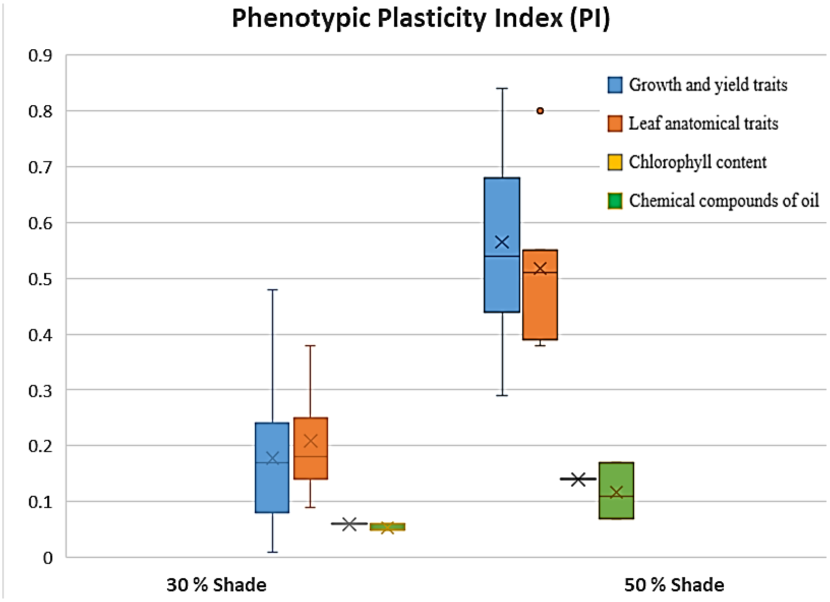

Figure 2. Box plots showing the phenotypic plasticity index (PI) distribution for each of the measured traits between 30% shade and 50% shade-grown patchouli plants

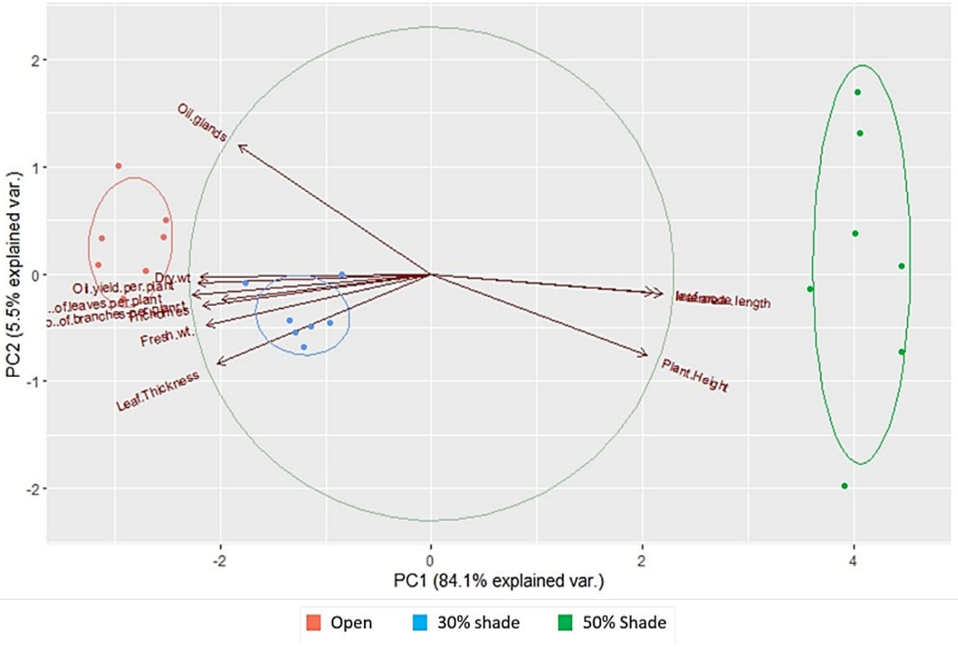

Figure 3. Biplot diagram of principal components

Conclusions

The patchouli plants when grown under more than 50% shade conditions exhibited the highest phenotypic plasticity in all the traits compared to 30% shade grown plants as well as open condition grown plants. The expression of a higher level of phenotypic plasticity in patchouli plants under shade (50%) has shown a negative impact on essential oil yield production and a positive effect on the ability to thrive under limited light intensity conditions.

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Research Article

NUTRITIONAL AND OIL PROFILE OF THE SEEDS OF *SESAMUM INDICUM* L. AND *VITELLARIA PARADOXA* C. F. GAERTN.

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Abstract: Nutritional and oil profile of the seeds of *Sesamum indicum* and *Vitellaria paradoxa* from Sokoto, Nigeria was carried out using standard biochemical procedures. Proximate, ascorbic acid, mineral analysis as well as extraction, quantification and the physicochemical analysis of the seed oil were assessed. On proximate and ascorbic acid analysis. The seeds were found to contain appreciable amounts of crude proteins with 18.23% and 8.70% obtained in *S. indicum* and *V. paradoxa* respectively. Lipid contents revealed 42.51% in *S. indicum* and 51.34% in *V. paradoxa* respectively. Total carbohydrates revealed that 39.03% and 52.12% were obtained in *S. indicum* and *V. paradoxa* respectively. Crude fiber contents revealed 5.31 g/100 g in *S. indicum* while in *V. paradoxa*, 7.11 g/100 g was recorded. Percentage ash contents revealed 6.62% and 4.61% in *S. indicum* and *V. paradoxa* respectively. Available energy (kj) revealed that *S. indicum* had 603.04 kj/100 g while *V. paradoxa* had 614.12 kj/100 g respectively signaling them as formidable energy sources. Ascorbic acid analysis revealed 97.36 mg/100 g and 179.16 mg/100 g in *S. indicum* and *V. paradoxa* respectively. With significant difference ($P \leq 0.05$) in the contents of crude proteins, crude lipid, crude carbohydrate, calorific value and ascorbic acid between the two sampled seeds. Minerals analysis revealed that the two seeds were rich in most of the essential minerals required for healthy growth and development. For instance, sodium was found to be 2.67 mg/100 g in *S. indicum* while 2.23 mg/100 g was identified in *V. paradoxa*. Potassium, magnesium and phosphorus results indicate that *S. indicum* had 56.96 mg/100 g, 62.12 mg/100 g and 76.72 mg/100 g and *V. paradoxa* had 46.72 mg/100 g, 31.64 mg/100 g and 116.74 mg/100 g respectively. With significant difference ($P \leq 0.05$) in the contents of potassium, magnesium and phosphorus oil extraction and its physicochemical properties revealed that the two seed contained substantial amounts of oil with 38.23% in *S. indicum* and 43.14% in *V. paradoxa* respectively. Acid value analysis showed that *S. indicum* had 32.14 mg KOH/kg while *V. paradoxa* had 33.24 mg KOH/kg. Saponification value analysis revealed that 131.86 mg KOH in *S. indicum* while 154.74 mg KOH was found in *V. paradoxa*. Iodine value analyzed showed that 116.86 g I_2 /100 g in *S. indicum* while 124.77 g I_2 /100 g was obtained in *V. paradoxa* while % Free Fatty Acid analysis revealed that *S. indicum* had 7.63% while *V. paradoxa* had 8.74% respectively. With significant difference ($P \leq 0.05$) between the two species in percentage yield, saponification and iodine values between the two seeds. The two studied seed types are endowed with natural food reserves and other much needed raw materials that could have industrial application making the seeds as formidable ingredients required for use by man.

Keywords: nutritional, physicochemical, seed-oil, *Sesamum indicum*, *Vitellaria paradoxa*.

Introduction

Nigeria, a country with a population exceeding 200 million, grapples with a multifaceted challenge: the intersection of energy and dietary problems within its food supply system. From food insecurity and malnutrition to agricultural practices and cultural dynamics, the factors influencing energy and dietary problems are explored in-depth. At the heart of

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Nigeria's energy and dietary issues lies food insecurity, affecting a substantial portion of the population [BENNETT & VOKOUN, 2023]. Many Nigerians lack consistent access to sufficient and nutrient-dense foods, resulting in chronic under nutrition and malnutrition. A significant consequence is the prevalence of deficiencies in essential vitamins, minerals, and macronutrients that are crucial for maintaining optimal health and well-being [ADEYEMI & al. 2023]. A defining feature of the Nigerian diet is its heavy reliance on staple crops such as cassava, yam, and maize. While these crops provide energy, they often fall short in providing a diverse range of nutrients necessary for a balanced diet [JACOB & al. 2023]. This dietary imbalance contributes to both under nutrition and over nutrition, as individuals may consume high quantities of carbohydrates while lacking essential vitamins and proteins. Nigeria's agricultural practices are marred by low productivity, traditional farming methods, and inadequate storage facilities. These challenges result in reduced yields, post-harvest losses, and limited availability of fresh produce. Inefficient agricultural practices hinder the country's ability to provide an ample and varied food supply, further exacerbating energy and dietary issues. The energy and dietary problems associated with Nigeria's food supply system are complex and interlinked, driven by a combination of factors spanning from agriculture and economy to culture and infrastructure. Addressing these challenges necessitates a multi-pronged approach that involves government policies, community engagement, education, agricultural innovations, and healthcare interventions. By acknowledging the depth of these issues and implementing comprehensive strategies, Nigeria can pave the way towards a more nourished and healthier future for its population. Rapid urbanization has introduced shifts in dietary habits, as individual transition from traditional diets to processed and convenience foods. Urban dwellers often face challenges in accessing fresh and affordable produce, leading to a higher consumption of energy-dense but nutrient-poor foods. This trend contributes to rising rates of diet-related non-communicable diseases such as diabetes and obesity. The term oil is used in general sense to describe all substances that are greasy or oily fluid at room temperature. There are abundant vegetable oils, namely; coconut oil, ground nut oil, rubber seed oil, cotton seed oil, olive oil, soy bean oil and cotton seed oil, etc. [DAWODU, 2009]. Vegetable oils are normally extracted from fruits, seeds kernel and nuts either by mechanical press or by use of solvents [AKPABIO & al. 2011].

Sesame (*Sesamum indicum* L.) is an herbaceous annual plant that belongs to the family Pedaliaceae genus *Sesamum*. Sesame seed is also known as benniseed (Africa), benne (Southern United States), gingerly (India), gengelin (Brazil), sim-sim, semsem (Hebrew) and tila (Sanskrit) [HASSAN, 2012]. Sesame has many species; most are wild and are native to sub-Saharan Africa. Sesame is highly tolerant to drought like conditions, and grows where other crops may fail. It is well suited to smallholder farming with a relatively short harvest cycle of 90-140 days allowing other crops to be grown in the field [USDA, 2005]. The world harvested about 4.76 million metric tons of sesame seeds in 2013, with Burma as the largest producer. The world's largest exporter of sesame seeds was India, while Japan was the largest importer [FAO, 2012]. Nearly 70% of the world production is from Asia. Africa grows 26% of the world’s sesame, with Sierra Leone, Sudan, Nigeria and Uganda being key producers [MAKINDE & AKINOSO, 2013]. Sesame production in Nigeria probably began in the middle belt region of the country and later spread out between latitudes 6° and 10°N. The major producing areas in order of priority are Nasarawa, Jigawa and Benue States. Other important areas of production are found in Yobe, Kano, Katsina, Kogi, Gombe and Plateau States [OJIAKO & al. 2010]. The black and white cultivars are grown basically in Nigeria. The white cultivar is grown around Benue (Oturkpo), Nassarawa (Doma), Jigawa (Malam-Madori) and Taraba states while the black cultivar grows in the Northern Nigerian region; Kano (Dawanau), and Jigawa (near Hadejia) states and in some parts of Katsina state [MAKINDE & AKINOSO, 2013].

S. indicum, known as sesame is an herbaceous annual plant that belongs to the family Pedaliaceae [AMOO & al. 2017]. Sesame has one of the highest oil contents of any seed. It is probably the most ancient oilseed used by humans as a food source [ABOU-GHARBIA & al. 2000]. Having nutty flavor, it is a common ingredient in cuisines across the world. In Nigeria, sesame seeds are used as soup ingredient, and constitute a useful source of vegetable oil for cooking [BEDIGAN, 2006]. At the local level it is processed into *Kantun ridi* and *Kunun ridi.* Oil is also extracted from the seed and the cake is made into *kulikuli* which together with the leaves are used to prepare local soup known as *Miyar taushe* and also used for cooking [MAKINDE & AKINOSO, 2013].

Sesame seeds have the highest oil content than rapeseed, groundnut, soybean and other oilseed crops [ANILAKUMAR & al. 2010]. Its oil and protein contents are estimated to be 50-60% and 18-25% respectively. To this can be added carbohydrates, fibers and ash [ALPASLAN & al. 2001]. This composition is supplemented by methionine (3.2%) which is often the limiting amino acid in legume-based tropical diets; tryptophan and the wide range of minerals such as phosphorus, calcium, iron, magnesium etc. as well as vitamins B1 and Vitamin E [OJIAKO & al. 2010].

Vitellaria paradoxa C. F. Gaertn. was cited three decades ago as the second most important oil crop in Africa after oil palm and is probably the most economically and culturally important tree species in the Sudan Sahelian region of Africa where oil palm does not grow. Across the distribution area, the estimated actual number of productive trees ranges from several hundred million [LOVETH, 2014] to a couple of billion respectively making it one of the largest population size of an economic tree species in the region. The dried kernel of fruit is used to produce oil or fat (Shea butter) for local consumption and is commercially sold as an ingredient in cosmetics, pharmaceutical and edible product. Shea was reported as a traded commodity by the Arab traveler, Ibn Battuta as early as the 14th century. The magnitude of its distribution and local importance caught the attention of early explorers such as Mungo Park in 1798 and has been a subject of research since colonial times. It is interesting to note that, the Shea is estimated to serve as the primary source of edible oil for more than 80 million rural people [NOUGHTON & al. 2014].

V. paradoxa is among the major tree species in African agro forestry systems. It is an indigenous oil producing wild plant that belongs to the family Sapotaceae and spontaneously grows in Africa. The plant is adjudged to play an important role in the provision of edible oil or fat that is traditionally used for frying, adding to sauces, as a skin pomade, for medicinal applications, for soap making, oil for lanterns and for cultural purposes during ceremonies, such as births, weddings as well as for funerals. In fact, the seed kernels produce oil content which is highly nutritious with unsaturated fatty acids such as oleic and linoleic fatty acids and fat-soluble vitamins [KARIN, 2004; KAPSEU & al. 2007]. The tree is the main indigenous oil producing wild plant spontaneously growing in Africa [HONFO & al. 2012]. HEE (2011) reported that Shea tree begins to bear fruit of commercial quantity after approximately 20-50 years. Butter extracted its nuts offers an opportunity for sustainable development in Sudanian countries and an attractive potential for the food and cosmetics industries [DAVRIEUX & al. 2010].

Materials and methods

Sample collection and preparation

Freshly harvested ripe seeds of *S .indicum* were procured from vegetable market at Kasuwar Dankure while *V. paradoxa* seed kernels were obtained from Kasuwan Ramin Kura all within Sokoto metropolis. The seeds were carried to Departmental Herbarium Department of Plant Biology, where voucher specimens were deposited. The seeds were dehulled, cleaned and dried under the sun for a day and later dried in the oven for three hours at 50 ºC to ensure that moisture content was highly removed. The seed samples were made into powder using cleaned pestle and mortar and kept in labeled bottles until used.

Proximate and ascorbic acid composition analysis

The micro-kjeldal method [PEARSON, 1976] was followed by the determination of crude proteins. Crude fats, crude fibre, moisture % and ash % were determined using the methods of [AOAC, 2005], while carbohydrate was determined by difference. The calorific values in kilo joule (kj) were calculated by multiplying the crude fat, protein and carbohydrate by Atwater factors of (k) 37, 17, and 17 respectively. Ascorbic acid was determined according to the method described by MUSA & al. (2010).

Mineral composition analysis

The minerals were analyzed by first dry ashing the samples at 550 ºC in the muffle furnace. The filtered solutions were used to determine Na, K, Mg, Ca, Fe, Cu, Zn, Co, Cd, and Ni by means of Atomic Absorption Spectrophotometer [AAS] (Buck Scientific Model-200A/210, Norwalk, Connecticut [06855]) and phosphorus was determined calorimetrically by Spectronic 20 (Gallenkamp, UK) using the phosphovanado molybdate method [AOAC, 2005].

Oil extraction and its physiochemical properties

Percentage oil yield and physicochemical properties of the Seed oil were determined in accordance with the protocols of ASTM D 189, ASTM-D 974, kinematic viscosity of the seed oil was carried out in accordance with the ASTM D 445 while iodine value and oil specific gravity were determined using the procedure as reported by AJIBOLA& al. (2018). Determination of the cetane number of the biodiesel was determined by the use of empirical formula in the literature using the result of saponification number (SN) and the iodine value (IV) of biodiesel. Oil colour and its physical state at room temperature were determined by organoleptic method [AOAC, 1975]. While physical state of the oil was determined by sensory evaluation [IBETO & al. 2012].

Data analysis

Treatments were replicated three times and the results obtained has been presented as means± S.E. of the means. The data collected was subjected to Analysis of Variance (ANOVA) using GenStat$^{(R)}$ 18th edition. Where the treatments were found to be significantly different, mean separation was carried out using Duncan's Multiple Range Test (DMRT) at 5% level.

Results

Proximate and ascorbic acid composition

Proximate composition of *S. indicum* and *V. paradoxa* in Sokoto, Nigeria has been presented in Table 1 below. From the results, there was significant difference ($P \leq 0.05$) in the composition of crude proteins, total carbohydrates and ascorbic acid in the two samples. Crude protein content obtained in *S. indicum* was 16.96% while 7.72% was obtained in *V. paradoxa*. For total carbohydrates, 39.03% was recorded for *S. indicum* while 52.12% was obtained in *V.*

paradoxa. Ascorbic acid analysis revealed that *S. indicum* had 97.36 mg/100 g while *V. paradoxa* had 179.16 mg/100 g respectively.

Table 1. Proximate and ascorbic acid composition of *Sesamum indicum* and *Vitellaria paradoxa*

Parameters	Units	*Sesamum indicum*	*Vitellaria paradoxa*
Moisture Content	%	2.67±0.63^a	2.23±0.53^a
Crude Protein	g/100 g	16.96±2.89^a	7.72±1.26^b
Crude lipid	g/100 g	42.12±2.86^a	41.64±1.62^a
Total carbohydrates	g/100 g	39.03±2.19^a	52.12±.2.10^b
Crude Fibre	g/100 g	5.31±0.86^a	7.11±0.99^a
Ash	%	6.62±0.07^a	4.61±0.05^a
Calorific Value	kj/100 g	603.04±5.88^a	614.12±4.67^a
Vitamin C	mg/100 g	97.36±2.43^a	179.16±3.19^b

Values are means ± standard deviation of three replications. Values within a row with different superscripts were significantly different ($p \leq 0.05$).

Table 2. Mineral composition of the seeds of *Sesamum indicum* and *Vitellaria paradoxa*

Mineral (mg/100g)	Symbol	*Sesamum indicum*	*Vitellaria paradoxa*
Sodium	Na	2.67±0.63^a	2.23±0.53^a
Potassium	K	56.96±2.89^a	46.72±1.26^b
Magnesium	Mg	62.12±2.86^a	31.64±1.62^b
Phosphorus	P	76.72±1.69^a	116.74±2.83^b
Manganese	Mn	32.76±1.05^a	48.66±2.36^a
Calcium	Ca	116.64±1.14^a	132.54±1.98^b
Iron	Fe	33.06±1.62^a	15.07±1.02^b
Cupper	Cu	6.24±0.06a	0.31±0.04^a
Chromium	Cr	0.06±0.03^a	0.02±0.01^a
Zinc	Zn	13.36±0.09^a	11.74±0.08^a
Nickel	Ni	0.17±0.04^a	0.19 ±0.02^a

Values are means ± standard deviation of three replications. Values within a row with different superscripts were significantly different ($P \leq 0.05$).

Results of mineral analysis have been presented in Table 2. The table revealed that there was significant difference ($P \leq 0.05$) in the composition of potassium, magnesium, phosphorus and iron. Calcium was the most abundant mineral with 116.64 mg/100 g and 132.54 mg/100 g in the seeds of *S. indicum* and *V. paradoxa* respectively. Phosphorus was the other mineral element with appraisable abundance in both the seeds with 76.72 mg/100 g obtained in *S. indicum* while 116.74 mg/100 g was obtained in *V. paradoxa.* Potassium was the third mineral with appreciable abundance with 56.96 mg/100 g while *V. paradoxa* had 46.72 mg/100 g.

Oil yield (%) and the physico-chemical properties of the seed oil of *S. indicum* and *V. paradoxa*

Results of the physico-chemical properties of the seed oils from *S. indicum* and *V. paradoxa* have been presented in Table 3. From the results, it can be seen that the two seed samples were endowed with quantifiable biofuel reserves with 48.23% in *S. indicum* and 53.14% in *V. paradoxa*. Acid value was analyzed with 32.14 mg KOH/kg obtained in *S. indicum* while 33.24 mg KOH/kg was obtained in *V. paradoxa*. Saponification value revealed that *S. indicum* had 131.86 mg KOH while *V. paradoxa* had 154.74 mg KOH and iodine value with

116.86 g I_2/100 g recorded for *S. indicum* while 124.77 g I_2/100 g was recorded for *V. paradoxa*. Cetane number analysis revealed 32 and 34 for *S. indicum* and *V. paradoxa* respectively. Oil colour was determined and it was yellow to pale brownish for the *S. indicum* and *V. paradoxa* respectively. State of the oil at room temperature revealed that the oils were liquid and semi-solid at room temperature. With significant difference ($P \leq 0.05$) in the percentage yield, saponification value and iodine value between the sampled seeds.

Table 3. Percentage oil yield and its physico-chemical properties

Parameters	**Unit**	***Sesamum indicum***	***Vitellaria paradoxa***
Oil Content	%	38.23±1.26^a	43.14±2.54^b
Acid Value	mg KOH/kg	32.14±1.12^a	33.24±1.17^a
Saponification Value	mg OH/g	131.86 ±2.84^a	154.74±3.08^b
Kinematic Viscosity	mm^2 /S	0.89±0.09^a	0.96±0.07^a
Specific Gravity	g/cm^2	0.82±0.05^a	0.89±0.08^a
Free Fatty Acid	%	7.63±0.89^a	8.74±0.66^a
Iodine Value	g/100 g	116.86±2.36^a	124.77±4.38^b
Cetane Number	-	32.00±1.06^a	34.00±1.12^a
Oil Color	-	Yellow	Pale brown
State of Oil at Room Temp.	-	Liquid	Semi solid

Values are means ± standard deviation of three replications. Values within a row with different superscripts were significantly different ($p \leq 0.05$).

Discussions

A moisture content of value 2.67-3.16% (Table 1) indicate that the seeds have a long shelf life. That is to say that they can be stored for a long time. The values obtained in the current study were in close agreement to the moisture content of 2.84% as reported by RAIMI & al. (2014) on the seeds of *V. paradoxa*, and 3.70% as reported by NZIKOU (2009), on *S. indicum* seeds. The moisture content compares favorably with the report of UGESE & al. (2010) who reported 2.2 to 3.8%. Protein content obtained in the current study is lower than 9.3% as reported by RAIMI & al. (2014) on *V. paradoxa*. Also, a bit lower protein with 7.6 to 8.9% was reported on *V. paradoxa* seeds by UGESE & al. (2010). Protein content as reported by NZIKOU & al. (2009) is a bit lower than obtained in the current study. Proteins are for growth and repair of tissues, and also serve as alternative energy sources in the absence of carbohydrate and fats. Crude fats contents of the seed types could be considered as being highly good. Results obtained compares favorably with the reported oil contents of 49.16% in *V. paradoxa* by RAIMI & al. (2014). Results obtained in the current study also compares favorably with the 46.95% fat contents of *Jatropha curcas*. More so, higher moisture content of 9.97% and 9.40%, high protein of 35.95% and 17.13% were reported on raw and defatted *Moringa oleifera* seeds. More so, high values of protein and crude fats were reported as 30.63% and 49.05% on the seeds of watermelon by JACOB & al. (2015). These values were higher than obtained in the current study. Crude fibre content obtained in the current study could be considered good but lower than the reported 12.57% reported on *Moringa oleifera* by OKIKI & al. (2015). Vitamin C with the ranges from 97.36 to 179.66 mg/100 g was reported by AFOLAYAN & al. (2014), also reported was high vitamin C content in locust bean pulp at a value of 542 mg/100 g quite higher than values obtained from the current study but this variation could be attributed to environmental condition and post-harvest handling.

The two seeds could be considered as being highly good in terms of nutrients composition especially when compared with other reports. For instance, very low minerals were reported by NWEZE & al. (2014) on *Moringa oleifera* leaf extracts with Ca, Mg, P, Zn, Cu and K with 2.29, 0.48, 0.44, 0.05, 0.01 and 1.62 mg/100 g respectively. The above values were highly low when compared with values obtained in the current study. In another study HASSAN & UMAR (2004), higher values of Ca, P, Mg and K were reported to be 342.50 mg/100 g, 400.00 mg/100 g, 215.38 mg/100 g and 600.00 g/100 g on whole seeds of African locust bean (*Parkia biglobosa*) by OLUWAGBENLE & al. (2019) on comparative assessment of the proximate, minerals composition of cucumber (*Cucumis sativus*), higher values than obtained in the current study were reported for Ca with 177.00 mg/100 g; Na with 156.00 mg/100 g and K with 541.00 mg/100 g while lower values of 2.21 mg/100 g, 0.56 mg/100 g and 9.08 mg/100 g reported for Cu, Mn and Fe respectively. Variation in the mineral composition could be attributed to the environmental factors such as soil and climatic conditions; this is in addition to species variability as well as the method of extraction.

Results of oil analysis revealed that the two seeds could be considered as highly good in terms of the percentage yield and physicochemical parameters analysis of the seed oil. According to EGBEKUN & EHIEZE (1997), variation in oil yield may be attributed to the differences in plant variety, cultivation pattern, climate, ripening stage as well as the extraction technique. Results obtained in the current study were however, higher than the percentage yield of 32.21% as reported on *Momordica balsamina* seed oil by AJIBOLA & al. (2018). In addition, values obtained in the current study were a bit lower than the range of values 44.72 to 53.88% as reported by KARAYE & al. (2021) on seeds of selected cucurbits seeds. The obtained values were higher than the 36.70% as reported on seeds of calabash (*Lagenaria vulgaris*) by SOKOTO & al. (2013). More so, iodine value of 153.40 g/ 100 g as reported by AKINONSO & RAJI (2010) was higher than values obtained in the current study. In another report by NZIKOU & al. (2009) on Sesame (*Sesamum indicum*) grown in Congo-Brazzaville, yield was 52.00%, higher than the value obtained in the current study, iodine value reported to be 112.40 g/100 g a bit less than the values obtained in the current study.

Conclusion

In conclusion, results in the current study on proximate, ascorbic acid and mineral composition of *S. indicum* and *V. paradoxa* seeds show that the seeds have high nutritional and energy values, and would be in addition to consumption by humans, serve as useful raw materials for industries. The seed oil could be described as having a sterling quality grade with high commercial value. It is edible and would find applications in food, soap and cosmetics production. The utilization of these types of seeds could be considered as valuable raw materials for inclusion in animal feed formulation so that the conventional oil seeds presently used as raw materials for animal feed production would be better utilized for human food.

Conflict of interest

Authors hereby declare that there is no competing interest of any sort among them.

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Research Article

EFFECT OF IRRIGATION INTERVAL ON GROWTH AND ARTEMISININ CONTENT OF WORMWOOD (*Artemisia annua* L.) CHEN YOUNG VARIETY IN SOKOTO

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Abstract: Artemisinin is the main antimalarial compound in *Artemisia annua*, used in the formulation of artemisinin-based combined therapies (ACT) to treat malaria. Artemisinin is largely obtained from *A. annua* plant but the content is very low and its production commercially is not cost effective worldwide. In view of the importance of this phytomolecule and plants being the only source of its production, this study evaluated the effect of irrigation interval on the growth and artemisinin content of *A. annua*. A greenhouse experiment was conducted at Botanical Garden of Usmanu Danfodiyo University Sokoto. The experiment was laid out in completely randomized design with 4 replications. The two factors examined were: (1) irrigation interval at different growth stage and (2) artemisinin content of *A. annua*. Irrigation interval (W) was taken at four levels (W_1, W_2, W_4, and W_6 days interval) while growth stage at three levels: early vegetative stage (EVS), mid vegetative stage (MVS) and late vegetative stage (LVD) and artemisinin were determined and quantified using High Performance Liquid Chromatography (HPLC). The calibration curve was constructed by plotting the peak area against the concentration by the external standard method on five concentration levels of artemisinin standard (5, 10, 15, 20, and 25 µg/ml), with three injections per level. Linear regression was used to establish the calibration curve. Results were calculated using the peak areas with determination coefficient (R^2) of 0.951. Results revealed that W_2 days irrigation interval had significantly ($p<0.05$) affected fresh weight, dry weight, stem diameter and root length during the EVS. At MVS, irrigation interval had no significant ($P>0.05$) effect on plant height and number of branches but significantly reduces stem diameter. During the late vegetative stage (LVD), irrigation intervals have no significant effect on all parameters evaluated. Artemisinin content was not significantly ($P>0.05$) affected by irrigation intervals during the EVS but significantly affected at mid and late vegetative stage and our result demonstrated that prolong mild irrigation interval (W_2) could significantly enhanced artemisinin content in *A. annua*.

Key word: Artemisinin, early vegetative stage (EVS), mid vegetative stage (MVS), late vegetative stage (LVD), High Performance Liquid Chromatography (HPLC).

Introduction

Artemisia annua L. (family Asteraceae), can be found in the temperate regions of the northern hemisphere, in arid and semiarid climates areas. The genus has a great economic importance as medicinal resource, flavouring agent, antibacterial, antifedant and antimalarial activities [IHSAN-UL-HAQ & al. 2012]. *A. annua* is the only planta medical that has been recognized to research and developed as the standards of western medicine research by the WHO in China. It is a famous herb, known for its highest efficiency and lowest toxicity in treating ague [WANG & al. 2011]. It is aromatic annual herbaceous plant [ZANJANI & al. 2012; LIU & al. 2013; MISRA & al. 2013] and belongs to genus *Artemisia* [LIU & al. 2013], family Asteraceae (Compositae) [ZANJANI & al. 2012] and commonly known as sweet

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wormwood or Qinghao [HUANG & al. 2010; EMADI, 2013]. It's the only member of genus *Artemisia* with an annual growth cycle [WILLCOX & al. 2004].

Artemisinin is an effective antimalarial compound that is synthesized in the glandular trichomes of medicinal plant *Artemisia annua* which has saved millions of lives. It is used in the formulation of Artemisinin based combination therapies (ACTs) recommended by the World Health Organization [WHO, 2017] for treatment of uncomplicated malaria caused by the *Plasmodium falciparum* parasite [DUFFY & MUTABINGWA, 2006]. Currently, the supply of ACTs is reliant on the agricultural production of artemisinin. However, plant-based production sometimes cannot meet the global demand due to the low amount of artemisinin produced in *A. annua* leaves (0.1%-2.0% of dry weight). Alternatively, a semi-synthetic system can be used for the production of artemisinin, in which yeast are engineered to synthesize its precursor, artemisinic acid [RO & al. 2006; PADDON & al. 2013]. However, the semi-synthetic production of artemisinin is expensive and thus cannot replace its agricultural production at present [PEPLOW, 2016]. Besides its anti-malarial activity, many other therapeutic effects of artemisinin on diseases such as cancer [EFFERTH, 2006; TIN & al. 2012], tuberculosis [ZHENG & al. 2017], and diabetes [LI & al. 2017] have been reported. The use of the whole Artemisia plant as a malaria therapy was found to be more effective than a comparable dose of pure artemisinin, and was shown to be able to overcome resistance to pure artemisinin in a rodent malaria model and human clinical trial [ELFAWAL & al. 2015; DADDY & al. 2017]. Recently Madagaska's Institute of Applied research has produced Artemisia-containing tonic that supposedly prevent and treat COVID 19 [MWANGI, 2020]. Hence, artemisinin is a potential multi-functional compound and is of high medicinal value. There is a considerable interest in increasing the artemisinin content of *A. annua* and an urgent need to identify other potential method for it production. Despite many malaria cases in savanna region, few or none has been tried to increase the artemisinin content in Savanna region of Nigeria. The research aimed to evaluating the effect of irrigation interval on growth and artemisinin content of *A. annua* in Sokoto.

Material and methods

Collection of plant material and soil

Artemisia annua seeds of Chen Young variety were sourced from Artemisia Programme Unit at the Institute for Agricultural Research (I.A.R) Ahmadu Bello University, Zaria.

Equipment and chemicals

High Performance Liquid Chromatography equipment Soxhlet extraction apparatus, Beakers, Syringe and measuring cylinder etc. Artemisinin standard was purchased from Sigma Aldrech through Bristol Scientific Company. Ethanol, methanol, n-hexane, sodium hydroxide, acetonitrial, and Indole butyric acid and all the solvents used were of analytical-grade.

Experimental design and inducement of irrigation interval

This experiment investigated the effects of irrigation interval on growth and artemisinin content of *A. annua*. Five weeks old seedlings of approximately of the same height (12-15 cm) were selected and subjected to four different watering regimes: control (W_1), mild (W_2), moderate (W_4) and severe (W_6) irrigation interval in completely randomised design (CRD) with four replicate. Soils with mild, moderate and severe irrigation interval were watered once at 2, 4 and 6 days interval respectively and daily as control. Four seedlings (stand) per each experimental unit.

Data were taken at early vegetative stage (EVS), mid vegetative stage (MVS) and late vegetative stage (LVS) after one, two and three-month post irrigation interval respectively. At each stage, plant height, number of branches, fresh weight, dry weight, stem diameter and roots length were determined and Leaves from each stand in the treatment were collected and analyzed. Each treatment was irrigated with one liter of water on it corresponding watering regime throughout the plant developmental stages. At each stage, samples were taken for artemisinin analysis to find out if artemisinin is affected by irrigation interval [YADAV & al. 2014].

Preparation of extracts for soxhlet extraction

Fresh leaves of *A. annua* were dried for two weeks at room temperature, pulverized into powder using mortar and pestle and homogenized. Adopting the method of CHRISTEN & VEUTHEY (2001), 5 g of pulverized sample was extracted with 200 ml of *n*-hexane at 60 °C in Soxhlet apparatus. The hexane was then evaporated under a vacuum and the samples reconstituted in 10 ml acetonitrile then filtered through Whiteman filter paper no.1.

Preparation of standards and high-performance liquid chromatography

Artemisinin solution was obtained by dissolving 10 mlg of artemisinin standard in 100 ml of acetonitrile to form the stock solution and from the stock solution 5 different concentrations of 5, 10, 15, 20 and 25 µg/ml were obtained. Each was run 3 times in HPLC from the results, standard calibration curve was drive. The HPLC analyses were performed with Agilent technologies 1200 series on Eclipse XBD-C18 (4.6 × 150 mm) column and detection was conducted at 214 nm wavelength. The acetonitrile were used as a mobile phase with 0.8 ml/min flow rate [LAPKIN & al. 2009]. Injection column was 10 µL; run time of 20 min at 30 °C.

Calibration curve

The calibration curve was constructed by plotting the peak area against the concentration by external standard method on five levels of concentration of artemisinin standard (5, 10, 15, 20, and 25 µg/ml), with three injections per level. Linear regression was used to establish the calibration curve. Results were calculated using the peak areas and determination coefficient (R^2) of 0.951.

Data analysis

The data obtained were analyzed using one-way analysis of variance with Minitab Statistical Software version 17. Significant means were compared using Turkey simultaneous test at $P<0.05$.

Results

Effect of irrigation interval on the growth of *Artemisia annua* at early, mid and late vegetative stages

The results of irrigation interval on the growth parameters of *A. annua* at early, mid and late stage vegetative stage are presented in Table 1. The results show that Irrigation Interval has no significant ($P>0.05$) effect on plant height and branch number at all the three vegetative stage evaluated. However, irrigation interval significantly affects fresh weight, dry weight and stem diameter at early and mid-vegetative stage while root length was only affected at early vegetative stage with the highest values (23.75 cm) on W_2 days irrigation interval. At mid vegetative stage, irrigation interval significantly reduced stem diameter which is directly proportional to the severity of the interval and the highest root length was observed at 2 days irrigation interval with (17.00 cm) followed by four days interval (12.50 cm) and then control (11.33 cm). At late vegetative stage, irrigation interval had no significant effect on all the growth parameters evaluated (Table 1).

Effect of irrigation interval on artemisinin content of *Artemisia annua* at early, middle and late vegetative stage

The result on effect of Irrigation Interval at early, mid and late vegetative stage on artemisinin content in *A. annua* is presented in Table 2. The result revealed that irrigation interval had no significant effect ($P>0.05$) on artemisinin content during the early and late vegetative stage of *A. annua*. However, irrigation interval significantly ($P<0.05$) affected artemisinin content at mid vegetative stage. The linear calibration curve obtained from High Performance Liquid Chromatography (HPLC) was presented in Figure 1.

Table 1. Effect of irrigation interval on the growth of *A. annua* at early (EVS), mid (MVS), and late (LVS) vegetative stage

Plant growth stage	Irrigation interval	Plant height (cm)	No. Branch	Fresh weight (g)	Dry weight (g)	Stem diameter (cm)	Root Length (cm)
EVS	W$_1$	42.75±10.24	49.00±10.42	25.07±4.67^{b}	6.45±1.07^{b}	2.02±0.22^{b}	13.50±1.2^{b}
	W$_2$	50.75±16.88	50.00±8.30	37.27±6.94^{a}	10.43±3.03^{a}	3.10±0.36^{a}	23.75±3.03^{a}
	W$_4$	43.50±14.20	39.00±10.14	19.68±2.12^{bc}	5.22±0.43^{b}	1.17±0.22^{c}	12.75±2.01^{b}
	W$_6$	38.00±14.17	35.00±12.97	11.65±3.03^{c}	4.27±0.60^{b}	1.02±0.22^{c}	11.00±0.82^{b}
MVS	W$_1$	62.00±7.21	66.00±5.20	21.23±5.20^{ab}	5.133±1.36	1.67±0.37^{a}	11.33±2.31
	W$_2$	77.50±12.40	67.00±10.83	34.02±10.83^{a}	8.18±4.40	0.95± 0.31^{b}	17.00±4.24
	W$_4$	76.25±2.50	38.00±4.53	15.93±4.53^{b}	4.37±0.39	0.700±0.14^{b}	12.50±2.38
LVS	W$_1$	101.00±1.73	83.00±20.20	95.1±70.3	18.63±15.43	2.00±0.20	25.43±4.41
	W$_2$	129.70±29.0	85.00±27.10	73.0±41.0	28.00±14.38	3.17± 0.40	19.33±5.13
	W$_4$	119.00±9.54	40.00±10.00	77.50±13.16	20.8±17.60	1.100±0.200	16.00±3.46

Means followed by same superscript in a column are not significantly different (P>00.5).

Table 2. Effect of irrigation interval on artemisinin content in *A. annua* (µg/ml)

Treatment	EVS	MVS	LVS
Daily irrigated (W$_1$)	5.65 ±1.20^{c}	13.95 ± 5.37^{b}	18.54 ±4.69^{b}
2 days interval (W$_2$)	5.22 ± 1.62^{c}	10.62 ± 3.91^{b}	16.02 ±3.31^{b}
4 days interval (W$_4$)	5.96 ± 2.49^{c}	33.60 ±19.6^{a}	33.5 ±17.5^{b}
6 days interval (W$_6$)	5.99± 2.32^{c}	-	-

EVS: Early vegetative stage. MVS: Mid vegetative stage. LVS: Late vegetative stage
Means followed by same superscript in a column are not significantly different (P>00.5). n=3

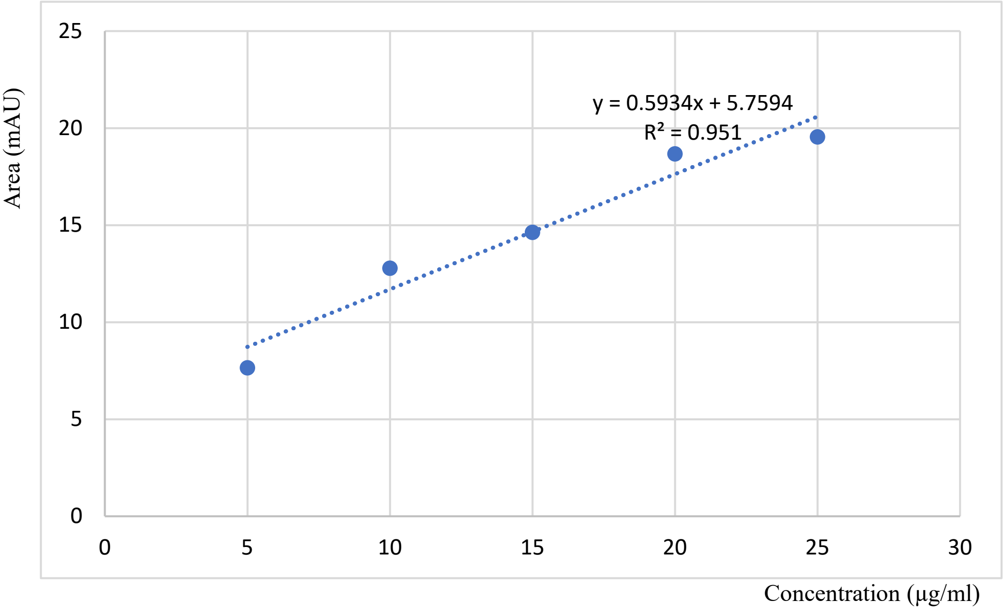

Figure 1. Linear calibration curve for Artemisinin standard

Discussion

The effect of irrigation interval at early vegetative stage on growth parameters of *A. annua* shows that W_2 day's interval has significantly ($p<0.05$) affected the fresh weight, dry weight, stem diameter and root length during the first two month of imposed irrigation interval. This was in agreement with the work of MARCHESE & al. (2010) that treatment of 38 hours water deficit induced the highest foliar biomass accumulation and artemisinin content per plant on dry weight basis, compared with control. Plant height and branch number was also higher at this level, this increase in growth parameters was un-expected because water stress generally decline or seize plant growth and biomass accumulation. This indicate that *Artemisia annua* require moderate moist water environment. Treatments that were watered once after two days interval grow better than any other treatment including control. Plant height decreased with increase in irrigation interval in this irrigation interval order once after two days interval > once after four days interval > once after six days internal. Similarly AZHAR & al. (2011) indicated that plant height, fresh and dry weight of plants was reduced significantly with increasing water stress levels in *Trichyspermum ammi* L. BETTAIEB & al. (2011) reported that *Cuminum cyminum* exposed to water stress caused reduction in growth attributes like plant height, number of branches, fresh and dry matter as well as yield components like number of umbels/plant and umbellets per umbel.

In moderate (W_4 days) and severed (W_6 days) irrigation interval, branch number, fresh weight, stem diameter and root length decrease greatly with increase in irrigation interval imposed. This result agreed with YADAV & al. (2014) who state that water stress significantly affected the plant growth parameters at all four stages of development (early vegetative, mid stage, late vegetative stage and full bloom stage). Irrigation intervals significantly ($p<0.05$)

affect the stem diameter. The extent of decline progressed proportionally to the severity of irrigation interval.

At mid vegetative stage, mild (W_2) irrigation interval greatly influences the plant height and branch number of *A. annua.* Plant height and branch number observed on this treatment was higher than all other treatment including control. Mild irrigation interval was found to significantly increase fresh weight ($P<0.05$). This contradict the result of BRISIBE & al. (2012) who reported that *A. annua* plant that were watered automatically using time controller performed better than those with intermittent periods of water stress and stem diameter is significantly affected by irrigation interval ($P<0.05$) which is directly proportional to the severity of irrigation interval. The highest root length was also observed at mild irrigation interval (17.00 cm) follow by moderate (12.50 cm) and then control (11.33 cm). This is because availability of moisture on the soil surface limits the roots extension. All stands on severe irrigation interval (W_6) die after one month of the imposed irrigation interval in green house which is contrary with our field result that survived for two weeks without water at forestry research farm.

At late vegetative stage, mild irrigation interval (W_2) has a positive influence on plant height, branch number, branch length, dry weight and stem diameter. At this stage, control had the lowest plant height and branch number among the treatment tested following by treatment with four days interval. This result was un-expected because water stress generally affects all plant parameters but the highest plant height and branch number were observed at W_2 treatment. Root length of *A. annua* decreased with increase in irrigation intensity with the highest in control followed by W_2 and W_4 days' interval respectively.

The effect of irrigation interval on artemisinin content of *Artemisia annua* showed a contrasting pattern of artemisinin concentration at all the three stage of development. Our finding revealed that irrigation interval has no significant effect on artemisinin content during early vegetative stage of *A. annua* (one month of imposed irrigation interval). Similar report were made by CHARLES & al. (1993) who found no significant correlation between artemisinin concentration and the water deficit applied for two weeks before harvesting, although there was a trend for artemisinin concentration to decrease with increased negative water potential. However, artemisinin content was significantly ($P<0.05$) affected by irrigation interval at mid vegetative stage. The significant increase in the leaf artemisinin content resulting from the treatment with moderate irrigation interval (W_4 days interval) can be attributed to the decrease in all the growth parameters evaluated on this treatment (plant height, branch number, fresh weight, dry weight, stem diameter and root length while photosynthesis is still occurring. Thus, the excess photo-assimilates not used in growth, were expected to be directed towards artemisinin biosynthesis. Many researchers like; MARCHESE & FIQUIRA, (2005) are on the above opinion. Similar result was reported by MARCHESE & al. (2010) that 38 hours water deficit significantly increase artemisinin content by 29% more than control. This also agrees with HERMS & MATTSON (1992), who reported that terpenes tend to accumulate under moderate water deficit. STAUDT & CHUINE (2005) found that increase in temperature and water deficit had significant effect on mono and sesquiterpenes emissions of *Artemisia* species. However, DUHL & al. (2007) reported that severe drought reduced the emissions of sesquiterpenes.

At late vegetative stage of *Artemisia annua*, artemisinin content was significantly affected by irrigation interval and increase significantly with the advancement in plant developmental stage. They increase in this order EVS>MVS>LVS at control and mild irrigation interval. This was in conformity with the work of YADAV & al. (2014) that artemisinin content increase with the progression in plant developmental stage. However, *A. annua* plants can tolerate some changes in water availability and temperature without a drastic decrease in artemisinin content. Thus, it is important to note that results in artemisinin content may vary depending on the origin of *A. annua* cultivar and on the regional environmental conditions.

Conclusion and Recommendation

This study concluded that irrigation interval had an effect on both growth and artemisinin content and suggested two days (W_2) irrigation interval for better growth performance and biomass production and four days (W_4) interval for high artemisinin content in *A. annua* at Savannah region of Nigeria.

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Research Article

PESTICIDAL PLANT SPECIES IMPACT ON WHIP SMUT REDUCTION AND SUGARCANE (*SACCHARUM OFFICINARUM* L.) PRODUCTION IN NIGERIA

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Abstract: Yield decline due to Whip smut is an issue that has plagued sugarcane production in Nigeria. The objective of the study was to evaluate the inhibitory effect of pesticidal plants at varying rates of application on whip smut disease for sugarcane genotypes production. Green house experiment was conducted in 2018 and 2019 at National Cereals Research Institute, Badeggi in the Southern Guinea Savanna ecology of Nigeria. The treatments consists of factorial combination of two sugarcane genotypes, B47419 and NCS 008, three plant extracts, (Moringa, Neem, Cassia) and three levels of application rates (40, 60, 80 g/l) arranged in a completely randomized design (CRD) replicated three times. The results showed that genotype B47419 suppressed *Sporisorium scitamineum* germination and penetration than NCS 008, and gave a higher growth and yield of sugarcane than NCS 008. Brix content was highest under B47419 than NCS 008. The application of Cassia plant extracts at the rate of 80 g suppressed *Sporisorium scitamineum* germination and penetration, produced higher growth and yield of sugarcane. In conclusion, application of Cassia plant extracts at 80 g was the best in managing *Sporisorium scitamineum*, effectively boast brix content and sustain the productivity of sugarcane in the study area and related ecologies.

Keywords: *Sporisorium scitamineum*, sugarcane disease, pesticidal plants, whip smut.

Introduction

Sugarcane (*Saccharum officinarum* L.) is a significant commercial cash crop widely grown by farmers in most tropical countries grown for the production of sugar, molasses, livestock feeds, alcohol, bagasse for fuel [BASSEY & al. 2021], and trash for mulching and as organic fertilizer [BASSEY & al. 2022]. Sugarcane contributes 60% of the total world sugar requirement, while the remaining 40% comes from sugar beet [BASSEY & al. 2023]. Whip smut (*Sporisorium scitamineum*) of sugarcane is the most serious and widely spread disease of sugarcane and causes a significant reduction in cane quantity and quality [WADA & ANASO, 2016; RAJPUT & al. 2021]. The severity of this disease often depends on the pathogen races, environmental conditions, cultivar genotype and the interaction among these three factors.

In addition biotic factors especially diseases effects limit optimum sugar cane yield [BASSEY & WADA, 2024]. Reduction in yield components and cane yield losses due to whip smut disease has been reported by WADA (2018). Besides, in this agroecology in Nigeria, the common sugar cane types under production are the chewing cane for human consumption and commercial cane as raw materials for the sugar and sugar-related industries. One potential way

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to manage smut incidence and improve sugarcane production among small land holders to meet the demand for sugar is by the use of pesticidal plant species.

There is an urgent need to investigate pesticidal plants as options to safe and boost the production of sugarcane genotypes in order to meet the needs of increasing population. Hence, the objective of the study was to evaluate the inhibitory effect of pesticidal plants at varying application rates on whip smut disease for sugarcane genotypes production in this study area.

Materials and methods

Green house trial was conducted at the sugarcane experimental screen house of the National Cereals Research Institute, Badeggi (latitude 9°45′ N, longitude 0.6°07′ E) in the Southern Guinea savanna agro-ecological zone of Nigeria in 2018 and 2019 rainy season.

At the commencement of the experiment, fresh smut whips were collected in the early hours of the day between 6.30-7.30 a.m. following the method of NASR (1977) and modified by WADA (2005). They were dried for one hour under the shade, scrubbed with hands covered with sterile gloves to obtain smut teliospores. The teliospores were sieved using a mesh. Ten grams (10 g) of the sieved teliospores were weighed and sealed in cellophane bags and stored in the refrigerator in the laboratory for inoculation process at a later date.

The smut teliospore suspension was prepared by adding one liter of sterilized distilled water which was subsequently emptied into 25 liters of sterilized water and stirred vigorously to obtain a homogenous suspension of the teliospores. The suspension aseptically inoculated on 3-budded sugar cane sets for 1 hour. They were then removed and incubated overnight in wet sterile jute for 14 hrs and subsequently planted out in the green house for further investigations.

Fresh leaves of *Azadirachta indica* (neem), *Moringa oleifera*, *Senna alata* (syn. *Cassia alata*) were washed with tap water and then with distilled water weighed (100 g) before grinding into paste with piston and mortar. The paste obtained were used to prepare three concentrations 40 g/l, 60 g/l and 80 g/l. The plant extracts were prepared by soaking the earlier prepared *Azadirachta indica* (neem), *Moringa oleifera*, *Cassia alata* leaves powder in sterile water overnight.

The inoculated cane cuttings were immersed in each of the extract [WADA, 2005].

The total rainfall during the experimental period was 1500.5 mm in 2018 and 1015.8 mm in 2019, respectively. The mean air temperature during the sugarcane plant cropping season was 33 to 34 °C in 2018 and 33 to 36 °C in 2019 plant cropping seasons (Table 1).

The treatments consists of factorial combination of two sugarcane genotypes, B47419 and NCS008, three plant extracts (Moringa, Neem, Cassia) and three levels of application rates (40, 60, 80 g/l) arranged in a completely randomized design (CRD) replicated three times. Tender healthy young stalks of six months old sugarcane were used as planting material. The stalks were cut into setts each containing three eye buds and planted in pots filled with soil. Agronomic practices from fertilizer application, weeding and watering were carried out at the required growth stages of the sugarcane until termination of the experiment. Weeding was done by hand pulling one week before the application of fertilizer. Irrigation was done twice a week.

All data collected were subjected to analysis of variance (ANOVA). The means were separated using Duncan multiple range test at 5% level of probability using SAS version 9.0 statistical package.

Results and discussions

Smut incidence at three months after planting (MAP) was significantly ($p<0.01$) observed on NCS008 than B47419 in 2018 season. There was no difference found in plant extracts used. Application rate of 80 g/l obtained the lowest smut incidence than the lower rates. There was no interaction between the treatment combinations on smut incidence at 3MAP in 2018 (Table 2). In 2019, smut incidence was lowest on B47419. *Moringa* plant extract significantly ($p<0.01$) had the least incidence at 3 MAP. Application rate of 80 g/l produced the least incidence of smut than the lower rates tested. There was no interaction between the treatment combination tested on smut incident in 2019 (Table 2). Smut incidence at 6 MAP was significantly affected by varieties in 2018. Plant extract had no effect on smut incident at 6 MAP. Application of 80 g/l of extract had the least incidence of smut compared to the lower rates (Table 2). In 2019, there was no significant effect of varieties on smut incident at 6 MAP. *Moringa* plant extract produced the least incident than the other extract tested. Application of 60 g/l of extract produced the least smut incident (Table 2). The superiority of B47419 in having lower Smut disease incidence than NCS008 can be attributed to the differences in the genetic makeup, fast establishment, higher tillering ability and quick canopy formation of the variety which was influenced by adequate rainfall and temperature. This result is in accordance with those of BASHIR & al. (2012) and FIGUEROA-RODRÍGUEZ & al. (2019) who reported that significant difference exist among Smut incidence of sugarcane genotypes due to genetic makeup and conductive environment.

Table 1. Temperature and rainfall distribution from 2018 to 2019 cropping season at Badeggi

Months	**Temperature (°C)**		**Rainfall (mm)**	
	2018	**2019**	**2018**	**2019**
January	36	35	0.0	0.0
February	38	37	0.0	0.0
March	39	40	95.4	4.0
April	40	39	18.7	4.5
May	36	34	220.6	274.9
June	32	35	286.8	146.4
July	31	32	346.1	161.8
August	31	31	203.8	251.7
September	30	30	273.5	202.1
October	33	32	59.2	28.2
November	34	35	0.0	0.0
December	35	33	0.0	0.0
Total	415	375	1504.1	1073.6

Source: Weather station, National Cereals Research Institute Badeggi.

Table 2. Effects of sugarcane genotypes, plant extracts and application rates on Smut disease incidence at 3 and 6 MAP in 2018 and 2019 seasons

Treatments	Whip Smut incidence			
	3 MAP		6 MAP	
	2018	2019	2018	2019
Genotypes (S)				
B47419	2.78	4.65	3.00	4.50
NCS008	4.61	5.15	3.00	4.22
LSD (0.05)	0.71	0.4	0.41	0.5
Extract (E)				
Moringa	3.69	4.17	3.00	2.92
Neem	3.69	5.03	3.00	5.42
Cassia	3.69	5.50	3.00	4.75
LSD (0.05)	0.81	0.5	0.41	0.6
Rates (R)				
40	3.83	5.33	3.25	4.92
60	3.52	4.81	2.92	4.53
80	3.43	4.56	2.83	3.83
LSD (0.05)	0.81	0.5	0.48	0.7
Interaction	NS	NS	NS	NS

LSD – least significant difference, NS – not significant, MAP – months after planting

Sugarcane stalk height at 6 MAP was significantly affected by varieties in 2018 (Table 3). NCS008 significantly produced higher stalks than B47419 in 2018 season. The application rate of 80 g/l had more stalk than the lower rate applied (Table 3). The interaction between varieties, plant extract, solvent and rate was not significant in 2018. In 2019 season, varieties had no effect on stalk high at 6 MAP. Similarly, Cassia plant extract significantly had higher stalks than the other extract tested. The application rate of 80 g/l had more stalk than the lower rate applied (Table 3). The interaction between varieties, plant extract, solvent and rate of application on stalk height was not significant in 2019 (Table 3). The production of consistently taller sugarcane plant and stalk in plots with NCS008 can be attributed to their erect stools at maturity, exhibition of heavy stool habit influenced by good environmental conditions, efficient moisture use, better weed competitiveness, tolerant to drought and variation in the leaf architecture. This finding is also in agreement with the findings of CHEEMA & al. (2010) who reported that, industrial sugarcane establishes faster because of its ability to withstand drought and diseases, this in turn translated into faster growth and development.

The effect of varieties, plant extract and rate of application on stalk girth at 6 MAP in 2018 season was not significant. Also, the interaction between the treatments on stalk girth at 6 MAP was not significant (Table 3). Significant effect was found in 2019 on stalk girth at 6 MAP. NCS 008 was significantly (P<0.01) higher than B47419. Furthermore, Cassia plant extract produced thicker stalk than the other extract tested in 2019 (Table 3). The rate of application 80 g/l significantly (P<0.01) produced thicker stalk than the lower rates. There was no significant effect on the interaction among the treatments on stalk girth at 6 MAP in 2019 (Table 3). The superiority of NCS008 in having bigger stalk girth than B47419 can be attributed to low stool habit, more robust and soft stem with high water and less sucrose content impacted by favorable rainfall and temperature. This confirms the finding of AHMED & al. (2014) who reported that, local canes has more robust, soft stem with high water and less sucrose content than the industrial canes.

Brix content at 8 MAP was significantly affected by varieties such that NCS008 had higher brix than B47419 in 2018. There was no difference among the plant extract tested. Furthermore, application rate of 80 g/l significantly produced higher Brix content than the lower rates at 8 MAP in 2018. In 2019, brix content at 8 MAP was higher in NCS008. Cassia plant extract significantly had higher brix than other extract tested. The application rate of 80 g/l produced higher brix than the lower rate tested. There was no interaction between the treatments (Table 3). The higher brix content observed in the genotype NCS 008 compared with the B47419 could be attributed to growth habit affected by beneficial environmental conditions, efficient use of applied inputs, better competitive ability to suppress weeds and tolerance to drought. This is in agreement to the findings of AHMED & al. (2014), HASSAN & al. (2017) and FIGUEROA-RODRÍGUEZ & al. (2019) who reported that, improved sugarcane (industrial canes) genotypes have thin stems with high sucrose content and less water due to the varied morphological differences that exist among the genotypes.

B47419 significantly produced more single stalk weight (SSW) than NCS008 in 2018. There was no significant difference among the plant extract used. SSW was higher when 80 g/l application rate was used than the other lower rates (Table 3). There was no interaction between the treatment combinations on SSW in 2018 season. In 2019, SSW was higher in NCS008 than B47419. Cassia plant extract performed better than other plant extracts used. Similarly, application of 80 g/l produced more SSW than the lower rates. There was no interaction between the treatment combinations on SSW in 2019 season (Table 3). Cane yield of NCS008 was significantly ($P<0.01$) higher than B47419 in 2018 season. There was no difference among the plant extract used on cane yield in 2018. The application rate of 80 g/l produced more cane yield than the lower rates tested (Table 3). In 2019, the yield of NCS008 was higher than that of B47419. Similarly, Cassia plant extract had more yield than the other extracts tested. The 80g application rate produced more cane yield than the other lower rate used (Table 3). Cassia plant extract in combination with application rate of 40-80 g/l consistently produced higher cane yield in 2018 season.

In 2019 season, cane yield was higher in combination with Neem/Cassia extract at 80 g/l rate of application (Table 3). The higher sugarcane yield of NCS 008 than that of B47419 in each year of this study could be due to its genetic make up, fast establishment influenced by favourable environmental conditions (Rainfall and temperature), higher tillering ability, quick canopy formation, heavy residue production, heavy stooling ability and better weed competitive ability, which was more efficient in exploiting growth contributing factors than B47419. This result is in accordance with those of BASHIR & al. (2012) and FIGUEROA-RODRÍGUEZ & al. (2019) who reported that significant difference exist among cane yield of sugarcane genotypes due to genetic makeup and good environmental conditions.

Table 3. Effects of sugarcane genotypes, plant extracts and application rates on Stalk height, Girth, Brix content, Single stalk weight and Cane yield at harvest in 2018 and 2019 seasons

Treatment	**Stalk height (cm)**		**Stalk girth (cm)**		**Brix content (%)**		**Single stalk weight**		**Cane yield ($t\ ha^{-1}$)**	
	2018	**2019**	**2018**	**2019**	**2018**	**2019**	**2018**	**2019**	**2018**	**2019**
Genotypes (S)										
B47419	111.1	124.3	1.21	1.33	17.04	18.02	0.73	0.95	19.35	22.08
NCS008	137.2	123.3	1.47	1.44	19.02	18.98	1.19	1.19	32.39	26.04
LSD (0.05)	7.6	4.1	0.86	0.1	0.49	0.1	0.6	0.1	1.25	0.95
Extract (E)										
Moringa	124.1	102.8	1.34	1.20	18.03	17.29	0.96	0.67	25.87	17.97
Neem	124.2	128.4	1.34	1.40	18.03	18.00	0.96	1.04	25.87	24.25
Cassia	124.1	140.1	1.34	1.54	18.03	20.21	0.96	1.32	25.87	29.96
LSD (0.05)	9.4	6.1	0.11	0.1	0.6	0.1	0.8	0.1	1.5	1.17
Rates (R)										
40	107.0	121.7	1.30	1.34	17.64	18.36	0.80	0.92	23.26	22.88
60	125.4	125.0	1.26	1.38	17.44	18.46	0.97	1.03	26.91	24.34
80	139.9	124.3	1.46	1.42	19.02	18.68	1.12	1.08	27.45	24.95
LSD (0.05)	9.4	5.0	0.11	0.1	0.6	0.1	0.82	0.1	1.52	1.17
Interaction										
S x W	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

LSD – least significant difference, NS – not significant.

Conclusion

Application of Cassia plant extracts at 80 g was the best in managing *Sporisorium scitamineum*, effectively boast brix content and sustain the productivity of sugarcane in the study area and related ecologies.

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Research Article

COMPARATIVE EFFICACY OF NPK FERTILIZER AND POULTRY DROPPINGS ON THE GROWTH AND YIELD OF *AMARANTHUS HYBRIDUS* L.

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Abstract: The persistent reduction in soil fertility as a result of continuous cultivation of the soil has become a problem to vegetable farmers, both inorganic and organic fertilizers have been recommended as good sources of nutrients. This study sought to compare the performance of NPK (15:15:15) and poultry manure at different application rate on the growth and yield of *Amaranthus hybridus*. The experiment was done in duplicate and laid out using a Randomized Complete Block Design (RCBD) with each plot measuring 1.2 m by 1.2 m. The plant height (117 cm), leaf length (22.70 cm), leaf width (14.60 cm), leaf area index (12.10 cm^2), fresh weight (387.58 g) and dry weight (54.20 g) were significantly ($P<0.05$) higher in plant with application rate of 15 g poultry dropping compared to NPK fertilizer and the control. Poultry droppings (15 g) is more efficient to be used by farmers for optimum growth and yield of *A. hybridus*.

Keywords: leaf, inorganic, organic, plant, weight.

Introduction

Amaranthus hybridus L., commonly referred to as amaranth, is a member of the Amaranthaceae family, which includes over 60 species [ANJALI, 2013]. They are primarily monoecious but, few are dioecious. The high nutritional value of this crop has attracted the attention from various nations globally [SRIVASTAVA, 2011]. Its leaves constitute a cheaper and rich source of protein, carotenoids [SHUKLA & al. 2006] and minerals and vitamins [MUYONGA & al. 2008]. Due to farmers' desire to fulfill the rising demand for agricultural products, there has been a surge in the need for mineral fertilizers in emerging nations in order to raise crop production. The need to sustain and increase yields is one of the agricultural difficulties that are becoming more prominent due to the growing global population and changing eating preferences [ADEDEJI & al. 2019].

Inorganic fertilizers are produced using synthetic substances or extracted from mineral deposits and, are easily accessible nutrients to plants. They include: Nitrogen (N), Phosphorus (P) and Potassium (K) which are suitable for crop production in the tropics to improve the fertility of the soil. However, their use has not always been successful in the tropics due to increased soil acidity, easy nutrient leaching, low organic matter status, decreased crop yield, and degradation of soil physical properties. The ingredients of NPK fertilizer are important and necessary, for plant growth. The usage of NPK fertilizer is crucial to the agriculture sector's ability to feed the world's population and guarantee healthy crops but, because of its high cost

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and negative effects on the environment, impoverished farmers cannot afford it and, are therefore not a desired option [EKWEALOR & al. 2021].

Organic manure contains high amount of nitrogen, phosphorus, potassium and other essential nutrients which are essential for soil fertility [OYEWOLE & OYEWOLE, 2011]. In contrast to inorganic fertilizer, organic fertilizer adds organic matter to the soil which improves soil structure, nutrient retention, aeration, soil moisture-holding capacity and water infiltration [DEKISSA & ALLEN, 2008]. Organic manure such as cow dung and chicken manure help to improve the soil by providing nutrients for growing crops and it also improves the soil quality because of its high organic matter content with available nutrients for plant growth. Although organic manure exists in readily available forms, cheap and easy to access, they need to be applied in large amounts to meet the nutrient requirement of crops [PRABU & al. 2003].

The persistent reduction in soil fertility as a result of continuous cultivation on the soil is becoming a problem to vegetable farmers. Application of poultry droppings to crops have been found to be an effective and cheap source of nutrient for sustainable production of vegetable crops [SHAGUFTA, 2012]. Poultry droppings contain significant amount of the macro and micro nutrients as well as organic matter that is capable of improving the soil aeration capacity, microbial activities, texture, structure and porosity levels that are very crucial for growth and yields of vegetables [MUSA & al. 2020]. ANO & AGWU (2005) reported that vegetables cultivated using organic manures such as poultry droppings are gaining popularity because of less chemical residue and better taste. This research compared the performance of inorganic fertilizer (NPK 15:15:15) and organic fertilizer (poultry manure) at different application rate on the growth and yield of *Amaranthus hybridus*.

Materials and methods

Study location

This study was carried out at the Botanical Garden (9°20'59"N, 12°29'7"E), of the Department of Plant Science, Modibbo Adama University, Yola. *Amaranthus hybridus* seeds and NPK (15:15:15) fertilizer were obtained from an agro-allied store while the poultry droppings used for the study was obtained from a poultry farm in Jimeta, Adamawa State Nigeria.

Sowing of seeds and experimental design

Seeds of *Amaranthus hybridus* were sown on a well-prepared nursery bed. The seedlings were transplanted one week after germination. Field experiment was done in duplicate and laid out using a Randomized Complete Block Design (RCBD) with a total of sixteen plots, each measuring 1.2 m by 1.2 m. Eight plots each were assigned to poultry and NPK treated seedlings.

Different mass of NPK fertilizer and poultry droppings (5, 10, 15 g) were applied to the soil beside each seedling on each plot except for the control (0 g) plots where neither NPK fertilizer nor poultry droppings was applied. The experiment was carried out for a total duration of seven weeks.

Data collection

Plant height: height (cm) of three randomly tagged amaranth plants was measured and recorded using a meter rule at five weeks after transplanting of the seedlings.

Number of leaves: number of leaves on three randomly tagged amaranth plants were counted and recorded at five weeks after transplanting of the seedlings.

Leaf length: leaf length (cm) of three randomly tagged amaranth plants was measured from the leaf apex to the leaf base using a meter rule at five weeks after transplanting of the seedlings.

Leaf width: leaf width (cm) of three randomly tagged amaranth plants was measured from the leaf apex to the leaf base using a meter rule at five weeks after transplanting of the seedlings.

Leaf area: leaf area was determined using a portable leaf area meter (AM 350 model).

Leaf area index: leaf area was calculated by dividing the leaf area (m^2) with the ground area (m^2) according to the method of AHMAD & al. (2015).

Fresh weight: whole plants were uprooted for each application rate in each replication and weighed using a digital weighing balance (Sartorius ED2245 model) to obtain the fresh weight in gram, five weeks after transplanting.

Dried weight: whole plant was oven dried at a temperature of 70°C for 55 minutes and weighed using a digital weighing balance (Sartorius ED2245 model) to obtain the dry weight in gram, five weeks after transplanting.

Data analysis

One-way analysis of variance was used to compare the growth performance of amaranth subjected to different application rate of NPK and poultry fertilizer using R software version 4.05, Tukey's Honest Significant Difference was used to separate means where significant. All tests were done at $P \leq 0.05$.

Results and discussion

The comparative effect of NPK fertilizer and poultry droppings on the growth and yield of *Amaranthus* is shown in Table 1.

Plant height of *Amaranthus* varied significantly across ($P<0.05$) the different treatments. The plant with the highest height (117 cm) were those on the soil that was treated with 15 g of poultry droppings while the lowest (68.80 cm) was in the control. The increase in height may be due to favourable nutrient mineralization. A similar report has been made by OLOWOAKE & OJO (2014) in *Amaranthus caudatus* where compost manure favoured plant height over NPK fertilizer. Number of leaves in each treatment did not vary significantly ($P>0.05$), however the highest number of leaves (36.80) was noted in plants treated with 15 g of poultry droppings and the lowest (24.20) was in the control treatment. Number of leaves in *Amaranthus* is an indication of the yield and the high number of leaves produced by 15 g of poultry droppings could be due to sustained release of nutrients in 15 g of poultry droppings over the other treatments. It depicts the efficiency of organic fertilizers in promoting the vegetative growth and yield of *Amaranthus*. This conforms to the report of YASSEN & al. (2009) that organic fertilizers (chicken manure and farm yard manure) improve the vegetative characters of spinach.

Table 1. Effect of NPK and poultry droppings on the growth and yield of *Amaranthus hybridus*

	App. rate	**PH (cm)**	**NL**	**LL (cm)**	**LW (cm)**	**LA (cm^2)**	**LAI**	**FW (g)**	**DW (g)**
NPK	**0g**	68.8 ± 0.75^{b}	24.2 ± 0.75^{a}	16.1 ± 0.125^{c}	9.28 ± 0.225^{b}	112 ± 1.85^{a}	7.01 ± 0.120^{b}	103 ± 0.900^{c}	15.0 ± 0.850^{e}
	5g	88.8 ± 8.25^{ab}	47.2 ± 19.2^{a}	20.8 ± 2.00^{abc}	12.9 ± 1.07^{ab}	202 ± 36.0^{a}	12.6 ± 2.26^{ab}	256 ± 10.2^{abc}	34.7 ± 1.07^{c}
	10g	73.0 ± 7.5^{b}	30 ± 1.00^{a}	19.9 ± 0.125^{abc}	12.2 ± 0.25^{ab}	183 ± 2.58^{a}	11.4 ± 0.160^{ab}	137 ± 16.3^{bc}	21.3 ± 0.890^{d}
	15g	90.8 ± 7.75^{ab}	35.2 ± 0.75^{a}	22.1 ± 0.875^{a}	12.6 ± 0.0250^{ab}	286 ± 69.1^{a}	11.6 ± 1.93^{ab}	246 ± 44.3^{abc}	35.5 ± 1.18^{c}
PD	**5g**	69.5 ± 5.00^{b}	25.8 ± 1.75^{a}	16.6 ± 0.625^{bc}	9.38 ± 0.125^{b}	117 ± 5.95^{a}	7.31 ± 0.370^{b}	127 ± 16.2^{bc}	25.3 ± 1.36^{d}
	10g	100 ± 9.25^{ab}	31.2 ± 1.25^{a}	21.4 ± 0.625^{ab}	12.1 ± 0.675^{ab}	194 ± 16.5^{a}	12.1 ± 1.03^{ab}	276 ± 18.5^{ab}	46.5 ± 1.15^{b}
	15g	117 ± 5.50^{a}	36.8 ± 3.25^{a}	22.7 ± 0.190^{a}	14.6 ± 1.88^{a}	249 ± 29.8^{a}	15.5 ± 1.86^{a}	387 ± 58.8^{a}	54.2 ± 1.20^{a}
	***P*-value**	0.0112 *	0.449	0.00634 **	0.0274 *	0.0591	0.031 *	0.00266 **	4.37e-07 ***

KEY: App.: application, PH – plant height, NL – number of leaves, LL – leaf length, LW – leaf width, LA – leaf area, LAI – leaf area index, FW – fresh weight, DW – dry weight, NPK: Nitrogen, Phosphorus, Potassium; PD: Poultry Droppings. Means with different superscript along each column are significantly different. * – significant, ** – highly significant, *** – very highly significant

Leaf length and width varied significantly across the different application rates of the fertilizer. The highest in leaf length (22.70 cm) and leaf width (14.60 cm) was recorded in plants treated with 15 g of poultry droppings and lowest in the control plant with values of 16.10 cm and 9.28 cm respectively. Leaf area did not vary significantly ($P>0.05$) in the accessions studied, the highest (286 cm^2) was recorded in 15 g of NPK treated plants and the lowest (112 cm^2) was in the control treatment. The increased leaf length and leaf width due to treatment with organic fertilizer could in turn lead to a large surface area of the plant which will affect the photosynthetic ability of the plant and in turn the general productivity of the plant. This is in conformity with the findings of EKWEALOR & al. (2021) which showed that application of organic fertilizer improved the leaf length, width and area of cabbage.

Leaf area index, fresh weight and dry weight was highest (15.5, 387 g and 54.2 g) in plant treated with 15 g poultry and lowest (7.01, 103 g and 15 g) in the control plant. This report indicates that poultry droppings increased the leaf production and as well as improved the biomass production of *Amaranthus*. The leaf area index (LAI) is an important variable used to evaluate many processes such as canopy, photosynthesis and evapotranspiration which play an important role in the transformation of energy and mass between the atmosphere and plant canopy [AHMAD & al. 2015]. The report of MUSA & al. (2020) that poultry manure significantly enhanced the growth and yield of okra is in conformity with this study.

Conclusion

Both NPK fertilizer and poultry dropping significantly improved the growth and yield of *Amaranthus*. However, 15 g application rate of poultry dropping was the best for most of the growth and yield parameters studied. It is recommended that 15 g of poultry droppings be used by farmers for each plant stand to obtain optimum growth and yield of *Amaranthus hybridus*.

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Research Article

SOIL AND SUGAR QUALITY IMPROVEMENT INFLUENCED BY MULCHING AND WEED MANAGEMENT PRACTICES IN NIGERIA

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Abstract: Yield decline is an issue that has plagued sugarcane production in Nigeria. The objective of the study was to evaluate the effects of sugarcane trash mulch and weed management practices on soil C and N, and sugar quality production. The treatments consisted of factorial combination of two sugarcane genotypes (Bida local and NCS 001), four sugarcane trash mulching levels (0, 3, 6, 9 t ha^{-1}) and four weed management practices: weedy check, 5 monthly hoe weeding (5MHW), pre-emergence (PE) application of diuron at 2 kg a.i./ha + Post-Emergence (POE) of 3-Maize force at 179.2 g/ha + Two hoe weeding (2HW), and PE Diuron + POE 3-Maize force arranged in a split plot design and replicated three times. The results showed that application of 9 t ha^{-1} trash mulch significantly ($P<0.05$) produced the highest organic carbon, N, which in turn enhanced sugar yield. Similarly, the application of 9 t ha^{-1} trash mulch plus PE+POE produced the highest NCS 001 sugar quality. Application of trash mulching at 9 t ha^{-1} plus PE+POE effectively enhanced soil C and N, and sustained the productivity of NCS 001 sugar quality in the study area and related ecologies.

Keywords: genotypes, soil properties, sugar quality, trash mulch, weed management.

Introduction

Sugarcane (*Saccharum officinarum* L.) family Poaceae is widely grown crop in Nigeria. It provides employment to over a million people directly or indirectly besides contributing significantly to the national exchequer [FAOSTAT, 2019]. It is widely grown in several tropical and subtropical countries of the world accounting approximately, 75% of world's sucrose production from sugarcane. Besides the production of raw sugar, of which sugarcane is mainly produced for, sugarcane also represents an important source of renewable energy which has recently gained attention because of ethanol production [PRIYANKA & al. 2019]. In Nigeria, it is grown on an estimated land area of over 500,000 hectares with a yield potential of over three million metric tons of sugarcane [BASSEY & al. 2021].

In the presence of climatic change, land degradation and biodiversity loss, soils have become one of the most vulnerable resources in the world [KANE, 2015]. Soils are a major carbon reservoir containing more carbon than the atmosphere and terrestrial vegetation combined. Soil organic carbon (SOC) is the main component of soil organic matter [KEILUWEIT & al. 2015]. As an indicator for soil health, SOC is important for its contribution to food production, mitigation and adaptation to climatic change. A high soil organic matter content provides nutrients to plants and improves water availability, both of which enhance soil fertility and ultimately improves food productivity [BALDOCCI & al. 2016]. Moreover, SOC

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improves soil structural stability by promoting aggregate formation which, together with porosity, ensures sufficient aeration and water infiltration to support plant growth [FAO & ITPS, 2015].

The gap between domestic production and the demand for sugar can be attributed to many factors. This include rapidly increasing population, increased demand for food, limited scope for extension of cultivation to new areas, diversified low yield potential, food scarcity, heavy importation and not self – sufficient in sugar production. The conventional cropping systems are exhaustive and depleting the soil badly, cultivable lands is decreasing due to urbanization and industrialization, enlarged families, and the current system of monocropping is not able to keep pace with increasing demands of farmers due low yield and subsistence farming is alarming [GEETHA & al. 2015; MOHAMMED & al. 2017].

One potential way to improve sugarcane production among small land holders and meet demand for sugar is by sugarcane trash mulching. Sugarcane genotypes vary in the production of trash which could be attributed to its high in-situ retention of trash as organic manures, replenishing soil quality by increasing soil nutrient status and enhancing chemical properties of the soil [CHOUDHARY & SINGH, 2016]. Weed management practices influences the addition of weed vegetation to the soil which decayed with time. This has also led to high accumulation of organic materials in the release of nutrients from decomposing microbial biomass, which translates to improved physical and chemical soil properties [AZADBAKHT & al. 2017; ABILOYE & al. 2018]. There is an urgent need to integrate these weed management options with trash mulching for improved soil fertility and boost the production of these sugarcane genotypes in order to meet the needs of increasing population. Hence, the objective of the study was to evaluate the effect of varying trash mulch and weed management practices on soil C and N, field sucrose and sugar quality production in the study area.

Material and methods

Field trial was conducted at the upland sugarcane experimental field of the National Cereals Research Institute, Badeggi (latitude 9°45ˈN, longitude 0.6°07ˈE) in the Southern Guinea savanna agro-ecological zone of Nigeria in 2016 and 2017 rainy season. At the commencement of the experiment, a composite sample from ten random points was collected, using a soil auger at 0-15 cm depth for both years. The initial soil properties of the experimental site are shown in Table 1. The total rainfall during the experimental period was 1504.1 mm in 2016 and 1045.4 mm in 2017, respectively. The mean air temperature during the sugarcane plant cropping season was 35 to 38 °C in 2016 and 34 to 36 °C in 2017 plant cropping seasons (Table 2). Prior to cultivation, the vegetative cover of the experimental site was manually cleared, ploughed and harrowed with a tractor. Tender healthy young stalks of six months old sugarcane were used as planting material. The stalks were cut into setts each containing three eye buds. The PE diuron was applied a day after planting at the rate of 2.0 kg a.i/ha while the POE 3-Maize force was applied at five weeks after planting (WAP) at rate of 179.2g a.i/ha. The weeds were identified using the hand book of West African Weeds [AKOBUNDU & al. 2016]. NPK fertilizer was applied at 150 kg N, 60 Kg P and 90 Kg K in equal halves at planting and 8 – 10 WAP. The treatments consists of factorial combination of two sugarcane genotypes, Bida local and NCS 001, four cane trash mulching levels, (0, 3, 6, 9 t ha^{-1}) and four weed management practices: weedy check, 5 monthly hoe weeding (5MHW), Pre-emergence of diuron at 2 kg a.i./ha (PE) + Post-Emergence (POE) 3-Maize force at 179.2 g/ha + Two hoe weeding (2HW), and PE diuron + POE 3-Maize force arranged as a split plot and replicated three times. Herbicides were applied with knapsack (CP3) sprayer at a spray volume of 4 l/ha. Weed management practices and mulching were allocated in the main plot while sugarcane genotypes in the subplot. The gross plot size was 35 m^2 (7 m x 5 m), while the net plot size was 17.5 m^2 (3.5 m x 5 m). Each net plot consists of four rows of 5 m long. All data collected were subjected to analysis of variance (ANOVA). The means were separated using Duncan multiple range test at 5 % level of probability using SAS version 9.0 statistical package.

Results and discussions

The physical and chemical properties of the soil before planting in 2016 and 2017 showed that the soil was sandy loam and slightly acidic (Table 1). The soils were low in nitrogen, phosphorus and other essential nutrients which were inadequate for sugarcane production. In 2016, higher organic carbon was found in plots grown with NCS 001 (Table 2). However, in 2017, organic carbon content was higher in Bida local than NCS 001. The result also shows that NCS 001 had significantly higher total nitrogen in 2017 (Table 3). Application of 9 t ha^{-1} trash mulch was associated with higher soil C and total N contents than that of the lower trash mulch rates (Table 3).With the exception of the weedy check, there was a significant increase in organic carbon and total nitrogen contents in all the other weed management practices in both years (Table 3). The application of Pre-emergence + Post-emergence herbicide produced the highest organic carbon and N. The interaction effects between trash mulch and weed management practices on soil nitrogen was significant (Table 4). Application of 9 t ha^{-1}mulch in combination with PE + POE and PE + POE + 2MHW had the highest Soil N in plant crop in 2016. The significant increase in organic carbon and total N in planted NCS 001 genotype, and only organic carbon content in Bida local genotype suggest their ability in producing and retaining sugarcane crop residues which was influenced by adequate rainfall and temperature. The retention of these residues can be considered as a method of arresting soil organic matter, which has the capacity to hold plant nutrients for sugarcane production [DU PREEZ & al. 2011].

Table 1. Initial soil physical and chemical properties in 2016 and 2017 cropping seasons

Parameter	2016 Site	2017 Site
Sand (g kg^{-1})	722	765
Silt (g kg^{-1})	135	156
Clay (g kg^{-1})	143	79
Textural class	Sandy loam	Sandy loam
pH (H_2O) (g kg^{-1})	5.80	6.40
Organic Carbon (g kg^{-1})	2.37	3.45
Total Nitrogen (g kg^{-1})	0.06	0.33
Available Phosphorus (mg kg^{-1})	20.29	23.15
Ca++ (cmol kg^{-1})	2.48	4.18
Mg++ (cmol kg^{-1})	1.38	3.68
K+ (cmol kg^{-1})	0.16	0.30
Na+ (cmol kg^{-1})	0.09	0.22
Exchangeable acidity (cmol kg^{-1})	1.03	1.07
ECEC (cmol kg^{-1})	5.14	9.45

Analyzed at National Cereals Research Institute Laboratory

Table 2. Temperature and rainfall distribution from 2016 and 2017 cropping season at Badeggi

	Temperature (°C)		**Rainfall (mm)**	
Months	2016	2017	2016	2017
January	36	35	0.0	0.0
February	38	37	0.0	0.0
March	39	39	95.4	4.0
April	40	39	18.7	4.5
May	36	35	220.6	274.9
June	32	33	286.8	146.4
July	31	31	346.1	161.8
August	31	30	203.8	251.7
September	30	30	273.5	202.1
October	33	33	59.2	28.2
November	34	35	0.0	0.0
December	35	34	0.0	0.0
Total	415	375	1504.1	1073.6

Source: Weather station, National Cereals Research Institute Badeggi

Sugarcane genotypes significantly influenced field sucrose (% brix) at 12 MAP (Table 3). Industrial sugarcane recorded the highest sucrose than the local chewing cane. This may be due to the fact that industrial sugarcane has relatively thin and hard stem, thick ring (nodes) and usually contains more sucrose and less water content impacted by favorable rainfall and temperature. This confirms the findings of BUSARI & al. (2009) and SHAH & al. (2009) which states that improved sugarcane (industrial canes) variety usually have thin stems with high sucrose content and less water due to their varied genetic potential of sugarcane genotypes. Furthermore, the highest Brix content was found in sugarcane plants given 6 t ha^{-1}, but similar to that in 9 t ha^{-1} in both years. Field sucrose was highest with the application of monthly hoe weeding which was similar to Pre-emergence + Post-emergence herbicide + 2 hoe weeding and application of Pre-emergence + Post-emergence herbicide only. The interaction effects between trash mulch and weed management practices on brix content was lowest in the non- application of mulch and weedy check treatment while mulch rate at 9 t ha^{-1} mulch in combination with PE + POE produced the highest brix content in plant crop in 2017 (Table 5). This may be attributed to effective weed control which resulted in increasing yield promoting attributes. Our result is in agreement with the previous findings of SMITH & al. (2009) and SINGH & al. (2011) which reported that all the weed control treatments favorably influenced the yield contributing characters such as stalk height, stalk girth and brix.

Sugarcane genotypes and trash mulching had no significant influence on percent polarity at harvest in both years (Table 3). Polarity at harvest was significantly ($P<0.05$) affected by weed management practices (Table 3). Polarity was highest with the application of Pre-emergence + Post-emergence herbicide which was similar to monthly hoe weeding in 2016 and 2017 cropping seasons. The difference in percent polarity of sugarcane genotypes was due to their varied genetic potential which exploit edaphic and aerial factors of crop production. These results are in accordance with those of SHAH &. al. (2009) and BASHIR & al. (2012) who reported significant difference among the sugarcane genotypes for cane polarity. Sugarcane genotypes had significant influence on percent purity at harvest. Industrial sugarcane recorded higher purity than the local chewing cane in both cropping seasons (Table 3). Furthermore, the highest percent purity was found in sugarcane plants given 9 t ha^{-1}, but similar to that in 6 t ha^{-1} in both years. Percent purity was highest with the application of Pre-emergence + Post-emergence herbicide which was similar to monthly hoe weeding in 2016 and 2017 cropping seasons. There was no significant influence of Sugarcane genotypes and weed management practices on percent fibre in both years of study. The variation in sugar quality for sucrose, polarity, purity and fibre could be attributed to heavy tillering, quick canopy formation and weed suppression which were enhanced by incorporation of sugarcane residues resulting in high SOC under the prevailing agro-ecological conditions. These results are in line with those of RASOOL & al. (2011) and GEETHA & al. (2015) who found significant variation in sugar quality for different residues/ sugarcane intercropping. The observed increase in sugar quality content might also be attributed to decrease in weed infestation, increased soil organic matter, improved physical and chemical properties and soil water regimes, which translates into better crop growth. This is in agreement with the work of NG CHEONG & TEELUCK (2015) and DE AQUINO & al. (2017) who reported that variation in sugar quality in sugarcanes could be attributed to varied varietal morphology and weed suppression under the prevailing agro-ecological conditions.

Table 3. Effects of sugarcane genotypes, mulch rates and weed management practices on organic C and N, field sucrose, polarity, purity and fibre at 12 MAP in 2016 and 2017 cropping seasons

Treatment	**Organic C (g kg^{-1})**		**Total N (g kg^{-1})**		**Field sucrose (% brix)**	
	2016	2017	2016	2017	2016	2017
Genotypes (S)						
Bida local	40.47	43.34	0.30	0.38	16.49	16.74
NCS 001	40.49	43.36	0.31	0.39	18.28	18.29
LSD (0.05)	0.005	0.007	0.02	0.005	0.62	0.58
Mulch rate (t ha^{-1})						
0	2.40	3.51	0.09	0.35	16.7	16.6
3	2.47	3.66	0.23	0.47	16.2	17.1
6	2.52	3.82	0.31	0.59	17.8	18.6
9	2.53	3.99	0.50	0.74	18.6	19.3
LSD (0.05)	0.29	0.006	0.009	0.006	0.8	0.7
Weed management (W)						
Weedy check	40.36	43.32	0.28	0.36	16.60	16.30
5 MHW	40.48	43.34	0.30	0.37	18.15	18.38
PE + POE + 2MHW	40.49	43.33	0.32	0.39	17.11	17.38
PE + POE	40.50	43.42	0.33	0.41	17.68	18.0
LSD (0.05)	0.01	0.006	0.005	0.004	1.29	0.82
Interaction						
M x W	NS	NS	*	NS	NS	*
Treatment (cont.)	**Polarity (%)**		**Purity (%)**		**Fibre (%)**	
	2016	2017	2016	2017	2016	2017
Genotypes (S)						
Bida local	19.3	19.3	83.2	82.2	13.0	12.7
NCS 001	19.2	19.4	85.6	85.8	13.4	13.6
LSD (0.05)	0.7	0.6	1.7	1.2	0.9	0.6
Mulch rate (t ha^{-1})						
0	19.1	19.2	83.0	83.5	13.9	13.4
3	19.4	19.2	83.8	83.3	13.2	13.3
6	19.5	19.5	84.9	84.0	12.9	13.4
9	19.2	19.5	85.2	85.2	12.6	12.4
LSD (0.05)	1.0	0.9	2.2	1.7	1.3	0.9

Weed management (W)						
Weedy check	18.4	18.8	83.4	82.4	12.2	12.9
5 MHW	19.6	19.8	85.0	84.8	13.6	13.1
PE + POE + 2MHW	18.7	18.7	83.6	83.7	13.4	13.1
PE + POE	20.2	20.1	86.6	85.2	13.5	13.6
LSD (0.05)	1.0	0.9	2.5	1.7	1.3	0.9
Interaction						
M x W	NS	NS	NS	NS	NS	NS

LSD – least significant difference, MHW – monthly hoe weeding, PE – pre-emergence (Diuron at 2 kg a.i./ha) herbicide, POE – post-emergence (3-Maize force at 179.2 g/ha) herbicide, NS – not significant, * – significant.

Table 4. Interaction between trash mulch and weed management practices on soil total nitrogen (g kg^{-1}) in 2016 cropping season

Mulch rate (t ha^{-1})	**Weed management practices**			
	Weedy check	**5 MHW**	**PE+POE+2MHW**	**PE+POE**
Mulch rate (t ha^{-1})	**Plant Crop 2016**			
0	0.08	0.08	0.09	0.12
3	0.19	0.22	0.24	0.25
6	0.26	0.30	0.32	0.36
9	0.42	0.50	0.54	0.56
LSD (0.05)	0.06			

LSD – least significant difference, MHW – monthly hoe weeding, PE – Pre-emergence (Diuron at 2 kg a.i./ha) herbicide, POE – Post-Emergence (3-Maize force at 179.2 g/ha) herbicide

Table 5. Interaction between trash mulch and weed management practices on Brix (%) at 12 MAP in 2017 cropping season

	Weed management practices			
	Weedy check	**5 MHW**	**PE+POE+2MHW**	**PE+POE**
Mulch rate (t ha^{-1})	**Plant Crop 2017**			
0	13.52	17.87	16.00	18.05
3	15.42	17.63	16.93	16.43
6	17.88	18.67	17.60	18.40
9	18.38	19.33	18.98	19.12
LSD (0.05)	6.68			

LSD – least significant difference, MHW – monthly hoe weeding, PE – pre-emergence (Diuron at 2 kg a.i./ha) herbicide, POE – post-Emergence (3-Maize force at 179.2 g/ha) herbicide

Conclusion

This study has shown that the application of 9 t ha trash mulch with 5 MHW or PE diuron plus POE 3-maize force effectively increased some soil C and N, field sucrose and cane sugar quality, especially NCS 001 in this agroecology of Nigeria.

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Research Article

ASSESSING DIFFERENT STRATEGIES FOR CONTROLLING WEEDS IN MAIZE CULTIVATION WITHIN THE DERIVED SAVANNA AGRO-ECOLOGY IN NIGERIA

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Abstract: Maize is the second most cultivated crop in Nigeria in terms of hectares of land. In the world it ranks fourth amongst cultivated cereals. However, its cultivation is faced with different challenges ranging from biotic to abiotic factors. Weeds are one of the biotic factors that threaten maize yield.in the field because of the cost and labor associated with it control. Hence, using Randomized Complete block Design, a two-year study was carried out to assess different strategies that are used in controlling weeds in the derived savanna agroecological zone of Nigeria at Ogbomoso Girls High School, Ogbomoso (8°9′N, 4°15′E) and Surulere North LCDA Igbon (8°14′N, 4°18′E). The total land area used was 13 m x 23 m with plot size 2 m x 3 m in three replicates. Eight treatments were used which are weedy plots, weed free, manual I, manual II, Pre-emergence herbicide (Glyphosate-1.08 kg a.i ha^{-1}), Post-emergence herbicide (Nicrosulfuron- 0.12 kg a.i ha^{-1}), Cowpea+Maize and Potato+Maize) to give 24 plots. Data collected were on weed parameters and maize yield. Weed parameters include: weed population, dry weed biomass and weed control ratio at 3, 6 and 9 weeks after sowing. Parameters on maize yield include number of seeds per cob, grain weight of seeds per cob, cob length, weight of 100 seeds per plot, grain weight per plot and grain weight per hectare. The result on weed parameters shows that there is no significant difference in the weed control rating and the dry weed biomass of manual weeding, pre-emergence herbicide, post emergence herbicide, cowpea+ maize, and potato + maize. Though there was significant difference in the weed population among the treatments. For maize yield (GWH), there was no significant difference between Manual II and the use of post emergence herbicide. Also the yield obtained from pre-emergence herbicide, potato+maize, cowpea+maize were not significantly different. Hence, it is recommended that legumes and potatoes can be used rotationally to control weeds in maize plots.

Keywords: maize, maize yield parameters, weed control strategies, weed parameters.

Introduction

Maize (*Zea mays* L.) is a staple crop extensively cultivated in tropical regions, where various environmental factors, farming practices, and genetic considerations shape its performance. It is a staple food in Nigeria, where maize occupies the largest area under cereal cultivation [ABAH & al. 2021; WOSSEN & al. 2023]. The production area for maize in Nigeria continues to expand [OLANIYAN, 2015], driven by technological advancements [HARUNA & al. 2023]. Globally, maize production has consistently averaged over 1,000 million metric tons (MMT) in recent decades. With an annual output of 11 MMT, Nigeria is considered the second-largest maize producer in Africa, following South Africa, while Ethiopia ranks third.

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Together, these three countries – South Africa, Nigeria, and Ethiopia – accounted for approximately 39% of Africa's total maize production in 2019 [ERENSTEIN & al. 2022; MAKAMA & al. 2022].

Maize serves as a staple food for millions of people, particularly in Africa, Latin America, and Asia, where it is consumed in various forms, such as maize flour, tortillas, polenta, and whole grain. According to POOLE & al. (2021), maize provides essential nutrients like carbohydrates, proteins, and fats, making it a vital food source for ensuring food security in many developing nations. BOUIS & al. (2011) underscore the role of biofortified maize in addressing malnutrition. A large proportion of global maize production is also used as animal feed. NUSS & TANUMIHARDJO (2010) noted that maize's high starch content makes it an excellent feed for energy-intensive livestock production, contributing to the global meat and dairy industries. Additionally, maize is a key feedstock for ethanol production, especially in the United States, where it plays a significant role in biofuel production. LU & MOSIER (2008) pointed out that the biofuel industry increases demand for maize, promoting energy security and reducing greenhouse gas emissions. SINGH & al. (2003) highlighted maize starch's versatility in products such as sweeteners, thickeners, adhesives, and biodegradable plastics. In Nigeria and across Africa, maize serves as a crucial income source. SMALE & MASON (2014) noted that maize farming supports the livelihoods of millions of rural households, contributing to poverty reduction and economic growth. FAO (2020) reported that maize is one of the most globally traded cereals, with exports driven by its use in food, feed, and industrial purposes. REVILLA & al. (2022) discussed maize's cultural significance, emphasizing its role as a symbol of heritage and identity in indigenous cultures.

Despite maize's significant economic importance, several limitations impact its production, particularly in developing countries like Nigeria. One of the main challenges is weed interference in maize fields. Weeds have a significant impact on maize cultivation, affecting crop growth, yield, and overall productivity. These unwanted plants compete with maize for essential resources such as sunlight, water, nutrients, and space, which can severely hinder crop development [SOLTANI & al. 2016]. The extent of weed interference depends on factors such as the type of weed species, their density, and the timing of weed emergence relative to the maize crop. Herbicide application is a widely used method for controlling weeds in maize cultivation. LUKANGILA & al. (2024) demonstrated that selective herbicides, when applied at the appropriate growth stage, can effectively reduce weed competition and improve maize yields. In addition to chemical methods, non-chemical approaches, such as crop rotation and intercropping, are also being explored. [LIEBMAN & ZIMDAHL, 2018] suggested that these practices can substantially reduce weed pressure and enhance maize yields, especially in organic farming systems. Given the critical role that weed pressure plays in maize production, this study aims to evaluate various weed control methods, including herbicides, in a derived savanna ecosystem.

Materials and methods

The experiments were carried out on arable fields of Ogbomoso North Local Government farms in Oyo State, Nigeria between April to August 2023 and 2024 in Ogbomoso North (8°9′N, 4°15′E). The climate of Ogbomoso is mostly influenced by the Northeast trade wind and Southwest trade wind. The annual rainfall of the area is between 1000 mm - 1286 mm. Temperature of the area ranges between 28 °C to 33 °C with a relative humidity of about 78% all year round except in January when the dry harmattan is at its peak. The soil type at the location where the experiment was set up was sandy loam with 14% clay content as described by EWETOLA & OSHUNSANYA (2015).

The experiment consisted of eight treatments (Table 1) laid out in a randomized complete block design with three replicates making a total of 24 experimental plots. Each plot size was 3 m × 2 m (6 m^2). Each plot was separated with 1m space between them and 2 m space in between the replicates. The total land area was 13 m by 23 m. Soil samples were collected before sowing at 0-15 cm depth randomly to determine the physico-chemical properties of the soil.

The result indicates that the soil was slightly acidic, sandy loam with nitrogen level that is well below the critical level of 0.15%. Sites were cleared and ploughed to pulverize the soil manually. Oba Super 6, a pro-vitamin A hybrid maize variety was the maize variety grown. It was planted at a spacing of 0.6 m × 0.5 m. Two seeds were planted per hill and thinned down to one seedling per hill at 14 DAS to give a plant density of 20 plants per plot.

In plots intercropped with cowpeas, the variety IT18 was planted one seed per hill in at 0.10 m from maize planting hills at two weeks after planting of maize. In the plots intercropped with potato, potato vines were planted at two weeks before planting maize. All plots including the control received basal application of Fertilizer N:P:K, (15:15:15) which was applied basally two weeks after planting maize at the rate of 250 kg /ha and Urea (46% N) at the rate of 100 kg/ha at six weeks after planting to meet the nutrient requirement of the maize.

Pre-emergence herbicide (PeEH) plots were planted as previously described and then sprayed with herbicides on the day of sowing. Post-emergence herbicides (PoEH) were applied after three weeks of planting. Herbicides were applied using a hand-pumped Knapsack sprayer which delivers 380 liters/ha of spray liquids. Manual weeding in Manual I plots was done at three weeks intervals, for Manual II at two weeks intervals and weed free plot was weeded weekly. In the maize + cowpea plot, and maize + potato plot weeding was done only once at 3 weeks after planting. This simulated the smallholder farmer practice in Nigeria of planting maize.

Weed species composition, weed density and weed dry weight

Weed species composition and weed density were determined by placing two quadrats size of 50 cm × 50 cm diagonally on each plot and then the weeds within each quadrat were uprooted, and sorted into broadleaf weeds, grasses, and sedges. The weed types were identified to the species level with the aid of weed identification manual of AKOBUNDU & al. (2016). The weeds were counted to calculate the weed density. Weed dry weight was estimated after oven-drying weed samples at 80 °C for 48 hours to constant weight.

Data collection and analysis

Data were subjected to analysis of variance (ANOVA) to test for treatment effects and interactions using the statistical analysis system (SAS) computer software package version 9.4 (SAS Institute, 2011). A combined ANOVA was conducted on plot means for all treatments in the two years. Significant differences between varieties were compared using the Fisher's least significant difference (LSD) at 5% probability level. Afterwards, Multiple Linear Regression Model (MLRM) was used to establish the linear relationship of dependent and independent variable [JEFFER, 1967] using PROC REG in SAS. The general linear model for MLRM in which response is related to a set of independent variables (X1) is given:

$$Y = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \ldots \beta_k X_k + \varepsilon_i$$

Where Y = dependent variable, β_0 is the intercept, β_1, β_2 … β_k are coefficients of the variables, X_1, X_2 … X_k are k^{th} independent variables and ε_i error term.

Principal Component Analysis (PCA) was performed to determine the parameters that account for most of the variations using R statistical software (Version 4.2.2) and was plotted using the package 'FactoMineR'.

Table 1. Treatments and their description

Treatments	Description
T1 (Weedy)	No weeding was done throughout the experiment
T2 (Weedy free)	Hoe weeding was carried out every week
T3 (Manual I)	Hoe weeding was done at (3weeks interval) 3, 6, and 9 WAP
T4 (Manual II)	Hoe weeding was done at (2weeks interval) 2, 4, 6, and 8 WAP
T5 (PeEH)	Pre emergence herbicide Glyphosate at 1.08 kg a.i ha $^{-1}$
T6 (PoEH)	Post emergence herbicide Nicosulfuron at 0.12 kg a.i .ha $^{-1}$
T7 (Cowpea)	Maize was relayed into cowpea at 2 WAP
T8 (Potato vine)	Maize was relayed into potato at 2 WAP

Results

Weed biodiversity

The species list of weeds encountered during the study is presented in Table 2. Altogether, sixteen weed species were identified during the two years when this experiment was conducted. The overview of various weed species, their families, life cycles, and densities over two years are shown in Table 3. Understanding weed density and relative density is crucial for managing weed populations and ensuring healthy crop growth. The weeds belong to nine different families, with a dominance of species from the Asteraceae, Poaceae, and Euphorbiaceae families. 50% of these weeds belong to broadleaves while the rest 50% are grass family. Seven of these weeds were annual while eight were perennial and only *Tithonia diversifolia* can either be annual or perennial depending on various environmental factors that enhance the survival of this weed species. The relative densities are provided for Year 1 and Year 2, showing significant variations across the years. *Euphorbia heterophylla* exhibited the highest weed density in Year 1, with a relative density of 54.82%, indicating it was the most dominant weed. *Imperata cylindrica* which is a grass weed also showed significant presence with relative densities of 23.35% in Year 1 while *Tithonia diversifolia* dominated the field in Year 2, with a relative density of 37.43%. This broadleaf weed is both an annual and perennial, making it more resilient and harder to control.

Means separation across all the treatments for Weed density, dry weed biomass, weed control effectiveness {Rated (1-10)}, and Phytotoxicity also rated 0-5 were presented in Table 2. The highest weed population (46.778) was observed in the weedy treatment, where no control measures were applied and this shows the natural weed proliferation without intervention. Weed-free treatment had the lowest weed population (17.722); Manual I (27.222) and Manual II (24.389) plots were manually weeded at different interval, and both significantly reduced the weed population compared to the weedy treatment, although they did not perform as well as the weed-free treatment. All other treatments showed relatively weed control efficiencies. Cowpea (24.333) and Potato vine (25.389) treatment plots had moderate weed populations, similar to the manual weeding methods, showing that they provided reasonable control of weeds. The overall mean for weed population was 28.59, with significant variation between treatments as indicated by the LSD (Least Significant Difference) value of 6.45. This suggests that differences greater than 6.45 between treatment means are statistically significant. Similar inferences was drawn from weed biomass production from all the treatment plots. The average dry weed biomass across all treatments was 0.04 kg/ha, with a significant difference between treatments at the LSD of 0.01 kg/ha. The average weed control rating was 7.28, indicating generally good control across treatments, with an LSD of 1.38, meaning differences in weed control ratings greater than 1.38 are statistically significant.

The overall analysis suggests that both manual and organic treatments (cowpea and potato vine) offer effective weed control with no phytotoxicity, while PoEH provides good control but with some crop damage. Weed-free and manual methods remain the most reliable in terms of reducing weed populations and biomass.

Table 2. Weed species list at the experimental site during study periods

Weed Species	**Family**	**Class**	**Life Cycle**	**Weed Density (m^2)**		**Relative Density %**	
				Year 1	Year 2	Year 1	Year 2
Acanthrospermum hispidum DC.	Asteraceae	Broadleaf	A	7		3.55	
Tithonia diversifolia (Hemsl.) A.Gray	Asteraceae	Broadleaf	A/P		64		37.43
Tridax procumbens L.	Asteraceae	Broadleaf	A	1		0.51	
Euphorbia heterophylla Desf.	Euphorbiaceae	Broadleaf	A	108		54.82	
Cleome rutidosperma DC.	Cleomaceae	Broadleaf	A	11		5.58	
Ipomoea triloba L.	Convolvulaceae	Broadleaf	A		5		2.92
Desmodium scorpiurus (Sw.) Poir.	Fabaceae	Broadleaf	P		12		7.02
Sida acuta Burm.f.	Malvaceae	Broadleaf	P		5		2.92
Mimosa invisa Mart. ex Colla.	Fabaceae	Broadleaf	P		19		11.11
Phyllanthus amarus Schumach. & Thonn.	Phyllanthaceae	Broadleaf	A		11		6.43
Imperata cylindrica (L.) Raeusch.	Poaceae	Grass	P	46		23.35	
Andropogon gayanus Kunth	Poaceae	Grass	P	17		8.63	
Chloris pilosa Schumach.	Poaceae	Grass	P	2		1.02	
Chrysopogon aciculatus (Retz.) Trin.	Poaceae	Grass	P	5		2.54	
Pennisetum violaceum (Lam.) Rich.	Poaceae	Grass	P		22		12.87
Setaria barbata (Lam.) Kunth	Poaceae	Grass	A		33		19.30

Table 3, shows the effect of different treatments caused substantial variation in the number of seeds per cob, grain weight per cob in kg, cob length in m, weight of 100 seeds per plot in kg, grain weight per plot in kg and grain weight per hectare in kg/ha. Weedy free had the highest yield parameters when compared with all the treatment: number of seeds per cob (547.67), grain weight per cob (0.206 kg), cob length (27.00 m), number of seeds per plot (0.033 kg), grain weight per plot

(3.843 kg) and grain weight per hectare (6377.70). Manual I was significantly higher than Manual II only in terms of number of seeds per cob (452.67) and weight of seed per plot (0.029 kg). All other yield parameters in Manual I and Manual II were not significantly different. Treatment PoEH which is a post emergent herbicide had yield parameters that were significantly higher than treatments PeEH across all the yield parameters. This implies that when yield is being considered post emergence herbicide will give higher yield than pre emergence herbicides. However, for treatments Cowpea and Potato, there is no significant difference between the two treatments except in terms of seeds per plot in which, cowpea (0.026 kg) than potato (0.024 kg). This implies cowpea and potato interchangeable for weed control and still have the same yield.

Table 3. Mean value for weed parameters of the evaluated treatments across the two years in kg/ha

Treatments	Weed parameters			
	Weed Population ($0.5m^2$)	**Dry Weed Biomass (kg/ha)**	**Weed Control Rating (1-10)**	**Phytotoxicity Rating (0-5)**
Weedy	46.78a	0.07a	3.44c	0.00b
Weed free	17.72c	0.02c	9.72a	0.00b
Manual I	27.22b	0.04b	7.22b	0.00b
Manual II	24.39b	0.04b	7.94b	0.00b
PeEH	40.61a	0.04b	6.89b	0.00b
PoEH	22.28bc	0.04b	7.89b	1.00a
Cowpea	24.33b	0.04b	7.61b	0.00b
Potato vine	25.39b	0.04b	7.50b	0.00b
Mean	28.59	0.04	7.28	0.13
LSD(0.05)	6.45	0.01	1.38	0.19

Means followed by the same letter in a column are not significantly different at 5% probability level. PeEH – pre emergence herbicide; PoEH – post emergence herbicides; LSD – least significant difference at 5% probability levels.

Multivariate analysis of weed and maize parameters

The correlogram illustrates the strength and direction of the linear relationships between parameters. In Figure 1, the dry weed biomass had a linear positive significant ($p<0.05$) relationships with weed population ($r = 0.81$). The weed population had a strong negative linear significant ($p<0.01$) relationship with the number of seeds per cob ($r = -0.91$). A similar trend was seen in grain weight per cob ($r = -0.87$), Dry weed biomass also had a strong negative linear significant ($p<0.01$) relationship with the number of seeds per cob ($r = -0.91$). A similar trend as that of weed population was seen in cob length ($r = -0.85$). Weed population also had a negative linear significant ($p<0.05$) relationship with cob length ($r = -0.83$) in similar manner as dry weed biomass with grain weight per plot ($r = -0.82$) for weed population and grain weight per plot ($r = -0.83$). For grain weight per hectare $r = -0.83$ and $r = -0.81$ respectively. However, there was very strong negative high correlation relationship ($p<0.001$) of dry weed biomass with weed control rating ($r = -0.98$) and grain weight per cob ($r = -0.93$).

Regression analysis examining the relationship between dry weed biomass and differences in yield parameters of maize (Table 5). It further indicates that dry weed biomass as controlled by the treatments in this experiment affects maize production negatively. This is further indicated by the strong significance of the regression coefficient ($p<0.001$ and $p<0.01$) and coefficient of determination ($R^2>0.9$) in Table 4. Consistent with the important differentiating parameter, a regression analysis examining the relationship between dry weed biomass and yield in maize. This is indicated in the strong significance in number of seed per cob, grain weight per cob, length of cob, weight of seed per cob and grain weight per plot for each treatment are not left out.

Principal component analysis (PCA) was based on the measured weed and maize parameters. The first two principal components (PCs) with eigenvalues >1 accounted for approximately 91% of the total variation among the accessions. The first and second PCs explained 80.7% and 10.3% of the total variation among the accessions, respectively. The proportion of variance explained by the third PC was 4.1% and the fourth PC accounted for 3.2% of the total variation. The PCs loading visualized by the PCA biplot shows the contributions of the measured parameters to PC1 and PC2 (Figure 2). The vectors of phytotoxicity rating, grain weight per hectare, cob length, weed population, dry weed biomass, grain weight per cob and grain weight of seed per plot point in the direction of PC1. The strength of the vectors weed population and dry weed biomass denotes a strong positive influence on PC1. Conversely, the vectors weed control rating, phytotoxicity, number of seed per cob, grain weight per cob, cob length, weight of seed per plot, and grain weight per hectare points to the negative side of PC1, indicating a strong negative influence on PC1. Dry weed biomass and weed population had a strong positive influence on PC1 while only weed population influenced PC2 positively.

Table 4. Mean value of yield parameters on maize for the evaluated treatments in kg/ha

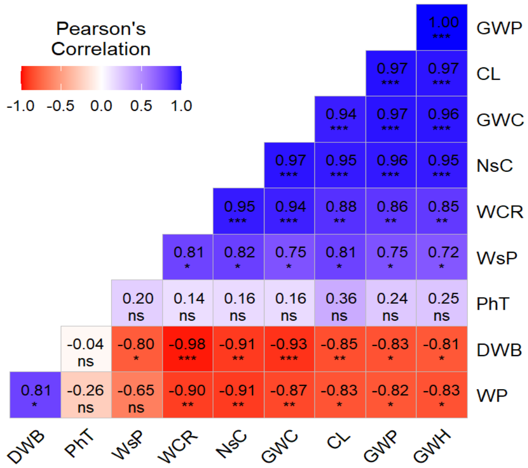
Treatments	**Yield Parameters on Maize**					
	Number of Seeds per Cob	**Grain Weight per Cob (kg)**	**Cob Length (m)**	**Weight of Seed Per plot (kg)**	**Grain Weight Per Plot (kg)**	**Grain Weight per hectare (kg/ha)**
Weedy	177.00c	0.05d	13.33c	0.01d	0.24d	399.30d
Weedy free	547.67a	0.21a	27.00a	0.03a	3.84a	6377.70a
Manual I	452.67bc	0.15b	24.67a	0.03abc	2.90b	4750.50b
Manual II	493.83ab	0.15b	24.50a	0.03bc	2.99b	4970.00b
PeEH	346.17d	0.12c	20.33b	0.03bc	1.52c	2140.70c
PoEH	449.67bc	0.15b	26.00a	0.03ab	2.86b	4761.80b
Cowpea	400.67cd	0.12c	19.67b	0.03bc	1.56c	2593.30c
Potato	382.33cd	0.12c	20.33b	0.02c	1.55c	2473.70c
Mean	406.25	0.13	21.98	0.03	2.18	3558.37
LSD (0.05)	22.94	0.02	3.28	0.01	0.50	750.07

Means followed by the same letter are not significantly different at 5% probability.

Table 5. Contributions of Dry Weed Biomass (DWB) to differences in yield parameters of maize based on stepwise regression

Agronomic trait	**Mean ± SE**	**Coefficient of determination (R^2)**	**Regression coefficient (β)**	**Intercept (α)**	**Significant F level**	**Regression equation y = βx + α**
Number of seeds per cob	406.25±28.95	0.40	-7082.25^{***}	696.18	15.09	y =-7082.25x + 696.18
Grain weight per cob (kg)	0.13±0.01	0.87	-2.78^{***}	0.25	37.60	y = -2.784x + 0.25
Cob length (cm)	21.979±1.144	0.769	-261.62^{**}	32.689	15.33	y = -261.62x + 32.689
Weight of seed per cob (kg)	0.026±0.002	0.847	-0.354^{**}	0.040	8.45	y = -0.354x + 0.040
Grain weight per plot (kg)	2.182±0.173	0.727	-65.765^{**}	4.874	44.91	y = -65.765x + 4.874

*,**,*** = significant at 0.05, 0.01 and 0.001 probability levels, respectively.



	DWB	PhT	WsP	WCR	NsC	GWC	CL	GWP	GWH
GWP									1.00 ***
CL								0.97 ***	0.97 ***
GWC							0.94 ***	0.97 ***	0.96 ***
NsC						0.97 ***	0.95 ***	0.96 ***	0.95 ***
WCR					0.95 ***	0.94 ***	0.88 **	0.86 **	0.85 **
WsP				0.81 *	0.82 *	0.75 *	0.81 *	0.75 *	0.72 *
PhT			0.20 ns	0.14 ns	0.16 ns	0.16 ns	0.36 ns	0.24 ns	0.25 ns
DWB		-0.04 ns	-0.80 *	-0.98 ***	-0.91 **	-0.93 ***	-0.85 **	-0.83 *	-0.81 *
WP	0.81 *	-0.26 ns	-0.65 ns	-0.90 **	-0.91 **	-0.87 **	-0.83 *	-0.82 *	-0.83 *

ns p >= 0.05; * p < 0.05; ** p < 0.01; and *** p < 0.001

Figure 1. Correlogram showing the relationship between average values of weed and maize parameters. Dark blue denotes a high positive correlation, whereas dark red represents a high negative correlation. The cell value denotes correlation coefficient (r) values. WP = Weed population; DWB = dry weed biomass; PhT = phytotoxicity rating; WsP = weight of 100s seed per plot (kg); WCR = weed control rating (1-10); NsC = number of seed per cob; GWC = grain weight per cob; CL = cob length (cm); GWP = grain weight per plot (kg); GWH = grain weight per hectare (kg/ha); *,**,*** = significant at 0.05, 0.01 and 0.001 probability levels, respectively; ns = nonsignificant.

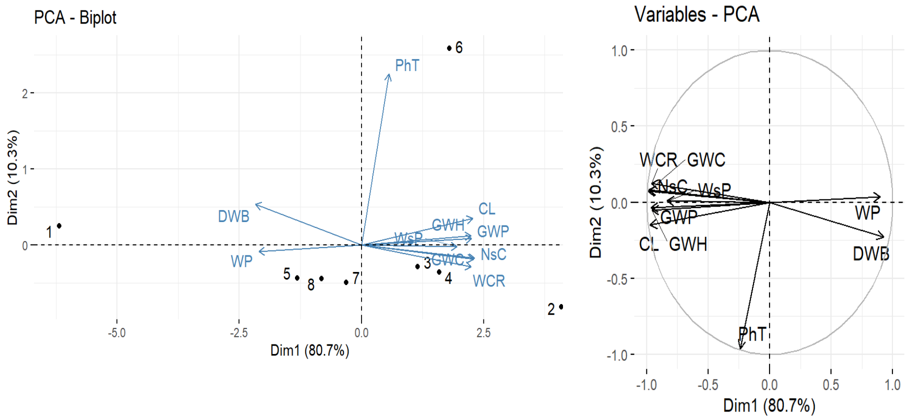


Figure 2. A two-dimensional principal component analysis (PCA) showing the relationships weed and maize parameters and checks evaluated. The first two components, PC1 (80.7%) and PC2 (10.3%) explaining the highest variance were plotted on the x-axis and y-axis, respectively. The arrows indicate weed and maize parameters contributing to the respective PCs and the correlation between parameters can be determined by the close arrow proximity. WP = weed population; DWB = dry weed biomass; PhT = phytotoxicity rating; WsP = weight of seed per plot (kg); WCR = weed control rating (1-10); NsC = number of seed per cob; GWC = grain weight per cob; CL = cob length (cm); GWP = grain weight per plot (kg); GWH = grain weight per hectare (kg/ha).

Discussions

The variation in weed density and species dominance between the two years indicates the dynamic nature of weed populations, likely influenced by environmental factors, crop rotations, or herbicide applications. The dominance of broadleaf species like *Euphorbia heterophylla* in Year 1 and *Tithonia diversifolia* in Year 2, as well as the emergence of grass weeds like *Imperata cylindrica* and *Setaria barbata*, suggests that integrated weed management strategies are required to target both broadleaf and grassy species.

The result shows that Manual I, Manual II, PeEH, PoEH, Cowpea, and Potato vine are all effective methods of weed control when compared to weedy plots as supported by IMOLOAME & OSUNLOLA (2017) and NGONADI & al. (2023). The weed control rating of this experiment also followed the same trend as they are not significantly different at 5% probability level. The effects of treatments on yield and yield components of maize shows that the use of post emergence herbicide (PoEH) method of weed control works effectively as Manual I and Manual II methods of weed control since there is no significant difference among them for all yield and yield components of maize. This finding is in accordance with the work of UGBE & al. (2016) and TIZHE & al. (2023). The yield in post emergence herbicide-PoEH was also significantly higher as a method of weed control when compared to that in Cowpea and Potato vine. This aligns with the findings of IMOLOAME (2017) and FALADE & al. (2023), who documented that the use of herbicides supports yield in maize. However, cowpea and potato vine treatments (cover crops) there was no significant difference in all the yield and yield components in maize production which implies they can be used interchangeably. This agrees with the works of NAYAN & al. (2020) and MAS-UD & al. (2021).

Regression analysis shows a significant effect between dry weed biomass and the yield and yield components of maize. It highlights the impact of the linear trends of the response of yield and yield components in maize. This signifies that a unit increase in dry weed biomass will likely lead to a decrease in yield and yield components in maize which is following the work of KOLAWOLE & OLAYINKA (2023) whose result on regression analysis shows that a unit increase in an independent variable can lead to an increase in the dependent variable (yield). The two-year combined data (2023-2024) in the correlogram shows that a positive highly significant correlation was revealed in the weight of seed per plot and weed control rating which is in accordance with the findings of VERMA & al. (2023). However, dry weed biomass, and weed population were highly negatively correlated with yield. This result supports the findings of VERMA & al. (2023) and DANIYA & al. (2013) who reported negative correlations between weed components and yield in cowpea and sesame respectively.

The PC analysis identifies two PCs accounting for 91% of the variations observed in which dry weed biomass and weed populations are the major parameters responsible as descriptors of yield and yield components in maize. This finding aligns with that of OLAYINKA & al. (2024) whose work identified four PCs to be collectively responsible for 73% of total variations in the research.

Conclusion

The different weed control efficacy assessment varied for all treatments-weed control methods. The weed-free plot stood out regarding maize yield and yield components. Post-emergence weed control PoEH gave a yield that had no significant difference from that of Manual I and Manual II which may be laborious and expensive in areas where availability of manpower is a limiting factor. This experiment affirms the reason why the use of herbicide is preferred over other control methods. Also, the findings from this experiment from the regression, correlation, and PC analysis show that weeds generally are principal factors determining yields in maize the derived savanna agroecology of Nigeria.

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Research Article

VARIABILITY AND ASSOCIATION STUDIES OF PHENOTYPIC CHARACTERS CONTRIBUTING TO YIELD IN FONIO MILLET (*DIGITARIA IBURUA* STAPF) ACCESSIONS

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Abstract: The study on fourteen fonio accessions explored the available variability, investigated component association and grouped the total variability into Principal components. The experiment was conducted with three replications using Randomized Complete Block Design. Most characters showed Significant differences among the accessions at 5% probability level. Phenotypic coefficient of variability was more significant than genotypic coefficients, indicating environmental impacts, though minimal. Grain yield was highly influenced by number of tillers and height, as indicated by correlation and path coefficients. Plant height and days to maturity showed positive impact on fonio yield. Among the nine principal components realized, three had eigen values greater than one, contributing a total variation of about 79.2%. PC1 had the highest Eigen value of 3.77 with the highest load (-0.442) in days to maturity. Selection and hybridization with attention on plant height, number of tillers per plant and panicle length of the population could help improve on the grain yield.

Keywords: correlation, fonio, principal components analysis, variability, yield.

Introduction

Fonio (*Digitaria* spp.), sometimes called fundi in most West African Countries but specifically known as acha in Nigeria [KALAT, 2014], is indigenous to West Africa. The cereal (*Digitaria* spp.) represents a unique component of millets biodiversity. It is regarded as the oldest cultivated African cereal [CRUZ & BEAVOGUI, 2016; DACHI & al. 2016]. Over 300,000 ha is devoted to fonio cultivation yearly in West Africa [BEZPALY, 1984]. FAO (2009) statistics showed that 448,247 ha of fonio was harvested and 480,227 tons of grains produced during the 2008/2009. In Nigeria, two cultivable species (*D. iburua* and *D. exilis*) are recognized. *Digitaria iburua*, also called black fonio, is less recognized for improvement [ADOUKONOU-SAGBADJA & al. 2010]. *D. iburua* has a high amount of magnesium, zinc and manganese compared to other cereals. It also contains a significant amount of thiamine (Vitamin B1), riboflavin (Vitamin B2), calcium and phosphorous compared to white rice [NRC,

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1996]. *Digitaria iburua* is autogamous, variability is mostly among component lines with little adaptation and less flexibility. According to BUDAK & al. (2004), there are changes in the population's genetic structure by selection because of the preserved superior alleles. When selection is applied, the genetic variance may be reduced to attain specific breeding objectives. According to SUNESON (1956), the population's improvement will be slow but in the long run, sufficient for obtaining attractive plant material. Assessing variability in *D. iburua* population is essential step towards its improvement [MISHRA & al. 2015], because environment is reported to have a high influence on yield, and polygenes are a complex character in most crop plants [ISONG & BALU, 2017].

Partitioning of the variability into heritable and non-heritable can be done using the genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), heritability (H^2), and genetic advance expressed as a percentage of the mean (GAM%). The combination of heritability and genetic advance is more effective and reliable for predicting breeding value by selection [MISHRA & al. 2015; RAMANJINAPPA & al. 2011; PATIL & al. 1996]. Fonio yield depends on its components and their interaction. The relationship may result either from developmental or genetic factors [BALOCH & al. 2014]. The contributing components of the complex characters are obtained from the correlation, however, path analysis is accurate in analysing the direct and indirect components of a character [ISONG & BALU, 2017]. Some authors [LIAQAT & al. 2015; IQBAL & al. 2006] also pointed out that in majority of crop species, the selection criterion for complex traits is developed using path coefficient analysis and the interaction between the traits. Hence, parameters affecting yield improvement can be analyzed using correlation and path coefficient analysis [REDDY & al. 2015]. Canonical or vector analysis (Principal Component Analysis) can also be used to indicate the character(s) contributing most of the variability in the population. In lieu of the aforementioned, this investigation seeks to analyse the variability as well as component association and to group the total variability into Principal components for effective selection in the population.

Material and methods

Location of the experiment

The location for the investigation was Acha (Fonio) research field of Acha Research Programme, National Cereals Research Institute, Badeggi, Nigeria. Badeggi is located at Latitude 9°45' N and Longitude. 6°07' E in the Southern Guinea Savannah of Nigeria.

The genetic materials and field experimentation

Fourteen (14) *D. iburua* accessions; Asaogba, Eburi iki, Eburi okwa, Eburi istimu, Eburi suum, Eburu Anime, iacho, egboba, ikiota, Kojegicho, Kojegichoonobi, Nachelenchia, Naram, Nashelleng and Tuwonta were raised in three replications in Randomized Block Design (RBD). The genetic materials were planted in plots of 3 m x 4 m each by broadcasting method with a seed rate of 25 kg/ha. Cultural practices like irrigation, soil conditioning, fertilizer application, weed and pest control were followed. The nutrient requirement was 15:15:15 NPK inorganic fertilizer, applied in two splits of 30 kg N/ha, 30 kg P_2O_2 and 30 kg/ha K_2O at 4 and 8 weeks after planting (WAP), respectively.

Data collection

The observations from randomly tagged 5 plants per plot were made on number of tillers per plant (NTPP), number of nodes per tiller (NNPT), spike length (SL), number of panicles per plant (NPPP), panicle length (PL), plant height (PH), days to 50% flowering (DFPF), days to maturity (DM) and grain yield (GY).

Statistical Analysis

AGRISTAT statistical analyses software was used to obtain the mean data for *per se* performance, analysis of variance (ANOVA) and variability parameters viz; phenotypic, genotypic and environmental variations, according to LUSH (1940), phenotypic and genotypic coefficient of variation, according to BURTON (1952). Broad sense heritability (Hb%) according to LUSH (1940) and Genetic advance over mean (%) as described by JOHNSON & al. (1955). In ANOVA, where the mean showed significant difference at a 5% probability level, Least Significant Difference (LSD) was used for mean separation. Also, association studies were carried out using correlation matrix and Path coefficient analyses. Statistical Tools for Agricultural Research package (STAR), version 2.0.1 of 2014 was employed to group the variability into different Principal components as described by SAEED & al. (2014).

Results and discussions

Analysis of variance, per se performance and descriptive statistics results are presented in Table 1, Genetic and Phenotypic Variability are in Table 2, Correlations are presented in Table 3, Regression and Direct Path are in Table 4 while Principal Components analysis is indicated in Table 5.

At 5% probability level, all the traits studied showed significant differences among the mean values implying that sufficient variability existed amongst the traits, breeders have a high opportunity to improve the traits by selection and hybridization according to [UMAR & al. 2020]. The accession Eburi iki recorded the highest yield value (836.10kg/ha) while Naram had the lowest yield of 425.00 kg/ha. Nashelleng, a *D. iburua* species recorded the tallest plants, corroborating the reports of ISONG & al (2022). Nashelleng also had the highest number of tillers per plant and number of nodes per tiller but required more days for 50% flowering and are late maturing. The phenotypic coefficient of variation ranged from 7.09 in plant height to 19.60 in grain yield. Also, genotypic coefficient of variation (GCV) values were from 7.0981 in plant height to 19.5633 in grain yield. Following the classification by SIVASUBRAMANIAN & MADHAVAMENON (1973), plant height was low for both parameters; grain yield was high for both parameters, while the other seven traits were moderate for the two variance parameters. In all the traits under study, the phenotypic coefficient of variability was higher than the genotypic coefficients of variation, indicating that the environment imparted though minimally on all the traits TEJASWINI & al. (2017).

Heritability, the proportion of genotypic variance in the phenotypic variance, was determined broadly by a method prescribed by LUSH (1940). According to JOHNSON & al. (1955) classification, all the traits recorded high heritability.

The expected genetic advance under selection indicated that all the traits under study had high genetic advance over mean (GAM) [JOHNSON & al. 1955]. By the findings of SAKTHI & al. (2007), heritability of all traits in this population is associated with high genetic advance, implying that cumulative gene effects are related to the heritability, also selection could be effective. Correlation analysis provided the basis to predict the corresponding change in one character at the expense of proportionate change in the other character. Genotypic correlation co-efficient between fonio grain yield with eight (8) biometric traits and inter-correlation among themselves were analyzed, and the result showed that phenotypic characters are higher than the magnitude of genotypic correlation. The fonio grain yield has positive correlation with number of tillers, nodes, height and length of panicle. At 5% probability level, panicle length was significantly correlated with grain yield, while number of tillers per plant and plant height were significantly correlated with grain yield at 1% probability for both phenotypic and genotypic correlations. According to AGUADO & al. (2008), the selection of these desired traits could help improve the grain yield of the fonio. The correlation among the yield component traits is important in deciding which trait to be given due attention in exercising selection. The association between the quantitative yield contributing traits like tillers with length of panicle and height; spike length with panicle length, plant height, days to 50% flowering and days to maturity; panicle length with plant height, days to 50% flowering and days to maturity; plant height with days to 50% flowering and days to maturity; days to 50% flowering with days to maturity are all positively and significantly correlated at 1% probability level at N minus 2 degrees of freedom for both the genotypic and phenotypic associations. This inter-correlation result corresponds with the findings of BASBAG & GENCER (2007) working on interspecific cotton hybrids. Therefore, selection based on these important yield contributing traits could bring about a breakthrough in fonio grain yield under interspecific hybridization.

Path coefficient analysis was derived following the methods described by DEWEY & LU (1959) to differentiate the genotypic correlation coefficients into direct and indirect effects. Based on the direct and indirect effects classification scale given by LENKA & MISHRA (1973), spike length and days to 50% flowering had very high but negative direct path effects on fonio grain yield. Conversely, plant height and days to maturity also demonstrated very high but positive direct path effects on fonio grain yield. The implication for direct selection of plant height and days to maturity as yield components will be directly felt in the early generation of population development. Panicle length was high and positive, while panicles in each plant had low direct, but positive path effects on fonio grain yield. From the study of grain yield and its components, high genetic variability, as indicated by the analysis of variance and variance components, existed amongst the components in fonio. KHAN (2003) suggested how to indicate the variability pattern and possibly group them into components. Following the method described in ISONG & al. (2017), mean data of fourteen (14) *D. iburua* accessions were subjected to Principal Component (PC) analysis. Nine (9) principal components were realized, and only three (3) of them had eigen values greater than one, contributing variations of about 79.2% of the total variation in the population. Therefore, the three (3) principal components are important in explaining the total variability and grouping of the components. The highest variability of 41.9% recorded by the first PC was contributed mostly by spike length, panicle length, days to 50% flowering and days to maturity. The second and third PCs having 23.4% and 13.9% respectively were mostly associated with the grain yield, number of tillers per plant and number of panicles per plant.

Table 1. *Per se* performance, descriptive statistics and analysis of variance for yield and yield attributes of 14 *Digitaria iburua* accessions

Accessions	**NTPP**	**NNPT**	**SL (cm)**	**NPPP**	**PL (cm)**	**PH (cm)**	**DFPF**	**DM**	**GY (Kg/ha**
Asaogba	11.00	16.40	12.23	8.00	11.40	123.43	68.33	89.67	741.67
Eburi iki	20.33	13.60	15.70	9.33	19.50	131.43	74.67	99.67	836.10
Eburi okwa	16.00	13.40	15.50	7.00	15.53	120.80	78.33	101.00	516.67
Eburi istimu	14.00	15.43	14.50	9.00	13.43	110.40	69.33	100.00	541.67
Eburi suum	15.00	11.80	14.50	8.00	12.80	129.70	84.33	100.33	538.87
Eburu Anime	13.00	11.90	16.10	10.00	14.33	118.43	81.00	104.00	480.53
iacho-egboba	16.00	14.80	9.70	9.00	11.23	117.63	69.00	89.00	608.33
ikiota	18.00	11.80	15.10	10.00	14.43	116.20	73.33	103.00	686.13
Kojegicho	15.00	15.10	12.10	8.00	10.50	119.80	68.33	98.00	538.87
Kojegichoonobi	19.00	12.40	11.50	7.00	11.80	112.80	72.67	103.00	650.00
Nachelenchia	17.00	16.87	10.90	9.00	12.70	113.80	57.67	84.00	756.73
Naram	12.00	16.57	11.10	7.00	11.50	106.40	69.00	91.67	425.00
Nashelleng	21.00	16.87	11.83	8.00	14.50	134.40	88.00	108.67	758.33
Tuwonta	19.00	16.80	8.90	10.00	11.20	109.60	60.67	84.00	666.67
Mean	**16.17**	**14.55**	**12.83**	**8.52**	**13.20**	**118.92**	**72.48**	**96.86**	**624.80**
Max	**21.00**	**16.87**	**16.10**	**10.00**	**19.50**	**134.40**	**88.00**	**108.67**	**836.10**
Min	**11.00**	**11.80**	**8.90**	**7.00**	**10.50**	**106.40**	**57.67**	**84.00**	**425.00**
SE	**0.13**	**0.37**	**0.35**	**0.13**	**0.03**	**0.09**	**1.69**	**0.84**	**6.44**
CV%	**0.95**	**3.08**	**3.35**	**1.81**	**0.27**	**0.09**	**2.86**	**1.06**	**1.26**
LSD 5%	**0.46**	**1.35**	**1.29**	**0.46**	**0.11**	**0.32**	**6.25**	**3.08**	**23.71**

N/B: NTPP = number of tillers per plant; NPPP = number of panicles per plant; SL= spike length; NNPT = number of nodes per tiller; PL= panicle length; PH = plant height; DFPF = days to 50% flowering; DM = days to maturity and GY= grain yield.

Table 2. Variance components, heritability and genetic advance for grain yield and yield components in 14 accessions of *Digitaria iburua* studied

Components	**Phenotypic variance**	**Genotypic variance**	**Environmental variance**	**Phenotypic coefficient of variation**	**Genotypic coefficient of variation**	**Broad sense herita-bility (%)**	**Genetic advance over mean (%)**
NTPP	9.43	9.41	0.02	18.99	18.97	99.75	39.03
NNPT	4.24	4.04	0.20	14.16	13.82	95.25	27.78
SL	5.69	5.51	0.19	18.59	18.29	96.75	37.05
NPPP	1.24	1.22	0.02	13.07	12.95	98.08	26.42
PL	5.64	5.64	0.001	17.99	17.98	99.98	37.05
PH	71.26	71.25	0.01	7.09	7.09	99.98	14.62
DFPF	73.74	69.43	4.31	11.85	11.49	94.16	22.98
DM	61.10	60.06	1.05	8.07	8.00	98.29	16.34
GY	15002.53	14940.41	62.12	19.61	19.56	99.59	40.22

N/B: NTPP = number of tillers per plant; NPPP = number of panicles per plant; SL = spike length; NNPT = number of nodes per tiller; PL = panicle length; PH = plant height; DFPF = days to 50% flowering; DM = days to maturity and GY = grain yield.

Table 3. Genotypic and phenotypic correlations of yield and yield attributes of 14 *D. iburua* accessions

		NTPP	**NNPT**	**SL**	**NPPP**	**PL**	**PH**	**DFPF**	**DM**	**GY**
NTPP	g	1.000	-0.051	-0.106	0.192	0.429**	0.324*	0.141	0.244	0.621**
	p	1.000	-0.060	-0.104	0.197	0.428**	0.324*	0.135	0.238	0.619**
NNPT	g		1.000	-0.690**	-0.089	-0.357*	-0.195	-0.519**	-0.596**	0.226
	p		1.000	-0.665**	-0.115	-0.347*	-0.191	-0.483**	-0.562**	0.219
SL	g			1.000	0.119	0.719**	0.383*	0.580**	0.675**	-0.133
	p			1.000	0.117	0.707**	0.376*	0.559**	0.662**	-0.130
NPPP	g				1.000	0.224	-0.054	-0.215	-0.159	0.295
	p				1.000	0.222	-0.053	-0.212	-0.166	0.291
PL	g					1.000	0.523**	0.447**	0.482**	0.368*
	p					1.000	0.523**	0.434**	0.479**	0.367*
PH	g						1.000	0.697**	0.480**	0.443**
	p						1.000	0.676**	0.475**	0.442**
DFPF	g							1.000	0.864**	-0.141
	p							1.000	0.831**	-0.139
DM	g								1.000	-0.111
	p								1.000	-0.110
GY	g									1.000
	p									1.000

N/B: NTPP = number of tillers per plant; NPPP = number of panicles per plant; SL = spike length; NNPT = number of nodes per tiller; PL = panicle length; PH = plant height; DFPF = days to 50% flowering; DM = days to maturity and GY = grain yield; g = genotypic; p = phenotypic; ** = significant at 1% probability; * = significant at 5% probability

Table 4. Path coefficients of grain yield with yield components in *D. iburua* accessions studied

	NTPP	NNPT	SL	NPPP	PL	PH	DFPF	DM	**Correlation Coefficient**
NTPP	**-0.356**	0.015	0.137	0.036	0.385	0.358	-0.223	0.268	0.621**
NNPT	0.018	**-0.293**	0.892	-0.016	-0.321	-0.215	0.817	-0.656	0.226
SL	0.038	0.202	**-1.293**	0.022	0.646	0.423	-0.914	0.744	-0.133
NPPP	-0.068	0.026	-0.154	**0.185**	0.202	-0.060	0.340	-0.175	0.295
PL	-0.153	0.104	-0.929	0.041	**0.899**	0.578	-0.704	0.531	0.368*
PH	-0.115	0.057	-0.495	-0.010	0.470	**1.106**	-1.098	0.529	0.443**
DFPF	-0.050	0.152	-0.750	-0.040	0.402	0.770	**-1.576**	0.951	-0.141
DM	-0.087	0.175	-0.873	-0.029	0.433	0.531	-1.361	**1.101**	-0.111

RESIDUE = 0.3738. **Bold figures in Diagonals** indicate direct effect on fonio grain yield

N/B: NTPP = number of tillers per plant; NPPP = number of panicles per plant; SL = spike length; NNPT = number of nodes per tiller; PL = panicle length; PH = plant height; DFPF = days to 50% flowering; DM = days to maturity and GY = grain yield.

Table 5. Principal component analysis (PCA) for 9 variables in 14 *D. iburua* accessions

Variables	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**	**PC9**
NTPP	-0.172	0.509	0.124	-0.643	0.164	-0.122	0.240	0.294	0.311
NNPT	0.340	0.229	0.349	0.408	0.116	-0.691	-0.116	0.121	0.161
SL	-0.417	-0.193	-0.328	0.318	0.264	0.028	-0.203	0.305	0.613
NPPP	-0.017	0.307	-0.698	0.012	-0.540	-0.351	-0.021	0.015	-0.061
PL	-0.400	0.233	-0.190	0.254	0.537	-0.176	0.372	-0.242	-0.412
PH	-0.367	0.214	0.349	0.388	-0.431	0.227	0.215	0.478	-0.189
DFPF	-0.432	-0.174	0.289	-0.033	-0.353	-0.260	0.203	-0.588	0.346
DM	-0.442	-0.167	0.144	-0.280	0.012	-0.326	-0.625	0.146	-0.400
GY	-0.067	0.632	0.082	0.153	0.022	0.358	-0.521	-0.390	0.114
Statistics									
Proportion of variance	0.419	0.234	0.139	0.076	0.056	0.041	0.023	0.009	0.004
Cumulative proportion	0.419	0.653	0.792	0.868	0.924	0.965	0.988	0.996	1.000
Eigen Values	3.769	2.108	1.252	0.685	0.499	0.372	0.205	0.078	0.034

N/B: NTPP = number of tillers per plant; NPPP = number of panicles per plant; SL = spike length; NNPT = number of nodes per tiller; PL = panicle length; PH = plant height; DFPF = days to 50% flowering; DM = days to maturity and GY = grain yield.

Conclusions

Sufficient positive genetic variability was recorded in the fonio population, all the traits under study showed significant differences at 5% probability level. Environment had minimal impact on all the traits under study. The phenotypic coefficient of variability showed higher figures than the genotypic coefficients of variation. High heritability and high Genetic Advance over mean (GAM) associated with all traits, indicates that heritability is largely due to additive gene effects. Selection based on correlation, path coefficient and Principal component analyses results will bring breakthroughs in breeding for fonio grain yield under interspecific hybridization. Plant height and days to maturity showed a direct positive effect on the grain yield, direct selection of these traits would be directly felt in the early generation of population development.

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Research Article

STUDIES ON CRITICALLY ENDANGERED GENUS *CEROPEGIA* FROM WESTERN GHATS OF NASHIK DISTRICT, MAHARASHTRA, INDIA

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Abstract: *Ceropegia* is tuberous herb, mostly found growing on grassy slopes of hills and plateaus. Species of *Ceropegia* are recognized mostly for its peculiar ornamental and/or medicinal properties. Most species from the genus *Ceropegia* are included in threatened category because of special requirements in terms of climate and habitat. Earlier studies have indicated that habitat destruction and collecting of tubers for local consumption are the major threats. However; during recent years; their populations have been found declining rapidly due to habitat modifications and anthropogenic pressures like grazing, tourism and trampling, land use changes, etc. Since the majority of the reported *Ceropegia* species were previously classified as vulnerable or endangered by the IUCN, conservation efforts ought to be pursued in order to slow the rate at which these species are becoming extinct. Present research article deals with taxonomy, flowering and fruiting period, present status, threats of critically endangered *Ceropegia* species found in and around Western Ghats region of Nashik district. The present study would help in identifying the species habitats and the areas to be prioritized for their conservation.

Key words: *Ceropegia,* Western Ghats, Nashik, Conservation.

Introduction

The Western Ghats is also called the 'Sahyadri' and the northern part of Western Ghats is the 'hotspot' having very high biological diversity. The Western Ghats range begins south of the Tapti River, close to the Gujarat-Maharashtra boundary. It passes 1,600 km across the states of Maharashtra, Goa, Karnataka, Tamil Nadu, and Kerala before terminating in Kanyakumari, which is located at the southernmost point of India [VENCATESAN & DANIELS, 2008]. The north-south run of Western Ghats is about 750 km and average breadth is 85 km. In Maharashtra, there are numerous naturally occurring vulnerable, rare, endangered and threatened (RET) plant species found in districts of northern Western Ghats, like Dhule, Jalgaon, Nandurbar, and Nashik [KSHIRSAGAR & PATIL, 2008; SANGALE & al. 2023]. Vegetation of the Northern Western Ghats can be divided into altitudinal zones. At elevations of 200-500 m, there is semi-deciduous and schrub vegetation. The moist deciduous forests with pockets of evergreen type in areas with more rainfall are found on the windward side of the Ghats, which receives the most rainfall [GAIKWAD & al. 2014].

Genus *Ceropegia* L. is tuberous herb and found growing on grassy slopes of hills.

It belongs to the Apocynaceae family and is indigenous to Australia, southern Asia, and Africa. Carl Linnaeus gave it its name; he originally described this genus in volume 1 of his 1753 publication *Species plantarum.* The blooms appeared to Linnaeus like a wax fountain. The

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scientific word, “keros” (wax) and “pege” (spring) were taken from this. There are 160-200 different species of genus *Ceropegia.*

It has been reported as critically endangered and endemic to Western Ghats of Maharashtra [MISHRA & SINGH, 2001]. It is one of the genera in the Apocynaceae family's subfamily Asclepiadoideae (milkweeds). The genus *Ceropegia* comprises 244 species distributed in Africa and Madagascar, extending in the east to Arabia, India and China, northern part of Australia as far as Canary Islands [BRUYNS, 2014; WFO Plant List, 2024; NIKAM & al. 2023]. Highest diversity of the genus occurs in South Africa, followed by Kenya, Madagascar and India [MURTHY & al. 2012]. In India, the genus is represented by 53 species, 2 subspecies and 6 varieties of which 37 are endemic to Peninsular India [AHMEDULLAH & NAYAR, 1986]. Majority of *Ceropegia* species in India occurs along steep hill slopes, rock crevices at low to high elevation lateritic plateaus, along with bushes, forest margins, grasslands of dry deciduous forests, shola forest margins and still others prefer to grow at slightly drier habitats. The northern Western Ghats region of Nashik district, includes places with dense vegetation like Peth, Baglan, Igatpuri, Trambakeshwar, and Sinnar which are exhibiting the presence of *Ceropegia* species.

Due to the habitat destruction or destructive collecting, most of the *Ceropegia* species are threatened. Not only these are genetically rare, but also scarcely available. It is noted that the majority of the species listed were previously classified as vulnerable or endangered by the IUCN RED list; as a result, conservation efforts ought to be made to slow the rate of extinction of these species [SUBBAIYAN & al. 2015].

Diverse conservation measures are available viz. *in situ*, *ex situ*, *in vitro*, participatory approach, local biodiversity boards etc. for critically endangered plants species like *Ceropegia*; However; there is a need for more research in terms of adoption of microhabitat/climatic specific, most suitable and feasible strategies [PATEL & al. 2017; AVHAD & al. 2023].

Germplasm conservation measures are being undertaken effectively so as to conserve narrowly endemic and critically endangered *Ceropegia* species. For example, *C. andamanica* acclimatized successfully through an *ex situ* conservation measures at Andaman & Nicobar Islands of India [DHOLE & al. 2023]. Recently a new species of *Ceropegia* namely *C. shivrayiana* is described at the Vishalgad fort in the Kolhapur district of Maharashtra state, India that resembles *Ceropegia lawii* Hooker f., which is distinguished by its hairy peduncles, obovate corolla cage, and reflexed corolla lobes [JANGAM & KAMBALE al. 2024].

Material and methods

Study area

Nashik district is located between latitudes 19°35' and 20°50' and the longitudes 73°30' and 74°55'. It covers the area of 15,582 sq. km. The prominent areas surveyed repeatedly, include mainly hilly areas like Anjaneri, Salher-Mulher, Ramshej, Saptashringi hill, Peint, Surgana, Chambhar leni, Sinnar ghat, and Igatpuri etc. (Figure 1).

Field visits and observations

Extensive field visits and botanical excursions were conducted during monsoon months (June to December) of year 2020-2022 covering to different corners and pockets of the northern part of Western Ghats of Nashik district. Systematic enumeration of observed *Ceropegia* species within their natural habitat, was done with reference to taxonomy, flowering and fruiting period, precise locations, ecology, threats, present status, etc. (Table 1). The identification was done with the help of floras and available standard literature [COOKE, 1957; NAYAR & SASTRY, 1988; LAKSHMINARASIMHAN & SHARMA, 1991; PRADHAN & SINGH, 1999; MISHRA & SINGH, 2001]. Voucher specimens have been deposited in the Herbarium, Department of Botany, Kr. V. N. Naik Arts Commerce & Science College, Nashik, Maharashtra.

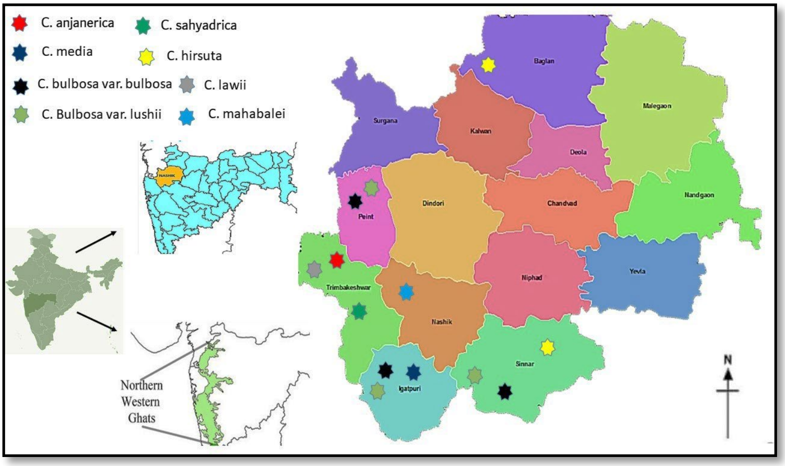


Figure 1. Location map showing distribution of *Ceropegia* species within Nashik district (Source: Maps of India.com)

Results and discussions

Systematic Enumeration

1. *Ceropegia anjanerica* Malpure, M. Y. Kamble & S. R. Yadav, Curr. Sci. 91(9): 1141. 2006; Karthik. *et al*., Fl. Pl. India 1: 160. 2009; Kambale & S. R. Yadav, Asklepios 115: 29. 2012. *Type*: INDIA, Maharashtra, Nashik district, Anjaneri hill, 10.09.2005. *N.V. Malpure* Malpure 1 (holo CAL! iso K! BSI! BLAT! SUK!).

Herb, flowers 2.5-3.5 cm long, usually slightly curved greenish yellow. Flower-tube is 1.2-1.5 cm gradually dilated at base, channelled with purple lines inside, lower inflated part deep purple. Petals are narrowly obovate. Flowers arise singly, on stalks 1-3 mm ling. Bracts are solitary, linear, sepals linear long-pointed, rough. Stem is round, rough, usually unbranched, up to 20 cm high. Leaves are opposite narrowing into stalks 2-5 mm long. Seeds 5×2 mm, ovate, coma white, silky. The tubers are edible and consumed by locals is one of the major threat observed during the study. The status of *C. anjanerica* recorded as endangered and endemic [PETHE & TILLU, 2016; NIKAM & al. 2023].

2. *Ceropegia bulbosa* var. ***bulbosa*** Roxb, cor. Pl. v. 1 (1795) p. 11, t. 7. Fl. B. I. v. 4, p. 67; Woodr. In Journ. Bomb. V. 12(1898) p.168; Watt, Dict: Econ. Prod. V. 2, p. 262.

Twiner, root tuberous, the size of a small turnip, a little flattened, with several fibres from its base, stem very slender, usually glabrous. Leaves excessively variable. Flowers pedunculated, umbellate cymes. peduncles long, arising from between the petioles; pedicels long slender. Corolla long greenish tube inflated at the base lobes long linear from deltoid base, hairy, connate at the tips, violet-purple inside outer corona-lobes inner corona-lobes narrowly linear, in long follicles, cylindrical, tapering towards an acute apex. Seeds long, ovate-oblong, flattened, with a broad sub membranous margin.

Over exploitation and habitat degradation are the important threats recorded during study. The status of *C. bulbosa* var. *bulbosa* is recorded as endangered

3. *Ceropegia bulbosa* var. ***lushii*** (Grah.) Hook. *f.* Fl. Brit. India 4: 68. 1883; Cooke, Fl. Pres. Bombay 2: 241. 1958 (Repr.); Ansari in Fasc. FL India 16: 11. 1984; Jagtap et al. Singh, N.P. in Fasc. Fl. India 24: 218. 1999. *C. lushii* Grah. in Bot. Mag. t. 3300. 1834. *C. acuminata* Roxb. Pl. Corom. 1: 12, t. 8. 1795; Hook. *f.* Fl. Brit. India 4: 78. 1883.

Tuberous twiners. Leaves 6-7 x 1.2-2.1 cm, acuminate. Flowers in axillary cymes; corolla 1.0-1.1 cm long; corona biseriate. Major threats were recorded as its over exploitation and habitat degradation. The status of C. *bulbosa* var. *lushii* is endangered.

4. *Ceropegia hirsuta* Wight &Arn. in Wight*,* Contrib. (1834) p.30. Fl. B. I. v. *4,* p. 71; Woodr. in Journ. Bomb. Nat. v. 12 (1898) p. 168. *Ceropegia jucquemontiana* Dalz. and Gibs. Bo. Fl.p.153. *C. ophiocephala* Dalz. in Kew Journ. Bot. V. 2 (1850) p. 259; Dalz. *&* Gibs. p. 154.

Saffruticose, twining, hirsute or pubescent roots tuberous, membranous, 5-10 cm. long, varying from ovate-cordate, obtusely cuminate, to narrowly lanceolate, acute, petioles long. Flowers umbellate cymes, peduncles long, arising from the petioles, acute, hirsute with rigid hairs. Corolla greenish, blotched with a very large club-shaped head tube inflated at base, enlarged at the mouth lobes as long as the tube. Seeds long, narrowly oblong.

Over exploitation and habitat degradation are the major threats recorded and the status of *C. hirsuta* is endangered.

5. *Ceropegia lawii* Hook*f.* Fl. Brit. India 4: 67, 1883; Cooke, Fl. Pres. Bombay 2: 240. 1958 pp. (Repr,ed.); Blatt, in J. Bombay nat. Hist. Soc. 36 (3): 534. 1933; Sant. Fl Purandhar 80. 1958, Sant. & Irani in Bull. Bot. Soc.

Herbs, erect, tuberous; stems pubescent above. Leaves opposite, petiolate, ovate-lanceolate, usually rounded at base, puberulous above. Cymes many flowered; peduncles and pedicels hairy. length of corolla 1.8-4 cm, with a tube length of 1.5 - 3.1 cm., inside a ring of hairs at the bottom of inflated base, rest glabrous; lobes 5-9 x 3-5 mm, ovate-cordate, hairy or glabrous inside. Outer corona of 10 obtuse lobes, hairy; inner linear, erect, 3-4 times as long as outer. Major threats recorded was its over exploitation and the status of *C. lawii* is endangered.

6. *Ceropegia media* (Huber) Ansari in Bull. Bot. Surv. India 11 (1 & 2): 199. (1969) 1971; Raghavan & Singh in Jain & Sastry (eds.), P1. Cons. Bull 3: 9. 1983; Ansari in Fasc. Fl. India 16: 24, t. 4 (18), *f.* 13. 1984; Singh & Raghavan in J. Econ. Tax. Bot. 8(1): 31. 1986.

Herbs, twining, tuberous; stems glabrous. Leaves opposite, petiolate, 5-15 x 1.5 cm, linear-lanceolate, puberulous above. Cymes 2-4-flowered; peduncles 1-2 cm long, pubescent; pedicels up to 1 cm long, hairy; length of bracts and bracteoles ranges 1-4 mm, lanceolate, glabrous. length of corolla 2.8 cm, with a tube length 2 cm, base slightly inflated, glabrous inside; lobes ca 8 x 2.5 mm, oblong, glabrous. Major threats were recorded as its over exploitation and the status of *C. media* is endangered.

7. *Ceropegia mahabalei* Hemadri et al. & Ansari in Indian For. 97(2)7 105. t, 1 (b). *f.* 1-4, 1971. Raghavan Singh in lain & Sastry (eds.). Pi. Cons. Bull, 3: 9. 1983; Amain in Fast Fl.

India 16: 24. t. 3 (17). 1984: Raghavan & Singh in J. Econ. Tax. Bot. 5(14: 159. 1984; Singh & Raghavan in ibid. 8 (1): 31. 1986.

Herb, erect ca 20-65 cm high, tuberous; stem hairy. Leaves opposite. subsassile, 3-15 x 0.3-1 cm, linear to linear-lanceolate, hairy above. Cymes 1-floweted; peduncles 1,3 mm long, hairy; pedicels 5-10 mm long. hairy bracts 5-15 mm long, subulate. Calyx 5-partite, lobes 1.1.75 cm long. hairy. Corolla 5.5-10 cm long; tube 3.5-6.5 cm long, base largely inflated. Narrowed in the neck, enlarging towards mouth, glabrous inside; lobes 1. 5-3 cm long, linear, elongated above from ovate deltoid base, hairy within. Seeds many, ovoid; coma ca 6 mm long, white. Major threats were recorded as its over exploitation and the status of *C. mahabalei* is endangered.

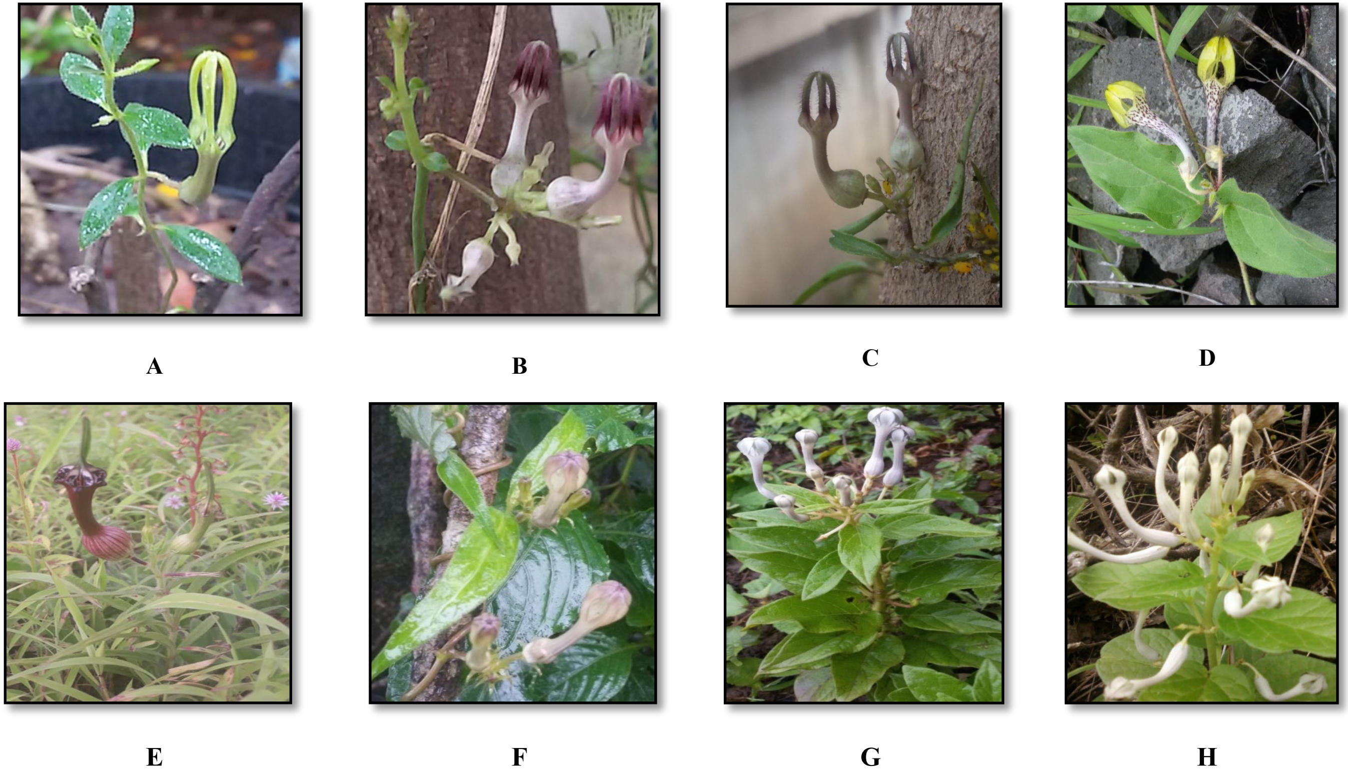
8. *Ceropegia sahyadrica* Ansari and B.G. Kulk. in Indian For. 97 (12): 68S, t.1, f. 1-4, t. 2, f. A (1, 2). 1971; Raghavan & Singh in Jain & Sastry (eds.), Pl. Cons. Bull. 3: 10. 1983 et in J. Econ. Tax. Bot. 5 (I): 159. 1984: Ansari in Fasc. Fl. India 16: 29, t. 4 (24). 1984; Mistry, Fl. Ratnagiri. I: 391. 1986 (*PhD. Thesis*); Ahmedullah & Nayar, Endemic Pl. Indian Reg. 1: 122. 1987.

Herbs, erect, 30-100 cm high, tuberous; stems, pubescent above, leaves opposite, 4-11 x 2-8 cm, ovate or ovate-lanceolate, cordate or rounded at base. Cynics few to ninny flowered; peduncles 1-5.5 cm long, hairy; pedicels 10-17 mm long, hairy; bracts 5-7 x ca I mm, linear, Sepals 5-7 mm long, glabrous. Corolla slightly curved, tube up to 4.4. cm long, base inflated with a ring of hairs inside at bottom, rest glabrous; lobes up to 2 x 8 mm, ovate, subcordate, glabrous. Follicles in pairs, up to 1.5 cm long, many seeded. Major threats were recorded as its over exploitation and the status of *C. sahyadrica* is endangered.

The present record of *Ceropegia* species are from different locations across the Nashik district like Sinnar, Anjaneri, Chambhar leni- Nashik, Igatpuri etc. which is restricted to small biogeographical areas and are rare in occurrence. (Table 1, Figure 1). Moreover, their populations have been declining rapidly due to habitat modifications and anthropogenic pressures. Therefore, *ex situ* and *in situ* conservation practices are recommended so as deal with various threats recorded during the study. Out of total *Ceropegia* species recorded, most species have been reported earlier from the Konkan region of Western Ghats. [PUNEKAR & al. 2006; KAMBALE & YADAV, 2019]. Species like *C. mahabalei*, *C. sahyadrica*, *C. bulbosa*, *C. hisrsuta* and *C. media* were discovered to be mostly growing on the dry cliffs and hill passes of the Western Ghats of Nashik district; which are known as ecologically specialized microhabitat. [DATAR & WATVE, 2018; SANGALE & al. 2021.

Table 1. Checklist of *Ceropegia* species observed at different locations within Nashik district

Species	Local Name	Habit	Habitat	Flowering Season	Locations	Latitude	Longitude
Ceropegia anjanerica Malpure, M.Y. Kamble & S.R. Yadav	Lahani Kharpudi	Herb	High altitude, Moist soil, Open Plateaus Rocky areas among the grasses.	August-September	Anjaneri, Trymbakeshwar	19.921 N	73.570 E
Ceropegia bulbosa var. *bulbosa* Roxb,	Hamil	Twiner	Moist soil, among the bushes and cactus plants	August-September	Igatpuri	19.704 N	73.568 E
					Sonambe	19.767 N	73.927 E
Ceropegia bulbosa var. *lushii* (Grah.) Hook.	Hamil	Twiner	Moist soil, among the bushes and cactus plants	July-October	Peth forest	20.258 N	73.510 E
Ceropegia hirsuta Wight &Arn	Khutti, Haaman	Twining Climber	Hilly slopes, among Cactus	August-September	Sonambe	19.767 N	73.927 E
					Adwadi	19.737 N	73.922 E
					Dubere	19.794 N	73.958 E
					Salher	20.723 N	73.945 E
Ceropegia lawii Hook *f.*	Moti Kharpudi	Twining Herb	High altitude, hilly slopes, moist soil, rocky areas	August-October	Adwadi,	19.737 N	73.922 E
					Anjaneri hill	19.921 N	73.570 E
Ceropegia media (Huber) Ansari	Medi Kharchudi	Twining Herb	High altitude, hilly slopes, moist soil, rocky areas	July-October	Igatpuri	19.704 N	73.568 E
Ceropegia mahabalei Hemadri et al. & Ansari	Kharchudi	Herb	Moist soil, Open Plateaus Rocky areas among the grasses.	August-October	Chambhar leni Nashik	20.068 N	73.788 E
Ceropegia sahyadrica Ansari & B.G. Kulk.	Kharpudi	Herb	Moist soil, Open Plateaus among the grasses.	August-October	Advadi Sinnar	19.737 N	73.922 E
					Anjaneri, Trymbakeshwar	19.921 N	73.570 E



A B C D E F G H

Figure 2. *Ceropegia* species records in Western Ghats region of Nashik district

A – *Ceropegia anjanerica* Malpure, M. Y. Kamble & S. R. Yadav; **B** – *Ceropegia bulbosa* Roxb. var. *bulbosa*; **C** – *Ceropegia bulbosa* Roxb. var. *lushii* (Graham) Hook. f.; **D** – *Ceropegia hirsuta* Wight & Arn.; **E** – *Ceropegia mahabalai* Hemadri & Ansari; **F** – *Ceropegia media* (H. Huber) Ansari; **G** – *Ceropegia lawaii* Hook. f.; **H** – *Ceropegia sahyadrica* Ansari & B. G. Kulk.

Conclusions

It is found that except the places like Anjaneri, Trymbakeshwar and Igatpuri in the northern part of Nashik Western Ghats, most of the locations in the study area are not densely forested. These pockets were explored in earlier similar works and most of the *Ceropegia* species recorded have already been documented during earlier studies. However, few *Ceropegia* species were observed at places like Advadi, Sonambe, Dubere of Sinnar tehsil and Chambhar leni from Nashik City area, where the forest cover is relatively low and the rainfall is also less.

Moreover, human intervention is increasing on a large scale in all the above areas due to local development projects and continuously changing rainfall patterns etc. Thus the habitats of few *Ceropegia* species are also found changing to a large extent. Similarly, there are some new habitats that have not been recorded in such previous studies.

It is found that insufficient study has been done in case of recorded critically endangered and endemic species especially, *C. anjanerica*. Prompt conservational actions should be undertaken by the concerned authorities as well as NGO’s and other conscious peers of the society. Also, the *Ceropegia* species enumerated in the present study require a species recovery program, whereby these are conserved in botanical gardens and in order to ensure their establishment; these can be reintroduced in their original habitats under careful observation. This study would help in identifying the species habitats and the areas to be prioritized for conservation.

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Research Article

IN-SITU CONSERVATION OF *EPIPACTIS PALUSTRIS* (L.) CRANTZ IN THE REPUBLIC OF MOLDOVA

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Abstract: The given paper provides recent data on the rare species, *Epipactis palustris* (L.) Crantz, (family Orchidaceae) from the "Codrii" Scientific Reserve, Republic of Moldova, including the current state of the taxon population, the degree of threat, the endangered category (according to IUCN requirements). Likewise, it contains the morphological description of the species, the ecological and chorological particularities and the characteristics of the habitat under study. Limiting factors are identified and protective measures are proposed.

Keywords: *Epipactis palustris* (L.) Crantz, rare taxa, bioecology, conservation, Republic of Moldova.

Introduction

In the flora of the Republic of Moldova genus *Epipactis* Zinn represents the most numerous and interesting genus in the family Orchidaceae, especially from a taxonomic perspective. Until 1979, 2 species of the genus *Epipactis* were known in the spontaneous flora of the Republic of Moldova – *E. helleborine* (L.) Crantz and *E. palustris* (L.) Crantz. In the same year, 1979, V. KIRTOKA as a result of field research and analysis of herborized materials identified two more species: *Epipactis purpurata* Smith and *E. atrorubens* (Hoffm.) Schult [KIRTOKA, 1979]. Recently, in 2023, a new species has been identified – *Epipactis leptochila* (Godfery) Godfery [SFECLĂ & al. 2023]. Thus, currently, in the flora of the Republic of Moldova there are 5 species of *Epipactis*, all of which are endangered taxa.

Rare species research is included both in the provisions of national laws and strategies, as well as in the international obligations of the Republic of Moldova, and the protection *in situ* of threatened species is a priority objective, included in the Biodiversity Conservation Strategies. Periodic assessment of the ecological status of rare plant species, as well as monitoring of populations of endangered floristic taxa is necessary, the data obtained provide updated information on the real state of species and the dynamics of their populations [IONIȚA & JARDAN, 2023]. This study refers to *Epipactis palustris* (L.) Crantz – Critically Endangered species (CR), protected by law, included in the Red Book of the Republic of Moldova (2nd and 3rd ed.). Beyond the borders of the republic, the species is spread from Europe to the Caucasus and Mongolia [powo.science.kew.org]. *Epipactis palustris* is distributed throughout Europe, but it is extremely rare in the southern Mediterranean region [JACQUEMYN & al. 2014; DJORDJEVIC & al. 2017] (Figure 1).

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Figure 1. *Epipactis palustris* (L.) Crantz distribution worldwide powo.science.kew.org

🟩 – Native 🟪 – Introduced

Material and methods

The study is based on floristic field research of the species *Epipactis palustris* (L.) Crantz (family Orchidaceae) conducted during 2021-2023 within the Scientific Reserve "Codrii", located in the center of the Republic of Moldova. During the investigations, scientific publications containing information about the bioecology and chorology of the species *Epipactis palustris* were reviewed, the existing herborized collections kept in the Herbarium of the National Botanical Garden (Institute) "Alexandru Ciubotaru" and the Museum of Natural Sciences of the State University of Moldova were critically processed, in order to specify the chorology of the species. Floristic research and critical analysis were carried out in accordance with the classical comparative-morphological method [KOROVINA, 1986]. The nomenclature and description of the species is presented according to current databases (The International Plant Names Index [IPNI, 2023], Plants of the World Online [POWO, 2023], The World Flora Online [WFO, 2023]) and fundamental works in the field [GHEIDEMAN, 1986; MOSER & al. 2002; PÂNZARU & al. 2002; NEGRU, 2007; MATCHUTADZE, 2014].

During the study, the area of the population was determined, its number was established, specifying the ontogenetic stage of each individual, vegetative or generative. The geographic coordinates were recorded using the GPSMAP® 64s GPS device CARMIN. The population assessment of *Epipactis palustris* species was carried out, the degree of threat was estimated and the endangered category established, according to the requirements of the International Union for Conservation of Nature [BILZ & al. 2011; IUCN, 2012] and the Legislation of the Republic of Moldova [Legislația, 1999], identifying limiting factors and proposing protection measures. The color photos are original, taken by authors with a Nikon D 3100 digital camera.

Results and discussions

Epipactis palustris (L.) Crantz, Stirp. Austr. Fasc., ed. 2. 2(6): 462 (1769).

Basionym: *Serapias helleborine* var. *palustris* L. Sp. Pl. 2: 950 (1753).

Long, recurrent, branched stoloniform rhizome. Stem 15-60 (80) cm long, erect, pubescent in the upper part, at the base with 1 or more scale-shaped, sheathed leaves. Leaves 4-8, spirally arranged, 5-17 cm long, oblong-ovate to oblong-lanceolate, acute, adaxially concave, abaxially, often purple; the upper ones narrow, acuminate, bracteiform; all ± erect, folded, abaxially prominently veined. Inflorescence – raceme 6-15 (20) cm long, lax, with 7-14 flowers. Bracts 5-25 mm long, lanceolate, acute, the lower ± equal to or slightly longer than the ovary. Flowers inodorous, zygomorphic, bisexual, initially campanulate, nutant, later wide-open, horizontal, finally pendulous, cream-white or, rarely white, more or less purplish-red or brown streaked. Outer perianth segments 7-12 mm long, lateral segments slightly longer than median segment, rounded on one side, brown, whitish or dull green outside, purple-red inside, lanceolate, obtuse to acute apex, concave, prominently 3-5-nerved, abaxially pubescent, adaxially glabrous. Inner perianth segments shorter and narrower than outer, whitish with purple veins at base, lanceolate, obtuse, glabrous, with branched veins. Label 10-12 mm long, equal to the external segments of the perianth. Gynostemium short, yellowish-green, extended upwards. Anther elliptic or subtetraangular, slightly bilobed. Pollen granular, oval, yellowish-white, friable; caudicles missing. Stigma transverse-elliptic or subtetraangular, bilobed. Glandular rostellum; viscidium obvious, white, sticky, present in open flowers. Ovary 9-16 mm long, narrow-pyriform, puberulent. Fruit – capsule 13-18 mm long, pendent. Seeds ovoid, 1.2-1.5 mm long, light brown [PAUCĂ & MORARIU, 1972; MOORE, 1980; CIUBOTARU & al. 2007; JACQUEMYN & al. 2014; OPREA, 2019; FLORA OF CHINA, 2024] (Figure 2).

Figure 2. *Epipactis palustris*: flower; habitus, inflorescence in fruit.

According to literature data, in the flora of the Republic of Moldova, over the last 50 years the state of the population has fluctuated considerably. Although in some publications *E.*

palustris is quoted from two locations: Răciula village, Călărași district and Lozova commune, Strășeni district [CHIRTOACĂ, 2002; PÂNZARU & al. 2002; POSTOLACHE, 2015], currently the presence of the species in the former remains uncertain, because neither concrete data nor herborized materials have been found from this point of distribution. According to the results of studies published by GHEIDEMAN T. (1986) and KIRTOKA V. (1988) stated that on the territory of the republic *Epipactis palustris* they grow only in the "Codrii" Reserve [GHEIDEMAN, 1986; KIRTOKA, 1988] (Figure 3).

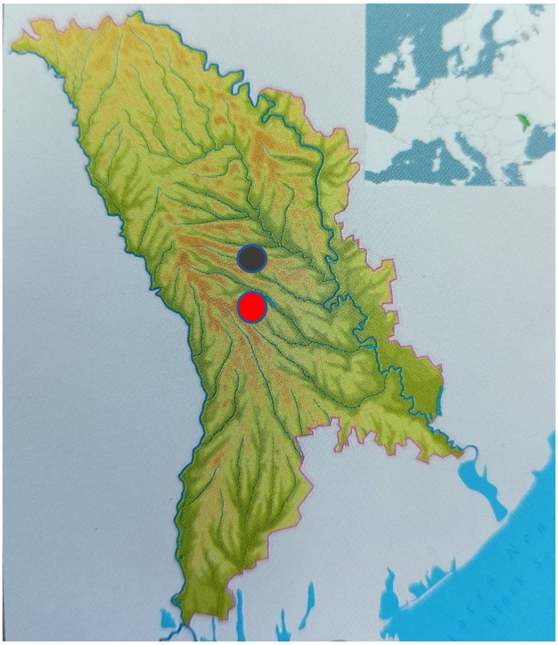
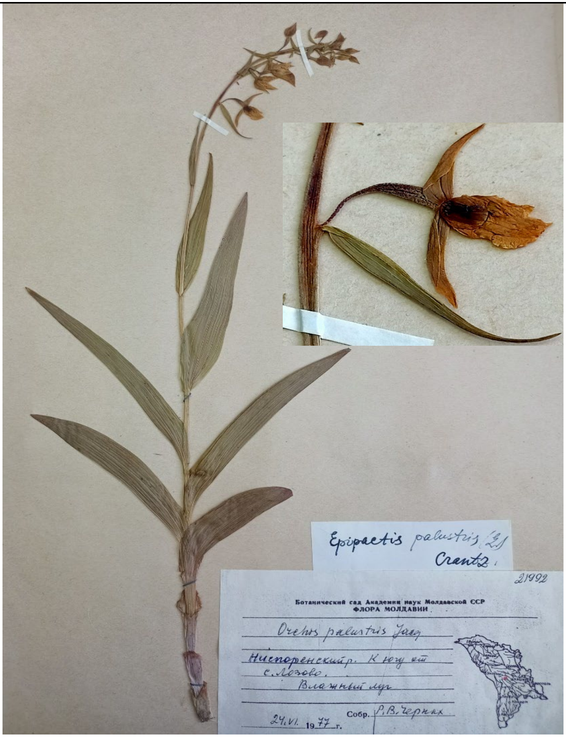


Figure 3. Distribution of *Epipactis palustris* in the Republic of Moldova:

🔴 – currently existing population 🔵 – extinct or probably extinct population

As a result of the critical analysis of the exsiccatae kept in the herborized collections of the country, in the Herbarium of the National Botanical Garden, 11 specimens of *Epipactis palustris* has been identified, three of which were collected from southern Bessarabia in 1927 and 1929 by the renowned scientists C. ZAHARIADI and N. ZELENETSKII, but not part of our study. The other 8 specimens were collected from the same location as follows: 4 specimens collected near the commune Lozova, Strășeni district (Figure 4), from the wet meadow with *Eriophorum latifolium* Hoppe near the Forestry Department (NICOLAEVA, GHEIDEMAN, 17.07.1952); 1 specimen each collected from the same location by other researchers (TEPLOVA, 25.06.1955; BOGONINA, 07.05.1956; CIORNÎH, 24.06.1977; KIRTOKA, 21.07.1977).



Epipactis palustris (L.) Crantz.

21992

Ботанический сад Академии наук Молдавской ССР
ФЛОРА МОЛДАВИИ

Orchis palustris Jacq

Ниспоренский р. К югу от с. Лозово.
Влажный луг

24.VI. 1977 г. Собр. Р.В. Черных

Figure 4. Specimen of *Epipactis palustris* collected from Lozova, in 1977

Thus, as a result of field research in recent years, the population reported in the vicinity of Lozova commune, from the "Codrii" Scientific Reserve, was assessed, the first collections here dating back to 1952. *E. palustris* grows through wet meadows of easily floodplain phytocoenoses. On the territory of the reserve it grows in the meadow located in the wide valley of the river between the forested slopes (N 47º06'16.7", E 28º21'46.9"), with an abundance of 1-2, on a wet meadow with vegetation dominated by grasses and various species of meadow. *Epipactis palustris* is the only species of the genus *Epipactis* that in the spontaneous flora of the Republic of Moldova grows outside the forest habitat and does not tolerate shading.

The sector in which *Epipactis palustris* grows was declared in 1975 a protection zone, being a representative sector with meadow vegetation "Meadow with *Eriophorum latifolium* Hoppe", association which includes other relict species, such as: *Dactylorhiza incarnata* (L.) Soó, *Thelypteris palustris* Schott, *Hypericum tetrapterum* Fries [KIRTOKA, 1982], all being included in the Red Book of the Republic of Moldova, 3rd ed. In the composition of the grassy carpet were also reported species such as: *Angelica sylvestris* L.f, *Arctium lappa* L., *Cirsium canum* (L.) All., *Cirsium vulgare* (Savi) Ten., *Inula helenium* L., *Orchis palustris* Jacq., *Symphytum officinale* L., *Valeriana officinalis* L., etc. (Figure 5).

Figure 5. *Epipactis palustris* in natural habitat

Bioecology. Perennial plant, Eurasian geophyte [CIOCÂRLAN, 2009], mesohygrophyte species. It blooms in June-July, capsules ripen in August. It is pollinated by insects. Multiply vegetatively, from buds on the rhizome and generatively, but young seedlings, provenient from seeds, were practically not reported. The rhizomes are thin, branched and located close to the soil surface, The tip of the rhizome grows upward each year to produce the aerial stem, while horizontal growth is continued by a new bud (or 2) emerging from the base of the vertical stem. Thus, over the course of a few years, a single plant can emit a considerable number of floriferous stems and vegetative shoots [JARDAN & CHIRIAC, 2008; JACQUEMYN & al. 2014; http://www.orchidsofbritainandeurope.co.uk/Epipactispalustris.html].

Decorative plant during flowering. It grows in groups, the herd and morphological parameters vary from year to year. In the summer of 2023, the population of the species *E. palustris* covered an area of about 56 m^2 with ± 100 phytoindividuals, predominating mature, floriferous specimens reaching 30-70 cm in height. Taking into account the data from the literature, according to which in 2001 the population contained 20-30 phytoindividuals [CHIRTOACĂ, 2002], and in 2015 there were 10-30 specimens [POSTOLACHE, 2015], we find with certainty that the conservation status of the taxon within the habitat is relatively favorable, stimulating the increase of the numerical population. Among the basic threats that can cause a decrease in the number of individuals we can mention: the change in the degree of soil moisture, often caused by long drought, which leads to drying of the biotope and negatively influences plant development, floodplain invasion by woody and/or invasive species, grassland valorization.

Sozologic status. In the Republic of Moldova *Epipactis palustris* is an endangered species, categorized, according to the guide developed by the International Union for Conservation of Nature as Critically Endangered [CR B2ab(ii,iii,v)].

At the same time*, Epipactis palustris* is a rare species in the countries bordering the Republic of Moldova, being included in the Red Book of Ukraine, in the category “Вразливий” – vulnerable [TIMCHENKO & KUZYARIN, 2009], as well as in the Red List of superior plants in Romania [OLTEAN & al. 1994].

At the European level, *Epipactis palustris* is widespread and often forms dense populations. Although populations are decreasing, existing threats are unlikely to cause them to decline severely in the near future. Therefore, in 2014, *Epipactis palustris* was assessed and included in the IUCN Red List as a low-risk threat [LC] species [MATCHUTADZE, 2014]. *Epipactis palustris* is also listed in *Annex II* of the Convention on International Trade in Endangered Species of Wild Fauna and Flora [CITES]. Several national red lists are included in European countries *Epipactis palustris* and are Endangered in Bulgaria [PETROVA & VLADIMIROV, 2009], Czech Republic [HOLUB & PROCHÁZKÁ, 2000], Norway and Finland; Vulnerable in Germany, Near Threatened in France and Hungary [KIRÁLY, 2007], Low risk in Denmark, Switzerland [MOSER & al. 2002] and the United Kingdom [MATCHUTADZE, 2014].

Protective measures. *Epipactis palustris* is an important species from the point of view of biodiversity conservation. Territorially, in the Republic of Moldova, it is protected in the Scientific Reserve “Codrii”. As effective protection measures, we propose the rigorous observance of the reserve regime, an essential condition for the conservation *in situ* of endangered species, the protection of the only growing place, the monitoring of the population status, the management of grazing and regulated mowing to control the excessive growth of vegetation, ensuring the water regime by avoiding water leakage, the conservation of the species in *ex situ* conditions, preservation of germplasm in gene banks.

Conclusions

It is important to note that although the species has been evaluated by the International Union for Conservation of Nature and categorized as LT (Least Concern) – at low risk of extinction, still requires compliance with conservation and population monitoring measures. Even if currently in the spontaneous flora of the Republic of Moldova the population of the species *Epipactis palustris* apparently it seems stable, its protection should not be reduced, because it is precisely the fact that conservation measures have been rigorously observed that has led to stopping the decline and the favorable status of the population. Estimated regional conservation status in Republic of Moldova: Criticaly Endangered [CR B2ab(ii,iii,v)].

Because the species is very rare, definitely identified in only one locality, it is necessary to comply with effective conservation measures such as: surveillance and monitoring of the existing population, study of its dynamics, favoring the conditions of expansion. Thus, limiting factors can be quickly identified and urgent and effective measures can be taken to halt the decline of populations of species threatened with their extinction and conservation.

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Research Article

RECONFIRMATION OF RARE PLANTS ON THE NORTHWESTERN BLACK SEA COAST

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Abstract: Rare plant species are particularly vulnerable to anthropogenic influences, which contribute to reducing their populations and restricting their distribution areas. The objective of this study was to reconfirm the information on the distribution of three rare plant species – *Tripidium ravennae*, *Trachomitum venetum*, and *Stachys maritima*, within the Danube Delta Biosphere Reserve, Romania. The research was carried out between 2019 and 2023. The results highlighted that the respective species are rare by showing limited distribution and small populations. Among the main threats to the survival of these species are tourism development, overgrazing, and soil compaction. *Trachomitum venetum* is a rare plant species in specific communities, showcasing its selective habitat preferences. *Tripidium ravennae* is a rare species in specific plant subassociations and plant associations, indicating its limited distribution. *Stachys maritima* is rare within different plant communities, reflecting its adaptability to varied environmental conditions within the Danube Delta Biosphere Reserve.

Keywords: Danube Delta, Romania, *Stachys maritima*, threats, *Trachomitum venetum*, *Tripidium ravennae*.

Introduction

At the global level, biodiversity faces threats [BUTCHART & al. 2010] due to impacts stemming from human activities [REICH & al. 2001; FAGERLI & al. 2011]. Rare species confront an elevated risk of extinction [CHICHORRO & al. 2019]. The reasons behind the persistence of certain plant species as rare, while others thrive abundantly, remain a subject of investigation and debate [TITTENSOR & al. 2010; WAMELINK & al. 2014]. This rarity may result from intrinsic factors, limitations in dispersal capacity, the impact of management practices, or abiotic conditions. Understanding the habitat preferences of rare plants is essential for effective conservation and management strategies. Rare plants often have specific habitat requirements or they are at the limit of their spreading area that can be influenced by factors such as soil *p*H, moisture levels, light availability, and specific symbiotic relationships. Recognizing these preferences helps identify critical habitats that require protection and can guide restoration efforts in degraded areas [WAMELINK & al. 2014].

The distribution of a species may also be constrained by its dispersal capacity [VERBERK, 2011]. This occurs, for instance, when the conditions of a habitat align with a species' needs, but it fails to reach that suitable habitat due to natural limitations in its spread [NORMAND & al. 2011]. Anthropogenic impact on rare species and their distribution is often

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exacerbated by habitat alterations, such as urbanization, infrastructure development, changes in water management practices, and intensified agriculture [NEWBOLD & al. 2015; CEBALLOS & al. 2017]. These human actions can disrupt ecological balance and drastically limit the natural spaces essential for the survival of rare species.

Understanding threats to rare plant species is important for developing biodiversity conservation and management strategies [McCLINTON & al. 2022]. This understanding relies on three main pillars, namely: 1) individual species assessments [GASTON & FULLER, 2009; IUCN, 2020]; 2) general knowledge about threats [SALA & al. 2000; PIMM & al. 2014], and 3) the role of protected areas [RODRIGUES & al. 2004; FELIX RIBEIRO & al. 2022].

The conservation strategies are essential for addressing these species' specific needs and vulnerabilities. For example, habitat destruction, whether through urbanization or agriculture, removes the unique conditions many rare plants rely on for survival. Similarly, climate change can alter the temperature and precipitation patterns critical to the life cycles of these plants, further endangering their existence [CORLETT, 2016]. Invasive species pose another significant threat: they can outcompete native flora for resources, alter habitat structures, and introduce diseases. The resilience of rare plant species to these threats is often low due to their limited distribution and specialized habitat requirements, making them more susceptible to decline or extinction [EARLY & al. 2016]. Rare plant species often serve as indicators of the overall status of an ecosystem, as they may be susceptible to changes in their environment. Studying these plants can provide valuable insights into the effects of climate change, habitat destruction, and the impact of invasive species. Understanding rare plants' distribution, ecology, and conservation status is essential for effective biodiversity conservation strategies.

The Danube Delta, recognized as a UNESCO World Heritage site, Biosphere Reserve, and Natura 2000 site, stands as proof of the complexity and richness of natural ecosystems, offering a habitat for a myriad of plant species, among which rare and endemic species hold a place of significant interest for conservationists and scientists alike [CLAUDINO-SALES, 2019; IUCN 2017]. The conservation and study of these rare plant species are not merely acts of environmental stewardship but are essential for maintaining the biodiversity and ecological balance that underpin the status and function of the ecosystem.

Material and methods

Fieldwork for this study was conducted in the Danube Delta Biosphere Reserve (Figure 1), spanning from March 2019 to July 2023. Plant species identification followed CIOCÂRLAN (2009), and species nomenclature adhered to the Euro+Med PlantBase standards [EURO+MED, 2024]. COLDEA & al. (2012) guided the categorization of plant communities (as plant associations), and habitat classification was aligned with the EUNIS [CHYTRY & al. 2020]. This research offers an in-depth examination of the species studied, encompassing their distribution, factors affecting their presence, conservation status, and plant communities. Geographic mapping of the findings utilized QGIS software, version 3.28.3 [QGIS Development Team, 2024]. To assess the rarity of species within the plant communities wherein they are situated, we evaluated based on Gaston's measure of rarity method [GASTON, 1994]. Gaston’s measure of rarity is a method used to assess the rarity of plant species based on their geographic distribution, habitat specificity, local abundance, and population size and fragmentation as follows: + - very rare or occasional, present but insignificant in coverage; 1 – rare but noticeable, with low density and limited impact on vegetation cover; 2 – more frequent but still sporadic, coverage is less than 25%; 3 – common and widespread, coverage is between

25% and 50%; 4 – dominant, occupying significant space, with coverage between 50% and 75%; 5 – very dominant, overwhelmingly present, with coverage exceeding 75%. This involved recording the aggregate abundance of individual species within one-meter relevés at sites where the respective plant species were identified. Notably, irrespective of a species' high numerical presence within a specific quadrat, it was categorized as rare if its abundance did not demonstrate consistency across multiple relevés within a given area.

Acronymes: DDBR is for Danube Delta Biosphere Reserve; ass. is for plant community/plant association.

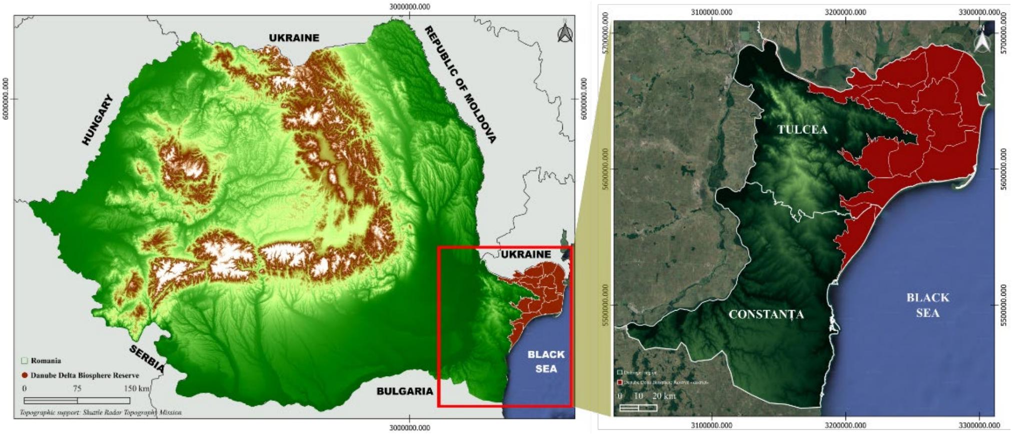

Figure 1. Maps of the study area: a) at the national level; b) at the regional level

Results and discussions

- ***Trachomitum venetum*** **(L.) Woodson - Fam. Apocynaceae Juss.**

Syn. *Apocynum venetum* L., *Trachomitum venetum* (L.) Woodson subsp. *venetum*

During the study period, the species was identified on the Sand Islet of Perișor (Tulcea County; Figure 2). The species was considered rare in the protected area and was identified in the following plant communities/plant associations: ass. *Artemisio santonici-Juncetum maritimi* Šeljag-Sosonko *et al.* 2000, ass. *Schoeno nigricantis-Plantaginetum maritimae* Horvatić 1934, and ass. *Salsolo ruthenicae-Xanthietum strumarii* Oberd. *et* Tx. 1950. The *Salsolo ruthenicae-Xanthietum strumarii* association has been identified as neighboring *Artemisio santonici-Juncetum maritimi*, with the studied species found in both associations, although evidently more abundant in the latter mentioned association. In the *Salsolo ruthenicae-Xanthietum strumarii* association, the species *Trachomitum venetum* is grazed; the specimens are small, permanently consumed, and exhibit a creeping plant appearance. The main factors limiting the survival of this species include the grazing, as well as the diminution and degradation of natural habitats, such as the dunes area, caused by these activities. Pollution is a significant negative factor as well.

At the site level, *T. venetum* was found in three plant communities, two of them being framed in a single Natura 2000 habitat, as follows: 1410 Mediterranean salt meadows (*Juncetalia maritimi*) within the ass. *Artemisio santonici-Juncetum maritimi* Šeljag-Sosonko *et al.* 2000 - 15 out of 27 relevés (55%), the species is considered frequent and ass. *Schoeno nigricantis-Plantaginetum maritimae* Horvatić 1934 - 5 out of 21 relevés (23%), the species is considered rare. In ass. *Salsolo* *ruthenicae-Xanthietum strumarii* Oberd. *et* Tx. 1950 the species was found in 4 out of 15 relevés (26%), which is considered rare.

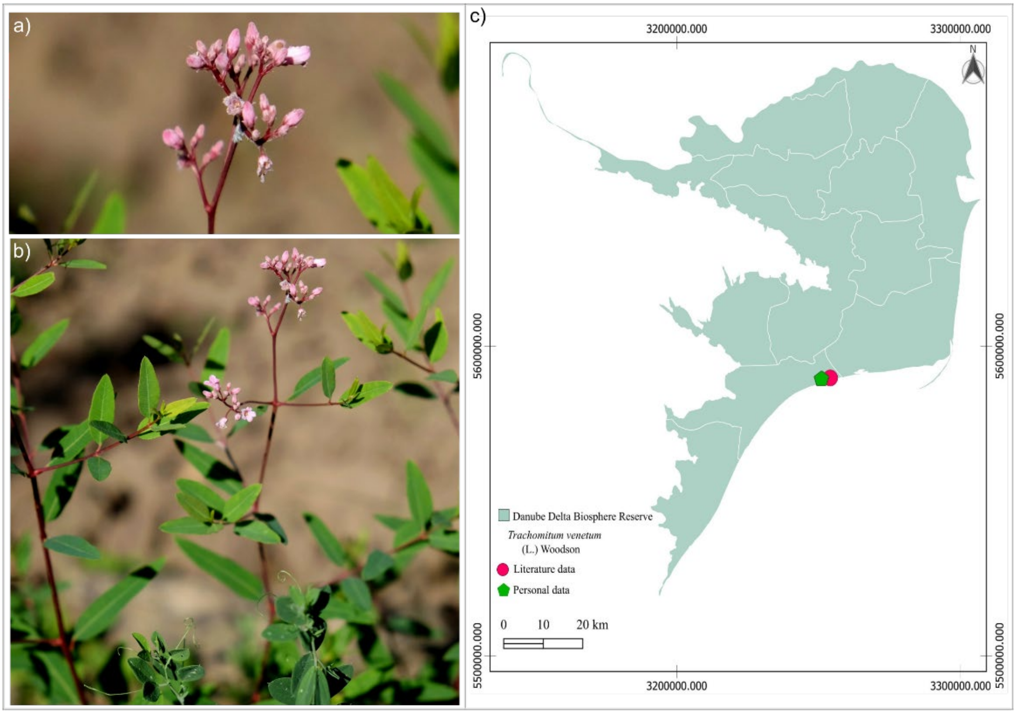

Figure 2. *Trachomitum venetum*: a) stems and leaves, b) habitat, and c) the distribution of the species in DDBR (photos a, b – Adrian Oprea, figure c – Simona Chirilă)

Description. *T. venetum* is a perennial species [NYÁRÁDY, 1961; CIOCÂRLAN, 2009] with a spreading rhizome [NYÁRÁDY, 1961; DIHORU & NEGREAN, 2009]. The stem is erect and richly branched, with a height of 50 (-70) cm [NYÁRÁDY, 1961; FloraVeg, 2024]. The leaves are elongated or elongated-lanceolate, very short petiolate, glabrous, mucronate [NYÁRÁDY, 1961; DIHORU & NEGREAN, 2009], with a length between 20-40 cm and a width between 5-10 cm. The flowers, small in size and with short pedicels, are surrounded by bracts and are pale pink; fruits are pendulous follicles between 5-15 cm long. The plant contains toxic latex [NYÁRÁDY, 1961; DIHORU & NEGREAN, 2009] and reproduces by seeds. Insects carry out pollination (is an entomophilous plant species), and the action of the wind disperses the seeds. The species is considered a relict of Romanian flora [DIHORU & NEGREAN, 2009].

Distribution, conservation status, ecology, and coenology

The species *T. venetum* is widespread in Southern, Eastern, and Central Europe up to Eastern Asia [NYÁRÁDY, 1961; KOZUHAROV & PETROVA, 1982; GBIF, 2024a] (it is cited to grow, also, in China [NYÁRÁDY, 1961], but it is not yet mentioned in Flora of China [FLORA OF CHINA, 2024]. In Romania, the species was reported in Dobrogea, Tulcea County, at grindul Perișor [BRÂNDZĂ, 1898; PRODAN, 1939; CIOCÂRLAN, 1994; SÂRBU & al. 2013]. The species is not evaluated at the Global and European levels [EEA, 2024a]. In

Romania, the species was considered “extinct” [OLTEAN & al. 1994] or “critically endangered” [OPREA, 2005; DIHORU & NEGREAN, 2009]. At the Danube Delta Biosphere Reserve level, the species was considered “endangered” [OȚEL, 2000]. The species *T. venetum* would grow in marshes, sands, sandy places, and on well-drained, moist soil [NYÁRÁDY, 1961; DIHORU & NEGREAN, 2009]. In fact, on Perişor Sand Islet, the only location in Romania, it grows along wet depressions, on salty sands, among the *Juncus maritimus*/*J. littoralis* tufts; marginally, it will be met within *Plantago maritima* and *Salsola kali* subsp. *ruthenica* plant communities.

- ***Tripidium ravennae* (L.) H. Scholz – Fam. Poaceae Barnhart**

Syn. *Andropogon ravennae* L., *Erianthus ravennae* (L.) P. Beauv., *Ripidium ravennae* (L.) Trin., *Saccharum ravennae* (L.) Murray

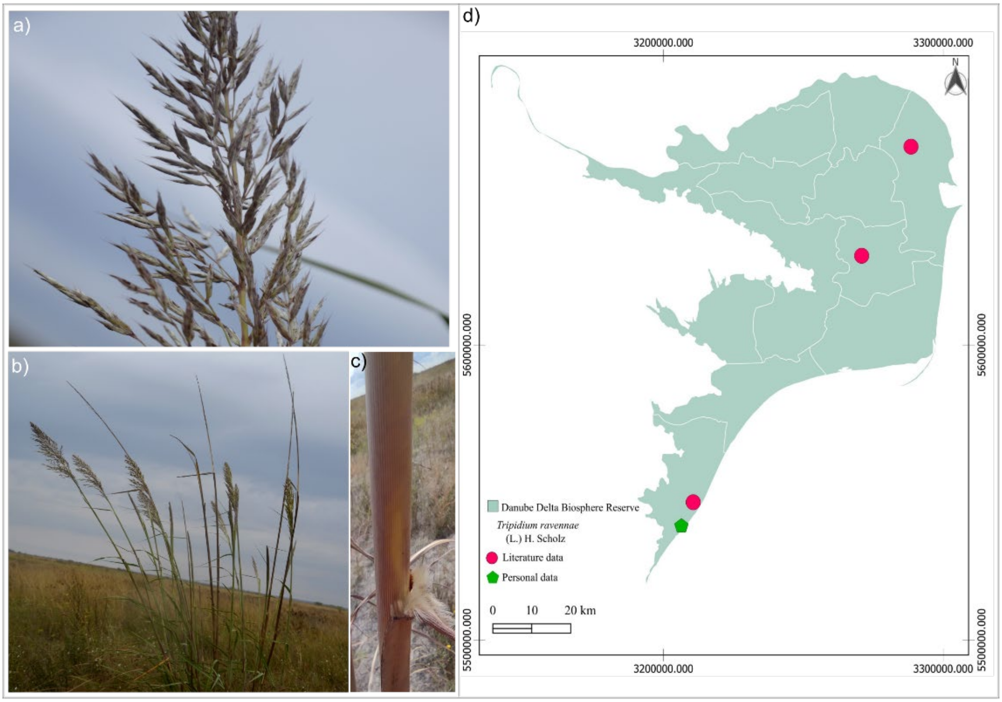

Figure 3. *Tripidium ravennae*: a) inflorescence, b) habitat and plant habitus, c) ligule very short and hairy, and d) the distribution of the species in DDBR (photos a, b and figure d – Simona Chirilă, photo c – Silviu Covaliov)

The species *T. ravennae* was reconfirmed on Chituc Sand Islet (Figure 3) in 2023. During the study period, the species was identified in the following plant communities/plant associations: ass. *Lactuco tataricae-Glaucietum flavae* Dihoru *et* Negrean 1976 [DIHORU & NEGREAN, 1976; NEGREAN & DIHORU, 1976.], and ass. *Puccinellietum limosae* Magyar ex Soó 1933 subass. *elymetosum elongati* (Şerbănescu 1965) Coldea *et al.* 2012. Limiting factors include intensive grazing and invasive species such as *Xanthium orientale* subsp. *italicum* (Moretti) Greuter, and dune subsidence. These conditions can lead to the degradation of the species' natural habitat, negatively affecting its survival and dissemination. At the site level, *T. ravennae* encountered the above-mentioned two plant communities, each of them being framed in two different Natura 2000 habitat types as follows: a) 1210 Annual vegetation of drift-lines, the ass. *Lactuco tataricae-Glaucietum flavae* Dihoru *et* Negrean 1976 – 4 out of 11 relevés (36%), the species is considered rare, and b) 1530* Pannonic salt-steppes and salt-marshes, the ass. *Puccinellietum limosae* Magyar ex Soó 1933 subass. *elymetosum elongati* (Şerbănescu 1965) Coldea *et al.* 2012 – 6 out of 17 relevés (35%).

Description. *T. ravennae* (Figure 3) is a perennial plant [GHIŞA, 1972; CIOCÂRLAN, 2009], with a height between 1 and 2 m. The stem is smooth and vigorous. The leaves are linear, with a width between 1 and 1.5 cm. The ligule is short, with long, dense, white hairs. The inflorescence is ovoid, panicle-type, white-hairy, up to 60 cm long. The lower glumes are pale, long, finely pointed, and distinctly 3-nerved. The lema presents an arista two times longer (than the lema) [GHIŞA, 1972].

Distribution, conservation status, ecology, and coenology

The species is distributed across Europe, North America, South America, Asia, and Africa [LANSDOWN, 2013; GBIF, 2024b]. It has been documented in Romania in the Constanța and Tulcea counties [GHIŞA, 1972]. In Dobrogea, the species has been reported in Tulcea County, in Danube Delta [GHIŞA, 1972], on Letea Sand Islet [NEGREAN & DIHORU, 1976], and in Constanța County, on Chituc Sand Islet [CIOCÂRLAN & SÂRBU, 1998; CIOCÂRLAN, 2009; SÂRBU & al. 2013], and between Caraorman (Tulcea County) and Capul Midia (Constanța County) [ZAHARIADI, 1940; GHIŞA, 1972]. At the Global [LANSDOWN, 2013], European [LANSDOWN, 2011], and European Union [EEA, 2024b] levels, the species is considered “Least Concern”. In the DDBR level, the species was included in the “endangered” category. This plant species is in danger of extinction, whose survival is unlikely if the causal factors continue to operate. The number of individuals in the reported populations has been reduced to a critical level [OȚEL, 2000]. The species is usually found in sandy or marshy lands, river banks, and alluvium [GHIŞA, 1972]. The DDBR grows on salty, moist sands, more or less close to the Black Sea beach.

- ***Stachys maritima*** **Gouan – Fam. Lamiaceae Lindl.**

In 2010, the species was reconfirmed at Portița (Fig. 4) and was identified in the plant communities/plant associations as ass. *Cakileto euxiniae-Salsoletum ruthenicae* Vicherek 1971 and ass. *Crambetum maritimae* (I. Șerbănescu 1970) Popescu *et al.* 1980 (*Cakiletea maritimae* Tx. et Preising in Tx. ex Br.-Bl. et Tx. 1952). On the total evaluated surface of 200 m^2, the number of individuals was between 30 and 40; on the total assessed surface of 400 m^2, the number of individuals was between 10 and 40. The limiting factors identified were marine erosion, grazing, and vehicle traffic. In 2022, the species was identified on Corbu beach sands (Constanța county) and between Sulina and Câșla Vădanei (Tulcea county). At Corbu, the species was identified within the ass. *Artemisietum tschernievianae* (*arenariae*) Popescu *et* Sanda 1977. On the total evaluated surface of 100 m^2, the number of individuals is between 10 and 20. The limiting factors identified were uncontrolled tourism that destroyed vegetation and grazing in the beach area. At the beach sector between Sulina and Câșla Vădanei, the species was identified within the ass. *Lactuco tataricae-Glaucietum flavae* Dihoru *et* Negrean 1976, the limiting factors are the accentuated marine erosion. The species is limited in development due

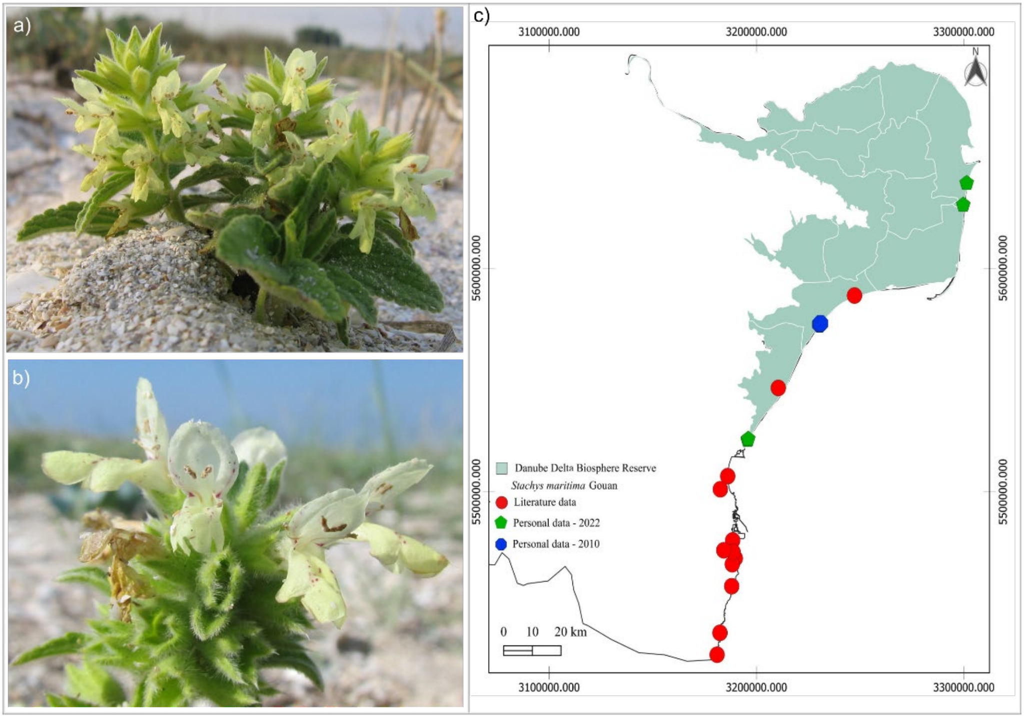

Figure 4. *Stachys maritima*: a) habitat, b) inflorescence, and c) the distribution of the species in DDBR (photos a, b - Mihai Doroftei, figure c - Simona Chirilă)

to the reduced specific areas of habitat (coastal region) between the shoreline and marsh vegetation.

At the site level, *S. maritima* was identified within four plant communities, each of them being framed in two different Natura 2000 habitat types as follows: a) 1210 Annual vegetation of drift-lines with ass. *Cakileto euxiniae-Salsoletum ruthenicae* Vicherek 1971 - 8 out of 35 relevés (22%), the species is considered rare and ass. *Lactuco tataricae-Glaucietum flavae* Dihoru *et* Negrean 1976 - 10 out of 31 relevés (32%), the species is considered rare; b) 2110 Embryonic shifting dunes with *Crambetum maritimae* (I. Şerbănescu 1970) Popescu *et al.* 1980 – 7 out of 11 relevés (63%), the species is considered frequent and ass. *Artemisietum tschernievianae* (*arenariae*) Popescu *et* Sanda 1977 – 5 out of 36 relevés (14%), the species is considered very rare.

Description. *S. maritima* is a perennial species [RĂVĂRUŢ, 1961; DIHORU & NEGREAN, 2009], with a height between 15 and 30 cm [RĂVĂRUŢ, 1961]. The rhizome is red, and the leaves are oblong, obtuse, and crenate [RĂVĂRUŢ, 1961; DIHORU & NEGREAN, 2009], with a length between 2 and 3 cm and a width between 7 and 15 mm. The flowers are almost sessile, 4-6 in the whorls, distant in the lower part, and close in the upper part. The calyx is grey-wooly tomentuous, tubular bell-shaped, between 5 and 8 mm long, with ovate-lanceolate and mucronate teeth, and the corolla is yellow, twice as long as the calyx. The stamens are slightly longer than the corolla tube, with hairy filaments in the lower part, and the fruit is a black nut with three edges rounded in the upper part [RĂVĂRUŢ, 1961].

Distribution, conservation status, ecology, and coenology

S. maritima is widespread in Mediterranean Europe and SE Asia Minor [RĂVĂRUŢ, 1961]. In Romania, the species was reported in Constanța County [RĂVĂRUŢ, 1961; DOLTU & al. 1983], in Mamaia [PRODAN, 1939; SÂRBU & al. 2013], Eforie Sud [MORARIU, 1957; NYÁRÁDY, 1959; SÂRBU & al. 2013], Eforie Nord and Eforie Sud [DIHORU & NEGREAN, 2009], Tuzla [PRODAN, 1939; SÂRBU & al. 2013], Palazu Mare and Agigea [SĂLĂGEANU, 1978; SÂRBU & al. 2013], Vama Veche [POP, 1969; SÂRBU & al. 2013], Techirghiol [PRODAN, 1939], Chituc Sand Islet [FĂGĂRAȘ & GOMOIU, 2002; SÂRBU & al. 2013], Costinești and Mangalia [DIHORU & NEGREAN, 2009], and Tulcea county: between Portița and Perișor [DIHORU & NEGREAN, 1976; SÂRBU & al. 2013], Periboina and Periteașca [FĂGĂRAȘ, 2014]. It occurs on littoral sands [RĂVĂRUŢ & al. 1961; DIHORU & NEGREAN, 2009]. In Romania, the species *S. maritima* was considered “critically endangered” [DIHORU & NEGREAN, 2009], “endangered” [OLTEAN & al. 1994; DIHORU & DIHORU, 1994] or rarely [BOȘCAIU & al. 1994]. The species is not evaluated at the Global and European levels [EEA, 2024c]. At the DDBR level, the species was included in the “endangered” category; thus, the species is in danger of extinction, whose survival is unlikely if the causal factors continue operating; the number of individuals in the reported populations has been reduced to a critical level [OȚEL, 2000].

Conclusions

Trachomitum venetum is considered rare in the plant communities *Salsolo ruthenicae-Xanthietum strumarii*, and *Schoeno nigricantis-Plantaginetum maritimae*, and frequent in *Artemisio santonici-Juncetum maritimi*. Regarding the species *Tripidium ravennae*, this is rare in the *Elymetosum elongati* subassociation (*Puccinellietum limosae* association) and the *Lactuco tataricae-Glaucietum flavae* association. In the case of the species *Stachys maritima*, this is considered frequent in the *Crambetum maritimae* association, rare in the *Lactuco tataricae-Glaucietum flavae* association and very rare in the *Artemisietum tschernievianae* (*arenariae*) association. When a plant’s natural habitat is at risk due to development, climate change, or other anthropogenic pressures, transplanting it to a safer location can help preserve the species.Transplanting species can be one of the measures for rare plant protection, and it is a recognized conservation strategy known as assisted migration or plant translocation. However, it is a complex process that must be approached with careful planning and consideration of ecological, genetic, and practical factors.

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Research Article

NEW CONTRIBUTIONS TO VEGETATION KNOWLEDGE OF THE DANUBE DELTA, ROMANIA (I)

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Abstract: New investigations on the flora, vegetation, and natural habitats of the Danube Delta (Romania) were recently carried out. Thus, more than 1,200 vascular plant species were inventoried, phytocoenoses of 253 plant communities, and 32 Natura 2000 habitats were recorded, all mapped on a large scale.

As part of the reviewing process of the Standard Form of the Natura 2000 site ROSCI0065 Danube Delta - Romania, the natural habitats included within it were also revised. Thus, it was found that black alder forests were omitted, which was one the purposes of this work, namely to fill this gap.

Following the recordings of all black alder forests in the Danube Delta, a phytocoenotaxon is proposed as new for science, namely Ass. *Periploco graecae-Alnetum glutinosae* ass. nov.

The Natura 2000 habitat in the Danube Delta that include this newly proposed plant community is: 91E0* Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion*, *Alnion incanae*, *Salicion albae*).

Key words: *Alnus glutinosa*, Natura 2000 habitats, new coenotaxon, *Periploca graeca*, Romania.

Introduction

As a result of the many surveys on the flora and vegetation of the Danube Delta, Romania, more than 1,200 vascular plant species [DIHORU & NEGREAN, 1976a; CIOCÂRLAN, 1994, 2011; DOROFTEI & al. 2011] and 157 plant communities [SIMON, 1960; DIHORU & NEGREAN, 1975, 1976b; POPESCU & al. 1997; SÂRBU, 2002] have been inventoried so far.

However, new phyto- and coenotaxa are still being identified, or new plant communities are being proposed for science.

Thus, in addition to those already inventoried plant communities so far, phytocenoses of another 96 plant communities were identified [Revision of the Management Plan ..., 2014-2021]. Those 253 plant communities identified so far are assigned to 32 natural habitats (it should be noted that a complete inventory of the existing natural habitats in the Danube Delta, with the allocation of plant community's characteristic of each natural habitat, has not been yet published).

Under provisions of the Article 17 of the Habitats Directive 92/43/EEC, each Member State must report each six years to the European Commission "on the measures taken under this Directive..." and the conservation status of both plant species and natural habitats on their territories [European Commission, Council Directive 92/43/EEC, 1992]. Thus, by analyzing the

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respective plant species, as well as the habitats within the Standard Form of ROSCI0065 Danube Delta, it was found that the black alder forests were completely omitted [Formularul Standard al ROSCI0065 Delta Dunării, 2008].

Alnus glutinosa (L.) Gaertn. (Syn. *Betula alnus* L. var. *glutinosa* L.), black alder or common alder, is a tree (Ph as life form) to 20 m high, flowering in March-April in Romania. It is frequently distributed across the country, from the pedunculate oak zone to the sessile oak zone, along the rivers, as isolated trees or in stands, sometimes growing in eutrophic swamps. It could be defined as a mesotrophic, hygrophyllous, and heliosciophyle species. The black alder prefers humid places, with groundwater on the surface, rich in humus and mineral substances, characterized by short-term floods and with as little amplitude as possible of the water level during floods [DRAGOMIR & BĂRBAT, 1961; LEANDRU, 1971]. It belongs to the alliances *Alnion glutinosae* or *Alno-Padion*, and class *Alnetea* [SANDA & al. 1980] or to alliance/suballiance *Alnion glutinosae/Alnenion glutinoso-incanae* [CHIFU & al. 2006; CHIFU & IRIMIA, 2014].

Distribution in Romania: *A. glutinosa* is natural distributed in historical provinces of Transylvania, Sătmar-Maramureş, Crişana, Banat, Oltenia, Muntenia, Moldavia, Bukovina, and Dobrogea [COLDEA & URSU, 2016] - from 1-2 m a.s.l. as minimum altitude to 1,250 m a.s.l. as maximum altitude in Southern Carpathians [GEORGESCU, in: SĂVULESCU, 1952].

The presence of the black alder in the Danube Delta, Romania, must be related to the smaller variations in the water level. The trees grow there on the high mounds, where the water is maintained for a large part of the year, their growth being quite vigorous [LEANDRU, 1970, 1971].

It should be mentioned that the marshes with black alder in the Danube Delta are part of a group of periodically flooded phytocoenoses [POPESCU & al. 1997].

According to MUNTEANU & CURELARIU (1996), surface deposits of *Alnus glutinosa* sites in the Danube Delta, are as follows:

- inside of the Nature Reserve "Arinişul Erenciuc" and along the right side of the Sf. Gheorghe arm: clay-loamy, loamy-clay (32-60% clay content), weakly carbonate, of fluvial and fluvial-lakes deposits; organic stratifications (sometimes marly) are frequently present in the low areas; the presence of peat deposits between 20 and 50 cm thick is indicated in several places;
- inside of the "Gârla Turcească" area: clayey, fluvial carbonate, and fluvial-lacustrine deposits, sometimes with thin sandy or organic stratifications (<10 cm thick).

It has been found that black alder trees within the Danube delta prefers poor freshwater habitats, settling on mollic/histic sandy soils, and constant high-water tables [HANGANU & al. 1994].

To mention, the Danube Delta itself, on the territory of Romania, covers an area smaller than the entire Danube Delta Biosphere Reserve [GÂŞTESCU & al. 2006]. The distribution area of black alder within the Danube Delta includes only the maritime delta, east of the Periprava-Crișan-Crasnicol line.

Study area

The study area is the Danube Delta (Romania), which is entirely made up of the Danube River, one of the most important European waterways. The Danube River flows into the Black Sea through a delta, situated in the north-western part of the Black Sea, between 44°25' and 45°30' northern latitude and 28°45' and 29°46' eastern longitude, being bordered by the Bugeac Plateau to the north and by the Dobrogea orogenic area to the south. The Danube Delta is one of the main elements of the Danube River - Danube Delta - Black Sea Geosystem. This delta can be divided into three major depositional systems, as they are: delta plain, delta front, and prodelta [PANIN, 1989; PANIN & RĂDAN, 2011].

The delta plain is separated into 2 large parts by an old chain of littoral cordons (today the large sandy islets of Letea, Caraorman, etc., which mark a former coastline – Periprava-Crişan-Crasnicol), in: 1) fluvial delta (upstream) and 2) maritime delta (downstream). The fluvial delta is more developed and older (among other reasons, it has well-developed reed swamps), while the maritime delta is younger, sandier, having a higher saline potential [HANGANU & al. 2002].

Climate

The climate of the Danube Delta (according to the meteo station of Sulina town) is excessively continental-temperate [PAŞCOVSCHI & DONIŢĂ, 1967] or of the so-called "dry steppe" [DONIŢĂ & IVAN, 1992].

The meteorological station of Sulina is placed at an altitude of 3 m a.s.l. (above sea level); thus, the average annual temperature is 11.1 °C, while the quantity of precipitations is of 359 mm/year, respectively. The number of recorded years is 61 for temperatures and 55 for precipitations [PAŞCOVSCHI & DONIŢĂ, 1967; PAŞCOVSCHI, 1976-1977].

Materials and methods

Data collection

The aquatic and terrestrial vegetation research was performed in several points of the Danube Delta during the summers of 2020 to 2024. They consisted in field trips, on which occasion there were made relevés within varied phytocoenoses. Also, all the peculiarities of the vegetation encountered on the field were recorded. Phytosociological relevés were made in the field following the methodology of Central European Zürich-Montpellier School; the field data were processed in the laboratory, according to the principles of the same Central-European floristic-sociological criteria to characterize the floristic composition of plant communities [BRAUN-BLANQUET, 1964].

The similar phytocoenoses were grouped in synthetic tables based on which were achieved the description of the vegetation considering several features, as: chorology, floristic composition and synmorphology, synecology, syndynamics, coenotaxonomy, importance, conservative value, and so on. The hierarchical syntaxonomy and syntaxon nomenclature is according to COLDEA & al. (2012, 2015, 2017).

The nomenclature of plant species follows the opera *Flora Europaea* [TUTIN & al. 1964-1980, 1993], Euro+Med Plant bases [https://ww2.bgbm.org/EuroPlusMed/PtaxonDetail …; europlusmed.org/cdm_dataportal/taxon …], and a field identification book [SÂRBU & al. 2013].

The authors' abbreviation of the Latin names of vascular plants in paper followed, in this work, BRUMMITT & al. (1992).

The new syntaxon was named taking into account the provisions of the International Code of Phytosociological Nomenclature [WEBER & al. 2000].

The maps were designed on a raster downloaded for free from the internet [https://www.geamap.com/en/ ...]. Some of the geographical maps were made with the help of the unmanned aerial vehicles (UAV)/aerial drones, which flew at low altitudes (between 50 m and 100 m).

Indices of soil moisture (humidity=U), air temperature (T), and soil reaction (*p*h, R) were given following certain works dealing with the indices of ecological characteristics of the wild vascular plant species of Romania [SANDA & al. 1983; SÂRBU & al. 2013].

Aim of the study

The purposes of this study are as follow: i) to demonstrate, once again, that *Alnus glutinosa* is present in the Danube Delta, Romania, and this species has been forming well-structured forests for decades in the lower part of the maritime delta, ii) to inform scientists, authorities responsible for biodiversity management and conservation, as well as the public or interested tourists, about the entire distribution of black alder (*Alnus glutinosa*) within the Danube Delta, and iii) to propose a new phytocoenotaxon for science, which includes black alder (*Alnus glutinosa*) plant communities, constantly accompanied by the silkvine (*Periploca graeca*), communities distributed exclusively on the territory of the Danube Delta.

Results and discussion

Black alder (*Alnus glutinosa*) is a native species to almost all continental Europe (except for the extreme North and South), the United Kingdom, and Ireland [https://europlusmed.org/cdm_dataportal/taxon/…]. Its range in Asia includes Turkey, Iran, and Kazakhstan; in North Africa, it is found in Tunisia, Algeria, and Morocco [GEORGESCU, in: SĂVULESCU, 1952].

Black alder is pretty common spread in the hilly and submontane-montane areas of Romania [GEORGESCU, 1952]. But, the plant communities dominated by black alder are frequently distributed in valleys along rivers and streams in hilly and submontane areas, being grouped in Ass. *Stellario nemorum-Alnetum glutinosae* Lohmeyer 1957 [COLDEA & URSU, 2016].

Although black alder is a common species in the vegetation belts mentioned above, in the plain area of Romania it is a fairly rare species, being cited as such in:

- fragmentary in the Muntenia Plain - in Chitila forest, Ilfov County, along the numerous meanders that the Colentina River makes there [SANDA & POPESCU, 1972; POPESCU & al. 1984], being considered as a scientific curiosity over there, with the trees growing on high mounds, around which the water never dries up [PAŞCOVSCHI & LEANDRU, 1958];
- in the forests of Frasinu and Spătaru, Buzău County [SANDA, 1970];
- small patches of black alder (grouped in Ass. *Alnetum glutinosae* Meijer-Drees 1936, in orig.) are found scattered in the Tecuciului Plain, as part of the northeastern Romanian Plain, along the banks of the Siret River, close to Cosmeşti-Vale and Cosmeşti-Deal villages, Galaţi county [MITITELU & al. 1993];
- along the lower part of the Siret River, in Galaţi and Vrancea Counties [MONAH, 2001].

Although *Alnus glutinosa* has also been cited in other areas of the Steppic Bioregion, sensu Habitat Directive EUR 27 of the European Union [INTERPRETATION MANUAL …, 2007], such as they are along the Colentina River, on the banks of the Siret River, or the forests of Frasinu and Spătaru and so on, black alder forms true groves, so characteristic on the embankments of the Danube River, in fluvial-maritime section of the Danube Delta, which presents a series of particularities as will be seen below.

Origins and Natural Distribution of *Alnus glutinosa* in the Danube Delta

A. On the fluvial-maritime sand islets

On the fluvial-maritime sand islet of Letea, in the northern part of it, towards the Chilia arm, between the villages of C. A. Rosetti (former Satu Nou), Letea, and Periprava, *A. glutinosa* enters the composition of the stands with naturally established species, which together form the "Hasmacul Mare" forest body. The low percentage of black alder existing today in the composition of that forest is explained by the gradual extraction of many trees by the inhabitants of the neighboring settlements, the bark being highly sought after for dyeing fabrics, and the wood for the construction of small boats, houses, and furniture [DRAGOMIR & BĂRBAT, 1961]. Another reason for the rarity of black alder on the fluvial-maritime sand islets is the presence of so-called "wild horses", who eats the bark especially in the winter season, so the trees dry out over time.

B. On the fluvial sand islets

The black alder has also settled naturally on the fluvial sand islets formed on both banks of the Sf. Gheorghe arm, in the downstream area, towards its discharge into the Black Sea, over a length of about 20 Km, where the amplitude of the water level during floods registers the minor variations. The first natural grove of black alder appeared on the low fluvial, left bank, of the Sf. Gheorghe arm, at Km 20, in the southeastern part of the Erenciuc Lake. At this point, the black alder forms pure stands as a floodplain, riparian forests. Thus, a nature reserve was designated, called "the Forestry reserve Arinișul Erenciuc", with an area of 50 ha [LAW No. 5 …, 2000], the trees being about 110 years old and average diameters between 28 and 50 cm, and average heights between 10 and 20 m; the consistency of the stand is between 0.7 and 0.9, with an average increase of about 8 m^3/year/ha; it is mentioned that current trees derive from shoots due to the exploitation of the initial trees, all of which originated from seeds [INCEF, 1960].

It is worth noting that due to the very low terrain in front of the Erenciuc Lake (2.5 hydro-degrees), the specimens of black alder developed adventive roots (as pneumatophores, more or less), which caught the annually deposited sediments, fixing themselves in the soil like giant stools placed on the ground. It was mentioned that downstream from the Erenciuc stand, there are isolated specimens or small clumps of black alder, aged up to 80 years, scattered among the specimens of white willow (*Salix alba* subsp. *alba*), grey willow (*Salix cinerea*), crack willow (*Salix fragilis*), almond willow (*Salix triandra*), the narrow-leaved ash (*Fraxinus angustifolia* subsp. *oxycarpa*), downy ash (*Fraxinus pallisae*), alder buckthorn (*Frangula alnus*), etc., located on the very low sand islets, of more recent formation, on both banks of the Sf. Gheorghe arm, until close to its discharge into the Black Sea [DRAGOMIR & BĂRBAT, 1961].

The locations already mentioned with *Alnus glutinosa* from the Danube Delta are: Letea forest [KANITZ, 1879-1881; BRÂNDZĂ, 1898]; Caraorman forest [PRODAN, 1923]; along the Danube River [PRODAN, 1935]; Erenciuc [PAŞCOVSCHI & LEANDRU, 1958; LEANDRU & al. 1960; DONIȚĂ & IVAN, 1992; MITITELU & al. 1997]; Sf. Gheorghe [MITITELU & al. 1968]; North of Cardon [ŞTEFUREAC & MOHAN, 1969]; "Hasmacul Mare" forest on Letea Sand Islet [IANCULESCU, 1970; DONIȚĂ & IVAN, 1992]; Sulina (without an exact distribution inhere) [DIHORU & NEGREAN, 1976a] (Figure 1 - black dots). In the last years there were identified new locations with black alder within the Danube Delta (Figure 1 - red rhombic signs), as they are: around Erenciuc lake (Figure 2), nearby and inside of "Cotu Ivancea" - on the right side of Sf. Gheorghe arm, along Sf. Gheorghe arm toward Sf. Gheorghe village (Figure 3), South of Sf. Gheorghe village (within the "Gârla Turcească" area)

(Figure 4), and Periprava - at the "tail" of Nebunu (also called Durnoi by the locals) lake. The geographical maps (Figures 2-4) were made with the help of aerial drones, which flew at low altitudes (between 50 and 100 m).

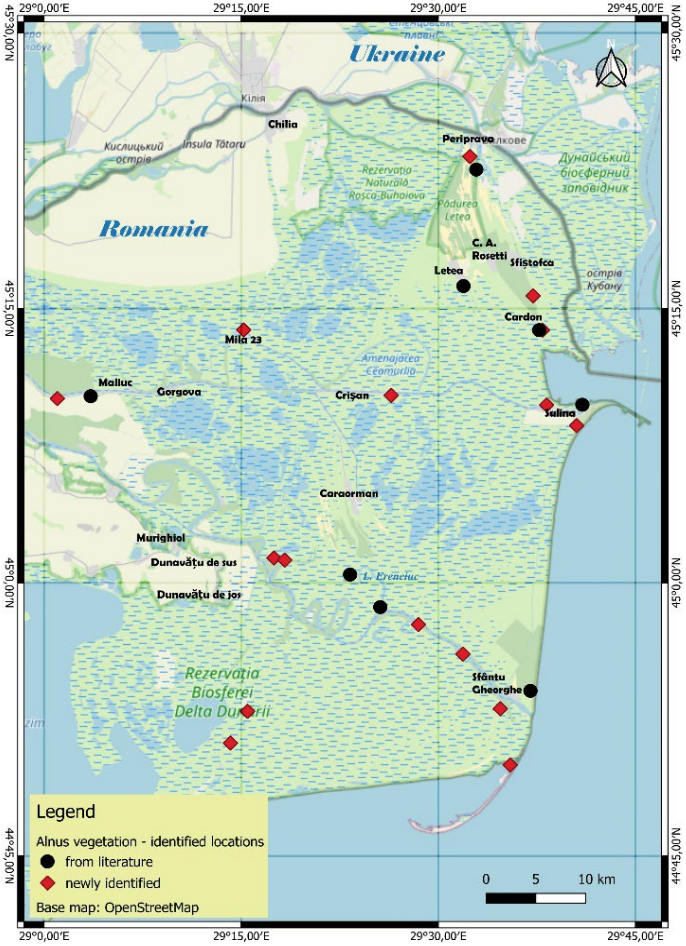

Figure 1. Distribution map of the black alder (*Alnus glutinosa*) in the Danube Delta (legend: ● old locations; ♦ newly identified locations; explanations above in the text)

Figure 2. Locations of black alder with silkvine communities - the area of Erenciuc lake

Figure 3. Locations of black alder with silkvine communities - along Sf. Gheorghe arm toward Sf. Gheorghe village

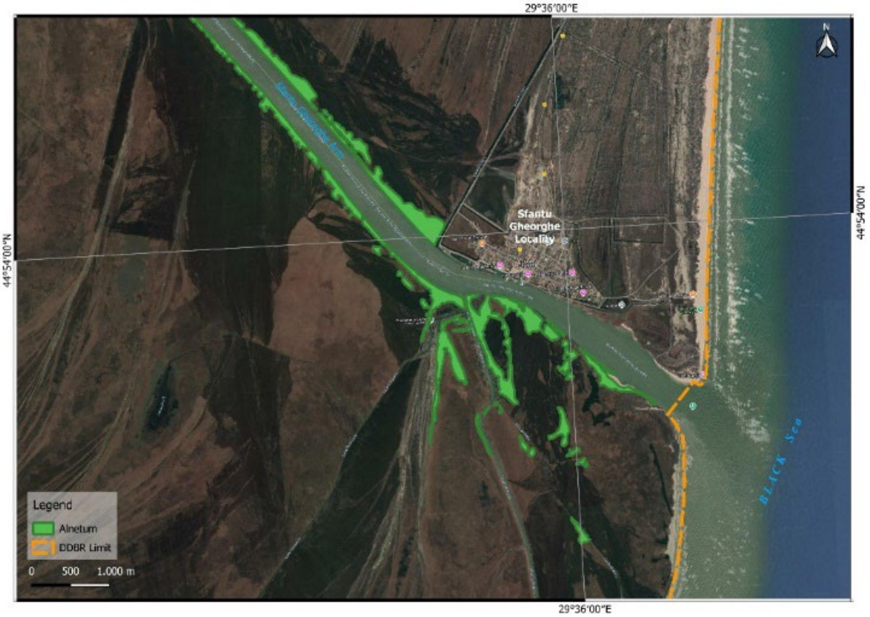

Figure 4. Locations of black alder with silkvine communities - south of Sf. Gheorghe village toward "Gârla Turcească" area

In some places of the Danube Delta, the black alder forms pure natural stands (locally called "aninişuri" or "arinişuri") while in other places it enters into association with other species, such as pedunculate oak (*Quercus robur* L.), grayish oak (*Quercus pedunculiflora* K. Koch), downy ash (*Fraxinus pallisae* Willm.), white poplar (*Populus alba* L.), quaking poplar (*Populus tremula* L.), and so forth [DRAGOMIR & BĂRBAT, 1961; DONIŢĂ & IVAN, 1992]. It is added that the black alder does not tolerate large variations in the amplitude of the flood level, from annual or periodic floods [RĂDULESCU & al. 1960]. Thus, the black alder could only settle close to the Danube River branches flowing into the Black Sea, on the fluvial-maritime beds of older formations, or, sometimes, on the fluvial beds of more recent formations [DRAGOMIR & BĂRBAT, 1961].

In recent years of our scientific trips, a lot of observations have been made on the companion plant species of black alder. Thus, one of them is the Balkan-east Mediterranean species *Periploca graeca* (silkvine, as its English vernacular name), which constantly accompanies the black alder in the Danube Delta. Thus, a new plant community/plant association is proposed here for science, to better characterize these plant communities from the Danube Delta (Romania), that is classified from a syntaxonomic point of view as follows:

Cl. ALNETEA GLUTINOSAE Braun-Blanq. & Tüxen ex V. Westh., J. Dijk, Passchier & G. Sissingh 1946
    Ord. Alnetalia glutinosae Tüxen 1937
        All. Alnion glutinosae Malcuit 1929

Ass. *Periploco graecae-Alnetum glutinosae* ass. nov. (syn. pro parte Ass. *Carici acutiformis-Alnetum* (Soó 1957) Dostal 1933 subass. *periplocaetosum graecae* Mititelu & al. 1997) (Table 2)

Relevé no. 1 is the type relevé for this newly proposed plant community (Table 2 includes 15 phytosociological relevés with the occurrence of *Alnus glutinosa* as the dominant species, in different points of the Danube Delta, Romania).

Why was it necessary to propose a new plant community for science?

Considering the plant structure of this newly proposed plant community (Table 2), we only partially agree with the point of view of MITITELU & al. (1997). According to the provisions of the International Code of Phytosociological Nomenclature, this syntaxon was not validly published, because it is neither accompanied by a vegetation table with relevés, nor is a type relevé previously identified in any described valid taxon indicated before 1.1.1979 (Art. 7, Rec. 7A) [WEBER & al. 2000]. Here's why it was proposed a new phytocoenotaxon for science, with relevés made in various points on both sides of the Sf. Gheorghe arm, Danube Delta, including those from the Nature Reserve "Arinişul Erenciuc", under the name: Ass. *Periploco graecae-Alnetum glutinosae* ass. nov.

The diagnostic species for this newly proposed syntaxon are the silkvine (*Periploca graeca*) and the black alder (*Alnus glutinosa*).

It is mentioned that on river levees in the maritime part of the Danube Delta, where the Sf. Gheorghe arm cuts through extensive peaty landscapes, black alder trees mix into the white willows (*Salix alba*) on the narrow clayey river levees. Often, they grow together with narrow leaved ash (*Fraxinus angustifolia* subsp. *oxycarpa*) and downy ash (*F. pallisae*). In such cases, the ashes grows in the highest part of the levees, accompanied by bushes of guelder rose (*Viburnum opulus*). It has been found that the silkvine (*Periploca graeca*), a Balkan-east Mediterranean plant species, constantly accompanies the river levee forests where the black alder is present in Danube Delta. The plant communities of black alder and silkvine prefers base-poor freshwater habitats with mollic/histic sandy soils, and constant high-water tables. Due to the precipitation (rain and snow) deficit recorded in the Danube Delta, the black alder habitat is normally too salinized to be suitable for this species. This is reflected in the isolated character and small to medium size of the tree stands with *Alnus glutinosa* in the Danube Delta, as well as the apparently weak vitality of black alder trees in some stands, such as those found along the Sf. Gheorghe arm. Nevertheless, this position at the margin of the distribution area makes the black alder stands of the Danube Delta important sites, both from geobotanic and conservation points of view [HANGANU & al. 1994].

It is worth noting that there have been several attempts to describe, or just cite, by various authors, the presence of black alder plant communities located in the Danube Delta, under the same name, more or less, as:

1. Ass. *Carici acutiformis-Alnetum* (Dostál 1933) Soó 1957 - a plant community cited without an exact locations in the Danube Delta; in fact, the cited work intended to present only an inventory list of all the plant communities in the vegetation of Romania, with scattered locations by historical provinces, mountain ranges, etc., but nothing else; the work did not include any vegetation table to argue the presence of the above indicated phytocoenotaxon in the Danube Delta vegetation [SANDA & al. 1980];
2. Ass. *Carici acutiformis-Alnetum* Soó 1963 - the authors cited the presence of this phytocoenotaxon only in a few locations in the Danube Delta, such as "Hasmacul Mare" forest at Letea and Erenciuc point, being characterized by the edifying species *Alnus glutinosa* and other hygrophilous species; the work did not include any vegetation table that would argue the presence of the indicated phytocoenotaxon in the Danube Delta, either [DONIŢĂ & IVAN, 1992];

3. Ass. *Carici* (*acutiformis*)-*Alnetum* (Soó 1957) Dostal 1933 subass. *periplocaetosum graecae* Mititelu, Baisan, Dumitraşcu & Parincu 1997 - the authors said that the black alder phytocoenoses from Erenciuc point were taken over in a conservation area under the name "the Forestry Reserve Arinișul Erenciuc", with an area of 11.6 ha at those times, where the substrate (soil) is permanently wet and represents an eutrophic peat, in a layer up to 1 m deep; the authors present only a synthetic vegetation relevé (no analytical table of vegetation is included in the original work), where *Alnus glutinosa* and *Carex acutiformis* only have AD indices (AD=4); the other species in the list do not have attached AD indices [MITITELU & al. 1997];

4. Ass. *Thelypteridi-Alnetum* Klika 1940 - this plant community was mentioned in the following areas: 1) Erenciuc point [POPESCU & al. 1997; SANDA & ARCUŞ, 1999]; 2) along St. Gheorghe arm to near its mouth into the Black Sea [POPESCU & al. 1997]; 3) Letea - at "Hasmacul Mare" [POPESCU & al. 1997]. There is no vegetation table included in the two works cited above.

MITITELU & al. (1997) observing that the black alder forests of the Danube Delta (Romania), being constantly accompanied by the Balkan-east Mediterranean liana *Periploca graeca*, are clearly different from other types of black alder forests (such as those included in Ass. *Carici acutiformis-Alnetum* and/or Ass. *Thelypteridi-Alnetum*); that why, the authors gave those forest communities a new name - "*Carici* (*acutiformis*) *alnetum* (Soó 57) Dostal 33 subass. *periplocaetosum graecae*" (in original).

According to the most recent synthesis on the woody vegetation of Romania, Ass. *Carici acutiformis-Alnetum* (Dostál 1933) Soó 1957, Ass. *Carici acutiformis-Alnetum* Soó 1963, and Ass. *Carici* (*acutiformis*)-*Alnetum* (Soó 1957) Dostal 1933, are all synonyms for Ass. *Thelypterido palustris-Alnetum glutinosae* Klika 1940 [COLDEA & al. 2015].

Comparing the presence of those species with the highest K (constancy classes K=III-V) within the given association (Ass. *Thelypterido palustris-Alnetum glutinosae* Klika 1940) in the other provinces of Romania [COLDEA & al. 2015] and the plant communities of *A. glutinosa* in the Danube Delta (Table 1) it can be easily observed that:

1) Ass. *Periploco graecae-Alnetum glutinosae* ass. nov. has as characteristic species *Periploca graeca*, with a maximum constancy (K=V) and the dominat species *Alnus glutinosa*, also with a maximum constancy (K=V); this combination of plant species is completely absent in the other regions, according to current knowledge regarding the vegetation cover of Romania; it is not excluded that this newly proposed plant community will be identified in the future in other areas, perhaps along the Danube River, where the silkvine (*Periploca graeca*) has been identified over time [ŢOPA, 1961]; we assume that the silkvine is widespread at its northernmost limit, in Romania, along the Danube River (see the species distribution map in Euro+Med PlantBase [https://europlusmed.org/cdm_dataportal …].

2) *Carex acutiformis* (cited within the Ass. *Carici acutiformis-Alnetum* (Dostál 1933) Soó 1957) has a low constancy (K=I) and a quite random presence, nowadays, in black alder phytocoenoses in the Danube Delta, while achieving the greatest constancy (K=V) in the other provinces, as well as in other plant communities in Romania.

One can observe some similarities of those plant communities with *Alnus glutinosa* and *Thelypteris palustris* recorded in different regions of Romania, as well as in central and eastern European area, excluding the Danube Delta [COLDEA & al. 2015] with the new plant community proposed here (see the analytical Table 2), namely:

1. a series of taxa, identified in the black alder communities of the Danube Delta, such as *Periploca graeca*, *Iris pseudacorus*, *Stachys palustris*, *Phalaroides arundinacea* subsp.

arundinacea, *Fraxinus pallisae*, *Rumex hydrolapathum*, *Jacobaea paludosa* subsp. *angustifolia*, could be considered as diagnostic taxa for this new plant community.

2. the characteristic taxa for the Ass. *Thelypterido palustris-Alnetum glutinosae* Klika 1940 seems to be the next ones: *Thelypteris palustris*, *Carex acutiformis*, *Galium palustre*, *Eupatorium cannabinum*, *Lemna minor* [COLDEA & al. 2015] (Table 1).

3. there is no black alder plot in the Danube Delta where the fern *Thelypteris palustris* is present nowadays, though it has been reported in the past works [MITITELU & al. 1997; POPESCU & al. 1997].

Table 1. Synoptic table reflecting the difference between the Ass. *Periploco grecae-Alnetum glutinosae* ass. nov. and Ass. *Thelypterido palustris-Alnetum glutinosae* Klika 1940; only species with a constancy of at least III (presence of at least 41%) in each association were recorded

Species	P-A	T-A
Periploca graeca	**V**	-
Rubus caesius var. arvalis	V	II
Salix alba subsp. alba	V	-
Carex riparia	V	II
Iris pseudacorus	**V**	I
Stachys palustris	**IV**	-
Amorpha fruticosa	IV	-
Phalaroides arundinacea subsp. arundinacea	**IV**	I
Humulus lupulus	III	I
Fraxinus angustifolia subsp. oxycarpa	III	I
Viburnum opulus	III	II
Fraxinus pallisae	**III**	-
Rumex hydrolapathum	**III**	-
Jacobaea paludosa subsp. angustifolia	**III**	-
Phragmites australis subsp. australis	III	-
Ranunculus repens	V	II
Calystegia sepium	IV	I
Symphytum officinale subsp. uliginosum	III	I
Urtica dioica subsp. pubescens	**II**	-
Alnus glutinosa	V	V
Solanum dulcamara	IV	IV
Lycopus europaeus	IV	III
Salix cinerea	III	III
Thelypteris palustris	-	V
Carex acutiformis	I	V
Galium palustre	II	IV
Eupatorium cannabinum	II	IV
Lemna minor	II	III
Poa palustris	I	III
Urtica dioica subsp. dioica	-	III

Legend. **P-A**: Ass. *Periploco grecae-Alnetum glutinosae* ass. nov. (15 relevés, the Danube Delta, personal data - see Table 2); **T-A**: Ass. *Thelypterido palustris-Alnetum glutinosae* Klika 1940 (34 relevés, in different regions/provinces of Romania, excluding the Danube Delta, according to COLDEA & al. 2015)

The tree layer, with a coverage of (50-) 75 (-90)%, is dominated by *Alnus glutinosa*. *Salix alba* subsp. *alba* is also a constant species in the tree layer, but with rather reduced coverage (up to 20%). Other tree species relatively frequent (K=III) are *Fraxinus angustifolia* subsp. *oxycarpa* and *F. pallisae*.

The shrub layer, with a coverage of up to 20%, is mainly composed by *Rubus caesius* var. *arvalis*, *Salix cinerea*, *Viburnum opulus*, and *Amorpha fruticosa*, the latter one being an invasive alien species [ANASTASIU & NEGREAN, 2007; SÎRBU & OPREA, 2011; ANASTASIU & al. 2019].

In the herbaceous layer, with a variable coverage between 15% and 80%, the species *Carex riparia* is mainly noticeable, with values of abundance-dominance (AD) between 1 and 4. *Solanum dulcamara*, *Stachys palustris*, *Iris pseudacorus*, *Phalaroides arundinacea* subsp. *arundinacea*, *Lycopus europaeus*, *Ranunculus repens*, *Calystegia sepium* are also constant species.

Periploca graeca grows abundantly both in the herbaceous layer (as many juvenile individuals) and in those of the shrub and the tree layers, giving, through its twisted stems, the most characteristic aspect of the physiognomy of these forests (Figure 9).

It can be observed that within this newly proposed plant community, i.e. Ass. *Periploco grecae-Alnetum glutinosae* ass. nov., there are some species with high constancy (Table 1 and 2), such as: *Carex riparia* (K=V), *Rubus caesius* var. *arvalis* (K=V), *Salix alba* subsp. *alba* (K=V), *Ranunculus repens* (K=V), etc. One might think that the species above mentioned could better characterize this plant community than *Periploca graeca*. Of course, the silkvine is indeed present in other plant communities in the Danube Delta, such as the forests located on Letea and Caraorman sand islets, for instance. But, this is not about the dominant species within the association; the species chosen now, namely *Periploca graeca*, as a characteristic species, characterizes best the black alder forests, accompanying the latter species throughout its distribution area in Danube Delta, reflecting the habitats/soil type (base-poor freshwater habitats, with constant high-water tables, mollic/histic sandy soils, and periodically flooded phytocoenoses) and local/particular climatic conditions (permanent deficit of precipitation, rain and snowfall) in the easternmost part of Romania, a climate called as an excessively continental-temperate or of "dry steppe". The other species having high constancy indices in Table no. 2 (e.g. *Carex riparia*, *Rubus caesius* var. *arvalis*, *Salix alba* subsp. *alba*, *Ranunculus repens*, etc.) are interesting, but these have too large distribution areas and large ecological preferences, so they cannot be considered as characteristic taxa.

Analysing the life forms spectrum of this newly plant community one can remark: out of a total of 82 species, hemicriptophytes (H) represents 42.68%; phanerophytes (Ph)=15.85%; terophytes (T)=14.63%; geophytes (G)=6.09%; geophytes-helohydatopyhtes (G-HH)=6.09%; hemitherophytes (Ht)=3.65%; epiphytes (Ep)=2.43%; chamaephytes (Ch)=1.21%; hydrophytes (Hd)=2.43%; geophytes-hemicriptophytes (G-H)=1.22%; hemicriptophytes-chamaephytes (H-Ch)=1.22%; hemicriptophytes-helohydatopyhtes (H-HH)=1.22%; helohydatopyhtes (HH)=1.22% (Figure 5).

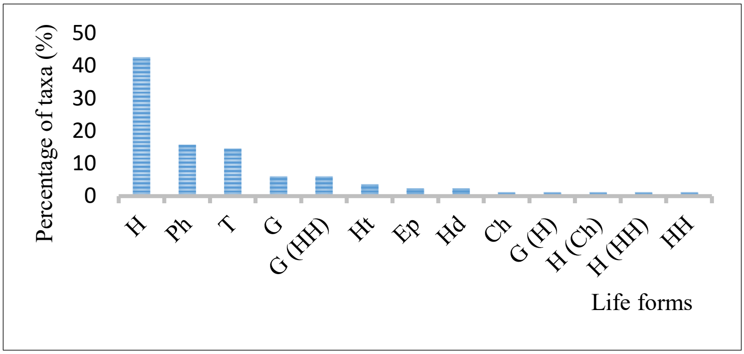

Figure 5. Life forms spectrum of the ass. *Periploco graecae-Alnetum glutinosae* ass. nov.

Concerning the floristics elements (geoelements), the Eurasian species represent 46.34%, circumpolar 19.51%, adventitious 9.75%, European 7.31%, cosmopolites 4.87%, atlanto-mediterranean 1.22%, balkano-danubian-pontics 1.22%, central-east European 1.22%, central European 1.22%, central European-Sub-Mediterranean 1.22%, Eurasian (Sub-Mediterranean) 1.22%, Eurasian-Africa 1.22%, Eurasian-north American 1.22%, Mediterranean 1.22%, pontic-pannonian-Mediterranean 1.22% (Figure 6).

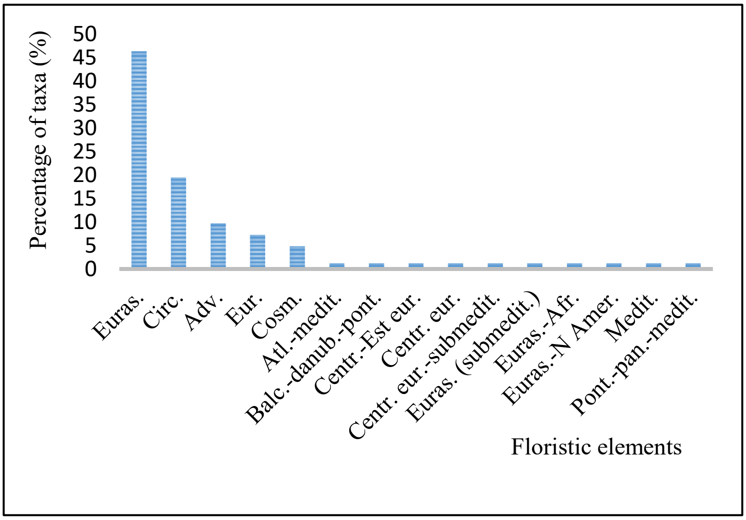

Figure 6. The floristics elements/geoelements spectrum of the ass. *Periploco graecae-Alnetum glutinosae* ass. nov.

The ecological factors were compiled accordind to SÂRBU & al. (2013); they are like the next:

- soil humidity (**U**) - U_{10} (plants of flooded soils)=**22.2%**; U_8=19.8%; U_9=16%; U_6=12.3%; U_5=12.3%; U_7=7.4%; U_{11}=4.9%; U_3=2.5%; U_4=1.2%; U_x=1.2%;
- air temperatures - **T_6** (moderately thermophilic plants)=**49.4%**; T_x=40.7%; T_7=7.4%; T_5=2.5%; T_x=40.7%);
- soil *p*H - **R_x** (euryonic plants)=**50.0%**; R_7=46.3%; R_6=3.7% (Figure 7).

The soil type is a gleysoil - it has the water table close to the earth's surface, being a “permanently wet soil”... [MARIAN MIERLĂ, 2023, pers. comm.]. Within the Forest Reserve “Arinişul Erenciuc” there are also soils with histic characters, i. e. they present a peaty horizon on the surface, of approximately 40 cm thickness (0-40 cm depth) [MUNTEANU & CURELARIU, 1995].

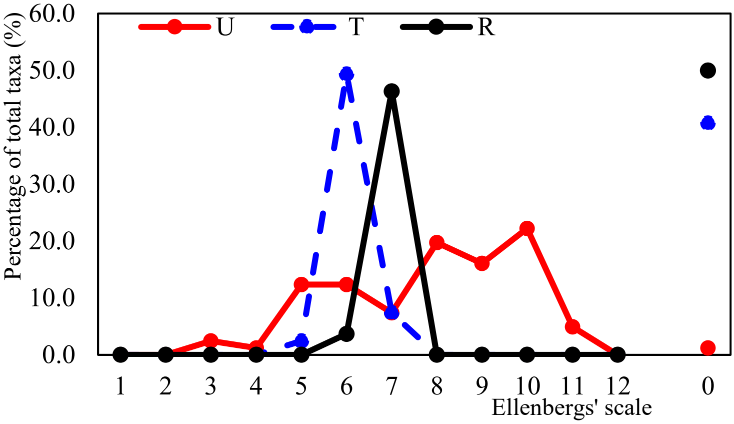

Figure 7. The ecological factors (soil humidity – U, air temperature – T, soil *p*H – R) chart of the ass. *Periploco graecae-Alnetum glutinosae* ass. nov.

As for the sozological categories, within the phytocoenoses of this newly proposed syntaxon, the following plant species on the national and international red lists, were registered: VU/R *Hottonia palustris*, R *Periploca graeca* (Figure 9), R *Cyperus serotinus*, R *Petasites spurius* [OLTEAN & al. 1994; IUCN CRITERIA..., 2024].

Aslo, the following alien plant species were recorded: *Amorpha fruticosa*, *Bidens connatus*, *B. frondosus*, *Eclipta prostrata*, *Sicyos angulatus*, *Fraxinus pennsylvanica*, *Prunus cerasifera*, *Aster lanceolatus* [SÎRBU & OPREA, 2011].

Forestry typology

According to the forestry typology, black alder communities in the Danube Delta were classified according to various works/authors as follows:

i) the great unit - **Elm, ash, and alder tree stands**; type 218. Alder trees stands of ponds and lakes - a scarce type of swamp forest, reported so far in two places in the Danube Delta and fragmentarily in the Muntenia Plain (e.g. along Colentina river valley), as a scientific curiosity, with the trees growing on high mounds, around which the water never dries up [PAŞCOVSCHI & LEANDRU, 1958]

ii) unit **L₁₂ₐ Danubian-Pontic psammophilic steppes** (with *Stipa borysthenica*, *Carex colchica*, *Scabiosa ucranica*), in complex with oak, ash, and poplar forests (*Quercus robur*, *Q. pedunculiflora*, *Fraxinus angustifolia*, *F. pallisae*, *Populus alba*, *P. tremula*, *P. canescens*), subunit **Low Depressions**, represented by Ass. *Carici acutiformi-Alnetum* Soó 1963. It is stated: "the *Carici acutiformi-Alnetum* association is found only in a few places ("Hasmacu Mare" forest, Erenciuc point), being characterized by the edifying species *Alnus glutinosa* and other hygrophilous species. It is found at the bottom of larger depressions where the water stagnates until summer, and the soil has a peaty character" [DONIŢĂ & IVAN, 1992].

Figure 8. Black alder stands at Erenciuc

Figure 9. *Periploca graeca* in black alder stands

Concerning the Natura 2000 habitats (under the typology of *Habitat Directive*, *European Communities Council*, 1992), the black alder communities in the Danube Delta can be attributed to a single and singular type of natural habitat, namely: **91E0*** Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion*, *Alnion incanae*, *Salicion albae*) (* means a priority habitat for conservation by Annex I of the Habitat Directive [EUROPEAN COMMISSION…, 1992; FORMULARUL STANDARD…, 2008; GAFTA & MOUNTFORD, 2008] (Figures 8-9).

From consulting the Natura 2000 Standard Form for the ROSCI0065 Danube Delta site [FORMULARUL STANDARD …, 2008], it was found that this habitat type was omitted from the inventory; through this work and through this proposal of a new phytocoenotaxon, a contribution is made to the correct identification of this Natura 2000 habitat type within the Danube Delta, on valid and pertinent scientific bases.

It is worth noting that riparian forests are under-researched in southeastern Europe. [DOUDA & al. 2016], and many of these forests have already been destroyed, for example, in Albania [KÁRPÁTI & KÁRPÁTI, 1961].

Conclusions

- the entire distribution of black alder (*Alnus glutinosa*) in the Danube Delta (Romania) is presented based on the geographical maps, including some made with the help of unmanned aerial vehicles (UAV)/aerial drones, within this work;
- the marshes with black alder in the Danube Delta are part of a group of periodically flooded phytocoenoses and the presence of this species within the Danube Delta is related to the smaller variations in the water level; the trees grow there on the high mounds, where the water is maintained for a large part of the year;
- a new phytocoenotaxon for science was proposed here, namely Ass. *Periploco graecae-Alnetum glutinosae* ass. nov., for the description of the vegetation built by the black alder in the Danube Delta and characterized by the presence of the Balkan-east Mediterranean species, *Periploca graeca*;
- the new phytocoenotaxon characterize the best those black alder communities in the Danube Delta, having the silkvine (*Periploca graeca*) as its characteristic species;
- the black alder communities in Danube Delta can be assigned to the Natura 2000 habitat 91E0* Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion*, *Alnion incanae*, *Salicion albae*), under the provisions of Annex I to the Habitats Directive of the European Union no. 92/43/EEC;
- there are some threatened plant species identified within black alder phytocoenoses, such as: *Hottonia palustris*, *Periploca graeca*, *Cyperus serotinus*, and *Petasites spurius*, which are included on national/international red lists, under different sozological categories (VU or R);
- some of the species identified within black alder forests are aliens, such as: *Amorpha fruticosa*, *Bidens connatus*, *B. frondosus*, *Eclipta prostrata*, *Sicyos angulatus*, *Fraxinus pennsylvanica*, *Prunus cerasifera*, *Aster lanceolatus*.

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Conflict of interest

There is no actual or potential conflict of interest in relation to this paper.

Table 2. Ass. *Periploco graecae-Alnetum glutinosae* ass. nov.

Relevé area (sq. m)	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	
Canopy	0.9	0.7	0.7	0.7	0.65	0.7	0.5	0.7	0.7	0.65	0.65	0.5	0.65	0.8	0.45	
Tree layer height (m)	16-17	18-20	18-20	10 -12	8-10	8-10	8-9	10 -12	8-10	10-11	10-11	10 -12	8-10	10-11	10 -12	K
Tree diameter (cm)	22-50	20-45	25-45	15-50	20-30	15-35	15-25	15-40	20-30	20-40	20-45	20-45	15-40	20 -45	10-30	
Shrub layer cover + regeneration (%)	30	15	20	15	15	5	15	7	25	25	7	10	10	30	10	
Herbaceous layer cover (%)	70	70	70	70	90	50	75	75	55	30	10	50	50	45	50	
Relevé no.	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	V
Charact. ass.																
Alnus glutinosa	5	4	4	4	4	4	4	4	4	4	4	3	4	4	3	V
Alnus glutinosa juv.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V
Periploca graeca	1	1	1 (-2)	.	+	+	1	+	+	1	+	+	+	1	1	V
Alnion glutinosae, Alnetalia glutinosae et Alnetea glutinosae																
Rubus caesius var. *arvalis*	2	1 (-2)	1	1 (-2)	1	+	1	1	1	.	1	1	+	2	+	V
Solanum dulcamara	+	+	+	+	+	+	+	+	.	+	+	.	+	.	.	IV
Stachys palustris	+	+	+	+	.	+	+	+	+	.	+	+	+	.	+	IV
Humulus lupulus	.	.	.	.	1	+	+	.	+	.	+	+	+	.	+	III
Salix cinerea	1	+	+	1	+	+	.	.	.	.	.	.	.	1	+	III
Fraxinus angustifolia subsp. *oxycarpa*	.	.	.	.	+	+	+	.	+	+	+	1	.	.	+	III
Glechoma hederacea	.	.	.	.	.	.	+	+	+	.	+	+	.	.	+	II
Eupatorium cannabinum	.	.	.	.	+	+	+	.	.	.	.	.	+	+	+	II
Lysimachia vulgaris	+	.	.	.	+	.	.	+	+	.	.	+	+	.	.	II
Salix cinerea juv.	.	.	.	1	.	.	.	.	.	.	.	.	.	+	+	I
Frangula alnus	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	I
Fraxinus angustifolia subsp. *oxycarpa* juv.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	I
Carpino-Fagetea																
Viburnum opulus	.	.	.	.	.	+	.	+	+	+	.	+	+	+	+	III
Viburnum opulus juv.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	+	III
Fraxinus pallisae	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	III

Salicetea purpureae															
Salix alba subsp. *alba*	+	1	1	1	+	1	+	1	1	+	.	1	1	2	1	V
Amorpha fruticosa	.	.	+	1	+	+	1	+	2	2	+	+	.	+	+	IV
Salix triandra subsp. *triandra*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	I
Salix triandra juv.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	I
Salix fragilis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	I
Phragmito-Magnocaricetea															
Carex riparia	4	4	4	4	4	3	4	4	3	2	1	3	2	3	3	V
Iris pseudacorus	+	+	+	+	+	+	+	+	.	+	+	+	+	.	+	V
Phalaroides arundinacea subsp. *arundinacea*	+	.	+	.	+	+	+	.	+	+	+	+	+	+	+	IV
Lycopus europaeus	+	+	+	.	+	+	+	1	+	+	+	+	+	.	.	IV
Rumex hydrolapathum	.	.	.	.	+	.	+	+	.	+	+	+	.	+	+	III
Jacobaea paludosa subsp. *angustifolia*	+	+	+	.	.	.	+	.	.	+	.	+	.	.	+	III
Phragmites australis subsp. *australis*	+	+	+	+	+	+	+	.	.	.	+	.	.	.	+	III
Galium palustre subsp. *palustre*	.	.	.	.	+	.	+	.	.	.	+	.	+	.	+	II
Mentha aquatica subsp. *aquatica*	.	.	.	.	.	.	+	+	.	.	.	.	+	+	+	II
Rorippa amphibia	.	.	.	.	.	.	.	.	+	+	+	+	.	+	+	II
Sium latifolium	+	+	+	.	+	.	.	.	.	.	.	.	.	.	+	II
Sium sisarum var. *lancifolium*	.	.	.	.	.	+	+	.	.	+	+	+	.	+	.	II
Sparganium erectum subsp. *erectum*	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	I
Stellaria palustris	.	.	.	+	.	.	.	.	.	+	.	.	+	.	.	I
Leersia oryzoides	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	I
Lythrum salicaria	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	I
Scrophularia umbrosa subsp. *umbrosa*	.	.	.	.	.	.	.	.	+	.	.	.	+	.	.	I
Oenanthe aquatica	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	I
Alisma plantago-aquatica	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	I
Carex acutiformis	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	I
Poa palustris	.	.	.	.	+	.	.	.	+	.	.	.	.	.	.	I

Sagittaria sagittifolia	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	I
Molinio-Arrhenatheretea																
Ranunculus repens	+	+	.	+	1	+	+	+	+	+	+	+	+	+	+	V
Calystegia sepium	+	+	.	.	+	+	+	.	+	+	+	.	+	.	+	IV
Symphytum officinale subsp. *uliginosum*	.	.	.	.	+	+	+	+	+	+	+	+	.	.	.	III
Equisetum arvense	.	.	.	.	.	.	.	.	+	.	+	+	.	.	+	II
Symphytum officinale subsp. *officinale*	+	+	+	+	.	.	.	.	.	.	.	.	.	.	+	II
Myosotis scorpioides subsp. *scorpioides*	.	.	.	.	+	.	+	.	.	+	+	+	.	+	.	II
Glyceria maxima	.	.	.	.	+	.	.	+	.	+	+	.	.	.	+	II
Alopecurus pratensis subsp. *pratensis*	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	I
Agrostis stolonifera	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
Berula erecta	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	I
Cardamine pratensis subsp. *pratensis*	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	I
Potentilla reptans	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	I
Scutellaria galericulata	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	I
Veronica beccabunga	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	I
Rorippa sylvestris subsp. *sylvestris*	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	I
Carex hirta	.	.	.	.	.	.	+	.	.	.	.	.	2	+	.	I
Apium graveolens	.	.	.	.	+	.	.	.	.	.	.	.	+	+	.	I
Bidentetea																
Bidens frondosus	.	.	.	.	.	.	+	.	+	+	.	.	+	.	+	II
Persicaria dubia	.	.	.	.	+	+	.	+	.	+	.	+	+	.	.	II
Bidens tripartitus	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	I
Echinochloa crus-galli	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	I
Eclipta prostrata	.	.	.	.	.	.	.	+	+	.	.	.	+	.	.	I
Persicaria hydropiper	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	I
Bidens connatus	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	I
Ranunculus sceleratus	.	.	.	.	+	.	.	+	.	.	+	.	.	.	.	I

Isoëto-Nanojuncetea																
Cyperus michelianus	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
Cyperus serotinus	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	I
Plantago major subsp. *major*	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	I
Potametea																
Persicaria amphibia var. *aquaticum*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	I
Hottonia palustris	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	I
Stellarietea mediae																
Chenopodium polyspermum	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
Artemisietea																
Arctium lappa	.	.	.	.	.	.	+	.	+	.	+	.	+	.	.	II
Cirsium vulgare	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	I
Tanacetum vulgare	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	I
Galio-Urticetea																
Urtica dioica subsp. *pubescens*	.	.	.	+	+	+	.	.	+	.	+	.	.	.	.	II
Sicyos angulatus	.	.	.	.	.	+	+	.	+	.	.	.	.	.	.	II
Myosoton aquaticum	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	I
Aliae																
Petasites spurius	.	.	.	.	.	+	+	.	.	+	.	.	.	+	.	II
Fraxinus pennsylvanica	.	.	.	.	.	.	+	.	+	+	.	+	.	.	.	II
Prunus cerasifera juv.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	i
Aster lanceolatus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	I
Loranthus europaeus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	I

Place and date of relevés: Rel. no. 1 - Nature Reserve "Arinişul Erenciuc" - left bank of Erenciuc Canal, N44.97672/E29.42572, August 1, 2023; Rel. no. 2 - Nature Reserve "Arinişul Erenciuc" - right bank of Erenciuc Canal, N44.97648/E29.42553, August 1, 2023; Rel. no. 3-4 - Nature Reserve "Arinişul Erenciuc" - right bank of Erenciuc Canal, N44.97546/E29.42616, August 1, 2023; Rel. no. 5 - right bank of Sf. Gheorghe arm - N44.89254/E29.58388, August 2, 2023; Rel. no. 6 - right bank of Sf. Gheorghe arm, N44.89299/E29.58480, August 2, 2023; Rel. no. 7 - right bank of Sf. Gheorghe arm, N44.89462/E29.57823, August 2, 2023; Rel. no. 8 - right bank of Sf. Gheorghe arm, N44.89429/E29.57844, August 2, 2023; Rel. no. 9 - right bank of Sf. Gheorghe arm, N44.89469/E29.57760, August 2, 2023; Rel. no. 10 - right bank of Sf. Gheorghe arm, N44.89622/E29.57538, August 2, 2023; Rel. no. 11 - right bank of Sf. Gheorghe arm, N44.89954/E29.57095, August 2, 2023; Rel. no. 12 - right bank of Sf. Gheorghe arm, N44.90058/E29.56935, August 2, 2023; Rel. no. 13 - South of Sf. Gheorghe arm in "Gârla Turcească" area, N44.88921/E29.57936, August 2, 2023; Rel. no. 14 - South of Sf. Gheorghe arm - "Gârla Turcească" area, N44.88843/E29.57857, August 2, 2023; Rel. no. 15 - left bank of Sf. Gheorghe arm, N44.92436/E29.55073, August 4, 2023.

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Research Article

SILENE MULTIFLORA IN MOLDOVA (ROMANIA)

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Abstract: *Silene multiflora*, a critically endangered species from the vascular flora of Romania, was first reported in the historical province of Moldova (east of the country) since the first half of the 19th century. However, the presence of this species in Moldova was later on contested (confusion with *S. nemoralis* or with *S. viscosa*?), and in the national floristic syntheses published up to now, the species has not been mentioned in the flora of this province. In this work, we confirm the occurrence of the species *S. multiflora* in northeastern Romania (Moldova), based on the specimens collected in the field, as well as on some older specimens kept in two public herbaria in Iași.

Keywords: botanical description, Caryophyllaceae, endangered species, new records, vascular flora.

Introduction

Silene multiflora (Ehrh.) Pers. Syn. Pl. 1: 496. 1805 (*Cucubalus multiflorus* Ehrh.; *Viscago multiflora* (Waldst. et Kit.) Hornem.; *V. multiflora* (Waldst. et Kit.) Baumg.; *Silene steppicola* Kleop., *S. syvashica* Kleop.) (Caryophyllaceae) is an Eurasian plant species, with a native area stretching from Central Asia and Siberia to Central Europe (Austria) [SHISHKIN, 1936; CIOCÂRLAN, 2009; CHATER & al. 2010; IZVERSCAIA, 2016].

Originally published with a short diagnosis, as *Cucubalus multiflorus*, by EHRHART (1792), it was more detailed described and illustrated, under the same name, by WALDSTEIN & KITAIBEL (1799-1802: Table 56) and later on transferred to the genus *Silene* by PERSON (1805).

Relevant iconography of this species has also been published by (in a chronological order): REICHENBACH (1844) (*Icones Florae Germanicae et Helveticae*, VI, Table CCXCI, Figure 5098); JÁVORKA & CSAPODY (1934) (*Iconographia Florae Hungaricae*, pp. 145, Figure 1160); GUȘULEAC (1953) (*Flora R. P. Române*, II, pp. 168, Pl. 22, Figure 3); DIDUKH (2002) (*Ecoflora of Ukraine*, Figure 143a); Izverscaia (2016) (*Flora Basarabiei*, II, pp. 284, Pl. 59, Figure 2); HOCK, in KIRÁLY & al. (2011) (*Új magyar füvészkönyv. Ábrák*. pp. 100, Figure 349); MEREĎA & al. (2012) (*Flora Slovenska*, VI(3), pp. 465, Table 33) etc.

According to the literature data, the presence of the species on the territory of Romania has been confirmed so far only from a few localities, as follows:

- Satu Mare County (Crișana; north-west Romania): Carei ("Nagy-Károli") – "*in pratis*" (as *Cucubalus multiflorus* Ehrh.) [WALDSTEIN & KITAIBEL, 1799-1802] – the first report of this species in the country; *idem* (Herb. Kitaibel, BP) [NEGREAN & DIHORU, 2009]; Sanislău - Vermeș marsh [KARÁCSONYI & NEGREAN, 1979, cited by NEGREAN & DIHORU, 2009] and Pișcolt [KARÁCSONYI, 1995];

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- Hunedoara County (Transylvania; central Romania): Mintia ("Marós Némethi"), Deva – hayfields and forest edges (as. *Viscago multiflora*) [BAUMGARTEN, 1816] – data confirmed by SIMONKAI (1887), UNGAR (1925), GUȘULEAC (1953) and NEGREAN & DIHORU (2009);
- the Transylvanian plain (Mezőség) [SCHUR, 1866] – hill meadows, especially on moorland. According to SIMONKAI (1887), Schur's indication is based on a specimen labelled with "Marós Némethi", so "Mezőség" should be read "Marós Némethi" (i.e. Mintia), the same locality mentioned by BAUMGARTEN (1816);
- Timiș County (Banat; west Romania): Timișoara [GUȘULEAC, 1953];
- Tulcea County (the Danube Delta; south-east Romania): Sfântu Gheorghe – wet meadows near Caraorman [UECHTRITZ & SINTENIS, 1838-1876, cited by KANITZ (1879-1881add); BRANDZA (1898); GUȘULEAC (1953)]; Caraorman – wet meadow, south of the forest (*legit*. G. Negrean 1980-HGN) [NEGREAN & DIHORU, 2009].

The presence of the species in the Muscel Mountains, on the Motoiu hill (Argeș County; Muntenia), as indicated by HOFFMANN (1862) – cited by BRANDZA (1879-1883), has not yet been confirmed and is considered doubtful, according to recent references [*e.g.* NEGREAN & DIHORU, 2009].

Controversial data about this species was also mentioned from Moldova (eastern Romania), in the 19th century, as follows:

- Moldova, as *Viscago multiflora*: "C.XIX.64" (CZIHACK, 1836, XIX, p. 64) [KANITZ, 1877-1881]; *idem* "*et Exsic*.!" [BRANDZA, 1879-1883] (the sign "!" means - specimen seen by Brandza);
- Vrancea County: Focșani, as *Viscago multiflora* Baumg. – hayfields and forest edges [SZABO, 1841];
- the upper Moldova ("Moldova super."), as *Silene multiflora* Pers., "*in pratis mont.*": "G hj" (GUEBHARD, 1842-1848, seen in the Herbarium of the Iași Museum)] [KANITZ, 1877-1881]; *idem* [BRANDZA, 1879-1883];
- Iași County, at Popricani, as *Viscago multiflora*: "Sz. man.?" (SZABO, 1873?) [BRANDZA, 1879-1883];

KANITZ (1877-1881) and BRANDZA (1879-1883) did not recognize, however, the presence of *S. multiflora* in Moldova, and considered that the data reported by CZIHACK, GUEBHARD and SZABO refers to either *S. viscosa* (L.) Pers. [KANITZ, 1877-1881] or *S. nemoralis* Waldst. & Kit. [BRANDZA, 1879-1883]. In addition, subsequent national or regional floristic syntheses from the last century [PRODAN, 1939; BORZA, 1947-1949; GUȘULEAC, 1953; BELDIE, 1977) or published more recently [OPREA, 2005; CHIFU & al. 2006; CIOCÂRLAN, 2009; NEGREAN & DIHORU, 2009; SÂRBU & al. 2013) do not confirm or even mention *S. multiflora* in the flora of Moldova.

Given its rarity in the country and the limiting factors ("located to the southern limit of its world range"; "small populations, with discontinuous distribution"; disturbance of habitats through "hydrotechnical works and grazing"; attack of the pathogenic fungus *Puccinia behenis* (DC.) Otth and the parasite *Eudarluca caricis* (Fr.) O. Erikss.), NEGREAN & DIHORU (2009) included this species in the *Red Book of Vascular Plants from Romania*, as critically endangered (CR).

The aim of this work is to confirm the presence of *S. multiflora* in Moldova, based on field data and herbarium specimens.

Material and methods

The species has been identified by analysing herbarium specimens collected during our own field work (2023), but also of existing specimens in two public herbaria in Romania: I (Herbarium of the Faculty of Biology, University "Alexandru Ioan Cuza" of Iași) and IASI (Herbarium of the "Ion Ionescu de la Brad" Iaşi University of Life Science). Many relevant floristic references at national and international level were used in the process.

Since it was assumed that *Silene multiflora* was mentioned in the flora of Moldova in the old references because of misidentification with either *S. nemoralis* or *S. viscosa*, we analysed the differences between these three species using both data from the literature and voucher specimens from the I and IASI herbaria.

The geographic coordinates were recorded on the field using the OsmAnd application, available at https://osmand.net/. To highlight morphological details, we used a OPTIKA binocular magnifier and a OPTIKA digital camera with CCD Sensor.

Plant nomenclature followed SÂRBU & al. (2013).

Voucher specimens were deposited in the Herbarium of the "Ion Ionescu de la Brad" Iaşi University of Life Science (IASI).

Results and discussions

During our recent field work (in the year 2023), we identified a population of *Silene multiflora* (Ehrh.) Pers., of about 50 specimens, to the southwest of the Horlești village (Rediu commune, Iași County, north-eastern Romania).

Figure 1. Hayfield meadow with *Silene multiflora*, near the Horlești village (Iași County)

Specimens of *S. multiflora* are scattered in this place, on an area of approx. 100 m^2, in a hayfield meadow (Figure 1) which lies at the base of the steep coast that borders the Horlești village to the southwest, and where a small tributary of the Bogonos stream originates.

In this meadow, together with *S. multiflora* grow other meso-hygrophytes (*Agrostis stolonifera*, *Carex distans*, *Inula helenium*, *Rorippa austriaca*, *Rumex crispus*, *Scorzonera cana*), also mesophytes (*Centaurea jacea*, *Dactylis glomerata*, *Elymus repens*, *Lotus corniculatus*, *Poa pratensis*, *Rhinanthus rumelicus*, *Tragopogon pratensis* subsp. *orientalis*) and even helophytes (*Phragmites australis*), all these being favoured by the higher soil moisture during the springtime.

Because of the increasing drying of the soil in summer, many xerophytes / xero-mesophytes originating from the steppic grasslands of the adjacent slopes, also occur in this meadow, such as: *Festuca valesiaca*, *Dianthus membranaceus*, *Galium octonarium*, *G. verum*, *Koeleria macrantha*, *Lathyrus pallescens*, *Medicago falcata*, *Melampyrum arvense*, *Onobrychis viciifolia*, *Stachys recta*, *Trinia ramosissima*, *Verbascum phoeniceum*, *Veronica austriaca*.

The occurrence of some halophytes (*Carex distans*, *Scorzonera cana*, *Aster oleifolius*) and salinity-tolerant species (*Iris brandzae*, *Agrostis stolonifera*, *Rorippa austriaca*, *Phragmites australis*, *Rumex crispus*) reveals the presence of soluble salts in the soil.

Figure 2. *Silene multiflora*: habitus (**a**) and details of stem: densely short hairy below (**b**), glabrous and viscid toward the inflorescence (**c**) (scale bar: 4 cm - **a**; 2,5 mm - **b**; 1,5 mm - **c**)

Herbarium specimens: IASI, no. 18056: *legit.* & *det*. C. Sîrbu & A. Oprea, the southwest of the Horlești village, Iași County, N 47.26407, E 27.43801, hay meadow, 03.06.2023; IASI, no. 18057, 18058, 18059 and 18060: *idem*, 08.06.2023.

After identifying the specimens collected on field, we have surprisingly discovered three other specimens of *S. multiflora*, in two public herbaria of the Iași city (IASI and I). These specimens were collected 80 years before, by M. Răvăruț, along the Valea Rea stream, a tributary of the Miletin River (Botoșani County). The three specimens were labelled as follows:

-IASI, no. 2231 (2 specimens): "*Silene multiflora* (Ehrh.) Pers.?"; Valea Rea (Botoșani County); meadows, hayfields; M. Răvăruț; 18.06.1943; "must be seen: the calyx is not hairy!";

-I, no. 4489 (1 specimen): "*Silene multiflora* (Ehrh.) Pers.?"; Valea Rea (Botoșani County); M. Răvăruț; 18.06.1943.

Following this finding, in June 2023, we searched for *S. multiflora* along the entire course of the Valea Rea stream, but we did not manage to identify any specimen. It should be noted however that most of the meadows in the valley of this stream were disturbed by intensely sheep grazing, which greatly reduces the chances of this species still surviving in that place.

Figure 3. *Silene multiflora*: the inflorescence

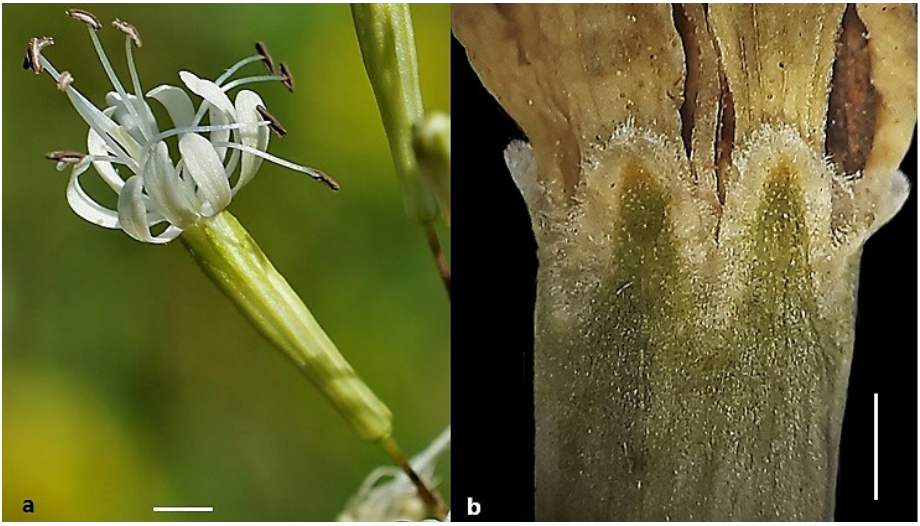

Figure 4. *Silene multiflora*: a single flower (**a**) and details of calyx teeth (**b**). (scale bar: 3 mm - **a**; 1 mm - **b**)

Figure 5. *Silene multiflora*: a dried petal, with ciliate claw (scale bar: 1 mm)

Botanical description (based on specimens collected by us and M. Răvăruț). Perennial. Stem simple, 50-80 cm tall, with 6-8 nodes, densely short hairy (hairs 0.1-0.2 mm long, bent downward), glabrous and viscid towards the inflorescence (Figure 2). Radical and lower cauline leaves long petioled; petioles short pubescent (hairs longer towards the sheath); lamina 15-40 × 8-10 mm, oblanceolate-spatulate, short pubescent especially on the edges and midvein (hairs similar to those on the stem), margins entire, apex obtuse. Middle and upper cauline leaves sessile (joined at the sheath level), lance-linear, gradually decreasing and narrowing upward, densely short hairy. Inflorescence narrowly paniculate to pseudo-verticillate (Figure 3); branches short, erect, opposite, glabrous, (1-) 3 (-6) flowered; bracts and bracteoles wide membranous and ciliated, especially in the sheath area. Flowers bisexual, pedunculate, oblique-ascending; pedicels glabrous, 3-7 mm long, much longer than bracteoles (lateral flowers). Calyx cylindric-clavate, truncate at the base, 10-12 mm long, glabrous (except the teeth margins), 10-veined; teeth ovate, obtuse, membranous and ciliated on margins (Figure 4). Petals white; claws 8-10 mm long, margins membranous, ciliate (Figure 5); limb of petals 4–6 mm long, deeply incised, with 2 broadly linear segments; coronal scales barely distinct, as 2 very short tubercles. Androgynophore 4-5 mm long, densely pubescent, with backward, very short, hairs. Stamen much longer than petals; filaments glabrous, anthers purple. Ovary ca. 5-6 mm long; styles white, 10-12 mm long. Capsules elongated-ellipsoidal, 8-9 mm long, 1.6-1.8 times as long as carpophore, with 6 recurved teeth (Figure 6). Carpophore (the former androgynophore) ca. 5.0 mm long (Figure 6). Seeds grey-black, reniform, 1 mm wide; lateral sides slightly concave, testa papillae radially elongated; dorsal side flat to slightly concave, testa papillae less elongated (tubercles-like); all papillae toothed at base (Figure 6).

Plant identification. The identification of this species was quite difficult, because there are some (seemingly?) controversial data in the literature, regarding its morphological features, such as: (Table 1: * features that match specimens examined by us): stem not viscid or viscid above*; calyx glabrous*, or sparsely to densely hairy, not glandular* or glandular; calyx teeth acute or obtuse*; petal claws - not ciliate or ciliate*; capsule equal to, shorter or longer* than the carpophore.

Figure 6. *Silene multiflora*: capsule with carpophore (**a**) and seeds (**b**)

We suppose that, excepting some statements that are most likely wrong (e.g., according to GUȘULEAC, 1953: the carpophore *usually* longer than capsule – as opposed to *S. chlorantha*; calyx sometimes with glandular hairs etc.), many of these controversial data in the literature reflects a large variability of the populations of *S. multiflora*, in various regions of its wide natural area. Some populations of this plant were circumscribed to different species (*S. steppicola* Kleopow; *S. syvashica* Kleopow), which subsequently were considered to the level of subspecies and/ or varieties [TZELEV, 2004; TZVELEV & GELTMAN, 2012] or just variations with no taxonomic value [PROKUDIN & al. 1987; MOSYAKIN, 1999; DIDUKH, 2002; CHATER & al. 2010; FEDORONCHUK, 2023], which strengthens this supposition. This variability deserves to be investigated more carefully in the future!

All the morphological features observed by us in the specimens from north-eastern Romania, as shown in the description above, fall within the range of variability of the species *S. multiflora*, as described in various references (Table 1). The viscosity of stem is, however, a feature rarely associated with this species: *Viscago multiflora* – "*caule* […] *viscoso*" [BAUMGARTEN, 1816]; *Viscago multiflora* – "*cotor* […] *cleios"* (*stem* […] *viscid*) [SZABO, 1841]; *Silene multiflora* – "*not viscid except in inflorescence*" (position 149 *bis* in the key) [CHATER & al. 2010]; *idem* – "*stem pubescent at the base, often glabrous and viscid above*" – for both *S. multiflora* and *S. italica* (position 19a in the key) [PROKUDIN & al. 1987].

Table 1. Some morphological features of the species *Silene multiflora*, according to various references (* features that match specimens examined by us)

Morphological features		**References**
Stem	**viscid above***	BAUMGARTEN, 1816 (*Viscago multiflora*); SZABO, 1841 (*idem*); PROKUDIN & al. 1987 (both *S. multiflora* and *S. italica*)
	not viscid	NEILREICH, 1867; BRANDZA, 1898; GUȘULEAC, 1953; BELDIE, 1977; FISCHER & al., 2008; CHATER & al., 2010: "except in inflorescence"!; KIRÁLY, 2011; MEREĎA & al. 2012
Calyx	**glabrous***	BELDIE, 1977; TZELEV, 2004 (*S. steppicola* subsp. *steppicola*; *S. syvaschica* var. *glabra*); TZVELEV & GELTMAN, 2012 (*idem*); BELKIN, 2009
	glabrous* or sparsely hairy	ROHRBACH, 1868; SHISHKIN, 1936; FISCHER & al. 2008; CIOCÂRLAN, 2009; CHATER & al. 2010; SÂRBU & al. 2013; KIRÁLY, 2011; IZVERSCAIAM 2016
	densely to sparsely hairy	MEREĎA & al. 2012
	pubescent	EHRHART, 1792; PERSON, 1805; WALDSTEIN & KITAIBEL, 1799-1802; NEILREICH, 1867; BRANDZA, 1898; PRODAN, 1939; GUȘULEAC, 1953; TZELEV, 2004 (*S. steppicola* subsp. *pubescens*)
	glandular	GUȘULEAC, 1953
	not glandular*	PRODAN, 1939; GUȘULEAC, 1953
Calyx teeth	**acute**	CHATER & al. 2010; IZVERSCAIA, 2016
	obtuse*	WALDSTEIN & KITAIBEL, 1799-1802; NEILREICH, 1867; ROHRBACH, 1868; BRANDZA, 1898; SHISHKIN, 1936; PROKUDIN & al. 1987; MEREĎA & al. 2012
Petal claws	**ciliate***	SHISHKIN, 1936; BELKIN, 2009; IZVERSCAIA, 2016
	not ciliate	MEREĎA & al. 2012
Fruit (*F*) versus carpophore (*C*)	***F* equalling *C***	EHRHART, 1792: "*capsula longitudine thecapodii*"; WALDSTEIN & KITAIBEL, 1799-1802: *idem*; NEILREICH, 1867: *idem* ("*carpophoro capsulam aequante*"); ROHRBACH, 1868: ≈ *idem* ("*subaequans*"); BRANDZA, 1898; PRODAN, 1939; CIOCÂRLAN, 2009: 8-10 mm both; NEGREAN & DIHORU, 2009: ≈ 8-10 mm both; IZVERSCAIA, 2016: 6-7 mm both

	***F* equalling C or somewhat longer**	CHATER & al. 2010: (7-)8-10 mm both or carpophore a little shorter; SÂRBU & al. 2013
	F* longer than *C*	SHISHKIN, 1936: 7-8 and 6 mm, respectively
	F* equalling or shorter than *C	GUȘULEAC, 1953: "capsule as long as carpophore or slightly shorter"; *S. multiflora* (Ehrh.) Pers. "differs from the similar species *S. chlorantha* (Willd.) Ehrh., by […] *carpophore longer than capsule*"
	F* slightly shorter, equalling, or much longer* than *C	MEREĎA & al. 2012: fruit 0.8-1.8 times as long as carpophore

By the stems with 6-8 nodes and the pedicels and calyx glabrous, the specimens we have analysed fit better to the east European (Ukrainian) populations designated as *S. multiflora* var. *glabra* (Kleop.) Fedor. (*Silene steppicola* Kleop. subsp. *glabra* Kleop.) [see TZELEV, 2004; FEDORONCHUK, 2023].

Has been *S. multiflora* actually found in Moldova since the 19th century? As shown in the *Introduction* section, according to BRANDZA (1879-1883) the distribution data of *Silene multiflora* (*Viscago multiflora*) reported by Czihack, Guebhard or Szabo from Moldova, must be taken into account for *S. nemoralis* Waldst. & Kit., while according to KANITZ (1877-1881), the same data must be considered for a much more distinct species – *S. viscosa* (L.) Pers. Certainly, the two authors could not be both right. On the other hand, the supposedly

Figure 7. Differences between *S. multiflora* (**Sm**), *S. nemoralis* (**Sn**) and *S. viscosa* (**Sv**) regarding the stem pilosity (scale bar: 1 mm)

misidentification of *S. multiflora* by confusion with *S. nemoralis* or with *S. viscosa* is rather unlikely, given the obvious differences among the three species [SHISHKIN, 1936; CHATER & al. 2010; MEREĎA & al. 2012; SÂRBU & al. 2013 etc.; see also: TALAVERA, 1990 for *S. nemoralis*]. *S. multiflora* differs from *S. nemoralis* especially by: stem densely puberulent below, hairs very short, 0.1-0.2 mm long (*versus* stem pubescent, hairs less dense, much longer, up to 1 mm) (Figure 7); inflorescence narrow; branches non viscide, short, erect (versus panicle much wider; branches viscide, long, spreading) (Figure 8); pedicels much longer than bracteoles at the time of flowering (*versus* pedicels shorter than or equalling bracteoles) (Figure 8); calyx 12-15 mm long, glabrous (to ± pubescent), not glandular (*versus* 14-24 mm; scattered hairy, often glandular); carpophore of 5-8 (-9) mm long, up to 1.8 times shorter than the mature capsule (*versus* 9-12 (14) mm, usually slightly longer than mature capsule) (Figure 9). Compared to *S. viscosa* it differs even more, especially by: the stem pubescence (stem, as well as the whole plant, glandular-tomentose, in *S. viscosa*) (Figure 7); basal leaves spatulate, margins entire, apex obtuse (*versus* leaves ovate-lanceolate, margins undulate-crenate, apex acute, in *S. viscosa*); calyx length and pilosity (14-24 mm, glandular-tomentose, in *S. viscosa*); carpophore-to-capsule ratio (carpophore 3-4 times shorter than the mature capsule, in *S. viscosa*) (Figure 9), etc.

Figure 8. Differences between *S. multiflora* (**Sm**) and *S. nemoralis* (**Sn**), regarding the inflorescence (left) and bracteoles to pedicel ratio (right) (scale bar: 2,5 cm – left; 2 mm – right)

Given the lack, nowadays, of herbarium specimens collected by Czihack, Guebhard or Szabo, no one can prove which of the two approaches (Kanitz versus Brandza) was wrong, if not both. In these circumstances, we cannot exclude the possibility that, in fact, Czihack, Guebhard and Szabo, or at least one of them, were right when mentioning *S. multiflora* from this historical province. The best proof of this is that the species is still present here, in a natural habitat whose characteristics match data from the literature (see below). In addition, it is worth mentioning that the place where we identified *S. multiflora*, near Horlești village (Rediu commune), is not far away from Popricani (about 6 km in a straight line) – the locality from where Szabo (1873) – according to BRANDZA (1879-1883) mentioned "*Viscago multiflora*". So, why wouldn't Szabo's data, although not confirmed by Brandza, refer to the same population as that we found, or to a neighbouring one, meanwhile disappeared?

Figure 9. Differences between *S. multiflora* (**Sm**), *S. nemoralis* (**Sn**) and *S. viscosa* (**Sv**), regarding the carpophore to capsule ratio (scale bar: 5 mm)

Coming back to more recent times, most likely, because of the glabrous calyx ("must be seen: the calyx is not hairy!"), Rǎvǎruț has considered, in 1943, that the identification of specimens collected by him as *S. multiflora* is not certain (hence the question mark on the herbarium labels: "*Silene multiflora* (Ehrh.) Pers.?"). Consequently, he did not publish his herbarium data, and the presence of this species in the flora of Moldova has remained unconfirmed until now.

Ecological requirements and habitats. According to TICHÝ & al. (2023), *S. multiflora* prefers soils rather moist ($M_{5.5}$), alkaline (R_8), with low salinity ($S_{3.5}$) which is consistent with data provided by other references [JÁVORKA, 1925; PRODAN, 1935;

SHISHKIN, 1936; BELKIN, 2009; CIOCÂRLAN, 2009; KIRÁLY, 2011; TZVELEV & GELTMAN, 2012; MEREĎA & al. 2012; SÂRBU & al. 2013] and is largely confirmed by our field data.

S. multiflora usually occurs on moist to marshy soils, dry in summer, rich in minerals and often slightly saline, in meadows and saline pastures in the lowland area, most often in communities of *Deschampsion cespitosae* and *Festucion pseudovinae* [MEREĎA & al. 2012]. It also grows on sandy soils [JÁVORKA, 1925; TZVELEV & GELTMAN, 2012; CIOCÂRLAN, 2009; SÂRBU & al. 2013], steppes [SHISHKIN, 1936; BELKIN, 2009; TZVELEV & GELTMAN, 2012], outcrops of chalk and limestone [TZVELEV & GELTMAN, 2012], and open woods [SHISHKIN, 1936; BELKIN, 2009].

According to the ecological data provided by CIOCÂRLAN (1994, 2009) and SÂRBU & al. (2013), among the *Silene* species in the flora of Romania, *S. multiflora* has the highest tolerance to seasonal water excess and soil salinity.

A proposal to protect this species. As shown in the introduction, *S. multiflora* is a very rare, critically endangered species in the flora of Romania. It is also very rare [TOFAN-BURAC & CHIFU, 2002; IZVERSCAIA, 2016; IZVERSCAIA & al. 2022], supposedly extinct [IZVERSCAIA & al. 2022] in the eastern neighbourhood of Romania (the Republic of Moldova). Therefore, we consider that the protection of this species in Romania is very necessary. In the northeastern Romania, this could be achieved by expanding eastward the Nature 2000 site ROSCI0058 "Dealul lui Dumnezeu", which is located nearby, to the southwestern limit of the Horlești village.

Conclusions

Based on our own field research and specimens stored in public herbaria of Iași (Romania), we confirm in this paper the occurrence of the species *S. multiflora* in northeastern Romania (Moldova).

This is a very rare, critically endangered plant species in the flora of the country. In order to protect population of *S. multiflora* in this part of the country, we propose the extension eastward of the Nature 2000 site ROSCI0058, such that this should also include the meadow where this species grows, near the village of Horlești.

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Research Article

THE STATE OF THE POPULATIONS OF *NECTAROSCORDUM BULGARICUM* IN THE STRICTLY PROTECTED ZONE OF THE "CODRII" RESERVE, REPUBLIC OF MOLDOVA

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Abstract: *Nectaroscordum bulgaricum* – Honey garlic, a vulnerable species, included in the Red Book of the Republic of Moldova, is territorially protected in the "Codrii" Reserve. The aim of the study was to identify and describe the Honey garlic populations, as a result the trend of the species will be estimated. As a result, 11 populations were identified and described in subplots: 40E, F, K; 41B, J, K; 42H; 46A, E, D and 49E. The geographical coordinates were taken and the map with the location of the identified populations was developed. The state of the populations of Honey garlic in the strictly protected zone of the "Codrii" Reserve was evaluated.

Keywords: Honey garlic, population, protected area, rare species.

Introduction

The „Codrii" Reserve occupies a special place among the protected areas of the Republic of Moldova and is the first reserve created with the status of a scientific research unit, by the Decision of the Council of Ministers of 27.09.1971, in order to preserve the most representative sectors of forests typical of the area of the Central Plateau of Codrii (Figure 1). The territory of the reserve has a special protection status, delimited into three functional zones: the strictly protected zone, the buffer zone and the transition zone.

The strictly protected area of the "Codrii" Reserve includes the sectors with the habitats of rare animal and plant species of universal value from a scientific and conservation point of view. It occupies an area of 723 ha and is intended for scientific research. The general character of the integral protection zone is determined by the presence of hills that follow each other in waves, and the general exposure determined by the relief and water drainage is south-east. The maximum altitude is 353 m, and the minimum 193 m. The territory of the integral protection zone is within the hydrological basin of the Botna River (tributary of the Dniester). The hydrographic network consists of multiple streams with seasonal flow - tributaries of the Botna, accumulating the surface runoff that forms after the rains. The soil, station and forest types are characteristic of the phytoclimatic floor – *Hilly oak forest with sessile oak and mixed forests on hills and beech forests of the inferior limit*. Light gray soils predominate. Mixed forests with sessile oaks on hills (51%) and sessile oak-mixed forests (19%) are representative for the forests of the integral protection zone [Forestry Research and Development Institute - Institutul de Cercetări și Amenajări Silvice (ICAS), 2020].

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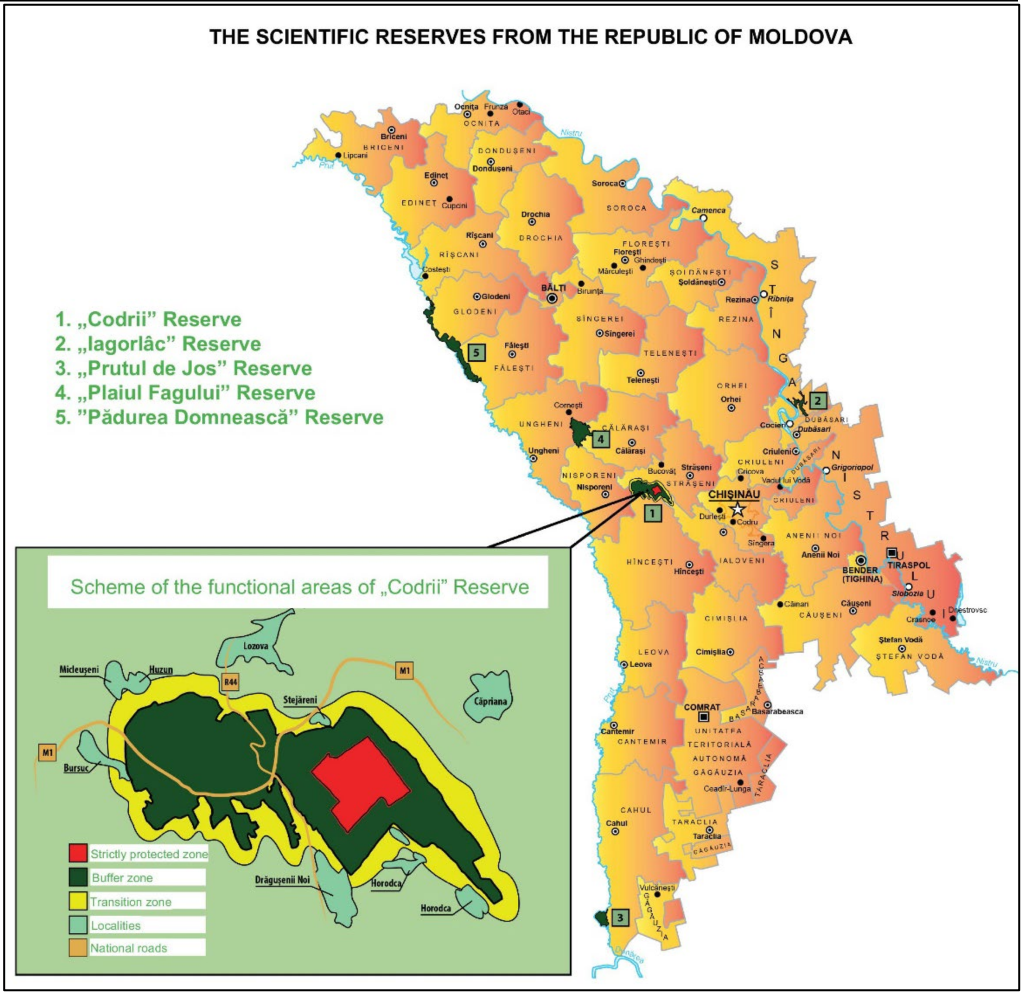

Figure 1. Location of the „Codrii" Reserve on the territory of the Republic of Moldova

The floristic inventory of the strictly protected area consists of 222 species of vascular plants, of which 32 are state protected plant species, which constitutes 53% of the total species protected by law on the territory of the "Codrii" Reserve [JARDAN, 2018]. Of these, 10 species are included in the Red Book of the Republic of Moldova (3rd edition): *Athyrium filix-femina* (L.) Roth, *Cephalanthera damasonium* (Mill.) Druce, *Cephalanthera longifolia* (L.) Fritsch, *Dryopteris filix-mas* (L.) Schott, *Epipactis purpurata* Sm., *Nectaroscordum bulgaricum* Janka, *Ornithogalum flavescens* Lam., *Polypodium vulgare* L., *Scopolia carniolica* Jacq. and *Thelypteris palustris* Schott [Red Book of Republic of Moldova, 2015].

Periodic assessment of the ecological status of rare plant populations is a necessity of major importance, in order to highlight the long-term trend of rare species. The purpose of the study was to identify and describe the populations of *Nectaroscordum bulgaricum* Janka in the strictly protected area of the "Codrii" Reserve.

Nectaroscordum bulgaricum is a Ponto-Balkan species, widespread in the Balkan Peninsula, Cyprus, Romania, Crimea, the Western Caucasus, Asia Minor [OMELICIUC-MEACUŞCO, 1979; STEARN, 1980]. In the Republic of Moldova it grows, at the northern limit of its Ponto-Balkan area, in the Teleneşti, Străşeni, Cimislia, Leova, Hinceşti and Cantemir districts [GHENDOV & CIOCÂRLAN, 2015].

Nectaroscordum bulgaricum Janka (honey garlic) – is a perennial, glabrous plant. Ovoid bulb, with a specific smell. Stem erect, unbranched, smooth. Loose inflorescence, consisting of 2-3 uniparous cymes, with 20-40 green-yellowish-violet flowers. Tepals or Perianth segments fleshy or thin, after flowering gathered in a cone. Fruit an ovoid capsule. Black seeds, relatively large, 3-edged. Blooms in May-June, fruits in June-July. Mesophilic, mesothermic, acid-neutrophilic species. Vulnerable species, included in the Red Book of the Republic of Moldova. Protected in the "Codrii" Reserve, in the landscape reserves "Hânceşti", "Căpriana-Scoreni", "Cărbuna" and in the natural reserve of medicinal plants "Logăneşti" [CIUBOTARU & al. 2007; GHENDOV & CIOCÂRLAN, 2015].

Materials and methods

The present study is based on the field researches carried out during the years 2022-2023, by following some itineraries that cover as representative as possible the strictly protected area within the "Codrii" Reserve.

The nomenclature of the species is presented according to fundamental works in the field [GHEIDEMAN, 1986]. In the population study of the species *Nectaroscordum bulgaricum*, the GPS coordinates were taken, the numerical effectively of the populations, the ontogenetic stage of each individual, the vitality of the individuals as well as the populations, and the accompanying plant species were recorded. The population size has been determined by extrapolating the average density of individuals to the total area of the population. The average density of individuals was determined in sample areas of 1 m^2 (3-5 sample areas, depending on the population size). The geographic coordinates were taken over using the GPSMAP64s GPS device, GARMIN. The map regarding the location of *Nectaroscordum bulgaricum* populations in the strictly protected area of the reserve was developed using the application QGIS.

Results and discussions

According to information from the literature, the presence of the species *Nectaroscordum bulgaricum* within the limits of the "Codrii" Reserve was reported in 1976 by GHEIDEMAN (1976). In 1984 it was indicated in plots 46, 48 and 49 of the strictly protected area, occupying areas of 2-3 m^2 [NIKOLAEVA, 1984; GHEIDEMAN & al. 1980]. To the inventory of the area with integral protection from 1993, STURZA & al. (1994) also reported the species in the plots 41 and 42.

As a result of our field research, in the strictly protected area of the "Codrii" Reserve, 11 populations of Honey garlic were identified in the subplots: 40E, F, K; 41B, J, K; 42H; 46A, E, D and 49E, type of station, *Hilly oak forest with sessile oak*, *mixed forests with sessile oak on plateaus*, *sunny and partly sunny slopes*, *with gray soils* [JARDAN, 2023]. The geographical coordinates were taken and the map with the location of the identified populations was drawn up (Figure 2).

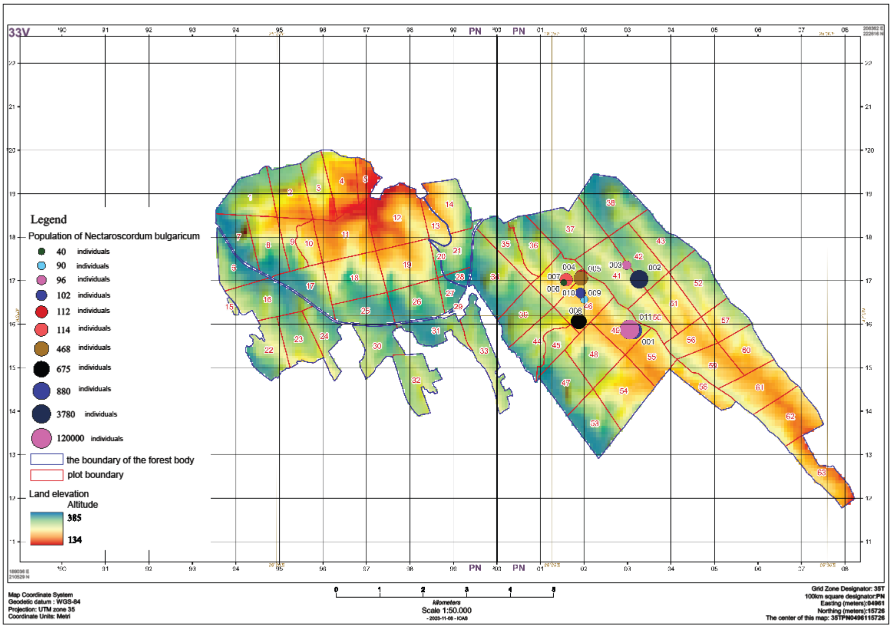

Figure 2. Map with the location of *Nectaroscordum bulgaricum* populations

The first recorded population of *Nectaroscordum bulgaricum* (on the date of 19.05.2022) in subplot 49E grows on an undulating slope with SW exposure, altitude 282 m, in a mixed forest with sessile oaks on hills – fundamental natural stand of superior productivity, consistency 0.8, age average 90 years. The upper floor of the trees consists of *Quercus petraea*, *Quercus robur*, the lower floor of *Fraxinus excelsior*, *Tilia cordata*, *Acer campestre*, *Carpinus betulus*, the layer of shrubs is represented by *Cornus mas*, *Crataegus monogyna*, *Corylus avellana*, *Viburnum lantana*. The herbaceous layer covers about 85% of the soil and includes: *Alliaria petiolata*, *Anemonoides ranunculoides*, *Arum orientale*, *Asparagus tenuifolius*, *Carex brevicollis*, *Carex pilosa*, *Carex sylvatica*, *Dentaria bulbifera*, *Fallopia dumetorum*, *Geum urbanum*, *Glechoma hirsuta*, *Lamium maculatum*, *Lapsana communis*, *Lunaria annua*, *Melica uniflora*, *Mycelis muralis*, *Polygonatum hirtum*, *Scutellaria altissima*, *Stellaria holostea*, *Stellaria media*, *Symphytum tauricum*, *Viola odorata*.

The population occupies an area of 220 m^2, with a density of 4 specimens per m^2. The share of juvenile individuals is 98% and generative ones – 2%.

The second population grows at the intersection of three subplots 41J, K and 42H (registered on 03.05.2023), on an upper undulating slope with SW and SE exposures, altitude 312 m, in a mixed forest with sessile oaks on hills and sessile oak-mixed forest - fundamental natural stand of superior productivity, consistency 0.8, average age 95 years. The upper layer of the trees is made up of *Quercus petraea*, the lower layer of *Fraxinus excelsior*, *Tilia cordata*, *Acer platanoides*, *Carpinus betulus*, the layer of shrubs is represented by *Cornus mas*. The grass cover covers the soil on about 80% of the surface and includes: *Allium ursinum*, *Anthriscus cerefolium*, *Asparagus tenuifolius*, *Carex sylvatica*, *Corydalis marschalliana*, *Dentaria bulbifera*, *Ficaria verna*, *Galium aparine*, *Geum urbanum*, *Glechoma hirsuta*, *Lamium* *maculatum*, *Melica uniflora*, *Mercurialis perennis*, *Polygonatum hirtum*, *Pulmonaria officinalis*, *Stellaria media*, *Veronica hederifolia*, *Viola odorata*.

The given population occupies an area of 1260 m^2, with a population density of 3 individuals per m^2. Generative specimens predominate (97%), but juvenile ones constitute only 3%.

The third population grows in subplot 41B, on an undulating slope, with SW exposure, altitude 306 m, in sessile oak-mixed forest - fundamental natural stand of superior productivity, consistency 0.8, average age - 90 years. The upper floor is represented by *Quercus petraea* and *Fraxinus excelsior*, the lower one of *Tilia cordata*, *Acer campestre*, *Carpinus betulus*, the shrub layer is represented by *Cornus mas* and *Crataegus monogyna*. The herbaceous cover covers the soil on about 70% of the surface and includes: *Allium ursinum*, *Dentaria bulbifera*, *Ficaria verna*, *Galium aparine*, *Glechoma hirsuta*, *Lamium maculatum*, *Melica uniflora*, *Polygonatum hirtum*, *Stellaria holostea*, *Stellaria media*, *Veronica hederifolia*.

This population occupies an area of 56 m^2, with a population density of 1.6 specimens/m^2, predominating generative individuals with 75%, juvenile ones constitute 25%.

The fourth population was recorded in subplot 40F, on an upper undulating slope with SW exposure, altitude 307 m, in a mixed forest with sessile oaks on hills – total stand derived of superior productivity, consistency – 0.8, average age 95 years. The upper layer of the trees is made up of *Quercus petraea*, the lower layer of *Fraxinus excelsior*, *Tilia cordata*, *Acer campestre*, *Carpinus betulus*, the layer of shrubs is represented by *Cornus mas*. The grass cover covers the soil on about 70% of the surface and includes: *Alliaria petiolata*, *Anthriscus cerefolium*, *Asparagus tenuifolius*, *Corydalis marschalliana*, *Fallopia dumetorum*, *Galium aparine*, *Glechoma hirsuta*, *Lamium purpureum*, *Melica uniflora*, *Polygonatum hirtum*, *Stellaria holostea*, *Veronica hederifolia*.

The population covers an area of 153 m^2, with a population density of 0.7 individuals/m^2, predominating the generative specimens (97%).

The fifth population was identified in subplot 46E, on an upper undulating slope with SW exposure, altitude 289 m, in mixed forest with sessile oaks on hills – fundamental natural stand of superior productivity, consistency – 0.8, average age 100 years. The upper layer of the trees is made up of *Quercus petraea*, *Fraxinus excelsior*, the lower layer of *Acer campestre* and *Carpinus betulus*, the layer of shrubs is represented by *Cornus mas*. The grass cover covers approximately 75% of the soil from the surface and includes: *Alliaria petiolata*, *Anemonoides ranunculoides*, *Anthriscus cerefolium*, *Arum orientale*, *Asparagus tenuifolius*, *Corydalis marschalliana*, *Fallopia dumetorum*, *Galium aparine*, *Glechoma hirsuta*, *Lamium maculatum*, *Mycelis muralis*, *Polygonatum hirtum*, *Stellaria media*, *Veronica hederifolia*.

The population covers an area of 383 m^2, with a population density of 1.2 individuals/m^2, predominating the generative specimens (96%).

The sixth population of *Nectaroscordum bulgaricum* recorded in subplot 40E grows on a medium undulating slope with SW exposure, altitude 293 m, in sessile oak-mixed forest – fundamental natural stand of superior productivity, consistency – 0.8, average age 100 years. The tree layer is made up of *Quercus petraea*, *Fraxinus excelsior* and *Tilia tomentosa* on the upper floor, and the lower one from *Acer platanoides*, *Sorbus torminalis*, the shrub layer is represented by *Cornus mas*, *Viburnum lantana*, *Crataegus monogyna*. The herbaceous cover covers the ground on about 80% of the surface and includes: *Alliaria petiolata*, *Allium ursinum*, *Anemonoides ranunculoides*, *Corydalis marschalliana*, *Dentaria bulbifera*, *Galium aparine*, *Glechoma hirsuta*, *Mercurialis perennis*, *Polygonatum hirtum*, *Stellaria holostea*, *Veronica hederifolia*, *Viola hirta*.

The population extends over an area of 25 m^2, with a population density of 3.8 individuals/m^2, generative specimens predominating (97%), juveniles constituting 3%.

The seventh population was recorded in subplot 40K, on an undulating lower slope with SW exposure, altitude 276 m, in mixed forest with sessile and pedunculate oaks on hills – high productivity fundamental natural stand, almost full consistency – 0.9, average age 95 year old. The upper floor of the trees is made up of 135-year-old *Quercus robur*, the lower floor of *Quercus petraea*, *Fraxinus excelsior*, *Acer campestre* and *Carpinus betulus*, the layer of shrubs is represented by solitary specimens of *Cornus mas*. The herbaceous cover covers the ground on about 75% of the surface and includes: *Anthriscus cerefolium*, *Dentaria bulbifera*, *Ficaria verna*, *Galium aparine*, *Geum urbanum*, *Glechoma hirsuta*, *Lamium maculatum*, *Melica uniflora*, *Polygonatum hirtum*, *Stellaria holostea*, *Viola hirta*.

The population occupies an area of 14 m^2, with a population density of 2 individuals/m^2, generative specimens predominating (95%).

Population number eight was identified in subplot 46A, on an undulating lower slope with SE exposure, elevation 229 m, in mixed forest with sessile and pedunculate oaks on hills – partially derived stand, consistency – 0.8, average age 90 years. The upper floor of the trees consists of *Quercus robus*, *Quercus petraea* with 120 years old, the lower one – *Fraxinus excelsior*, *Acer campestre*, *Acer platanoides*, *Tilia tomentosa* and *Carpinus betulus*, the shrub layer is represented by *Viburnum lantana*, *Cornus mas*. The grass cover covers the soil on about 75% of the surface and includes: *Anthriscus cerefolium*, *Asparagus tenuifolius*, *Galium aparine*, *Geum urbanum*, *Glechoma hirsuta*, *Lamium maculatum*, *Polygonatum hirtum*, *Pulmonaria officinalis*, *Stellaria holostea*, *Stellaria media*, *Veronica hederifolia*, *Viola hirta*.

The population occupies an area of 222 m^2, with a population density of 3 individuals/m^2, generative specimens predominating (95%).

The ninth population of *Nectaroscordum bulgaricum* registered in subplot 46D grows on a flat lower slope with SW exposure, altitude 215 m, in oak-sessile oak-mixed forest - artificial stand of superior productivity, consistency – 0.9, average age 40 years. The layer of trees is composed of *Quercus robur* on the upper floor, and the lower layer of *Fraxinus excelsior*, *Tilia cordata*, *Carpinus betulus*, *Acer campestre*, the layer of shrubs is represented by *Crataegus monogyna*. The herbaceous cover covers the ground on about 80% of the surface and includes: *Ajuga reptans*, *Asarum europaeum*, *Dentaria bulbifera*, *Ficaria verna*, *Galium aparine*, *Galium odoratum*, *Geum urbanum*, *Glechoma hirsuta*, *Hedera helix*, *Polygonatum hirtum*, *Sanicula europaea*, *Stellaria holostea*, *Viola hirta*.

The population extends over an area of 24 m^2, with a population density of 3 individuals/m^2, with generative specimens predominating (99%), juveniles constituting only 1%.

Population number ten was recorded in subplot 46E, on an undulating slope with SW exposure, altitude 231 m, in mixed forest with sessile oaks on hills – high productivity fundamental natural stand, consistency – 0.8, average age 100 years. The upper floor of the trees is made up of *Quercus petraea*, the lower floor of *Fraxinus excelsior*, *Acer campestre*, *Tilia tomentosa* and *Carpinus betulus*, the layer of shrubs is represented by *Crataegus monogyna*, *Viburnum lantana*, *Cornus mas*. The grass cover covers the soil on about 70% of the surface and includes: *Aegopodium podagraria*, *Asparagus tenuifolius*, *Dentaria bulbifera*, *Ficaria verna*, *Hypericum hirsutum*, *Lamium purpureum*, *Mercurialis perennis*, *Stellaria holostea*, *Veronica hederifolia*.

The population consists of three clumps and occupies an area of 34 m^2, with a population density of 3 individuals/m^2, predominating the generative specimens (98%).

The 11th population of *Nectaroscordum bulgaricum* recorded in subplot 49E grows on an undulating slope with SW exposure, altitude 262 m, in mixed forest with sessile oaks on hills – fundamental natural stand of superior productivity, consistency – 0.8, average age 90 years. The layer of trees is made up of *Quercus petraea* on the upper floor, and the lower one of *Quercus robur*, *Fraxinus excelsior*, *Tilia cordata*, *Carpinus betulus*, *Acer campestre*, the shrub layer is represented by *Cornus mas*, *Viburnum lantana*, *Crataegus monogyna*. The herbaceous cover covers the soil on about 85% of the surface and includes: *Aegonichon purpureo-caeruleum*, *Alliaria petiolata*, *Allium ursinum*, *Anthriscus cerefolium*, *Asparagus tenuifolius*, *Carex brevicollis*, *Dactylis glomerata*, *Dentaria bulbifera*, *Fallopia dumetorum*, *Galium aparine*, *Glechoma hirsuta*, *Hedera helix*, *Lamium maculatum*, *Lapsana communis*, *Melica uniflora*, *Mercurialis perennis*, *Polygonatum hirtum*, *Pulmonaria officinalis*, *Stellaria holostea*, *Symphytum tauricum*, *Urtica dioica*, *Veronica hederifolia*, *Viola hirta*.

The population extends over an area of 3 ha, with a population of 4 individuals/m^2, with generative specimens predominating (98%), juveniles constituting only 2% (Figure 3).

Figure 3. The population of *Nectaroscordum bulgaricum* in subplot 49E

Phenological observations were made on the given species, recording the following phases: vegetative, budding, flowering, fruiting and senescence (Figure 4, Table 1).

vegetative growth   bud development   budding

flowering   fruiting   senescence

Figure 4. The development phases of sp. *Nectaroscordum bulgaricum*

Table 1. Phenological phases of sp. *Nectaroscordum bulgaricum*

The characteristic of the phenological phases	Phenological phases				
	The beginning of vegetation	Budding	Flowering	The beginning of fruiting	Senescence
2022					
Date	14.02	16.05-20.05	20.05-06.06	01.06	16.05-04.07
Phase duration (days)		5	17		49
The sum of positive temperatures at the beginning of the phenological phases (°C)	25.8	700.0	758.4	965.5	700.0
2023					
Date	17.02	18.05-22.05	22.05-9.06	06.06	15.05-10.07
Phase duration (days)	-	4	17	-	56
The sum of positive temperatures at the beginning of the phenological phases (°C)	17.2	757.2	826.7	1089.7	704.3

The Honey garlic started its vegetative period on February in the territory of the reserve, when the sum of positive average temperatures reached 17-26°C. Budding lasted 4-5 days, after 90 days from the initiation of the vegetative phase. The beginning of the flowering period was recorded in the third decade of May, when the sum of positive temperatures exceeded 750°C and lasted for 17 days. The drying of the vegetative organs started during the formation of buds, on May, ending the vegetative phase on July with the complete drying of the plants.

As a result of the study, the state of the populations of Honey garlic species in the strictly protected area of the “Codrii” Reserve was evaluated. In the coming years, the monitoring of the populations will continue with the estimation of the long-term trend of the species *Nectaroscordum bulgaricum*.

Conclusions

11 populations of *Nectaroscordum bulgaricum* Janka were identified and described in the strictly protected area of the “Codrii” Reserve with the location of the growth points on the map.

The presence of the Honey garlic in plot 40 was reported for the first time.

The largest population of *Nectaroscordum bulgaricum* in the strictly protected area extends over an area of 3 ha, with an effective of 4 individuals/m^2.

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Research Article

MAIN THREATS AFFECTING *ARNICA MONTANA* L. IN THE NORTHEASTERN REGION OF ROMANIAN CARPATHIANS

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Abstract: The current paper is focused on an emblematic medicinal species, *Arnica montana*, and on identification of the main threats it faces in the northeastern region of Romanian Carpathians. The species was investigated in 30 localities, in various habitats, characterized by different land management types, and different floristic composition of plant communities. The most important threat for *A. montana* in NE Carpathians was the habitat loss, mainly caused by abandonment of the traditional meadows management and natural succession of vegetation. Accumulated data underline the importance of management measures for *A. montana*, measures that have controlled and maintained the habitats of montane grasslands over time. The most suitable method of maintaining habitats in a favorable state of conservation could be represented by a combination of mowing and moderate intensity grazing while maintaining a level of fertilization as low as possible. Collection of flower heads for medicinal purposes must be avoided in small size populations.

Keywords: *Arnica montana*, conservation, grasslands, management, threats.

Introduction

The extinction rate of numerous plant species has accelerated [HUMPHREYS & al. 2019] in the last decades as a result of degradation of natural habitats due to (among others) their inadequate management, overexploitation, urbanization or climate change. Detailed knowledge about threats to plant species is mandatory in elaborating and implementing conservation strategies [HERNÁNDEZ-YÁÑEZ & al. 2016]. Comprehensive assessment of threats to a certain plant species will enable specific approaches in development and implementation of conservation actions [GALLAGHER & al. 2023]. While conservation efforts are mainly focused on endemic species or those with narrow distributions, conservation of medicinal plants is essential because of their role in human health [SHUKLA, 2023].

Arnica montana L. is one of the approximately 30 species of the *Arnica* spp. genus, distributed in Europe, Asia and North America [SCHMIDT, 2023]. It is a medicinal, perennial forb, with European areal. It grows in nutrient-poor pastures and hayfields, forest glades, or heathlands from lowlands up to the mountain or subalpine vegetation belts [CIOCÂRLAN, 2000; MAURICE & al. 2012; DUWE & al. 2017; PĂCURAR & al. 2023], up to 3000 m a.s.l. [DUWE & al. 2017]. From an ecological point of view, it is a heliophilous species, but it can tolerate a certain degree of shading, preferring moderately moist, acidic and nutrient-poor soils

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[ELLENBERG & al. 1992; SÂRBU & al. 2013]. It is a diagnostic species for secondary grasslands on nutrient poor soils classified in class *Nardetea strictae* Rivas Goday et Borja Carbonell in Rivas Goday et Mayor López 1966 (more frequent in plant communities within *Nardetalia strictae* Preising 1950 order), but significant populations can be identified in class *Calluno-Ulicetea* Br.-Bl. et Tx. ex Klika et Hadač 1944 (in vegetation of *Vaccinio myrtilli-Genistetalia pilosae* Schubert ex Passarge 1964 order) or *Molinio-Arrhenatheretea* Tx. 1937 (in particular in plant communities classified in order *Arrhenatheretalia elatioris* Tx. 1931) [CHIFU & al. 2015; MUCINA & al. 2016]. The species has both the capacity for vegetative propagation by the long-living rhizomes as well as for sexual reproduction, but it is fully self-incompatible [LUIJTEN & al. 1996; BEGEMANN, 2022]. Despite pappus presence, the fruits have a limited dispersal ability [STRYKSTRA & al. 1998], while the seeds generally germinates in the autumn and do not form a bank in the soil [BEGEMANN, 2022].

The decline of *A. montana* populations made it a species of European Community interest whose sampling from nature and exploitation should be carried out considering certain management measures according to the Habitat Directive (1992). It was also included in the Red List of cormophytes from Romania, as a vulnerable species [OLTEAN & al. 1994]. Cultivation of *A. montana* as a form of *ex-situ* conservation or as resource for pharmaceutical industry or reintroduction programs is challenging. A series of studies highlighted that it is a recalcitrant species in culture, the establishment and maintenance of plantations being relatively difficult and strongly dependent on climatic and edaphic conditions [SUGIER, 2007; SURMACZ-MAGDZIAK & SUGIER, 2012]. Also, differentiation and propagation potential of achenes depend on the morphological diversity of flowers and position of flower heads on the plant [SUGIER & al. 2022]. Similar studies carried out in Bulgaria [BALABANOVA & VITKOVA, 2016] showed that *A. montana* can only be successfully cultivated at altitudes above 1400 m, on acid soils, while in Serbia the species was cultivated at altitudes above 1000 m [PLJEVLJAKUŠIĆ & al. 2014], in contrast to northern Europe (Finland, Poland) where the species was cultivated at lower altitudes [SUGIER & al. 2013; SUGIER & al. 2022]. In addition, for some low altitude, and more isolated populations there was highlighted the genetic erosion phenomenon, a restricted gene flow among populations, and an increase of vegetative reproduction [DUWE & al. 2017; MAURICE & al. 2016]. Still, a large geographical distance and consequently increased genetic distance between populations can have negative effects generated by outbreeding depression, and the projects of reintroduction or restocking must be locally implemented [BEGEMANN, 2022].

Land management, abandonment of traditional practices, habitat loss and collection of flower heads for medicinal purposes are some of the various threats for persistence of this species [KATHE, 2006; PĂCURAR & al. 2007; ROTAR & al. 2010; MARDARI & al. 2019; HOLLMANN & al. 2020]. But, as HOLLMANN & al. (2020) emphasized, the threat types can be different depending on the location of *A. montana* populations and conservation actions may differ. Thus, the main objective of this article is to identify and characterize the major threats faced by *A. montana* in the northeastern region of Romanian Carpathians.

Material and methods

Identification and inventory of the natural populations of *Arnica montana* in the northeastern region of the Romanian Carpathians was carried out during 2014-2017 vegetation seasons. Most of locations were revisited in 2023-2024. Numerous transects were performed in different localities in several mountainous massifs, transects along which the populations of *A.*

montana were identified, counts were made in sample plots, and the main threats were registered and characterized. For all identified populations were registered floristical and phytosociological data, while the species' abundance and its population structure were also investigated.

Results and discussions

In the studied region, *Arnica montana* was identified in three types of habitats including six plant communities in 30 locations from Suceava, Neamţ, and Bistrița-Năsăud counties: Sadova, Lucina, Tihuţa Pass, Valea Putnei, Cârlibaba, Piatra Fântânele, Călimanul Cerbului (three stations), Pietrosul Bistriței, Panaci, Șaru Dornei, Gura Haitii (two stations), Sabasa, Coverca, Drăgoiasa, Ortoaia (two stations), Chiril, Obcina Feredeului, Ceahlău, Dorna Arini (three stations), Rarău, Tarnița, Stulpicani, valleys of Farcașa și Neagra Broștenilor rivers (in Bistriţei, Călimani, Stânişoarei, Rarău, Suhard, Bârgăului, Obcina Mestecănişului, Obcina Feredeului Mountains). *A. montana* was a part of the floristic compositions of some mesophilic secondary grasslands (pastures and meadows), acidophilous shrub communities (mainly in subalpine belt), grasslands - scrublands ecotones, abandoned pastures, etc. (Figure 1). More detailed information for each of these, aspects related to the location and characteristics of the populations, the floristic and phytosociological composition, the plant communities that define the type of habitat and references to the abundance of the species were presented in MARDARI & al. (2015, 2019).

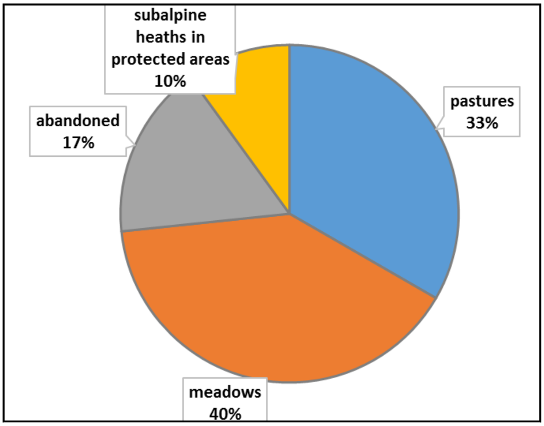

Figure 1. Distribution of *Arnica montana* sites depending on land management

The most important threats for *A. montana* in NE Carpathians are the habitat loss and fragmentation, mainly caused by abandonment of the traditional meadows management and secondarily caused by the expansion of human settlements (resulting in the destruction of the natural habitat and the extinction of local populations located on private properties, near or inside localities on lands suitable for various constructions). No threats from non-native invasive plant species were recorded in the studied habitats while the effect of tourism and recreation activities was negligible.

Land abandonment is perhaps the most significant threat for *A. montana* in the investigated areas because, through natural succession, it induces changes, in both the floristic composition of grasslands and on their functional and specific diversity. Abandoning mowing in grasslands (and even rational grazing) favors the establishment of some species of shrubs or trees (e.g. *Picea abies*) that, in turn, facilitates the colonization of meadows by other more competitive herbaceous species, changing their floristic composition (Figure 3). Maintaining the knowledge and practices of traditional use of meadows (mowing and grazing) represents the best solution for reducing the effect of abandonment in the studied region. Accumulated data underline the importance of management measures for *A. montana*, measures that have controlled and maintained montane grasslands over time. Mowing ensures the periodic removal of dominant and competitive species and thus diminishes the competition relations, maintains the diversity of mountain meadows and favors species with leaves arranged in basal rosettes (Figure 2). Mowing also improves the light regime at ground level and prevents the accumulation of litter (which facilitates the establishment of juvenile *A. montana* individuals).

Figure 2. Mountain hay meadow with *Arnica montana* in Gura Haitii (Suceava county)

Figure 3. Abandoned grasslands and ecological succession in Călimani Mountains

In addition, a moderate grazing regime results in a decrease in the abundance of some dominant *Gramineae* species (animal selectivity) with an effect on the vertical structure of meadow communities, a positive effect that favors rosettes species. Grazing also reduces competition in meadows and animals can represent vectors of seed and fruit dispersal of certain plant species, thus ensuring connectivity between populations and the recolonization of degraded habitats [ROSENTHAL & al. 2012]. The decrease in the density of *A. montana* individuals on unused or abandoned land can be interpreted as an effect of natural succession, whereby shrub species or young trees which facilitate the establishment of other grass species which through competition can eliminate the species typical of meadows developed on nutrients poor soils. In addition, in abandoned meadows there is a significant accumulation of litter, with an inhibitory effect on young plants mainly through physical obstruction and modification of the light regime.

Another effect of land abandonment is represented by the invasion of meadows by a series of native and highly competitive species that have the ability to build dense, compact communities, with rapid expansion, communities that in a short period of time modify the floristic composition of initial grasslands, and the diversity and abundance of species in mountain meadows.

Intensive grazing, especially by sheep, results in the invasion of meadows by some nitrophilous ruderal species that build dense communities (e.g. *Rumex alpinus*), sometimes over large areas, which gradually eliminate the semi-natural meadow communities. Because *A. montana* is a species with a rosette of basal leaves, it tolerates grazing well, but soil compaction by animals can have a negative effect on its populations. Respecting the grazing period, rotational grazing (because animal species are selective), correct determination of the pastoral value of meadows, and of maximal number of animals grazing per unit area of grassland are some ways to reduce the impact of this traditional activity on *A. montana* populations.

A usual practice in the investigated area is that of soil fertilization using natural manure. Increasing the amount of fertilizers also has negative effects, as the species prefers nutrient-poor soils. This effect can be explained by the fact that an increase in the amount of nutrients in the soil can determine the dominance of one-two-three species and the generation of an asymmetric competition, manifested against smaller (in size) and slower-growing species. Another possible explanation is related to the fact that ammonium ions are, in particular, toxic for this species because they prevent the formation of mycorrhizae and reduce the competitive abilities of the species [DUECK & ELDERSON, 1992; MAURICE & al. 2012]. This aspect could be important in the development of possible conservation strategies for the species, strategies that could suggest maintaining a low level of soil fertilization that is practiced in the studied region in order to increase the vegetal biomass production.

In most locations (e.g. Tihuţa Pass, Valea Putnei, Piatra Fântânele, Gura Haitii, Sabasa, Coverca, etc.) the habitat of mountain hay meadows presents a favorable state of conservation (because there are no major disturbances in terms of their surface, in structure and composition of plant communities). Their management – the lands are mowed – ensures the prospects of preserving these parameters in the future. For other populations (e.g. Sadova, Ortoaia, Obcina Feredeului, Dorna Arini, etc.), the habitat condition is inadequate (because of the abandonment of traditional management favoring the establishment of woody species, and of irrational grazing causing changes in the floristic composition of the meadows and their transition towards low-value *Nardus* grasslands). Also the species rich *Nardus* grasslands on siliceous substrates, in most locations (e.g. Chiril, Panaci, etc.) present a favorable state of conservation (the lands are mowed or moderately grazed). The threats to the habitat of subalpine heaths are minor as they are located in the perimeter of some protected areas (in strictly protected perimeters or inaccessible stations from Ceahlău National Park and Călimani National Park).

From another perspective, the main reason why the species is considered threatened is the excessive collection for medicinal use. The excessive collection of inflorescences, for an income source – as a type of disturbance, can significantly influence the populations of *A. montana*, in the long term, even in areas where the species was very abundant (e.g. Sabasa, Gura Haitii, Dorna Arini, Șaru Dornei, etc.). However, the effect of collecting inflorescences can be significant in the long term, by decreasing the genetic diversity and even causing the disappearance of smaller populations. Thus, the conservation strategy of this species must include a rational collection of flower heads only in areas where the species is abundant and only based on resource assessment and is strongly connected to habitat conservation. A project developed in central and northern regions of Apuseni Mountains (Romania) represent an excellent example of sustainable collection of *A. montana* and conservation of its habitat by maintaining the traditional management of grasslands, based on the participatory approach of both landowners and harvesters [PĂCURAR & al. 2023].

Conclusions

Overall, the results of this study are consistent with the hypothesis that the conservation of the species *A. montana* is dependent on the management measures applied to the habitat. The species requires that grassland mowing measures and low intensity grazing to be implemented after the achenes have matured. Mowing removes competitive species and prevents natural succession. Under these conditions, the most suitable method of maintaining habitats with *A. montana* in a favorable state of conservation could be represented by a combination of mowing and moderate intensity grazing and maintaining a level of fertilization as low as possible. Collection of flower heads for medicinal purposes must be avoided in small size populations.

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COMPUTATIONAL SCREENING TO IDENTIFY GENES INVOLVED IN DNA REPAIR IN *ARABIDOPSIS THALIANA*

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Abstract: The repair of damaged DNA is an essential function for living organisms. While great strides have been made in understanding this process in animal and yeast models, our knowledge in plant DNA repair is not as developed. Plants face many sources of DNA damage which they cannot so easily avoid: UV radiation from sunlight, reactive oxygen species produced endogenously by their mitochondria and chloroplasts, reactive oxygen species accumulated while under conditions of cold, heat, or salt stress. Understanding plant DNA repair is particularly relevant as the accumulation of DNA damage can negatively impact the growth and yield of agronomically important species. In this study, a broad classification of genes related to DNA repair in the model dicot *Arabidopsis thaliana* was conducted using gene ontology and gene enrichment analysis. The results of this broad classification serve to elucidate pathways for further study in plant DNA damage response and repair.

Keywords: cell death, DNA repair, gene enrichment analysis, gene ontology, stress response.

Introduction

DNA encodes the necessary instructions for life [CHATTERJEE & WALKER, 2017]. Yet organisms inevitably encounter various sources of damage to their DNA from their environment and their metabolism [EKER & al. 2009], which threaten the genomic integrity of their cells. Damaged DNA, if not repaired, can lead to the impairment of important cellular processes and ultimately cell death [SZURMAN-ZUBRZYCKA & al. 2023]. Thus, the evolution of mechanisms to detect and repair damaged DNA are essential for the survival and perpetuation of living organisms [CHATTERJEE & WALKER, 2017; MANOVA & GRUSZKA, 2015]. This process is believed to be highly conserved across animals, fungi, plants and yeast, although key distinctions remain [GRIN & al. 2023; SZURMAN-ZUBRZYCKA & al. 2023; YOSHIYAMA & al. 2013].

Much of our understanding of the DNA damage response (DDR) pathway comes from models of mammalian cells due to its pertinence in understanding the development of cancer, the cellular aging process, and the development of certain diseases [GIMENEZ & MANZANO-AGUGLIARO, 2017; GRIN & al. 2023] as well as from work with single celled yeast, but our knowledge in plant DDR lags behind [GIMENEZ & MANZANO-AGUGLIARO, 2017; SINGH & al. 2010]. Nonetheless, important work in the past a few decades, primarily in the model plant *Arabidopsis thaliana*, has significantly enhanced our understanding of plant DDR [SZURMAN-ZUBRZYCKA & al. 2023].

As the accumulation of DNA damage in plants can negatively impact their growth and yield [MANOVA & GRUSZKA, 2015; SZURMAN-ZUBRZYCKA & al. 2023], gaining a comprehensive understanding of the plant DDR network has many key applications for the improvement of agronomically important species. Additionally, understanding the plant DDR

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network has important applications for precise and efficient gene targeting, insertion, and incorporation of *Agrobacterium*-mediated T-DNA for genome editing and modification [SHEN & LI, 2022].

In contrast to animals, plants are challenged with many additional sources of DNA damage due to their unique life history [GRIN & al. 2023]. As organisms anchored to their environment by their root systems, they are unable to move away from many environmental sources of DNA damage [SPAMPINATO, 2017]. Reliant on photosynthesis, plants are also constantly exposed to the DNA-damaging UV radiation of the sun [YOSHIYAMA & al. 2013]. ROS can accumulate and lead to DNA damage while plants experience various forms of abiotic stress: salt stress, conditions of drought, cold or heat stress, or acidic soils [SZURMAN-ZUBRZYCKA & al. 2023]. Biotic stress from the pathogen attack by fungal, bacterial, or viral pathogens, as well as insect herbivores can also lead to the accumulation of DNA damage for plants [MANOVA & GRUSZKA, 2015; TUTEJA & al. 2008]. Additionally, exposure to genotoxic pollutants or heavy metals in the soil can contribute to the damage of plant DNA [GRIN & al. 2023; SZURMAN-ZUBRZYCKA & al. 2023, WATERWORTH & al. 2011]. Plants can also produce a wide variety of genotoxic secondary metabolites, of which psoralens, topoisomerase inhibitors (camptothecins, podophyllotoxins), and aristolochic acid are probably the best-studied examples [see GRIN & al. 2023]. And some widespread plant protein toxins, such as ribosome-inactivating proteins, also damage DNA [STIRPE & al. 2006].

DNA damage can be classified into two primary categories: damage, breaks, or errors that occur on a single strand of DNA, single stranded breaks (SSB), or damage or breaks to both strands of DNA, double stranded breaks (DSB) [SZURMAN-ZUBRZYCKA & al. 2023]. The latter carries more severe consequences for the cell due to chromosomal fragmentation and the loss of considerable amounts of genetic information [MANOVA & GRUSZKA, 2015]. In response to this damage, plants initiate the DDR pathway. First, DNA damage must be detected. Two sensor kinases of the phosphoinositide-3-kinase-related protein kinase (PIKKs) family, ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad3-related) are involved in this process [SHEN & LI, 2022; WATERWORTH & al. 2011]. With ATM being recruited to the sites of DSBs while ATR being recruited to the sites of SSBs or general replication stress [NIMETH & al. 2020; HIRAKAWA & al. 2017]. Both of these proteins then phosphorylate suppressor of gamma 1 (SOG1) which serves as a key regulator of the DDR process in plants and a functional homolog of the animal p53 tumor suppressor protein [YOSHIYAMA, 2015]. From the sensing of DNA damage and the activation of SOG1, an important checkpoint is reached. The cell cycle is arrested to prevent serious genetic errors from being passed forward to daughter cells and to provide the cell with time to repair [GIMENEZ & MANZANO-AGUGLIARO, 2017; LAZZARO & al. 2009].

The main regulator of the DDR pathway in plants, SOG1, activates not only many genes related to DNA repair, but also those related to cell cycle regulation [SZURMAN-ZUBRZYCKA & al. 2023]. Cell cycle arrest is the first effect of DDR activation and is crucial to allow time to repair to avoid transmission of lesions to daughter cells . If an extreme number of DNA lesions occur and DNA repair machinery is not able to fix them, the endoreduplication could be activated [SZURMAN-ZUBRZYCKA & al. 2023]. In this case DNA replication is not followed by mitotic division. This could cause an increase in ploidy level and usually leads to enlargement and differentiation of the cell [LANG & SCHNITTGER, 2020]. Endoreduplication is known to be implicated in various stres responses in plants [LANG & SCHNITTGER, 2020]. If the DNA damage is sensed to be repairable, a variety of DNA repair mechanisms are available to address the specific kind of DNA damage or lesion that has occurred. Or the severity of the

DNA damage may be sensed to be sufficient to warrant progression to programmed cell death [SZURMAN-ZUBRZYCKA & al. 2023].

SSBs may be addressed by nucleotide excision repair (NER), base excision repair (BER), or mismatch repair (MMR) [NIMETH & al. 2020]. Base excision repair addresses modified, damaged, or missing bases [GRIN & al. 2023]. Nucleotide excision repair addresses UV induced lesions and bulky adducts that distort the conformation of the DNA helix [MANOVA & GRUSZKA, 2015]. Mismatch repair addresses various errors that occur during DNA replication, mismatched, wrongly inserted, or deleted bases [SPAMPINATO, 2017]. For SSB repair by NER, BER, or MMR the complementary strand serves as a template for the accurate recovery of sequence information [SZURMAN-ZUBRZYCKA & al. 2023].

DSBs may be addressed through homologous recombination (HR) or through non-homologous end joining (NHEJ). In the case of homologous recombination, the availability of sister chromatids or homologous chromosomes is required for accurate repair without the loss of sequence information. In the case of non-homologous end joining, fragmented DNA stranded are repaired without the availability of a template and are the prone to errors or loss of sequence information [SHEN & LI, 2022].

With the progress in our understanding of the genetic and biochemical details of these repair processes in plants, the molecular checkpoints and decision between repair, endoreduplication, and programmed cell death in response to DNA damage have yet to be fully understood. Towards this, a more comprehensive network analysis or systems biology view will certainly be a plausible approach as indicated in our previous work in various plant species [ARMAS & XING, 2022; CONROY & al. 2013; YOUNG & al. 2018].

Gene ontology (GO) allows for the classification of genes and gene products into functional categories, i.e. at the level of molecular function, which describes the biochemical activity; at the level of cellular component, which describes the location within the cell; and at the level of biological process, which describes the wider pathway and biological operation [ASHBURNER & al. 2000]. A variety of tools are available for the sorting of gene lists into functional categories in accordance with gene ontology [GE & al. 2020]. In this study we aim to investigate plant DDR using a broad computational screening approach including the widely available and abundant genomic data for *Arabidopsis thaliana,* gene enrichment analysis, and gene ontology. It is the hope that the identification of genes related to DDR in *Arabidopsis thaliana* and their classification into functional categories in accordance with gene ontology will aid in mapping out the DDR process in plants.

Methods

Literature search

A literature search was preformed to obtain a reference list (n=30) of known DNA repair genes in *Arabidopsis thaliana* from reputable journal publications by querying the search terms '*Arabidopsis thaliana* anti-cell death genes', '*Arabidopsis thaliana* DNA repair genes', and '*Arabidopsis thaliana* pro-survival genes' into Web of Science and Google Scholar. This list was then used to obtain protein-protein interaction data, gene co-expression data, and genetic interaction data for each reference gene.

Protein-protein interactions

Protein-protein interactions for each of the reference genes were obtained through the use of STRING database (https://string-db.org) [SZKLARCZYK & al. 2023] with a default cutoff score of (score >= 0.400).

Gene co-expressions

Gene co-expression data were obtained using the Bio-Analytic Resource for Plant Biology (https://bar.utoronto.ca) [WAESE & al. 2017] and ePlant expression angler tool therein with a cutoff of the top 25 results for each reference gene.

Genetic interactions

Genetic interactions were obtained using BioGRID Database of Protein, Genetic, and Chemical Interactions (https://thebiogrid.org) [OUGHTRED & al. 2021] with all unique interactions collected for each reference gene.

Gene enrichment analysis and categorization (GO and KEGG)

The protein-protein interaction data, gene co-expression data, and genetic interaction data were compiled together. Genes were ranked according to how many approaches they were found in. Those found in all three approaches were assigned 1st priority, those found in two approaches were assigned 2nd priority, and those found in only one approach were assigned 3rd priority. The compiled data were then entered into the ShinyGO gene enrichment tool (http://bioinformatics.sdstate.edu/go) [GE & al. 2020] with an FDR cutoff of 0.05 for gene ontology enrichment and classification. KEGG (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg) analysis is integrated and describes how genes and proteins interact in specific pathways and systems.

Results and discussions

DDR-related gene identification

Through the use of STRING database examining protein-protein interactions, BAR ePlant expression angler examining gene co-expression, and BioGRID database examining genetic interactions, a gene list of potential DDR or DDR related genes within the DDR network in *Arabidopsis thaliana* (n=852) were identified from an original list of known DDR genes through literature extraction (n=30 and see Supplementary data Table 1). In the rest of this work, all the related genes in this network will be call DDR-related genes or DDR for simplicity.

The potential *Arabidopsis* DDR genes were ranked by priority in accordance with the number of approaches they were identified in (see Methods section) (Supplementary data Table 2). Of these, an insufficient amount (n=4) fell into 1st priority, being identified in all three approaches, to proceed to gene enrichment analysis. As a result, 1st and 2nd priority (n = 58) were combined for the purposes of enrichment analysis.

The potential *Arabidopsis* DDR genes with the strongest evidence (combined 1st and 2nd priority) were compared to all identified potential *Arabidopsis* DDR genes (1st, 2nd, and 3rd priority) during the following enrichment analysis.

GO Enrichment Analysis

Enrichment analysis and classification into functional GO terminology of our *Arabidopsis* DDR genes provided an interesting mixture of results with some seeming to provide support to previous knowledge in plant DDR while others indicating potential areas for further analysis (Supplementary data Table 2, Figures 1, 2, 3, and 4).

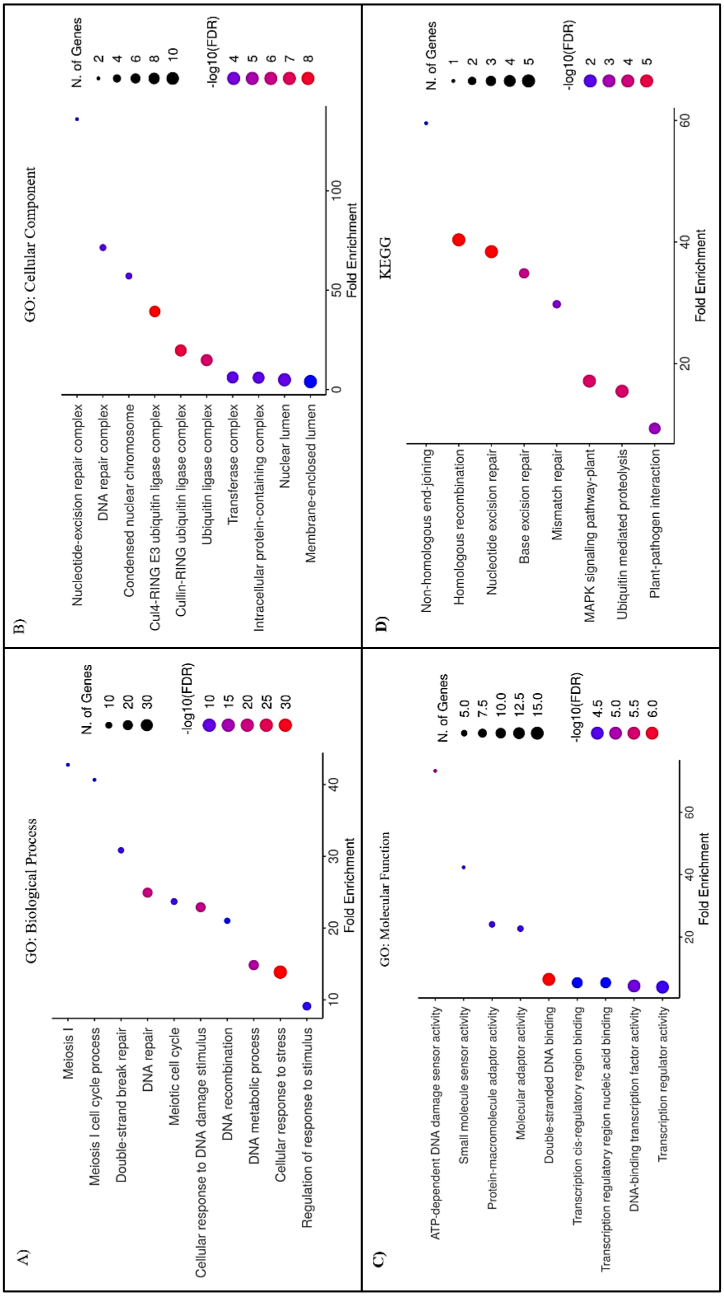

Figure 1. Gene ontology analysis for genes ranked 1st and 2nd priority. Dot plot illustrating the top ten categories by fold enrichment with an FDR cutoff of 0.05 for the following classification systems: A) biological process, B) cellular component, C) molecular component, and D) KEGG (Kyoto Encyclopedia of Genes and Genomes).

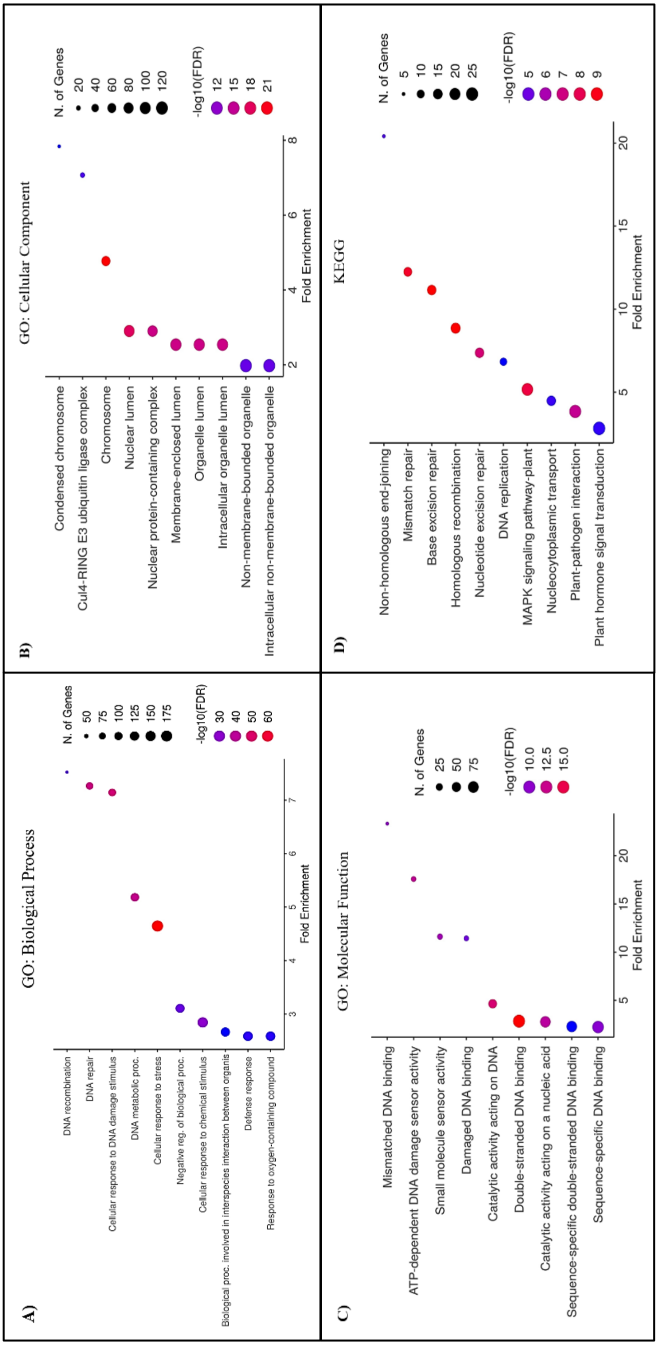

Figure 2. Gene ontology analysis for genes of all priorities. Dot plot illustrating the top ten categories by fold enrichment with an FDR cutoff of 0.05 for the following classification systems: A) biological process, B) cellular component, C) molecular component, and D) KEGG.

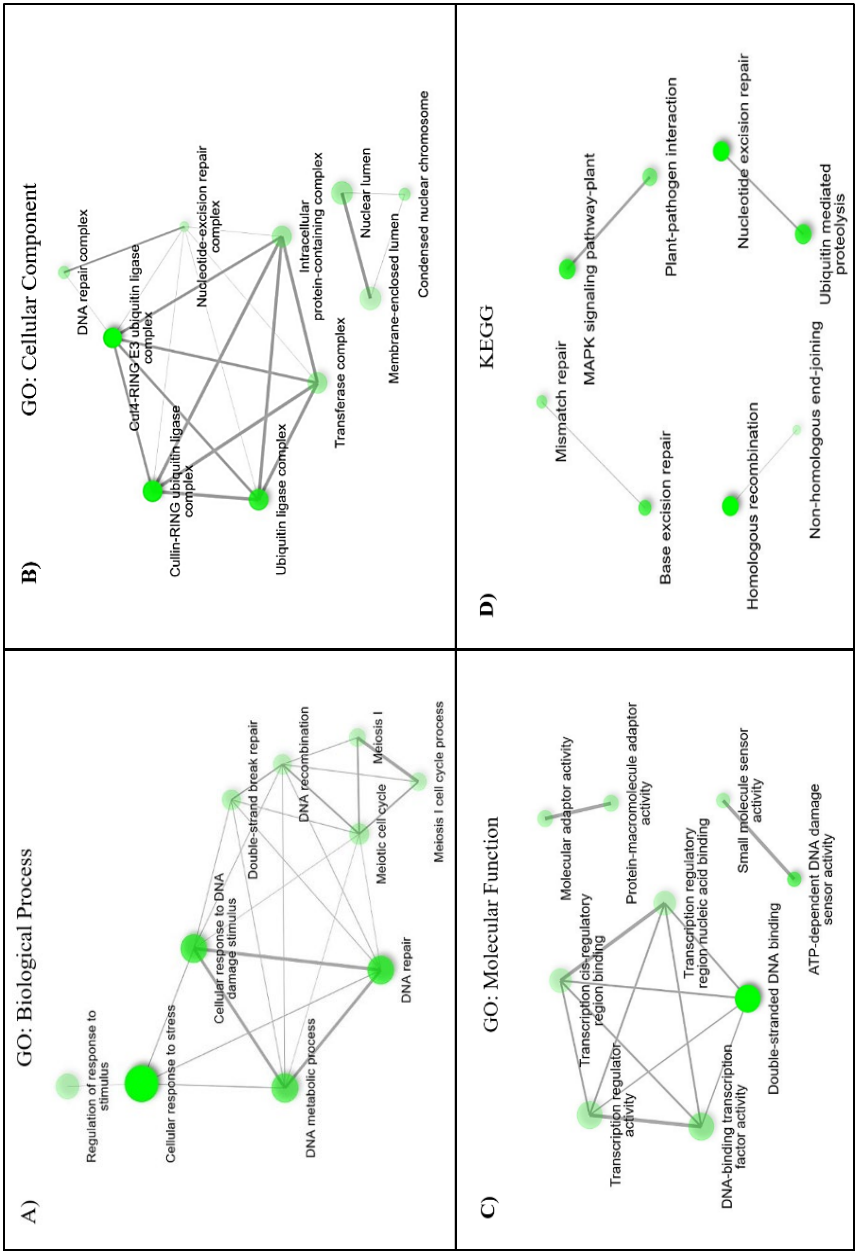

Figure 3. Gene ontology analysis with network plot for genes ranked 1st and 2nd priority. Network plot illustrating the connections between the top ten categories by fold enrichment with an FDR cutoff of 0.05 and an edge cutoff of 0.3 for the following classification systems: A) biological process, B) cellular component, C) molecular component, D) KEGG. Edge thickness indicates greater shared genes between pathways. Darker nodes indicate higher enrichment. Node size indicates the number of genes.

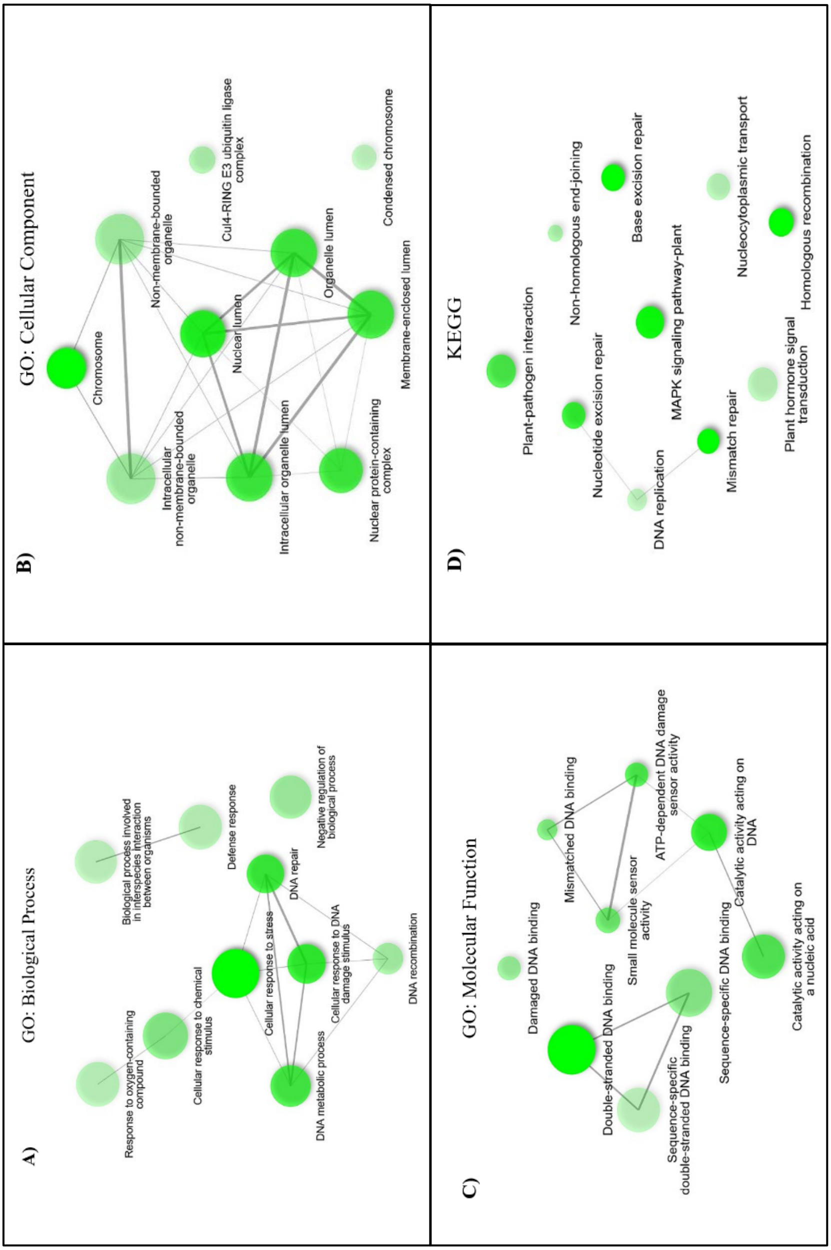

Figure 4. Gene ontology analysis with network plot for genes of all priorities. Network plot illustrating the connections between the top ten categories by fold enrichment with an FDR cutoff of 0.05 and an edge cutoff of 0.3 for the following classification systems: A) biological process, B) cellular component, C) molecular component, D) KEGG. Edge thickness indicates greater shared genes between pathways. Darker nodes indicate higher enrichment. Node size indicates the number of genes.

Biological process

At the level of biological process, when analysing genes with the greatest support (1st and 2nd priority), the top 10 GO terms by fold enrichment were: meiosis I, meiosis I cell cycle process, double strand break repair, DNA repair, meiotic cell cycle, cellular response to DNA damage stimulus, DNA recombination, DNA metabolic process, cellular response to stress, and regulation of response to stimulus.

When analyzing genes from all priorities, the top 10 GO terms by fold enrichment were: DNA recombination, DNA repair, cellular response to DNA damage stimulus, DNA metabolic process, cellular response to stress, negative regulation of a biological process, cellular response to chemical stimulus, biological process involved in interspecies interaction between organisms, defense response, and response to oxygen containing compound.

The return of GO terms such as 'DNA repair', 'DNA recombination', 'double strand break repair' and 'cellular response to DNA damage stimulus' among others, provide support that our identified genes fall within the realm of DDR network at the biological process level.

The return of GO terms 'meiosis I', 'meiosis I cell cycle process', and 'meiotic cell cycle' in combination with 'DNA recombination' and 'double strand break repair' when analysing genes with the greatest support (1st and 2nd priority), seems to be suggestive of the homologous recombination DNA repair network. As during meiosis, sister chromatids become available as a template for homologous recombination repair of double strand breaks [LUI & al. 2022]. This pathway is even more clear when observing the bubble figure (Figure 3A).

Cellular Component

At the level of cellular component, when analysing genes with the greatest support (1st and 2nd priority) the top 10 GO terms by fold enrichment were: nucleotide excision repair complex, DNA repair complex, condensed nuclear chromosome, cul4-RING E3 ubiquitin ligase complex, cullin-RING ubiquitin ligase complex, ubiquitin ligase complex, transferase complex, intracellular protein-containing complex, nuclear lumen, and membrane enclosed lumen.

When analyzing genes from all priorities, the top 10 GO terms by fold enrichment were: condensed chromosome, cul4-RING E3 ubiquitin ligase complex, chromosome, nuclear lumen, nuclear protein-containing complex, membrane enclosed lumen, organelle lumen, intracellular organelle lumen, non-membrane-bounded organelle, and intracellular non-membrane-bounded organelle.

The return of the GO terms 'condensed nuclear chromosome', 'chromosome', 'nuclear lumen', 'organelle lumen' among others provides support that our genes and gene products are active at the sites of DNA within the cell - the nucleus, chloroplasts, and mitochondria.

The appearance of the GO terms 'cul4-RING E3 ubiquitin ligase complex', 'cullin-RING ubiquitin ligase complex', and 'ubiquitin ligase complex' presents an interesting opportunity for further study and may be related to cell cycle regulation through ubiquitination [SZURMAN-ZUBRZYCKA & al. 2023]. This process also appears to have a relation with nucleotide excision repair (Figure 3B).

Molecular Function

At the level of molecular function, when analysing genes with the greatest support (1st & 2nd priority), the top 10 GO terms by fold enrichment were: ATP-dependant DNA damage sensor activity, small molecule sensor activity, protein-macromolecule adaptor activity, molecular adaptor activity, double stranded DNA binding, transcription cis-regulatory region binding, transcription regulatory region nucleic acid binding, DNA-binding transcription factor activity, and transcription regulator activity.

When analysing genes from all priorities, the top 10 GO terms by fold enrichment were: mis-matched DNA binding, ATP-dependant DNA damage sensor activity, small molecule sensor activity, damaged DNA binding, catalytic activity acting on DNA, double stranded DNA binding, catalytic activity acting on a nucleic acid, sequence specific double stranded DNA binding, sequence specific DNA binding.

The return of the GO terms ‘ATP-dependant DNA damage sensor activity’, ‘double stranded DNA binding’, and ‘catalytic activity acting on a nucleic acid’ among others provides further support that our identified genes are within the realm of DDR and their potential action via DNA. The network seen in (Figure 4C) for all gene priorities appears to show a pathway-like relationship for mismatch repair.

KEGG (Kyoto Encyclopedia of Genes and Genomes)

Enrichment analysis using KEGG terminology, when analysing genes with the greatest support (1st & 2nd priority) the top ten GO terms by fold enrichment were: non-homologous end joining, homologous recombination, nucleotide excision repair, base excision repair, mismatch repair, MAPK signaling pathway, ubiquitin mediated proteolysis, and plant-pathogen interaction.

In the enrichment analysis of genes of all priorities using KEGG terminology, the top 10 GO terms by fold enrichment were: non-homologous end joining, mismatch repair, base excision repair, homologous recombination, nucleotide excision repair, DNA replication, MAPK signaling pathway, nucleocytoplasmic transport, plant pathogen interaction, and plant hormone signal transduction.

The return of the KEGG terms ‘non homologous end joining’ and ‘homologous recombination’ for our high priority genes (1st and 2nd priority) provides support for the importance of addressing DSBs in plant DDR. The return of the KEGG terms ‘MAPK signaling pathway’ and ‘plant pathogen interaction’ suggests a possible involvement of MAP kinase pathway in cell death and DDR in *Arabidopsis* biotic stress response and offers an interesting area for further study.

NHEJ was observed as the KEGG term with the greatest fold enrichment for both data sets, which appears indicative of the importance of NHEJ in plant DDR. DSBs are the most serious form of DNA damage [MANOVA & GRUSZKA, 2015], and our results appear supportive of NHEJ as the dominant repair mechanism in addressing them over HR in *Arabidopsis thaliana.* This is supportive of previous knowledge of plant DDR in which plants share the predominance of NHEJ in addressing DSBs over HR with animals and in contrast with yeast, *Saccharomyces cerevisiae* [SHEN & LI, 2022].

In Figure 3 D, for genes with the greatest support (1st and 2nd priority), the nodes for NHEJ and HR were connected, which indicates >20% of shared genes between the two pathways. This may be due to both addressing the repair of DSBs [SHEN & LI, 2022]. Relations were also shown between mismatch repair and base excision repair, both of which act on SSBs and damaged or mismatched bases [GRIN & al. 2023; NIMETH & al. 2020]. Relations were shown between NER and ubiquitin mediated proteolysis, which was also observed previously at the level of cellular component. In addition, the return of the KEGG terms ‘non-homologous end joining’, ‘homologous recombination’, ‘nucleotide excision repair’, ‘base excision repair’, and ‘mismatch repair’ for both gene lists provide further support that our identified genes fall within the realm of the DDR network.

Conclusions

The appearance in multiple approaches (protein-protein interactions, gene co-expressions, and genetic interactions) as well as the results of GO gene enrichment analysis at the levels of biological process, cellular component, molecular function, and KEGG provide compounding support that our identified genes in *Arabidopsis thaliana* fall within the realm of the DDR network.

Results from GO enrichment analysis such as the relation between NER and ubiquitin mediated proteolysis or the appearance of MAPK signaling pathway provide interesting areas for future study. We have identified a select number of genes (n = 20, Supplementary data Table 3) of high priority (1st and 2nd priority) related to DDR that fall into the GO categories of 'cell cycle process' or 'meiotic cell cycle' or are MAP kinases that may be of interest for immediate future study in relation to the DDR network in *Arabidopsis thaliana*.

With the wide availability of genomic data on *Arabidopsis thaliana* as well as programs for GO enrichment analysis the results of this brief study only begin to scratch the surface of investigation into the plant DDR network through computational means. It is the hope that the results of this broad scale screening and classification of DNA repair related genes in *Arabidopsis thaliana* will serve as an important basis for future studies in plant DNA repair.

Understanding the DNA repair network is critical in improving the resistance of plants to a wide variety of abiotic and biotic stresses [GAO & al. 2022; SZURMAN-ZUBRZYCKA & al. 2023] as well as for providing a means of efficient genome modification [MANOVA & GRUSZKA, 2015; SHEN & LI, 2022]. By gaining a strong understanding of the plant DDR network in *Arabidopsis thaliana,* we set the stage to progress to applying this knowledge for the improvement of agriculturally important crop species [GRIN & al. 2023].

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Research Article

ARABIDOPSIS ATMKK1 KNOCKOUT MUTANT FLOWERS UNDER SHORT DAY CONDITIONS

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Abstract: There are a variety of conditions that regulate flowering time in *Arabidopsis*, but there are no reported instances mitogen-activated protein kinase pathways playing a decisive role in flowering time. Our work has indicated that when long-day plant *Arabidopsis* mitogen-activated protein kinase kinase 1 (*AtMKK1*) was knocked out, *Arabidopsis* plants flowered under short day conditions. Possible mechanisms are discussed.

Keywords: flowering time, mitogen-activated protein kinase, photoperiod.

Introduction

Mitogen-activated protein kinase (MAPK) pathways represent a crucial regulatory mechanism in plant development [JAGODZIK & al. 2018; RODRIGUEZ & al. 2010]. Light, as a signal, is critical in plant growth and development and plants are acutely sensitive to seasonal, daily and moment-to-moment variations in solar radiation [MATSUBARA & al. 2014]. The light environment can convey information through variations in at least four dimensions, i.e. quality (the balance of photons of different wavelengths), quantity (energy flux), direction, and periodicity (relative length of day and night). However, with evidenced critical roles of both MAPK pathways and photoperiod in plant development, the involvement of MAPK pathways in photoperiod regulation is lacking. AtMKK1 (accession number AY050774; unique gene ID AT4G26070) is a stress response kinase that can activate the MAP kinases AtMPK3, AtMPK4 and AtMPK6 [RODRIGUEZ & al. 2010; MENG & ZHANG, 2013]. In our previous study, knockout of *AtMKK1* enhanced salt tolerance during both germination and adulthood and proteomic analysis indicated that the level of the α subunit of mitochrondrial H^+-ATPase, mitochrondial NADH dehydrogenase and mitochrondrial formate dehydrogenase was enhanced in *AtMKK1* knockout mutants upon high salinity stress [CONROY & al. 2013]. Here we report our observation of bolting in *Arabidopsis* AtMKK1 knockout line under a short day condition.

Material and methods

Selection of mutant plants and plant growth conditions were as described previously [CONROY & al. 2013]. Primers for SALK_027645 (*mkk1-2*) were selected from approximately 40 bp upstream of the insert, forward 5'-TATTTGGAGCTTGGGACTGG-3' and downstream of the insertion reverse 5'-GCGAGATGAAGGAGCAAAAC-3'. The third primer used to identify knockouts was the Signal LBA1 primer 5'-TGGTTCACGTAGTGGGCCATCG-3'. There were two rounds of PCR. One using the left and right primers to identify wild-type alleles

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and the second using the left primer with the LBA1 primer to identify the T-DNA insertions. RT-PCR was carried out under the following conditions: 94 °C for 1 min; 1 min at 94 °C, 1 min at 61 °C, and 1 min at 72 °C for 25 cycles; and then 10 min at 72 °C.

For long day conditions the chamber was set to sixteen hours of light and eight hours of dark all at 22 ºC. For short day conditions, the lights were on for eight hours and off for sixteen hours again at a constant temperature of 22 ºC. Wild type and A31 knockout line seeds were all sterilized using 70% ethanol for two minutes followed by a solution of 30% bleach and 0.02% Triton X-100 for eight minutes. After surface sterilization was complete the seeds were rinsed eight to ten times with sterile water and the seeds were then stratified by placing the seeds on MS medium at 4 ºC for four days. They were then moved to growth chamber A (short day) and chamber B (long day). After ten days the plants were transferred from the plates into sterile soil and continued to grow in growth chambers.

Results and discussions

Arabidopsis development is photoperiod sensitive [GUO & al. 1998]. *Arabidopsis* as a long day plant followed normal growth patterns, bolting at 4-5 weeks and seeds were ready to be harvested by 8 weeks (Table 1). In contrast the short day plants followed normal short day growth and the wild type plants did not enter reproductive growth at any point. This can be contrasted to the appearance of the mutant plants. As can be seen in Figure 1C there was evidence that the plants had indeed entered reproductive growth. There was no significant difference in rosette leaf development (Table 1).

Table 1. Rosette leaf numbers (35-day-old) and percentage of plants that flowered (at 8 weeks). Data based on three repeats of experiments with standard deviation. LD: long-day; SD: short-day.

Plants	Rosette leaf number	Flowering plants (%)
Col-0 LD	13.84±0.26	100±0
A31 LD	13.36±0.47	100±0
Col-0 SD	13.45±0.83	0±0
A31 SD	13.62±1.06	41±2.16

The earlier flowering of AtMKK1 mutants under short days was unexpected. *AtMKK1* is commonly considered a defense responsive gene with few members of kinase cascades impacting upon the development of *Arabidopsis* [GAO & al. 2008; COLCOMBERT & HIRT, 2008]. This being said, there are still many roles and functions for MAPK pathway components that have yet to be identified. The impact of AtMKK1 on plant development has not been examined on any significant level, but the preliminary results showing the ability of *AtMKK1* mutant A31 to respond to changes in photoperiod indicated a significant phenotypic variation. More importantly, all of the bolted plants displayed fully formed siliques, flowers, as well as browning siliques ready for harvest.

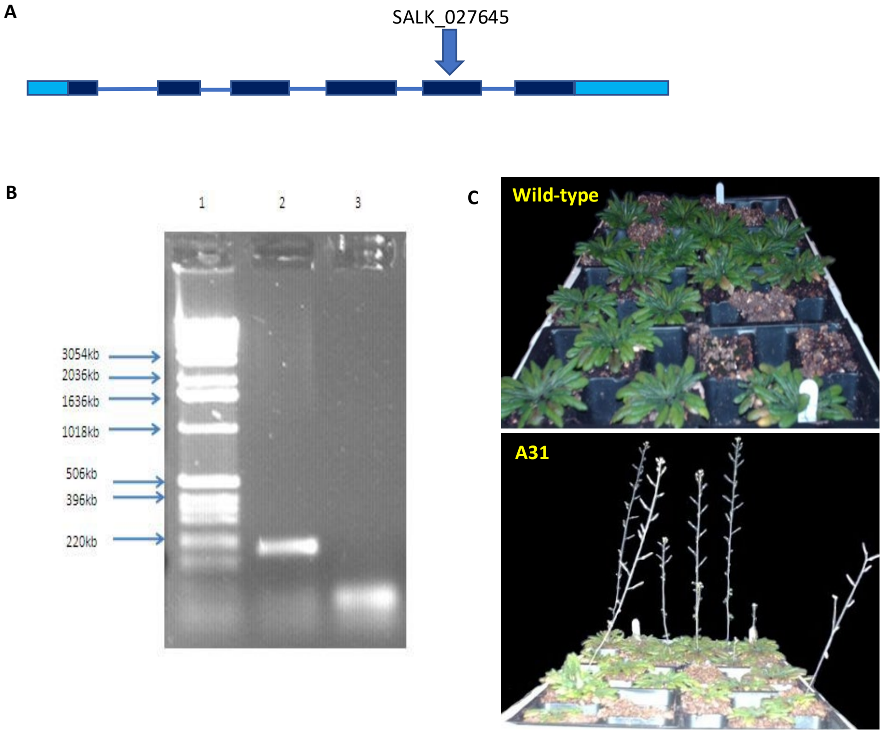

Figure 1. A. Insertion site of knockout mutant of *AtMKK1* (SALK_027645) is in the fifth intron. Line segments represent exons, dark boxes represent introns, and light boxes represent untranslated 5' or 3' flanking regions. **B.** Homozygous mutants for SALK_027645. Lane 1 shows the 1 kb ladder. Lane 2 shows the presence of the wild type AtMKK1 gene as seen in the band near the 220 kb marker. Lane 3 with no band shows that this sample contains the T-DNA insertion preventing PCR from running due to the size of the insert. PCR was performed three times to confirm the results. **C.** Wild type and knockout line A31 plants grown in 8 hours light and 16 hours of darkness after 8 weeks. This experiment was performed three times with similar results.

There are a variety of conditions that regulate flowering time in *Arabidopsis*, but there are no reported instances MAPKs, MAPKKs, or MAPKKKs playing a decisive role in flowering time. There are several environmental conditions, hormonal responses, and genetic variables that can lead to changes in the ability of *Arabidopsis* to thrive, or at least reproduce faster under short day conditions. These conditions are notable. Changes in light conditions, such as altering photoperiod or switching from short day to long day, can trigger bolting or planting in long day and then switching to short day can also affect bolting time [GUO & al. 1998; NAKATSUKA & al. 2009; SONG & al. 2013]. Changes to the intensity or wavelength of light can also cause *Arabidopsis* to bolt [CERDAN & CHORY, 2003]. Changes in the vernalization of *Arabidopsis*, through lowered temperatures during growth, or by increasing stratification time can also play a role in altering the ability of *Arabidopsis* to bolt [SONG & al. 2013]. Plants in this study, both wild type and *AtMKK1* mutants, were grown under the same temperature conditions and all seeds were stratified for the same amount of time and under the same conditions, 4 °C in the dark. This rules out changes in the vernalization as the triggering factor in the early bolting of the *AtMKK1* mutants. Once the environmental effectors have been ruled out the only remaining possibilities are that there needs to be a genetic or hormonal response in the *AtMKK1* mutant plants allowing the plants to break free from their short day induced vegetative state.

The primary hormones responsible for the initiation of bolting under short day conditions are gibberellins (GA) [WILSON & al. 1992; SONG & al. 2013]. There is little support for AtMKK1 interacting with GA. AtMKK1 is primarily linked with defensive hormones such as jasmonic acid or salicylic acid, as well as some recent links to ABA [Xing & al. 2008]. AtMKK1 appears to have no relationship with GA and in fact there are few if any MAPK cascade kinases that have been linked to GA [COLCOMBERT & HIRT, 2008]. The lack of characterization of MAPK cascades however does not rule out the possibility of AtMKK1 playing a role in a GA pathway directly or indirectly. AtMKK1 does play a role in regulating ABA sensitivity and *AtMKK1* mutants show reduced sensitivity to ABA [Xing & al. 2008]. There are ABA deficient and ABA insensitive mutants that exhibit early signs of bolting [BERNIER & al. 1993; BERNIER & PÉRILLEUX, 2005; DOMAGALSKA & al. 2010]. The ability of ABA deficient or insensitive mutants to bolt under short day conditions provides an interesting potential hypothesis to explain the effects of AtMKK1 upon early bolting. Due to the large numbers of short day AtMKK1 mutant plants that were capable of bolting, and due to the reported positive regulatory link to ABA in AtMKK1, the reduced sensitivity towards ABA could be the source of the early bolting.

Environmental factors, hormonal responses and genetic components all play a role in controlling the flowering time in *Arabidopsis*. There is a possibility that AtMKK1 does impact upon the expression level of photoperiod sensitive genes but there is little to no evidence to support that possibility at this time. A study with CRISPR gene editing of multiple members of MAPK cascade components in rice showed that loss-of-function mutations in *OsMPK1* and *OsMPK6* are unfavorable and an enrichment of inherited open reading frame-preserving mutations for *OsMPK1* and *OsMPK6* genes was found in T1 plants [MINKENBERG & al. 2017]. We could assume that a mutation of an essential MAPK pathway component may have a pleiotropic effect, which may include our observations in this study.

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Review Article

USE OF BIOSTIMULATING *SALIX* SOLUTIONS FOR THE VEGETATIVE PROPAGATION OF *CHRYSANTHEMUM*: A REVIEW

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Abstract: In the last decades, in horticulture, improving the propagation methods for decorative plants and raising their efficiency became a high priority. Preserving and maintaining the desired plants traits are essential and thus, vegetative reproduction *via* cuttings is usually the preferred way to obtain high quality material. Today, there are plenty of commercially available synthetic products that promise better and faster rooting and development of cuttings, but in most cases the price is very high and the environmental impact, due to their chemical composition, is another thing that must be taken into consideration. Finding new, eco-friendly and natural solutions that promote a better yield, which can be valued both economically and scientifically, determined research in the field and the results are promising. In this paper we aim to compile the present findings from relevant literature, still very scarce, underlining the importance of diversifying the solutions involved in increasing the natural growing and development of roots in plant cuttings, leading to a better percentage of rooting individuals with a minim impact towards the substrate in particular and the environment in general.

Keywords: *Chrysanthemum*, cuttings, natural extracts, rhizogenesis, willow extract.

Introduction

The chrysanthemum (*Chrysanthemum* s.l.) is one of the most important ornamental species, its production value increasing exponentially because of the fast improvement of the society's living conditions and lifestyle. The commercial varieties of chrysanthemum, especially the ones cultivated in pots, are usually vegetatively propagated *via* cuttings.

Chrysanthemum s.l. belongs to the Asteraceae (Compositae) family and is one of the economically important and favored floricultural crops, ranking second in the cut flower trade after roses [TEIXEIRA DA SILVA & al. 2020]. *Chrysanthemum* has a long history of cultivation; it was first cultivated in China as an herb since the 15th century BC and was then successively introduced in Japan, Europe, and the United States. The ancestry of modern chrysanthemum is still uncertain, but the plant is thought to have emerged mainly as a result of long-term artificial selection of variants belonging to several wild species, including *Chrysanthemum vestitum* ($2n = 54$), *C. indicum* ($2n = 18$, 36), *C. lavandulifolium* ($2n = 18$), *C. nankingense* ($2n = 18$), and *C. zawadskii* ($2n = 54$). Cultivated chrysanthemum is a complex hexaploid that also exhibits aneuploidy, in which chromosome numbers vary from 47 to 67 [DOWRICK, 1953; ROXAS & al. 1995]. However, a chromosome number of 54 is the most frequent and stable conformation ($2n = 6x = 54$) [CHEN & al. 1996; DAI & al. 1998; LIU & al. 2012].

In the last years, research was undertaken to accelerate the rhizogenesis process in many species that show difficulties in developing roots. This aspect mostly focused on using different chemical products (growing or rooting stimulators) to encourage rhizogenesis. The

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successful rooting of *Chrysanthemum* s.l. cuttings was reported under the influence of various factors, including type of cutting, rooting substrate, season, and environmental conditions, such as temperature, light and humidity.

In Romania, very few studies were conducted regarding alternative propagation methods and the conditions that affect the propagation in chrysanthemums [COJOCARIU & al. 2018; COJOCARIU & TĂNASE, 2019]. A part of these studies focuses on both the morpho-anatomical and biometric aspects, and on a series of modern approaches of *in vitro* multiplication of this species. After reviewing the relevant literature, we noticed that the research in the field of chrysanthemum cultivation was centered on the improvement of various flower traits, to increase their ornamental value. These characteristics include color, size and shape of the inflorescence and the quality of production. Although through classical sexual propagation methods the deserved results were obtained, there are some limits, such as restricted genetic diversity, limited cross breeding due to incompatibility and differences in polyploidy between the genitors, different flowering times and unequal growth. As a result, sexual multiplication can alter the balance between the factors that influence the plants' growth and development. A way to create and keep new varieties of plants is by using biotechnological methods. In micropropagation, the most common methods used for these purposes are the stimulation of axillary ramification and *in vitro* nodal sections cultivation [ROUT & al. 1996; ROUT & al. 2006].

The main methods of vegetative propagation through cuttings involves the stimulation of the plant's fragment tissue, using humidity and light, until the adventitious roots appear (AR) [OWEN & MAYNARD, 2007]. The AR development is a complex process that usually requires special environmental conditions and hormonal stimuli, which are specific to both species and tissue type [AGBO & OBI, 2007]. The modern propagation methods *via* cuttings use complex technologies, such as synthetic vegetal hormones, steam, and fine spraying systems, aeroponics, advanced hygiene systems and protocols to optimize the AR formation and to maximize the reproduction efficiency [PREECE, 2003].

Research history regarding the use of biostimulators in rhizogenesis

Plant biostimulators can be either microorganisms, microbial derivates or mineral, marine and vegetal materials that stimulate the physiological responses of plants, including induced systemic resistance (ISR) and improve nutrient and water absorption, promote plant growth and health, or offer tolerance towards biotic and abiotic stress factors, without having nutritive or phytosanitary properties. This definition covers both the technical and the regulatory aspects of biostimulators. However, there are some exceptions among the bio-stimulating products available on the market, because of the inherent nutritional and pesticide properties of some of their active ingredients [DARA, 2021].

YAKIN & al. (2017) offers a more complex definition, a synthesis of all the variants in the field and describes a biostimulator as a formulated product of biological origin that improves the plants' productivity as a result of the new and emergent properties of its ingredients and not as a unique consequence given by the presence of the plants' essential nutritional substances, growth regulators or plants' protection compounds.

In dicotyledonous plants in general, the AR can be defined as roots that can develop under specific conditions, from organs such as leaves or stems. AR formation in cuttings derived from stem tips with leaves is a crucial physiological process in the propagation of many ornamental plant species. Despite the intensive control over the environmental factors in the modern ornamental plants propagation industry, important economic losses still appear, due to an insufficient rooting [LIU & al. 2013].

Chrysanthemums (*Chrysanthemum* s.l.) have a significant ornamental value and a great economic importance, but their cultivation also involves losses caused by inadequate rooting of cuttings from young stems, which represents the main propagation method used for this culture. The insufficient understanding of the mechanisms that control the AR formation limits the use of efficient technologies for improving the AR development in ornamental plants' cuttings.

The adventitious rooting is a genetic trait and is influenced by many factors, both internal (endogenous), but also environmental (exogenous). One of the endogenous factors with a key role in the AR formation is the auxin. Numerous authors have proved that auxin has the capacity to initiate AR formation. PAGNUSSAT & al. (2002) showed that nitric oxide mediates the auxin response that leads to the development of AR in cucumber, and LIAO & al. (2010) studied the role of nitric oxide in the AR formation process in *Chrysanthemum*. The progressive accumulation and the local concentration of auxin at the base of the cuttings seems to play an important role in rooting initiation. [ACOSTA & al. 2009]. There are clearer studies that demonstrate that AR formation in many ornamental and industrial plant species also depends on the ethylene action [CLARK & al. 1999; SHIBUYA & al. 2004] that is produced following the injury of the plant tissue in cuttings making.

The plants' response to stress created by cutting the stem is a necessary step in the AR formation process [DA COSTA & al. 2013]. Once detached from the mother plant, the cutting must redistribute the remaining resources as fast as it can, to form AR and to reestablish the physiological balance, which allows the resource transfer in various parts of the cutting. Some studies showed that the distribution of carbohydrates in detached cuttings is probably more important than the intrinsic substance content [RUEDELL & al. 2013].

The origin of AR primordia varies anatomically and histologically depending on the species. In *Chrysanthemum* genus, for stem tip cuttings their formation is initiated in the interfascicular regions of the connection flanks of the pericyclic parenchyma [STANGLER, 1956]. The temperature at which the mother plants and cuttings are being kept influence the metabolism of AR. In the experiment done on *Camellia japonica* and *Chrysanthemum morifolium*, OOISHI & al. (1978) observed that the temperature effect on the cuttings can be mediated by carbohydrates' metabolization. The rooting took place in close connection with the temperature: 16%, 36% and 87% of rooted cuttings at 17 °C, 23 °C and 30 °C respectively. Chrysanthemums root earlier at 23 °C or 30 °C, but the root development doesn't continue, probably due to the different effects of the temperature on the primordia initiation and their following development. DYKEMAN (1976) tested the rooting of *Chrysanthemum* sp. and *Forsythia* sp. cuttings at 25 °C and 30 °C and observed a faster rooting and more roots forming at 30 °C, but the lengthening of the root, its diameter and the development of root hairs was superior at 25 °C. Another proven fact was that high temperatures favor the initiation of root primordia, while lower temperatures especially encourages the root development. The beneficial influence of higher temperature on the rhizogenesis initiation can be explained by the increase of respiration [OOISHI & al. 1978] and simple sugars catalysis, which are being stored at lower temperatures [HAISSING & al. 1986].

Considering the aspects of the root initiation and development stimulation, it is well known that AR formation in plants can be stimulated by the exogenous application of nitric oxide (NO) and hydrogen peroxide (H_2O_2), but the mechanism of this physiological response is still unclear. Research regarding the chrysanthemums were undertaken to understand the effects of NO and H_2O_2 on the cuttings rooting process and the biochemical changes inside the rooting area during rhizogenesis. The results showed that NO and H_2O_2 influence the rooting of chrysanthemums cuttings depending on their doses, with a maximum biological response at 50 μM sodium nitroprusside (SNP) as donor of NO and 200 μM H_2O_2. Also proven was a synergic effect between NO and H_2O_2 on the rooting mediation. NO and H_2O_2 treatments at adequate doses could increase the activity of polyphenol oxidase (PPO) and indoleacetic acid oxidase (IAAO) as well as the soluble carbohydrate content and total nitrogen, and at the same time decrease the total amount of polyphenols in the chrysanthemum cuttings. Moreover, the rooting percentage was significantly correlated with these activities or contents of the biochemical constituents. Together, these results indicated that NO and H_2O_2 treatments improved the AR development synergically and independently through the stimulation of PPO and IAAO activities and the carbohydrates and nitrogen content, simultaneously repressing the production of polyphenols [LIAO & al. 2010]. Similar biostimulating effects were observed for *Aloe vera* extract, including an accelerated plant and biomass growth, root development and oil production [EL SHERIF, 2017; HAMOUDA & al. 2012].

Research history regarding the use of *Salix* sp. extract

Salix genus includes mostly trees and sub-trees belonging to Salicaceae family, which comprises over 450 species, distributed all over the world and colonizing riparian habitats, including wet areas and water banks [ARGUS, 1997].

The willow bark extracts were extensively studied because of their medicinal implications and their role in plant-herbivores interactions. It was reported the presence of various secondary metabolites, such as salicin, triandrin, (+)-catechin, picein and salicortin, as well as polymeric tannins, but the content of these compounds varies a lot depending on the species, plant age, growth season and the solvent used for extraction [DOU & al. 2018]. The extraction with warm water represents a practical and cheap method to isolate the non-cellular substances from willow bark, having a significant yield, in a brief time. Time wise, it was proven that the extraction yield was high even after only 5 minutes, increasing up to 60 minutes and then remained relatively constant during long-time treatments. Raising the temperature from 60 to 80 °C led to a significant yield increase and further, raising the temperature up to 100 °C lowered the yield because of the partial degradation and/or extract precipitation at elevated temperature [DOU & al. 2018].

The willow extract was used for thousands of years in medicine for its analgesic effect and traditionally as a treatment for muscular osteoarthritic rheumatism, inflammation and pain, flu and respiratory problems, arthritis, gout, spondylitis, and rheumatoid arthritis, as well as for many other systemic diseases associated with inflammation [EMA, 2017]. Assyrians and Egyptians already knew the analgesic effects of a willow leaves decoct (extract) for relieving articular pain. At the beginning of the 19th century, salicin (an alcoholic ß-glycoside of the salicylic acid) was extracted from the willow bark and purified: the active extract, named salicin was isolated in its pure form by Henri Leroux, a Franch pharmacist and by Raffaele Piria an Italian chemist, who managed to separate the acid in its pure form [LÉVESQUE & LAFONT, 2000]. Later, a Franch chemist, Charles Frédéric Gerhardt synthesized the acetylsalicylic acid from salicin, which became the active compound of the medicine called aspirin [FUSTER & SWEENY, 2011].

Many plant extracts are used especially as biopesticides, and less as biostimulators. Some products based on soybean and other plant oils are used both as biostimulators and biopesticides. However, there are few examples of vegetal extract that are used exclusively as biostimulators and indirectly contributed to the disease and pest control. Willow bark extract, which is used as a plant growth regulator and biostimulator, also shows fungicide properties [DENIAU & al. 2019].

Besides its use in medicine, many researchers tried to use willow bark extract in plant protection, because plant diseases represent a key problem in the economically important crops. KARST (2002) proved the efficacy of this extract in the protection of vineyards against vine downy mildew (*Plasmopara viticola*), and ARIF & al. (2009) also described its antifungal properties to explain this potential phytosanitary efficiency. For this purpose, the Ecological Institute for Food and Agriculture from France (ITAB) conducted the experimental field-testing program "4P" to align with the European Union (EU) general regulation regarding pesticides and reducing the use of copper. The "4P" program was initially designed to test plant extract as complementary solutions to overcome the use of copper in the horticultural and agricultural fields. Furthermore, a lot of work was put into homologating the willow bark extract as plant protection method by the EU. The willow bark extract was approved by the EU regulations regarding pesticides (EC Regulation 1107/2009) applicable in agriculture as base compound with antifungal properties [MARCHARD, 2016].

The *Salix* sp. bark extract is used to control foliar fungal diseases caused by *Taphrina deformans*, *Venturia inaequalis*, *Plasmopara viticola*, *Erysiphe necator* and *Podosphaera leucotricha*. Regarding the fruit trees (peach tree, apple tree) and vine (*Vitis vinifera*), several studies were conducted in France. A significant antifungal effect was observed when treatment with willow bark was applied (at a concentration of 220 g/ha). The effect of the aqueous extract is due to the high content of salicylic glycosides or salicylate that act by reducing the stress impact on the plant and by activating some defense mechanisms. However, the salicylic acid alone, compared to the willow extract, did not yield the same results. The *Salix* sp. bark decoct shown antifungal properties also by inhibiting spore germination [MARCHARD, 2015]. Many studies proved that salicylic acid has antifungal activity [Da ROCHA NETO & al. 2015; TOSUN & al. 2003].

The interest towards this compound as natural resource for plant protection is multiple; it is useful for plant protection acting as elicitor for defense and resistance mechanisms [DEMPSEY & KLESSIG, 2017]. This extract is also environmentally friendly, because the active molecules do not have biocide properties, being classified as safe for human consumption. The willow infusion inhibits the germination and spreading of fungal disease without killing the fungus, as the chemical pesticides do. Using the same recipe that is used in cultivating fruit trees and vine [MARCHARD, 2016], it was proven that *Salix* extract has antifungal activity towards *Botrytis cinerea* and *Penicillium expansum*, species that mainly contaminate post-harvest fruits [ANDREU & al. 2018; HUSSAIN & al. 2011].

MUTLU-DURAK & YILDIZKUTMAN (2021) demonstrated the potential of willow bark extracts (WBE) and willow leaves extracts (WLE) as vegetal biostimulators with key roles in improving the early growth in corn (*Zea mays*) in control and salinity induced stress conditions. In 3 days, the treatment of seeds with salicylic acid and willow extract increased the biomass of corn plantlets with 130% and 225%, respectively. The root surface was also improved by 43% with salicylic acid and with 87% with willow extract. Moreover, these extracts increased the protein concentration in leaves and reduced the negative effects of salinity during early growth. The decrease in lipids oxidation processes and the specific activity of antioxidant enzymes in willow extract treated seeds suggest a remission of the oxidative stress caused by salinity.

During saline stress conditions, it was proved that in corn leaves, the protein concentrations were improved by 50% to 80% when applying high doses of willow extract (leaves and bark). The plants treated with WLE presented a lower Na/K ratio compared to other treatments. So, the stress limiting effect of willow extracts can be partially attributed to the decrease in Na accumulation and to keeping a relatively low Na/K ratio. The results indicate the fact that willow can represent a valuable resource, and the leaves and bark aqueous extracts can be used as effective and eco-friendly biostimulators [MUTLU-DURAK & al. 2023].

Recent research proved that willow bark extract, which contains high amounts of salicylic acid (SA) as phytohormone, have biostimulating properties on plant growth, with known effects on flowering, callus formation, stress mediation and rooting. AL-AMAD & QRUNFLEH (2016) show that the percentage of callus formation in olive tree cuttings (*Olea europaea* L. 'Nabali') was lower (40.5%) under the effect of willow (*Salix babylonica*) leaves and bark extract compared to control (55.5%), probably due to the high quantities of cytokines accumulated in plant at the end of the vegetative season, while the bark and wood extract showed better results. The average number of formed roots/rooted cuttings was significantly higher in all experiments.

A series of studies demonstrated that SA and other salicylates in the willow extracts stimulate root growing in cuttings, seedlings and mature plants, in many species of economically important plants, such as: soybean (*Glycine max*) or carrot (*Daucus carota*) [BASU & al. 1969; GUTIÉRREZ-CORONADO & al. 1998; HAYAT & al. 2010].

KAWASE (1970) studied the presence of rooting stimulating compounds from young or lyophilized white willow (*Salix alba*) aqueous extracts. These molecules were split through paper chromatography or chemical fractionation, and their role in the rooting process was tested on mung beans (*Vigna radiata*) cuttings. GESTO & al. (1977) studied the content in rhizogenesis stimulating substances of two extracts: chestnut (*Castanea sativa*) and willow (*Salix viminalis*), proving that from September to June, the total phenolic content showed a similar model in both species, although for chestnut were observed two clear maximum peaks in February-March and June. Furthermore, in both extracts were identified compounds such as: p-hydroxybenzoic acid, p-coumaric acid, ferulic acid, gentisic acid, salicylic acid and cinnamic acid, while scopoletin and p-hydroxyphenylacetic acid were specific to the chestnut extracts, and caffeic acid, esculetin, catechol and saligenin were only found in willow extracts, (the last two compounds identified in large doses). Catechol proved to be efficient in increasing the number of roots and showed a synergic root growth effect with the indole-acetic acid (IAA). In the willow extract, IAA was present from April to June and contributed to a good rooting performance. IAA was identified exactly when the extract showed the highest rooting activity.

Research history regarding the use of *Salix* sp. extract in the vegetative propagation of chrysanthemums

The rhizogenesis biostimulating compounds represent an optimized method of vegetative propagation *via* cuttings, and the right application concentrations are, most of the time, species-specific. The use of willow extract as biostimulator on chrysanthemum cuttings was investigated especially for its capacity to stimulate AR formation and ramification, improving the propagation efficiency through cuttings in this species.

There are very few studies regarding the use of *Salix* sp. extracts for the rooting stimulation in chrysanthemums, although for other species the results were obvious and encouraging after using this solution.

Weeping willow (*Salix babylonica*) extracts contain a variety of bioactive compounds, including salicylates and phenolic compounds. Indole-3-butyric acid (IBA), present in willow extract, is a phytohormone which initiates and accelerates root formation. As a result of modern research, the rhizogensis stimulating effect of the commercial biostimulator Nutrifield Complex Root Nectar® (Nutrifield Pty Ltd., Melbourne, VIC) and willow bark extract were assessed on chrysanthemum (*Chrysanthemum* sp.) and lavender (*Lavandula* × *hybrida* 'Frills') cuttings. The commercial product applied at a concentration of 1 ml/L improved the rooting capacity in both species compared with the control, while a concentration of 1.06 µL/L of willow bark extract exceeded the results of the commercial product for both species [WISE & al. 2020]. The best results for chrysanthemum were observed when applying willow bark extract at a concentration of 1.06 µL/L, which led to root ramification in 13.9 days, while in the control cuttings the root ramification occurred after 18.4 days (12.83% faster in the case of willow bark extract). For lavender, all levels of willow extract treatment showed equivalent results compared with the control for both AR formation ($P<0.001$) and AR ramification ($P<0.001$). The AR biostimulating effects showed the willow bark extracts can be successfully used to accelerate the propagation in semi-woody species, underlining their applicability in increasing the process efficiency in the horticultural industry, dependent on fast vegetative propagation at a large scale [WISE & al. 2020].

Considering the fact that *Salix* stems produce an auxin named indole butyric acid (IBA), a hormone that naturally stimulates root growth, in the last few years (2022-2024) we conducted a series of studies within the chrysanthemum collection of "Anastasie Fătu" Botanical Garden of Iași – Romania, which aimed at testing the influence of a *Salix babylonica* water extract (SE) on the rooting process in *Chrysanthemum* s.l. cuttings.

The first step was represented by testing the SE in a controlled environment and establishing the optimal concentration for rooting stimulation. Within the second step several commercial products known for their rotting stimulating properties (Atonik, Clonex) were used to obtain comparative data related to the efficiency of *Salix* water extract [URSACHI, 2024]. The commercial rooting stimulating hormones are mainly made from synthetic forms of IBA. Alongside the rooting hormone, the willow stems also produce salicylic acid that are involved in wound healing, allowing the sap to circulate, giving roots the opportunity to develop. The aim of the research was to perfect the technology for obtaining chrysanthemum cuttings, improving the reproductive material yield, and raising the percentage of rooted cuttings, and thus the quality of planting material.

Based on direct visual observations and on measuring the newly formed AR, we were able to notice that the most efficient practical method is represented by treating the chrysanthemum cuttings with *Salix babylonica* extract at a concentration of 5%. An increase in SE concentration (10% or 20%) led to a decrease in root development, having mostly an inhibitor effect [URSACHI, 2024].

The new methods proposed after the research conducted within the "Anastasie Fătu" Botanical Garden chrysanthemum collection, following the testing of several *Salix babylonica* water extracts, aimed at raising the economic yield of the collections and can lead to a decrease of the total production costs, by using vegetal biostimulators, easy to obtain compared to the available commercial products. Testing the *Salix babylonica* extract in the experiments focused on obtaining viable cuttings on a standard multiplication substrate (peat + sand v/v) drove us to conclusions regarding its efficiency in rooting initiation and development, with results that can recommend this variant as a biostimulator treatment for cuttings rooting.

Conclusions

Chrysanthemums (*Chrysanthemum* s.l.) represent one of the major groups of ornamental plants that bring a significant contribution to the horticultural industry. To preserve the desired characteristics (general aspect, plant height, inflorescence color, size, shape, etc.) of the thousands of varieties created so far, the main propagation method used for this species is vegetative reproduction using cuttings. In practice, this classic method, optimized over time, still has some drawbacks, especially when referring to the cuttings capacity to form a powerful root system that can ensure the further development of the future plant.

Research in this field led to the commercial production of various chemical solutions that promise to resolve this issue, many of them with noticeable results. The two main problems of these products are the high production and selling price and their environmental impact, derived from their chemical composition. Because of these aspects, studies that test the biostimulating rooting capacity of natural products (especially vegetal extracts) started to be developed in the last few years, with encouraging outcomes.

One of the most promising plants in this direction is considered to be the willow (*Salix* sp.). Its extracts have been used for centuries in the medicinal and pharmaceutical industries for their analgesic effect, being known to treat muscular osteoarthritic rheumatism, inflammation and pain, flu and respiratory problems, arthritis, gout, spondylitis, rheumatoid arthritis, etc. A biochemical analysis of the willow extracts (bark, leaves, stems) showed the presence of many bioactive compounds such as salicin, triandrin, (+)-catechin, picein and salicortin, polymeric tannins, etc. which are used for their antifungal properties, but also for their capacity to promote plant growth, and to stimulate root formation and development.

Regarding the use of willow extracts as a biostimulator for roots formation in chrysanthemums, the studies are very few and relatively new, despite the promising results obtained for other economically important species, a fact that encouraged us to further investigate this aspect.

The outcomes of our research showed a positive influence of the willow bark extract on the rooting process in chrysanthemum cuttings, proving that a natural product, with no impact on the environment and cheap to produce can solve a problem that negatively affects the efficiency and propagation cost of this horticulturally significant species.

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Review Article

THE BENEFICIAL EFFECTS OF OLIVE TREE (*OLEA EUROPAEA* L.) IN THE NUTRITIONAL, PHARMACEUTICAL AND INDUSTRIAL APPLICATION: A REVIEW

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Abstract: *Olea europaea* L. (olive tree / zaitoon tree / zaitoon) belong to family *Oleaceae*. This is a valuable and an economically commercial oil woody shrub or small evergreen tree plant species and especially popular in diet of people of Mediterranean region. The olive tree oil is extracted from the fruit of *O. europaea* and used for cooking, agricultural, biological, biochemistry, genetics, microbiology, immunology, pharmacology, toxicology, food technology, pharmaceutical sciences, agro-food sector and environmental sustainability. The whole parts (leaves, branches, fruit) of this plant traditionally used and cultivated for food and medicine in Europe, Mediterranean, warm temperate region, Asian, African, Kingdom of Saudi Arabia, Tunisian regions and in different parts of Pakistan. *O. europaea* also provide an excellent material of polyphenol, abundant unsaturated fatty acids and flavonoids.

O. europaea seedling also possesses different types of resistance and tolerance to biotic and abiotic stress. There are several publications available which has highlighted the influence of waste product, polluted and contaminated air, water and soil on olive tree growth. *O. europaea* also contains many bioactive compounds and oil have been reported to control many ailments. A lot of work has been published on the biological compounds derived from olive tree in recent years for the treatment of different diseases. The potential of *O. europaea* plant extract showed antidiabetic, anticancer, asthma, cholesterol, uric acid, diarrhea, inflammation, blood pressure, antiheart and antiarthritis treatment.

This review study was aimed to assess the beneficial characteristics of olive tree in terms of its nutritional, pharmaceutical properties and application in industries. The outcome will help to researchers working in the field of the nutritional, pharmaceutical and industrial sectors for examining the fruitful results. The 650 research articles were searched for review and 166 were selected using electronic search engine likewise google, google scholar, research society and development and science direct.

Keywords: Antioxidant, antidiabetic, cultivar, environmental pollution, industrial activities, oleuropein, olive tree, photosynthesis, zaitoon tree.

Introduction

Olea europaea L. (olive tree / zaitoon tree / zaitoon) belongs to family *Oleaceae* and is traditionally used as a functional food in Europe and Mediterranean countries and also to treat different types of diseases. The olive tree represent an important economic position in the Mediterranean area due to their wide distribution and representativeness such as in Tunisia with 90 million olive trees covering 1.8 million hectares of land, where it is grown traditionally in rain fed conditions, such as the case of Sfax region that was retaining 25% of national olive tree oil production [DGPA, 2018]. These oil trees, in fact, are capable of maintaining relatively high yield with symbol of longevity [JAFRI, 2021; CHIESI & al. 2022]. ESPESO & al. (2021) believed that olive tree cultivation began in Mesopotamia (4,000 B.C.), with the first records of olive tree crops dating to the Minoan civilization, at around 2,500 B.C. The olive tree is one of

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the most beloved trees in the history of mankind and considered it sacred since Ancient Greek times, throughout the Mediterranean region and now spread over the subtropical region globally, including Pakistan [EFLORA, 2023]. The global trend towards greater population will give rise to substantial increases in world demand for food animal protein (meat, milk and dairy products), vegetable oils and processed foods [PERONE & al. 2022] in developed and developing countries.

O. europaea has many ethnopharmacological [HONG & al. 2021] and nutritional relevance. The fallen herbal waste of olive tree leaves are a good source of antibacterial activity, color and in dyeing of cotton fabrics [YILMAZ & BAHTIYARI, 2020]. Olive tree is the main ingredient associated with many health benefits for treatment of Alzheimer's disease, heart health, cancer prevention, lower blood sugar, cholesterol, uric acid levels, as a laxative, mouthwash, and as a vasodilator, it has also been used to treat diabetic ketoacidosis, inflammation, diarrhea, respiratory and urinary tract infections, stomach and intestinal illnesses and asthma, and also range from fighting inflammation to reducing the growth of microorganisms [HASHMI & al. 2015; ZHAO & al. 2023]. In Mediterranean folk medicine, *O. europaea* leaf preparations are used as a common remedy for gout [FLEMMIG & al. 2011].

The use of natural bioactive molecules extracted by-products of the olive tree oil supply chain for the production of functional feeds may represent a possible source of circular economy in view of environmental sustainability [DI MEO & al. 2023]. This oil trees also helps in combating global warming, climate change, soil erosion, soil contamination, salinity, waterlogging and desertification problems. The byproducts of table olive tree oil industry are of great value but also to the environment and to the human health. Since leaves represent around 10% of the total weight of olive tree arriving at the mill, it is worth obtaining high added-value compounds from those materials for the preparation of dietary supplements, nutraceuticals, functional food ingredients or cosmeceuticals [ŞAHIN & BILGIN, 2018].

Olive tree leaves have recently been recognized as a valuable source in cosmetic and pharmaceutical industry as well as in preparation of health supporting beverages [PONGRAC & al. 2022]. The nutritional status of olive tree (zaitoon) orchards from the central region of the Rio Grande do Sul with fruit yield recorded. Soil, leaf, and fruit from eight orchards for the contents of N, P, K, B, pH, TOC, $Al^{+3,}$ and soil texture analyzed. The available P content in soil were found low. In leaf tissue, K and N presented high contents [FIGUEIREDO & al. 2022].

The different aspect of olive tree growth, pollen development, structural changes, mesocarp formation, alternate bearing behavior by the balance between growth of vegetative and reproductive organs growth was observed [RODRÍGUEZ-GARCÍA & al. 2003; GOMES & al. 2009; ZIENKIEWICZ & al. 2011; ZUCCHINI & al. 2023]. The leaves of olive tree can provide a greener alternative for the recovery of heavy metals from the products of waste and raw materials (mobile phone, keyboards, circuits, computer accessories). Printed circuit boards (PCBs) is becoming a source of precious metals and polyphenols rich plant extract was obtained from olive tree leaves, and its ability to contribute to reducing four metals, namely, Ag, Cu, Cr, and Sn, that are present in scrap PCBs [ALEXANDRE-FRANCO & al. 2022]. There are predictions in Tunisia about the increase of temperature to 2.7 °C on the horizon of 2050, which may contribute to the degradation of agro-ecosystems and particularly the olive tree sector. The physio-biochemical feature, oil quality and growth behavior of "Zelmati" olive tree in Kebili-Rjim Maatoug region, which is a desert oasis in southwestern Tunisia characterized by a hot desert climate was carried out [ROUINA & al. 2019]. The authors recorded the decrease in chlorophyll content, with an increase of malondialdehyde, soluble proteins, proline and soluble sugars contents in leaf tissues due to hot climate. The published result showed evidence about the plant tolerance or resistance to biotic or abiotic stresses due to disturbances of nutritional status [HUBER & al. 2012; SANZANI & al. 2012]. Olive tree is reported a moderately salt tolerant crop. Recent studies suggestd that olive tree can be irrigated with water containing 3200 mg/l of salt (ECw of 5 dS/m) producing new growth at leaf Na levels of 0.4-0.5% dry weight, fruit weight, and moderate salinity is associated with reduction of CO_2 assimilation rate, stomatal and mesophyll conductance [CHARTZOULAKIS, 2005]. In natural environment the growth of olive tree being reported to abiotic stresses, such as water deficit [FERNÁNDEZ-ESCOBAR, 2019; TEKAYA & al. 2022]. Olive tree oil extraction processes generate significant amount of wastes likewise, olive tree mill solid waste, and olive tree mill wastewater, which are rich in precious compounds and reported toxic to the environment [MECHNOU & al. 2021].

Table 1. Botanical description of olive tree (*Olea europaea* L.)

Kingdom	Plantae
Order	Lamiales
Genus	Olea
Family	Oleaceae
Botanical name	*Olea europaea* Linn. (fruit oil)
Tree	tree grow up to 7 m high, greyish-green
Bark	grey, on branchlets whitish
Leaves	lanceolate, sometimes ovate, c. 4 cm long, 1 cm broad, coriaceous; upper surface dark green, with few scales, ventral silvery-whitish due to scaly hairs; petiole 5 mm.
Flowers	whitish, in terminal or lateral cymes. Calyx truncate or with 4 little teeth. Corolla tube short; lobes 4, 1-2 mm long. Drupe blackish-violet when ripe, ovoid, 1-2 cm in diam.; pulp oily.
Flowering period	April-May. Fruit: September-October.
Genetic variability	Olive tree cultivars represent an invaluable heritage of genetic variability selected over more than 5500 years of cultivation
Soil	well drained
Average life span	500 years, current oldest recorded for 2000 years
Origin / distribution	probably in Asia Minor. Cultivated since ancient times throughout the Mediterranean region (Algeria, Argentina, China, Cyprus, France, Greece, Israel, Italy, Jordan, Libya, Morocco, Portugal, South America, Spain, Syria, Turkey, Tunisia), Europe, now spread over the subtropical regions of the entire globe.
Nutritious value	Fat, fibre and water, calorie, olive oil is rich in monounsaturated oleic acid. Vitamin E.
Olive tree oil grade	(1) virgin, from first pressings that meet defined standards; (2) pure, or edible, a mixture of refined and virgin; (3) refined, or commercial, consisting of lampante from which acid, colour, and odour have been removed; (4) lampante, high-acid oil, named for its use as a lamp fuel, obtained from a second pressing of residual pulp with hot water (some inferior virgin oils are classed as lampante); and (5) sulfide, extracted with solvents and refined repeatedly.
Constituents	Oleic acid, Linoleic acid, Palmitic acid, Stearic acid.Phenolic compounds (oleuropein, hydroxytyrosol, tyrosol and oleocanthal).
Food industry	The extraction of olive tree oil obtained by crushing, pressing and centrifuging, generating byproducts that can be reused for recovery of compounds or generation of new products which is a new approach in the food industry.
Miscellaneous properties / uses	They have been used in the human diet as an extract, an herbal tea, and a powder, and they contain many potentially bioactive compounds that may have antioxidant, antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic, blood pressure, and hypocholesterolemic properties. Olive oil has a high content of antioxidants, which assists in the repairing of cell membrane and moisturizing the skin. Its chlorophyll content aids in the healing of skin conditions and reducing the signs of aging.

References: RUGINI & FEDELI, 1990; RAINA, 2003; EL & KARAKAYA, 2009; SAAD, 2015; RALLO & al. 2018; WANG & al. 2019; DOOLY, 2020; SILVA & SCHMIELE, 2021; FERREIRA & al. 2021; BRITANNICA, 2023; EFLORA, 2023; AGMRC, 2023; PP, 2024.

This olive tree plant species was not growing well in Pakistan in the past. Recently different varieties have been planted together with some proper pollinators, and the results are quite satisfactory. There is hope now that the cultivation of the olive tree will spread all over the northern regions of Pakistan in the near future [EFLORA, 2023]. FRÖLECH & al. (2021) evaluated about basal lesion and the forms of application of indolebutyric acid (IBA) in 'Arbequina' olive (*Olea europaea* L.) minicut. It was concluded that minicutting of olive 'Arbequina' can be done with lesion at the base and without indolebutyric acid. The micropropagation of the olive tree used to obtain several healthy seedlings in less time, compared to traditional techniques, from an explant. Thus, this work was defined a protocol of contamination and oxidation for Oliveira (*Olea europaea* L.) cv. Koroneiki explants [OLIVEIRA & al. 2021]. STANNER & WEICHSELBAUM (2013) studied antioxidants role for human nutrition.

The fruit and oil of olive tree has many nutritional, medicinal and industrial application in many ways for living organisms. In recent years its cultivation is increasing in the agricultural area due to public demands on global scale. The aim of this review is to highlight the nutritional and pharmaceutical importance of *O. europaea* in industrial sector. The information was gathered from different research articles published in national and international scientific journals.

Table 2. Different varieties of olive tree (*Olea europaea* L.)

Name of olive oil tree varieties	Reference
Tunisian olive tree varieties, namely, Chemchali, Chemlali, Chétoui, Gerboui, Sayali, Zalmati and Zarrazi.	ABAZA & al. 2007.
Jordan cultivars (Nabali, Improved Nabali and Abo-shoka)	AL-MAAITAH & al. 2009
Greek cultivars *koroneiki*, *megaritiki* and *kalamon*	KIRITSAKIS & al. 2010
Olea europaea var. *mastoidis* and *O. europaea* var. *Koroneiki*	KAVROULAKIS & NTOUGIAS, 2011
Konservolea and Kalamata	ABUSAFIEH & al. 2011
Corregiola and Frantoio	GOLDSMITH & al. 2015
Kalamata	BASAHI & al. 2016
IFAPA Centro 'Alameda de Obispo', Cordoba, Spain	KYÇYK & al. 2016
Zelmati	ROUINA & al. 2019
O. europaea var. *sylvestris*	HAROUAK & al. 2021
Olive tree cultivars (Chemlali, Manzanilla, Picaul and Toffahi)	RASHED & al. 2022
Italian cultivars ('Biancolilla', 'Nocellara Etnea', 'Nocellara Messinese', 'Nocellara Siracusana', 'Zaituna')	PALMERI & al. 2022
Leccino	SODINI & al. 2023

Material and methods

The information was sighted from the national and internationally published research work using different online electronic search engine tools such as ACS Publications, BioOne, EGU (European Geosciences Union), Europe PMC, Google Scholar, Google, Hindawi, IDEAS/RePEc, PebMed, Plos One, Science Direct, Springer Link, Taylor & Francis Online, and with different keywords which includes: antioxidant, anticancer, cultivar, ecology, environmental pollution, germination, growth, oils, oleuropein, olive tree, pollution, polyphenol, virgin olive oil and explained in Tables 1-4.

Discussions

Phytomedicine - therapeutical or pharmaceutical benefits

Olive tree leaves in traditional remedies for different types of ailments in European and Mediterranean countries such as Greece, Spain, Italy, France, Turkey, Israel, Morocco, and Tunisia were used. There are many publications available on the use of olive tree that showed health benefits for the treatment of hypertensive, inflammatory, diabetic, cancer, heart, stroke and gout (Table 2). The phenolic compounds present in olive tree leaves confer benefits to the human health especially the oleuropein, are associated to antioxidant, antihypertensive, hypoglycemic, hypocholesterolemic, gastroesophageal reflux, aommon disorder disease, cardioprotective activity and as a support in the treatment of obesity [VAKIL, 2004; PICHE & GALMICHE, 2005; TACK, 2005; KARAMANOLIS & al. 2006; CORON & al. 2007; VOGEL & al. 2014; MONE & al. 2016]. The oldest and traditional methods of prevention and cure of known diseases in Greek, Arab, Islamic health systems based on method diet therapy (Plants and animal product) and early Muslims utilized many plants and animal products mentioned in the Holy Quran and in the Hadith of the Prophet Muhammad (peace be upon him) for health promotion, for example, dates, black seeds, olive tree leaf and olive oil, honey, and camel milk, respectively [SAAD, 2015]. The positive effects of olive tree leaf extract and its key phytochemical constituents have been reported on blood pressure, cancer, respiratory infections, inflammation, insulin resistance and incidences of cardiovascular disease [SOMOVA & al. 2003; AL-AZZAWIE & ALHAMDANI, 2006; POUDYAL & al. 2010; BOSS & al. 2016; BREAKSPEAR & GUILLAUME, 2020; ANTONIOU & HULL, 2021; USMANI & ALMOSELHY, 2023].

Table 3. Therapeutic treatment from different parts of *Olea europaea*

Plant part/product	Treatment	Reference
Olive extract	Inhibited the activities of amylases from human saliva and pancreas	KOMAKI & al. 2003
Leaf	Human leukemia HL-60 cells	ABAZA & al. 2007
Plant	Anticancer	DAI & MUMPER, 2010
Leaves	Antihypertensive, hypoglycemic, hypocholesterolemic, cardioprotective activity and support treatment of obesity	VOGEL & al. 2014
Extra virgin olive oil	Anti-osteoporosis, anti-inflammatory properties, anticancer properties in patients	LIU & al. 2014
Oleuropein	Anti-proliferative activity against a number of cancer types (pancreatic cancer cells)	GOLDSMITH & al. 2015
Leaf	Infectious diseases	EFENTAKIS & al. 2015
Olive oil	Affected bone and uterus in ovariectomized rats	ZHENG & al. 2016
Leaf	blood pressure and inflammatory markers	LOCKYER & al. 2017
Olive oil	Cardiovascular disease, cancer, neurodegenerative disease, osteoporosis, anti-proliferative, pro-apoptotic, and anti-inflammatory activities	GARCIA-MARTINEZ & al. 2018
Extra virgin olive oil	Lowering the incidence of cardiovascular events, including myocardial infarction and stroke	NOCELLA & al. 2018
Extra virgin olive oil	Linked to ageing and age related diseases related to a common chronic low grade inflammation	GAMBINO & al. 2018
Olive oil	Positively associated with a better volumetric bone mineral density treatment in Spanish women	RONCERO-MARTÍN & al. 2018
Olive oil	Fatty liver disease	ABENAVOLI & al. 2019

Olive oil, fruit	Seem to be beneficial for preventing osteoporosis disease and its progression	CHISARI & al. 2019
Leaf extract	Cardiovascular, metabolic disease, blood pressure, respiratory infections, inflammation, and insulin resistance	BREAKSPEAR & GUILLAUME 2020
Olive oil	Beneficial effect on some bone characteristics	DÍAZ-CURIEL & al. 2020
Olive product	Reduced incidences of cardiovascular disease	ANTONIOU & HULL, 2021
Leaves	Cancer cell	ALBOGAMI & HASSAN, 2021
Olive product	Antitumor, tumor genesis, tumor suppression and chronic inflammation, delaying the development, progression or recurrence of various types of tumors	FERREIRA & al. 2021
Leaves	prostate cancer cell lines (HT29 and PC3, resp.)	ALBOGAMI & HASSAN, 2021
Olive oil	Prevent bone mineral density loss	RONDANELLI & al. 2021
Product	Cure the symptoms of Gastroesophageal reflux disease	DA SILVA & al. 2022
Leaves	phenols, secoiridoids, hydroxycinnamic acids, flavonoids	PALMERI & al. 2022
Plant	Human lung cancer cell lines (A549)	NAJIBULLAH & al. 2023
Fruit	Cancer therapy	ALHARBI & FELIMBAN, 2023
Leaves	Exhibiting anti-photoaging activity	XU & al. 2023
Olive oil	Hepatic steatosis and liver enzymes	MA & al. 2023
Olive oil	Cardiovascular	TARABANIS & al. 2023

Anticancer properties

Olive tree leaves have a long history of medicinal and therapeutic use [ALBOGAMI & HASSAN, 2021]. Cancer is one of the most serious public health issues worldwide and might be an important cause of death. A nontoxic strategy for producing silver nanoparticles (AgNPs) for cancer therapy was established in green synthesis method using (OFE) *Olea europaea* fruit extract [ALHARBI & FELIMBAN, 2023]. The AgNPs-OFE was reported highly cytotoxic against the T47D cancer cell line with a 50% inhibitory concentration (IC50) of 77 µg/mL. The leaf extracts of the four olive tree cultivars (Chemlali, Manzanilla, Picaul and Toffahi) as a source for some anticancer agents was examined. The olive tree leaf extract has anti myeloma activity and *in vitro* cytotoxic activity of olive tree leaf extract against mouse metastatic melanoma B16F10 cell lines [MAJUMDER & al. 2019]. In another study, NAJIBULLAH & al. (2023) examined the anticancer potential of chemically characterized *O. europaea* extract in MTT assay and EB/AO double staining method using Human lung cancer cell lines (A549). The study outcome showed that *O. europaea* extract significantly inhibited cell proliferation and apoptosis in human lung cancer (A549) cell lines, and it also explores the chemical composition of *O. europaea* leaves extract. ALBOGAMI & HASSAN (2021) investigated the anticancer properties of an olive tree leaf extract *in vitro* using colorectal and prostate cancer cell lines (HT29 and PC3, resp.)

The phenolic compounds from olive tree products have antitumor potential through their effects in the prevention of tumor genesis and tumor suppression in different cancer models. The phenolic compounds from olive tree products (oleuropein, hydroxytyrosol, tyrosol and oleocanthal) may exert tumor genesis inhibiting effects, such as repair and protection against damage from oxidative stress and chronic inflammation, and thus could help in reducing the risk of cancer and delaying the development, progression or recurrence of various types of tumors [FERREIRA & al. 2021]. Oleuropein has been shown to exhibit anti-proliferative activity against a number of cancer types. The treatment of water, 50% ethanol and 50% methanol extracts of Corregiola and Frantoio variety *O. europaea* leaves showed slight differences in their phytochemical properties, and at 100 and 200 µg/mL, all decreased the viability of the pancreatic cancer cells relative to controls [GOLDSMITH & al. 2015].

Anti-heart disease

Extra virgin olive oil is the most representative component of this diet and seems to be relevant in lowering the incidence of cardiovascular events and stroke [NOCELLA & al. 2018]. Atherosclerosis is characterized by a chronic low grade inflammatory process which can result in atherothrombosis and a number of cardiovascular diseases. The consumption of extra-virgin olive oil is associated with a reduction in inflammatory biomarkers and molecules implicated in atherosclerosis as well as cardiovascular diseases incidence and mortality as well as other complications such as heart failure and atrial fibrillation [WONGWARAWIPAT & al. 2018]. The presence of monounsaturated fats in olive oils favor the heart health. BREAKSPEAR & GUILLAUME (2020) identified the olive tree leaf extract, prepared from the fresh or dried leaves of *O. europaea* is generating interest as a cardiovascular and metabolic disease risk modifier. Positive effects for the olive tree leaf extract and its key phytochemical constituents have been reported on blood pressure, respiratory infections, inflammation, and insulin resistance. The olive tree which is rich in olive products has positive effects on health, associated with reduced incidences of cardiovascular disease [MARTÍNEZ-GONZÁLEZ & al. 2019; ANTONIOU & HULL, 2021].

Infectious – inflammation, liver function and bone health

Olive oil has been associated with bone health and overall data suggested a protective impact of virgin olive oil as a source of polyphenols in addition to vitamin D3 on bone metabolism through improvement of oxidative stress and inflammation was recorded [TAGLIAFERRI & al. 2014]. Olive tree can help with reduction in inflammation which is a root cause of many diseases. The olive tree leaf is considered an important traditional herbal medicine utilized against infectious diseases [EFENTAKIS & al. 2015]. The presence of polyphenols found in olive tree can help reduce the risk of chronic inflammation. The liver is an organ susceptible to a multitude of injuries that causes liver damage, like steatosis, non-alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma, ischemia-reperfusion injury and extra virgin olive oil presented several protective effects on the liver, reducing hepatic steatosis, hepatocyte ballooning, fibrogenesis, preventing lipid peroxidation, among other effects [SOTO-ALARCON & al. 2018]. Osteoporosis is a metabolic disease affecting the bone mineral density thus compromise the strength of the bones and suggested that the intake of phenols seems to influence bone mineral density by acting as free radical scavengers, preventing oxidation-induced damage to bone cells [CHISARI & al. 2019].

Anti-diabetic potential

Diabetes is a lethal disease in both developed and developing countries and fourth leading cause of death in the most develop countries and diabetic foot ulcer [RAO & al. 2010; WAINSTEIN & al. 2011; ARUMUGAM & al. 2013; BUOWARI, 2013; MOGHADDAM & al. 2013; SALAH & al. 2017]. This developed due to inadequate regulation of the blood sugar which imposes a serious health issue [SALEHI & al. 2019] and affect almost every part of the body and often leads to blindness, heart and blood vessel disease, stroke, kidney failure, amputations, insulin and nerve damage [SOUMYA & SRILATHA, 2011; SHARMA & ARYA, 2011; SINGAB & al. 2014; WANG & al. 2014].

The regular consumption of extra virgin olive oil as a main source of fat altered the inflammatory response characterizes chronic immunemediated inflammatory diseases (IMID) such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, systemic lupus erythematosus, and psoriasis. It is also associated with a reduced risk of developing chronic degenerative disorders such as cardiovascular diseases, type 2 diabetes and cancer [SANTANGELO & al. 2018].

***Gastroesophageal reflux disease* (*GERD*)**

Gastroesophageal reflux disease (GERD) is a common clinical problem that affects millions of people worldwide [CLARRETT & HACHEM, 2018; GYAWALI & FASS, 2018; KATZKA & KAHRILAS, 2020; CHAPELLE & al. 2021; DE SANTIAGO & al. 2021; SHARMA & YADLAPATI 2021; MALFA & al. 2021]. It was found that the use of *O. europaea* in traditional chinese medicine in the main symptoms of GERD [SILVA & al. 2022].

Antibacterial and fungal potential

Seven aerobic bacterial strains capable of degrading several of the monocyclic aromatic compounds occurring in the phenolic fraction of olive mill wastewaters (OMWs) were isolated from an Italian OMW [DI GIOIA & al. 2002]. *Colletotrichum acutatum* is a cosmopolitan and damaging plant pathogen of temperate, subtropical, and tropical fruits and causes anthracnose on *O. europaea*. The antifungal activity of residues from the olive industry (bagasse) in the Picual variety and of vegetative parts of olive tree cultivars of the Arbequina and Picual varieties, against *Candida* spp., *Microsporum gypseum* and *Sporothrix brasiliensis* was evaluated [MARTINS & al. 2022]. The hydroalcoholic extracts of the bagasse of the Picual variety and the leaves of the Picual and Arbequina varieties showed promising fungistatic activity against the isolates of *Microsporum gypseum*, with CIM ranging from 100 mg/ml to 200 mg/ml.

Bioactive – nutritional compounds composition

Olive oil is composed of TGs, esters of oleic, linoleic, palmitic, fatty acids, along with rich in polyphenols, phenolic compounds, amongst which elenolic acid, alpha tocopherol, flavonoids, pinoresinol and lignans are the main constituents of its oil [OWEN & al. 2000; TAAMALLI & al. 2012; DE BOCK & al. 2013; HASSEN & al. 2015; SASAKI & ISODA, 2022]. Phenolic compounds playing a potential role in public health and reducing risk of chronic diseases [ABAZA & al. 2015].

The inhibitory action of an ethanol extract of *O. europaea* inhibited the activities of amylases from human saliva and pancreas with IC50 values of 4.0 and 0.02 mg/ml, respectively recorded [KOMAKI & al. 2003]. Olive tree leaves are well known for their high polyphenol content and two main phenolic compounds oleuropein and 3-hydroxytyrosol were investigated [GUGLIELMOTTI & al. 2020]. Olive tree fruits, leaves and oils are consider a sources of nutritional and other antioxidants compounds and gaining increasing interest in recent years. It has several pharmacological properties, including antioxidant, cardioprotective, anti-heterogeneous, neuropathic, obesity, anti-cancer, heart, liver, brain, kidney and other effects. The information about the use oleuropein obtained from olive tree leaves in the treatment and prevention of diseases was reviewed [OTERO & al. 2021].

O. europaea constitutes a source of many bioactive compounds and showed many benefits for human health and used in technological, pharmaceutical and industrial purposes (Table 3). RASHED & al. (2022) evaluated the biological activity of oleuropein (an ester of 2-(3, 4-dihydroxyphenyl) ethanol (hydroxytyrosol) which has the oleosidic skeleton that is common to the secoiridoid glucosides of *Oleaceae*.

Table 4. Bioactive compound derived from different parts of *Olea europaea* L.

Plant part	**Bioactive compound**	**Reference**
Leaves	Secoiridoids	GARIBOLDI & al. 1986
Olive oil	TGs, esters of oleic, linoleic, palmitic, fatty acids, along with rich in phenolic compounds, amongst which elenolic acid, alpha tocopherol, flavonoids, pinoresinol and lignans are the main constituents of its oil	OWEN & al. 2000
Leaves	secoiridoids, flavonoids, and triterpenes	EL & KARAKAYA, 2009
Leaves	secologanoside, dimethyloleuropein, oleuropein diglucoside, luteolin-7-*O*-glucoside, rutin, oleuropein, oleuroside, quercetin, ligstroside and verbascoside	KIRITSAKIS & al. 2010
Olive tree waste water	hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid, vanillic acid, syringic acid, gallic acid, luteolin, quercetin, cyanidin, verbascoside and some polymeric compounds	D'ANTUONO & al. 2014
Leaves	phenolic compounds – oleuropein	VOGEL & al. 2014
Leaves	oleuropein, total phenolic compounds, total flavonoids and oleuropein	GOLDSMITH & al. 2015
Olive oil	The main sterols found in olive oil were β-sitosterol, Δ(5) -avenasterol, campesterol and stigmasterol, Over the group of cultivars, total sterol contents ranged from 855 to 2185 mg kg$^{(-1)}$	KYÇYK & al. 2016
Leaves	Phenolic	LOCKYER & al. 2017
Fruit	Fatty acid (98-99%)	ROMANI & al. 2019
Fruit	Phenolics, phytosterols, tocopherols, and squalene (11-2%)	ROMANI & al. 2019
Leave	High polyphenol content and two main phenolic compounds oleuropein and 3-hydroxytyrosol	GUGLIELMOTTI & al. 2020
Leaves	Phenolic compounds from olive products (oleuropein, hydroxy tyrosol, tyrosol and oleocanthal)	FERREIRA & al. 2021
Oils	Primary source of mono- and poly-unsaturated fats	SALAH & al. 2021
Seed extract	The antioxidant and antidiabetic potential of Indian olive seed extracts. Treatment with MEOE and AEOE reduced the aggravated liver and kidney function biomarkers.	AKHTAR & al. 2022
Olive	The biological activity of oleuropein (an ester of 2-(3,4-dihydroxy phenyl) ethanol (hydroxytyrosol) which has the oleosidic skeleton that is common to the secoiridoid glucosides	RASHED & al. 2022
Leaves	Bioactive compounds, including 9,12-octadecadienoic acid (Z,Z)-, n-hexadecanoic acid, 9-octadecenamide, (Z)-, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, squalene, 2-(2-Hydroxy-2-phenylethyl)-3, 5,6-trimethylpyrazine, Benzoic acid, 4-formyl-, methyl ester, 2-Methoxy-4-vinylphenol, Vitamin E	SYED & al. 2022
Olive	Identified 37 fatty acid and 35 flavonoid compounds	NIU & al. 2022
Extra virgin oil	May exert beneficial effects on bone by modulating marrow adiposity, which would support their protective effect against bone pathologies	LIU & al. 2022
Leaves	Oleuropein and luteoloside	XU & al. 2023
Fruit	Fatty acids, sterols, erythrodiol, uvaol, and chlorophylls	GAGOUR & al. 2024

KIRITSAKIS & al. (2010) determined the olive tree leaf phenolic composition of the Greek cultivars *koroneiki*, *megaritiki* and *kalamon* using LC/MS and the ability of phenolic compounds to inhibit the lipoxygenase (LOX) activity was investigated. The ten main components in the olive tree leaf extracts for the cultivars *koroneiki* and *kalamon* were: secologanoside, dimethyloleuropein, oleuropein diglucoside, luteolin-7-*O*-glucoside, rutin, oleuropein, oleuroside, quercetin, ligstroside and verbascoside reported. Olive tree leaves contain several potentially bioactive compounds that may have hypoglycemic and hypolipidemic properties [ACAR-TEK & AĞAGÜNDÜZ, 2020]. HAROUAK & al. (2021) tested *O. europaea* var. *sylvestris* against oral diseases and for phytochemical screening. The total flavonoides (195.80 ± 2.91 mg CE/g decocated extract) significantly ($p<0.0001$) obtained from decocted extract of *Olea europaea* L. subsp. *europaea* var. *sylvestris*, total phenolic (167.71 ± 12.52 mg GAE/g d.e.) and total condensed tannins (250.44 ± 10.18 mg CE/g d.e.) from soxhlet extract and infused extract of *Tetraclinis articulata* L. whereas; The correlation analysis using Principal Component Analysis (PCA) was found positively between infusion and decoction, between total flavonoids and total phenols. The bioactive constituents activity of a cold methanolic extract of *Olea europaea* leaves was identified [SYED & al. 2022]. Several unique bioactive compounds, including 9,12-octadecadienoic acid (Z,Z)-, n-hexadecanoic acid, 9-octadecenamide, (Z)-, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, squalene, 2-(2-Hydroxy-2-phenylethyl)-3, 5,6-trimethylpyrazine, Benzoic acid, 4-formyl-, methyl ester, 2-Methoxy-4-vinylphenol, Vitamin E reported.

The bioactive components of *Olea europaea* leaves extract (OLE), was determined by high Performance Liquid Chromatography (HPLC). The results showed that oleuropein and luteoloside inhibited their activity by directly interacting with MMP-1, MMP-3, and MMP-9, thereby exhibiting anti-photoaging activity [XU & al. 2023].

The published documented data reported in this review demonstrated that the main product of *O. europaea* fruits, leaves and byproducts having bioactive compounds also can be used for food, nutritional and pharmaceutical applications according to the requirement of economic strategy. *O. europaea* fruit is a peculiar vegetal matrix contain high levels of fatty acids (98-99% of the total weight of extra virgin olive oil, EVOO) and low quantities (1-2%) of phenolics, phytosterols, tocopherols, and squalene. Among these minor components, phenolics are relevant molecules for human health [ROMANI & al. 2019].

Industrial application

Olive oil industry playing an important role in the economic activities of some countries, especially in Mediterranean basin sector due to the publications of positive evidences from scientific literature related to public health. An estimated 3.2 million metric tons of olive oil was produced worldwide in 2020 [CHRAIBI & al. 2022]. Eight different categories of olive oils and olive-pomace oils exist namely, extra virgin olive oil, virgin olive oil, virgin lampante olive oil, refined olive oil, olive oil composed of refined olive oil and virgin olive oils, olive pomace oil, crude olive-pomace oil, refined olive pomace oil and only extra virgin olive oil, virgin olive oil, olive oil composed of refined olive oil [EC, 2024]. The demand for the olive oil production is increasing rapidly in recent years. Olive products is fulfilling many promising roles in olive oil and pharmaceutical industry. Olive fruits are significant source of phenolic compounds. In addition to eating of this delicious fruit, it is used for oil industry and in the ancient time Kingdom's wealth was determined among other things, was their olive oil industry [RIVULIS, 2024]. Olive is an evergreen xerophytic tree grown for its drupes, which yield oil and has been closely associated with human religious, sociocultural, medicinal, important part of nutritional needs and agroindustry and olive oil industry [SHETTY & WATERLOW, 2003; RAINA, 2003; BALASUNDRAM & al. 2005; PALMERI & al. 2007; PERALBO-MOLINA & al. 2013; ORTEGA & al. 2016; ŞAHIN & BILGIN, 2018; WANG & al. 2019; SILVA & al. 2021; JURADO-CONTRERAS & al. 2022].

Processing industries have been successfully reported for manufacturing and marketing jarred baby foods with the use of vegetable oils, including olive oil, as well as other sources of visible fat added fat in jarred infant foods supplied to the Polish market [HOZYASZ & al. 2010]. Olive biophenols considered a valued class of natural products used for the practical application in the food, pharmaceutical, beverage, cosmetic and nutraceutical industries due to their powerful biological activity which includes antioxidant and antimicrobial properties [DELISI & al. 2016]. The processes for extracting and refining edible oils are well-established in industry at different scales [GABER & al. 2023]. The olive oil industry represents one of the fastest growing industrial sectors worldwide, such as Spain, Greece, and Italy, and becoming an important industry in countries, such as Chile, South Africa, or Argentina [ALONSO-FARIÑAS & al. 2020]. The commercial olive production generally reported to occur in two belts around the world, between 30° and 45° N latitude and between 30° and 45° S, where the climatic requirements for growth and fruitfulness available [BRITANNICA, 2023]. The most abundant agrifood residues in the Mediterranean basin derive from the traditional crop, olive trees. Olive leaves are also an abundant byproduct in the olive oil industry and characterized by high quantities of phenols [PALMERI & al. 2022]. The olive oil industry generates large amount of byproducts (leaves, branches) which negatively influenced on the economic and local environment due to incomplete processing [GULLÓN & al. 2020; ESPESO & al. 2021; GARCÍA-PASTOR & al. 2023].

The extraction of phenolic compounds through membrane technologies for their application in the food, cosmetic and/or pharmaceutical industry reported [CASSANO & al. 2011; CASSANO & al. 2013; GALANAKIS & al. 2010; PARASKEVA & al. 2007]. The olive mill wastewaters may be a suitable source of valuable compounds that could be used to transform an agro-industrial wastewater into useful and relevant ingredients [OBIED & al. 2009] and olive mill wastewater has been reported in the preparation of functional beverages [ZBAKH & EL ABBASSI, 2012]. However, the table olive industry generates large amounts of wastewater (liquid and solid form) due to the alkaline treatment, fermentation and washing steps and high content of organic matter, a high percentage of suspended solids and fats, an acidic or alkaline pH, high conductivity due to its high salt content, and colored waters due to the presence of polyphenolics compounds in wastewaters make their treatment difficult, leading to a relevant environmental problem with a complicated technological, economic, and social solution [DE LA CASA & al. 2009; BANIAS & al. 2017; FERRER-POLONIO & al. 2017; CAMPUS & al. 2018; FERNÁNDEZ-GONZÁLEZ & al. 2018; ELARIDI & al. 2020; MESSINEO & al. 2020; HADIDI & al. 2021; ARANEDA & al. 2023; GARCÍA-PASTOR & al. 2023).

The disturbances related issues in environment influences the production of olive crop yield and fruit color. Olive mill waste water (OMWW) is an acidic (pH 4-5), saline (EC ~ 5-10 mS cm^{-1}), blackish red aqueous byproduct of the three phase olive oil production process [AHARONOV-NADBORNY & al. 2017]. The effect of OMWW spreading on leaching of metal cations (Na, K, Mg, Mn, Fe, Cu, Zn) in four non-contaminated agricultural soils having different textures (sand, clay loam, clay, and loam) and chemical properties was determined. The results presented in this study demonstrate that OMWW spreading on agricultural soils, due to its organic load and the saline content, may mobilize various indigenous soil metal cations, including major elements such as Na and Mg, as well as heavy metals such as Cu and Zn and redox active metals such as Fe and Mn.

KAVROULAKIS & NTOUGIAS (2011) noted the bacterial diversity in *O. europaea* var. mastoidis generated olive mill waste waters consisted mainly of members of *Acetobacteriaceae*, *Prevotellaceae* and *Lactobacillaceae*, while the majority of β-proteo bacteria identified in *O. europaea* var. *koroneiki*-generated olive mill wastewaters were placed within the families *comamonadaceae Oxalobacteraceae*, *hydrogenophilaceae* and *rhodocyclaceae*. MARI & al. (2016) evaluated the occurrence of yeast populations during different olive oil extraction processes in three consecutive years in Tuscany (Italy), by analysing crushed pastes, kneaded pastes, oil from decanter and pomaces. The results showed yeast concentrations ranging between 10(3) and 10(5) CFU/g or per mL. These findings suggest a phenomenon of contamination of the plant for oil extraction that selects some yeast species that could affect the quality of olive oil.

Conclusions

This review study contributes to the understanding about the ecological, pharmacological, vegetative growth potential in different environmental habitat of a woody plant species, *O. europaea* at global scale. There is a special care required for conserving and cultivating of olive tree according to current trend of climate change around the world and serious effort is required for protection of this important plant species. Phytochemical studies revealed the presence of iridoids, flavonoids, volatile oil, and other metabolites. Available scientific references revealed that this plant species has many biological properties for health benefits for the treatment of inflammation, piles, cancer, diabetic, blood pressure and diarrhea, aches, jaundice, dysentery. This review was tried to cover the comprehensive knowledge of the traditional medicinal uses, phytochemistry, pharmacology, toxicology and ecology of *O. europaea* for researchers, farm manager and agriculturist working for the increase in cultivable areas. These ideas supported the keen importance of olive tree cultivation to human kind since ancient time.

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JOURNAL OF PLANT DEVELOPMENT
GUIDE TO AUTHORS

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