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DOWNREGULATION OF ARABINOGALACTAN PROTEINS DURING SPERMATOGENESIS IN THE MOSS *PHYSCOMITRIUM PATENS*

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Abstract: Seedless plants utilize flagellated sperm cells for reproduction that develop using a series of cell walls resulting in naked motile cells. Arabinogalactan Proteins (AGPs) have been shown to play an important role in the maturation of sperm in ferns by an unknown mechanism. We sought to identify AGPs expressed in the spermatids of the moss *Physcomitrium patens* to identify this mechanism because it is amenable to genetic manipulation. We tracked the expression of 121 putative AGP-encoding genes across three time points of development with RNAseq and quantified total AGPs to compare to the fern *Ceratopteris richardii*. Unexpectedly, AGP genes and proteins were significantly downregulated in *P. patens*. BURP domain-containing genes, which are expressed in pollen of angiosperms, were highly upregulated and may serve similar roles to the AGPs of ferns. This study shows that the fern cell walls do not share as significant of a need for AGPs of developing sperm in bryophytes and this may be related to the number of flagella found in the respective lineages.

Keywords: bryophyte, BURP-domain proteins, cell wall, flagella, gametogenesis, spermatid development.

Introduction

Throughout plant evolution, key adaptations arose in major lineages allowing them to thrive in drier conditions. One feature that has undergone dramatic changes among lineages is the morphology of the sperm cell [SOUTHWORTH & CRESTI, 1997]. The male gamete of the oldest land plants form a naked coiled cell containing a pair of flagella reminiscent of sperm from related algae. The pollen grains in most seed plants represent the entire microgametophyte and only produce sperm nuclei housed within. As new lineages emerged from ancestral bryophytes, sperm cells increase their number of flagella whereas ferns have dozens, but a few pollen-bearing seed plants, Cycads and Ginkgo, can have upwards of 50,000 flagella. In these, sperm travel through a pollen tube that bursts open just shy of the egg and the flagella drive the sperm across the short distance within the ovule to the egg [RENZAGLIA & GARBARY, 2001; SOUTHWORTH & CRESTI, 1997].

Hydration of mature antheridia in seedless plants cue sperm release, initiating their journey to the egg-bearing archegonium to form the zygote. In the later stages of development, sperm transform from the characteristic boxy plant cell to take on their coiled shape while the locomotory apparatus forms at the cell anterior concurrent with the deletion of cytoplasm, facilitated by the chemically unique extraprotoplasmic matrix (EPM) [BERNHARD & RENZAGLIA, 1995; GENAU & al. 2021; LOPEZ & al. 2017]. The locomotory apparatus contains a number of microtubule based structures including the lamellar strip, the overlying

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flagella, and their anchoring basal bodies all attached to a row of connected microtubules, known as a spline [RENZAGLIA & al. 2000]. Sperm of most gymnosperms and all flowering plants, lack any need for flagella as pollinators and pollen tubes cooperate to deliver sperm nuclei directly to the egg.

Arabinogalactan Proteins (AGPs) are excreted glycoproteins containing large branching sugar residues consisting primarily of arabinose and galactose. They are encompassed within the hydroxyproline-rich glycoprotein family, along with extensins and proline-rich proteins [SEIFERT & ROBERTS, 2007]. Historically, they are defined by their small proteinaceous backbones with high proportions of hydroxyproline that act as *O*-glycosylation points [TAN & al. 2003, 2010]. However, a survey of plant genomes found much higher AGP diversity, including the formation of chimeras with kinases, formins, or cell wall modifying domains among other subfamilies [MA & al. 2017]. The authors of this survey ultimately define AGPs by the proportion of "glycomodules" to gene length. Glycomodules are the dipeptides enriched in AGP sequences consisting of Pro + [Ala, Ser, Thr]. While most have a signal peptide and many are predicted to be GPI-anchored, these do not strictly define the proteins. Those that are GPI-anchored have the ability to remain linked to the outer surface of the plasma membrane, but may also render them susceptible to cleavage by phospholipases that would release them into the wall where they may play myriad roles [BORNER & al. 2003; ELORTZA & al. 2003, 2006]. The nature of this is, however, consistently vague with little direct evidence of a molecular mechanism [LAMPORT & al. 2014].

AGPs are known to play roles in many aspects of plant biology such as cell fate determination, plant microbe-interactions, gravitropism, and more [SEIFERT & ROBERTS, 2007], but they commonly facilitate aspects of reproduction across most land plants. They are produced at various developmental events and in key tissues involved in pollen and sperm production in a diverse sampling of flowering plants [CHUDZIK & al. 2014; COSTA & al. 2015; LI & al. 1992; MA & al. 2019; QIN & al. 2007; SOUTHWORTH & KWIATKOWSKI, 1996] and gymnosperms [MOGAMI & al. 1999; YATOMI & al. 2002]. Both the generative cell, giving rise to two sperm cells, and vegetative cell, controlling the growth of the pollen tube, strongly label for AGPs in most angiosperms and they are believed to function in both pollen tube elongation and pollen-pistil interactions affecting compatibility and efficient delivery of the sperm cells in non-flagellated plant lineages [LESZCZUK & al. 2019; SEIFERT & ROBERTS, 2007]. In the fern *Ceratopteris*, varying AGP populations are differentially expressed through spermatogenesis and are intimately associated with flagella throughout their elongation in the extracellular matrix [LOPEZ & RENZAGLIA, 2014]. When the AGPs are chemically removed late in development, flagella become haphazardly arranged and the cell fails to eliminate cytoplasm or develop the microtubular backbone. The purpose of this study was to directly identify AGP encoding genes associated with spermatogenesis in flagellated plants using the model moss *Physcomitrium patens* by tracking AGP expression, family-wide, throughout antheridium development in order to test for a more specific mechanism of AGP activity.

Materials and methods

Tissue growing conditions. *Ceratopteris richardii* spores were obtained and grown according to the manufacturer (Carolina Biological, Burlington, NC, USA). *Physcomitrium patens* culture was maintained under 24 °C 24hr light at 400-500 lux every 7-12 days growing on PpNH4 media. In preparation for inducing gametangia, 2 mm balls of protonemal tissue were arranged in a 9x9 grid on BCD+Ammonium Tartarate (5 mM) media [RESKI & COVE, 2004]

and grown for 5 weeks to allow gametophore development. Gametangia were induced as described [HOHE & al. 2002]. Antheridia development was confirmed by microscopy prior to harvest. For RNA-seq samples, tissue was frozen in liquid nitrogen, ground to a fine powder and stored at -80 °C before RNA extraction. For qPCR samples, gametophore tips only were dissected, frozen, and stored at -80 °C. These samples were homogenized in RNAzol (Molecular Research Center, Cincinnati, OH, USA) using microfuge pestles followed by an application of the homogenate to a shredder spin column (Lamda Biotech, St. Louis, MO, USA).

RNA prep, RNA-seq, and analysis. RNA was extracted from quadruplicate samples of each timepoint using RNAzol, according to the manufacturer's protocol. Subsequently, samples were DNAse I treated and concentrated using a silica column (Zymo research, Irvine, CA, USA). Samples were assessed using a Qubit and 2100 Bioanalyser, ribo-depleted with a RiboZero Plant kit. Libraries were created with NEXTflex Rapid Directional qRNA-seq kit and sequenced with a HiSeq4000 (75pb paired-end, 30M reads per sample) performed by ACGT, Inc (Wheeling, IL, USA). Bcl2fastq was used to de-multiplex the raw reads. These data were further analyzed by the University of Virginia Bioinformatics Core, including quantitation and expression analyses.

For qPCR analysis, RNA quality was assessed visually on a gel and equivalent quantities were converted to cDNA using Protoscript II Reverse Transcriptase (NEB, Ipswich, MA, USA). qPCR was conducted using the Pfaffl method, after primer pair efficiencies were calculated in triplicate [PFAFFL, 2001], using PowerUp SYBR green master mix (Life Technologies, Carlsbad, CA, USA) on a QuantStudio3 thermocycler. Primer pairs are listed in Table 1.

Table 1. Primers used in qPCR analyses

Primers	Gene Locus		Efficiency	Sequence
E-256	Pp3c7_430	Fasciclin-like AGP	2.14	TCCTTCTCTCTCTACTCTTCCCACTTC
E-257				CCTGATACCTCCAATCGCCAAC
E-258	Pp3c21_10620	Periostin-Related	1.94	AGCCTTCACCATCACCAG
E-259				TAGAGCGACAACAGCGGAC
E-252	Pp3c1_4130	Fasciclin-like AGP	1.94	AGCAGCGAAGGTCTACAG
E-253				GTCGATACCATGAACAGCAAC
E-254	Pp3c13_8280	Fasciclin-like AGP	2.06	TTGCGCCCTTCTCGTTTC
E-255				GCAACAACTTTCTCGTGCAG
E-476	Pp3c10_22850	Periostin-Related	1.97	GCTTCCTAACACTACCTTGAG
E-477				CTCCAGTGAGAGCAAATACC
E-478	Pp3c26_8120	IRAK1	*ΔΔCt	GCCTACTGCTTCTTGTATTGC
E-479				TTGCATGGAGTTGTGTCTG
E-480	Pp3c5_14830	Probable LTP_2	*ΔΔCt	TCGCACCGCTTGTATCTG
E-481				CGAGCGTGAAACAAGGAAC
E-484	Pp3c16_19400	BURP domain	1.94	GATCAGAGCAAGGAAAGTCG
E-485				TATCCTTTGAGTGGGACCTG
E-470	Pp3c5_9210	AGP-31	1.96	TGAAAGATGCCGAGGTGG
E-471				TTTCGCCTTACATCCATTGC
E-272	Pp3c13_2360	Reference Gene; RP-L21e	1.94	TTTCTCTTCTTCCTCTCGCTC
E-273				TTGTGCCTGAAGGGTCTG
E-533	Pp3c8_1210	LTP_2	*ΔΔCt	AATCGGACATCATGCCTTC
E-534				CTGGAATAACTCTGCCATTGC

E-535	Pp3c12_11710	PLAT/LH2 domain	*ΔΔCt	GAGTGCCATGCAGAGCAAC
E-536				TGCGTCTATCCCACGATGA
E-537	Pp3c2_4510	BURP domain	*ΔΔCt	ACGCATCACCACTCTACAGC
E-538				CGTCCATCACCTTCCATCTC

AGP extraction. Soluble AGPs were extracted according to [POPPER, 2011]. Total protein was quantified using the BCA method and total AGPs were quantified using a radial gel diffusion assay [VAN HOLST & CLARKE, 1985]. The gel diffusion assay showed an R^2>0.99 using Gum Arabic as a standard with a dilution series between 0.031-2 µg.

Results and discussions

MA & al. (2017) identified AGPs from across the plant kingdom, and found 104 putative AGPs in the *P. patens* genome. Dynamic expression of this suite of genes was assessed using RNA-seq in plants at two points during spermatogenesis compared against non-induced samples. *P. patens* produces antheridia when exposed to low light, low temperature and long day conditions (16 dark/8 light) [HOHE & al. 2002]. Upon induction, new antheridia initials form and mature over 10 defined stages [LANDBERG & al. 2013]. However, additional antheridia initials form in succession after the previous. While the oldest antheridia may mature by 16 days after induction (stage 9), there will be a greater percentage of stage 9 antheridia later as additional sets of antheridia form and then mature. With this in mind, we sampled plants at 0 days past induction (dpi), showing only vegetative growth, at 15 dpi, when the first antheridia are nearly mature, and 28 dpi, when 2-4 antheridia in each cluster are mature and relatively few young antheridia apparent. When expression of all 104 putative AGPs were assessed, it was found that the majority were either downregulated or saw no significate change in expression (Figure 1A and archived data [JOHNSON, 2023]). Only two putative AGPs saw moderate upregulation during spermatogenesis in the initial screen. These include Pp3c26_8120 which showed a mere 1.28 Log_2 fold change (Log_2FC) at 15dpi that was sustained in the 28 dpi samples; and Pp3c10_22850 showed a 1.55 Log_2FC increase in 28 dpi vs. 0 dpi samples. A third putative AGP, Pp3c5_9210, not identified as an AGP by MA & al. (2017) was expressed strongly only in the 28dpi sample with a 2.96 log_2FC (Figure 1A). It is not surprising that some AGPs were missing from the target list as the authors acknowledged using a high threshold that likely excluded some genes. In validating these results with qPCR, we found that Pp3c26_8210 was too lowly expressed to accurately quantify in most replicates. Pp3c10_22850 saw a gradual increase in expression at 15dpi, but then a gradual downregulation at 28dpi. The third putative AGP, Pp3c5_9210 did see increased expression at both 15dpi at 1.82 Log_2(FC) and a much stronger increase of 5.77 Log_2(FC) at 28dpi (Figure 1B). The RNA-seq samples contained whole gametophores in an attempt to harvest tissue quickly, though this may have resulted in a dilution of spermatid-derived RNA with vegetative tissue. Subsequent qPCR analysis contained only gametophore tips, where antheridia are bore, giving a higher concentration of the tissues/cells of interest so a larger Log_2(FC) in our qPCR analysis is not unexpected.

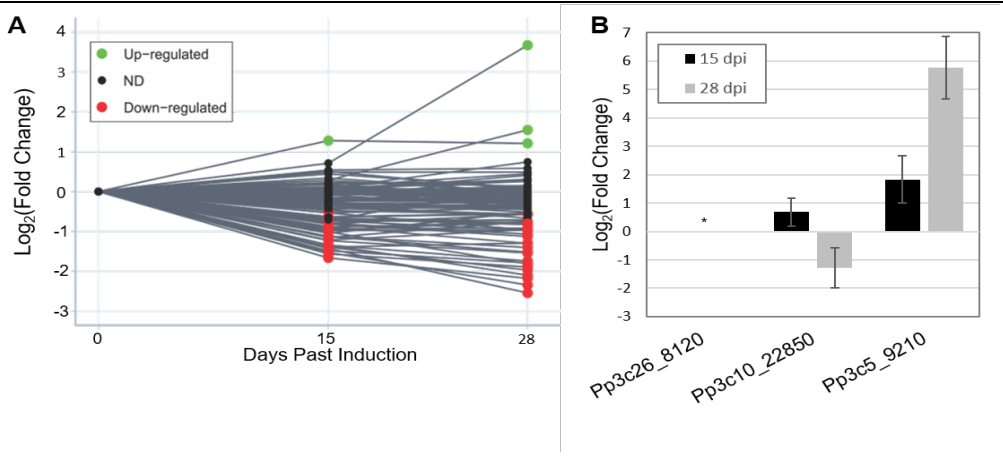

Figure 1. AGP-transcriptome dynamics during spermatogenesis in *P. patens*. **A**. normalized relative transcript counts of 104 putative AGP-encoding genes 0, 15 or 28 days past induction. Only three AGPs show a significant up-regulation, while the majority show no change (ND) and many show down-regulation. $p > 0.05$. **B**. qPCR analysis of AGPs in antheridia-induced tissue at 15 and 28 dpi.
* Pp3c26_8120 was not expressed at sufficient enough levels to measure in 3 out of 4 replicates. Bars are +/- SE. N=3-4.

Since the drop in AGP expression was initially unexpected, we wanted to confirm whether this change was reflected in total protein changes over the same time period. Total soluble AGPs were extracted and quantified (Figure 2A). While there was no significant change between 0- and 15-dpi samples, there was a significant decrease in AGPs during the maturation of sperm cells at 28 dpi, whereas we found 0.065, 0.076, and 0.036 µg AGP/µg extracted protein, respectively. We then quantified AGPs in the fern *C. richardi* to ensure that the total protein extract was reflective of the ultrastructural analysis that initially identified the increase in spermatogenesis-related AGPs [LOPEZ & RENZAGLIA, 2014]. As expected, a significant increase in AGPs was noted when sperm cells matured and at the same timepoint point that swimming sperm were observed, 11 days after sowing (das), compared to 8 das; $p<0.05$ (Figure 2B). While it should be noted that *C. richardii* sperm mature more rapidly and the gametophytes are structurally simpler and short-lived, there was more than 10 times the mass of AGPs in *C. richardii* compared to *P. patens*. AGP levels increased from 0.310 to 0.569 µg AGP/µg extracted protein as sperm matured. In conjunction with our gene expression analysis, this suggests that AGPs play a far reduced role in spermatogenesis in moss compared to *C. richardii*. However, a pair of studies investigating the cell wall makeup of maturing moss sperm cells in *P. patens* and *Aulacomnium palustre* note the presence of dynamic populations of AGP-epitopes in the spermatid walls that wanes some as maturation commences [HENRY, 2021; LOPEZ-SWALLS, 2016]. From our data, it is unclear how or when these AGPs were expressed, though there is an emerging theme that the presence of particular AGPs may arise from the presence of the particular glycotransferases that build them under given conditions rather than the AGP-encoding genes themselves [SILVA & al. 2020]. It is possible that one/some of the non-differentially expressed genes in our study codes for the AGPs found in mature sperm and the changing epitopes labelled by [HENRY, 2021] result from the differential expression of AGP-modifying glycotransferases. Our data, however, clearly show a reduction in AGP expression during spermatogenesis.

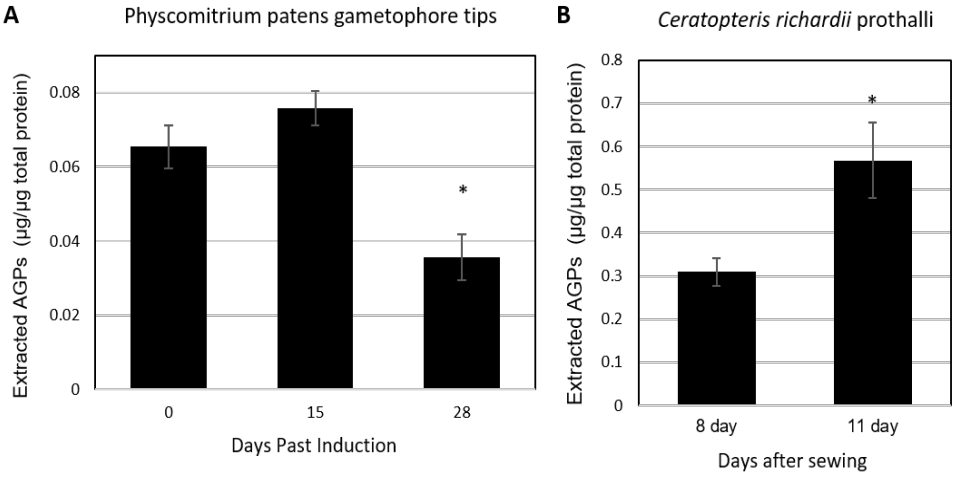

Figure 2. Arabinogalactan protein production during spermatogenesis. **A**. Extracted AGPs from developing *P. patens* gametophore tip quantified by radial diffusion assay. Note decrease in AGP levels during the maturation of sperm cells. At 28 dpi significantly fewer AGPs ($p<0.05$) are produced compared to non-induced tissue **B**. Extracted AGPs from developing *Ceratopteris richardii* prothalli, a flagellated plant known to require AGPs in sperm development. AGP levels are significantly increased ($p<0.05$) at 11 days after sewing compared to 8 days. Bars = SE; n = 3-4.

What was upregulated? Of the transcripts that were up regulated, no GO terms were significantly enriched. However, when the most up-regulated genes were assessed individually, some anticipated and unanticipated genes were identified (See Table 2 and archived data [JOHNSON, 2023]). For example, a β-tubulin component and two histone associated transcripts (Pp3c13_14080 and Pp3c26_7260) were among the top upregulated genes comparing 15 dpi to 28 dpi samples, as would be expected with the production of key microtubule-based structures and the spiral shaping of the nucleus. A key component to their maturation is the deletion of cytoplasm and organization of flagella into the extraprotoplasmic matrix. Thus, we would expect to find an upregulation of genes encoding secreted proteins, cell wall modifying enzymes and transport proteins, or possibly catabolic enzymes for cytoplasmic deletion and lysosome-related enzymes. A catabolic PLAT/LH2 protein (Pp3c12_11710), and four putative secreted proteins: a Lipid Transfer Protein (Pp3c8_1210), a pollen Ole e I Allergen (Pp3c5_9210) and two BURP domain containing proteins PpBURP4 (Pp3c16_19400) and PpBURP5 (Pp3c2_4510) were among the most highly upregulated.

What was downregulated? The genes most downregulated throughout sperm cell development are mostly associated with photosynthesis (Table 2 and archived data [JOHNSON, 2023]). This is unsurprising as induction of antheridia involves a low light, short day, low temperature treatment. However, there may be a more direct link to spermatogenesis or antheridia development as male fern gametophytes also show lower levels of photosynthesis compared to their hermaphroditic counterparts [CHEN & al. 2019]. This is also in agreement with the observation that plastids are reduced early during spermatogenesis in the liverwort *Blasia pusila* [RENZAGLIA & DUCKETT, 1987].

Table 2. Most up-regulated genes during late antheridium development. Log2 (fold-increase) in samples, ranked compared 15dpi vs. 28 dpi to emphasize late-stage spermatid development.

Gene code	Putative function	Sig rank[1]	15 dpi vs. 28 dpi	0 dpi vs. 15 dpi	0 dpi vs. 28 dpi	Basemean
Pp3c16_19400	BURP domain-containing protein	1	5.06	0.62	5.68	59.03
Pp3c12_11710	Lipase/lipooxygenase, PLAT/LH2 family protein	2	5.06	0.00	5.21	41.05
Pp3c13_14080	Linker histone H1 and H5 family	3	4.85	0.15	5.00	35.19
Pp3c8_1210	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	4	4.30	0.51	4.81	39.20
Pp3c2_4510	BURP domain-containing protein	6	3.98	0.15	4.13	19.05
Pp3c5_9210	Pollen Ole e 1 allergen and extensin family protein (A subclass of AGPs)	9	2.96	0.71	3.67	43.46
Pp3c26_470	Dehydrin family	12	2.62	0.22	2.84	251.42
Pp3c26_7260	histone H1-3	13	2.52	0.00	2.66	7.61
Pp3c8_2420	Tubulin beta chain 2	16	2.41	0.25	2.66	6.66
Pp3c10_19280	SF5-Stromal Interaction Molecule Homolog	17	2.30	0.49	2.79	63.49

[1]: Sig rank = rank of most significant (Padj) comparing 15dpi vs. 28dpi samples

Table 3. Most down-regulated genes during late antheridium development. Log2 (fold-increase) in samples, ranked compared 15dpi vs. 28dpi to emphasize late-stage spermatid development.

Gene code	Putative function	Sig rank[1]	15 dpi vs. 28 dpi	0 dpi vs. 15 dpi	0 dpi vs. 28 dpi	Basemean
Pp3c13_15990	Global Transcription Factor	5	-1.54	NA	-2.28	1106.97
Pp3c13_15980	ribulose bisphosphate carboxylase small chain 1A	7	-1.96	-2.55	-4.51	39257.61
Pp3c13_16130	ribulose bisphosphate carboxylase small chain 1A	8	-1.94	-2.03	-3.97	4418.33
Pp3c13_15790	NA	10	-1.99	-1.19	-3.18	199.05
Pp3c13_15786	ribulose bisphosphate carboxylase small chain 1A	11	-1.90	-3.28	-5.17	304.44
Pp3c13_15800	ribulose bisphosphate carboxylase small chain 1A	55	-1.24	-3.73	-4.97	7098.13
Pp3c12_22350	carotenoid cleavage dioxygenase 1	312	NS	-4.69	-3.68	101.34
Pp3c13_2310	photosystem I subunit D-2	925	NS	-1.71	-2.12	291.15
Pp3c27_2340	photosystem II subunit R	1757	NS	-1.73	-1.46	2064.85
Pp3c13_16000	ribulose bisphosphate carboxylase small chain 1A	1799	NS	-2.53	-3.01	1919.28
Pp3c18_7850	NA	3120	NS	-5.19	-5.72	259.74
Pp3c14_380	Disease resistance-responsive (dirigent-like protein) family protein	7060	NS	-2.53	-2.73	2644.46
Pp3c8_7680	NA	8096	NS	-3.67	-3.47	33208.92
Pp3c19_21160	plastocyanin 1	10180	NS	-1.44	-1.53	5099.99
Pp3c3_18750	ribulose bisphosphate carboxylase small chain 1A	18734	NS	-2.25	-2.31	3203.72

[1]: Sig rank = rank of most significant (Padj) comparing 15dpi vs. 28dpi samples

It has become clear that AGPs can play a major role serving as a Ca^{2+} store in the apoplast in a pH dependent manner through the negatively charged GlcA residues common to AGPs [LAMPORT & VÁRNAI, 2013; LOPEZ-HERNANDEZ & al. 2020; ZHANG & al. 2021]. The high proportion of AGPs found in *Ceratopteris* spermatids were suggested to support flagella positioning and elongation through this calcium signaling mechanism [LOPEZ & RENZAGLIA, 2018]. Cortical microtubule dynamics are influenced by Ca^{2+} [NICK, 2013] as are some interactions between AGPs and microtubules [NGUEMA-ONA & al. 2007; SARDAR & al. 2006], though a direct role in the polymerization of the stable microtubules found in flagella is not known. However, this mechanism could help explain why moss spermatocytes, with only 2 flagella show reduced AGP gene expression and protein as compared to the sperm of *Ceratopteris* with approximately 80 flagella [RENZAGLIA & al. 2017]. The diversity in AGP-glycosylation patterns found in tracheophytes [MUELLER & al. 2023] gives the possibility that AGPs evolved to provide a chemical environment facilitating development of these more complex sperm cells after the split from bryophytes. Furthermore, AGPs are also well known to be involved in tip growth, for which *P. patens* would have displayed significant protonema growth under vegetative conditions [LEE & al. 2005; TEH & al. 2022]. As the plants shift away from vegetative growth, the need for broad production of AGPs would also wane. [MIGNONE & BASILE, 2000] found that a strong increase in AGP production occurs as *P. patens* produces buds and transitions into gametophore growth, but in agreement with our data, the plants shift away from their production as gametophores transition into gametangia production.

Physcomitrium patens ecotypes. It was recently discovered that the most commonly studied ecotype of *P. patens*, Gransden (Gd), has accumulated (epi-)genetic changes resulting in spermatozoids with poorly formed flagella [HISS & al. 2017; MEYBERG & al. 2020]. This was attributed to consistent subculturing and encouraged the characterization of the Reute (Re) ecotype which shows healthy sperm production. To determine the nature of this in Gd, differentially expressed genes were determined and the key flaw in Gd seems to involve both the polymerization of its axoneme microtubules within the flagella as well as the adhearence of the plasma membrane to the flagella, roles AGPs could perceivably be involved in. However, off the 15 putative AGPs we identified as down-regulated in Gd, they were found to be similarly down-regulated or show no change in Re between juvenile and adult gametophore tissue, according to PEATmoss [FERNANDEZ-POZO & al. 2020]. This confirms our primary finding that AGP downregulation during gametangia development occurs in both Re and Gd. The only strongly up-regulated AGP we identified, Pp3c5_9210V3.1, was similarly upregulated in Ru and it was subsequently shown to contain a premature stop codon in Re [MEYBERG & al. 2020]. Clearly it is not necessary for normal sperm cell development. Furthermore, we confirmed that the two BURP domain containing genes were also strongly upregulated in the Reute ecotype of *P. patens* during sperm cell maturation (Figure 3). Although our developmental approach to characterizing changes in gene expression was conducted in Gd, the genes identified show similar expression patterns in both ecotypes.

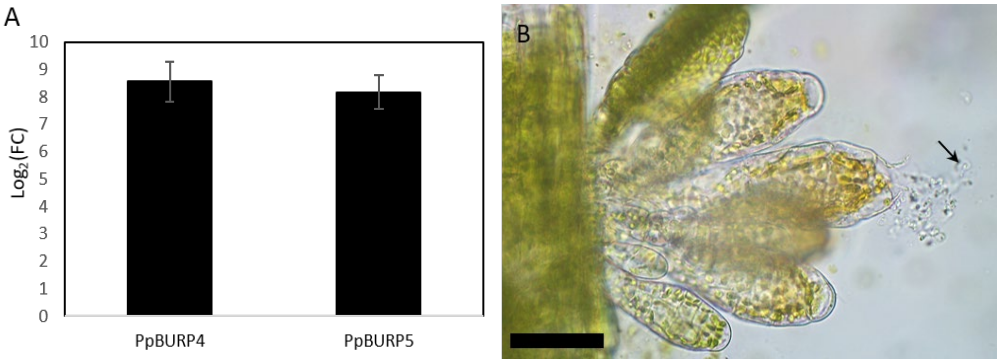

Figure 3. PpBURP4 and PpBURP5 are highly expressed during antheridia maturation in Reute ecotype. **A**. qPCR analysis of PpBURP4 and PpBURP5 in tissue 28 days past induction of antheridia formation. **B**. Image is representative of 28dpi tissue with mature swimming sperm (arrow) and a series of younger antheridia. Bar = 50 µm.

BURP-domain containing proteins. Two of the most highly upregulated genes in this study belonged to a family of genes that shows some similarities to AGPs in that they are extracellular proteins [WANG & al. 2012; XU & al. 2013] that play roles in many stress and developmental pathways. There are also a number of reports of involvement of these genes with male gametes of seed plants [KHLAIMONGKHON & al. 2021; SUN & al. 2019; TREACY & al. 1997; WANG & al. 2003]. It could be that these proteins are produced and secreted to help modify the cell walls or extra protoplasmic matrices that serve a homologous function to the AGP-rich layer in *C. richardii* spermatogenesis [LOPEZ & RENZAGLIA, 2014].

The BURP domain itself was initially characterized by its presence in BMN2, a microspore protein from *Brassica napus* [BOUTILIER & al. 1994]; USP, a nonstorage seed protein from *Vicia faba* [BASSÜNER & al. 1988]; RD22, a dehydration responsive protein from Arabidopsis [YAMAGUCHI-SHINOZAKI & SHINOZAKI, 1993]; and *PG1*β, a noncatalytic subunit of polygalacturonase isozyme I from *Solanum lycopersicum* [ZHENG & al. 1992]. The domain is located at the C-terminus and built from a pair of spaced cystine residues followed by four cysteine-histidine repeats ($CHX_{10}CHX_{23\text{-}27}CHX_{23\text{-}26}CHX_8W$) [DING & al. 2009; WANG & al. 2015]. This protein family emerged with the migration onto land but broad functional diversification only occurred after the split from lycophytes and likely showed strong diversification more recently among angiosperms [WANG & al. 2015; YU & al. 2022]. Phylogenetic analyses show most moss and lycophyte BURPs segregating into their own subfamily, BURP-IV, giving some question as to broad functional comparisons of these proteins between major plant lineages. To compound on that, each of the namesake genes for the family cluster into the three other BURP-domain containing gene subfamilies; including BMN1-like, PG1β-like, and BURPIII that includes RD22- and USP- homologues, which are all absent from lower land plants and limiting our ability to extrapolate functions of the identified genes. Regardless, the functional characterization of any BURP domain containing protein has only recently occurred whereas some members show autocatalytic peptide cyclase activity to form bioactive cyclopeptides [CHIGUMBA & al. 2022]. These are largely considered to be specialized metabolites that play defensive roles across a number of vascular plant lineages and it is unclear how these might contribute to the development of sperm cells in bryophytes. These

data highlight the need to further characterize BURP domain containing genes from seedless plants to determine their importance in spermatogenesis.

Conclusions

Sperm cells represent brief time in an organism's life cycle in which the fate of a lineage falls onto a single cell. With this, it is not surprising that plant evolution shows only incremental changes in overall structure when comparing the major lineages. It was surprising to find major differences in the importance of AGPs during spermatogenesis comparing the model moss and fern. AGPs were hypothesized to facilitate the formation of flagella in ferns and our data may actually agree as the pair of flagella in bryophytes wouldn't require as great a need for these glycoproteins as the 80+ flagella found in *Ceratopteris* sperm. This begs to determine the quantity of AGPs found in the development of Cycads and Ginkgo and their 1000s of flagella. As an alternative explanation, findings that seedless plants show the most structural diversity of AGPs among plant lineages could have provided ferns a mechanism to evolve and change the makeup of the spermatid extraprotoplasmic matrix during maturation to provide roles that other cell wall components may be serving in bryophytes, such as BURP-domain containing proteins.

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NEW DATA ON THE DISTRIBUTION AND INVASION STATUS OF SOME ALIEN PLANTS IN ROMANIA

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Abstract: In this paper we report new chorological data for 20 alien plant taxa from the vascular flora of Romania. A total of 9 species (*Campsis radicans*, *Euphorbia glyptosperma*, *Grindelia squarrosa*, *Impatiens balfourii*, *Oenothera suaveolens*, *Robinia* × *ambigua*, *Rudbeckia triloba*, *Sedum sarmentosum*, *Setaria faberi*) are reported as new to the regional floras within the country. *Oenothera pycnocarpa* and *Dittrichia graveolens* are reported in their second and the third sites in Romania, respectively. For the remaining 9 species (*Cytisus scoparius*, *Dysphania pumilio*, *Eleusine indica*, *Erigeron sumatrensis*, *Eriochloa villosa*, *Oenothera depressa*, *Paspalum distichum*, *Rosa rugosa* and *Sicyos angulatus*), we provided new field data, to improve knowledge on their current distribution and invasion status. All taxa are neophytes, introduced either accidentally (11 taxa) or deliberately (9 taxa), more than half of which are currently invasive or potentially invasive in the country.

Keywords: chorological data, invasion status, neophytes, new floristic records, vascular flora.

Introduction

Research on alien flora, mainly on neophytes (*i.e*. alien plant species introduced after the year 1500, according to RICHARDSON & al. 2000), has registered notable development in recent decades in Romania. For all neophytes cited in the vascular flora of Romania until 2011, historical data on their distribution and invasive status in the country were previously extensively documented by ANASTASIU & NEGREAN (2009) and SÎRBU & OPREA (2011). After 2011, new data on neophytes from Romania were added by reporting on some previously unrecorded species [e.g.: ANASTASIU & MEMEDEMIN, 2012; OPREA & al. 2012; NAGODĂ & al. 2013; CAMEN-COMĂNESCU & al. 2016; NEGREAN & al. 2017; SÎRBU & OPREA, 2017; SÎRBU & ȘUȘNIA, 2018; SZATMARI & HURDU, 2020, etc.], but also by new contributions to knowledge on their distribution, habitat preferences and invasive status in the country [e.g.: SÎRBU & al. 2012; NAGODĂ, 2015; NEGREAN & al. 2017; OPREA & al. 2021; SÎRBU & al. 2021; ȘUȘNIA, 2022, etc.]. An extensive literature review on alien plant species (primarily neophytes) in Romania, covering the 1778-2018 time-period has been compiled recently [SÎRBU & al. 2022], resulting in a list of 102 invasive or potentially invasive alien plant species in the country.

Field research on this topic increased substantially especially during 2019-2022, when the project "*The adequate management of invasive species in Romania in accordance with EU Regulation*" [Code SMIS 120008; https://invasive.ccmesi.ro/], coordinated by Dr. P. ANASTASIU, was carried out at the national level.

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This work aims to update knowledge on distribution and invasive status of a number of 20 neophytes from Romania, some of which are here reported as new to the regional floras within the country.

Material and methods

Species of neophytes were recorded as a result of our field works between 2012 and 2022 (but particularly in the last 3 years), in various regions of the country. For species identification and nomenclature we used standard floras, such as: BRITTON & BROWN (1970), KOMAROV & al. (1968-2004), TUTIN & al. (1968-1980, 1993), SÂRBU & al. (2013) etc. The invasive status in Romania (*i.e.*, casual, naturalised or invasive) of neophytes listed in the paper was established according to RICHARDSON & al. (2000) and PYŠEK & al. (2004). The geographic coordinates (northern latitude, eastern longitude) were recorded in the field using the application OsmAnd (https://osmand.net/). Voucher specimens were deposited in the Herbarium of the University of Life Science "Ion Ionescu de la Brad" of Iaşi (IASI) and the Herbarium of the Botanical Garden "Anastasie Fătu", "Alexandru Ioan Cuza" University of Iaşi (IAGB). Phytosociological data for *Cytisus scoparius* were recorded and presented according to CRISTEA & al. (2004) and COLDEA (2012). Limits of the geographical-historical regions (provinces) of Romania mentioned for each species are consistent with CLEMENT & al. (1959).

Results and discussions

Campsis radicans (L.) Seem. (*Tecoma radicans* Juss.)

This is a liana native to North America, widely cultivated as ornamental [CIOCÂRLAN, 2009]. It has been cited as a casual alien (escaped from cultivation) in several localities of Dobrogea [ANASTASIU & al. 2009; MEMEDEMIN & al. 2016] – including the Danube Delta [ANASTASIU & al. 2014], Muntenia [NAGODĂ & al. 2014; NAGODĂ, 2015] and Transylvania [NEGREAN & al. 2017].

We report it now for the first time in three historical provinces of Romania, namely Moldova (Bacău, Botoșani, Iași, Neamț, Suceava and Vrancea counties), Oltenia (Gorj and Vâlcea counties) and Banat (Timiș County):

– Bacău County: Răcăciuni – roadsides and vacant grounds (N 46.33478, E 27.00289; *leg. et det*. C. Sîrbu, 27.VIII.2020);

– Botoșani County: Miorcani – derelict old fences (N 48.20180, 26.85153; *leg. et det*. A. Oprea, 13.XI.2020);

– Iași County: Bivolari – derelict fences and vacant grounds (N 47.51085, E 27.43692; *leg. et det*. A. Oprea, 06.X.2021), Hârlău – railway embankment and associated disturbed grounds (N 44.62100, E 26.92012; *leg. et det*. C. Sîrbu & A. Oprea, 09.X.2019), Scânteia – derelict old fences (N 47.33049, E 27.56485; *leg. et det*. A. Oprea, 30.VIII.2021);

– Neamț County: Bicaz – train station and associated disturbed lands (N 46.86142, E 26.09889; *leg. et det*. C. Sîrbu, 31.VII.2020), Tupilați – derelict old fences (N 46.88473, E 26.63539; *leg. et det*. A. Oprea, 10.X.2021), Ştefan cel Mare – derelict old fences (N 46.75446, E 26.51116; *leg. et det*. A. Oprea, 11.X.2021);

– Suceava County: Dolhasca – disturbed grounds, along the railway track (N 47.54022, E 26.60391; *leg. et det*. C. Sîrbu & A. Oprea, 10.VII.2021);

– Vrancea County: Râmniceni – disturbed grounds (N 45.53679, E 27.44885; *leg. et det*. A. Oprea, 13.VI.2021), Alexandru Vlahuță towards Cândești – roadsides (N 47.54172, E 27.10132; *leg. et det*. C. Sîrbu & A. Oprea, 27.VIII.2022).

– Gorj County: Roșiuța – derelict fences and walls (N 44.80089, E22.98152; *leg. et det*. A. Oprea, 13.VIII.2020);

– Vâlcea County: Căzănești – derelict fences and vacant grounds (N 45.93423, E 24.28324; *leg. et det*. A. Oprea, 12.VIII.2020), Copăcelu – derelict fences and vacant grounds (N 45.95138, E 24.30619; *leg. et det*. A. Oprea, 12.VIII.2020);

– Timiș County: Timișoara – at the edge of the "Green Forest" ("Pădurea Verde") (N 45.77837, E 21.25194; *leg. et det*. C. Sîrbu, 10.VI.2017).

New chorological data from other regions:

– Brașov County: Bod – vacant grounds (N 45.76760, E 25.64433; *leg. et det*. A. Oprea, 16.IX.2022), Cristian – derelict fences and walls (N 45.50592, E 25.48369; *leg. et det*. A. Oprea, 23.VIII.2020);

– Covasna County: Ozun – derelict fences and walls, vacant grounds (N 45.10467, E 25.84720; *leg. et det*. A. Oprea, 12.VIII.2020);

– Buzău County: Buzău, The Marghiloman Park – flower beds, edges of paths, spaces between pavers (N 45.14716, E 26.84534; *leg. et det*. A. Oprea & C. Sîrbu, 31.VIII.2022);

– Tulcea county: Tulcea – on the Danube right bank (N 45.17995, E 28.80316; *leg. et det*. C. Sîrbu & A. Oprea, 02.VIII.2020), vacant ground next to the "Monumentul Eroilor" (N 45.18646, E 28.81441 *leg. et det*. C. Sîrbu & A. Oprea, 02.VIII.2020); Periprava – derelict fences (N 45.40503, E 29.54127; *leg. et det*. C. Sîrbu, A. Oprea, M. Doroftei, S. Covaliov 03.VIII.2021), Mila 23 – the channel bank and roadside along the channel (N 45.21678, E 29.23539; *leg. et det*. C. Sîrbu, A. Oprea, M. Doroftei, S. Covaliov, 13.VIII.2021); Caraorman – roadsides, vacant grounds, walls (N 45.07740, E 29.39205; *leg. et det*. C. Sîrbu & A. Oprea, 01.VIII.2022); Plopu – roadside and derelict yard (N 45.78572, E 29.13367; *leg. et det*. C. Sîrbu & A. Oprea, 31.VII.2022); Sarinasuf – derelict yard (N 45.75258, E 29.06776; *leg. et det*. C. Sîrbu & A. Oprea, 31.VII.2022), Sulina – household yard (N 45.09212, E 28.38370; M. Doroftei); Crișan – derelict yard (N 45.10261, E 29.23556; M. Doroftei); Mila 23 – derelict yard (N 45.13419, E 29.15046; N 45.13393, E 29.15083; M. Doroftei); Gorgova – the bank of the Danube River within the locality, roadside (N 45.10423, E 29.09445; N 45.10414, E 29.09389; M. Doroftei); Mila 36 channel joint with Șontea (N 45.15101, E 29.55327; M. Doroftei); Gârla Șontea – "La Scăunele" (N 45.15100, E 28.58437; M. Doroftei).

Current status in Romania's flora: casual (or quasi-naturalized).

Cytisus scoparius (L.) Link (*Sarothamnus scoparius* (L.) W. D. J. Koch) (Figure 1)

This is a species originating from Central, Western and Southern Europe [CIOCÂRLAN, 2009], reported (outside the cultivation sites) as naturalised [ANASTASIU & NEGREAN, 2009] or invasive [SÎRBU & al. 2022] in almost all provinces of the country so far [SÎRBU & Oprea, 2011].

Although it is pretty widespread in Moldova (Eastern Romania) – see references in SÎRBU & OPREA, 2011 – very extensive populations of this species have not been reported from this country's province of the country, until now. During our recent fieldwork, we recorded vast populations of *C. scoparius*, covering entire mountain slopes south of the town of Moinești (Bacău County). We provide below the plant composition of a phytocoenosis of the association *Festuco - Agrostietum capillaris* Horv. 1951, recorded in the above-mentioned place, strongly invaded by *C. scoparius* (N 46.44947; E 26.46729; surface: 100 m^2; westerly aspect, slope 20%;

leg. et det. C. Sîrbu & A. Oprea, 08.VI.2020; plant species and abundance-dominance values – AD): ***Cytisus scoparius*** 4; **Car. ass.**: *Agrostis capillaris* ssp. *capillaris* 3, *Festuca rubra* 1; ***Cynosurion*** & ***Arrhenatheretalia***: *Cynosurus cristatus* +, *Gentianopsis ciliata* +, *Hypochaeris radicata* +, *Achillea millefolium* +, *Briza media* +, *Leontodon hispidus* +; ***Molinio-Arrhenatheretea***: *Agrostis stolonifera* ssp. *stolonifera* +, *Centaurea jacea* ssp. *jacea* +, *Festuca pratensis* ssp. *pratensis* +, *Helianthemum nummularium* ssp. *nummularium* +, *Holcus lanatus* +, *Polygala vulgaris* ssp. *vulgaris* +, *Thymus pulegioides* ssp. *pulegioides* +, *Viola canina* ssp. *montana* +; ***Festuco-Brometea***: *Carlina biebersteini* ssp. *brevibracteata* +, *Eryngium campestre* +, *Galium verum* ssp. *verum* +, *Pimpinella saxifraga* subsp. *saxifraga* +, *Plantago lanceolata* (+); ***Trifolio-Geranietea***: *Fragaria vesca* +, *Ranunculus polyanthemos* subsp. *polyanthemoides* +, *Seseli annuum* ssp. *annuum* +; ***Variae***: *Daucus carota* ssp. *carota* +, *Betula pendula* +, *Carpinus betulus* +, *Crataegus monogyna* +, *Pinus sylvestris* (juv.) +, *Rosa canina* +, *Salix caprea* +.

Current status in Romania's flora: invasive.

Dittrichia graveolens (L.) W. Greuter (Figure 2)

The first data concerning the occurrence in Romania of this Mediterranean species was published by SZATMARI & HURDU (2020), namely along the European road E68 (DN1), between the villages Vișteа de Jos and Ucea de Jos (Brașov County) and between the villages Arpașul de Jos and Scoreiu (Sibiu County), on the roadsides and in the cracks formed between the concrete tiles of a bridge.

Figure 1. *Cytisus scoparius*, south of the Moinești town, Bacău County

We have found hundreds of specimens of *D. graveolens* near Tohanu Nou (Brașov County), about 80 Km road from Vișstea de Jos, on compacted gravel, on both sides of the road E574 (N 45.55196, E 25.38871; *leg. et det.* C. Sîrbu, 22.X.2022). This is the third place where the species has been identified in Romania to date.

SZATMARI & HURDU (2020) pointed out the high invasive potential of this species due to its easy dispersal over long distances and broad ecological tolerance. According to PONTICELLI & al. (2022), *D. graveolens* "is currently undergoing a dramatic northward expansion of its native range related to climate change". For a detailed description of this species and taxonomy of the genus, see BRULLO & De MARCO (2000).

Figure 2. *Dittrichia graveolens*, Tohanu Nou, Brașov County

Current status in Romania's flora: naturalised, potentially invasive.

Dysphania pumilio (R. Br.) Mosyakin & Clemants (*Chenopodium pumilio* R. Br.)

Reported relatively recently in Romania [CHYTRÝ, 1993], previously assessed as a casual neophyte [ANASTASIU & NEGREAN, 2009], the species originating from tropical regions (Australia, New Zealand, New Caledonia), has been confirmed in the country, until now, only on fluvial sands of the Danube, in the Danube Delta [CHYTRÝ, 1993; COSTEA, 1994; OPREA, 2005; CIOCÂRLAN, 2009; SÎRBU & OPREA, 2011; SÂRBU & al. 2013] and in the Galați County [SÎRBU & OPREA, 2011]. We identified it between the pavement tiles in the central zone of the city of Focșani (N 45,69750, E 27.18303; *leg. et det.* C. Sîrbu & A. Oprea, 28.VII.2022). This is the first report of this species in anthropogenic habitats at a great distance from the Danube River, and the first for the Vrancea County. Current status in Romania's flora: invasive.

Eleusine indica (L.) Gaertn.

This is a species native to tropical and subtropical Asia, first mentioned in the country by RĂVĂRUŢ & MITITELU (1960). Although the population reported by the cited authors from north-eastern Romania (the city of Iași) did not survive over time, new populations have been reported in recent decades from other regions, as follows: southern Moldova [SÎRBU & OPREA, 2011; ȘUȘNIA, 2022], Dobrogea [COSTEA, 1996; SÎRBU & OPREA, 2011; MEMEDEMIN & al. 2016], Muntenia [NEGREAN & CONSTANTIN, 1999; OPREA & al. 2004; NAGODĂ, 2015], Oltenia [RĂDUŢOIU & STAN, 2013] and Transylvania [NEGREAN & al. 2017]. ANASTASIU & NEGREAN (2009) assessed it as naturalised in the country.

We report it now for the first time in Argeș, Buzău and Vrancea counties, as follows: Jgheaburi – roadside, sandy ground (N 45.27771, E 24.80556; *leg. et det.* C. Sîrbu & A. Oprea, 11.VIII.2022) (Argeș County); Buzău train station – near the platform (N 45.14269, E 26.84434; *leg. et det.* A. Oprea & C. Sîrbu, 31.VII.2022) (Buzău County); Cândești – roadside, vacant sandy ground (N 45.53985, E 27.08698; *leg. et det.* C. Sîrbu & A. Oprea, 27.VIII.2022) (Vrancea County).

Current status in Romania's flora: naturalised, potentially invasive in southern regions and casual elsewhere; dispersed over long distances by road and rail transport.

Erigeron sumatrensis Retz. (*Conyza sumatrensis* (Retz.) E. Walker)

An invasive species native to South America, reported in Romania for the first time by ANASTASIU & MEMEDEMIN (2012). After this first report, in just a single decade, it has been identified in several provinces of the country, namely: Dobrogea [ANASTASIU & MEMEDEMIN, 2012; MEMEDEMIN & al. 2016; OPREA & al. 2021], Banat, Moldova, Oltenia [OPREA & al. 2021] and Muntenia (Argeș County) [SOARE, 2021].

We report it here for the first time in three counties (Dâmbovița, Buzău and Iași), as follows: Târgoviște – train station, between the railway lines (N 44.91593, E 25.45447; *leg. et det.* A. Oprea & C. Sîrbu, 11.VIII.2022) and Curtea Domnească (The Royal Court) – on the lawns (N 44.93211, E 25.45832; *leg. et det.* A. Oprea & C. Sîrbu, 11.VIII.2022) (Dâmbovița County); Buzău train station – between the railway lines (N 45.142900, E 26.82873; *leg. et det.* A. Oprea & C. Sîrbu, 31.VII.2022) (Buzău County); Iași (the central area, near the Palace of Culture) – vacant land (N 47.15710, E 27.58806; *leg. et det.* C. Sîrbu, 05.IX.2022); Miroslava – disturbed ground, at the edge of the orchard (N 47.13922, E 27.54893; *leg. et det.* C. Sîrbu, 12.XI.2022) (Iași county); Corbu – seaside at abandoned industrial site (N 44.21209, E 28.41327; M. Doroftei) (Constanța County).

Current status in Romania's flora: naturalised to invasive.

Eriochloa villosa (Thunb.) Kunth

This is a weed of Asian origin [CIOCÂRLAN & SIKE, 2006; CIOCÂRLAN, 2009], quite common (invasive) in western and north-western Romania [CIOCÂRLAN & SIKE, 2006; FĂRCĂȘESCU & al. 2007, 2008; ARDELEAN & al. 2009, 2018; KARÁCSONYI, 2011; NEGREAN, 2011; SZATMARI, 2016; NEGREAN & al. 2017; SÎRBU & al. 2022].

We identified it in Alba County, at Blaj, near the "Eminescu's Linden Tree" – in a corn field (N 46.18062, E 23.93088; *leg. et det.* A. Oprea, 20.VIII.2021) and at Ighiu – in a corn field (N 46.14964, E 23.51041; *leg. et det.* A. Oprea, 21.VIII.2021). These reports seem to be the first from the Alba County and the easternmost from Romania.

Current status in Romania's flora: invasive.

Euphorbia glyptosperma Engelm. (*Chamaesyce glyptosperma* (Engelm.) Small)

Reported a few years ago [SÎRBU & ȘUȘNIA, 2018] for the first time in the flora of Romania, this neophyte of North American origin, identified so far only in Moldova (Galați, Vrancea and Vaslui counties) [SÎRBU & ȘUȘNIA, 2018; ȘUȘNIA & al. 2020; OPREA & al. 2021; ȘUȘNIA, 2022], seems to be much more widespread than initially thought, or is in full process of invasion.

We report it from new localities, as follows: East of Cornățel – river sands and pebbles in the Trotușului river bed, upstream of the bridge (N 46.13416, E 27.05252; *leg. et det.* C. Sîrbu & A. Oprea, 27.VIII.2022) (Bacău County); south of Adjud – vacant land, with compacted gravel (N 46.08645, E 27.19130; *leg. et det.* C. Sîrbu & A. Oprea, 27.VIII.2022), Bonțești – vacant land, with compacted gravel (N 45.67776, E 27.05255; *leg. et det.* C. Sîrbu & A. Oprea, 26.VIII.2022), Urechești – roadside, on compacted gravel (N 45.60420, E 27.08860; *leg. et det.*

C. Sîrbu & A. Oprea, 26.VIII.2022) (Vrancea County); Râmnicu Sărat, north of the town – vacant, stony land, with concrete slabs, in the yard of an out-of-use industrial hall (N 45.39778, E 27.04790; *leg. et det.* C. Sîrbu & A. Oprea, 06.XI.2022) (Buzău County). The species is reported here for the first time from Muntenia (at Râmnicu Sărat, Buzău County) and Bacău County (at Cornățel). We have to mention that the specimens collected from Râmnicu Sărat have atypical seeds, weakly ornamented.

Current status in Romania's flora: invasive.

Grindelia squarrosa (Pursh) Dunal.

This is a species native to North America, previously known in Romania only toward the eastern border of the country, between the cities of Iași and Galați [SÎRBU & OPREA, 1998, 2011; OPREA & al. 2021], at first assessed as casual [ANASTASIU & NEGREAN, 2009], later on as invasive, or potentially invasive [TRUȚĂ & al. 2012; SÎRBU & al. 2022].

We report it here from the Brăila port area – on the railway embankment (N 45.28964, E 27.98203; *leg. et det.* C. Sîrbu & A. Oprea, 11.VI.2022). This is the first report of *G. squarrosa* in Muntenia and the southernmost from Romania until now. From the current data, it is quite predictable that it will spread further to other regions of the country along the transport networks.

Current status in Romania's flora: invasive.

Impatiens balfourii Hook. f.

This is an Asian species (native to the Himalayas), introduced in Romania as an ornamental plant and first listed by NEGREAN (2011) as an alien plant species in the country. Until now, it has been cited from Banat [NEGREAN, 2011], Crișana [NEGREAN, 2011, 2012; KARÁCSONYI, 2011], Muntenia [NEGREAN, 2011, 2012; NAGODĂ, 2015; ANASTASIU & al. 2017], Oltenia [NEGREAN & CIORTAN, 2014] and Transylvania [KOVÁCS, 2012; NEGREAN & al. 2017].

We report it for the first time as a neophyte in Moldova (eastern Romania), from Pojorâta (Suceava County) – roadsides, vacant lands and ditches (N 47.48450, E 25.49068; *leg. et det.* A. Oprea, 03.IX.2022).

Current status in Romania's flora: naturalised (casual in eastern Romania).

Oenothera depressa E. Greene

This species, native to North America, has recently been reported in the alien flora of Romania [SÎRBU & OPREA, 2017], being known until now in Moldova [SÎRBU & OPREA, 2017; ȘUȘNIA & al. 2020; ȘUȘNIA, 2022], Transylvania [SÎRBU & OPREA, 2017] and the Danube Delta [OPREA & al. 2021].

We report it now from new localities, as follows: Cornățel – fluvial sands in the Trotuș river bed (N 46.13416, E 27.05252; *leg. et det.* C. Sîrbu & A. Oprea, 27.VIII.2022) (Bacău County); Smârdan – vacant land, with sandy-stone substrate (N 45.46556, E 27.95785; *leg. et det.* C. Sîrbu & A. Oprea, 05.VI.2021); Tecuci – the embankment of the ring road under construction, at the eastern end of the bridge over the railway (N 45.81380, E 27.43831; *leg. et det.* C. Sîrbu & A. Oprea, 06.VI.2021) (Galați County); Iași – at the Socola train station – between the railway tracks (N 47.13989, E 27.61237; *leg. et det.* C. Sîrbu, 24.IX.2022) (Iași County); Bogata – Moldova river bank (N 47.41124, E 26.20646; *leg. et det.* A. Oprea, 02.IX.2022) (Suceava County). We mention that this invasive species has not been reported from the counties of Bacău, Iași and Suceava, up to now.

Current status in Romania's flora: invasive.

Oenothera pycnocarpa G. F. Atk. & Bartlett

This is a North American species, very rare in Romania, known so far only from one locality, namely Răchiteni (Iasi County) – on the right, sandy bank of the Siret River [SÎRBU & OPREA, 2017]. We report it here from a second locality in the country, this time in an anthropogenic habitat: Dolhasca train station (Suceava County) – between the railway tracks and vacant lands nearby (N 47,4247, E 26,61059; N 47.42377, E 26.61126; *leg. et det.* C. Sîrbu & A. Oprea, 10.VII.2021).

Current status in Romania's flora: naturalised.

Oenothera suaveolens Person

A native to North America, this species has been recently reported from Romania [SÎRBU & OPREA, 2017], in both anthropogenic (vacant lands, train stations) and natural habitats (fluvial sands, river banks), in the counties of Iași [SÎRBU & OPREA, 2017], Galați [SÎRBU & OPREA, 2017; ȘUȘNIA & al. 2020], Vrancea [ȘUȘNIA, 2022] and Brăila [ȘUȘNIA & al. 2020].

We report it now for the first time in the Danube Delta (Tulcea County), as well as in Neamț and Bacău counties, as follows:

– Tulcea County (the Danube Delta): Caraorman – the edge of the path, on the sand dunes, near the "kneeling oak" ("stejarul îngenunchiat") (N 45.02986, E 29.40650; N 45.02905, E 29.40602; *leg. et det.* C. Sîrbu & A. Oprea, 01.VIII.2022), Letea (the village) – roadsides and associated vacant lands (N 45.29115, E 29.51412; N 45.29197, E 29.51127; *leg. et det.* C. Sîrbu & A. Oprea, 03.VIII.2022), Letea (the nature reserve) – sand dunes, dry interdune depressions (N 45.30099, E 29.51353; N 45.3028, E 29.51266; N 45.30838, E 29.52889; N 45.31012, E 29.53331; N 45.32166, E 29.51923; *leg. et det.* C. Sîrbu & A. Oprea, 04.VIII.2022);

– Bacău County: Galbeni – sandy land at the sand and gravel quarry, downstream of the dam (N 46.45031, E 26.95338; *leg. et det.* C. Sîrbu, 26.VII.2021), Coteni – fluvial sands on the left bank of Siret River (N 46.53564, E 26.98858; *leg. et det.* C. Sîrbu, 26.VII.2021);

– Neamț County: Adjudeni – fluvial sands on the right bank of the Siret River (N 46.79910, E 26.96416; *leg. et det.* C. Sîrbu, 26.VII.2021), Ion Creangă – fluvial sands on the left bank of the Siret River (N 47.30992, E 26.96227; N 47.30985, E 26.97140; *leg. et det.* C. Sîrbu, 26.VII.2021), Sagna – on the dyke bordering the Siret River bed (N 46.96501, E 26.99056; *leg. et det.* C. Sîrbu, 27.VII.2021).

The species has already been reported from Iași County [SÎRBU & OPREA, 2017], but new points of presence have been recorded recently, as follows: between Kogălniceni and Alexandru Ioan Cuza – along the Siret River, on the edge of the dyke (N 47.13119, E 26.82347; *leg. et det.* C. Sîrbu, 13.IX.2021) and inside of the riparian forest (N 47.12982, E 26.82249; *leg. et det.* C. Sîrbu, 13.IX.2021); Iași, near the "Frumoasa" Monastery – vacant land at the edge of a silted up pond (N 47.14051, E 27.59094; *leg. et det.* C. Sîrbu, 24.IX.2022); between Solonț and Zaboloteni – fluvial sands on the right bank of the Prut River (N 47.48581, E 27.47303 *leg. et det.* C. Sîrbu, 15.IX.2021).

Current status in Romania's flora: invasive.

Paspalum distichum L. (*Paspalum paspalodes* (Michx.) Scribn.) (Figure 3)

This neophyte of tropical origin, invasive in Romania [ANASTASIU & NEGREAN, 2009; SÎRBU & OPREA, 2011; SÎRBU & al. 2022], was first mentioned in the country by ROMAN (1992). Until now it is known from several localities along the Danube River, between the Nera micro-delta, in Caraș-Severin County (upstream) and Sulina, Danube Delta (downstream) [ROMAN, 1992; CIOCÂRLAN, 2000, 2009, 2011; OPREA, 2005;

ANASTASIU & NEGREAN, 2009; SÂRBU & al. 2007; DOROFTEI & al. 2011; SÎRBU & OPREA, 2011; NEGREAN, 2012; SÎRBU & al. 2021; CAMEN-COMĂNESCU & MIHAI, 2022].

From the Danube Delta, the species has been reported so far from three localities: the Letea forest [ROMAN, 1992], Mila 28 [SÎRBU & OPREA, 2011] and Sulina [DOROFTEI & al. 2011]. In our recent field work, we have identified extensive populations of *P. distichum* in many other localities in this protected area, namely: Cardon, in the south-eastern extremity of the Letea levee, near the bridge – on the channel edges (N 45.23006, E 29.63172; *leg. et det.* C. Sîrbu, A. Oprea, M. Doroftei, S. Covaliov, 04.VIII.2020); Cardon, at the monastery – on the channel edges (N 45.25828, E 29.62489; *leg. et det.* C. Sîrbu, A. Oprea, M. Doroftei, S. Covaliov, 04.VIII.2020); downstream of Crișan, at the wharf for picking up waste – marshy

Figure 3. *Paspalum distichum*, Sulina, Tulcea County

place (N 45.17274, E 29.43669; *leg. et det.* C. Sîrbu & A. Oprea, S. Covaliov, 06.VIII.2021); Mila 23 – on the channel edges (N 45.21612, E 29.23484; *leg. et det.* C. Sîrbu & A. Oprea, 13.VIII.2021); upstream of Vulturu – on the channel edges (N 45.17061, E 29.00346; *leg. et det.* C. Sîrbu & A. Oprea, S. Covaliov, 03.VIII.2020). The species has already been cited from the Sulina town, as shown before, but without a precise location. We identified a large population of *P. distichum* in this locality, around a pond on the left side of the Sulina chanel (N 45.16059, E 29.63885; N 45.16045, E 29.63828; *leg. et det.* C. Sîrbu, A. Oprea, S. Covaliov, M. Doroftei, 03.VIII.2020).

Current status in Romania's flora: invasive.

Robinia* × *ambigua Poir. (*R. pseudoacacia* × *viscosa*; *R. dubia* Fouc., *R. hybrida* Audib., *R. intermedia* Soul.-Bod.)

This is a hybrid between *R. pseudoacacia* and *R. viscosa*, of North American origin, cultivated for ornamental purposes. As a casual alien plant, it has been reported from Romania only from Alba County, at Sebeş (Viile Românilor and Calea Scufundată) [BORZA, 1959]. We report it for the first time in the flora of Moldova and the Danube Delta (as a casual alien plant, outside the cultivation sites), as follows: Lunca Asău – on the river bank and the roadside (N 46.42444, E 26.42993; *leg. et det.* A. Oprea & C. Sîrbu, 03.VII.2022) (Bacău County); Giurgeni – roadsides (N 47.20069, E 27.11683; *leg. et det.* A. Oprea, 26.VI.2022), Mărmureni – roadsides and vacant lots (N 47.21101, E 27.17894; *leg. et det.* A. Oprea, 26.VI.2022), Valea Enei – roadsides (N 47.79991, E 27.15270; *leg. et det.* A. Oprea, 26.VI.2022) and Valea Ursului – roadsides (N 47.20069, E 27.09544; *leg. et det.* A. Oprea, 26.VI.2022) (Neamț County); in the Danube Delta (Tulcea County): Lăstuni – household yard (N 45.01340, E 28.41335), Zebil – train halt (N 44.57539, E 28.43073; M. Doroftei).

Figure 4. *Rudbeckia triloba*, Soveja, Vrancea County

Current status in Romania's flora: casual.

Rosa rugosa Thunb.

An East Asian species cultivated in gardens as ornamental [PRODAN, 1956], it was reported as a casual alien from the counties of Satu Mare [KARÁCSONYI, 1995], Sălaj [NEGREAN & al. 2017] and Botoșani [HUȚANU, 1999]. We identified a small population of *R. rugosa* in the grassland of the "Poiana cu Schit" nature reserve – Grajduri, Iași County (N 46.98649, E 27.58479; *leg. et det.* C. Sîrbu, 10.VII.2021). This is the second record of its spontaneous occurrence outside the cultivation site in Moldova (Eastern Romania).

Current status in Romania's flora: casual.

Rudbeckia triloba L. (Figure 4)

This is a North American species, widely cultivated in Romania for its ornamental value, previously reported as a casual alien plant, from only a few localities, namely: Bocicoi (Maramureș County), Agapia (Neamț County), Ruginoasa and Iași (Iași County) [SÎRBU & OPREA, 2010]. Recently, numerous new points of presence of this species outside the cultivation sites have been registered, in some cases (Lepșa, Soveja) the populations being very extensive, of hundreds of individuals. Our new records are as follows:

– Covasna County: Imeni – roadsides (N 46.06089, E 26.16695; *leg. et det.* A. Oprea, 10.IX.2022), Bățanii Mari – vacant lots (N 46.08847, E 25.69137; *leg. et det.* A. Oprea, 10.IX.2022);

– Bacău County: Livezi – vacant lots (N 46.40294, E 26.73394; *leg. et det.* A. Oprea, 06.X.2020);

– Botoșani County: Lunca – on a deposit of river sand (N 47.88901, E 26.26002; *leg. et det.* C. Sîrbu & A. Oprea, 01.XI.2020);

– Iași County: Cârlig – vacant lots (N 47.20421, E 27.56004; *leg. et det.* C. Sîrbu, 10.VII.2013);

– Neamț County: Pângărați – ditch beside the road (N 46.92989, E 26.20125; *leg. et det.* A. Oprea., 10.XI.2021);

– Vrancea County: Adjud – roadsides (N 46.10162, E 27.17959; *leg. et det.* C. Sîrbu, 28.VIII.2020), Lepșa – uncultivated land, near the access road to "Piatra Ciutei", abundant (N 45.9421, E 26.5884; *leg. et det.* A. Oprea & C. Sîrbu, 26.VIII.2022), Răcoasa – clogged ditch on the roadside (N 45.99314, E 26.86780; *leg. et det.* A. Oprea & C. Sîrbu, 26.VIII.2022) and vacant lands (N 45.99300, E 26.86732; N 45.99282, E 26.86865; *leg. et det.* A. Oprea & C. Sîrbu, 26.VIII.2022), Soveja – the bank of the Dragomira stream, abundant (N 45.99830, E 26.64168; N 45.99905, E 26.66158; *leg. et det.* A. Oprea & C. Sîrbu, 26.VIII.2022);

– Vaslui County: Vaslui – roadside and on the Delea stream bank (N 46.65753, E 27.71786; *leg. et det.* C. Sîrbu, 5.VIII.2017).

According to our data, the species is now reported for the first time in Transylvania (Covasna County), as well as in some counties of Moldova (Botoșani, Bacău, Vaslui and Vrancea).

Current status in Romania's flora: naturalised.

Sedum sarmentosum Bunge (Figure 5)

An ornamental plant, native of East Asia, reported in the alien flora (as a refugee from culture) in almost all provinces of the country [DRĂGULESCU, 2010; NEGREAN, 2011; ANASTASIU & al. 2011, 2014; SÎRBU & OPREA, 2011; NEGREAN & CIORTAN, 2014; NAGODĂ, 2015; NEGREAN & al. 2017], except for Moldova (eastern Romania). We have registered it in several localities in this historical province, namely:

– Suceava County: Stulpicani – vacant land (N 47.47200, E 25.75902; *leg. et det.* A. Oprea, 06.IX.2022), Ițcani-Suceava – railway station (N 47.676667, E 26.216944; *leg. et det.* A. Oprea, 06.IX.2022);

– Neamț County: Piatra Șoimului – on river pebble and the concrete bank of the river Calu (N 46.79410, E 26.45704: *leg. et det.* C. Sîrbu & A. Oprea, 02.VII.2022);

– Bacău County: Valea lui Ion – stony ground (N 46.69963, E 26.62701; *leg. et det.* C. Sîrbu & A. Oprea, 03.VII.2022);

– Vrancea County: Soveja – stony ground on a stream bank (N 46.00010, E 26.66462; *leg. et det.* C. Sîrbu & A. Oprea, 26.VIII.2022);

In other provinces of the country, we have also recorded it in new localities as follows:

– Bihor County: Băile Felix – on the edge of the forest, next to the railway (N 46.99758, E 21,97893; *leg. et det.* C. Sîrbu, 2017);

– Sălaj County: Jibou – sidewalks (N 47.25856, E 23.25161; *leg. et det*. Sîrbu & Oprea 2012);

– Cluj County: Frăsinet – on the bank of the Valea Ierii rivulet (*leg. et det.* Sîrbu & Oprea, 2012);

– Covasna County: Bixad – vacant land (N 46.10217, E 25.85851; *leg. et det.* A. Oprea, 16.VII.2020);

– Dâmbovița County: Pucioasa – roadside, sidewalks (N 45.04481, E 25.43852; N 45.04439, E 25.43903; N 45.04468, E 25.43879; *leg. et det.* C. Sîrbu & A. Oprea 11.VIII.2022).

Current status in Romania's flora: naturalised.

Figure 5. *Sedum sarmentosum*, Piatra Șoimului, Neamț County

Setaria faberi J. Herrm.

This is a species native to East Asia, accidentally introduced into Romania, from where it was first cited by COSTEA (1996). So far it has been reported as a casual neophyte [ANASTASIU & NEGREAN, 2009] in anthropogenic habitats of railway stations only from Dobrogea [COSTEA, 1996; CIOCÂRLAN, 2000, 2009], Muntenia [CIOCÂRLAN, 2009; OPREA & al. 2012; CAMEN-COMĂNESCU & MIHAI, 2022] and Moldova [OPREA & al. 2012]. We identified it for the first time in Transylvania, in the Făgăraș railway station (Brașov County) – on the railway embankment and the associated vacant land (N 45.83046, E 24.97393; *leg. et det*. A. Oprea, 20.IX.2022).

Current status in Romania's flora: casual.

Sicyos angulatus L.

This is a neophyte native to North America, invasive in Romania [SÎRBU & al. 2022], reported to date from all provinces of the country [ANASTASIU & al. 2011; RĂDUȚOIU & STAN, 2013; NICULESCU & al. 2021; ARDELEAN & ROȘU, 2016; NEGREAN & al. 2017; for older references, see SÎRBU & OPREA, 2011].

From the Danube Delta, the species has been first mentioned by ANASTASIU & al. (2011), recorded along the Black Sea coast. We have recently identified this species in several places in the Delta, between Tulcea and Sulina, as follows: Litcov Channel – channel banks (N 45.12441, E 29.18134; *leg. et det.* A. Oprea, C. Sîrbu, S. Covaliov, 04.VIII.2021), Șontea Channel – channel banks (N 45.2031, E 29.17947; *leg. et det.* A. Oprea, C. Sîrbu, S. Covaliov, 04.VIII.2021), Caraorman – Channel Vătafu-Împuţita – on the willows and the water's edges (N 45.11919, E 29.42309; N 45.11741, E 29.39516; *leg. et det.* A. Oprea & C. Sîrbu, 03.VIII.2022), Crișan – on the Danube banks (N 45.17437, E 29.38675; *leg. et det.* A. Oprea, C. Sîrbu, S. Covaliov, M. Doroftei; 05.VIII.2021), Mila 23 – channel banks (N 45.21678, E 29.23539; *leg. et det.* A. Oprea, C. Sîrbu, S. Covaliov, M. Doroftei, 13.VIII.2021), Mila 28 – channel banks (N 45.17400, E 29.06185; *leg. et det.* A. Oprea, C. Sîrbu, S. Covaliov, M. Doroftei, 04.VIII.2020), Tulcea – on the Danube banks (N 45.18000, E 28.80097; N 45.17995, E 28.80316; *leg. et det.* A. Oprea, C. Sîrbu, S. Covaliov, M. Doroftei, 04.VIII.2020), Sulina – channel banks (N 45.15947, E 29.63745; N 45.15284, E 29.67612; *leg. et det.* A. Oprea, C. Sîrbu, S. Covaliov, M. Doroftei, 06.VIII.2021), Vulturu (upstream) – channel banks (N 45.17061, E 29.00346; *leg. et det.* A. Oprea, C. Sîrbu, S. Covaliov, M. Doroftei, 05.VIII.2020), Grindul Lupilor – roadside (N 44.41444, E 28.56265; M. Doroftei).

Other new chorological data:

– Caraș-Severin County: Baziaș – wet thickets (N 44.86299, E 21.39048; *leg. et det.* A. Oprea, 20.VIII.2020);

– Mehedinți County: the Mraconia bay – roadside (N 47.74677, E 22.29391; *leg. et det.* A. Oprea, 19.VIII.2020);

– Iași County: Hălăucești – unkempt land, on the bank of a stream (N 47.10111, E 26.81632; *leg. et det.* C. Sîrbu, 14.IX.2021), Șcheia – the steep bank of the Siret River (N 47.11073, E 26.88616; *leg. et det.* C. Sîrbu, 13.IX.2021);

– Neamț County: Borca – the bank of the homonymous stream (N 47.79752, E 25.77423; N 47.79752, E 25.77445; *leg. et det.* A. Oprea, 11.XI.2021).

The species has not been reported previously in Iași and Neamț counties.

Current status in Romania's flora: invasive.

Conclusions

The paper presents new chorological data and the current invasion status in Romania for a total of 20 species of neophytes;

Some taxa are reported for the first time in some regional floras within the country, as follows: *Campsis radicans* – the first record for Moldova (eastern Romania), Oltenia and Banat; *Impatiens balfourii*, *Robinia* × *ambigua* and *Sedum sarmentosum* – the first record for Moldova (eastern Romania); *Oenothera suaveolens* – the first record for the Danube Delta; *Euphorbia glyptosperma* and *Grindelia squarrosa* – the first record for Muntenia; *Rudbeckia triloba* and *Setaria faberi* – the first record for Transylvania;

Oenothera pycnocarpa and *Dittrichia graveolens* are reported in their second and third sites in Romania, respectively;

Based on current data, the invasive status in Romania of the taxa analyzed in the paper was assessed as follows: 4 species are casual (*Robinia* × *ambigua, Rosa rugosa*, *Setaria faberi*, *Campsis radicans*); 4 species are naturalised (*Impatiens balfourii*, *Oenothera pycnocarpa*, *Rudbeckia triloba*, *Sedum sarmentosum*); 3 species are potentially invasive (*Dittrichia graveolens*,

Eleusine indica, *Erigeron sumatrensis*) and 9 species are invasive (*Cytisus scoparius*, *Dysphania pumilio*, *Eriochloa villosa*, *Euphorbia glyptosperma*, *Grindelia squarrosa*, *Oenothera depressa*, *Oenothera suaveolens*, *Paspalum distichum*, *Sicyos angulatus*).

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RIPGUT BROME (*BROMUS DIANDRUS*) IN ROMANIA'S VASCULAR FLORA

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Abstract: The paper refers to *Bromus diandrus*, a vascular plant species identified in the spontaneous flora of the historical province of Muntenia, namely at the Buzău railway station during the summer of 2022 and autumn of 2023. Both the ecological conditions of the habitat, as well as some taxonomic and coenotaxonomic aspects of the species are presented in the paper. A key is also included to help identify the other species from the sect. *Genea*, *Bromus* spp., known so far in the flora of Romania.

Key words: identification key, natural distribution, relevées, ruderal vegetation, spontaneous plants.

Introduction

It is not uncommon for different sources to provide varying estimates of the number of species within a particular taxonomic group, as the exact number of species can be difficult to determine and can depend on factors such as the specific criteria used to define a species and the methods used to identify and classify them. So, estimates in the scientific literature of the number of species in *Poaceae* Barnhart (*Gramineae* Juss.) family is ca. 8,000, belonging to ca. 700 genera [BUIA & al. 1965; NYÁRÁDY & BELDIE, 1972]. Others estimates a number between 7,000 and 8,000 species [CRISTEA, 2014], while acording to other sources, family of *Poaceae* includes 9,000-10,000 species [https://ro.wikipedia.org/wiki/Poaceae]. It is important to note that these estimates are based on current knowledge and understanding of the group, and as research continues and new species are discovered, these estimates may change in the future.

Bromus L. [LINNÆUS, 1753] have ranged from 100 to 400 species, but plant taxonomists currently recognize around 160-170 species [https://ro.wikipedia.org/wiki/Poaceae]. The romanian botanist I. Todor stated that the genus *Bromus* consisted of more than 100 species worldwide distributed [TODOR, 1972].

Etymology of *Bromus*: 1. gr. bróma = food; 2. gr. bromos = the vernacular name of cultivated oat species (a name cited in this form for the first time appeared in Theophrastus's work, which is accessible to us in Italian [TEOFRAST, 1549]).

The genus *Bromus* is part of the cool-season grass lineage (subfamily Pooideae, which includes about 3,300 species). Within Pooideae subfamily, *Bromus* is classified in tribe Bromeae (the only genus in the tribe). *Bromus* species occur in many habitats worldwide, mostly in temperate regions, including Europe [CLAPHAM & al. 1952; SMITH, 1980; KON & BLACKLOW, 1989; SOMLYAY, 2001; STACE, 2010; https://europlusmed.org/cdm_dataportal/taxon/897057], Asia [CLAPHAM & al. 1952; COPE, 1982; KON &

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BLACKLOW, 1989; NADERI & al. 2012; MALIK & MOHAMMAD, 2015; NADERI & RAHIMINEJAD, 2015], Africa [CLAPHAM & al. 1952; KON & BLACKLOW, 1989; MEJRI & al. 2010; https://www.cabi.org/isc/datasheet/31167], North America [CLAPHAM & al. 1952; KON & BLACKLOW, 1989; SAARELA, 2008], Central America [O'CONNOR, 1990], South America [https://www.cabi.org/isc/datasheet/31167/], Australia [CLAPHAM & al. 1952; KON & BLACKLOW, 1989; MICHAEL & al. 2010; BORGER & al. 2021], and New Zealand [CLAPHAM & al. 1952; KON & BLACKLOW, 1989; BURGHARDT & FROUD-WILLIAMS, 1997; https://www.cabi.org/isc/datasheet/31167].

Bromus diandrus Roth

The most common vernacular name in english literature of *B. diandrus* is "the ripgut brome", according to WILLIAMS (1986). Other names are: great brome, ripgut grass, giant brome, slands grass, jabbers, Kingston grass, spear grass, brome grass [BOWCHER & al. 2019].

It is a species native to the Irano-Turanian, Pontic, and Mediterranean regions [CABI, 2020], but it has been widely introduced elsewhere (Europe, Asia, Africa, North America, South America, Australia, and Oceania) as an alien weed [KUNEV, 2021; BORGER & al. 2021; CABI, 2022]. Close to Romania, *B. diandrus* has been reported as a naturalized species in Bulgaria [KUNEV, 2021; STOYANOV & al. 2022], and Hungary, as doubfully introduced there, perhaps only cultivated [https://europlusmed.org/cdm_dataportal].

Among other locations with *B. diandrus* in the proximity of Romania, are the next ones: the coastline of the Black Sea and its surroundings, including "Insula Șerpilor" and Crimea, as well as Southwestern Russia and Georgia [GBIF, 2023; https://www.gbif.org/species/2703760]. Also, it has also been identified in the European part of Turkey [WEBB, 1966], Greece [GBIF, 2023], Montenegro [GBIF, 2023], and North Macedonia [KOSTADINOVSKI & al. 2019].

B. diandrus and *B. rigidus* Roth belong to the sect. *Genea* Dumort. [SALES, 1993; SAARELA & al. 2007; SAARELA, 2008; STACE, 2010; LLAMAS & ACEDO, 2019], and differ from the other species of this section mainly by their longer lemma and awn, which are of at least 20 and 30 mm, respectively [SMITH, 1980]. They are morphologically very similar and are often difficult to distinguish from each other [SALES, 1993; STOYANOV & al. 2022; VERLOOVE, 2022; http://alienplantsbelgium.be]. Some authors consider them to be conspecific [VELDKAMP & al. 1991; SALES, 1993; SAARELA, 2008], but their exact taxonomic classification is still under debate and subject to ongoing research [BORGER & al. 2021].

According to literature data [SMITH, 1980; SALES, 1993; STACE, 2010; BOWCHER & al. 2019; KUNEV, 2021], *B. diandrus* differs from *B. rigidus* mainly by the following combination of features: panicle usually lax, with branches spreading laterally or pendent, at least some branches being longer than spikelets; callus scar ovate or almost circular, rounded at the ends; presence of a conspicuous constriction at the base of lemma, in side-view; anthers longer than 0.7 mm (vs. panicle usually dense, stiff [BOWCHER & al. 2019], with erect, shorter branches than spikelets; callus-scar elliptic, pointed/acute at the ends; lemma straight at base; anthers up to 0,7 mm long, in *B. rigidus*). However, ecological conditions can induce variations in the features, which makes it difficult to correctly differentiate between the two species, and, in some cases, there can be significant overlap in their morphology. For instance, SALES (1993) observed in population with other *diandrus*-like features all variations from dense and stiffly erect panicles to lax and spreading ones. The same author reduced these two taxa to varietal rank, and pointed out that the differences between them are subtle enough that identification of many specimens beyond *B. diandrus sensu lato* is often impossible.

Therefore, a careful examination of multiple diagnostic features and a comparison with reliable reference specimens may be necessary for an accurate identification.

Even the ecology differs; while *B. diandrus* is a tolerating species to a wide range of climates, growing both on acidic and alkaline, sandy or loamy soils, on those soils plenty in nitrogen (N) and phosphorus (P), *B. rigidus* prefers the sandy calcareous soils [KON & BLACKLOW, 1995].

As a result of molecular and phylogenetic studies on populations of those 2 species collected from France, was stated that *B. diandrus* and *B. rigidus* are genetically similar, seeming to have a common maternal genome, while the paternal genomic background still remains unclear for now [FORTUNE & al. 2008].

The aim of the current paper is to: i) to bring evidences on the presence of *B. diandrus* in Romania, ii) made considerations upon the features to discriminate to the closely related species in sect. *Genea*, iii) to underline the phytosociological aspects of the species *B. diandrus* in Buzău railway station, aspects that can further help our understanding of the ecology of this species in Romania.

Material and methods

The plant names follow the well-known work *Flora Europaea* [TUTIN & al. 1968-1980; TUTIN & al. 1993], including the on-line *Flora Europaea* [http://euromed.luomus.fi/; http://ww2.bgbm.org/europlusmed]. For those species lacking in Europe, other floras were used, as: *Flora of the Northern United States and Canada* [BRITTON & BROWN, 1970], on-line floras of the *United States of America* [www.efloras.org/Flora of North America], the flora of Republic of China [www.efloras.org/florataxon ... Flora of China], or the flora of ex-URSS area [TZVELEV, 1976, 1983].

The author's name(s) of each plant species are abbreviated according to BRUMMITT & POWELL (1992).

The distribution of the taxa within Europe follow the same on-line *Flora Europaea* [http://euromed.luomus.fi/euromed map.php].

All the plant specimens collected were deposited in public herbaria, as: IAGB (located within the Botanical Garden "Anastasie Fătu", "Alexandru Ioan Cuza" University of Iaşi), and IASI (located within the University for Life Sciences "Ion Ionescu de la Brad", Iași).

Other herbaria collections were checked to have a complete view of the distribution of *B. diandrus* in Romania. The acronymes for herbaria collections follow THIERS (2022+).

Fieldwork was conducted in the summer of 2022 and early fall of 2023; the coordinates were registered in WGS84 system, using a Garmin's GPSMAP 60CSx and digital photos were taken.

Results

Bromus diandrus Roth (syn. *Anisantha diandra* (Roth) Tutin; *A. gussonei* (Parl.) Nevski; *Bromus gussonei/gussonii* Parl.; *B. maximus* Desf. subsp. *gussonei* (Parl.) Arcang.; *B. rigidus* Roth subsp. *gussonii* (Parl.) Maire; *B. rigens* L. subsp. *gussonei* (Parl.) Cout.; *Zerna gussonei* (Parl.) Grossh.) [http://ww2.bgbm.org/EuroPlusMed].

Analyzed specimens in public herbarium collections in Romania:

Herb. (Herbarium) I, no. 182824, collected in county of Timiș: Timișoara North railway station, *in ruderatis*, *ad viam ferream*, 45º45′00.83″N, 21º12′06.39″, alt. (altitude) ca.

91 m a.s.l. (above sea level), *leg. et det*. G. Negrean, 30 May 2014 (labelled as *Bromus rigidus* Roth);

Herb. I, no. 183800, collected in county of Mehedinți: Drobeta-Turnu Severin railway station, *in ruderatis*, 45º37´18.58″N, 22º38´03.39″, alt. ca. 52 m a.s.l., *leg. et det*. G. Negrean, 25 June 2013 (labelled as *Bromus rigidus* Roth);

Herb. CL, no. 665457, collected in county of Mehedinți: Drobeta-Turnu Severin railway station, *in ruderatis*, 44º37′18.58″N, 22º38′03.39″, alt. ca. 52 m a.s.l., *leg. et det*. G. Negrean, 25 June 2013 (labelled as *Bromus rigidus* Roth);

Herb. IAGB, no. 47735-47738, collected in county of Buzău: Buzău - railway station, between the railway tracks and the associated disturbed sites, N 45.14222/E 26.82994, *leg.* A. Oprea, 9 July 2022; *det.* A. Oprea & C. Sîrbu;

Herb. IASI, no. 18044, collected in county of Buzău: Buzău railway station, between the railway tracks and the associated disturbed sites, N 45.14222/E 26.82994, *leg.* A. Oprea, 9 July 2022; *det.* A. Oprea & C. Sîrbu;

Herb. IASI, no. 18045-18046, collected in county of Buzău: Buzău railway station, between the railway tracks and the associated disturbed sites, N 45.14237, E 26.83085, *leg. et det.* A. Oprea & C. Sîrbu, 31 July 2022.

Discussion

The presence of *B*. *diandrus* in the spontaneous flora of Romania was cited under this name for the first time in 1979, in a field identification book, as: "...? ...quoted from Banat, but unconfirmed... "!. This citation is "blind", without any reference given in there. But, following the features included in the identification key to the genus *Bromus* ["erect spikelets; lemma of 2-3 cm long, with a 3-5 cm awn long"] one can see that at as a matter of fact, the author actually referred to an other species, which it is now accepted as *B*. *rigidus* Roth (in fact, the author placed the latter name as a synonime for the first one) [BELDIE, 1979].

Later works did not included *B*. *diandrus* as being present in Romania's flora [POPESCU & SANDA, 1998; CIOCÂRLAN, 1990, 2000, 2009; SÂRBU & al. 2013]. Until, in 2022, when STOYANOV & al. (2022), as a result of the revision of some specimens, previously identified by Gavril Negrean as *B. rigidus* Roth, collected from the harbour of Constanța (Herb. SOM, no. 177292) and from Drobeta-Turnu Severin railway station (Herb. CL, no. 665457) reported the presence of this species in Romania's flora. In the Herbarium of the University "Alexandru Ioan Cuza" in Iași (I), there are other similar specimens collected from Drobeta-Turnu Severin railway station (I, no. 183800, *leg*. *et det*. Negrean, at 25 June 2013, labelled as *B. rigidus* Roth) and from Timișoara North railway station (Herb. I, no. 182824, *leg*. *et det*. Negrean, at 30 May 2014, labelled as *B. rigidus* Roth).

B. *diandrus* was fully described as a species new to science by A. W. Roth [ROTH, 1787].

Within this paper, the species is presented taking into account the characteristic features observed on the specimens collected in the Buzău railway station (Romania), in the summer of 2022 and the beginning of autumn, 2023: an annual plant, therophyte, 30-90 cm high, stem hairy below panicle, with leaves up to 10 mm wide, rough, with some long hairs; ligule prominent, 3-6 mm long, membranous, and jagged tipped; panicle loose, nodding, over 25 cm long, pale green becoming purple-red, ± lax, wide-ovate (Figure 1); branches of 1-7 cm long, laterally extended or ± pendulous when mature, with 1-2 (-3) spikelets each; spikelet branches longer than spikelet itself; spikelets pendulous, at maturity of 25-40 mm long, with 5-

8 flowers (even, 4-11 flowers/spikelet [http://beta.floranorthamerica.org /Bromus_diandrus]); glumes unequal, 1 (-3) nerved, lower glume of 15-25 mm, upper one of 25-35 mm (Figure 2); lemmas of 20-35 mm long, involute, with non-tangent edges, finely 2-toothed, with awn of 30-65 mm long, straight, flattened, scabrous; abscission scar/callus-scar (at the base of the lemma) subcircular-ovate, rounded at the end (Figure 3a) in accordance with GILL & CARSTAIRS (1988), STACE (2010) and BORGER & al. (2021), < 1 mm in accordance with KON & BLACKLOW (1989); lemma, at the base, with an obvious constriction (in lateral view) (Figure 3b) in accordance with SALES (1993); anthers of 0.7-5.9 mm [SALES, 1993]; grain of 9-11 mm long, hairy at the tip, in accordance with CLAPHAM & al. (1952) and CABI (2022).

Obs.: though the epithet *diandra* means "two male flowers" (or stamens) in fact, the number of stamens of *B. diandrus* vary from 2 [ROTH, 1787] up to 2-3 [SMITH, 1980; SALES, 1993; MALIK & MOHAMMAD, 2015]. The examined specimens collected from Buzău railway station had always 3 stamens; also, the length of the anthers was of ± 1.3 mm. SMITH (1980) refers to anther lengths between 1 and 5 mm.

The chromosome number: 2n=8x=56; it is an octoploid species [SMITH, 1980; GILL & CARSTAIRS, 1988; KON & BLACKLOW, 1988; OJA & LAARMANN, 2002]. The flowering period of *B. diandrus*, in Buzău railway station: June; the maturation of grains: August-September.

The chromosome number of *B. rigidus*: 2n=6x=42; it is a hexaploid species [GILL & CARSTAIRS, 1988; KON & BLACKLOW, 1988].

Regarding the general/worldwide distribution area of *B. diandrus*, there are several points of view, as the following:

- Mediterranean-Southwest Europe [SMITH 1980; https://europlusmed.org/cdm_dataportal/taxon/897057]
- Mediterranean region & South and Central-Western Europe [MALIK & MOHAMMAD, 2015]
- Mediterranean-Eurasiatic [CLAPHAM & al. 1952; KON & BLACKLOW, 1989; BORGER & al. 2021]
- Irano-Turanian, Pontic, and Mediterranean regions [KUNEV, 2021; CABI, 2020], but introduced elsewhere as an alien weed [https://www.cabi.org/isc/datasheet].

Within the native areas, *B. diandrus* grows in cultivated fields, vineyards, orchards, and roadsides, or various disturbed sites [CLAPHAM & al. 1952; KON & BLACKLOW, 1989]. Outside of the native area, *B. diandrus* is met in various habitats, including coastal sand dunes, pastures, hilltops, croplands, fallows, wastelands, roadsides, even in national parks and nature reserves [BOWCHER & al. 2019].

We were able to analyze specimens collected by Gavril Negrean in 2013 and 2014, which are preserved in the various official herbaria of Romania (CL, I). All these specimens have panicle dense, with erect branches, which explains why Negrean labelled them as *B. rigidus*. However, by the presence of conspicuous constriction at the base of lemma, more or less rounded callus scar, and anthers of about 1 mm long, they can be attributed to *B. diandrus*.

Figure 1. *Bromus diandrus* – panicle (a specimen photographed in Buzău railway station)
Photo: C. Sîrbu, 31 July 2022

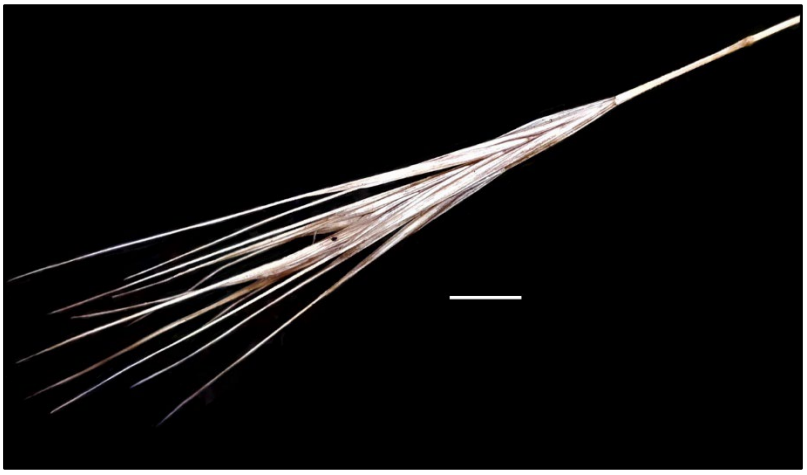

Figure 2. *Bromus diandrus* – spikelet. Scale: 0.5 mm
Photo: C. Sîrbu, 31 July 2022

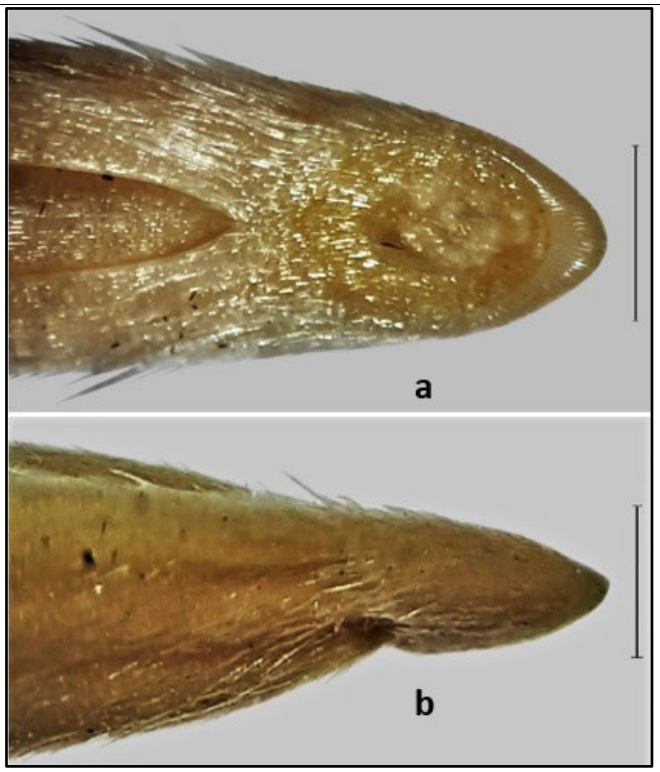

Figure 3. *Bromus diandrus* – callus scar (a) and base of lemma (b). Scale: 0.5 mm
Photo: C. Sîrbu, 22 September 2022

B. diandrus has been identified by us in Buzău railway station (N 45.14237, E 26.83085, ≈ 95 m a.s.l.): *leg. et det*. Adrian Oprea, 9 July 2022; a second collecting: Adrian Oprea & Culiță Sîrbu, 31 July 2022; voucher specimens: IAGB, no 47.735-47.738; IASI, no. 18044-18046. At the first date of registration, on 9 July 2022, the plants were at the anthesis stage, while at the later one, on 31 July 2022, they were already at the fruiting stage (it was at the same stage of development at the beginning of autumn 2023).

The specimens collected at Buzău railway station show all the specific traits of *B. diandrus* above mentioned from the literature, including loose and nodding panicle, spreading, with pendulous spikelets, and at least some branches longer than spikelets.

At the above mentioned place, *B. diandrus* grows abundantly (thousands of specimens), between the railway tracks in the railway station and the associated disturbed sites, along the entire extent of railway station area.

The EUNIS habitat type for ripgut brome in Romania's flora is identified as J4.3 *Rail networks* and E5.1 *Anthropogenic herb stands* [SCHAMINÉE & al. 2012], unlike the preferred habitats in Bulgaria, where *B. diandrus* species grows in habitat type identified as B1.324 *Pontic white dunes*, between non-vegetated sand beaches, at the foot of sea cliff slopes [KUNEV, 2021; STOYANOV & al. 2022].

This is the first report of *B. diandrus* from Muntenia (in Buzău county) and the fourth from Romania.

Place: Buzău railway station;
Vegetation: ruderal, between the railway lines;
Habitats: railway embankments;
Altitude: ≈ 95 m a.s.l.;
Surface: flat land;
Soils: superficial, usually acid;
Surveying date: 31 July 2022;
Fruits/caryopses collection: 10 September 2023.

For the purpose of this paper only two relevées are given (a more detailed phytosociological study will be carried out soon):

Relevé no. 1 - surface: 9 sq. m, vegetation height: 40 cm, vegetation cover: 85%.
N 45.14269, E 26.82876, ≈ 95 m a.s.l.

Bromus diandrus 4, *Euphorbia davidii* 1, *Erigeron canadensis* 1, *Ambrosia artemisiifolia* 1, *Amaranthus albus* +, *A. powelii* +, *Lepidium densiflorum* +, *Xanthium italicum* +, *Euphorbia maculata* +, *Chenopodium album* sensu lato +, *Erodium cicutarium* +, *Polygonum aviculare* +, *Echium vulgare* +, *Artemisia scoparia* +, *Tribulus terrestris* +, *Lactuca serriola* +, *Salsola kali* subsp. *ruthenica* +, *Portulaca oleracea* +, *Taraxacum officinale* +, *Fraxinus pennsylvanica* juv. +, *Ailanthus altissima* (juv.) + ...

Relevé no. 2 - surface: 9 sq. m, vegetation height: 45 cm, vegetation cover: 95%.
N 45.14237, E 26.83085, ≈ 95 m a.s.l.

Bromus diandrus 5, *Euphorbia davidii* 1, *Parthenocissus inserta* +, *Potentilla argentea* subsp. *argentea* +, *Echium vulgare* +, *Melilotus officinalis* +, *Tribulus terrestris* +, *Cephalaria transsylvanica* +, *Chondrilla juncea* +, *Geranium purpureum* +, *Eleusine indica* +, *Bassia scoparia* + ...

Identification key for *Bromus* (sect. *Genea*) in the flora of Romania is proposed as follow:

1a Lower glume 1 (rarely incompletely 3) veined, the upper one 3 (rarely incompletely 5) veined. Lemmae not aristate or aristate, with the arista inserted between 2 terminal teeth or below the level of the teeth 2

1b Lower glume 3-5-veined, upper (5-) 7-9-veined. Lemmae aristate, with the arista inserted on the back, slightly below its terminal teeth . .....

2a Annual plants, spikelets dilated towards the tip. Arista longer than lemma 3

2b Perennial plants, spikelets with parallel edges. Arista shorter than the lemma or missing .....

3a Lemma over 20 mm long. Panicle ± dense. Stamens 2-5 4

3b Lemma up to 20 mm long. Panicle loose. Stamens 2 or 3 5

4a Dense panicle, erect, 15-20 cm. Lemma 22-25 mm, deeply bifid, with a 30-50 mm arista. Stamens 2, with anthers of 0.7-1 mm long. Scars ± elliptical.
B. rigidus Roth (*Anisantha rigida* (Roth) Hyl.) – Annual, T, 20-40 cm, VI-VII. Cited from Timişoara North railway station (Herb. I, leg. G. Negrean 2014), Caraş-Severin county - along the Danube river banks [JAVORKA, 1925; CIOCÂRLAN, 2000, 2009] and the Constanţa harbour [ANASTASIU & al. 2009; MEMEDEMIN & al. 2016]. 2n=42. *Festucetalia valesiacae*. Medit.-pont.

4b Panicle ± lax, ± pendulous, over 25 cm. Lemma 20-35 mm, deeply bifid, with 4-5 mm teeth, with a 30-65 mm arista. Stamens 2-5, with anthers of 0.7-5.9 mm long. Scars almost circular.

B. diandrus Roth (*Anisantha diandra* (Roth) Tutin) – Annual, T, 30-90 cm, VI-VII. Ruderal. 2n=56. Pont.-iran.-turan.-medit. (Locations: above in this work, in sect. Analyzed specimens)

5a Panicle erect, lax, with branches of about 10 mm or more, shorter than the spikelets, 2-3 at a node. Spikelets loose, with 6-10 flowers. Lemmas of 12-20 mm, narrowly oblong-lanceolate, with the edges sometimes involute when mature and a 12-20 mm arista, straight or slightly divaricate. Stamens 2.

B. madritensis L. (*Anisantha madritensis* (L.) Nevski) – Annual, T, -60 cm, VI-VII. Very rare. Cited from Bucharest – railway station "Triaj" [ANASTASIU & NEGREAN, 2008], Chitila South – the railway station "Triaj" and Constanţa harbour [ANASTASIU & NEGREAN, 2008; ANASTASIU & al. 2009]. 2n=28, 42. S & W Eur.

5b Panicle pendulous, with branches of the same length or longer than the spikelets 6

6a Stem pubescent below the panicle. Panicle unilateral, with flexible and ramified branches. Lemma of 8-12 mm, lanceolate, inconspicuously veined, with a 10-18 mm arista. Stamens 3.

B. tectorum L. (*Anisantha tectorum* (L.) Nevski) – Annual, T, 20-50 cm, V-VI. Frequently, from steppe area to beach belt of vegetation, on dry, ruderal and sandy places. Cont. euras.

6b Stem glabrous below panicle. Panicle multilateral, lax, pendulous at the end, with simple and rough branches. Lemma linear-subulate, 12-20 mm, obviously veined, with a 15-30 mm arista. Ligule of about 4 mm. Stamens 3.

B. sterilis L. (*Anisantha sterilis* (L.) Nevski) – Annual, T, 20-90 cm, V-VI. Frequently, from steppe area to beach belt of vegetation, in acacia plantations, on ruderal places, sands or loess. Euras. (submedit.)

Regarding the possible provenance of *B. diandrus* in Romania's flora, it is reasonable to assume that it could be a contaminant of cereals or other goods transported via railways or arriving through the Black Sea harbours of Romania (also G. Negrean himself identified this species in railway stations of Timişoara, Drobeta-Turnu Severin, and Constanţa harbour). This idea is also supported by other authors [GLEICHSNER & APPLEBY, 1989; MICHAEL & al. 2010].

Considering the location of the species in disturbed habitats and the possible ways of arrival, through harbours or railways, it can be assumed that *B. diandrus* is an adventitious species in the spontaneous flora of Romania.

For the time being, *B. diandrus* does not seem to extend beyond the spaces of railway stations or harbours in Romania; however, preventive measures must be considered to control the invasion of this species outside the current ruderal areas. Some management measures as weed control, could include, for instance: a) burning residues on site, as it is already practiced in Australia and USA [HEENAN & al. 1990; SWEET & al. 2008; BOWCHER & al. 2019], b) complete inversion of the soil as it is practiced in Spain [GARCIA & al. 2013; RECASENS & al. 2016], c) biological control as it is practiced in Tunisia and United Kingdom [LAWRIE & al. 1998; MEJRI & al. 2010], d) non-selective herbicide as it is practiced in Spain [ROYO-ESNAL & al. 2018], e) pre-emergence herbicide as it is practiced in Australia and New Zealand [DASTGHEIB & al. 2003; KLEEMAN & GILL, 2008; KLEEMAN & al. 2016], f) post-emergence herbicide as it is practiced in Australia and Spain [GILL & BOWRAN, 1990; KLEEMAN & GILL, 2009; GARCIA & al. 2014; ROYO-ESNAL & al. 2018], g) spray-topping as it is practiced in Australia [BOWCHER & al. 2019], h) grazing as it is practiced in Australia and New Zealand [TOZER & al. 2007; BOWCHER & al. 2019], i) weed seed harvesting for

seed control as it is practiced in Australia [WALSH & POWLES, 2014; BORGER & al. 2020], and so forth.

Conclusions

The natural distribution of ripgut brome (*Bromus diandrus*) in the Romania's flora is presented here, in synthesis. This species was recorded in the fourth known place in Romania, namely Buzău (it is the first location in the historical province of Muntenia), in the city's train station. At the site, *B. diandrus* was estimated to grow in the thousands along and inbetween the railway tracks and the associated disturbed sites, throughout the railway station area.

Considering the habitat, ecology, and the ruderal behavior, it can be assumed that the ripgut brome is an adventitious species in Romania's flora.

An identification key of the species of the genus *Bromus*, sect. *Genea*, known up to now in the Romania's flora was compiled and included in this work.

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COLLECTION AND PHENOTYPIC CHARACTERIZATION OF SOME NIGERIAN BAMBARA GROUNDNUT (*VIGNA SUBTERRANEA*) GERMPLASM USING SEED MORPHOLOGY

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Abstract: Bambara groundnut is an indigenous African legume with great potential to tackle food insecurity in Nigeria. A germplasm collection mission was carried out in collaboration with the Agricultural Developments Project (ADP) Extension officers of Nigeria between October and December 2014. Bambara groundnut seeds were collected from farmers in Kaduna, Niger, Kogi, Benue, Plateau, Adamawa, Nasarawa, Jigawa, Enugu and Federal Capital Territoy (FCT) Abuja. Some seeds were also collected from National Centre for Genetic Resources and Biotechnology (NACGRAB). A total of 45 original seed lots were collected which comprised of mixed seeds (different seed coat colours) and pure seeded accessions (comprising of one seed coat colour). A total of 24 distinct seed morphotypes were identified from the collections. The highest number of accessions were collected from NACGRAB (11) followed by Niger State (10) and the lowest from Benue, Jigawa and Adamawa States (2). Niger State also had the highest number of mixed seeds. The different seed phenotypes observed in the study are important for field production of true to type lines and can be exploited for the genetic improvement of bambara groundnut.

Keywords: Bambara groundnut, characterization, collection, germplasm, phenotypic.

Introduction

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is a herbaceous plant with substerranean fruit-set cultivated by smallholder farmers in semi-arid region of Africa [AZAM-ALI & al. 2001]. It is an indigenous African leguminous crop belonging to the family Fabaceae and sub-family Faboideae [ABEJIDE & al. 2020]. It has different names by different language groups in Nigeria. In Hausa language bambara groundnut is called “Gurjiya” or “Kwaruru”. In Goemai language of Plateau State it is known as “Kwam”, and Kanuri people refer to it as “Ngamgala”. In Igbo language, it is commonly known as “Okpa” while the Yoruba’s call it “Epa-roro”or “Epa-kuta”. Although the name “bambara groundnut” was derived from the Bambara tribe which presently lives in Mali where the crop is also believed to have originated from [NWANNA & al. 2005].

Bambara groundnut is a high protein crop that is largely cultivated for its seeds used as human food. Chemical analyses showed that the seed contains 32.50-32.72% of total essential amino acids including lysine, histidine, arginine, leucine and isoleucine and 66.10-70.80% of the non-essential amino acids such as methionine, glycine, cysteine, tyrosine and proline [AMARTEIFIO & al. 2006]. It also contains 63% carbohydrate, 19% protein, 6.5% oil and minerals like calcium (95.5-99 mg/100 mg), iron (5.1-9 mg/100 mg), potassium (11447-14355

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mg/100 mg) and sodium (2.9-10.6 mg/100 mg) as reported by MAYES & al. (2012).

Bambara groundnut is a potential crop in contributing to world food security and reducing malnutrition [MSHELIA & al. 2004; OUEDRAOGO & al. 2008]. The seeds can be eaten fresh when boiled and can also be processed by milling to make flour; a paste is then made from the flour and then used in the preparation of various fried or steamed products like "akara" and "moin-moin" [OKPUZOR & al. 2010]. Another much loved Nigerian delicacy made from bambara groundnut is 'Okpa', which is produced by wrapping the doughy paste in banana leaves or polythene and then boiled. The seeds can also be used to produce vegetable milk that is comparable with soy milk. Following a protein functionality test on the ground seeds, BROUGH & al. (1993) indicated that bambara groundnut can compete with or replace other conventional flours in a range of processed products. Bambara groundnut seeds can be used as animal feed and the leafy shoots are also used as fodder [BRINK & al. 2006].

The demand for bambara groundnut is increasing due to its many uses, its high nutritional value and medicinal value as it is believed to be suitable for consumption by diabetic and hypertensive patients. The crop also serves as a major diet for poor subsistence farmers who cannot afford expensive animal protein. In Nigeria, despite the nutritional value, uses and agronomic advantages of bambara groundnut, it is still one of the less cultivated and underutilized legumes. This has been attributed to lack of improved varieties [MAYES & al. 2011]. The crop has received little attention by Scientists [HILLOCKS & al. 2012]. It is cultivated from local land races as there are no true varieties of the species bred for specific traits in Nigeria [ANCHIRINAH & al. 2001]

The increasing world population requires that efforts be made towards increasing food production. Bambara groundnut is a potential crop that can be used to tackle food insecurity and malnutrition in Nigeria. Being a highly nutritious crop, bambara groudnut is relevant to food security. Bambara groundnut also plays an important role in income generation for resource poor farmers in Nigeria as demand for the crop is on the increase due to increasing awareness of its nutritional value. It fetches a high market price and there is therefore a great need to increase its productivity.

Bambara groundnut germplasm is abundant in Sub-Saharan Africa, as the crop is grown in every tropical region of the continent. So far, wild relatives of cultivated bambara groundnut have only been found in North Eastern Nigeria and Northern Cameroon. It is believed that the crop originated from this part of the continent. Electrophoretic studies conducted by HOWELL & al. (1994) did not reveal a significant difference between the cultivated genotypes and the supposedly wild forms, and it was concluded that the wild plant might simply be an escape from the cultivated ecotype. The major germplasm collection held by IITA has been characterized and evaluated. A few other countries such as Zambia, Burkina Faso, Ghana have also characterized their germplasm.

The collections of bambara groundnut available in most national programs may not reflect all the diversity existing in the respective countries. The crop germplasm is often collected in an opportunistic manner. Plant collectors use a collecting mission for a major crop to include sampling of bambara groundnut. For example, scientists at IITA have usually collected bambara groundnut samples during collecting missions for cowpea or rice. Collecting missions primarily devoted to bambara groundnut need to be organized in many countries producing the crop in order to save those ecotypes that are in the process of extinction. It is based on this that Bambara groundnut germplasm were collected from major growing areas in Nigeria and characterized based on their seed morphology.

Materials and methods

Collection of bambara groundnut germplasm

A germplasm collection was carried out in collaboration with the Agricultural Development Projects' (ADP) Extension Officers of Nigeria between October and December 2014. The major bambara groundnut producing States in Nigeria such as Kaduna, Niger, Kogi, Benue, Plateau, Adamawa, Nassarawa, Jigawa, Enugu and Federal Capital Territory (FCT) Abuja were visited in order to collect the germplasm. Some germplasm was also collected from National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan. The seeds were collected packed and sealed in thick paper envelopes each of which was given an entry number, information regarding the locality and local name were also recorded.

Characterization of bambara groundnut germplasm using seed morphology

The seeds collected from farmers and NACGRAB were characterized based on their seed coat colour and pattern and also eye colour and pattern according to the descriptor list of *Vigna subterranea* produced by the International Plant Genetic Resource Institute (IPGRI, 2000). Each original seed lot collected comprising of mixed seeds were sorted out into their distinct seed morpho-types. Royal Horticultural chart was used to identify the colours.

Results

A total of 45 original seed lots were collected during the survey comprising of 34 seed lots collected from farmers and 11 from National Centre for Genetic Resources and Biotechnology (NACGRAB) Ibadan (Table 1). The highest number of accessions were collected from NACGRAB (11) followed by Niger State (10). The least number of accessions were collected from Benue, Jigawa and Adamawa States (2). It was observed that farmers possessed both pure seeds (with same seed coat colour pattern and eye colour pattern) and mixed seeds (comprising of a variety of seed coat colour pattern and eye colour pattern). Out of the original seedlots collected during the survey, Niger State had the highest number of mixed seeds (4) and the least was observed in Nassarawa State (0) with all the accessions having pure seeds and no mixed seeds. NACGRAB had the highest number of original seed lots comprising of pure seeds (9) and the least was observed in Benue, Jigawa and Adamawa States having one accession that is pure seeded (Table 1).

Table 1. Number of bambara groundnut accessions collected during the survey

Geopolitical zone	State	Number of accessions	Number of pure seeds	Number of mixed seeds
North-Central	Kogi	3	2	1
	Benue	2	1	1
	Plateau	3	2	1
	Niger	10	6	4
	Nassarawa	2	2	0
	FCT	3	2	1
North-West	Kaduna	3	2	1
	Jigawa	2	1	1
North-East	Adamawa	2	1	1
South-East	Enugu	4	2	2
NACGRAB	NACGRAB	11	9	2
Total		45	30	15

The 45 bambara groundnut accessions collected during the survey were made up of 24 distinct seed morphotypes (Figure 2) with varying seed coat colours and eye colours. A total of 21 descriptors were used to describe their seed coat colours. They are cream (B, C, M), cream purplish spots (D), cream brown spots/stripe (R), cream black stripe (S), cream dark brown patches (U), cream light grey spots (T), cream black patches (K), black (A), red (N), light red (I), dark red (E, F), brownish red (Q), brown speckled with black (H), red speckled with black (G), brown (J), brown with brown pattern below hilum (L), brown with black pattern below hilum (O), cream black (P), grey brown (V), grey black (W), variegated red (X) (Figure 2).

Figure 1. Mixed and pure seeds of bambara groundnut accessions.
A, B, C: samples of mixed seeds of bambara groundnut accessions collected during the survey
D, E, F: samples of pure seeds of bambara groundnut accessions collected during the survey

Figure 2. Variations in bambara groundnut seed coat colour collected during the survey.
A – black; B, C, M – cream; D – cream purplish spots; E, F – dark red; G – red speckled with black; H – brown speckled with black; I – light red; J – brown; K – cream black patches; L – brown with brown pattern below hilum; N – red; O – brown with black pattern below hilum; P – cream/black; Q – brownish red; R – cream brown spots/stripes; S – cream purplish stripes; T – brown with grey spots; U – cream with brown patches; V – grey brown; W – variegated red; X – grey black

Table 2. Characteristics of bambara groundnut accessions (original seed lots) collected from peasant holders in Nigeria

S/N	Accession number	Local government	State	Seed type	Local name	Diversity score
1.	NG-KG-O1	Dekina	Kogi	Brownish red	Okpakpikpa	1.0
2.	NG-KG-02	Ankpa	Kogi	Mixed	Jatoaka	3.0
3.	NG-KG-03	Ofu	Kogi	Cream	Okpafufu	1.0
4.	NG-EN-O4	Igbo-Etiti	Enugu	Brownish red	Aki naukwaa	1.0
5.	NG-EN-05	Igbo-Etiti	Enugu	Mixed	Eyo	3.0
6.	NG-EN-06	Igbo-Etiti	Enugu	Mixed	Oddy	4.0
7.	NG- EN-07	Igbo-Etiti	Enugu	Cream	Caro	1.0
8.	NG-KD-08	Zaria	Kaduna	Mixed	Kwaruru/ gurjiya	7.0
9.	NG-KD-09	Jemaá	Kaduna	Cream	Kwaruru	1.0
10.	NG-KD-10	Sanga	Kaduna	Cream purplish spots	Kwaruru	1.0
11.	NGR-PL-11	Jos	Plateau	Mixed	Kwam	2.0
12.	NGR-PL-12	Jos	Plateau	Brownish red	Kwam	1.0
13.	NGR-PL-13	Jos	Plateau	Cream purplish spots	Kwam	1.0
14.	NGR-BN-16	Ogbadibo	Benue	Mixed	Karo	2.0
15.	NGR-NS-14	Laffia	Nassarawa	Cream	Ikpeyi	1.0
16.	NGR-NS-15	Obi	Nassarawa	Cream black stripes	Kirikiri	1.0
17.	NGR-JG-17	Kazaure	Jigawa	Mixed	Kwaruru	3.0
18.	NG-NI-18	Chanchaga	Niger	Black	Kwaruru	1.0
19.	NG-NI-19	Chanchaga	Niger	Ceam	Kwaruru	1.0
20.	NG-NI-20	Bida	Niger	Mixed	Edzu	11.0
21.	NG-NI-21	Katcha	Niger	Cream	Edzubokun	1.0
22.	NG-NI-22	Kotangora	Niger	Variegated cream black	Yarkasa	1.0
23.	NG-NI-23	Kotangora	Niger	Mixed	Kwaruru	3.0
24.	NG-NI-24	Kotangora	Niger	Mixed	Kwaruru	1.0
25.	NG-NI-25	Shiroro	Niger	Mixed	Kwaruru	5.0
26.	NG-NI-26	Shiroro	Niger	Cream	Yarkasa	1.0
27.	NG-NI-27	Shiroro	Niger	Cream purplish spots	Kwaruru	1.0
28.	NGR-AD-28	Yola	Adamawa	Mixed	Kwaruru	2.0
29.	NGR-AB-29	Zuba	FCT	Cream purplish stripes	Kwaruru	1.0
30.	NGR-AB-30	Dakwa	FCT	Cream purplish spots	Kwaruru	1.0
31.	NGR-AB- 31	Yaba	FCT	Mixed	Kwaruru	2.0
32.	NGR- BN-32	Kwande	Benue	Cream	Sisi	1.0
33.	NGR-JG-33	Hadejia	Jigawa	Cream	Gurjiya	1.0
34.	NGR-AD-34	Yola	Adamawa	Cream	Kwaruru	1.0
35.	NGB-01486	-	NACGRAB	Cream	-	1.0
36.	NGB-01493	-	NACGRAB	Cream	-	1.0
37.	NGB-01492	-	NACGRAB	Cream	-	1.0
38.	NGB-01496	-	NACGRAB	Cream purplish spots	-	1.0
39.	NGB-01489	-	NACGRAB	Cream	-	1.0
40.	NGB-01491	-	NACGRAB	Cream	-	1.0
41.	NGB-01311	-	NACGRAB	Cream	-	1.0
42.	NGB-01646	-	NACGRAB	Mixed	-	3.0
43.	NGB-01645	-	NACGRAB	Mixed	-	2.0
44.	NGB-01488	-	NACGRAB	Cream	-	1.0
45.	NGB-01487	-	NACGRAB	Cream	-	1.0

Discussion

The seeds collected from farmers during the survey revealed that farmers possess and grow both pure and heterogeneous mixtures of seeds that hold distinctive and divergent genetic attributes (Table 1). ALHASSAN & EGBE (2013) in a participatory rural appraisal of bambara groundnut in Kogi and Benue States observed that farmers possess both pure and mixed seeds. MOHAMMED & al. (2013) also reported the same in Kano State that bambara groundnut landraces exist as heterogeneous mixtures of seeds of few to several morphotypes that embraces a wide genetic potential. Farmers grow mixtures of different seed types probably because of the absence of improved varieties which has resulted into variable yields between years and localities [ABU & BUAH, 2011].

The highest number of pure seeded accessions was observed in NACGRAB (9), this could be because, being a National Centre for Genetic Resources, some level of sorting of the accessions must have been carried out. Accessions, which have cream seed coat colour, are most homogeneous due to market demand. This is because at the time of harvesting, farmers avoid mixture on Bambara groundnut seeds which possess creamy seed coat colour.

The bambara groundnut seed collections (45 seedlots) collected from farmers in Nigeria and NACGRAB phenotyped using visual techniques to describe seed morphology revealed that bambara groundnut possesses distinguishable morphological identities that can be exploited through breeding. The 24 distinct seed morphotypes (Table 2) distinguished are important for field production of true to type lines that can be used for further genetic improvement of the crop. Variations in seed feature have been previously reported by other authors such as MASSAWE & al. (2005) and ABU & BUAH (2011). ABU & BUAH (2011) reported that seeds of bambara groundnut landraces possess identifiable morphological features such as seed testa colour, seed shape, eye and hilum colour and pattern. While PADULOSI & al. (2002) reported that variations in seed coat colour and eye colour and patterns displayed by the landraces are useful to differentiate among bambara groundnut genotypes.

Conclusion

It can be concluded from the study that bambara groundnut germplasm in Nigeria embraces a wide genetic pool with distinct seed morphological identities that can be exploited and used in crop improvement. The different seed phenotypes observed in the study are also important for field production of true to type lines that can be used for further genetic improvement of bambara groundnut.

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PHYTOCHEMICAL SCREENING OF DHAMAN (*GREWIA TILIIFOLIA* VAHL) FRUIT IN SUB-TROPICAL REGION OF INDIA

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Abstract: The present study was conducted to determine the availability of bioactive phytomolecules in the Dhaman (*Grewia tiliifolia*) fruit collected from a sub-tropical region of India. Methanolic and ethanolic extracts were prepared from processed Dhaman fruits. The extracts were screened for phytomolecules using the Gas chromatography – mass spectrometry (GC-MS) method. Results found that the methanolic extract has alkaloids at 5%, esters at 21%, triterpenoids at 2%, fatty alcohol at 5%, acid chloride at 2%, hydrocarbons at 26%, and steroids at 2%. However, the ethanolic extract has alkaloids (2%), ester (27%), fatty alcohol (6%), hydrocarbons (9%), steroids (13%), amide (4%), and triglycerides (2%) in different amounts. From this study, it has been concluded that the Dhaman fruit is highly enriched in different phytomolecules. As a result, Dhaman fruit has a high potential for curing various human diseases and well-being.

Keywords: bio-active molecules, ethanolic extract, GC-MS, methanolic extract, phytomolecules.

Introduction

Ayurveda, an ancient system of medicine originating in India, recommends the utilization of various components of the *Grewia tiliifolia* plant for treating conditions such as irritation, burning sensations, fever, blood disorders, excessive menstrual flow, and diabetes [ZIA-UL-HAQ & al. 2013]. It is known to be effective in addressing gastric issues, hyperdipsia, rhinopathy, ulcers, skin diseases, haematemesis, and general debility. Traditional healing practices involve employing the leaves, bark decoction, and infusion of this medicinal plant to treat snakebites in livestock through wound drenching [OWUOR & KISANGAU, 2006]. The application of fresh leaf paste aids in burn treatment, while the powdered bark is utilized for its aphrodisiac properties [MRABTI & al. 2022]. Furthermore, the ripe fruits of *Grewia tiliifolia* are consumed for their nutritional value [AHMAD & al. 2013]. DIXIT & GEEVAN (2000) documented the historical use of *Grewia tiliifolia* as an agricultural tool and food source.

Grewia tiliifolia, commonly known as Dhamani or Dhaman, is a medium-sized tree reaching a height of approximately 20 meters, with a trunk length of 8 meters and a diameter of 65 cm. The plant bears small yellow flowers on thick axillary peduncles. It holds a significant place in the Ayurvedic system of medicine. *Grewia tiliifolia* is primarily found in various regions of India, including Punjab, Himachal Pradesh, Uttar Pradesh, Chennai, Andhra Pradesh, and Mumbai. It belongs to the Malvaceae family, which encompasses approximately 150

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species of small trees or shrubs distributed across subtropical and tropical regions, including tropical Africa, Arabia, the Himalayas, India, Pakistan, China, Bangladesh, Myanmar, Thailand, and Northern Australia [ULLAH & al. 2012]. India alone hosts around 40 different species, such as *G. asiatica*, *G. tenax*, *G. hirsuta*, *G. damine*, *G. lasiodiscus*, *G. optiva*, *G. biloba*, *G. bicolor*, and many others [KUMAR & al. 2022].

Grewia tiliifolia, known as Dhaman, exhibits various medicinal properties. The powdered wood of *G. tiliifolia* is used as an emetic to counteract opium poisoning. Additionally, the stem bark acts as a coagulant and shows cardiovascular effects [KUMAR & al. 2022]. Studies have demonstrated the analgesic and antipyretic properties of *G. tiliaefolia* bark [PAVIAYA & al. 2013]. The plant is often employed in the treatment of burning sensations, blood disorders, and as an aphrodisiac and tonic. Furthermore, the methanolic extract and a constituent called gulonic acid gamma-lactone obtained from *G. tiliifolia* have shown in vivo wound healing activity [AHAMED & al. 2009]. ADHIKARI & al. (2010) reported the use of flowers, fruits, bark, and leaves of *G. tiliifolia* for treating syphilis. Notably, *G. tiliifolia* exhibits cholinesterase inhibitory properties and demonstrates anti-amyloidogenic and neuroprotective effects in *in-vitro* and *in silico* conditions [MALAR & al. 2017]. *G. tiliifolia* is utilized in cases of inflammation and burning sensations [JUVEKAR & al. 2007]. It is employed for the treatment of skin diseases, inflammatory bowel diseases, diarrhea, and pruritus [BADAMI & al. 2002]. Previous researchers have isolated three triterpenoids, namely betulin, friedelin, and lupeol, from the stem bark of *G. tiliifolia* [BADAMI & al. 2002, 2004]. Moreover, the methanolic extract of *G. tiliifolia* stem bark demonstrates potent antioxidant and antibacterial properties [JUVEKAR & al. 2007]. The same extract has shown significant wound healing activity in various cutaneous wound models in rats [AHAMED & al. 2009].

In traditional Indian folk medicine, *Grewia asiatica* fruit, also known as Dhaman fruit, is used to alleviate blood disorders, as well as cardiac and respiratory diseases [POONAM & SINGH, 2009]. Research suggests that *G. asiatica* possesses anticancer [GUPTA & al. 2014], antioxidant [ZIA-UL-HAQ & al. 2013], radio-protective, hepatoprotective [SHARMA & SISODIA, 2009], and antihyperglycemic [KHATTAB & al. 2015] activities. *Grewia asiatica* fruit is rich in nutrients, including vitamins and minerals, and contains various bioactive compounds such as anthocyanins, tannins, phenolics, and flavonoids [ZIA-UL-HAQ & al. 2013].

Although the utilization of Dhaman trees in traditional medicine is well-established, there remains a paucity of studies focusing on the phytochemical screening of Dhaman fruit. Further investigations in this area may unveil specific curative properties associated with Dhaman fruits, offering new avenues for research. Consequently, these findings hold potential implications for promoting human well-being in the Post-COVID-19 era.

Materials and methods

Experimental site

The experiment was performed in Rehan Khas village, situated in the Kangra district in Himachal Pradesh. The experimental site lies between 32°9'49.02" North Latitude and 75°54'49.30" East Longitude (Figure 1).

Figure 1. Experimental site make by Google 3D Earth Pro software

Environmental conditions

The experiment was conducted under average temperatures of 22-37 °C in Rehan Khas village in District Kangra HP. In Himachal Pradesh, over 30 years of records, average air temperatures were 0.7 to 2.4 °C higher than in the 1980s, as against the global average of 0.5 °C; the Himachal Pradesh trend indicates an increase of 0.06 °C per year.

Plant material collection

Fresh fruits were collected from the Dhaman tree (Figure 2). The fruits of Dhaman were dried for 30 days in the shade at room temperature. Then grounded, the fruit and a powder were obtained. Powdered samples were kept in a refrigerator (4 °C) for further experiments.

Figure 2. Dhaman tree; the experimental plant from where fruit samples were collected

Plant ethics, guidelines, and source

We hereby confirm that our utilization of plants in this study adheres strictly to international, national, and/or institutional guidelines. Our research specifically excludes the involvement of genetically modified plants, as well as any genetic plant resources procured from local suppliers or collectors. This encompasses species obtained from protected areas, endangered species possessing medical significance, or quarantine organisms (e.g., harmful pests or plant pathogens). Consequently, we did not require legal authorization from the appropriate governing body or the need to adhere to standard protocols. Our sample collection occurred within the confines of Rehan Khas village, located in the Kangra district of Himachal Pradesh. The experimental site's geographical coordinates fall between 32°9'49.02" North Latitude and 75°54'49.30" East Longitude (Figure 1).

Preparation of fruit extract

The extraction of Dhaman tree fruit was conducted following the method proposed by FATOPE & al. (1993), with minor adjustments. Briefly, 20 g of fruit powder was percolated at room temperature using 400 mL of methanol for methanolic extraction, as well as for aqueous extraction. Another 20 g of powdered fruit was mixed with 400 mL of hot water, starting at an initial temperature of 98 °C. The mixture was then placed in conical flasks, tightly sealed, and subjected to agitation in a shaker at room temperature for 24 hours, operating at 100 rpm. After the 24-hour period, the extract was filtered using a muslin cloth, followed by further filtration using Whatman filter paper no. 1. The filtrates were subsequently concentrated using a water bath set at a temperature range of 35-40 °C. The resulting extracts were appropriately labelled and stored in a refrigerator at 4 °C. To prepare a stock solution, the extract was dissolved in DMSO to achieve a concentration of 0.5 g/mL.

Phyto-characterization of fruit extract

Salkowski test (Terpenoid test): a sample weighing 0.5 g was combined with 2 mL of chloroform, followed by the addition of 3 mL of concentrated sulfuric acid to form a distinct layer. The presence of terpenoids was indicated by the formation of a reddish-brown coloration at the interface.

Flavonoid test: to 0.5 mL of the extract filtrate, 5 mL of dilute ammonia was added, followed by the addition of 1 mL of concentrated sulfuric acid. The presence of flavonoids was indicated by the appearance of a yellow coloration in the solution, which faded over time.

Saponin test: 0.5 g of the extract was mixed vigorously with 5 mL of distilled water. After the formation of a stable and persistent froth, 3-4 drops of olive oil were added, and the mixture was vigorously shaken again. The formation of an emulsion indicated the presence of saponins.

Saponin glycosides test: 0.5 mL of the extract was treated with 80% H_2SO_4, resulting in a deep yellow coloration, confirming the presence of saponin glycosides.

Tannin test: a sample weighing 0.5 g was mixed with 10 mL of distilled water and boiled. The solution was then filtered, and a few drops of 0.1% ferric chloride were added. The presence of tannins was indicated by the formation of a brownish-green or blue-black color.

Phenolics test (Ferric chloride test): small amounts of the aqueous and alcoholic extracts were separately dissolved in 2 mL of distilled water, followed by the addition of a few drops of 10% aqueous ferric chloride solution. The presence of phenols was confirmed by the development of a blue or green color.

Carbohydrate test (Molisch's test): 0.5 g of the extract was mixed with 5 mL of distilled water, and the resulting solution was filtered. To the filtrate, 1 mL of α-Naphthol and

concentrated H_2SO_4 were added, leading to the formation of a purple color, indicating the presence of carbohydrates.

Chlorogenic acid test: 0.5 mL of the extract was treated with aqueous ammonia and exposed to the air. The resulting solution exhibited a green color, suggesting the presence of chlorogenic acid.

Coumarin test: 0.5 mL of both aqueous and alcoholic extract solutions were treated with 10% sodium chloride. The yellow coloration of the solution indicated the presence of coumarin.

Flavone test: two methods were employed to detect the presence of flavones. In the first method, 0.5 mL of the extract was treated with sodium hydroxide, and the appearance of a yellow coloration indicated the presence of flavones. In the second method, 0.5 mL of the extract was treated with sulfuric acid, resulting in a yellowish-orange coloration, also indicating the presence of flavones.

Anthocyanin test: two methods were utilized to determine the presence of anthocyanin. In the first method, 0.5 mL of the extract was treated with aqueous sodium hydroxide, and the formation of a blue or violet coloration confirmed the presence of anthocyanin. In the second method, 0.5 mL of the extract was treated with sulfuric acid, resulting in a yellowish-orange coloration, also indicating the presence of anthocyanin.

Phytochemical screening using GC-MS: the phytochemical analysis was conducted utilizing a Thermo Trace 1300GC gas chromatography system coupled with a Thermo TSQ 8000 Triple Quadrupole MS, following the procedure described by BHARDWAJ & al.(2019), with minor adjustments. Data processing was performed using XCalibur 2.2SP1 software with Foundation 2.0SP1. The analysis employed a BP 5MS column (30 m X 0.25 mm, 0.25 µm). The total program duration was 60 minutes, and the temperature program for the column oven was as follows: starting at 80 °C (maintained for 1.0 minute), followed by an increase to 236°C at a rate of 6 °C/min, with a 5-minute hold, and finally reaching 300 °C at a rate of 8 °C/min with a 20-minute hold. The carrier gas (Helium) flow rate was set at 1.10 mL/min, and the injection volume and injector temperature were 2.0 µL and 280 °C, respectively. The mass range analyzed was 50-650 m/z using electron ionization (EI) mode. Identification of phytocomponents was based on comparison of their mass spectral data with the NIST 2.0 database. The results were expressed as percentage areas.

Methodology limitations: due to limitations in logistical support and funding, certain essential parameters such as antioxidant analysis and in vitro studies to assess biological effects could not be conducted. However, despite these limitations, our study successfully revealed unique and significant findings regarding the percentage availability of various phytomolecules in Dhaman fruit, which was the main objective of this investigation.

Results and discussions

Phytochemical characterization of extracts of *Grewia tiliifolia* fruits

After extracting herbal plants using different solvents, the extract with the highest yield (aqueous extract of *Grewia tiliifolia*) was subjected to phytochemical characterization to identify various bioactive phytomolecules. The analysis revealed the presence of terpenoids, flavonoids, saponins, tannins, phenols, carbohydrates, coumarin, and anthocyanin in three of the extracts. However, chlorogenic acid and saponin glycoside were not detected. These findings are consistent with a study conducted by HARIDAS & al. (2017), which also reported the presence of terpenoids, flavonoids, and phenols in the tender leaves of *Grewia tiliifolia* Vahl.

It is worth noting that terpenoids, flavonoids, and phenols possess notable antioxidant activity, suggesting their potential as antioxidants.

Gas chromatography (GC-MS) analysis

The present study covers the GC-MS analysis of methanol and ethanol extracts of *Grewia tiliifolia* (Figure 3 and 4). Both extracts obtained using the soxhlet extraction showed the presence of 48 compounds in each. Most compounds found in these two extracts were of the ester class. In contrast, hydrocarbons, fatty alcohol, alkaloids, triglyceride, amide, steroids, triterpenoid, sesquiterpenoid, acid chloride, phenic acid derivatives, and fatty acid classes of secondary metabolites were also identified (Table 1 & 2, Figure 5). The maximum peak area was found in methanol extract for 9,12-octadecadienoic acid, methyl ester, trans-13-Octadecenoic acid, and methyl ester.Further, in ethanol extract, 2-methyl-Z, Z-3,13-octadecadienol was found at a maximum, followed by n-Hexadecanoic acid.

Grewia tiliifolia bark has long been employed in traditional medicine. Previous investigations [BADAMI & al. 2002, 2004] successfully isolated three triterpenoids, namely betulin, friedelin, and lupeol, from the stem bark of *G. tiliifolia*. These studies also assessed the in vitro cytotoxic properties of *G. tiliifolia* bark against various cell lines [BADAMI & al. 2003]. Phalsa, a member of the *Grewia* species, is another plant used in folk medicine. It contains essential mineral elements, carbohydrates, and various active metabolites such as flavonoids and alkaloids [PATIL & al. 2011; ULLAH & al. 2012]. Historically, plants have been a source of lead compounds for anticancer drugs like vincristine and taxol [GREENWELL & RAHMAN, 2015]. *G. villosa*, belonging to the *Grewia* genus, has been reported to possess anticancer activity, and compounds such as nitidanin, grewin, harman, and gulonic acid have been identified from different *Grewia* species [KHADEER AHAMED & al. 2010; WALIULLAH & al. 2011].

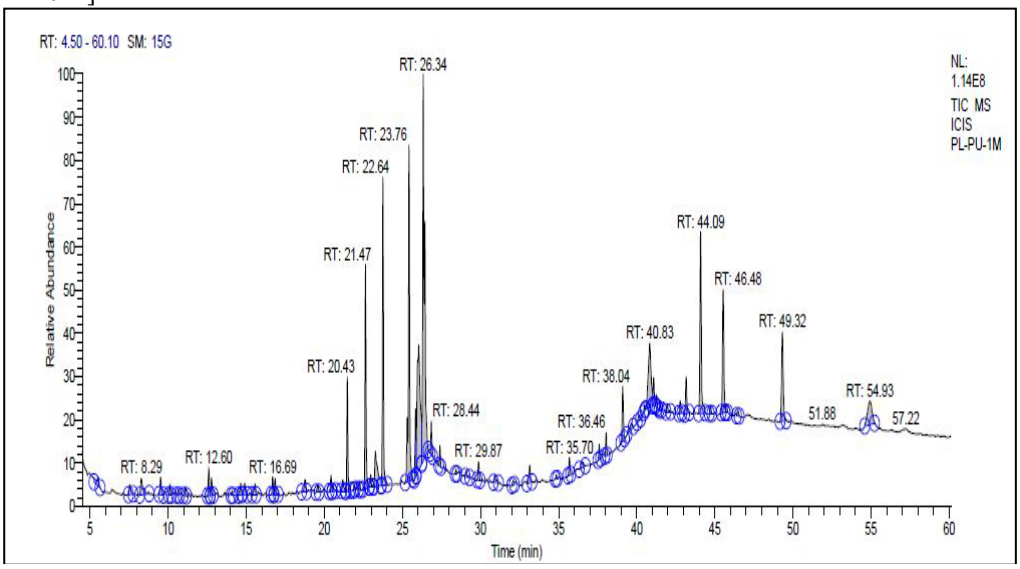

Figure 3. GC-MS Chromatogram of methanolic extract of *Grewia tiliifolia* fruit

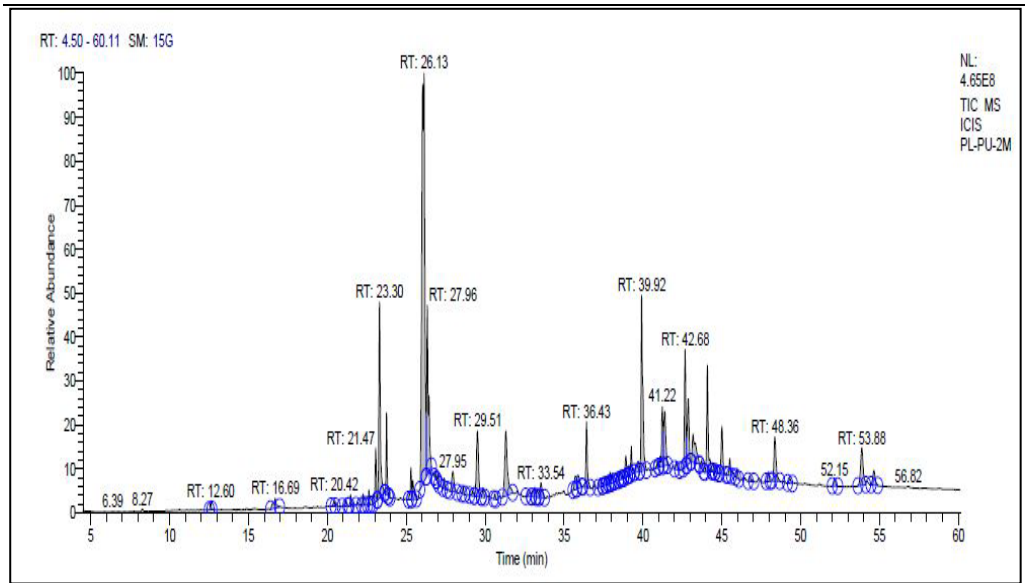

Figure 4. GC-MS Chromatogram of ethanolic extract of *Grewia tiliifolia* fruit

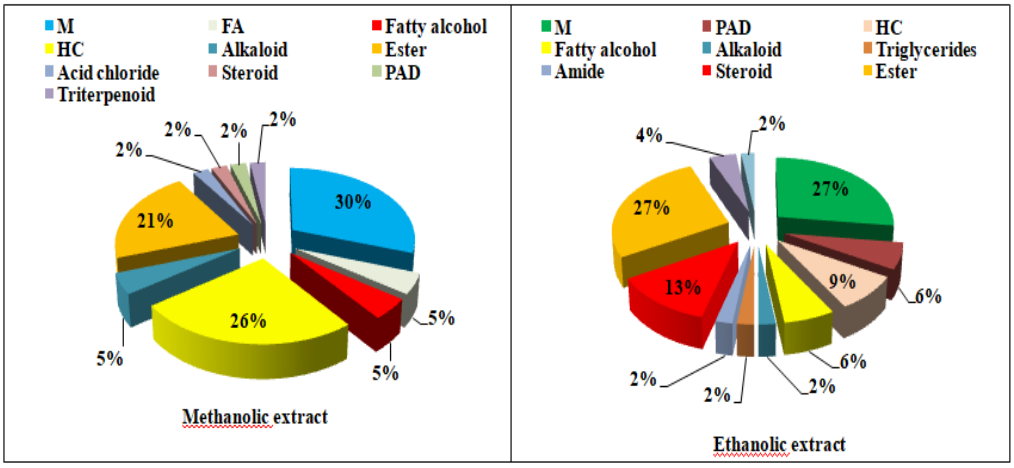

Figure 5. GC-MS Chromatogram of methanolic and ethanolic extract of *Grewia tiliifolia* fruit

Table 1. Compound identified in methanolic extract of *Grewia tiliifolia* fruit using GC-MS

S. No.	Compound name	Formula	M.W.	R.T.	% Area	CAS No.
1.	1,1,1,3,5,5,5-Heptamethyltrisiloxane	$C_7H_{22}O_2Si$	166.33	39.64	0.46	1873-88-7
2.	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	$C_{13}H_{40}O_5Si_6$	444.97	29.87	0.52	38147-00-1
3.	17-Octadecynoic acid	$C_{18}H_{32}O_2$	280.44	26.05	9.71	34450-18-5
4.	2-Hexyl-1-octanol	$C_{14}H_{30}O$	214.39	10.66	0.18	19780-79-1
5.	2-t-Butyl-1-methyl-3-phenyl-imidazolidin-4-one	$C_{14}H_{20}N_2O$	232.32	9.52	0.61	NA
6.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.53	21.19	0.34	102608-53-7
7.	3-Hexadecene,(Z)-	$C_{16}H_{32}$	224.42	12.60	0.73	34303-81-6
8.	4,2-Cresotic acid, 6-methoxy-, bimol. ester, methyl ester, 4,6-dimethoxyotoluate	$C_{29}H_{30}O_{10}$	538.54	29.09	0.22	19314-74-0
9.	4,4-Bis(dichlorofluoromethyl)-1,2-oxathietane-2,2-dioxide	$C_4H_2Cl_4F_2O_3S$	309.93	36.46	0.37	22721-88-6
10.	5-Octadecene,(E)-	$C_{18}H_{36}$	252.48	16.69	0.46	7206-21-5
11.	6,9,12-Octadecatrienoic acid, phenylmethyl ester,(Z,Z,Z)-	$C_{25}H_{36}O_2$	368.55	34.85	0.10	77509-03-6
12.	7,9-Ditertbutyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276.37	22.26	0.18	82304-66-3
13.	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294.47	26.34	17.01	2462-85-3
14.	9,12-Octadecadienoyl chloride, (Z,Z)-	$C_{18}H_{31}ClO$	298.89	30.92	0.34	7459-33-8
15.	ç-Sitosterol	$C_{29}H_{50}O$	414.70	44.09	6.23	83-47-6
16.	Cyclopropaneoctanoic acid,2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl], methyl ester	$C_{22}H_{38}O_2$	334.53	32.08	0.15	10152-71-3
17.	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	222.46	40.16	0.22	541-05-9
18.	Decane, 2,3,5,8-tetramethyl-	$C_{14}H_{30}$	198.39	15.57	0.38	192823-15-7
19.	Decane, 2-methyl-	$C_{11}H_{24}$	156.31	20.56	0.23	6975-98-0
20.	Di-n-decylsulfone	$C_{20}H_{42}O_2S$	346.61	39.11	1.61	111530-37-1
21.	Dodecane, 2,6,11-trimethyl-	$C_{15}H_{32}$	212.41	14.11	0.13	31295-56-4
22.	Dodecane, 4,6-dimethyl-	$C_{14}H_{30}$	198.39	14.65	0.36	61141-72-8
23.	Eicosanoic acid, ethyl ester	$C_{22}H_{44}O_2$	340.58	26.85	0.79	18281-05-5
24.	Hexadecane, 2,6,11,15-tetramethyl-	$C_{20}H_{42}$	282.55	19.58	0.28	504-44-9
25.	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284.48	2376.	7.95	628-97-7
26.	l-Proline, N-methoxycarbonyl-, isohexyl ester	$C_{13}H_{23}NO_4$	257.33	8.29	1.09	NA
27.	Methyl stearate	$C_{19}H_{38}O_2$	298.50	25.84	1.61	112-61-8
28.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	23.27	2.47	57-10-3
29.	Octadecane, 1-(ethenyloxy)-	$C_{20}H_{40}O$	296.53	20.43	0.42	930-02-9
30.	Octane, 2,4,6-trimethyl-	$C_{11}H_{24}$	156.31	12.78	0.51	62016-37-9
31.	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	579.25	37.60	0.38	19095-24-0
32.	Pentanoic acid, 5-hydroxy-,2,4-ditbutylphenyl esters	$C_{19}H_{30}O_3$	306.44	14.90	0.43	166273-38-7

33.	Phthalic acid, cyclobutyl isobutyl ester	$C_{25}H_{38}O_4$	402.57	21.47	3.01	NA
34.	Silane, cyclohexyldimethoxymethyl-	$C_9H_{20}O_2Si$	188.34	7.55	0.39	17865-32-6
35.	Silane, diethyldecyloxypentadecyloxy-	$C_{29}H_{62}O_2Si$	470.88	45.53	4.27	NA
36.	Silicic acid, diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	296.58	41.09	0.79	3555-45-1
37.	Squalene	$C_{30}H_{50}$	410.72	38.04	0.55	111-02-4
38.	Tetracosamethylcyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$	889.84	22.98	0.30	18919-94-3
39.	Tetradecanoic acid, 10,13-dimethyl-, methyl ester	$C_{17}H_{34}O_2$	270.45	22.64	5.89	267650-23-7
40.	trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.49	25.43	10.61	NA
41.	Undecane, 2-methyl-	$C_{12}H_{26}$	170.33	18.77	0.51	7045-71-8
42.	Undecane, 3,7-dimethyl-	$C_{13}H_{28}$	184.36	11.12	0.27	17301-29-0
43.	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	$C_{16}H_{28}O_3$	268.39	28.44	0.11	NA

Table 2. Compound identified in ethanolic extract of *Grewia tiliifolia* fruit using GC-MS

S. No.	Compound name	Formula	M.W.	R.T.	% Area	CAS No.
1.	1,1,1,3,5,5,5-Heptamethyltrisiloxane	$C_7H_{22}O_2Si_3$	222.50	42.11	0.35	1873-88-7
2.	1,1,1,5,7,7,7-Heptamethyl-3,3bis(trimethylsiloxy) tetrasiloxane	$C_{13}H_{36}O_4Si_4$	368.76	49.31	0.22	87867-97-8
3.	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	390.55	29.51	3.27	6422-86-2
4.	1,5,7,9,11,13-Hexamethyl-3,15-diphenyloctaprismo Octasilasesquioxane	$C_{18}H_{28}O_{12}Si_8$	661.09	42.87	2.62	NA
5.	10-Heneicosene (c,t)	$C_{21}H_{42}$	294.56	16.69	0.61	95008-11-0
6.	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	498.88	37.60	0.34	54284-45-6
7.	2-Bromotetradecane	$C_{14}H_{29}Br$	277.28	36.43	2.18	74036-95-6
8.	2-Cyclohexen-1-one, 3-(3-hydroxybutyl)-2,4,4-trimethyl	$C_{13}H_{22}O_2$	210.31	37.29	0.40	27185-79-1
9.	2-methyltetracosane	$C_{25}H_{52}$	352.68	27.00	0.19	NA
10.	2-Methyl-Z, Z-3,13-octadecadienol	$C_{19}H_{36}O$	280.49	26.13	27.34	NA
11.	2-Piperidinone, N[4-bromonbutyl]-	$C_9H_{16}BrNO$	234.13	38.39	0.28	195194-80-0
12.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.53	21.19	0.20	102608-53-7
13.	3-Hexadecene, (Z)	$C_{16}H_{32}$	224.42	12.60	0.20	34303-81-6
14.	3-Isopropyl-6a,10b-dimethyl-8-(2-oxo-2-phenyl-ethyl)-dodecahydro-benzo[f]chromen-7-one	$C_{26}H_{36}O_3$	396.56	38.72	0.10	NA
15.	4,8,12-Tetradecatrien-1-ol, 5,9,13-trimethyl-	$C_{17}H_{30}O$	250.42	38.04	0.25	NA
16.	6-Octadecenoic acid, methyl ester, (Z)-	$C_{19}H_{36}O_2$	296.49	25.42	0.74	2777-58-4
17.	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276.37	22.26	0.24	82304-66-3
18.	8-Methyl-6-nonenamide	$C_{10}H_{19}NO$	169.26	31.32	3.99	NA
19.	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol,(3á,5Z,7E)-	$C_{27}H_{44}O_3$	416.63	37.72	0.30	40013-87-4
20.	9,12,15-Octadecatrienoic acid, 2,3-bis(acetyloxy)propyl ester, (Z,Z,Z)-	$C_{25}H_{40}O_6$	436.58	32.84	0.31	55320-02-0

21.	9,12-Octadecadienoic acid (Z,Z), methyl ester	$C_{19}H_{34}O_2$	294.47	25.30	0.84	2462-85-3
22.	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	308.50	26.34	6.38	7619-08-1
23.	9-Octadecenoic acid, methyl ester	$C_{21}H_{40}O_4$	356.54	53.88	2.17	3443-84-3
24.	á-Amyrin	$C_{30}H_{50}O$	426.72	45.98	0.31	638-95-9
25.	Allopregnan-3á, 9à-diol-20-one, 3acetate	$C_{23}H_{36}O_4$	376.53	43.17	2.19	24587-69-7
26.	Androst-4-en-3-one, 17-acetoxy-19-(N-formylmethylamino)	$C_{23}H_{33}NO_4$	387.51	35.89	0.84	NA
27.	á-Sitosterol	$C_{31}H_{52}O_2$	456.74	43.73	0.28	915-05-9
28.	Benzoic acid, 2-(dimethylamino)ethyl ester	$C_{11}H_{15}NO_2$	193.24	23.06	1.40	2208-05-1
29.	Bisphenol, bis(tert-butyldimethylsilyl) ether	$C_{27}H_{44}O_2Si_2$	456.81	44.50	0.18	NA
30.	Butyl 9-tetradecenoate	$C_{18}H_{34}O_2$	282.46	38.54	0.23	NA
31.	Cedran-diol, (8S,14)-	$C_{15}H_{26}O_2$	238.36	47.95	0.32	62600-05-9
32.	Corymbolone	$C_{15}H_{24}O_2$	236.35	38.91	0.71	97094-19-4
33.	Ç-Sitosterol	$C_{29}H_{50}O$	414.70	39.70	0.63	83-47-6
34.	Cyclohexanepropanol, 2,2-dimethyl-6-methylene-	$C_{12}H_{22}O$	182.30	29.20	0.18	95452-04-3
35.	Cyclooctane, (methoxymethoxy)-	$C_{10}H_{20}O_2$	172.26	33.31	0.27	42604-11-5
36.	Di-n-decylsulfone	$C_{20}H_{42}O_2S$	346.61	40.93	0.19	111530-37-1
37.	E-11-Hexadecenoic acid, ethyl ester	$C_{18}H_{34}O_2$	282.46	26.85	0.13	NA
38.	Eicosanoic acid, phenylmethyl ester	$C_{27}H_{46}O_2$	402.65	35.71	0.58	77509-04-7
39.	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284.48	23.75	1.78	628-97-7
40.	Hexadecanoic acid, octadecyl ester	$C_{34}H_{68}O_2$	508.90	48.36	2.11	2598-99-4
41.	Lupeol	$C_{30}H_{50}O$	426.72	45.51	0.50	545-47-1
42.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	23.30	6.52	57-10-3
43.	Octadecanoic acid, octadecyl ester	$C_{36}H_{72}O_2$	536.95	54.64	0.79	2778-96-3
44.	Phthalic acid, cyclobutyltridecyl ester	$C_{25}H_{38}O_4$	402.57	21.47	0.33	NA
45.	Pyrimidine, 2-(4'-butyl[1,1'-biphenyl]-4-yl)5-ethyl-	$C_{22}H_{24}N_2$	316.44	41.38	2.46	130827-90-6
46.	Silicic acid, diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	296.58	44.71	0.26	3555-45-1
47.	Stigmastan-3,5-diene	$C_{29}H_{48}$	396.69	39.27	0.81	NA
48.	Sulfurous acid, 2-propyl tridecyl ester	$C_{16}H_{34}O_3S$	306.50	23.92	0.11	NA
49.	Tetracosamethyl-cyclododecasiloxane	$C_{24}H_{72}O_{12}Si_{12}$	889.84	27.40	0.30	18919-94-3
50.	Tetradecanoic acid, 10,13-dimethyl-, methyl ester	$C_{17}H_{34}O_2$	270.45	22.63	0.33	267650-23-7
51.	Vinyl 10-undecenoate	$C_{13}H_{22}O_2$	210.31	33.54	0.70	5299-57-0

LIU & al. (2008) isolated compounds including heneicosanoic acid, β-sitosterol, propyl palmitate, and catechin from *G. biloba*. AHAMED & al. (2009) and ULLAH & al. (2012) also identified lactone, gluonic acid, and derythro-2-hexenoic acid from the same plant. Additionally, NATARAJAN & al. (2015) reported compounds with anti-diabetic potential from *Grewia hirsuta*, and REHMAN & al. (2022) identified tridecanoic acid, octanoic acid, eicosanoic acid, and octadecatrienoic acid from *G. tenax*. Based on these findings, it is plausible to explore similar medicinal properties in Dhaman fruit.

Significance of this study

Medicinal plants have long been employed to prevent diseases, maintain health, and treat various ailments. Plants rich in compounds such as triterpenoids, flavonoids, hentriacontane, 16-hentriacontanone, adiantone, isoadiantone, ß-sitosterol, fernene, 2-methoxy-5,40-dimethylbenzenebutanal, methyl octadecanoate acid, kaempferol, quercetin, 3,4',7-trihydroxy-3',5-dimethoxyflavone, catechin, epicatechin, afzelechin, epiafzelechin, mesquitol, ophioglonin, aromadendrin, phenol, dichloromethane, phytol, coumarins, glycosides, etc., have demonstrated significant biological activities, which are gaining increasing attention [PRABHADEVI & al. 2012; ABAYOMI & al. 2014; LIU & al. 2015; AMAN & al. 2016; QAMAR & al. 2021]. The present study identified numerous phytomolecules in Dhaman fruit through GC-MS analysis. Consequently, this fruit holds potential for treating various human ailments such as dementia, exhibiting antimicrobial properties, antiandrogenic properties, antioxidant and neuroprotective activities, anti-diabetic and anti-leukemic effects, analgesic and anti-inflammatory properties, immune-stimulant and antitumor activities, and more. The local community has long revered this plant due to its important role in their daily lives. This study highlights the need for further research on specific plants to explore their unique curative properties. The implications of these findings for rural communities could contribute to their well-being and safety.

Conclusion and future perspectives

This study elucidated the presence of diverse phytomolecules in abundance in Dhaman fruit. GC-MS analysis revealed the identification of forty-eight distinct phytomolecules, including esters, hydrocarbons, fatty alcohols, and ester compounds. These bioactive molecules are well-known for their antimicrobial, antiandrogenic, antioxidant and neuroprotective, anti-diabetic, anti-leukemic, analgesic, anti-inflammatory, immune-stimulant, antitumor, anti-alopecic, lubricant, hemolytic, 5-α reductase inhibitory, diuretic, and antifungal properties. Hence, Dhaman fruit shows significant potential for combating various human diseases and promoting overall well-being. Moreover, the fruit could serve as a beneficial feed supplement in agro-animal sectors such as poultry farming, dairy cattle industry, and fish farming, enhancing health and productivity. Consequently, this phytochemical characterization study of Dhaman fruit lays the groundwork for future research in the development of pharmaceutical drugs and feed additives.

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CONTRIBUTIONS TO THE STUDY OF SOME AROMATIC SPECIES OF THE GENUS *NEPETA* L.

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Abstract: The paper presents a study on four species of aromatic plants: a native species from the spontaneous flora of the Republic of Moldova (*Nepeta cataria* L.), a species to be introduced (*Nepeta racemosa* Lam.) and two non-native species (*Nepeta melissifolia* Lam., *Nepeta grandiflora* M. Bieb.); little research has been conducted on these species, but they are therapeutically valuable. The genus *Nepeta* L. is one of the largest and most important genera in the family Lamiaceae, subfamily Nepetoideae. The studies aimed at their evaluation and characterization from a bio-ecological and phytochemical aspect, in order to highlight the biomorphological peculiarities, the essential oil content and the possibility of using them in aromatherapy, perfumery, phytotherapy and gastronomy. The research highlighted the high adaptive potential and the prospects of cultivation of these species under the pedoclimatic conditions of the Republic of Moldova.

Key words: *Nepeta*, introduction, aromatic plants, composition, essential oil.

Introduction

The main purpose of each botanical garden is to work on introducing plants, in order to enrich the flora of a certain region with new promising species, valuable from economic point of view, with a wide but also rational use of genetic resources. The cultivation of aromatic plants in our country, after a long period of stagnation, is slowly recovering. Currently, there is an active interest in studying these plants, as they are a natural and local source of raw material. People, knowing the properties of these plants, gradually expanded their scope of application in various branches of the national economy: aromatherapy, phytotherapy, perfumery and food. The growing demands contributed to the fact that many peoples tried to identify promising plant species in their homeland, others to introduce them, which led to the creation of collections of aromatic and medicinal plants specific to each country. Such a collection has also been created in the "Alexandru Ciubotaru" National Botanical Garden (Institute), Chișinău.

Among the many genera of aromatic plants present in the collection, the genus *Nepeta* L. is of particular importance, being represented in the spontaneous flora of the Republic of Moldova by three species: *N. parviflora* Bieb., *N. pannonica* L. and *N. cataria* L. The following species have been introduced and researched in the collection of the "Alexandru Ciubotaru" National Botanical Garden (Institute), Chișinău: *Nepeta cataria* L., *Nepeta racemosa* Lam., *Nepeta melissifolia* Lam., *Nepeta grandiflora* M. Bieb., *Nepeta kokanica* Regel, which have served as research subjects.

Materials and methods

The research was initiated in 2019 and included observations on the species of the genus *Nepeta* L., presented in the collection of aromatic plants: *Nepeta cataria* L., *Nepeta*

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racemosa Lam., *Nepeta melissifolia* Lam., *Nepeta grandiflora* M. Bieb., *Nepeta kokanica* Regel. The plants were grown in plots, on a south-facing field, under ecologically balanced conditions, on a general agrotechnical background. Phenological observations were made according to the method used in botanical gardens, with slight modifications depending on the peculiarities of each species, throughout the growing season [Metodica fenologhiceschih nabliodenii v botaniceschih sadah SSSR, 1979]. The essential oil content was determined by steam distillation [Gosudarstvenaia farmacopeia SSSR, 1968]. During the growing season, observations were made on the reaction of plants to late spring frosts, resistance to low temperatures, the influence of light intensity, the insufficiency and excess of atmospheric precipitation and the resistance of plants to diseases and pests. The phytochemical compounds of the essential oil were determined at "Stejarul" Biological Research Center, Piatra Neamț, Romania, with the help of the gas chromatograph Agilent Technologies tip 6890N coupled to the mass selective detector (MSD) 5975 inert XL MSD, by gas chromatography mass spectrometry (CG/MS).

Results and discussions

One of the species included in the research is *Nepeta cataria* L. (Figure 1, a) species introduced from the spontaneous flora of the Republic of Moldova, herbaceous, perennial plant, which has numerous fibrous roots in the soil starting from a woody, branched rhizome. The stem is erect, vigorous, and square, with short hairs. Under the conditions of the botanical garden, it reaches 40-60 cm in height. The leaves are triangular ovate with acuminate tip. The inflorescences are spikes, with flowers located in the axils of the leaves in the upper part of the shoots. The flowers are pink, grouped in dense whorls, arranged at the base of the leaves from the top of the stem. The flowering stage occurs between July 25 and August 15. The fruits are ellipsoidal, brown, smooth nutlets. The natural range of this species includes Western Europe and Asia, up to the Himalayas. In the spontaneous flora of the Republic of Moldova, it occurs sporadically, it grows in meadows and forest edges, thickets, ruderal and stony areas. The biologically active substance is the essential oil, which is contained in the non-lignified aerial part. The maximum content is recorded in the full flowering stage and is 0.24-0.30%, in inflorescences – 0.32-0.41%. The main compounds of the essential oil are: carvacrol, thymol, citral, nepetalactone, nepetalic acid, nepetal-glucoside-ether, limonene, geraniol, caffeic acid, ursolic and rosmarinic acid, coumarins and flavonoids. The plant product possesses therapeutic properties, such as: antispasmodic, antitussive, astringent, carminative, gastrointestinal stimulant, tonic, sedative, emmenagogue, diuretic and cholagogue [ARDELEAN & MOHAN, 2008]. It is beneficial in neurological disorders, chest pains, and improvement of digestion by stimulating gastrointestinal secretions, stimulation of salivation and toothache relief. The essential oil obtained from the plant is used in the perfume and cosmetic industry, in the production of soaps [TELEUȚĂ & al. 2008]. The aerial part of the plant is part of the aromatic herb mixture used in the production of vermouth. The leaves and shoots are used to flavour sauces and soups. The leaves are used to flavour tea.

Nepeta racemosa Lam. (Figure 1, b) is a perennial herbaceous plant, introduced from the Nikita Botanical Garden, Yalta, Crimea, which has, in the soil, fibrous roots starting from a woody, branched rhizome. The stem is erect, densely pubescent, vigorous, square, under the climatic conditions of our country, it reaches 30-40 cm in height. The leaves are cordate-ovate rarely oblong-ovate. The inflorescence consists of several false whorls, of which 3-4 grouped at the top and another 1-3 whorls are spaced. The corolla of the flower is blue-purple, white

pubescent outside, the lower lip is twice as long as the upper one, with a large central lobe and obliquely semicircular side lobes. It blooms all summer long. The fruits are ellipsoidal, smooth, brown-black nutlets that ripen at the end of June. The species occurs, under natural conditions, in the Caucasus. This species has been successfully introduced in Crimea and the Republic of Moldova. The perennial plants start the growing season at the beginning of March, they bloom in the period from June to August. The seeds ripen in the last days of September. The plants synthesize essential oil in all organs. The raw material harvested during the summer contains 0.20-0.22% essential oil and the material harvested in autumn – 0.15-0.18%. If cultivated, it grows well on calcareous, humic, structural soils. The plants are frost-tolerant. They withstand low temperatures, down to -25-30 °C. In the Republic of Moldova, if irrigated, this species gives up to three harvests. It can be propagated only vegetatively, by cuttings or by division. The essential oil contains nepetalactone, germacrene, citral, citronellol, geraniol, nerol, mucilage, saponins, etc., and it exhibited antioxidant activity and antibacterial properties against *Escherichia coli* microorganisms [MOLLOVA & al. 2023]. The plant product has diuretic, analgesic, anti-inflammatory, anxiolytic, sedative, hypnotic, antispasmodic, astringent, antitussive and antimicrobial effects [RABOTEAGOV & ACSENO, 2014]. The essential oil is used in the perfume and cosmetic industry, particularly to produce soaps. The leaves and tops of the plant stems harvested in the full flowering stage are used as a spice. The fresh and dried leaves are added to teas for a cooling and flavouring effect. They contain high amounts of vitamin C, therefore have calming effect, help treating colds and fever and improve appetite. In landscaping, it is used as an ornamental plant, resistant to drought, with long flowering period, which makes it possible to use this species successfully in the design of gardens, over which pollinators will gather, attracted by the honey flowers.

Nepeta melissifolia Lam. (Figure 1, c) is an herbaceous, perennial plant introduced into the Botanical Garden by seed exchange from the Bordeaux Botanical Garden, France. It has ascending stems, growing 40-60 cm tall, ovate-cordate leaves, up to 3.5 cm long, pubescent. The flowers are up to 1.5 cm long, blue with red dots, in spike inflorescences. They are native to Crete and the Aegean Islands [BHAT & al. 2018]. It grows among bushes on rocky slopes. Under the climatic conditions of our country, the plants start growing at the end of April. The budding stage occurs in the middle of June. It blooms between July and August. The growing season lasts for 135 days. The flowers are attractive to bees. The biologically active substance is the essential oil, which is contained in the non-lignified aerial part, in the full flowering stage reaching 0.20-0.30%, in inflorescences 0.25-0.35%. The basic compounds of the essential oil are: nepetalactone, nerol, geraniol, geranial, β-Caryophyllene, linalool, carvacrol, thymol, citral, nepetalic acid, nepetal-glucoside-ether, limonene, β-pinene, etc. (Table1, Figure 2).

Nepeta grandiflora M. Bieb. (Figure 1, d) is a perennial, hemicryptophyte, obtained by international seed exchange from Germany. The plant has a square, light green, slightly branched stem and fibrous root. The leaves are opposite, crenate, toothed. The flowers – violet, grouped in terminal whorls. Fruit – brown nutlet. It is researched as an aromatic and medicinal plant, which contains essential oil (0.31-0.42%). Under the conditions of our country, it reaches 50-75 cm in height. Plants go through the entire development cycle, synthesize essential oil (0.31-0.42%). In the essential oil obtained from the aerial parts of the plant, 25 chemical compounds were identified, the main ones being: germacrene D, eucalyptol, etc. (Table 1, Figure 3).

In folk medicine, it is a perfect remedy for reducing blood pressure, a good sedative for hyperactive children and it improves digestion [RABOTEAGOV & ACSENO, 2014].

Nepeta kokanica Regel. is an herbaceous, perennial species, obtained in 2021 by the International Seed Exchange from Germany. It has numerous stems, 10-35 cm tall, ascending

or erect, strongly branched, covered with long white hairs. The leaves are light green, 1.5-2.5 cm long and 1-2.2 cm wide, ovate, with serrated edges. The flowers are grouped in false whorls, forming an ovoid inflorescence of bright blue colour. The fruit is an elongated, brown nut. It blooms in July-August. It is able to complete the entire cycle of vegetation. It is currently under research.

Figure 1. Plant species of the genus *Nepeta* L.
a – *Nepeta cataria* L.; b – *Nepeta racemosa* Lam.;
c – *Nepeta grandiflora* M. Bieb.; d – *Nepeta melissifolia* Lam.

Table 1. The chemical composition of the essential oil of the species *Nepeta melissifolia* Lam. and *N. grandiflora* M. Bieb.

TR (min)	Kovats Index	Compounds	Area %	
			N. melissifolia	*N. grandiflora*
5.65	922	α-Thujene	0.1	0.1
5.85	930	α-Pinene	0.2	1.5
6.91	974	Sabinene	0.2	3.7
7.03	978	β-Pinene	0.9	5.6
7.19	985	3-Octanone	0.2	0.4
7.39	992	β-Myrcene	0.1	1.1
8.50	1026	p-Cymene	0.4	0.1
8.66	1030	Limonene	-	1.9
8.74	1033	Eucalyptol	0.2	27.1
8.93	1038	*trans*-β-Ocimene	0.2	2.2
9.31	1048	*cis*-β-Ocimene	0.5	2.3
11.21	1101	Linalool	2.0	0.3
14.35	1181	4-Terpineol	0.4	0.5
14.90	1194	α-Terpineol	0.2	0.2
15.57	1210	3-Terpinen-1-ol / *p*-Menth-3-en-1-ol	0.1	-
15.89	1217	Dihydro myrcenol acetate	0.2	-
16.47	1231	Nerol	8.5	-
16.98	1242	Neral	1.6	-
17.58	1256	Geraniol	5.7	-
18.23	1271	Geranial	2.0	-
19.17	1292	Thymol	0.1	-
19.57	1301	Carvacrol	0.8	-
21.99	1359	(4aS,7S,7aS)-*trans*,*cis*-	22.9	-
22.78	1378	α-Copaene	-	0.2
23.15	1388	β-Bourbonene	-	0.7
23.20	1388	(4aS,7S,7aR)-*cis*,*trans*-	47.6	-
23.39	1393	(4aR,7S,7aS)-*cis*,*cis*-Nepetalactone	2.1	-
23.44	1394	β-Elemene	-	1.0
24.56	1422	β-Caryophyllene	1.3	12.3
25.92	1455	α-Humulene	0.1	0.7
27.02	1482	Germacrene D	0.1	28.0
27.65	1498	Bicyclogermacrene	-	1.3
28.11	1510	α-Farnesene	-	0.4
28.70	1525	δ-Cadinene	-	0.3
30.95	1584	Spathulenol	0.9	4.1
33.61	1655	τ-Cadinol	-	0.5
		Other compounds	*0.5*	*3.5*

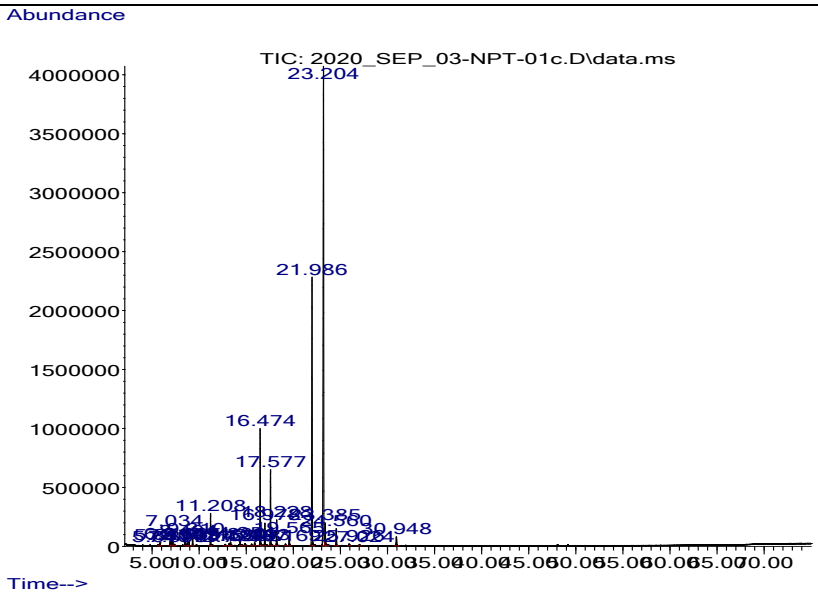

Figure 2. GC-MS chromatogram of *Nepeta melissifolia* Lam.

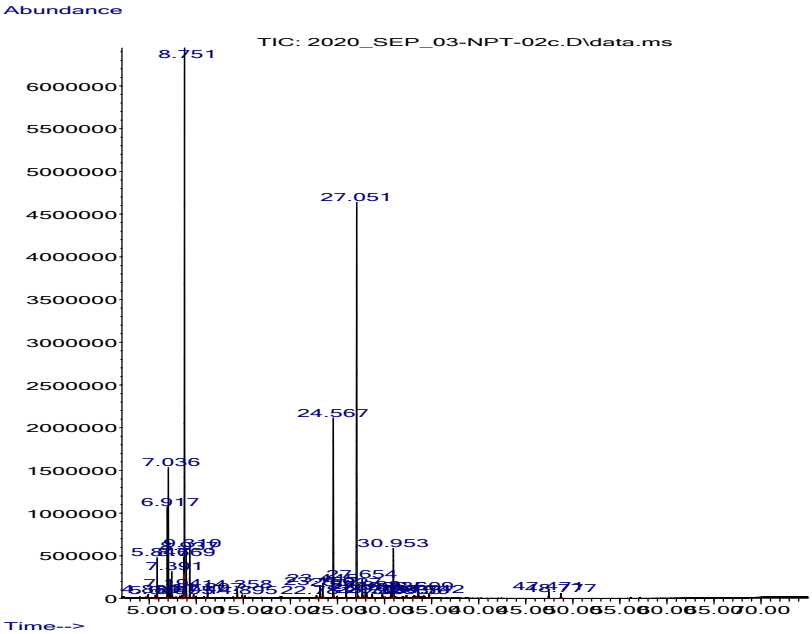

Figure 3. GC-MS chromatogram of *Nepeta grandiflora* M. Bieb.

Conclusions

The pedoclimatic conditions of Moldova are favourable for the growth and development of plants of the genus *Nepeta* L. They fully complete the ontogenetic cycle. The essential oil content varies among the plant species. The highest content was found in the species *Nepeta grandiflora* M. Bieb. (0.31-0.42%), followed by *Nepeta cataria* L. (0.29-0.40%). The species *Nepeta grandiflora* M. Bieb. is the tallest (50-75 cm), followed by *Nepeta melissifolia* Lam. (40-60 cm). The introduced species fully complete the ontogenetic cycle. The plants bloom and bear fruit starting from the 2nd year of vegetation. They synthesize essential oil.

The conducted research on the chemical composition of essential oil produced by the introduced species, under the conditions of the Republic of Moldova, indicates that in *Nepeta melissifolia* Lam. the main compound is (4aS,7S,7aR)-*cis*, *trans*-Nepetalactone (47.8%), followed by (4aS,7S,7aS)-*trans*, *cis*-Nepetalactone (22.9%), nerol (8.5%), geraniol (5.7%), geranial (2.0%). The presence of nepetalactones is frequent in the essential oils produced by species of the genus *Nepeta*. According to the obtained data, the composition of the essential oil from the species *N. grandiflora* M. Bieb. differs in the predominant presence of the compound Germacrene D (28%), followed by β-Caryophyllene (12.3%), β-Pinene (5.6%), Spathulenol (4.1), Sabinene (3.7%) etc.

The species of the genus *Nepeta* L. introduced and researched in the National Botanical Garden (Institute) can serve as sources of local raw material for the production and diversification of the range of natural cosmetic and pharmaceutical products of plant origin. All species are excellent honey plants.

Acknowledgements

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PHYTOCHEMICAL SCREENING AND ANTI-BACTERIAL ACTIVITY OF *ERYTHRINA VARIEGATA* LEAF, STEM AND ROOT EXTRACTS

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Abstract: *Erythrina variegata* is a potent medicinal plant belonging to the family Fabaceae. Present investigation was carried out the preliminary phytochemical screening of the *Erythrina variegata* to evaluate the presence of alkaloids, flavonoids, glycosides, phenols, tannins, steroids/triterpenoids, quinones, saponins by using different parts of the plant extracts such as leaf, stem and root in five different solvent systems (methanol, butanol, chloroform, ethanol, and distilled water) by cold maceration technique. According to our evaluation the high intensity of secondary metabolites like alkaloids and glycosides were strongly observed in leaf butanol extract and complete absence of saponins except in aqueous solvent was seen. In stem extracts butanol and chloroform were more efficient solvents for alkaloids, glycosides, tannins and moderate for phenols and steroids. The results of root extract revealed the strong presence of alkaloids, flavonoids, glycosides inbutanol extract. Due to its efficiency in butanolic extract *Erythrina variegata* was used to test anti-bacterial activity. Which showed the highest zone of inhibition against *Bacillus subtilis* in leaf and root extract whereas in stem butanolic extract highest zone of inhibition was against *Proteus vulgaris.*

Keywords: anti-bacterial activity, *Bacillus subtilis*, *Erythrina variegata*, plant extracts, *Proteus vulgaris.*

Introduction

Medicinal plants have the ability to cure many diseases which can be used as a resource formany drugs [PAVANI & SHASTHREE, 2022]. The presence of different phytochemicals in the plant determines the medicinal value of the plant [SANTHIYA & al. 2016]. Most of the developed countries use the compounds obtained from medicinal plants for the production oftraditional medicines [YADAV & AGARWALA, 2011]. However, these plants should be studied to betterunderstand their properties, and the safety upon their usage. The phytoconstituents identifiedfrom the plant material helps to predict the possible pharmacological activity of that plant [SHAIKH & PATIL, 2020]. Therefore, understanding the phytochemical profile of *Erythrina variegata* can provide valuable insights into its the rapeuticpotentialandleadto the development of novel natural remedies or pharmaceutical agents.

Phytochemical screening is a crucial process in the field of plant science and pharmacology. It involves the systematic analysis and identification of various biologically active compounds present in plants. These compounds, known as phytochemicals, are natural chemicals that contribute to the plant's characteristics and play essential roles in their growth, development, and defense mechanisms [DOUGHARI, 2012].

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Phytochemicals are of great interest to scientists and researchers because of their potential health benefits and medicinal properties. Many of these compounds have been found to possess antioxidant, anti-inflammatory, antimicrobial, and anticancer properties, among others, making them valuable for drug development and alternative medicine [RALTE & al. 2022].

The alkaloids extracted from the leaves of *E. variegata* are reported to have analgesic and anti-inflammatory activity whereas iso-flavonoids isolated have antibacterial activity [KUMAR & al. 2010], cytotoxic [NKENGFACK & al. 2001], anthelmintic [JESUPILLAI & PALANIVELU, 2009], antiulcer and diuretic properties [SACHIN & ARCHANA, 2009].

E. variegata holds immense potential as a natural source of antimicrobial agents. Its long-standing use in traditional medicine and recent scientificevidence of its efficacy against various pathogens highlight the importance of further exploration. The therapeutic properties of this plant may offer a sustainable and effective solution to combat infectious diseases and address the growing concern of antimicrobial resistance.The various parts of *E. variegata*, such as leaves, bark, flowers, and seeds, are used totreat a range of ailments, including microbial infections. The plant's therapeutic potential is attributed to its diverse phytochemical composition, which includes alkaloids, flavonoids, tannins, saponins, and other secondary metabolites [SANTHIYA & al. 2016].

Studies investigating the antimicrobial properties of *E. variegata* have shown promising results against a wide spectrum of pathogens. some studies suggest that *E. variegata* bioactive compounds may interfere with crucial enzymatic processes within microbial cells, further compromising their viability [BASKAR & al. 2010].

As a result, this study is being conducted to give phytochemical analysis, employing various solvents and *Erythrina variegata* anti-bacterial characteristics.

Material and methods

Collection of plant material

E. variegata plant was collected from the department of biotechnology, Kakatiya University, Warangal where the plants were grown and maintained in proper condition. The collected plant materials were washed thoroughly under running tap to remove dirt. After that the parts were separated and shade dried for 10 days and were made in to powder form for further use (Figure 1).

Preparation of extract

The 3 grams of each plant part powder was taken along with 30 ml of each solvent in separate conical flask the solvents used here were aqueous, butanol ethanol, chloroform and methanol and were incubated in orbital shaker for about 48 and later the extracts were filtered using Whatman filter paper. The final extracts were kept in a rotating shaker for 48 hours at 28 °C. After 48 hours, the extract was filtered subsequently subjected for preliminary screening by using standard methods protocol.

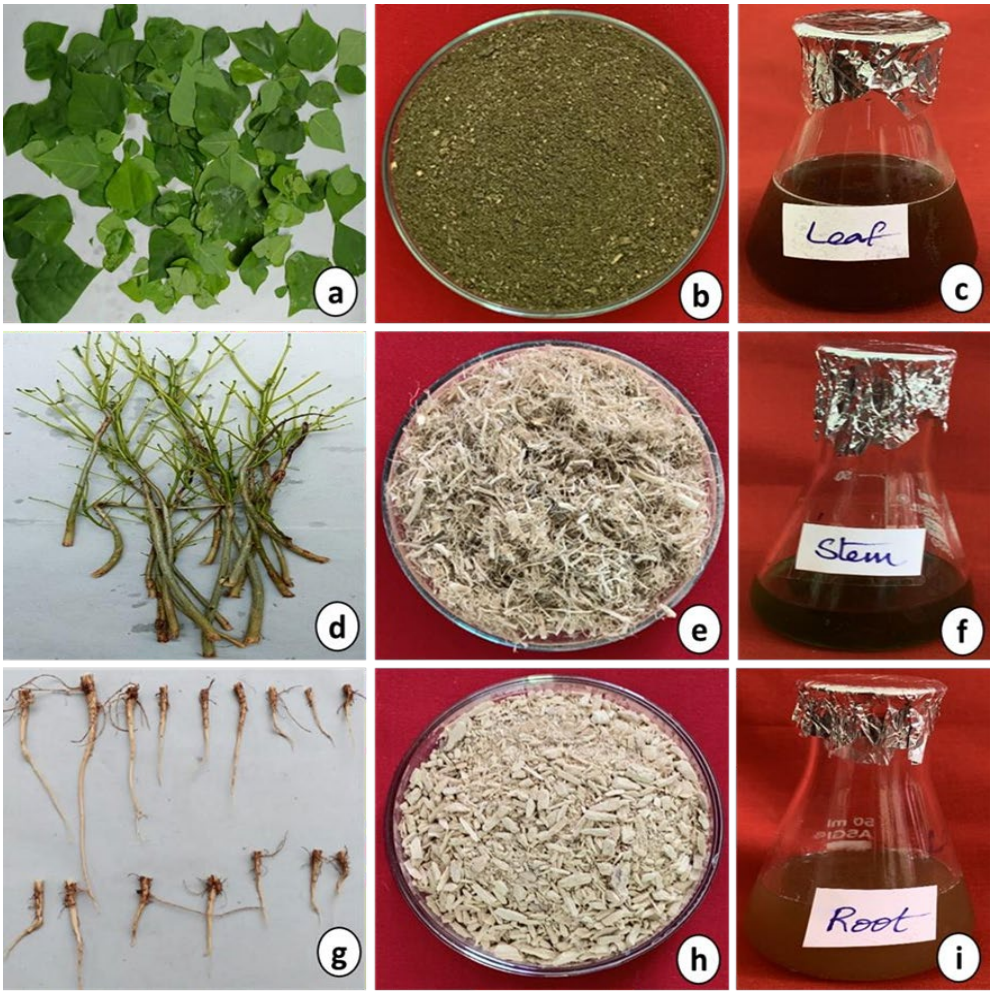

Figure 1. Parts of *Erythrina variegata*. (a) – Leaves; (b) – Leaves powder; (c) – Leaves extract; (d) – Stem; (e) – Stem powder; (f) – Stem extract; (g) – Root; (h) – Root powder; (i) – Root extract.

Preliminary screening (qualitative) of phytochemicals in plant *Erythrina variegata* is determined by using the following tests (Table 1-6)

Table 1. Tests for alkaloids

Test	Procedure	Observation
Mayers test	Few drops of filtered plant extract and 1 to 2 drops of Mayer's reagent (along the sides of test tube)	A creamy white/yellow ppt
Hager's test	0.5 ml filtered plant extracts and mixed with 1-2 drops of Hagers reagent	A creamy white ppt
Tannic test	0.5 ml filtered plant extracts and mixed with 10% tannic acid solution	Formation of buff color ppt

Table 2. Tests for flavonoids

Test	Procedure	Observation
Alkaline reagent test	1 ml filtered plant extracts and mixed with 2 ml of 2% NaOH solution (and add few drops dil. HCl)	An intense yellow color, becomes color less on addition of diluted acid
Ferric chloride	Extract aqueous solution mixed with few drops 10% Ferric chloride solution	A green precipitate was appeared

Table 3. Tests for glycosides

Test	Procedure	Observation
Molisch test	2 ml filtered plant extracts and mixed with 2 drops of alcoholic α–naphthol and 1 ml conc. H_2SO_4 (along the sides of test tube)	A violet ring was formed in the middle of the two layers of the liquids
Conc. H_2SO_4 test	5 ml filtered plant extracts mixed with 2 ml glacial acetic acid and few drop of 5% $FeCl_3$ + conc. H_2SO_4	A browning or red ppt was formed

Table 4. Tests for saponins

Test	Procedure	Observation
Foam test	0.5 gm plant extract mixed with 2ml distilled water and vigorously shaken for 10 to15 mints	Persistent foam layer was formed

Table 5. Tests for phenols

Test	Procedure	Observation
$FeCl_3$ test	Plant extract mixed with few drops 5% ferric chloride sol.	Dark green/bluish black color
Ellagic acid test	Plant extract was mixed with few 5% glacial acetic acid and 5% sodium nitrite Solution	Solution turns muddy/Niger brown precipitate

Table 6. Tests for tannins

Test	Procedure	Observation
Alkali reagent test	1 ml plant extract was added to 2 ml of 2% NaOH solution (and add few drops dil. HCl)	An intense yellow color, becomes color less on addition of diluted acid
Fecl3test	1 ml of plant extract mixed with few drops 5% ferric chloride sol.	Dark green/bluish black color
Gelatin test	1.0 ml Plant extract is dissolved in 5 ml distilled water and add 1% gelatin Solution and 10% NaCl	A white precipitate

Anti-bacterial activity

Using the well diffusion method, the plant extracts anti-microbial activity was evaluated in aseptic condition. the process in the well diffusion method, includes a volume of the microbial inoculum which is distributed over the entire agar surface. Next, a volume (20-80 µl) of the extract solution is put into the well by aseptically making a hole with a diameter of 6 to 8 mm using a sterilecork borer or tip. The test microorganism is then placed on an appropriate agar plate, and the incubation process is continued. The antibiotic ingredient spreads across the

agar media and stops the tested microbial strain from growing. All the plates were incubated at 37 °C for 24 hours. After incubation, the size (diameter) of the inhibition zones was measured.

Results and discussions

Phytochemical screening

Leaf extract

The phytoconstituents like alkaloids and glycosides in leaf extract of *E. variegata* were found to be in more concentration in butanol followed by chloroform extract and whereas the other phytoconstituents like flavonoids and phenols were found to be more in butanolic extract (Figure 1 and Table 7). Similarly phytochemical analysis of the ethanolic leaf extract of *E. senegalensis* showed the presence of alkaloids, saponins and flavonoids in moderate quantities [NNAMA & al. 2017].

Table 7. Phytochemical constituents of *Erythrina variegata*

Part of plant	Components	Methanol extract	Butanol extract	Chloroform extract	Ethanol extract	Aqueous extract
LEAF	Alkaloids	+	++	+	+	–
	Flavonoids	+	++	++	+	–
	Glycosides	+	++	+	+	+
	Phenols	+	++	+	+	+
	Tannins	+	+	+	+	+
	Steroids/triterpenoids	–	+	–	+	+
	Quinones	–	–	–	–	+
	Saponins	–	–	–	–	+
STEM	Alkaloids	+	++	+	+	++
	Flavonoids	+	+	+	+	+
	Glycosides	+	++	++	+	+
	Phenols	+	+	+	–	+
	Tannins	+	+	+	++	+
	Steroids/triterpenoids	+	+	+	+	–
	Quinones	–	–	+	–	–
	Saponins	–	–	–	–	++
ROOT	Alkaloids	+	++	++	+	+
	Flavonoids	++	++	+	++	–
	Glycosides	+	++	+	+	+
	Phenols	–	+	–	–	+
	Tannins	+	+	+	++	++
	Steroids/triterpenoids	+	–	+	+	+
	Quinones	++	+	++	++	–
	Saponins	–	–	–	–	+

Stem extract

The phytoconstituents such as alkaloids were more in concentration in butanol and aqueous extract whereas glycosides were found to be more in butanol extract along with them tannins were found more abundant in chloroform and ethanol extract. Similarly, results were observed in *Zingiber officinale* by AHMED & al. (2022).

Root extract

The phytoconstituents like alkaloids and glycosides were found to be more abundant inbutanol extract whereas flavonoids were in more concentration in methanol and butanol extracts. Similar, results were reported in *Mutuntingia calabura* leaf methanolic extract shown maximum zone of inhibition against *Escherichia coli* [SUVARCHALA & al. 2022]. Among all the extracts used saponins were found only in aqueous extract of all three plant parts like leaf, stem, root.

Anti-bacterial activity for leaf butanolic extract

The anti-bacterial activity of leaf, stem and root butanolic extracts (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml) and the control streptomycin (10 µg/ml) was evaluated. The butanolic leaf extract shown a maximum zone of inhibition at 80 µg/ml when compared to other concentrations. As the concentration of plant extract increased, the inhibition zone was also found to increase. The maximum antibacterial activity was shown towards *Bacillus subtilis* and *Escherichia coli* at 80 µg/ml concentration, followed by *P. vulgaris* and least zone of inhibition was shown by *B. Sphaericus* (Figure 2, 3). Similar work were reported in *M. cymbalaria* methanolic extract showed a high inhibition zone against *E. coli* [CHAITANYA & al. 2021] and the antibacterialactivity of *Moringa oleifera* ethanolic leaf extracts shown towards *Staphylococcus aureus* and *Escherichia coli* [JAHAN & al. 2022].

Anti-bacterial activity for stem butanolic extract

The stem butanolic extract revealed considerable anti-microbial activity with large extent ofinhibitory zones *against P. vulgaris*, followed by *E. coli* in contrast to the other bacterial strains tested (Figure 4, 5). However, it was shown that *B. sphaericus* have minimal anti-bacterial activity. The similar findings were reported in stem methanolic extract of *Sesbania grandiflora* [ANANTAWORASAKUL & al. 2011].

Anti-bacterial activity for root butanolic extract

The antibacterial properties of the various concentrations of root extract (20 µg/ml, 40 µg/ml, 60 µg/ml, and 80 µg/ml) were evaluated in comparison to the control (10 µg/ml). Compared tothe other concentrations, the 80 g/ml concentration displayed the largest zone of inhibition (Figure 6, 7). The root butanolic extract effectively inhibited *B. subtilis* in a zone. *E. coli*, *P. vulgaris* and *B. sphaericus*, however, demonstrated the least antibacterial action. Similar findings were reported in root extract of *Diploknema butyracea* [CHHETRY & al. 2022].

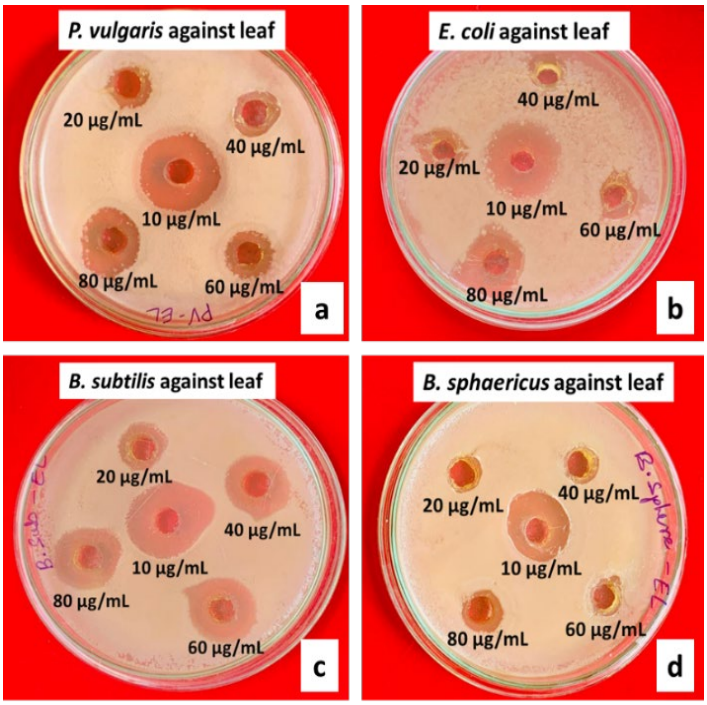

Figure 2. Anti-bacterial activity of butanolic leaf extracts of *E. variegata* against different bacterial strain
a. Zone of inhibition against *P. vulgaris*; b. Zone of inhibition against *E. coli*;
c. Zone of inhibition against *B. subtilis*; d. Zone of inhibition against *B. sphaericus*

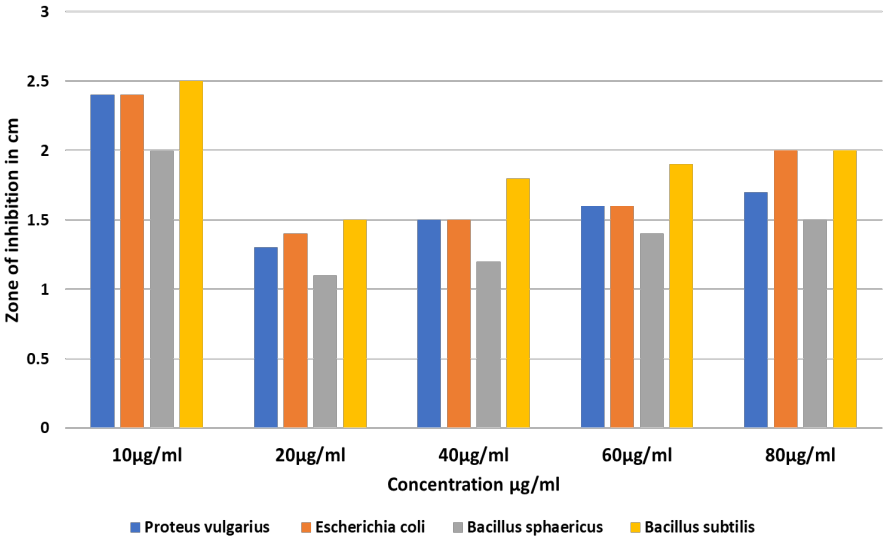

Figure 3. Zone of inhibition for butanolic leaf extract of *Erythrina variegata*

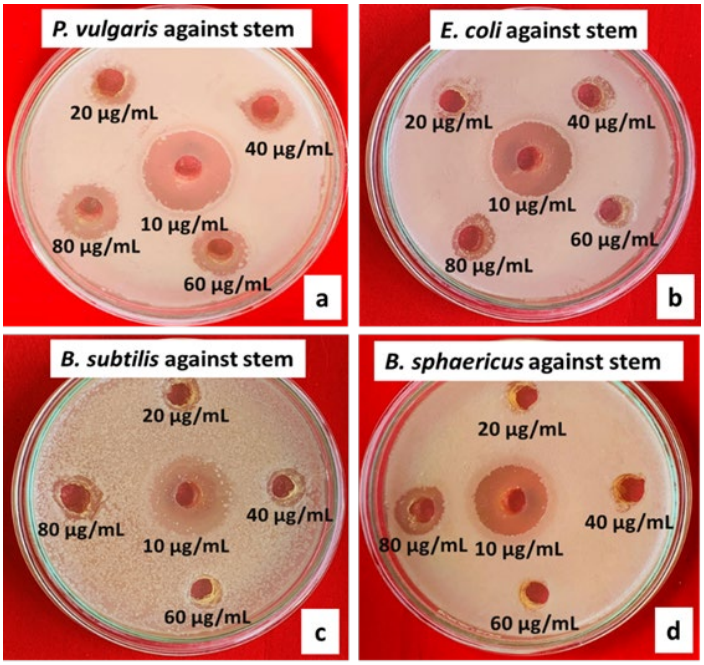

Figure 4. Anti-bacterial activity of butanolic stem extracts of *E. variegata* against different bacterial strain: a. Zone of inhibition against *P. vulgaris*; b. Zone of inhibition against *E. coli*; c. Zone of inhibition against *B. subtilis*; d. Zone of inhibition against *B. sphaericus*

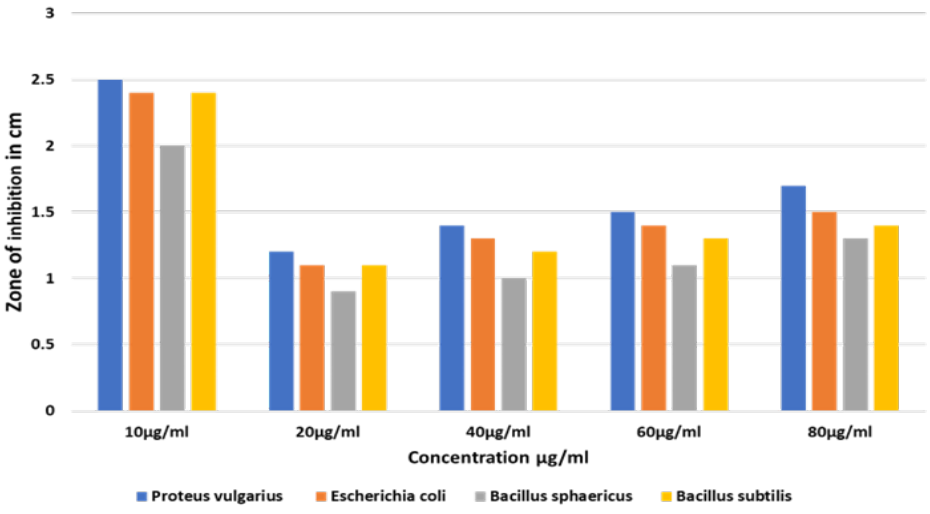

Figure 5. Zone of inhibition for butanolic stem extract of *Erythrina variegata*

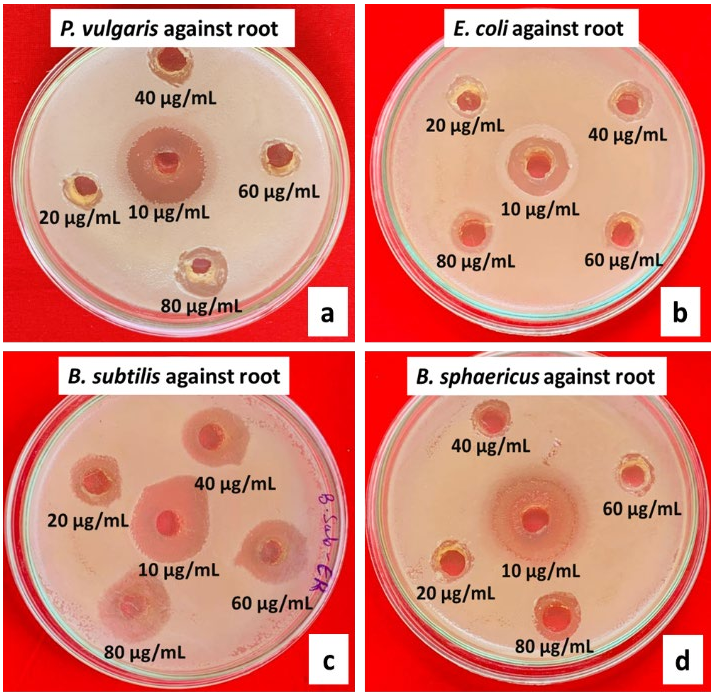

Figure 6. Anti-bacterial activity of butanolic root extracts of *E. variegata* against different bacterial strain. a. Zone of inhibition against *P. vulgaris*; b. Zone of inhibition against *E. coli*; c. Zone of inhibition against *B. subtilis*; d. Zone of inhibition against *B. sphaericus*

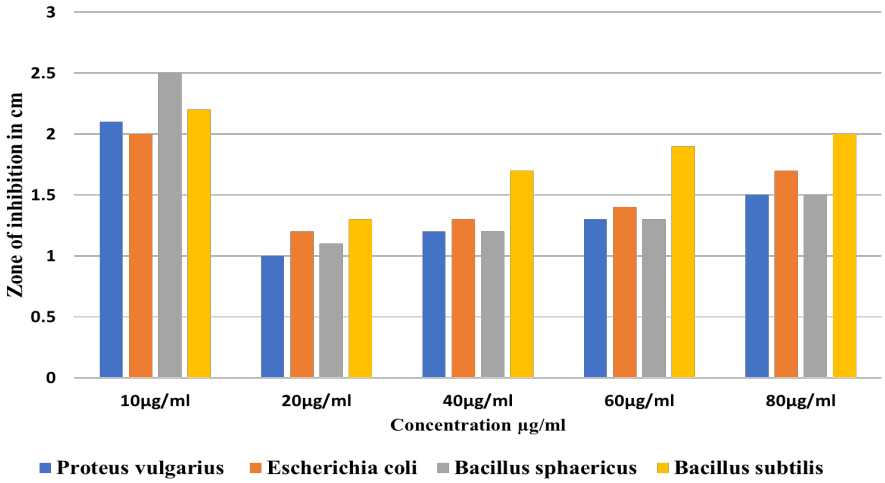

Figure 7. Zone of inhibition for butanolic root extract of *Erythrina variegata*

Conclusions

This study is concluded that the phytochemical screening of *E. variegata* showed positive results for the presence of phytochemicals like alkaloids, flavonoids glycosides, steroids and quinones in butanolic extracts of leaf stem and root. Anti-bacterial activity of butanolic extracts was carried out which showed a maximum zone of inhibition at 80 µg/ml compared to other concentrations. As the concentration of the plant extract increased, the inhibition zone was also found to increase, which concludes the anti-bacterial activity of *E. variegata* and secondary metabolites obtained from it have medicinal properties. It is useful for the production of medicine to treat various diseases.

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SUSTAINABLE OKRA (*ABELMOSCHUS ESCULENTUS* L.) PRODUCTIVITY AS INFLUENCED BY FORTIFICATION OF GOAT MANURE WITH RICE HUSK ASH IN NORTHERN GUINEA SAVANNA ECOLOGY OF NIGERIA

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Abstract: To assess the agronomic effects of sustainable okra production as impacted by goat manure (GM) and rice husk ash (RHA) on a community-based demonstration farm in Gombe State, field trials were carried out during the wet seasons of 2017 and 2018. The trails consisted of four levels of GM and RHA, each measuring 0.0, 5.0, 7.5, and 10.0 t ha^{-1} which were used on Kwadon's local cultivar of okra in a Randomized complete block design (RCBD) replicated four times. Findings revealed that days to 50% emergence and establishment count were not significantly ($P>0.05$) influenced by the application of GM and RHA, regardless of rates of application. Other growth-related indices were significantly ($P<0.05$) higher due to GM, such as plant height at 4, 5, and 6 WAS, number of leaves $plant^{-1}$ at 4 WAS, and pod diameter. On the other hand, RHA significantly ($P<0.05$) increased plant height, the number of leaves $plant^{-1}$ at 5 and 6 WAS, leaf area, shortened days to 50% flowering, and the number of pods $plant^{-1}$across the sampling periods and seasons. Similar to this, due to adequate mineralization of organic material through time and space, fresh okra's marketable weight increased in the 2018 season compared to the previous year. Thus, the application of 10.0 t ha^{-1} of both GM and RHA can be adopted in the production of okra in the study area to reduce greenhouse gas emissions resulting from the use of inorganic agrochemical inputs.

Key words: fortification, goat manure, growth, okra, rice husk ash, yield characters.

Introduction

In an effort to combat hunger and meet the demand for food from the world's ever-increasing human population, as part of the Sustainable Development Goals (SDGs), farming system depends on the use of synthetic fertilizers, pesticides, and growth regulators for enhancing crop productivity [SEARCHINGER *&* al. 2019]. Despite the increase in yield brought about by using these methods, the rate of pest incidence and resistance, as well as the degradation of the ecosystem arising from global warming, is still at an alarming rate. The need to find more sustainable methods of producing food becomes essential in light of the SDGs' aim to guarantee food security in a holistic manner without lowering the quality of the ecosystem and causing a loss of biodiversity [FOLEY *&* al. 2011; PAUSTIAN *&* al. 2016; SHAKOOR *&* al. 2020 a, b]. Instead of using chemical pesticides, regenerative agriculture, which is a subset of sustainable agriculture, promotes the use of strategies like crop and animal rotation, composting, no-till farming, agroecology, and agroforestry. Regenerative agriculture increases the amount of fertile topsoil that is accessible for farming, leading to a more wholesome and long-lasting food system [REGENERATIVE ORGANIC ALLIANCE, 2020]. RHODES (2017)

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asserts that implementing regenerative agriculture towards integrating crop and livestock residue for food production could lessen environmental degradation and improve household sustainability. By incorporating organic and inorganic nutrients into the soil, enhancing the fertility of the soil, increasing the organic matter content, and increasing the soil's ability to hold water, soil amendments are used in agriculture to support plant growth and development.

According to extensive research, adding organic manure to the soil can increase crop productivity for a variety of crops, including okra [ADEKIYA *&* al. 2018; OKEE, 2020], eggplant [MOURSY *&* al. 2021], pepper [ANTONIOUS, 2014; ROSA-MARTÍNEZ *&* al. 2021], kale and collard greens [ANTONIOUS *&* al. 2014], cabbage and broccoli [ANTONIOUS *&* al. 2012] among other crops. Okra (*Abelmoschus esculentus* L.), also known as lady's finger is a valuable multifunctional crop in Nigeria and around the world valued for its tasty green seed pods, immature fresh leaves, and dried fruits [PATIL *&* al. 2015; SANDHAM *&* al. 2019]. It also contains high amount of dietary fiber, antioxidants, folate, and vitamins A, B, and C, as well as minerals including sodium, calcium, potassium, zinc, and iron [DHRUVE *&* al. 2015]. Because of its home and industrial utility, its production demand continues to outstrip supply [AWOLU *&* al. 2014; DONG *&* al. 2014]. Given that it is typically eaten as a succulent vegetable, it becomes essential to enhance its output utilizing a regenerative strategy. Compared to other animal manures, there is less information available on the cultivation of solanaceous crops utilizing goat manure. There has also been little research done on using rice husk ash to increase food output. In light of this, the current study was carried out with the aim of evaluating the impact of goat manure and rice husk ash on the productivity of okra.

Materials and methods

Experimental site

Field trials were conducted during 2017 and 2018 cropping seasons at a Community-Based Demonstration farm (11°03′0.0″ E, 10°18′59″ N) within Gombe using different levels of Goat manure and Rice husk ash.

Treatments and experimental design

The experimental treatment comprised of four levels of Goat manure (0.0, 5.0, 7.5 and 10.0 t ha^{-1}) and four levels of Rice husk ash (0.0, 5.0, 7.5 and 10.0 t ha^{-1}) which were factorially combined and applied to okra and replicated four times in a Randomized complete block design.

Land preparation and sowing

The land was ploughed and ridged before plots were marked out of size 4 m x 3 m. Four seeds of okra (*var*. Kwadon local, a predominant cultivar of okra cultivated by farmers in the experimental area) were sowed on intra row spacing of 30 cm on each ridge. The organic materials (Crop livestock) used were thoroughly incorporated into the soil based on treatment combination after marking the individual plots a week before sowing. Weeding was carried out twice manually using hoe on an interval of three weeks.

Data collection and Data analysis

Five plants per treatment were randomly selected and tagged from which growth and yield related characters were assessed. The data collected on growth and yield characters were subjected to analysis of variance and differences between means were determined by Duncan Multiple Range Test (DMRT) in General Linear Model (GLM) of SPSS.

Results and discussion

In the farming seasons of 2017 and 2018, levels of goat manure (GM) and rice husk ash (RHA) had no significant (P>0.05) impact on the number of days to 50% emergence and crop establishment count (Table 1). Similarly, treatment interaction was also not significant in both seasons.

Table 1. Effect of goat manure and rice husk ash on number of days to 50% emergence and establishment count of okra at Gombe during 2017 and 2018 wet cropping season

Treatment	Days to 50% emergence		Establishment count 3 WAS	
	2017	2018	2017	2018
Goat manure (t ha^{-1})				
0.00	4.31	4.06	79.90	71.09
5.00	4.06	4.31	71.09	74.22
7.50	4.31	4.31	74.22	77.03
10.0	4.37	4.38	77.03	79.92
LS	NS	NS	NS	NS
SE±	0.11	0.12	2.87	3.22
Rice Husk Ash (t ha^{-1})				
0.00	4.19	4.18	69.53	69.53
5.00	4.25	4.25	77.27	77.27
7.50	4.37	4.38	77.73	77.73
10.0	4.25	4.25	77.73	77.73
LS	NS	NS	NS	NS
SE±	0.11	0.12	2.87	3.22
Interaction				
Gm x Rh Ash	NS	NS	NS	NS

NS – not significant; WAS – weeks after sowing; LS – level of significance, SE – standard error.

Plant height was significantly (P<0.05) influenced by application of GM at 3, 4, 5 and 6 WAS in both 2017 and 2018 farming seasons, respectively (Table 2). Application of 0-10 t ha^{-1} of GM results in a significant (P<0.05) increase in plant height, with 10 t ha^{-1} producing the tallest plant in both seasons. The rise in plant height supports ANIEFIOK & al. (2013) findings, which indicated that adding poultry manure to water leaves increased plant height. Similar to this, NAJAH & al. (2021) found that the application of organic fertilizer increased the height of the okra plants. Application of RHA did not differ significantly due to plant height at 3WAS in both seasons. However, it significantly (P≤0.05) differs at 4, 5, and 6 WAS, respectively, where the application of 5.0-10.0 t ha^{-1} of RHA gave significantly taller plants at 4 WAS in the 2017 and 2018 seasons. Additionally, plants grown in response to 10.0 t ha^{-1} of RHA were substantially taller than those grown in response to other treatments, which were at par at 5 and 6 WAS during the farming seasons of 2017 and 2018, respectively. Treatment interaction with respect to plant height was not significant in 2017 and 2018 farming seasons, across the sampling periods.

Table 2. Effect of goat manure and rice husk ash on plant of okra at Gombe during 2017 and 2018 wet cropping season

Treatment	Plant height (cm)							
	3 WAS		4 WAS		5 WAS		6 WAS	
	2017	2018	2017	2018	2017	2018	2017	2018
Goat manure (t ha^{-1})								
0.00	9.56^{c}	19.56^{c}	15.50^{b}	25.50^{b}	21.44^{b}	31.44^{b}	24.19^{b}	34.19^{b}
5.00	9.81^{c}	19.81^{c}	17.38^{ab}	27.38^{ab}	24.50^{b}	33.75^{b}	24.75^{b}	34.75^{ab}
7.50	10.50^{b}	20.50^{b}	17.56^{ab}	27.56^{ab}	23.75^{b}	34.50^{b}	25.93^{ab}	35.94^{ab}
10.0	11.31^{a}	21.25^{a}	19.38^{a}	29.38^{a}	29.50^{a}	39.50^{a}	28.81^{a}	38.81^{a}
LS	*	*	*	*	*	*	*	*
SE±	0.11	0.18	0.71	0.75	0.97	1.02	1.12	1.21
Rice Husk Ash (t ha^{-1})								
0.00	10.37	20.38	14.69^{b}	24.69^{b}	23.06^{b}	33.06^{b}	23.31^{b}	33.31^{b}
5.00	10.13	20.13	19.38^{a}	27.63^{a}	23.31^{b}	33.31^{b}	25.31^{ab}	35.31^{ab}
7.50	10.19	20.13	18.13^{a}	28.13^{a}	25.81^{ab}	35.81^{ab}	27.38^{a}	37.38^{ab}
10.0	10.50	20.50	17.63^{a}	29.38^{a}	27.00^{a}	37.00^{a}	27.69^{a}	37.69^{a}
LS	NS	NS	*	*	*	*	*	*
SE±	0.11	0.18	0.71	0.75	0.97	1.02	1.12	1.21
Interaction								
Gm x Rh	NS	NS	NS	NS	NS	NS	NS	NS

Means followed by common letter(s) in a column are not significantly different at 5% probability level, DMRT. NS – not significant; WAS – weeks after sowing; LS – level of significance; SE – standard error; * – significant at 5%

Number of leaves plant^{-1} was significantly affected by levels of GM at 4 WAS and rice husk ash at 5 and 6 WAS during 2017 and 2018 seasons (Table 3). At 4 WAS, application of 10.0 t ha^{-1} of GM ($P<0.05$) significantly resulted in highest number of leaves plant^{-1} compared with other levels which were at par in both years. However, number of leaves plant^{-1} due to levels of RHA at 10.0 t ha^{-1} significantly resulted in highest number of leaves plant^{-1} while other levels were at par at 5 and 6 WAS in both seasons, respectively. Nevertheless, treatment interactions due to number of leaves plant^{-1} were not significant in both years across the sampling periods. The increase in number of leaves due to GM application could be attributed to nutrient supplied by the manure compared with no nutrient as in control. ADESINA & al. (2014) who reported increase in pepper leaves number due to increase in levels of poultry manure application as organic soil amendment.

Table 3. Effect of goat manure and rice husk ash on number of leaves plant^{-1} of okra at Gombe during 2017 and 2018 wet cropping season

Treatment	Number of leaves plant^{-1}							
	3 WAS		4 WAS		5 WAS		6 WAS	
	2017	2018	2017	2018	2017	2018	2017	2018
Goat manure (t ha^{-1})								
0.00	4.37	18.75	6.50^{b}	21.00^{b}	6.06	21.06	6.81	21.38
5.00	4.44	19.13	6.00^{b}	21.00^{b}	6.31	21.06	6.75	21.50
7.50	4.13	19.44	6.00^{b}	21.63^{b}	6.06	21.13	6.19	21.75
10.0	4.44	19.44	8.00^{a}	23.00^{a}	6.13	21.31	6.13	21.81
LS	NS	NS	*	*	NS	NS	NS	NS
SE±	0.10	0.31	0.29	0.31	0.19	0.24	0.26	0.34

Rice husk (t ha^{-1})								
0.00	4.25	19.25	6.75	21.50	5.63^{c}	20.63^{b}	5.75^{b}	20.75^{b}
5.00	4.38	19.38	6.50	21.63	5.88^{bc}	20.88^{ab}	6.44^{ab}	21.75^{ab}
7.50	4.38	19.38	6.75	21.75	6.38^{ab}	21.38^{ab}	6.75^{a}	21.75^{ab}
10.0	4.38	18.75	6.50	21.75	6.69^{a}	22.69^{a}	7.19^{a}	22.19^{a}
LS	NS	NS	NS	NS	*	*	*	*
SE±	0.10	0.31	0.29	0.31	0.19	0.24	0.26	0.34
Interaction								
Gm x RH	NS	NS	NS	NS	NS	NS	NS	NS

Means followed by common letter(s) in a column are not significantly different at 5% probability level, DMRT. NS – not significant; WAS – weeks after sowing; LS – level of significance; SE – standard error; * – significant at 5%.

Application of GM significantly influenced leaf area plant^{-1}and pod diameter of okra in the 2017 season only. However, there was no significant influence observed on leaf area and pod diameter in 2018, days to 50% flowering, number of pods plant^{-1} and pod length in 2017 and 2018 seasons (Table 4). Leaf area, days to 50% flowering, and number of pods per plant were significant ($P \leq 0.05$) due to the application of RHA in both seasons.

Rice husk ash at 10.0 t ha^{-1} produced the larger leaves in both years of the trial (Table 4). On the other hand, the application of 10.0 t ha^{-1} of rice husk ash significantly ($P<0.05$) decreased the number of days to 50% flowering in both seasons compared to other rates that were at par. The number of pods plant^{-1} was significantly higher arising from the application of 10.0 t ha^{-1} rice husk ash compared to other rates, which were at par in the 2017 and 2018 seasons, respectively. Further, pod length and pod diameter were not significant due to RHS in both the 2017 and 2018 seasons. Similarly, treatment interactions remained insignificant ($P>0.05$) across all parameters in both the 2017 and 2018 farming seasons.

Table 4. Effect of goat manure and rice husk ash on leaf area, days to 50% flowering, number of pods plant^{-1}, pod length and pod diameter of okra at Gombe during 2017 and 2018 wet cropping season

Treatment	Leaf area (cm)		Days to 50% flowering		Number of pod plant^{-1}		Pod length (cm)		Pod diameter (cm)	
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
Goat manure (t ha^{-1})										
0.00	80.50^{b}	85.86	49.69	49.56	12.81	27.56	12.31	14.19	1.48^{b}	2.48
5.00	80.81^{b}	85.81	49.56	49.69	13.06	27.81	15.25	14.28	1.56^{ab}	2.56
7.50	84.81^{ab}	89.81	49.94	49.94	12.94	27.94	13.66	15.66	1.64^{ab}	2.64
10.0	102.69^{a}	107.81	50.13	50.13	12.56	28.06	12.28	17.13	1.73^{a}	2.73
LS	*	NS	NS	NS	NS	NS	NS	NS	*	NS
SE±	5.51	6.41	0.20	0.24	0.16	0.16	0.95	0.91	1.56	0.07
Rice Husk Ash (t ha^{-1})										
0.00	69.69^{c}	74.56^{b}	50.00^{a}	50.31^{a}	12.56^{b}	27.56^{b}	12.03	13.97	1.61	2.61
5.00	80.57^{bc}	85.75^{b}	50.31^{a}	50.00^{ab}	12.63^{ab}	27.63^{ab}	12.94	14.75	1.66	2.66
7.50	90.50^{ab}	95.69^{ab}	49.75^{ab}	49.75^{ab}	13.06^{ab}	28.06^{ab}	13.84	15.84	1.58	2.58
10.0	108.00^{a}	113.00^{a}	49.25^{b}	49.25^{b}	13.13^{a}	28.13^{a}	14.69	16.96	1.56	2.56
LS	*	*	*	*	*	*	NS	NS	NS	NS
SE	5.51	6.41	0.20	0.24	0.16	0.16	0.95	0.91	1.56	0.07
Interaction										
Gm x Rh Ash	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Means followed by common letter(s) in a column are not significantly different at 5% probability level, DMRT. NS – not significant; WAS – weeks after sowing; LS – level of significance; SE – standard error; * – significant at 5%.

Okra fresh marketable weight and dry weight were not significantly affected by the application of GM and RHA, irrespective of the rates of application, in the 2017 season, while in the 2018 season they were significantly influenced by both GM and RHA (Table 5). The application of 5, 7.5, and 10 t ha^{-1} of GM resulted in a higher fresh marketable weight of okra than the control, which resulted in a lower weight. On the other hand, application of RHA at 5.0 t ha^{-1} significantly produced heavier marketable pods, though statistically at par with 7.5 and 10.0 t ha^{-1} compared to the control that recorded a lower weight. Similarly, treatment interaction was not significant in both years (Table 5). Findings from this study can infer that the application of GM and RHA can significantly influence crop productivity, as depicted in the growth and yield attributes of okra. This corroborates the findings of GONZÁLEZ & al. (2023), who reported that goat manure fertilization resulted in an increase in grain yield and total protein content of two varieties of *Chenopodium quinoa* Willd. Grown at high altitude. Similarly, ABUKARI (2014) documented a higher number of nodules and nodule weight in cowpea arising from a 4.5 t ha^{-1} application of RHA. SARANYA & al. (2018) reported an increase in plant height, shoot and root weight, and dry matter due to the application of RHA at 10 t ha^{-1} + 50% of the recommended K rate. THIND & al. (2017) recorded a 25% increase in grain yield of wheat over control arising from a 10 t ha^{-1} RHA application. The 2018 growing season saw a marked improvement in growth and yield-related traits, which could be attributed to the organic materials' correct mineralization throughout time and space, which allowed the crop to utilize them for the formation of dry matter. Due to the rapid decomposition of organic materials, which influences how much rice husk ash is applied, this is the moment when nutrients are released. The results concurred with those of DE LA ROSA & al. (2023), who reported sustainable crop production by evaluating the agronomic impact of volcanic ash, rice husk ash, and other soil amendments as a means of lowering pollution and protecting the ecosystem.

Table 5. Effect of goat manure and intact rice husk on fresh weight and dry weight of okra at Gombe during 2017 and 2018 wet cropping season

Treatment	Fresh marketable weight (g)		Dry weight (g)	
	2017	2018	2017	2018
Goat manure (t ha^{-1})				
0.00	189.37	183.18^{b}	28.84	32.39
5.00	191.22	200.28^{a}	30.47	36.02
7.50	185.27	204.37^{a}	30.19	37.11
10.0	174.43	206.28^{a}	25.39	56.22
LS	NS	*	NS	NS
SE±	6.87	7.23	1.71	9.23
Rice Husk Ash (t ha^{-1})				
0.00	176.36	181.36^{d}	26.30	33.48
5.00	193.99	208.99^{a}	31.56	56.77
7.50	193.06	201.88^{ab}	30.40	38.56
10.0	177.28	198.28^{bc}	26.48	33.48
LS	NS	*	NS	NS
SE	6.87	7.23	1.71	9.23
Interaction				
Gm x Rh Ash	NS	NS	NS	NS

Means followed by common letters in a column are not significantly different at 5% probability level, DMRT. NS – Not significant; LS – Level of significance; SE – standard error; * – significant at 5%.

Conclusion and recommendation

The results of the experiments show that the application of GM and RHA up to 10.0 t ha^{-1} greatly improved the growth and yield indices of okra in the research area. Thus, GM can be exploited as a crucial part of organic manure, and when strengthened with RHA, its effectiveness can be enhanced towards the production of okra and other horticultural crops, lowering greenhouse gas emissions from the usage of synthetic agrochemicals in soils and the environment.

Conflict of interest

The authors declare no conflict of interest.

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EFFECTS OF CHROMIUM STRESS ON SEED GERMINATION AND EARLY SEEDLING GROWTH PERFORMANCES OF PEARL MILLET *PENNISETUM GLAUCUM* (L.) R. BR. (POACEAE)

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Abstract: Heavy metals are widely available in the environment due to the natural processes, industrial, anthropogenic activities and ultimately in the results contaminate the immediate environment. The presence of the heavy metals such as Pb, Cd, Cr, Ni, Fe and Cu in environment disturb the quality of ecosystems, soils, water, air and vegetation. The chromium metal at higher level in ecosystem is an alarming signal for both developed and developing countries. *Pennisetum glaucum* is an annual grass which is widely cultivated in drought, rain fed and high temperature areas. The limited amount of literature available on the impact of chromium stress on *P. glaucum.* This study was conducted to investigate the beneficial or harmful effect of chromium stress on seed germination and seedling growth performances of *P. glaucum* in *in vitro* conditions. The different concentration of chromium 0, 25, 50, 75 and 100 ppm was applied. In present study, the overall results suggests the variable response on the rate of seed germination percentage and plant growth of *P. glaucum* to chromium stress was recorded. Results showed that the chromium treatment at 25 ppm significantly ($p<0.05$) reduced root growth of *P. glaucum*. The chromium at 50 ppm level significantly decreased the rate of percentage of seed germination of *P. glaucum*. The seedling dry weight of *P. glaucum* seedling was decreased highly at 75 ppm chromium. Root / shoot ratio also decreased due to gradual increase in chromium from low (25 ppm) to higher (100 ppm) levels. Similarly, the chromium at 25 to 100 ppm gradually decreased the percentage of tolerance and seedling vigor index of *P. glaucum*. An effective efforts for minimize the chromium toxicity and tolerance in plants are required.

Keywords: heavy metal, pearl millet, root, shoot, toxicity.

Introduction

Chromium is an important environmental pollutant and commonly used in leather tanning industries [CICATELLI & al. 2017; GOMES & al. 2017] chrome plating and stainless steel industries. Chromium with atomic number 24 and atomic weight of 52 is known to cause carcinogen [KUMAR & CHOPRA, 2015]. The addition of metals in the environment due by human and industrial activities is a serious concern for germination and growth of flora and fauna. Metals at cellular level produced oxidative stress on plants [SMEETS & al. 2009; KAPOOR & al. 2022] and uptake at higher concentrations become toxic for plant growth. The leaching of metals via food chain caused human health hazards [KUMAR & al. 2013] and environment [KIMBROUGH & al. 1999; KOTAS & STASICKA, 2000].

It is recognized that heavy metals due to their toxicity, long persistence in nature can accumulate in the trophic chain and cause organism dysfunction [CHEN & al. 2021]. The harmful effects of chromium on photosynthesis, and water relations capabilities in plant was reported [SHANKER & al. 2005]. The hexavalent chromium toxicity under 10 and 50 µM stress for *Amaranthus viridis* and *Amaranthus cruentus* in hydroponic system was recorded [BASHRI

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& al. 2016]. The excess level of chromium produced harmful impact on germination and seedling growth of field Pea (*Pisum sativum* Malviya Matar-15 (HUDP-15), Pusa Prabhat (DDR-23), *Arabidopsis thaliana* and uptake of certain nutrients in *Citrullus*, rice, castor bean, food crops and some vegetables [PANDEY & PANDEY, 2008; DUBE & al. 2003; DU & al. 2003; de SILVA & al. 2021; ABDELGAWAD & al. 2022; ALI & al. 2022; BASIT & al. 2022; CHEN & al. 2022]. The research work on the risk assessment of chromium in date palm (*Phoenix dactylifera*) leaves as use in livestock feed recorded [PILLAY & al. 2003]. The chromium toxicity on germination and early seedling growth in melon (*Cucumis melo* L.), okra (*Abelmoschus esculentus*); *Sesbania sesban* L. (Merrill) and tolerance limit in *Hibiscus cannabinus* L., *Cannabis sativa* L., *Zea mays* L., wheat and alfalfa seeds was recorded [AKINCI & AKINCI, 2010; MOHANTY & al. 2015; DING & al. 2016; ANJUM & al. 2017; CITTERIO & al. 2003; KNEŽEVIĆ & al. 2021; LEI & al. 2021; MUSHTAQ & al. 2021, 2022; MURTHY & al. 2022]. Effect of chromium (VI) phytotoxicity on morpho-physiological characteristics, yield, and yield components of two chickpea (*Cicer arietinum* L.) varieties, *Brassica oleracea* L. var. *acephala* DC., *Brassica juncea* and *Vigna radiata*, peas, sunflower, tumble weed, wheat, rice seedlings, regulation of cell death, chromium uptake and proline metabolism in wheat, and pulses observed [NODELKOSKA & DORAN, 2000; FAISAL & HASNAIN, 2005; TORRESDEY & al. 2005; FOZIA & al. 2008; JUN & al. 2009; DIWAN & al. 2010; OZDENER & al. 2011; WAKEEL & XU, 2020; SINGH & al. 2020; 2022a; 2022b; WANI & al. 2022]. MUKHERJI & ROY (1977) studied the effect of $K_2Cr_2O_7$ solutions between 10-5 and 10-3 M on germination and seedling elongation of rice and on the content of reducing sugars and amino acids in potato tuber slices. There was considerable water loss, and the effect of chromium on the changes in permeability became intensified gradually with increasing time of exposure.

Pennisetum glaucum L. R. Br. is member to family Poaceae and known as Bajra. Pearl millet is found in Africa and in the sub-continent. This purple majesty species is the most widely grown millet in plain and hilly areas. *P. glaucum* consider as a multiple cereal grain crop food, fodder, fuels and prefer to grow in well-drained soil in full sun light. *P. glaucum* is naturally rich in nutrients, gluten free and is sold in products likewise whole as raw grain, breads, biscuits and pasta grown at more than 27 million hectares worldwide [SATURNI & al. 2010; NAMBIAR & al. 2011; MANWARING & al. 2016].

An ever increase of chromium metal pollution is affecting growth of agricultural crops in regional and global level. *P. glaucum* is economically an important grain crop of Pakistan and successfully growing in drought area. There seems little literature available on chromium toxicity on germination and early seedling growth of millet cereal as compared to wheat, rice and maize in Pakistan. This investigation was carried out to determine the influence of chromium on early seedling growth performances of an important agricultural cereal crop *P. glaucum* of growing for grain and fodder production.

Materials and methods

The legume seeds of *P. glaucum* were obtained from super store. The percentage of germination was first checked. The surface of seeds were sterilized with 1N NaOCl solution for one minute to avoid any fungal type of action and washed with double distilled water. Ten seeds were kept on filter paper in each Petri dishes having 90 mm diameter in equal distance. The Petri dishes were washed to drop off the chances of further fungal infectivity. Chromium salt was used as potassium chromate with five 0, 25, 50, 75 and 100 ppm concentrations and initially, five ml of chromium solution was applied. Old solution from each petri dish was changed after

two days and two ml of prepared chromium solutions were added in each set of treatment. The Petri dishes were placed at room temperature (32±2 °C) along with 240 Lux light intensity. The experiment was completely randomized with three replicates and terminated after ten days. Seed germination, root, shoot, seedling lengths and root / shoot ratios were noted. The treated three tallest seedlings were oven dried at 80 °C for 24 hours to get dry weight. The seedling vigor and tolerance indices was determined [BEWLY & BLACK, 1982; IQBAL & REHMATII, 1992].

T.I. = (Mean root length in metal solution / Mean root length in distilled water) x 100

Statistical analysis

The recorded data was analyzed for ANOVA and Duncan's Multiple Range Test on personnel computer using COSTAT version 3. The level of significance was found at $p < 0.05$ level.

Results and discussion

The abiotic stress conditions likewise addition of heavy metals (Cr) in the immediate environment affect plant growth and development. Chromium is a toxic heavy metal and higher level suppress the seed germination and plant growth. Increasing heavy metal concentrations in the soil, non-biodegradable nature, long biological life in ecosystem have become a significant problem in the modern industrialized world due to several anthropogenic activities which, ultimately pose a threat on human life also [THAKUR & al. 2016].

The damage in plant growth due to metal may result at any stage of growth of plant [PRODGERS & INSKEEP, 1981]. The biotic and abiotic factors influence on crop yield and abiotic stress such as drought, salinity, extreme temperatures and heavy metal (HM) contamination are the common factors reported in the scientific literature worldwide. GANGAIAH & al. (2013) demonstrated the effects of different heavy metals such as chromium (Cr), cobalt (Co) and lead (Pb) on pearl millet (*Pennisetum glaucum* (L.) R.Br.) seed germination and seedling growth using doses of 1, 10, 20, 50 and 100 ppm along with control cultures i.e. tap and distilled water media. This present study proved the decreased in rate of seed germination percentage as well as seedling growth of *P. glaucum* as the increased concentrations in the substrate. The treatment of chromium at higher concentration 100 ppm decreased growth characteristics of *P. glaucum*. Statistically analyzed data showed that seed germination, seedling growth and seedling dry weight of *P. glaucum* seedlings were reduced significantly ($p<0.05$) with increased concentrations of chromium (Table 1). Chromium treatment at 75 and 100 ppm significantly ($p<0.05$) affected seed germination percentage (76.66) and (73.33%) against control (100%). The crop yield production depends upon the prevailing conditions of biotic and abiotic stress. Chromium concentrations at 1, 10, 20, 50 and 100 ppm decreased seed germination of *P. glaucum* by in vitro conditions [GANGAIAH & al. 2013]. It was determined that inhibitory effects of Cr were more on all seedling growth variables of *P. glaucum* as compared to control treatment. Similarly, KABIR & al. (2011) studied the tolerance of *Samanea saman* (Jacq.) Merr. for Cu, Fe, Pb and Zn under laboratory conditions and showed that with increasing concentrations of metals reduced seed germination.

The use of genetic engineering to modify plants for metal uptake, transport and sequestration may open up new avenues for enhancing efficiency of plant [EAPEN & D'SOUZA, 2005]. The rapid industrialization, and modern agricultural practices have resulted in increased heavy metal contamination in the environment, which causes toxicity to the living

organisms [KAVAMURA & ESPOSITO, 2010; MIRANSARI, 2011]. Heavy metal stress adversely reduce growth and productivity of plant. The protective efficacy of seed priming with SiO_2 NPs (400 mg/L) in relieving the Cr (200 µM) phytotoxicity mainly in *Brassica napus* L. seedlings recorded. In this study an evidence was established about the Cr-detoxification by seed primed SiO_2 NPs in *B. napus*, indicated the potential of SiO_2 NPs as stress reducing agent for crops grown in Cr contaminated areas. The different Cr levels (200, 300, and 400 mg/kg soil) affected the growth of mung bean seedlings with the use of Azospirillum brasilense and salicylic acid [ALI & al. 2023]. Furthermore, the Cr treatment decreased shoot and root length, plant height, dry weight, and chlorophyll content of mung bean. 37.15% plant height, 71.85% root length, 57.09% chlorophyll contents, 82.34% crop growth rate was decreased when Cr toxicity was @ 50 µM. Furthermore, Mung bean seedlings reported severely damaged by Cr contamination, which limits their growth and physiological characteristics.

Table 1. Effects of chromium on germination and growth of Pearl millet

TRT (ppm)	**SG** (%)	**Root length** (cm)	**Shoot length** (cm)	**Seedling size** (cm)	**Seedling dry weight** (g)	**Root / shoot length Ratio**
00	100.00±0.0a	12.94±1.1a	6.32±0.4a	19.26±1.3a	0.026±0.004a	2.03±0.1a
25	100.00±0.0a	3.95±0.3b	2.15±0.3b	6.04±0.6b	0.026±0.004a	2.03±0.2a
50	83.33±3.3b	3.53±0.2b	1.47±0.1bc	5.01±0.3bc	0.020±0.004ab	2.53±0.3ab
75	6.60±3.3bc	2.91±0.4b	1.01±0.3c	3.40±1.6cd	0.013±0.001ab	3.21±1.0ab
100	73.33±3.3c	0.94±0.2c	1.55±0.2bc	2.48±0.4d	0.010±0.004b	0.63±0.1b
L.S.D. $p<0.05$	8.13	1.62	0.876	2.33	0.014	1.405

Symbol used: TRT = treatment; SG = Seed germination; Number followed by the same letters in the same column are not significantly different ($p<0.05$) according to Duncan's Multiple Range Test.

Potassium dichromate at concentrations above 0.5 mM suppressed growth of radicle and plumule of pea significantly. Its deleterious effect was more pronounced on the growth of roots than on shoots. In addition chromium treated plants a larger proportion of pods failed to set seeds and the average number of seeds per pod was lower [BISHNOI & al. 1993]. The root growth performances of *P. glaucum* was strongly affected by all concentration of chromium. The chromium at 25 ppm level produced a negative and significant impact on root and shoot growth of *P. glaucum*. Similarly, ISLAM & al. (2016) also found decrease in growth of *Zea mays* L. under chromium. Chromium is non-essential element for plant growth. The seedling size of *P. glaucum* showed similar trend of decline as recorded for root and shoot growth. The highest seedling size of *P. glaucum* was recorded for control (19.26 cm) and lowest (2.48 cm) at 100 ppm of chromium. The high uptake of chromium from the medium could be important reason of reduction in seedling height of *P. glaucum*. Seedlings of *P. glaucum* significantly decreased its dry weight with 100 ppm of chromium. Heavy metals produced toxic effects on the plant biomass [SINGH & al. 2015]. Phytotoxic effects of chromium on germination and seedling growth of different plant species investigated [JOUTEY & al. 2013; NAZ & al. 2013], root growth and environmental contamination [PRADAS & al. 2014; PRADAS & al. 2021].

Chromium stress influence on growth, nutritional quality and tolerance in plant [MOHAMMED & al. 2021; LÓPEZ-BUCIO & al. 2022]. The metal tolerance character found in well adapted plant species. The seedlings of *P. glaucum* also tested for tolerance to different level of chromium. The seedlings of *P. glaucum* responded differently for tolerance to chromium (Figure 1). Chromium at 25 ppm showed high percentage of tolerance for *P. glaucum* and lowest

to chromium at 100 ppm. The chromium pollution treatment showed the order of phytotoxicity tolerance 25>50>75>100 ppm in seedlings of *P. glaucum*.

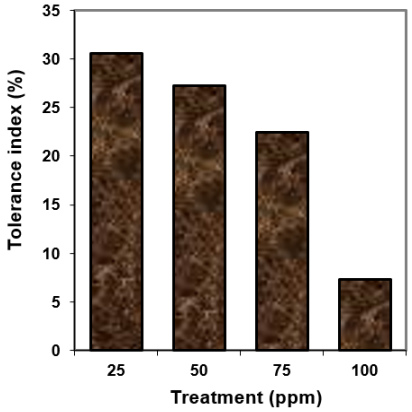

Figure 1. Percentage of tolerance in *P. glaucum* using different concentration of chromium (25, 50, 75, 100 ppm) as compared to control.

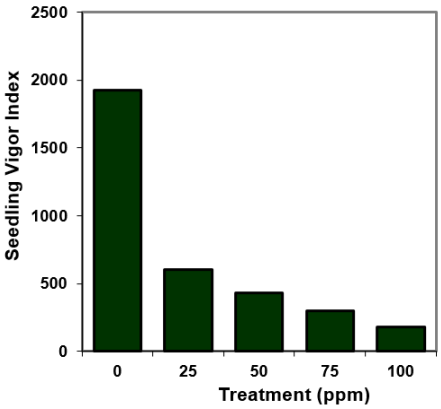

Figure 2. Seedling vigor index for *P. glaucum* using different concentration 0, 25, 50, 75, 100 ppm of chromium.

An association between seedling vigor index percentages in chromium was also recorded with similar trends (Figure 2). The results suggests that an increase in chromium concentration decreased seedling vigor index. Seedling vigor of *P. glaucum* was prominently decreased at 100 ppm of chromium and agreed with the findings of AMIN & al. (2013) who has been found that chromium treatment at 100 mg kg^{-1} adversely affected seedling vigor index of *Hibiscus esculentum* L.

Conclusion

It was concluded that excess level of chromium produced phytotoxic effects on seedling growth of *P. glaucum*. The chromium at 25 ppm significantly ($p<0.05$) affected root, shoot and seedling length of *P. glaucum*. The seedlings *P. glaucum* showed lowest tolerance and seedling vigor indices to chromium at 100 ppm level which might be due to disturbances in metabolic system. It is suggested that such types of ecotoxicological studies can be useful for cultivation of vegetation in chromium polluted areas based on tolerance index.

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BIODIVERSITY OF AQUATIC PLANTS AND MICROFLORA IN TAGWAI DAM, NIGERIA

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Abstract: Aquatic biodiversity of microflora and plants are the varieties of organisms and the ecosystems that make up the wetlands of the world and their interactions. Tagwai Dam is located in Chanchaga local government area of Niger State, Nigeria, located between longitude 60°39' to 60°44' East and latitude 34° to 90°37' North to South-west of Minna, Niger State, Nigeria. Transect sampling collection of aquatic flora and phytosociological method was adopted by using planktonic net, sterilized poly pots and plastic bottles from five sampling stations. Isolation and identification of microflora was conducted using serial dilution for bacterial species and biochemical tests for the identification. Agar pour plate method for the isolation and morphological characteristics for the identification of fungal species. The dominant families of aquatic plants included Araceae, Nymphaeaeceae, Alismataceae, Marsileaceae and Ceratophyllaceae. The identified bacterial species were *Salmonella* species, *Proteus* species, *Psuedomonas* species, *Enterobacter* species, while, the fungal species identified were *Aspergillus* species, *Mucor pusillus*, *Penicillum notatum* and *Candida albicans* were the most dominant microflora found from the sampling stations. The abundance and identification of these aquatic plants and microflora revealed there biodiversity and importance as they serve source of food and energy to the wetland. Aquatic plants and micro flora make up the ecosystem more reliable and comfortable for the aquatic animals and zooplanktons. They are the primary source of energy, the first organisms in food chain in a wetland community.

Keywords: aquatic plants, biodiversity, ecosystem, microflora, transect sampling, wetlands.

Introduction

Aquatic flora make water bodies more important, due to the introduction of non-native species from one part of the world. Some are beneficial or of horticultural interest while majority escaped cultivation leading to acid spread problems [EVERITT & al. 2011]. Documented of fresh water aquatic flora will encourage water chemistry, hydrologic regime, temperature, sedimentation and availability of biodiversity with rich species. The aquatic flora [DEGOOSH, 2014] are phytoplankton ecosystem with different descriptive morphology, importance and nutritive value for aquatic faunas and they serve as primary producer, medicinal, water purifiers, absorbance of carbon dioxide, hide out, spawning site for aquatic faunas and other terrestrial faunas.

Aquatic flora are floating and rooted flora of hydrophytes known as macrophytes and phytoplankton in the study of water ecosystem. They are herbs, shrubs, legumes, forage, fruits

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or fruitless producing flowers, leaves with or no trichome with swollen and stolon or rhizomes Some are perennial monocot found in all types of water bodies [RODGERS, 2014]. They are also grasses, vegetables and rooted plants with smooth succulent or rough pine stem and lamina. They have mechanism in leaf and root capable of receiving oxygen, nutrient and water from aquatic environment with modified stem and leaves for storage and absorbance of photosynthetic activities. Most of their roots are feathery, blunt and shallow to the soil or attachment for dangling freely in water, for example water hyacinth with rooted leaves. Though, some are vegetative in nature with leaves and fruit [RODGERS, 2014], for example *Elodea* sp., *Cabomba* sp., *Ceratopteris thalictroides*, *Mourera fluvialitis*, micro sword and rooted algae. They are of different species which constitute macrophytes and phytoplankton. The aquatic flora are grouped into flaunting leaves, free floating, surmerged plants and emergent flora though some are tall, minutes, short in nature [DEGOOSH, 2014].

Aquatic plants and microflora can be seen growing abundantly in differ water bodies, lakes and water ways in every side of the world [MOHAMMED & al. 2013]. Some are floating, submerged and others are emerged. However, some are producers of amino acid, presence of anti-nutritional factor (ANF's), some have excess crude fibers, presence of cellulose, hemicellulose and lignin [KHAN & GHOSH, 2012]. They also have proximate composition of ash, crude protein and crude fat.

They are the main source of freshwater retention, and their chemical constituent need adequate growth of aquatic flora for optimum concentration of much needed nutrients (nitrogen and phosphorus) with calcium and other nutrient element [MANAHAN, 2005]. Some aquatic macrophytes are used for aesthetics, drainage, fishing, flood control, hydropower generating, irrigation, navigation, recreation and ultimately land values. Assessment of aquatic flora is of more important in the previous years over the implementation of National Pollutant Discharge Elimination System (NPDES) to regulate aquatic plants management, mostly the qualitative assessment of nuisance plant. Aquatic flora might help in reducing floods, contribute to the development of climate and weather that will supply enough dissolved oxygen (DO) to the ecosystem and may tend to reduce the deflection of ozone layer. Many microscopic organisms infect fishes, causing spoilage of aquatic environment and loss of income either by the cost of medication treatment or direct loss of the organism to infectious diseases; while some are of zoonotic potential, causing diseases in animal and human, and some may produce metabolites that can cause positive effect to man and animal [KEDDY, 2010].

The aquatic flora have some new importance but undiscovered. Marine flora are consumed by human as macro-algae, used in pharmaceutical, use as biomass feedback while freshwater flora are demanded in water garden and aquarium planting [BHARATHIRAJA & al. 2015]. Hydrophytes are used as food source notably in south east Asia but uncooked ones are involve in transmission of Fasciolopsiasis and they are source of animal nutrition, for example *Eichornia* sp. (water hyacinth), *Lemna minor* (duckweed) and *Trichanthera gigantea* (suiban) [HILL & al. 2011].

Identification of aquatic plants and microflora are broadly disturbing in Tagwai Dam, Niger State due to poor management of the water body and destruction of native flora, over grassing and water pollution. The aquatic plants and microflora are the modest of ecosystem hence they are the primary producer and ecosystem repairer. The aim of this study is the assessment of biodiversity of aquatic plants and micro flora in Tagwai Minna, Niger State, Nigeria.

Material and methods

Study Area

The study was carried out at Tagwai Reservoir, Minna, Niger State, Nigeria. Tagwai reservoir has a total surface area of 44 hectares, and storage capacity of 28.3 million m^3 of water. It has a depth of 25 meters and a length of 18 km. The reservoir was constructed in 1980 on longitude 60°39' to 60°44' East, and latitude 34° to 90°39' North to South-West of Minna [MUHAMMED & al. 2019].

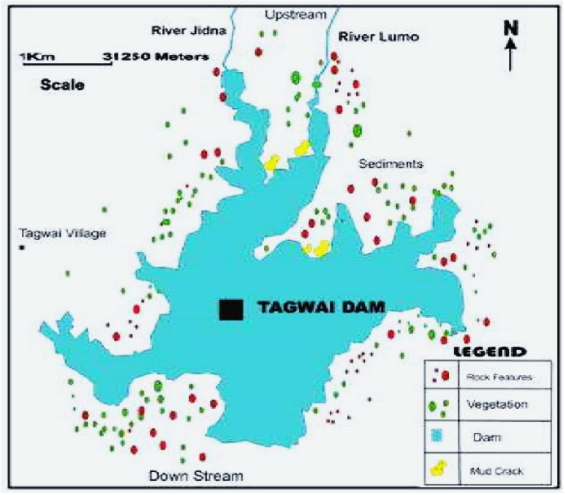

Figure 1. Map of Tagwai Dam. Source: Niger State water Board, Minna, Nigeria

Samples Collection

Transect sampling collection of aquatic flora and physical characteristic (Phytosociological) method of collection was adapted by using planktonic net, polypots and plastic bottles in five different locations of the dam with labeling p01a, p02b, p03c, p04d and p05eon weekly basis in the morning and evening for a period of four months. The map of the study area as shown in Figure 1. A 20 ml syringe Horst was attached at the bottom of the planktonic net with about 30 centimeters radius of iron rod which the net was hanged on and along (8 ft) foot stick in length for handling and taking water sample from the located point. The samples were used for the identification of aquatic flora in the Dam. More than 200 square meters away from each sampling point was use in order to avoid over distribution of aquatic the same aquatic flora species in any sampling point MADSEN & WERSAL (2017).

The water samples in the five sampling station were transported to the laboratory of Biological sciences department at Ibrahim Badamasi Babangida University Lapai for microbial analysis. The preserved samples were allowed to settled at room temperature for three days and the samples was shake for free phytoplankton and siphoned for the sample to be minimized to 10 ml for modified form for counting of cell and mount on Stereo microscope. The phytoplankton were counted identified and placed on their groups [KOLOANDA & OLADIMEJI, 2004].

Identification and the distribution of aquatic flora in Tagwai Dam

The aquatic flora were identified based on their root and leaves with the help of an atlas by [SPALDING, 2010]. The aquatic flora were identified, classified according to their species and types and also the rate of their percentage distribution / percentage frequency distribution of aquatic flora was determined by: sum = (no. of flora at a location x 100) / total number of flora.

Serial dilution of water samples

The water samples collected in the five different locations (p01a to p05e) were serially diluted in accordance to [ANDERSON, 2011].

Microbial analysis of the water samples

Prepared Potato Dextrose Agar (PDA) and Nutrient Agar were poured into 30 petri dishes, 15 petri dishes were used for each media in a sterile and conducive environment. After pouring of the medium, it was allowed to solidify and then spread plate the medium by inoculating the 10^{-3}, 10^{-4} and 10^{-5} test tube samples into the media in 3 petri dishes for each sample as described by [EFUNTOYE & al. 2012].

Characterization and identification of the microbial isolates

The fungal and the bacterial isolates from the water samples were characterized and identified based on their morphological characteristics, biochemical properties such as mannitol salt agar test, oxidase test, indole test, citrate utilization test, triple sugar iron test, gram staining and microscopic characteristics as described by CHEESBROUGH (2005).

Data analysis

The method adapted for the collection of aquatic flora was survey and transect sampling [JOHNSON & NEWMAN, 2011] where collection of data involved using of chart and tables assessment in field and laboratory. The determination of data was obtained using excel: formula = Average, mean (total number of species). The average percentage of aquatic plants distribution average was 3.1%, bacteria was with the average of 9, while fungi has average of 12% in frequency distribution.

Results

Identification and the distribution of aquatic flora

Thirty five (35) aquatic plants were identified and classified into ten (10) families, nine (9) orders and twelve (12) species and into habitat in accordance to plants habitat with *Mimosa pigra* and *Cyperus* species having the higher percentage of distribution while *Persicaria senegalensis* was less though invasive and nuisance flora accumulated into the eastern axis of the reservoir (Table 1). The distribution of aquatic flora in Tagwai Dam were: *Mimosa pigra*, *Persicaria senegalensis*, *Wolfia* sp., *Nymphaea* sp., *Ludwigia* sp., *Pistia stratiotes*, *Hydrocleys nymphoides*, *Ceratophyllum demersum*, *Lagarosiphon* sp., *Marsilea* sp., *Cyperus* sp., *Schoenoplectus* sp. (Table 2).

Table 1. Identification of aquatic flora of Tagwai Dam

Common name	Family	Order	Genus	Species
Giant sensitive tree	*Fabaceae*	*Fabales*	*Mimosa*	*pigra*
Snakeroot	*Polygonaceae*	*Caryophyllales*	*Persicaria*	*senegalensis*
Duckweed	*Araceae*	*Alimatales*	*Wolffia*	sp.
Water lily	*Nymphaeaceae*	*Nymphaeales*	*Nymphaea*	sp.
Alligator weed	*Onagraceae*	*Myrtales*	*Ludwigia*	sp.
Water lettuce	*Araceae*	*Alismatales*	*Pistia*	*stratiotes*
Water poppy	*Alismataceae*	*Alismatales*	*Hydrocleys*	*nymphoides*
Coontail	*Ceratophyllaceae*	*Ceratophyllales*	*Ceratophyllum*	*demersum*
Oxygen weed	Hydro*charitacea*	*Alismatales*	*Lagarosiphon*	*major*
Water clover	*Marsileaceae*	*Salviniales*	*Marsilea*	sp.
Giant sedge	*Cyperaceae*	*Poales*	*Cyperus*	*ustulatus*
Water reed	*Cyperaceae*	*Poales*	*Schoenoplectus*	*subterminalis*

Source: Authors collection

Table 2. Distribution of aquatic flora in Tagwai Dam

Aquatic flora	Number	Percentage (%)
Mimosa pigra	5	13.5
Persicaria senegalensis	1	2.7
Wolffia sp.	2	5.4
Nymphaea sp.	3	8.1
Ludwigia sp.	4	10.8
Pistia stratiotes	3	8.1
Hydrocleys nymphoides	1	2.7
Ceratophyllum dermersum	4	10.8
Lagarosiphon sp.	2	5.4
Marsilea sp.	3	8.6
Cyperus ustulatus	5	13.5
Schoenoplectus subterminalis	3	8.1
Total	37	99.9

Source: Authors collection

The total viable counts of the bacterial isolates from the five sampling stations of the Tagwai Dam was shown in Table 3.

Table 3. Total viable count of bacteria isolates

Location	Dilution factor	Number of colonies	Population (cf/ml)
P01a	10^{-3}	108	1.8^5
P01b	10^{-4}	64	6.4^5
P02a	10^{-3}	90	0.9^5
P02b	10^{-4}	47	4.7^5
P03a	10^{-3}	43	0.43^5
P03b	10^{-4}	19	1.9^5
P04a	10^{-3}	96	0.96^5
P04b	10^{-4}	50	5.0^5
P05a	10^{-3}	92	0.92^5
P05b	10^{-4}	40	4.0^5

Keys: P01a to P05e = station

The bacterial species identified, total number of colonies and the percentage distribution of the bacterial species in Tagwai Dam as shown in Table 4.

Table 4. Total number of colonies and percentage distribution of bacteria in Tagwai Dam

Bacteria species identified	Number of colonies	Morphology	Percentage (%)
Salmonella sp.	3	Rods	5.3
Proteus sp.	7	Rods	12.5
Escherichia coli	14	Rods	25
Pseudomonas sp.	8	Rods	14.3
Enterobacter sp.	16	Rods	28.6
Staphylococcus sp.	4	Cocci	7.1
Shigella sp.	4	Rods	7.1

Source: Authors collection

The fungal species identified and their frequency of occurrence from the sampling stations during the period of collection was shown in Table 5.

Table 5. Identification and their frequency of occurrence fungal isolates

Fungal species identified	First	Second Dish	Total count.	Percentage (%)
Aspergillus niger	4	8	12	16.9
Aspergillus flavus	5	10	14	19.7
Aspergillus fumigatus	6	3	9	12.7
Mucor pusillus	4	9	13	18.3
Penicillum notatum	3	7	10	14.0
Candida albicans	7	6	13	18.3

Source: Authors collection

Discussion

The percentage distribution of *Mimosa pigra* of the family Fabaceae, and *Cyperus ustulatus* of the family Cyperaceae as the most dominant hydrophytes with 13.5% each while, the least species of distribution was observed in *Persicaria senegalensis* of the family Polygonaceae and *Hydrocleys nymphoides* of the family Alismataceae with 2.7% each during the period of collection. This might probably be as a result of environmental factors such as rainfall and vegetation type of the plants habitat in Tagwai Dam. This is in agreement with the findings of ADESINA & al. (2011), who reported that hydrophytes with free floating and emergent plants such as the Fabaceae and Cyperaceae formed the highest frequency distribution of 93.3% with dominant species irrespective of season in Jebba Lake, the dominant species are being *Mimosa pigra* and *Vossia cuspidata*. DIENYE (2015) reported that Cyperaceae had the highest species abundance and lowest were recorded in other families in New Calabar River. Also, the findings of IDOWU & NGAMARJU (2011) reported that high abundance of species composition of aquatic hydrophytes at Lake Alau in Nigeria during the rainy season has increased the water level which had favoured the increased in aquatic free floating and emergent vegetation. The presence of *Nymphaea* sp. and *Pistia stratiotes* in some of the sampling stations might be due to the effects of transportation of wastes from domestic and industrial sources from the surroundings of the dam into these stations, these activities could enhanced the growth of these plants. ALFRED & al. (2014) reported that soil erosion, flooding, transportation of

nutrient-rich industrial and domestic sources can provide rich nutrients that can boost *Nymphaea* sp. and *Pistia stratiotes* plant growth parameters and there propagules. OBOT & MBAGWU (1988) also reported that anthropogenic changes caused eutrophication of lakes, altering aquatic vegetation species and abundance in water bodies.

The bacteria species identified in the sampling stations were *Salmonella* sp., *Proteus* sp., *Staphylococcus* sp., *Esherichia coli*, *Pseudomonas* sp., *Enterobacter* sp. and *Shigella* sp. The presence and abundance of some of these bacteria species could be attributed to human domestic activities such as wastes disposal, washing at the water banks, fishing with hazardous chemicals. This corroborated with the findings of CHUKWUEMAKA & al. (2019) who reported *Salmonella* sp., *Staphylococcus* sp. and *Esherichia coli* in Tagwai Lake, as responsible for water borne disease. Also, the reports of ADAMU & al. (2022) , reported *Esherichia coli* and *Pseudomonas* sp. as the common encountered species in water and other aquatic products in Lake Dangana, Nigeria. *Esherichia coli*, *Pseudomonas* sp. and *Staphylococcus* sp. identified in this study, have been previously reported by OBIRE & AGUDA (2015), ADAMU & al. (2022) as bacterial species that are actively involved in organic materials decomposition which might have been as a result of the water through the surface run-off from the lake surroundings. SINGLETON & SAINSBURY (2001) in their findings reported these bacteria species as causative agents of gastrointestinal disorders such as diarrhea and upper respiratory infections.

The fungi species isolated and identified in the sampling stations were *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Mucor pusillus*, *Penicillum notatum* and *Candida albicans*. The presence and abundance of some of the fungi species could be as a result of organic materials degradation in the water body. ADAMU & al. (2022) reported that *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Mucor pusillus*, and *Penicillum* sp. as waste degraders in Dangana Lake, Nigeria. Similar findings were reported by ARIYO & OBIRE (2016), SOKOLO & al. (2018), ADAMU & al. (2018) as waste degraders in aquatic environments.

Conclusion

The identification and determination of the aquatic plants distribution in Tagwai Dam revealed that *Mimosa pigra* and *Cyperus ustulatus* are the dominant distributed flora with 13.5% while *Persicaria senegalensis* and *Hydrocleys nympoides* are the least with 2.7%. Though, more abundance species of plants are in Station one (p01a), the eastern axis of the dam with high rate of invasive and nuisance flora species accumulating the site which might lead to eutrophication of the reservoir. Also in the isolation and identifying of microflora (fungal and bacterial species) from the five different locations of Dam showed that *Enterobacter* species have the high rate of distribution of 28.6%, while *Salmonella* species has the low distribution of 5.3%. For the fungi *Aspergillus flavus* have higher percentage of 19.7% and the *Aspergillus fumigatus* distributed at low percentage of 12.7%. The dam has average of 1% of aquatic flora distribution.

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Conflict of interest

Authors declare that they have no conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CULTIVAR AND WEED CONTROL STRATEGY INFLUENCING THE PRODUCTIVITY OF ROSELLE (*HIBISCUS SABDARIFFA* L.) IN A SEMI-ARID ENVIRONMENT OF NIGERIA

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Abstract: Field trial was conducted during the 2019/2020 cropping seasons to determine the effect of cultivar and weed control strategies on the productivity of roselle in a semi-arid environment. The trials comprised of two cultivars (deep red and white) and seven weed control strategies (pendimethalin at 2.0, butachlor 2.0, butachlor 1.5 + pendimethalin 1.5 kg a.i. ha^{-1}, butachlor 1.5 kg a.i. ha^{-1} + SHW at 6 WAS, pendimethalin 1.5 kg a.i. ha^{-1} + SHW at 6 WAS, hoe weeded twice at 3 and 6 WAS, and weedy check) which were factorially combined and replicated thrice in a Randomized complete block design. The application of pendimethalin at 2.0 kg a.i. ha^{-1}, as well as the deep red roselle cultivar, resulted in significant ($P<0.01$) increase in the number of days to 50% emergence, crop injury scores, and lowest stand count. The weedy check, on the other hand, recorded the lowest crop vigor score, plant height, number of leaves $plant^{-1}$, leaf area, and leaf area index. Similarly, weedy check had the lowest fresh fruit weight, fresh capsule weight, dried capsule weight, seed weight, seed yield, and 1000 seed weight when compared to the other weed control strategies. Weed cover and weed density were similar in weedy check compared to the other weed control strategies, but treatment efficiency index was significantly ($P<0.01$) higher with application of two hoe weeding's at 3 WAS and 6 WAS, butachlor 1.5 kg a.i. ha^{-1} + SHW at 6 WAS and pendimethalin 1.5 kg a.i. ha^{-1} + SHW at 6 WAS. Deep roselle cultivar outperformed white roselle in terms of growth and yield characters. Therefore, the cultivation of deep red roselle with butachlor and pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS can be adopted in place of hoe weeding for season long weed control in the study area.

Keywords: cultivar, productivity, roselle, yield, weed control strategy.

Introduction

The semi-arid ecology is an integral part of a dryland area found worldwide with an aridity index of 0.20-0.50 and with about 34% of its land under cultivation [SAFRIEL *&* al. 2005]. Dryland ecosystems are considered to be under threat [ADEEL *&* al. 2005; SAFRIEL *&* al. 2005] due to loss of biodiversity, increasing soil degradation, poverty, drought, and encroachment of invasive species which impede crop and livestock production [REYNOLDS *&* al. 2007; COTULA *&* al. 2006; 2009]. This can be mitigated by encouraging farmers to enhance their crop production ventures, especially those with household and industrial applications, since they have the potential to improve their socioeconomic standing. Crop production, on the other hand, faces a number of obstacles posed by biotic and abiotic stress, including determining which agronomic approaches are best for a given crop in a given ecosystem. Biotic variables are the most important component in ensuring a crop's predicted yield. Weed infestation becomes more significant among biotic variables depending on the weed

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flora and duration of weed competition [THAKUR *&* al. 2016]. Semi-arid dryland soil contains approximately 100-300 million buried weed seeds per hectare, of which only a percentage germinate and emerge each year, resulting in crop-weed competition for limited environmental resources [CHIKOYE *&* al. 2004]. Roselle (*Hibiscus sabdariffa* L.), of Malvaceae family, crop native to Central and West Africa. It is, however, mostly grown in tropical and subtropical places around the world for its tasty calyces [AMIN & al. 2008; PURSEGLOVE, 1991]. Apart from the nutritional and physiological benefits that the crop provides, it also provides a considerable source of revenue for subsistence rural farmers in Africa's drylands [NYARKO *&* al. 2006; ATTA *&* al. 2010]. The Roselle seeds until now do not have any commercial applications though they are a valuable food resource on account of their protein, calorie and substantial amount of fiber and valuable micro-nutrient [AKANBI *&* al. 2009; NORHAYATI & al. 2019]. The seeds contain an edible fixed oil (17-20%) similar with cotton seed oil properties [OTTAI *&* al. 2004; OTTAI & ABD-EL-KHAIR, 2004; HUSSEIN *&* al. 2010; HASSAN & al. 2014]. Roselle is more prone to weed infestation, especially at the beginning of its life cycle, because of its slow initial growth behavior, which reduces canopy cover. An effective weed management practices is necessary for higher crop production and better economic returns of any crop production venture. As a result, weed control in the crop has become increasingly important. The need to evaluate the effectiveness of some weed control strategies and choices of cultivars becomes imperative towards ensuring sustainable roselle production in the semi-arid dryland areas.

Materials and methods

Experimental site

The experiment was carried out during the 2019/2020 seasons at the Research and Training Farm of the Centre for Dry Land Agriculture, Bayero University Kano (12°43' N Latitude; 8°31'0.9" E Longitude; altitude of 481 m above sea level). The maximum temperature fluctuated between 29.7 °C and 40.2 °C, while the minimum temperature ranged between 20.7 °C and 26.5 °C, with a total amount of rainfall of 989.8 mm falling from June to October. The soil at the experimental site was sandy loam with a pH of 6.37, low organic carbon (0.49 g/kg), low available N (0.04 percent), low available P (31.7 mg/kg), and medium available K (0.19 cmol/kg).

Treatments and experimental design

The experiment included seven (7) weed control treatments and two roselle cultivars (white and deep red roselle) factorially combined and laid out in a randomize complete block design (RCBD) and replicated three (3) times.

Land preparation and sowing

Prior to marking out individual plots, the experimental field was harrowed twice. The gross and net plot was 3 x 4 m and 4.5 m^2 respectively, with 0.5 m and 1.0 m between subplots and replicates. Cultivars were sourced from the National Horticultural Research Institute (NIHORT) Kano Station, Bagauda, and 5 seeds of each cultivar were sowed per hole before being pruned to two plants per stand-1 at 2 WAS (weeks after sowing).

Treatment and fertilizer application

The herbicides were administered pre-emergence two days after sowing with a knapsack sprayer equipped with a blue deflector polyjet nozzle and set at 2.1 kg m^{-2} pressure to

provide a spray volume of 250 L ha^{-1}. To avoid wind drift, the spraying was done first thing in the morning. Herbicides were used in accordance with the treatment plan. Hoe weeding was performed at 3 and 6 WAS for hoe weeded treatments, respectively, and at 6 WAS for the treatments assigned to additional hoe weeding. Weedy check plots were left unweeded for comparison during the experiment period. The optimal fertilizer rate was applied at 2 WAS at the rate of 300 kg ha^{-1} of N, P_2O_5, and K_2O in the form of NPK 20-10-10.

Data collection and Data analysis

Data were collected on growth and yield characters of roselle, as well as on weed attributes. The data acquired in the field was subjected to analysis of variance (ANOVA) using GENSTAT (17^{th} edition). Significant means were separated using the Student Newman-Keuls Test (SNK) at a 5% level of probability.

Results and discussion

Effect of weed control strategy and cultivar on growth characters

Weed control strategy was only significant on days to 50% emergence; weed control strategy and cultivar were both significant on crop injury scores, days to 50% flowering and stand count at harvest, but the interaction was only significant on crop injury score (Table 1). When compared to other weed control strategy that resulted in a lower number of days to 50% emergence; application of pendimethalin at 2.0 kg a.i. ha^{-1} considerably ($P \leq 0.05$) increased the number of days to 50% emergence, but was par with butachlor 2.0 kg a.i. ha^{-1} and butachlor 1.5 + pendimethalin 1.5 kg a.i. ha^{-1}. Pendimethalin at 2.0 kg a.i. ha^{-1} produced the greatest crop injury scores, however it was comparable to other strategies' that caused less crop injury (Table 1). Similarly, pendimethalin 2.0 kg a.i. ha^{-1}, pendimethalin 1.5 + butachlor 1.5 kg a.i. ha^{-1}, and pendimethalin 1.5 kg a.i. ha^{-1} + SHW at 6 WAS resulted in a lower stand count as compared to other weed control strategies that led in a greater stand count. Weedy check significantly ($P \leq 0.05$) in increasing the number of days to 50% flowering though at par with application of pendimethalin at 2.0 kg a.i. ha^{-1}. The remaining weed control strategies, on the other hand, resulted in decreased number of days to 50% flowering (Table 1). In comparison to the white roselle cultivar, the deep red roselle cultivar produced the highest crop injury scores and longest days to 50% flowering. Stand count was significantly ($P \leq 0.01$) higher with white cultivar. Significant interaction was observed on crop injury scores (Table 2). The application of pendimethalin 2.0 kg a.i. ha^{-1} to deep red roselle resulted in the greatest injury score, closely followed by the same treatment applied to white roselle, while other treatments resulted with lower injury values throughout the cultivars. The increase in number of days to 50% emergence, crop injury scores, as well as low stand count obtained in pendimethalin applied at 2.0 kg a.i. ha^{-1} and other pendimethalin combinations might be due to the phytotoxic effect of the herbicide on the cultivars. IMOLOAME & al. (2011) in sesame; DANTATA & SHITTU (2014) in sorghum and SHITTU *&* al. (2021) in roselle, all observed phytotoxic effects of pendimethalin on small-seeded crops, as evidenced by reduced stand counts and high crop injury scores. This confirms the herbicide's ability to include mitotic poisons that impair cell division in susceptible crops and weed seeds. However, depending on their genetic make-up, some sensitive crop genotypes may be able to metabolize it. Weed control strategy was highly significant ($P \leq 0.01$) on crop vigor, whereas cultivars and their interaction did not differ significantly.

Table 1. Effect of weed control strategy and cultivar on days to 50% emergence, crop injury score, days to 50% flowering, stand count at harvest of roselle during 2019/2020 (pooled data)

Treatment	Days to 50% emergence	Crop injury scores	Days to 50% flowering	Stand count at harvest
Weed control strategy (W)				
Weedy check	4.17	-	88.17^{a}	18.50^{a}
Hoe weeded at 3 and 6 WAS	4.33	-	83.33^{b}	21.67^{a}
Pendimethalin at 2.0 kg a.i. ha^{-1}	5.00	5.50^{a}	85.33^{ab}	11.33^{b}
Butachlor at 2.0 kg a.i. ha^{-1}	4.67	2.88^{b}	83.67^{b}	21.67^{a}
Butachlor at 1.5 + Pendimethalin 1.5 kg a.i. ha^{-1}	4.67	2.17^{c}	85.50^{b}	12.83^{b}
Butachlor at 1.5 kg a.i. ha^{-1}+ SHW at 6 WAS	4.17	2.17^{c}	84.33b	22.33^{a}
Pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS	4.00	3.00^{b}	85.67^{b}	12.00^{b}
Level of probability	0.253	0.039	0.002	<0.001
SE(±)	0.303	0.134	0.731	1.139
Cultivar (C)				
White roselle	4.24	2.80^{b}	76.38^{b}	18.81^{a}
Deep red roselle	4.62	3.07^{a}	93.90^{a}	15.57^{b}
Level of probability	0.108	<.001	<0.001	<0.001
SE (±)	0.162	0.030	0.391	0.609
Interaction				
W x C	0.957	0.030	0.558	0.357

Means followed by the same superscripts in a column are not significantly different at 5% according to Student-Newman-Keuls test (SNK). WAS = weeks after sowing, SHW = supplementary hoe weeding, SE = Standard Error.

Table 2. Interaction between weed control strategy and cultivar on crop injury score of roselle during 2019/2020 (pooled data)

Cultivar	Weed control strategy						
	T1	T2	T3	T4	T5	T6	T7
White roselle	-	-	4.00^{b}	2.67^{cd}	2.00^{cd}	2.33^{cd}	3.00^{c}
Deep red roselle	-	-	5.00^{a}	3.00^{c}	2.33^{cd}	2.00^{d}	3.00^{c}
SE(±)	0.189						

Means followed by the same superscripts in a column are not significantly different at 5% according to Student-Newman-Keuls test (SNK). T1 = Weedy check; T2 = Hoe weeded at 3 and 6 WAS; T3 = Pendimethalin at 2.0 kg a.i. ha^{-1}; T4 = Butachlor at 2.0 kg a.i. ha^{-1}; T5 = Butachlor 1.5 + Pendimethalin 1.5 kg a.i. ha^{-1}; T6 = Butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS; T7 = Pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS; SE = Standard Error.

Plant height, number of leaves, leaf area, and leaf area index were also significant as a result of the weed control strategy and cultivar (Table 3). The application of Butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS was highly significantly (P≤0.01) and produced the most robust plant, followed by hoe weeding twice at 3 and 6 WAS and butachlor at 2.0 kg a.i. ha^{-1}. Other treatments, such as weedy checked, resulted in less vigorous plants. Application of butachlor at 2.0 kg a.i. ha^{-1}, butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS, and hoe weeded twice at 3 and 6 WAS increased plant height substantially more than other treatments that resulted in decreased plant height (Table 3). Hoe weeded twice at 3 and 6 WAS was highly significant (P≤0.01) and resulted in more number of leaves, followed by butachlor 2.0 and butachlor 1.5 kg a.i. ha^{-1} + SHW at 6 WAS, compared to other treatments that resulted in a significantly lower number of leaves, with weedy check recording the lowest number of leaves plant^{-1} (Table 3). Application

of butachlor at 2.0 kg a.i. ha^{-1} was highly significant ($P \leq 0.01$) and produced the widest leaves which were closely followed by hoe weeded twice at 3 WAS and 6 WAS, butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS in comparison with other treatments that resulted in wider leaves, while weedy check recorded the narrowest leaf (Table 3). Hoe weeded twice at 3 and 6 WAS, and butachlor at 2.0 kg a.i. ha^{-1} was highly significant ($P \leq 0.01$) and increased LAI, though at par with butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS and pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS, compared with weedy check that significantly resulted in the decrease LAI (Table 3). The white roselle cultivar produced significantly taller plants than the Deep red, which produced shorter plants. At 12 WAS, the deep red roselle cultivar considerably ($P \leq 0.05$) produced a greater number of leaves $plant^{-1}$, leaf area, and leaf area index, whereas the white roselle produced the lower values, respectively (Table 3). The increase in plant aspect caused by butachlor application at 2.0 kg a.i. ha^{-1} and butachlor application at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS might be attributed to more effective weed management with less phytotoxic effect as compared to pendimethalin rates. RAJU & MITRA (2020) observed that the use of Pretilachlor 50 EC (900 ml ha^{-1}) + one-hand weeding resulted in the best growth parameters and fibre output of roselle. Similarly, SHITTU *&* al. (2021) also reported the efficacy of butachlor at 2.0 kg a.i. ha^{-1} and butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS on calyx yield of roselle in Nigeria's Sudan savanna region.

Table 3. Effect of weed control strategy and cultivar on crop vigor scores, plant height, number of leaves, leaf area and leaf area index of roselle during 2019/2020 (pooled data)

Treatment	Crop vigor at 12 WAS	Plant height (cm) at 12 WAS	Number of leaves at 12 WAS	Leaf area (cm^2) at 12 WAS	Leaf area index at 12 WAS
Weed control strategy (W)					
Weedy check	3.83^{d}	56.98^{b}	59.67^{e}	137.8^{d}	1.57^{c}
Hoe weeded at 3 and 6 WAS	7.33^{b}	83.86^{a}	115.48^{a}	217.8^{a}	2.91^{a}
Pendimethalin at 2.0 kg a.i. ha^{-1}	4.83^{c}	58.70^{b}	69.63^{d}	160.5^{cd}	2.16^{b}
Butachlor at 2.0 kg a.i. ha^{-1}	7.17^{b}	90.79^{a}	104.43^{b}	221.9^{a}	2.81^{a}
Butachlor at 1.5 + Pendimethalin 1.5 kg a.i. ha^{-1}	5.00^{c}	60.56^{b}	75.87^{cd}	167.8^{cd}	2.16^{b}
Butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS	8.50^{a}	84.17^{a}	106.96^{b}	200.1^{ab}	2.66^{ab}
Pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS	5.50^{c}	65.19^{b}	79.62^{c}	183.0^{bc}	2.44^{ab}
Level of probability	<0.001	<0.001	<0.001	<0.001	<0.001
SE(±)	0.328	2.369	2.152	9.02	0.151
Cultivar (C)					
White roselle	6.10	76.54^{a}	85.60^{b}	132.6^{b}	1.77^{b}
Deep red roselle	5.95	66.39^{b}	89.16^{a}	235.6^{a}	2.99^{a}
Level of probability	0.570	<0.001	0.038	<0.001	<0.001
SE(±)	0.175	1.266	1.150	4.82	0.081
Interaction					
W x C	0.166	0.833	1.010	0.49	0.952

Means followed by the superscripts in a column are not significantly different at 5% according to Student-Newman-Keuls test (SNK). WAS = weeks after sowing; SHW = supplementary hoe weeding; SE = Standard Error.

Effect of cultivar and weed control strategies on yield and yield related characters

Table 4 presents the effect of cultivar and weed control strategy on number of fruits plant^{-1}, fresh fruit weight, fresh capsule weight, dry capsule weight, seed weight, seed yield and 1000 seed weight of roselle. Weed control strategy was highly significant ($P \leq 0.01$) with all the yield characters, cultivar was significant ($P < 0.05$) except with fresh capsule weight and 1000 seed weight while interaction was not significant ($P > 0.05$) across the yield and yield related characters (Table 4). The application of butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS was highly significant ($P \leq 0.01$) and produced the height number of fruits plant^{-1} compared with the remaining weed control strategy. However, it was at par with two hoe weeding's at 3 and 6 WAS and butachlor at 2.0 kg a.i. ha^{-1} compared with the rest of the treatment that produced lower number of fruits plant. Fresh fruit weight was highly significant ($P \leq 0.01$) with the application of hoe weeding twice at 3 and 6 WAS, though at par with other weed control strategy that resulted in higher values compared to weedy check that significantly produced the lowest fresh fruit weight (Table 4). Fresh and dry capsule weight were significantly ($P \leq 0.01$) highest with the application of hoe weeding twice at 3 and 6 WAS, butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS and pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS compared with other weed control strategies that produced lower while weedy check substantially produced the lowest fresh and dry capsule weight of roselle (Table 4). Seed weight and seed yield was significantly highest with hoe weed d twice at 3 and 6 WAS though statistically comparable with the remaining weed control strategy with the exception of weedy check which significantly produced the lowest seed weight and seed yield (kg ha^{-1}) of roselle (Table 4). All weed control strategy except weedy check significantly produced higher 1000 seed weight of roselle. Similarly, the deep roselle cultivar outweighed the white roselle by significantly ($P \leq 0.01$) producing the highest number of fruits plant^{-1}, fresh fruit weight, dry capsule weight, seed weight and seed yield ha^{-1}, respectively (Table 4). The increased yield and yield related characters in these treatments might be due to effective control of weeds during the critical period as evidenced by low weed cover scores, weed density and high treatment efficiency index, which favored the increased crop growth and ultimately on yield components and yield of the crop. This corroborates with the findings of AMARA JYOTHI *&* al. (2018) who reported high yield and yield components of roselle due to application of Butachlor 50% EC at 1.5 kg a.i. ha^{-1} at 45-48 hours of sowing + one hand weeding. This is also supported by the findings of SHITTU *&* al. (2021) who confirms the increased in calyx yield of roselle due to application of butachlor at 1.5 kg a.i. ha^{-1}. In another development by SHITTU (2023), the productivity of Tomatoes was greatly enhanced due to effective weed management owing to the application of butachlor at 1.5 and 2.0 kg a.i. ha^{-1} + SHW at 6 WAS.

Table 4. Effect of weed control strategy and cultivar on number of fruits, fresh fruit weight, fresh capsule weight, dry capsule weight, seed weight, seed yield and 1000 seed weight of roselle during 2019/2020 (pooled data)

Treatment	Number of fruits plant^{-1}	Fresh fruit weight (g)	Fresh capsule weight (g)	Dry capsule weight (g)	Seed weight (g)	Seed yield (Kg ha^{-1})	1000 seed weight (g)
Weed control strategy (W)							
Weedy check	7.00^{c}	62.9^{e}	29.20^{c}	11.06^{c}	7.32^{d}	822^{d}	23.67^{b}
Hoe weeded at 3 and 6 WAS	18.50^{ab}	165.9^{a}	75.17^{a}	25.06^{a}	17.08^{a}	2305^{a}	35.67^{a}
Pendimethalin at 2.0 kg a.i. ha^{-1}	8.33^{c}	70.5^{b-d}	42.69^{b}	15.64^{b}	10.21^{bc}	1196^{cd}	34.33^{a}
Butachlor at 2.0 kg a.i. ha^{-1}	13.67^{bc}	129.8^{abc}	45.64^{b}	16.08^{b}	12.20^{abc}	1753^{abc}	32.67^{a}
Butachlor 1.5 + Pendimethalin 1.5 kg a.i. ha^{-1}	9.67^{c}	76.4^{b-d}	40.45^{b}	14.55^{b}	10.63^{bc}	1368^{bcd}	31.33^{a}
Butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS	22.17^{a}	131.7^{ab}	73.80^{a}	24.01^{a}	15.28^{ab}	2033^{ab}	37.00^{a}
Pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS	10.17^{c}	83.2^{bd}	70.07^{a}	21.47^{a}	15.25^{ab}	1902^{ab}	32.50^{a}
Level of probability	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
SE (±)	1.737	15.21	3.39	1.397	1.394	173.1	1.581
Cultivar (C)							
White roselle	11.40^{b}	92.51^{b}	54.50	16.64^{b}	11.71^{b}	1513^{b}	32.62
Deep red roselle	14.43^{a}	113.31^{a}	53.21	19.89^{a}	13.42^{a}	1738^{a}	32.29
Level of probability	0.019	0.032	0.620	0.005	0.017	0.098	0.783
SE (±)	0.928	8.13	1.81	0.747	0.745	92.5	0.845
Interaction							
W x C	0.572	0.108	0.156	0.349	0.878	0.856	0.747

Means followed by the same superscripts in a column are not significantly different at 5% according to Student-Newman-Keuls test (SNK). WAS = Weeks after sowing, SHW = Supplementary hoe weeding; SE = Standard Error.

Effect of cultivar and weed control strategies on weed parameters

Table 5 presents the effect of cultivar and weed control strategy on weed cover scores, weed density and herbicide efficiency index of roselle. Weed cover cores was highly significant ($P \leq 0.01$) and highest in weedy check compared with other weed control strategy that resulted in lower weed cover scores. Similarly, weed density was highest in weedy check compared with the remaining weed control strategy. However, the application of hoe weeded twice at 3 and 6 WAS, pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS and butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS significantly ($P \leq 0.01$) produced the lowest weed cover scores in roselle (Table 5). Treatment efficiency index was highly significantly ($P \leq 0.01$) and highest in hoe weeded twice at 3 and 6 WAS compared with the remaining weed control strategy. However, it was closely followed by the application of butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS and pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS which had higher treatment efficient index compared with the rest of the weed control strategy that resulted in lower TEI (Table 5). Uninterrupted weedy conditions resulted in high weed cover scores and weed density being obtained in weedy check plots. However, application of herbicide either alone or supplemented with hoe weeding at 6 WAS resulted in lower weed cover scores, weed density, as well as an enhanced treatment efficiency index as compared with the weedy check. Similar findings were reported by various scholars on reduced weed cover score and weed density as well as increased weed control

efficiency in roselle due to pre-emergence herbicide application [AMARA JYOTHI *&* al. 2018; RAJU & MITRA, 2020; SHITTU *&* al. 2021; SHITTU & BASSEY, 2023; SHITTU, 2023].

Table 5. Effect of weed control strategy and cultivar on weed cover scores, weed density and treatment efficiency index of roselle during 2019/2020 (pooled data)

Treatment	Weed cover scores	Weed density (n m^{-2})	Treatment efficiency index (TEI)
Weed control strategy (W)			
Weedy check	4.45^{a}	185.50^{a}	-
Hoe weeded at 3 and 6 WAS	1.20^{d}	15.00^{d}	34.45^{a}
Pendimethalin at 2.0 kg a.i. ha^{-1}	2.90^{bc}	66.33^{c}	8.59^{d}
Butachlor at 2.0 kg a.i. ha^{-1}	3.05^{b}	132.50^{b}	9.28^{d}
Butachlor 1.5 + Pendimethalin 1.5 kg a.i. ha^{-1}	2.53^{bc}	89.33^{c}	9.58^{d}
Butachlor at 1.5 kg a.i. ha^{-1}+ SHW at 6 WAS	2.00^{bcd}	24.00^{d}	15.29^{b}
Pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS	1.67^{cd}	23.00^{d}	12.55^{c}
Level of probability	<.001	<.001	<0.001
SE (±)	0.334	11.03	0.445
Cultivar (C)			
White roselle	2.70	82.5	14.30^{b}
Deep red roselle	2.39	70.5	15.60^{a}
Level of probability	0.238	0.162	0.002
SE (±)	0.178	5.89	0.257
Interaction			
W x C	0.976	0.514	0.344

Means followed by the same superscripts in a column are not significantly different at 5% according to Student-Newman-Keuls test (SNK). WAS = weeks after sowing, SHW = supplementary hoe weeding; SE = Standard Error; Weed cover score by visual observation on a scale of 1-5; 1 = less weed cover and 5 = highly infested weed cover.

Conclusion and Recommendation

In arid places, the communities are highly unique. Others rely on natural resources less, while others rely on them more. Environmental variability, combined with rising population levels and increased competition for crop growth resources between crop and weed biotypes, emphasizes the need for an effective weed control strategy that can promote viable roselle growth and seed yield as an alternative to hoe weeding, which is a common farmer practice for combating weeds, thereby increasing farmers' income and investment options in roselle production. Based on the findings from this trial, it can be concluded that application of butachlor at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS and pendimethalin at 1.5 kg a.i. ha^{-1} + SHW at 6 WAS be recommended as alternative to two hoe weeding's, which is the farmer's practice in the dry land ecology of Nigeria, as it increases fresh fruit weight, fresh and dry capsule weight, and seed yield, which was also traced to the deep red cultivar, and effectively results in the lowest weed cover scores and weed density as well as enhances the treatment efficiency index. Hence, farmers in this area can be encourage to adopt this strategy of combating weed menace in roselle farm.

Conflict of interest

The authors declare no conflict of interest.

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COMPARATIVE NUTRITIONAL AND PHYTOCHEMICAL COMPOSITION OF *SORGHUM BICOLOR* (L.) MOENCH AND *ZEA MAYS* L.

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Abstract: This study was carried out to determine the nutritional and phytochemical properties of freshly harvested grains of *Sorghum bicolor* and *Zea mays* using standard biochemical procedures. Nutritional properties analyzed included proximate composition analyzed using Soxhlet extraction, AOAC Kjeldal methods. Mineral contents were determined using Atomic Absorption Spectrophotometry [AAS] while the phytochemicals were determined using standard procedures. All analyses were replicated three times. From the results, crude protein content was low, with values, 7.81% and 6.66% for *Sorghum bicolor* and *Zea mays* respectively. Lipid analysis showed that *Sorghum bicolor* had a higher lipid content of 16.57%. Crude carbohydrate content was higher in both seeds. Percentage of crude fiber was 11.20% and 8.11% for *Sorghum bicolor* and *Zea mays* respectively. Vitamin C analysis showed that *Zea mays* had a higher content of 122.14 mg/100 g. Available energy kj/100 g was also analyzed for *Sorghum bicolor* and *Zea mays* respectively. Significant differences ($P \leq 0.05$) was observed in crude lipid, crude fiber, vitamin C, and available energy between the two seeds. Mineral analysis revealed appreciable contents of minerals necessary for healthy growth and development. Calcium content was highest in *Sorghum bicolor*. Significant differences ($P \leq 0.05$) was observed in phosphorus, magnesium, calcium, and iron between the two seed types. Phytochemical contents of the seeds showed significant differences ($P \leq 0.05$) in tannins, nitrates and saponin with 12.57 mg/100 g, 18.18 mg/100 g and respectively in *Sorghum bicolor* and *Zea mays*. Nitrate and saponin content was higher in *Zea mays*. Alkaloid, flavonoids and phytate contents was detected in both seeds. Minute amounts of oxalate and cyanide were detected in both seed types. It is important to note that *Zea mays* is under pressure as one of formidable food source due to the increasing demand, and thus, increasing the cultivation of *Sorghum bicolor* could alleviate the over dependence on maize as the primary source of nutrition for humans and animal feed formulation.

Keywords: animal-feed, anti-nutritional, guinea-corn, maize, nutritional.

Introduction

Sorghum bicolor is the scientific name of guinea corn, in Hausa land, it is called Dawa Jan-Jare, is local name and is the one of the categories of guinea corn, which belongs to the family Poaceae. It is native to North Africa, where it is cultivated for its grains which are used for food for human beings and animals and for ethanol production. Guinea corn is a cereal grain that originated in Africa and is eaten throughout the world. It is especially valuable in arid terrain because of its resistance to drought, guinea corn is a nutrient rich grain that is often ground into flour to make bread, porridge and pancakes. Typical flowering plant placed in the grass family

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of Poaceae, seventeen of the twenty-five species are native to Austria 10th the range of some extending to Africa, Asia Mesoamerica and certain islands in the Indian and pacific oceans. One species is grown for its grains, while many others are used as fodder plants either cultivated in warm climates worldwide. *Sorghum bicolor* is an important food crop particularly in arid and semi-arid tropics. It is a dual-purpose crop providing staple food for humans and as a fodder for livestock, alcohol production as well as well as preparation of industrial; products many people in Africa and Asia depend on sorghum as the stuff of life. Being a drought tolerant crop, it can give dependable and stable yield in both raining and post raining seasons. It thrives with less rainfall than is needed for rice and maize and can be grown where no other major cereals can be cultivated. Altogether, *Sorghum* is one of the indispensable crops required for the survival of man. According to FAO (1995) report, sorghum was grown globally on area of about 46 million ha with a production of about 60 million tons. However, in India, sorghum is cultivated on an area of about 7.65 million tons. The role of plants in the maintenance of good health is well known. These plants constitute an enormous reservoir of wide varieties of compounds, which exhibit some medicinal and nutritive properties. The leading producers of this valuable crop include Nigeria (12%), USA (10%), Sudan (8%) and Mexico (8%) reported by USDA (2016). *Sorghum* food material is readily available in Nigeria and has promising nutritional attribute. Whole sorghum grain is an important source or B-complex vitamins and some minerals like phosphorus, magnesium, calcium and iron, the protein content of sorghum is similar to that of wheat and maize with lysine as the most limiting amino acid [FAO, 1995]. Maize (*Zea mays* L.), commonly known as corn. It is another important member of Poaceae family, is the third world most important cereal crop universally following wheat, rice, occupies a pivotal role in world's economy and is second among cereals for human consumption after wheat [MULI & al. 2016]. It is the main source of income for smallholder farmers in Africa in general [YAREGAL & FIREB, 2019]. Maize is the cereal widely grown for throughout the world in a range of agro-ecological environment; maize is produced annually than any other grain crops. In 2017, Africa produces 7.4% of the 1,135 million tons produced world-wide in 40 million hectares according to data from FAO (1995). Out fifty species of maize exist and consist of different colors, texture and grain shapes and sizes [AZEVEDO & LEA, 2005]. The white and yellow varieties are the most preferred by most people depending on the region. Maize was introduced in the 1500s and has since became one of African's dominant food crops. The grains are rich in vitamins A, C and E, carbohydrate as well as essential minerals. Maize is staple food for almost half the population of Sub-Saharan Africa and is important for carbohydrate, protein, iron, vitamin B and minerals. The produce, maize meal (ugali), porridge, pastes, and beer, and can be boiled or roasted as fresh as it comes from the farm. Maize is also produced to produce oils for cooking. It is also very important crop for animal feed. Most of maize production in Africa is rain fed. Over the years, maize has become an important crop taking over acreages from traditional crops such as millet and sorghum. In 2018, about 10.2 million tons of maize was produced from 4.8 million hectares, making Nigeria the highest producer in Africa [FAO, 2018]. Research effort by breeders and agronomists have led to the production of many technologies including the breeding of high yielding varieties that are tolerant to drought, diseases and striga infestation [KAMARA & al. 2014]. Maize is grown both (as sweet corn) for human consumption and (as field corn) for other uses such as animal feed and biofuels. Worldwide, only around 15% of maize production is used for human consumption with most production going to animal feed. However, the production of maize production for food production in developing countries is higher at 25 and even higher in regions such as South East Asia where it is estimated to 30-40% whilst in parts of Sub-Saharan Africa, it can be as high as 70-80%.

Materials and methods

Sample collection and preparation

Fresh grains of *Sorghum bicolor* and *Zea mays* were procured from Sokoto Agricultural Development Project [SADP] in Sokoto, Nigeria. The two seeds were taken to the Departmental Herbarium, Department of Plant Science for authentication, where voucher specimens were deposited. The two seeds were separately ground into fine powders and kept in a cleaned bottle until required for analysis.

Proximate and Ascorbic acid composition analysis

The micro-kjeldal method was followed for the determination of crude proteins. Crude lipids, 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) 4, 4 and 9 respectively. Ascorbic acid was determined according to the method described by MUSA & al. (2010).

Mineral composition analysis of *Sorghum bicolor* and *Zea mays*

The mineral composition of the samples was determined 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].

Phytochemical screening of seeds of *Sorghum bicolor* and *Zea mays*

Alkaloid, tannin and flavonoid contents were determined according to the method of TREASE & EVANS (1989) while the phytate contents was determined using the method as described by VAN-BUREN & ROBINSON (1981). Oxalate composition was analyzed following the method as described by YOUNG & GREAVES (1940). Cyanide contents was determined using the method of DAY & UNDERWOOD (1986), the nitrate content was determined following the method of WANG & al. (2005). Saponin was determined using the method of EL-OLEMY *&* al. (1994) while flavonoid was determined using the method of BOHM & KOPACI (1994).

Data Analysis

The results obtained has been presented as Means ± SE 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 and discussion

Proximate and ascorbic acid composition of *Sorghum bicolor* and *Zea mays*.

Proximate composition of *Sorghum bicolor and Zea mays* in Sokoto, Nigeria has been presented in Table 1 below. From the results, values for % crude protein contain were the least 7.81% and 6.66%, obtained in *Sorghum bicolor* and *Zea mays* respectively. Crude lipid (%) analysis revealed 16.51% in *Sorghum bicolor* while 17.18% was recorded for *Zea mays* respectively. Total carbohydrate (%) revealed the highest contents with 79.64% obtained in *Sorghum bicolor*, while 82.39% was recorded in *Zea mays*. Crude fibre (%) revealed 8.20% was recorded for *Sorghum bicolor* while 8.11% was obtained in *Zea mays* respectively. Vitamin C

contents revealed appreciable contents of the valuable ascorbic acid in both the seeds with 98.62 mg/100 g obtained in *Sorghum bicolor* while in *Zea mays* 122.14 mg/100 g was recorded. Available energy (kj/100 g) revealed that the two seeds were loaded with 396.90 k/cal per 100 g in *Sorghum bicolor* while 462.42 k/cal per 100 g in *Zea mays* respectively. The values of crude protein, % ash and crude fibre contents of 28.00%, 9.28% and 12.57% reported on *Moringa oleifera* by OKIKI *&* al. (2015) were higher than the obtained values in the current study. The difference could be attributed to species variability. However, obtained values obtained in the current study were higher than the reported value by the same author as above. Low value of ascorbic acid was reported to be 0.72 mg/100 g on *Moringa oleifera* while vitamin C contents as reported by RAIMI & al. (2014) was higher with 189.53 mg/100 g in seeds of *Vitellaria paradoxa*. In another study by AFOLAYAN & al. (2014), reported value of 542.40 mg/100 g was highest than the obtained values in the current study. Low value of vitamin C was however, reported to be 0.72 mg/100 g on *Moringa oleifera* by OKIKI *&* al. (2015). More so, the protein with 28.54% and lipid 22.47% reported by HASSAN & UMAR (2004) on dehulled seeds of *Parkia biglobosa* were higher than obtained values in the current study. Low value of carbohydrate was reported to be 37.87 %. Higher protein, % ash and % crude fibre contents of 28.00%, 9.28% and 12.87% were reported higher than obtained in the current study as reported by OKIKI & al. (2015) on *Moringa oleifera* and in a study by JACOB & al. (2015) on melon seeds. In another report on proximate traits of the seed and seed cake of Shea butter tree (*Vitellaria paradoxa*) in Nigeria's savanna ecozone, the range of crude carbohydrate as reported by the same author as above was 64.0% to 68.0% while that of crude protein ranged from 8.8% to 9.3% respectively.

Table 1. Proximate and ascorbic acid composition of *Sorghum bicolor* and *Zea mays*

Parameters	Units	*Sorghum bicolor*	*Zea mays*
Moisture content	%	5.90±0.63[a]	6.11±0.49[a]
Crude protein	% DW	7.81±0.99[a]	6.66±0.96[b]
Crude lipid	% DW	16.51±1.17[a]	17.18±1.17[a]
Total carbohydrates	% DW	79.64±2.19[a]	82.39±.2.10[a]
Crude fibre	% DW	8.20±0.86[a]	8.11±0.99[a]
Ash	% DW	6.23±0.63[a]	7.16±0.76[a]
Vitamin C	mg/100 g	98.62±3.46[a]	122.14±3.19[b]
Calorific value	kj/100 g	396.90±4.26[a]	462.42±4.46[b]

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 seeds of *Sorghum bicolor* and *Zea mays* respectively. Analysis for phosphorus revealed the highest contents of 116.74 mg/100 g obtained in *Zea mays* while 96.72 mg/100 g was recorded in *Sorghum bicolor*. Magnesium contents was 62.12 mg/100 g obtained in *Sorghum bicolor* while 41.64 mg/100 g was recorded in *Zea mays*. Analysis for potassium revealed the highest content 56.96 mg/100 g and 46.72 mg/100 g respectively recorded for *Sorghum bicolor* and *Zea mays*. Manganese analysis revealed 52.76 mg/100 g in *Sorghum*

bicolor while 48.66 mg/100 g was recorded for *Zea mays* respectively. Other essential minerals were zinc and iron with appreciable composition in the two seed types. In a report by MUNAHIRA & al. (2022), low values of iron, manganese and zinc were reported 1.87, 0.26 and 3.77 mg/100 g on *Lagenaria siceraria*. Higher values were reported for *Sesamum indicum* and *Moringa oleifera* by KARAYE & al. (2022). More so, low values of zinc and calcium were reported to be 0.58 mg/100 g and 82.50 mg/100 g on *Moringa oleifera* as reported by OKIKI & al. (2015). Also, low values of 30.24, 6.24, 0.30, and 0.72 mg/100 g on calcium, magnesium, manganese and zinc as reported by RAIMI & al. (2014) on seeds of *Vitellaria paradoxa*. Overall, the two seed types could be considered too well in terms of proximate and mineral profile. In a study by OKIKI & al. (2015), low values of iron and calcium were recorded 0.58% and 82.50%. However, higher values of zinc, magnesium and potassium were reported 64.17%, 643.33% and 430.00% reported by the same author as above. More so, higher iron and zinc contents of 144.70 mg/100 g and 21.05 mg/100 g as reported by JACOB & al. (2015).

Table 2. Mineral composition of the of seeds of *Sorghum bicolor* and *Zea mays*

Mineral (mg/100 g)	Symbol	*Sorghum bicolor*	*Zea mays*
Sodium	Na	7.72±0.93[a]	5.23±0.53[a]
Potassium	K	56.96±2.69[a]	46.72±2.26[b]
Magnesium	Mg	62.12±2.86[a]	31.64±1.62[b]
Phosphorus	P	76.72±2.99[a]	116.74±3.83[b]
Manganese	Mn	32.76±1.05[a]	48.66±276[b]
Calcium	Ca	116.64±3.14[a]	132.54±3.98[b]
Iron	Fe	23.06±1.62[a]	15.07±1.02[b]
Cupper	Cu	0.24±0.06a	0.31±0.04[a]
Chromium	Cr	0.06±0.03[a]	0.02±0.01[a]
Zinc	Zn	13.36±1.09[a]	11.74±0.98[a]
Nickel	Ni	0.07±0.04[a]	0.09 ±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 on phytochemical screening of *Sorghum bicolor* and *Zea mays* has been presented in Table 3. The results show a significant difference ($P \leq 0.05$) in the contents of tannins, nitrates, flavonoids and saponin between the two species. For instance, tannin was 12.51% DW in *Sorghum bicolor* while 18.18% DW in *Zea mays*. Nitrate content was 16.62% DW in *Sorghum bicolor* while it was 22.14% DW in *Zea mays*. Flavonoid content was 24.56% DW while it was 13.63% DW recorded in *Zea mays.* Saponin content was 22.23% DW obtained in *Sorghum bicolor* while it was 15.67% DW recorded in *Zea mays* respectively. In a report by KARAYE & al. (2013) on evaluation of selected Nigeria cucurbits, low values of phytate, cyanide and oxalate, compared to the obtained values in the current study, were reported on *Lagenaria aegyptiaca*, *Citrullus lanatus* and *Momordica balsamina* seeds. In another report by NWEZE & NWAFOR (2014), alkaloids were reported to be 3.56% DW and flavonoids 3.36% DW. However, it has been reported that climatic factors and stages of maturity could cause variation in the distribution of phytochemicals BAMISHAIYE & al. (2011) as well as the choice of solvent as different solvents have different extraction capabilities and spectrum of solubility for phytoconstituents [HANDA & al. 2008]. Low values of tannins, oxalate, saponin were reported to be 0.32, 0.93 and 1.67% DW as reported by AFOLAYAN & al. (2014) were lower than the obtained values in the current study. In a study by NWEZE & NWAFOR (2014).

Phytochemicals such as alkaloid was reported to be 3.56% DW, saponins 1.41% DW, tannins 9.36% DW and flavonoids 3.56% DW the values were lower than obtained in the current study. In addition, 19.1% DW and 13.80% DW were reported by FAGBEMI & al. (2005) to be higher than obtained in the current study. However, higher tannin and oxalate contents of 26.40% DW and 39.40% DW as reported on melon seeds by JACOB & al. (2015).

Table 3. Phytochemical composition of the seeds of *Sorghum bicolor* and *Zea mays* (presented as % DW)

Parameters	*Sorghum bicolor*	*Zea mays*
Alkaloids	11.23±0.78[a]	12.66±1.17[a]
Tannins	12.51±1.16[a]	18.18±1.42[b]
Phytate	9.64±0.94[a]	6.39±0.35[a]
Oxalate	6.12±0.46[a]	8.11±0.69[a]
Cyanide	0.03±0.07[a]	0.06±0.05[a]
Nitrate	16.62±1.36[a]	22.14±1.19[b]
Flavonoid	14.56±1.24[a]	13.63±1.11[a]
Saponin	22.23±1.78[a]	15.67±1.32[b]

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

Conclusion

In conclusion, it is importantly pertinent to note that *Zea mays* is under pressure as one of the main foods used by man from time immemorial. There is nowadays, an immense pressure on maize as the food source and for use as a formidable source of feeds for fish and poultry. More so, due to the fluctuating weather conditions occasioned by less downpour, there is the urgent need to get alternative food source that may augment the maize used. Therefore, increasing the cultivation of *Sorghum bicolor* could alleviate the over dependence on maize as the primary source of nutrition for humans and animal feed formulation strategies.

Conflict of interest

The authors affirm that there is no conflict of interest amongst them.

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ELECTRICAL SIGNALING AND ITS PHYSIOLOGICAL AND BIOCHEMICAL IMPACTS DURING HERBIVORE ATTACKS: A BRIEF REVIEW ON *ARABIDOPSIS THALIANA*

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Abstract: Electrical signals in plants were first documented in the mid-19th century. In response to insect attacks, plants generate electrical signals that spread throughout the plant body and trigger physiological, biochemical and molecular responses. *Arabidopsis* has been used as a model plant in the past several decades. In this mini review, we will address the current understanding of electrical signaling in *Arabidopsis* and its physiological and biochemical impacts during herbivore attacks.

Keywords: electrical signal, gene expression, herbivore attack, long-distance communication, plant response.

Introduction

Plants are constantly exposed to numerous stressors in nature such as heat, salt, flooding, pathogens and herbivores; therefore, they have evolved various defense mechanisms to protect themselves against these stressors. One of the most fascinating defense mechanisms utilized by plants is the use of electrical signals to combat insect attacks. Electrical signals are a means of rapid, long-distance communication within a plant, and they play a crucial role in coordinating various physiological and biochemical processes [FOTOUHI & al. 2022]. In response to insect attacks, plants can generate electrical signals that spread throughout the plant body, providing a means of communication between the attacked site and the remote sites of the plant. These electrical signals trigger a cascade of physiological and biochemical response, including the production of defensive compounds, which can deter herbivores [FÜRSTENBERG-HÄGG & al. 2013]. Considerable studies have shown evidence of electrical signals taking place distally, far from the site of herbivore damage [ZIMMERMANN & al. 2016].

The discovery of electrical signals in plants is often attributed to British physiologist, John Scott Burdon-Sanderson, who conducted pioneering experiments on the electrical properties of plant cells in the mid-19th century. In 1873, Burdon-Sanderson published a landmark paper, in which he described his experiments on the electrical responses of plant tissues to various stimuli, such as heat and mechanical pressure [BURDON-SANDERSON, 1873]. He utilized a technique known as capillary electrometer, which allowed him to detect and record weak electrical currents generated by plant cells [BURDON-SANDERSON, 1873].

Burdon-Sanderson observed that when a plant leaf was mechanically stimulated, such as touching or pinching, it generated a small electrical current that was detectable with his capillary electrometer. He also observed that the electrical response of the leaf varied depending on the nature and intensity of the stimulus. His experiments marked the first time that the electrical properties of plant cells had been systematically studied and documented. His findings

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suggested that plants may have a primitive form of nervous system, capable of generating and transmitting electrical signals in response to various stimuli [BURDON-SANDERSON, 1873]. Although his work was groundbreaking, it was not widely recognized or accepted by the scientific community at that time. Today, the discovery of electrical signals in plants by Burdon-Sanderson is considered a seminal event in the history of plant physiology, paving the way for further research on the electrical properties of plants and their role in various physiological and biochemical processes.

Types of electrical signals in plants

Due to the sessile condition of plants, it is critical for them to detect external cues and trigger long-distance intercellular signals for them to adapt to new environmental conditions. Plant cells have evolved numerous plant signals to convey information across the plant, such as reactive oxygen species (ROS), calcium ion (Ca^{2+}), nitric oxide (NO) and electrical signals (ES) [GILROY & al. 2016; CHOI & al. 2017]. As for electrical signals, three types have been observed to occur in plants, and these are action potential (AP), variation potential (VP) or called slow wave potential (SWP), and system potential (SP) [ZIMMERMANN & al. 2016].

i) AP has been associated with non-damaging stimuli such as cold and touch. It depends on a single transient depolarization of the plant plasma membrane and exhibits distinct dynamics in comparison to variation potentials [FROMM & BAUER, 1994].
ii) VP is a transient depolarization of the plant plasma membrane that has an irregular shape and can persist for several minutes. VP has been shown to be triggered by damaging stimuli such as wounding and burning [DZIUBIŃSKA & al. 2003].
iii) SP can be triggered by a wide range of external stimuli. In comparison with variation potential and action potential, system potential is consisting of a transient hyperpolarization of the plasma membrane, which is most likely driven by the activation of H^+-ATPases [ZIMMERMANN & al. 2009].

Among these three types of electrical signals, VP or SP are the most widely investigated herbivore-induced depolarizations that can move in long distances and last for minutes to hours, and is considered a unique electrical signal in higher plants [KLOTH & DICKE, 2022]. Studies have demonstrated that electrical signals in plants can regulate various physiological processes, including gene expression, phloem translocation, synthesis of hormones, etc. [FILEK & KOŚCIELNIAK, 1997; SUKHOV & al. 2012; VODENEEV & al. 2015]. In contrast with AP, VP is not subject to the 'all-or-none law', which means that the parameters of VP can directly impact plant physiological activities [VODENEEV & al. 2006; FELLE & ZIMMERMAN, 2007]. Burning is the most prevalent external stimulus known to induce VP in a diverse range of higher plants, including soybean, barley, and sunflower [VODENEEV & al. 2015]. Wounding or cutting can also trigger VP in some plants such as in pea, maize, and sunflower; however, not in plants such as wheat and tomato [VODENEEV & al. 2012; VODENEEV & al. 2015].

Consequently, burning has emerged as the most frequently utilized stressor to trigger VP. VP is prolonged depolarization of the plasma membrane that can last for up to several minutes and attain high amplitudes (up to tens of mV) with a propagation rate of $mm \cdot s^{-1}$ [VODENEEV & al. 2011, 2012]. VP can encompass two distinct components. The first is a sustained depolarization, and the second is the presence of spikes akin to AP [DZIUBIŃSKA

& al. 2003]. However, VP can also manifest without AP-like spikes [STAHLBERG & COSGROVE, 1997].

The generation of sustained depolarization and/or AP-like spikes may occur in the same plant and is dependent on the severity of the injury and the distance from the local zone of damage. The amplitude and speed of propagation of VP are inversely proportional to the distance from the local damage site [VODENEEV & al. 2015]. In wheat and pumpkin, it has been estimated that the amplitude decrement is 10% cm^{-1} [VODENEEV & al. 2011]. Indeed, the amplitude of VP is directly proportional to the severity of the injury [VODENEEV & al. 2012]. In addition, VP has been demonstrated to propagate even through dead and injured plant tissues [EVANS & MORRIS, 2017].

Generation of variation potential

The hypothesis that the inactivation of plant plasma membrane H^+-ATPases is crucial for VP generation is supported by pharmacological studies [JULIEN & FRACHISSE, 1992; FRACHISSE-STOILSKOVIĆ & JULIEN, 2006]. Sodium orthovanadate, a H^+-ATPase inhibitor, was found to decrease both VP amplitude and depolarization/repolarization rates [KATICHEVA & al. 2014]. On the other hand, VP amplitude was observed to increase upon administration of fusicoccin, a proton pump activator [VODENEEV & al. 2015]. VP generation is also influenced by changes in external and internal pH. Alkalinization of the apoplast (with a magnitude of 0.2-0.7 change in pH unit) accompanies VP generation, while a decrease of 0.3-0.6 pH unit was noted in the cytoplasm [GRAMS & al. 2009; SUKHOV & al. 2014]. In addition, an increase in plasma membrane permeability induced by administering the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was found to decrease VP amplitude, which supports the role of H^+-ATPase inactivation in VP generation [JULIEN & FRACHISSE, 1992; FRACHISSE-STOILSKOVIĆ & JULIEN, 2006]. These findings suggest that changes in H^+-ATPase activity and pH regulation are involved in the generation of VP. Depolarization of the plasma membrane is caused by the inactivation of proton pumps. Ca^{2+} also plays a role in the generation and regulation of VP. Inhibiting Ca^{2+}-permeable channels or dissipating the electrochemical gradient for Ca^{2+} blocks VP generation or decreases VP amplitude in various plants including pumpkins, wheat, barley, and tomatoes [JULIEN & FRACHISSE, 1992; FRACHISSE-STOILSKOVIĆ & JULIEN, 2006; ZIMMERMANN & al. 2009; KATICHEVA & al. 2014]. According to predictions, the activation of Ca^{2+}-permeable channels is the first step required for the depolarization of the plasma membrane and the inactivation of H^+-ATPases [VODENEEV & al. 2011; SUKHOV & al. 2013; KATICHEVA & al. 2014]. Ca^{2+} influx can also activate K^+ and Cl^- channels, generating AP-like spikes and rapid plasma membrane depolarization [SUKHOV & al. 2013]. On the other hand, long-lasting plasma membrane depolarization is caused by H^+-ATPases inactivation [SUKHOV & al. 2013]. It is noteworthy that Ca^{2+} influx is responsible for both proton pump inactivation and K^+ and Cl^- channel activation [VODENEEV & al. 2015].

Propagation of variation potential

As previously mentioned, VP or SWP/SP is a rapid electrical signal that is propagated within the plant vascular system and other plant tissues. There are two main hypotheses for VP propagation: hydraulic wave and chemical agent [MANCUSO, 1999; VODENEEV & al. 2015; EVANS & MORRIS, 2017].

The hydraulic wave hypothesis suggests that VP propagation is caused by a change in hydraulic pressure within the plant vascular system [MALONE, 1992; STAHLBERG & COSGROVE, 1997; MANCUSO, 1999; VODENEEV & al. 2012]. When a plant is damaged by an external stimulus, such as an insect attack or physical injury, the damaged tissue releases cellular contents and causes a sudden increase in local turgor pressure [VODENEEV & al. 2015]. This pressure wave then propagates through the plant vascular system, resulting in the propagation of VP [STAHLBERG & COSGROVE, 1997]. However, the propagation speed of the hydraulic wave is much faster than that of the VP, which suggests that other mechanisms may be involved in the VP propagation [VODENEEV & al. 2015].

The chemical agent hypothesis suggests that the VP is propagated through the release of chemical agents, such as ROS, calcium ions, and/or neurotransmitters. When a plant is damaged, it releases ROS, which are known to regulate ion channels in the plasma membrane, leading to the depolarization of the membrane potential [VODENEEV & al. 2015]. This depolarization triggers the opening of calcium channels, leading to an influx of calcium ions into the cytoplasm. The increase in cytoplasmic calcium concentration then triggers the release of neurotransmitters, which propagate the VP to adjacent cells. This hypothesis suggests that a combination of chemical agents may be involved in VP propagation, with different agents playing different roles in different plant species [VODENEEV & al. 2015].

Regardless of the mechanism involved, the VP is propagated along the plant vascular system and other plant tissues. VP travels through phloem, xylem, and apoplast, which are the interconnected networks of cells that transport water, nutrients, and other molecules throughout the plant [ZIMMERMANN & al. 2016]. VP is propagated by changes in the membrane potential of adjacent cells, with depolarization of one cell triggering the depolarization of the adjacent cells. The propagation speed of the VP varies between different plant species and tissues, but it generally ranges from a few millimetres to several centimetres per second [VODENEEV & al. 2015].

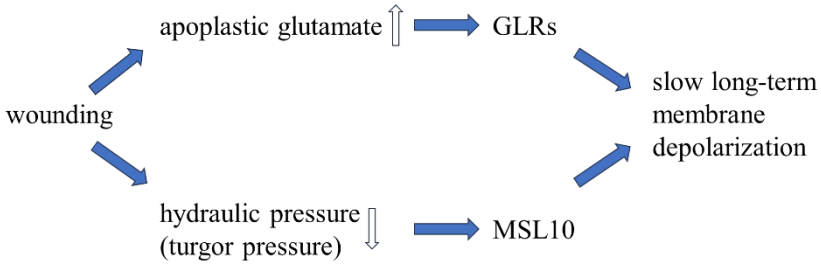

Figure 1. Wounding (chewing damage) – induced responses within the first minutes after infestation. Chewing damage induces a decrease in hydraulic pressure (turgor pressure) and an increase in apoplastic amino acids including the representative glutamate (Glu). These two main changes are perceived by mechanosensitive ion channels on plasma membrane (e.g., MSL10) and the glutamate receptor-like cation channels (GLRs including GLR3.1, GLR3.2, GLR3,3 and GLR3.6), respectively, leading to a slow long-term membrane depolarization. Open arrows indicate level changes and solid arrows indicate pathway directions.

Detection of electrical signals in long-distance communication in plants

Plants have developed sophisticated mechanisms to detect and respond to insect attacks. One such mechanism is the production of electrical signals that can be detected both intracellularly and extracellularly. Both intracellular and extracellular detection of plant electrical signals provide valuable information about the plant response to the attack.

Intracellular recording: The first step in intracellular detection of plant electrical signals is to prepare the plant tissue for electrode insertion. This typically involves removing the outer layers of the plant tissue to expose the cells of interests. Once the tissue has been prepared, the microelectrode is inserted into the cell or group of cells to be measured. The electrode is connected to an amplifier and recording device, which allows for the measurement and analysis of the electrical signal [ZHAO & al. 2013]. When an insect attacks a plant, it can cause the plant cell membrane to depolarize, leading to an influx of calcium ions and the production of electrical signals. This depolarization can be measured as a change in electrical potential between the inside and outside of the cell [ZIMMERMANN & al. 2016]. The microelectrode is able to detect these changes in potential and generate an electrical signal that is amplified and recorded for further analysis. Intracellular detection of plant electrical signals allows for the measurement of changes in membrane potential at a high spatial resolution; however, it requires invasive procedures and may damage the plant tissue [LI & al. 2021]. Intracellular detection also involves the use of voltage-sensitive dyes, which are fluorescent molecules that can be incorporated into the cell membrane and detect changes in membrane potential [MATAMALA & al. 2021]. These signals can also be detected by imaging the fluorescence of the voltage-sensitive dyes. Another method of intracellular detection involves the use of patch clamp electrophysiology [LI & al. 2021]. This technique involves the use of glass pipette to create a seal on the surface of a plant cell membrane. By controlling the voltage applied across the membrane, ion currents can be measured, providing information about the membrane potential and ion channel activity.

Extracellular recording: Extracellular detection involves the use of microelectrodes or non-invasive methods such as surface potential measurements that can detect the electrical signals produced by the plant during an insect attack [FOTOUHI & al. 2022]. This method involves the use of microelectrodes or surface electrodes placed on the surface of plant tissues. These electrodes are typically made of metal or glass and are small enough to be placed on the surface of the plant tissues without causing significant damage [LI & al. 2021].

During the attack, plant cells release ions and other charged molecules into the extracellular space, resulting in a change in the electrical potential of the surrounding tissue. This change in potential can be detected by the electrodes, which convert the electrical signal into a measurable voltage that can be recorded and analyzed [BRUCE & PICKETT, 2007]. One common method of extracellular detection is to use a glass microelectrode filled with a conductive solution such as saline or KCl. The tip of the electrode is placed on the surface of the plant tissue, and a reference electrode is placed in a nearby location [LI & al. 2021]. The voltage difference between the two electrodes is then measured using an amplifier and recorded by a data acquisition system. Another method of extracellular detection involves the use of surface electrodes, which are placed on the surface of the plant tissue and can detect changes in the electric field surrounding the tissue [LI & al. 2021]. This method is less invasive than microelectrode techniques and allows for the detection of electrical signals over a larger area.

A recent summary of common techniques for the detection of plant electrical signals at various ranges can be found in LI & al. (2021), including metal electrode, glass microelectrode, electrical penetration graph/aphid technique, voltage clamp, patch clamp, and self-reference ion-selective electrode technology (SIET)/microelectrode ion flux estimation (MIFE).

Initial and systemic response of plants following insect attack

When a plant is attacked by insects, it undergoes a series of complex defense responses to protect itself from further damage. These responses can be divided into two categories: local responses that occur at the site of insect attack and systemic responses that occur throughout the entire plant [MOSTAFA & al. 2022].

Locally, the first response is usually physical; however, this will not be further explained here. If the insect is successful in penetrating the plant's tissues, it causes local damage to the cells [MOSTAFA & al. 2022]. This damage triggers electrical signals in the form of VP or SWP at the wounded site. These electrical signals will trigger the opening of ion channels in the damaged cells which allows for the influx of calcium ions [VODENEEV & al. 2015]. The influx of calcium ions will stimulate the production of ROS and activates mitogen-activated protein kinase (MAPK) cascades [MOSTAFA & al. 2022]. This leads to the induction of defense-related genes and synthesis of defense-related compounds [WANG & al. 2013]. In this long-distance signaling mechanism, wounded leaves synthesize prosystemin in phloem parenchyma cells, and the prosystemin is proteolytically processed to systemin. Systemin is released from phloem parenchyma cells and binds to receptors on the plasma membrane of adjacent companion cells. This binding activates a signaling cascade involving phospholipase A2 (PLA2) and MAP kinases, which results in the biosynthesis of jasmonic acid (JA). JA is then transported via sieve elements to unwounded leaves. There, JA initiates a signaling pathway in target mesophyll cells, resulting in the expression of genes that encode protease inhibitors. Plasmodesmata facilitate the spread of the signal at various steps in the pathway [ERB & REYMOND, 2019; MOSTAFA & al. 2022].

In addition, SWPs can also activate other defense-related genes, including those involved in the biosynthesis of secondary metabolites such as alkaloids, terpenoids, and phenolics [DIVEKAR & al. 2022]. These metabolites can have toxic or deterrent effects on insects, providing further protection to the plant.

Hormone changes in *Arabidopsis thaliana* upon electrical signaling after insect attack

Studies have shown that electrical signals triggered by herbivore attacks can activate several different hormone signaling pathways in plants, such as JA, salicylic acid (SA), and abscisic acid (ABA).

The JA pathway is particularly relevant in plant defense against herbivores, as it plays a key role in regulating the production of defense compounds such as protease inhibitors, which can deter herbivores from feeding on the plant [HOWE & JANDER, 2008]. Electrical signals triggered by herbivore attacks have been shown to upregulate genes involved in JA biosynthesis and signaling as previously mentioned above, suggesting that electrical signals can activate this pathway to aid the plant defend against herbivores. In more detailed explanation, the influx of calcium ions can activate the JA signaling pathway by inducing the expression of JA biosynthesis genes and JA-responsive genes [MOUSAVI & al. 2013; FROMM & LAUTNER, 2007].

Arabidopsis mutants deficient in SA biosynthesis (*sid2-1*) or signaling (*npr1*) were shown to be more resistant to *S. littoralis* and *Bemisia tabaci* [BODENHAUSEN & REYMOND, 2007; ZARATE & al. 2007]. The SA pathway antagonizes JA signaling and can therefore act as a negative regulator of JA-dependent defenses in plants [PIETERSE & al. 2012]. However, more evidence is needed to support that electrical signaling leads to a rapid systemic accumulation of SA [KLOTH & DICKE, 2022].

Although the role of ABA in plant defense against herbivores is less understood, some studies have suggested that electrical signals can also activate the ABA pathway [FROMM & LAUTNER, 2007]. The expression of *Arabidopsis* chloroplast-localized glycerolipid A1 lipases *PLIP2* and *PLIP3* was induced by ABA and leads to JA accumulation and the work seemed to indicate a mechanistic link between ABA accumulation and downstream JA-defense responses [WANG & al. 2018]. It was also found that pea aphid performance is decreased on the ABA biosynthesis mutant *aba1-1* in *Arabidopsis* [HILLWIG & al. 2016].

Changes in gene expression in *Arabidopsis thaliana* upon electrical signaling after insect attack

At the remote site, signaling pathways activated by electrical signal can lead to changes in gene expression through the activation or repression of transcription factors that bind to certain sequences of DNA and modulate the expression of genes of interest. The activation or repression of transcription factors is typically mediated through post-translational modifications, such as phosphorylation, methylation, or acetylation, which can alter their activity or stability. For example, the calcium signaling pathway can activate a calcium-dependent protein kinase (CPK) that phosphorylates and activates a transcription factor called MYC2, which in turn activates the expression of genes involved in defense against herbivores, such as proteinase inhibitors and polyphenol oxidases [KAZAN & MANNERS, 2013; WASTERNACK & HAUSE, 2013].

Similarly, JA pathway can activate a transcription factor called MYC2 or MYC3, which binds to specific DNA sequences located in the promoter regions of the genes of interest and activate their expression. The SA pathway, on the other hand, can activate a transcription factor called NPE1, which is translocated to the nucleus, activating the expression of pathogenesis-related (PR) genes by interacting with other transcription factors [KAZAN & MANNERS, 2013; FU & DONG, 2013]. In addition, the ethylene pathways can also repress the expression of genes involved in growth and development, which can help the plant allocate resources towards defense [HEIL & TON, 2008]. Here, we highlight some well-studied genes.

Glutamate receptor-like (GLR) genes

Electrical signals triggered by insect herbivory have been shown to regulate the expression of glutamate receptor-like (GLR) genes in *Arabidopsis thaliana.* GLR genes encode for ion channels that have a structural similarity to ionotropic glutamate receptors in mammals. These genes play a crucial role in mediating the electrical signal propagation within plants, which is essential for the systemic communication between different parts of the plant in response to insect attack [TOYOTA & al. 2018].

Several studies found that GLR3.3 and GLR3.6 were upregulated in systemic leaves of *Arabidopsis* plant after local leaf wounding by herbivory, and this upregulation was dependent on the electrical signal triggered by the wounding. The upregulation of these genes led to increased JA levels in the systemic leaves, which is a key signal for plant defense responses. This study suggests that GLRs play a role in systemic defense signaling in plants [MOUSAVI & al. 2013; XUE & al. 2022].

Lipoxygenase 2 (LOX2)

This gene encodes a lipoxygenase enzyme that catalyzes the synthesis of JA. LOX2 expression is upregulated by electrical signaling triggered by herbivory, and its products are involved in the regulation of several defense-related genes, including those encoding proteinase inhibitors and threonine deaminase. LOX2 has been observed to play a role in the activation of JA-mediated defense responses in *Arabidopsis thaliana* [VISWANATH & al. 2020].

Pathogenesis related protein-1 (PR-1)

This gene encodes a pathogenesis-related protein that is induced by the SA pathway and is a marker of systemic acquired resistance (SAR). PR-1 expression is also upregulated by electrical signaling triggered by herbivory, indicating a potential crosstalk between SA and JA pathways [BRICCHI & al. 2012].

WRKY70 transcription factor

This gene is associated in the regulation of JA pathway and is induced by electrical signaling triggered by herbivory. Activation of WRKY70 leads to the upregulation of several defense-related genes, including proteinase inhibitors and polyphenol oxidase (POX). WRKY70 has also been shown to negatively regulate defense pathways [CHAKRABORTY & al. 2020].

Omics approaches

In various study systems, the dominant biological functions activated by herbivory are suggested to be responses to biotic and abiotic stress, production and response to ROS, calcium signaling, cell wall modification, secondary metabolism, hormone metabolism, and transcriptional regulation [KUŚNIERCZYK & al. 2008; REYMOND & al. 2004]. While considerable progress has been made, there is a growing need to better understand how the cascade components function in modules, complexes and signaling networks.

Omics approaches were taken in an investigation of the impact of aphid feeding on gene expression and epigenetic control in *Arabidopsis* plants [ANNACONDIA & al. 2021]. They found that aphid feeding induced changes in gene expression in *Arabidopsis* plants. These changes were visualized using volcano plot, which showed genes that were significantly upregulated. They also found that the upregulated genes were mainly associated with the defense response in the plant. This was confirmed by analyzing gene ontology (GO) terms and categories, which showed that, many of the genes that are upregulated were associated in biological processes related to defense responses [ANNACONDIA & al. 2021].

In addition, they also found that aphid feeding caused a relaxation of epigenetic control in *Arabidopsis* plants. This was shown by the upregulation of a single epigenetic component during aphid feeding. Furthermore, various transcription factors were significantly upregulated during aphid infestation. This suggests that they may play a role in regulating the plant's response to aphid feeding. Lastly, their study found that aphid feeding caused changes in chromatin accessibility. They identified the significant overexpression of a single component of the epigenetic regulatory pathways that was overexpressed under aphid attack, HIKESHI-LIKE PROTEIN1 (HLP1), a promoter binding protein that promotes chromatin acetylation [SHARMA & al. 2019]. Overall, their study suggests that aphid feeding induces changes in gene expression and epigenetic control in *Arabidopsis* plants, which in turn lead to the activation of the defense response [ANNACONDIA & al. 2021]. Clearly, network-wide approaches enable identification of groups of closely associated proteins with common biological functions, and

to further understand the regulation of these signaling components in cellular and physiological contexts. For example, transcriptomic approaches have been used in the analysis of the landscape of herbivore oviposition in *Arabidopsis* and have revealed considerable novel and potential components in the signaling process [OJEDA-MARTINEZ & al. 2022]. It is thus highly possible that omics analysis dedicated to electric signaling may have a significant potential to the further understanding of the response mechanisms upon herbivore attacks.

Conclusions and perspectives

In conclusion, the findings of this research have important implications for understanding plant-herbivore interactions and for developing new strategies for plant protection in agriculture and natural ecosystems. This research demonstrates that electrical signals play an important role in the physiological and biochemical responses of *Arabidopsis thaliana* during herbivore attacks. Furthermore, this research highlights the complexity and dynamic nature of plant defense responses and the role of electrical signals in shaping these responses. It is hoped that our work will further elucidate the function of electrical signals in herbivore-induced systemic response of plants. Future studies in this area could focus on elucidating the specific mechanisms by which electrical signals modulate gene expression and metabolic pathways in response to herbivore damage. For example, researchers could explore the roles of specific ion channels, signaling molecules, and transcription factors in mediating the effects of electrical signaling on plant metabolism and defense. Additionally, researchers could investigate the impact of electrical signals on the expression of epigenetic marks, such as DNA methylation and histone modifications, which can modulate gene expression and metabolic pathways in response to environmental stimuli.

Furthermore, future studies could also investigate the potential of electrical signaling in enhancing plant resistance to herbivores in agriculture and horticulture. For example, researchers could explore the efficacy of using electrical stimulation as a means of priming plants to respond more robustly to herbivore attacks or using electrical signaling to trigger the production of natural pesticides. Overall, the insights gained from this study have important implications for our understanding of the complex mechanisms underlying these effects and to explore their potential applications in sustainable agriculture and environmental management. The potential for using electrical signaling as a tool for enhancing plant resistance to herbivores has exciting implications for sustainable agriculture and could lead to novel strategies for managing pests in crop systems.

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THE EFFECTS OF ACID MIST ENVIRONMENT ON PLANT GROWTH: A REVIEW

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Abstract: The wet (rain, snow, fog, sleet, dew) and dry (transport of aerosol, particles and gases) deposition of acidic substance in environment results due to human, automobile, fossil fuel burning and industrial activities. Acid deposition is worldwide environmental degradation problems and in recent years these acidic materials are increasing at alarming scale in the environment both in developed and developing countries, including Pakistan. Some scientific literature survey reports suggest that plant growth and agriculture yield decrease due to consequence of acid rain. In addition, acid rain is found responsible for producing toxic effects on the morphological parameters of agricultural crop. The evidence collected from last more than fifty years showed the common significant effects of acid rain on seed germination percentage, seedling height, root hair and structure, alteration in leaf anatomy, size and area, stomatal structure, size, pollen germination, photosynthetic pigments and physiological changes in herbs, shrubs and trees. Still, little is known on the impact of acid rain on plant growth. This study was aimed to review the effects of acid mist on growth performances of some selected plant species. This review is contributed with the help of literature survey, research work published on the impact of acid rain on the plant growth.

Key words: fossil fuel, leaf damage, mineral nutrition, root, seed germination, shoot, water potential, yield.

Introduction

The rapid growth of economic development, industrial and automobile activities has given rise to many common ecological and environmental pollution problems [SANKA & al. 1995; IQBAL & SHAFIQ, 2006; SHAFIQ & IQBAL, 2012; LIU & al. 2016; QIAO & al. 2018; SHAFIQ & al. 2019; IQBAL & al. 2023]. Sources of pollution depends on specific industrial activities, anthropogenic emission due to fuel combustion, geographical, geological, environmental contamination, coal combustion, climatic and sociological conditions which ones alone or in combination influence all parts of the environments. The pollutants likewise sulfur and nitrogen oxides are chemically converted in the atmosphere to form strong acids (H_2SO_4 and HNO_3) and this chemical reaction in the presence of moisture formed acid rain and increase of acidity in the environment decrease the level of alkalinity. Therefore, sulfuric and nitric acids can form and fall as acid rains [JALALI & NADERI, 2012]. The pH value of a substance determines its acidity or alkalinity and is measured on a scale of 0.0 to 14.0. The pH values less than 7.0 acidic, more than 7.0 basic, pure water has a pH of 7.0 and making it neutral [CBEF, 2013]. There are tenfold differences between each unit recorded. The pH 6 is ten times more acidic than pH 7, pH 5 is 100 times more acidic than pH 7 [GRANAT, 1972; LIKENS & al. 1972]. The pH of acid rain usually ranged about 3.0 to 5.5 [REIQUAM, 1970; DAI & al. 2013]. The effects of acid rain on soil acidification, calcium nutrition, tree growth, environmental disaster, ecological system and forest health also reported [CAP, 1993; SVERDRUP & al. 1994; DeHAYES & al. 1999; LARSSEN & al. 2006; XU & al. 2015; GUO & al. 2016; DEBNATH

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& al. 2018; BARTELS & al. 2019; LIU & al. 2019]. The maximum sulfur dioxide concentrations, exceed WHO guideline in some areas of Pakistan [UNEP, 1992]. Acid rain in 2018 affected an area of 530,000 km^2 in China [Ministry of Ecology and Environment of the People's Republic of China, 2018]. Acid rain availability produces harmful impact on herbs, shrub and trees. In present review the variance between pH levels 6.0 to 2.0 indicates that seed germination, seedling growth, root system, plant dry weight, pollen germination and photosynthetic activities significantly behaved differently. JU & al. (2017) stated that the precipitations with pH values lower than 5.6 as acid rain and contribute to several key environmental issues, including acidification of soils and waters, leaf injury and forest decline, loss of biodiversity, and damage of buildings and metal materials. In the natural environment, soil pH has an enormous influence on soil biogeochemical processes. Soil pH that influences myriads of soil biological, chemical, and physical properties and processes that affect plant growth and biomass yield. The soil pH interlinked with the biological, geological, and chemical aspects of the soil environment as well as how these processes, through anthropogenic interventions, induce changes in soil pH [NEINA, 2019]. Acid rain describes any form of precipitation that contains high levels of nitric and sulfuric acid. It can occur in the form of snow, fog, and tiny bits of dry matter that settled on earth. Normal rain is slightly acidic with pH of 5.6 and acid rain generally range between pH 4.2 and 4.4 [NUNEZ, 2019].

The plants can be considered as the biggest victim of acid rain pollution in terrestrial ecosystem [RAMLALL & al. 2015]. It was found that simulated acid rain stress induced changes in root system, root morphology, yield and shoot: root ratio of seedlings; climate change, invertebrates, microorganism and soil respiration for nutrient uptake in forest [ERICSSON, 1995; KUPERMAN & EDWARDS, 1997; KUKI & al. 2008; REIS & al. 2012; LIANG & WANG, 2013; EL-MALLAKH & al. 2014; LIANG & al. 2015; LIANG & al. 2018; LIANG & al. 2020]. The impact of acid rain on pollen germination in corn, foliar nutrient concentrations for sugar maple, foliar injury and on the biogeochemical cycles of red spruce ecosystems noted [NEUFELD & al. 1985; LEITH & al. 1989; SHERMAN & FAHEY, 1994; HOGAN, 1998]. The disturbances in the chemical climate of earth, enzymatic and non-enzymatic antioxidant activities, ecosystem may also decrease in the pH and an increase in foliar leaching losses [COWLING, 1983; DU & al. 2020]. The possible impact of atmospheric acid deposition on leaf litters, tree leaves, root phenotypes and tree growth noted [SOLBERG & al. 2004; WANG & al. 2010; TOMAŠEVIĆ & al. 2011; SUN & al. 2013; BARTELS & al. 2019]. An important factor governing germination is the pH [HORA & BAKER, 1972]. Acid rain toxicity is deleterious to plant growth. Normally, rainfall is slightly acid, but its pH value lower than 5.6 induced high effects of on soil pH, soil microbial community, leaf injury, root, sapling and woody tree growth [ZHANG & al. 1996; OUYANG & al. 2008; PIETRI & BROOKES, 2008; MEENA, 2013; WANG & al. 2014]. Effects of simulated acid rain on the mineral nutrition, foliar pigments, biochemical attributes and photosynthetic rates of sugar maple, white spruce and wheat seedlings recorded [DIXON & KUJA, 1995; DOLATABADIAN & al. 2013]. Acidic deposition and inputs affected forest in northeastern US [DRISCOLL & al. 2001; 2003].

Acid rain pollution studies are a matter of utmost concern. Great concern has been expressed, in developed and developing countries about the toxicity role of acid rain on the immediate environment. The occurrence of incased precipitation acidity over wide areas of the city raises serious question, as it can effects on growth and vigor of plant species. This effort of research review work was carried out with the aim to highlight and understand the different types of effects of acidified rain on plant growth, soil and environmental with the help of available scientific literature covering 1970-2023. The searching was done using large database from different web sites, scientific journals, google, google scholar, scientific journals, PubMed, Hindawi, Sciencealert.net and Science Direct.

Effects of acid mist on seed germination and seedling growth of plant

The effects of acid rain on seed germination and seedling growth of different plant species is provided in Table 1. The interpretation of results showed a wide range of sensitivities of seed germination to acidic substrate conditions (pH 4.0, 3.0, 2.4) exists among five tree species characteristic (*Acer saccharum* L. Sapindaceae, *Acer rubrum* L. Sapindaceae, *Betula lutea* Britton Betulaceae, *Tsuga canadensis* (L.) Carrière Pinaceae and *Pinus strobus* L. Pinaceae of Adirondack mixed hardwood conifer forests [RAYNAL & al. 1982]. The rate of seed germination of Balsam fir and yellow birch showed significantly greater germination at pH 3 than at pH 4 or 5 [SCHERBATSKOY & al. 1987]. The acid rain treatment of *Vicia faba* L. cv. 'Con Amore', grown either in soil or quartz gravel in eight open top chambers to two levels of SO_2 (charcoal-filtered air and charcoal filtered air enriched with SO_2) and two artificial rain treatments (pH 5.6 and pH 3.0/4.0), alone or in combination resulted in a decrease of fresh and dry weight of whole plants, leaves, stalks, fruits and roots; number of leaves, stalks, blossoms, pods and seeds; leaf area; plant height; sulphur content total fresh and dry weight and fruit production of plants grown in soil, while, particularly at the beginning of the rain treatments, dry weight of whole potted plants and leaves as well as the number of leaves of plants grown in quartz gravel decreased [ADAROS & al. 1988].

A variable response of two years old red spruce (*Picea rubens* Sarg.) seedlings growth and foliar injury to varying pH acidity value (2.5-3.5) in concentrations of sulfur and nitrogen was observed [JACOBSON & al. 1990]. In a study, seeds and seedlings of five hardwood species were subjected to a simulated acid rain 2.0, 3.5, 5.0, 6.0 pH, and to distilled water (the control). Seed germination was remarkably inhibited by pH 2.0 treatment for three hardwood species while seedling growth was stimulated at pH levels between 3.5 and 5.0. The inhibition of seed germination and seedling growth for all the treated hardwood species was recorded by pH 2.0 treatment [FAN & WANG, 2000]. SINGH & AGRAWAL (2004) reports the effect of simulated acid rain of different pH 5.6 (control), 5.0, 4.5, 4.0 and 3.0 on two cultivars of wheat (*Triticum aestivum*, Malviya 213(M213) and Sonalika). Shoot and root lengths significantly declined at pH 3.0 in both varieties. Leaf area declined at pH 4.0 and 3.0 in M213 at both ages and at 75 days in Sonalika. Total biomass of 75 days plants declined significantly at pH range 4.5-3.0 in M213 and at pH 4.0 and 3.0 in Sonalika and concluded that acid rain has a significant negative effect on wheat plant performance.

LIU & al. (2011) reported the different effects of calcium on seed germination, seedling growth and photosynthesis of six forest tree species under simulated acid rain. The seed germination percentage, germination index of rice and wheat was absolutely inhibited with simulated acid rain stress at pH 2.0. Furthermore, rice and wheat seeds germinated abnormally at pH 2.5. An inhibition index of shoot and root length of rice, wheat and rape seeds decreased with increased pH values [ZENG & al. 2005]. Such types of studies are helpful in understanding the susceptibility of tree species to acid precipitation. Growth of five weeks old white ash (*Fraxinus americana*) was found the greatest for seedlings treated with pH 4.3 and the least for those treated with pH 5.6 or 3.0 simulated rain under controlled environmental conditions. Significant linear decreases in root dry weight, and root/shoot ratio occurred with increasing rain acidity [CHAPPELKA & CHEVONE, 2011]. Similar types of the effects of simulated acid rain (pH 2.5, 3.5,4.5 and 5.6) on the seedling growth of *Jatropha curcas* L. was recorded by [SHU & al. 2019]. The effect of varying simulated acid rain solutions treatment, one each at pH 5.6, 4.5, 3.5 and 2.5, on the growth of two crop plants, brinjal (*Solanum melongena* Linn.) and cowpea (*Vigna unguiculata* ssp. *cylindrica* (L.) Walpers was assessed [ARORA & al. 2022]. This study revealed that decrease in pH to 2.5 adversely affected almost all the growth parameters in brinjal. In case of cowpea, however, this depression was quite discernible even at pH 3.5.

Table 1. Effects of acid mist on seed germination and seedling growth of plant

Name of plant species	pH range 2.0-6.0	Symptoms	Reference
Betula alleghaniensis Britt. – Betulaceae	2.3	Seedling growth decreased	WOOD & BORMANN, 1974
Acer rubrum L. – Sapindaceae *Betula lutea* Britton – Betulaceae *Pinus strobus* L. – Pinaceae	4.0 3.0 3.0-2.4	Inhibition Inhibition Stimulation	RAYNAL & al. 1982
Balsam fir and yellow birch	3 than at pH 4 or 5	Significantly greater germination at pH 3 than at pH 4 or 5	SCHERBATSKOY & al. 1987
Vicia faba L. cv. 'Con Amore'	5.6 and 3.0/4.0)	Decrease of fresh and dry weight, stalks, fruits and roots; number of leaves, stalks, blossoms, pods and seeds; leaf area; plant height; sulphur content, fruit production, and leaves as well as the number of leaves of plants	ADAROS & al. 1988
Pinus taeda L.	5.30, 4.0 -	Seedling height and diameter growth decrease	EDWARDS & al. 1990
Acer accharum Marsh. *Picea glauca* (Moench) Voss	3.2	Decreased seedling height	DIXON & KUJA, 1995
Clitorea ternatea L. *Senna holosericea* (Fresen.) Greuter. *Adenanthera pavonina* L. *Senra incana Cav.*	4.0, 3.0	Seed germination and early seedling growth decreased Root/shoot inhibited	SHAUKAT & SHAFIQ, 1998
a=Cinnamomum camphora L. – Lauraceae *b=Castanopsis fissa* Rehd. et Wils. – Fagaceae *c=Koelreuteria bipinnata* Franch. – Sapindaceae	2.0	a=reduction [51.09%] b=reduction [76.61%] c=reduction [56.32%]	MUNZUROGLU & WANG, 2000 – China
Cinnamomum camphora L. *Castanopsis fissa* Rehd. et Wils. *Koelreuteria bipinnata* Franch. *Ligustrum lucidum* Ait. *Melia azedarach* L.	2.0	Seedling growth adversely decreased	FAN & WANG, 2000 – China
Two cultivars of wheat (*Triticum aestivum,* 'Malviya' 213(M213) and 'Sonalika')	5.6, 5.0, 4.5, 4.0 and 3.0	Shoot and root lengths significantly declined at pH 3.0 in both varieties. Leaf area declined at pH4.0 and 3.0 in M213. Total biomass declined significantly at pH range4.5–3.0 in M213 and at pH 4.0 and 3.0 in Sonalika	SINGH & AGRAWAL, 2004
Rice and wheat	2	Seedling inhibition	ZENG & al. 2005
Tomato	2.5	inhibition of growth	DEBNATH & al. 2018
Jatropha curcas L. – Euphorbiaceae	4.50 (+)	Seedling growth stimulated	SHU & al. 2019 – China
Two crop plants, brinjal (*Solanum melongena* Linn.) and cowpea (*Vigna unguiculata* ssp. *cylindrica* (L.) Walpers	5.6, 4.5, 3.5, 2.5	2.5 adversely affected almost all the growth parameters in brinjal. In case of cowpea, this depression was quite discernible even at pH 3.5.	ARORA & al. 2022

Effects of different concentrations of acid mist pH (T1-2.82, T2-3.45, T3-4.46, T4-5.55) on root, shoot, seedling height and seedling dry weight of *Albizia lebbeck*

The shoot growth of *Albizia lebbeck* (L.) Benth. at pH 4.46 was found promotory. A sharp decline in shoot growth of *A. lebbeck* was noticed in pH 5.55 and 3.45 followed by pH 2.82 and 4.46 treatment, respectively (Table 2). The maximum reduction in shoot growth of *A. lebbeck* at 5.5 pH was recorded. The acid rain not only affects the aerial parts of plant but also degrade the fertility of soil and increases the vulnerability of plants to toxic metals [DU & al. 2017].

Table 2. Effects of different concentrations of acid mist pH on seedling growth and dry weight of *Albizia lebbeck*

Treatments	Root length (cm)	Shoot length (cm)	Plant height (cm)	Seedling dry weight (g)
T1	16.00	12.20	28.10	2.856
T2	20.00	11.70	31.70	2.992
T3	15.40	11.70	27.10	2.878
T4	14.10	10.50	24.60	2.308
L.S.D. P<0.05	9.32	2.28	10.64	1.421
Source: IQBAL & SHAFIQ (2023) – Pakistan				

The effects of acid mist on leaf growth, anatomy and stomata of plant species

The relative sensitivities of foliage of foliage of several clones of *Tradescantia* sp., *Pteridium aquilinum*, *Quercus palustris*, and *Glycine max* to acid rain, and leaf surface and anatomical alterations to simulated acid rain at pH 5.7, 3.4, 3.1, 2.9, 2.7, 2.5, and 2.3 levels was investigated [EVANS & CURRY, 1979]. Sporophyte leaves of bracken fern (*P. aquilinum*) were most sensitive to simulated acid rain among the species tested. About 10% of the surface area of older leaves of *P. aquilinum* was injured after exposure to 10 rainfalls at pH 2.5 (a single 20-min rainfall daily). The gall formation that resulted from both cell hypertrophy and hyperplasia occurred in lesions of *Tradescantia*, and *Q. Palustris* [EVANS & CURRY, 1979]. In general, it was concluded that the tested plant species that show cell hyperplasia and hypertrophy of leaf tissues after exposure to simulated acid rain.

Acid rain can negatively impact on micromorphology, leaf function and anatomy of plant health [SILVA & al. 2005; SANT'ANNA-SANTOS & al. 2006; TONG & al. 2014; WU & LIANG, 2017; MA & al. 2021] and suppresses leaf function and mesophyll cell (Table 3). The more acute injury of acid rain to plant foliage includes variation in stomatal conductance [DONG & al. 2017]. Acid rain can affect the structure of plant leaves, destroy the cuticle, and leaves, cause leaves to lose a large amount of nutrients likewise potassium, calcium, and magnesium and cell building [SINGH & AGRAWAL, 2007; HU & al. 2019]. The treatment of pH 4.5 (H_2SO_4) altered the micro morphological changes in youngest leaves, wilting of epidermal common cells and stomatal guard cells of *Joannesia princeps* [ANDRADE & al. 2020]. The visible leaf damage and anatomical alterations in two urban trees, *Liquidambar styraciflua* L. and *Fraxinus uhdei* (Wenz.) Lingelsh growing in Mexico City with sulfuric acid solutions at pH 2.5 and 3.8 reported [RODRÍGUEZ-SÁNCHEZ & al. 2020].

As an important edible part of leafy vegetables, the leaf blade is also one of the more sensitive plant parts to environmental stresses [XIONG & al. 2016; YANG & al. 2018; GAO & al. 2020]. The extent and magnitude of acid rain in Vietnam and other Asian countries have become more apparent since over the past decade. In this study, the effect of simulated acid rain (pH 5.0, 4.0, and 3.0) and control treatment (pH 6.0) are observed for three species *Brassica*

integrifolia, *B. rapa*, and *B. juncea* in Hanoi. In pot experiment results showed that acid rain causes direct damage to leaves. Observations also revealed white spots on leaves; leaves getting discolored and gradually turning yellow, curling leaf marginal, and turning dark blue, with the most severe symptoms being necrotic leaves. Parameters of the shoot and root length, leaf area, biomass, and chlorophyll content all decrease as pH drops. In conclusion, *B. rapa* showed the highest resistance capability to acid rain compared with *B. integrifolia* and *B. juncea,* especially its proline content is the highest at pH 3.0 in three Brassicaceae species [PHAM & al. 2022].

Table 3. The effects of acid rain on leaf anatomy, damage and stomata of plant species

Name of Plant species	Acid rain pH 2.0 - 6.0, symptoms and reference		
	2.0-6.0	**Symptoms**	**Reference**
Betula alleghaniensis Britt – Betulaceae	3.0	Foliar tissue damage	WOOD & BORMANN, 1974
Pinus jeffreyi Grev. & Balf. – Pinaceae *Sequoiadendron giganteum* (Lindl.) Buchholz – Cupressaceae	3.4 2.0	Leaf chemical changes	WESTMAN & TEMPLE, 1989 – U.S.A.
Picea rubens Sarg.	3.5	Foliar injury	JACOBSON & al. 1990 – U.S.A.
Picea abies L. Karst. *Pinus sylvestris* L.	4.0 3.0	Alteration in the size of the ultrastructure of needles of mesophyll chloroplasts	BÄCK & HUTTUNEN, 1992
Both conifers and broadleaved tree seedlings	3.5	Subtle changes in the structural characteristics of leaf surfaces	CAPE, 1993
Shortleaf pine – *Pinus echinata* Mill.	5.3, 4.3, 3.3	Leaf area affected	SHELBURNE & al. 1993
Red spruce – *Picea rubens* Sarg.	3.0	Impaired stomatal functions, including a smaller maximum aperture, slower closure and an increased lag time between stomatal closure. Delayed stomatal closure	BORER & al. 2005
Liquidambar formosana *Schima superba*	3.0	Leaf necrosis	CHEN & al. 2013
Liquidambar styraciflua L. – Altingiaceae	2.5, 3.8	Cuticle alterations	RODRÍGUEZ-SÁNCHEZ & al. 2020 – Mexico
Fraxinus uhdei (Wenz.) Lingelsh. – Oleaceae	2.5, 3.8	Visible leaf damage, anatomical alterations	RODRÍGUEZ-SÁNCHEZ & al. 2020 – Mexico
Joannesia princeps Vell – Euphorbiaceae	4.5	Wilting epidermal and stomata guard cell	ANDRADE & al. 2020 – Brazil
Pak choi (*Brassica rapa* subsp. *chinensis*)	3.5	Growth retardation and leaf yellowing	ZHA & al. 2022
Brassica integrifolia *Brassica rapa* *Brassica juncea* in Hanoi	3.0	white spots on leaves; leaves getting discolored and gradually turning yellow, curling leaf marginal, turning dark blue, severe symptoms being necrotic leaves	PHAM & al. 2022

The effects of acid rain on root system (root phenotypes, growth, mineral content) of plant species

Root systems provide mechanical support and helps in nutrient uptakes and the addition of acid rain usually damage the plant root growth (Table 4). The diameter growth of white oak were significantly decreased to rains of pH 3.6 [WALKER & McLAUGHLIN, 1991]. Acid rain threatens the development of plant roots [HUANG & al. 2000; LIU & al. 2018b]. The research work of HUANG & al. (2019) shows that acid rain increases the accumulation of reactive oxygen species and inhibits roots growth and root system development of white oak (*Quercus alba* L.) and loblolly pine (*Pinus taeda* L.) were examined. The effects of topsoil heavy metal pollution (3,000 mg kg^{-1} Zn, 640 mg kg^{-1} Cu, 90 mg kg^{-1} Pb and 10 mg kg^{-1} Cd) and (synthetic) acid rain (pH 3.5) on tree growth and water use efficiency of young forest ecosystems consisting of Norway spruce (*Picea abies*), willow (*Salix viminalis*), poplar (*Populus tremula*) and birch (*Betula pendula*) trees and a variety of understorey plants was investigated. The fine root mass was significantly reduced by heavy metal pollution in *P. abies*, *P. tremula* and *B. pendula*. Above and below ground growth was strongly inhibited by acidic subsoil in *S. viminalis* and to a lesser degree also in *P. abies* [MENON & al. 2007].

Table 4. The effects of acid rain on root system (root phenotypes, growth, mineral content) of plant species

Name of plant species	Acid rain pH 2.0 - 6.0, symptoms and reference		
	2.0-6.0	Symptoms	Reference
White oak (*Quercus alba* L.) and Loblolly pine (*Pinus taeda* L.)	3.6	Growth and root system development reduced	WALKER & McLAUGHLI, 1991
Soybean (*Glycine max* L.)	3.0	Root phenotype	SUN & al. 2013
Rice *Oryza sativa* L.	2.5	Root length, surface area, volume and number of tips reduced	ZHANG & al. 2016
Rice *Oryza sativa* L.	2.0	Severe reduction in root growth	JU & al. 2017
Rice *Oryza sativa* L.	4.5 3.5	Reduced morphology and growth	LIU & al. 2018
Quercus acutissima and *Cunninghamia lanceolata*	4.5 2.5	Damage root length and area	LIU & al. 2022
Pinus massoniana Lamb	4.6	primary lateral root length, root dry weight and number of root tips in seedlings exposed to simulated acid rain at pH 4.6 were higher than that of the control (pH 6.6).	ZHOU & al. 2022

The effects of acid rain on biomass of different plant species

Some other studies that assessed similar pattern of decrease in biomass production in forests and agricultural areas (Table 5). The effects of simulated acid rain, at varying pH levels of 5.7, 4.0, 3.1 and 2.7 on yields of radish, garden beet, kidney bean, and alfalfa recorded. The results showed no significant difference in the yields of radish, kidney bean, and alfalfa when treated with simulated acid rain when compared to the yields of garden beet treated with pH 5.7 simulated rain [EVANS & al. 1982]. However, the combinations of ozone (carbon-filtered (control), ambient, 1.7 x ambient, and 2.5 x ambient) and acidic precipitation (pH 5.3, 4.3 and 3.3) for 16 months (1989 harvest) or 28 months (1990 harvest) showed trend of increased in aboveground biomass in seedlings of Shortleaf pine (*Pinus echinata* Mill.) and concluded that because N concentrations in the soils generally increased with decreasing pH [SHELBURNE &

al. 1993]. The reduction in forest productivity, water quality, the availability of nutrients due to acid stress are very common [DAHL & SKRE, 1971; SHEPPARD & al. 1993; NEAL & al. 2010]. The toxic impact of simulated acid rain on growth and yield of two cultivars of wheat noted SINGH & AGRAWAL (2004). LV & al. (2014) reported the effects of sulfuric, nitric, and mixed acid rain on litter decomposition, soil microbial biomass, and enzyme activities in subtropical forests of China. It also leads to further decreases in vertical growth, stem incremental growth, and in total plant biomass [ZHANG & al. 2016; LIU & al. 2018]. The inhibitory effects of acid rain on plant growth in general, aboveground and belowground plant parts responded differently. The interactions between acid rain pH and other acid rain characteristics and experimental characteristics indicating that there were pH dependent interaction patterns [SHI & al. 2021].

Table 5. The effects of acid rain on biomass of different plant species

Name of Plant species	Acid rain pH 2.0 - 6.0		
	2.0 - 6.0	Symptoms	Reference
Pinus taeda L.	5.3, 4.0	Biomass accumulation, seedling height and diameter growth, biomass accumulation and leaf pigment concentrations of loblolly pine	EDWARDS & al. 1990
Pinus echinata Mill.	5.3, 4.3, 3.3	Biomass less	SHELBURNE & al. 1993
Vigna sinensis L. and *Phaseolus mungo* L.	4, 2	Biomass accumulation, leaf chlorophyll, net photosynthesis, and photosystem activities. The level of chlorophyll on a unit fresh weight basis showed progressive reduction upon increasing acidity of mists treatment; the reduction was due to the loss of Chl a and Chl b. The increased stomatal diffusive resistance and reduced photosynthetic pigments lowered the net photosynthetic rate.	MUTHUCHELIAN & al. 1994
Zebrina pendula	5.6, 3.5 2.5, 1.5	Biomass, relative anthocyanin concentration, chlorophyll content, nitrate reductase activity, proline content, antioxidase activity. slightly inhibited antioxidant activity. visible injury symptoms on leaves, with a sharp decline in ornamental quality.	ZHANG & al. 2014

Effects of acid rain on pollen development

Acid rain produce inhospitable environment on pollen tube elongation, germination and growth in most of the plants. The information available on the impact of acid rain on pollen germination of plants. WERTHEIM & CRAKER (1987) evaluated the properties of an acid rain episode that could influence the germination of pollen in corn (*Zea mays* L.) by treating silks with a simulated acid rain and measuring the subsequent germination of pollen on the silks. The data indicated that acid rain creates an inhospitable environment for pollen germination on the silk surface. Reduced germination appeared directly related to the acidity of the rain. Rinsing silks with a pH 5-6 rain after treatment with a pH 2-6 rain did not increase pollen germination above that on silks treated only with a pH 2-6 rain. Pollen germination on silks was inhibited even when silk tissue was exposed to a simulated rain of pH 2-6 for <1-5 min. The seed yields of corn (*Zea mays* L.) plants were significantly reduced on where the silks had been exposed to an episode of simulated acid rain at pH 3.6 as compared with yields on plants with silks exposed to simulated rain of pH 5.6. The reduction in yield appeared related to a decrease in pollen germination and tube elongation associated with acidic conditions and limited quantities of

pollen available for pollination. Germination and tube elongation of pollen were also inhibited when grown on an agar medium acidified to pH 4.6 [CRAKER & WALDRON, 1989].

In the broad leaved species, pollen germination and pollen tube elongation showed sensitivities to detergent and acidity. The presence of 1 to 3 mg 1-1 sodium dodecyl-benzensulfonate detergent, or a growth medium pH of 4.0-5.0, inhibited pollen germination and pollen tube elongation more in broad leaved trees than in conifers. Pollen germination of most broad-leaved species was completely inhibited in the presence of detergent concentrations of more than 3-5 mg l-1; the only exceptions were some entomophilous species (*Salix caprea* L.) in which the ability of the pollen to germinate in high pollutant concentrations could be related to the presence of tryphyne [PAOLETTI, 1992]. The introduction of genetic material into the pollen and the production of transformed plants produced from seed formed after fertilization with treated pollen could have a tremendous impact on the improvement of economically important crops, tobacco [SMITH & al. 1994]. The effects of simulated acid fog (SAF) and temperature on stigmatic receptivity in two birch species were performed [HUGHES & COX, 1994]. Excised reproductive branches were sampled from representative individuals of mountain paper birch (*Betula cordifolia* Regel.) and paper birch (*Betula papyrifera* Marsh.) in populations adjacent to the Bay of Fundy, New Brunswick, Canada. Since 1979 these trees have exhibited branch dieback in association with abnormal foliar browning symptoms. This browning has been linked with acidity and nitrate deposited by fog, which is frequent in the area. In general, experimental results indicated that pollen germination increased with temperature, but pH effects were less obvious. Similarly, pollen tube growth responded positively to temperature and was little affected by fog acidity. ANOVA tests indicated a significant difference ($P< 0.05$) between species in their pollen germination response only at 12 °C, and not at the other three temperatures tested. For pollen tube growth, significant differences between species ($P< 0.05$) were demonstrated at 12 and 22 °C. A significant pH effect was demonstrated at 27 °C for germination, while pH effects on tube growth were significant at 27 and 12 °C ($P< 0.01$). A response surface regression analysis indicated that acidity significantly affected pollen germination in mountain paper birch ($P<0.001$) but not in paper birch. For pollen tube growth, however, temperature was more important than pH and produced highly significant effects in both species ($P<0.001$). Acidity was also a significant factor in pollen tube growth for paper birch. Effects of simulated acid precipitation (pH 5.6, 3.6, 2.6) on pollination in *Oenothera parviflora* L. from different populations were examined both *in vitro* and *in vivo*. The response of pollen *in vitro* indicated significant inhibitory effects of pH, and demonstrated that pH values ≤ 3.6 were inhibitory to both germination and tube growth, when compared with the treatment of pH 5.6. Dosages of LD50 for *in vitro* pollen germination, taken as the initial pH of cultures for the different pollens, ranged from pH 3.49 to 3.72. Stigma germination and initial tube growth on the stigmatic surface also declined significantly ($P<0.01$) in response to acid rain simulation prior to hand pollinations. Again simulants $\leq$ pH 3.6 significantly reduced stigma receptivity compared with the treatment at pH 5.6 [COX, 1984].

The effects of acid rain were observed on the development of anther and pollen grain in *Phaseolus vulgaris* L. (Table 6). The plants were irrigated with distilled water (pH 6.8) before treatments and considered as the control. Plants were treated by HNO_3 solution pH 4.5, 4, 3 and 2 separately. Plants were treated by mixed solutions of HNO_3 and H_2SO_4 pH 4.5, 4, 3, 2 separately, applying single spraying treatments. Results showed that number of pollen grains and fertile pollen were decreased in plants that treated by acid solutions. Pollen development was taking as other dicotyledonous plants. But in plants that were treated by different acidic solutions, some abnormalities were seen during pollen development. Tetrads were formed as

spherical shape in normal plants but changing of tetrad shape to polygonal form is one of the treated effects by acid solutions [CHEHREGANI & al. 2006]. Pollination is a key event for fruit set. There has been an increasing interest in acid mist impact on pollen germination. Environmental conditions such as temperature, rain and high wind speed negatively affect pollination [RAMÍREZ & DAVENPORT, 2013]. A plant biostimulant is any substance or microorganism which can be applied to plants to enhance seed germination and plant growth development along with its nutritional efficiency. Plant biostimulants collectively influence: plant growth development, pollen tube development, flower and fruit set, plant pigments, shoot and root development, nutritional efficiency, yield and shelf-life of crops, rhizospheric and soil microorganisms, general soil health and plant-environment interactions. Biostimulants are derived from natural origins and can help reduce the use of chemical products and also mitigate the negative impacts of harmful chemicals in the environment. The impacts on the shelf-life and efficiencies of commercial biostimulants, as compared to synthetic chemical products and highlights the opportunities and challenges of their market expansion [GUPTA & al. 2021].

Table 6. Effects of acid rain on pollen germination, growth and development

Name of Plant species	Acid rain pH 2.0 - 6.0		
	2.0 - 6.0	**Symptoms**	**Reference**
Corn (*Zea mays* L.)	2.6	Inhospitable environment reduced germination of pollen	WERTHEIM & CRAKER, 1987
Corn (*Zea mays* L.)	5.6, 4.6, 3.6	The reduction in yield appeared related to a decrease in pollen germination and tube elongation associated with acidic conditions and limited quantities of pollen available for pollination. Germination and tube elongation of pollen were also inhibited when grown on an agar medium acidified to pH 4.6	CRAKER & WALDRON, 1989
Broad leaved trees / conifers Conifer	5.0, 4.0 3.0-2.5	Inhibition Pollen tube elongation	PAOLETTI & al. 1992 – Italy
Malus sylvestris Miller Cv. 'Golden'	3.3, 3.4	Decreased by 41.75% Pollen tube elongation 24.30%	MUNZUROGLU & al. 2003 – Turkey
Phaseolus vulgaris L.	4.5, 4.0, 3.0, 2.0	The number, development of anther and pollen grain decreased. Tetrads were formed as spherical shape in normal plants but changing of tetrad shape to polygonal form is one of the treated effects by acid solutions	CHEHREGANI & al. 2006

The effects of acid rain on alterations and changes in photosynthetic pigments (chlorophyll a, b) in plant species

Photosynthesis is the basic metabolic process in plant growth and development, which is very sensitive to various abiotic stresses [ZHENG & al. 2009; DONG & al. 2017; LIU & al. 2022]. Acid rain found responsible for declining photosynthetic abilities [LIU & al. 2007]. It is well known that acidic precipitations are harmful for plants, in fact, they can damage the photosynthetic machinery, plant physiology, reduce the chlorophylls content and increase the production of reactive oxygen species, while at agroecosystem levels they are responsible for the crop yield losses, above and below ground plant parts [SHU & al. 2023]. Chlorophyll fluorescence characteristics and the growth response of *Elaeocarpus glabripetalus* to simulated acid rain [LIU & al. 2015]. Industrial activity has been threatening the environment for decades and this resulted in dramatic damage of forest covers in the south-west part of Poland [JABLOŃSKI & al. 2019]. This work investigates the response to simulated acid rain on

photosynthetic organs of 13 deciduous trees and 10 dicotyledonous plants (Table 7). The deleterious effects of simulated acid rain on chlorophyll contents, chlorophyll fluorescence, chlorosis, nutrient loss, enzyme activity changes in foliage of plant reported [REN & al. 2018]. Plants tolerance to stresses requires maintaining the photosynthetic apparatus [MA & al. 2019]. The application of simulated acid rains pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 to green leaves of 13 deciduous trees and 10 species of dicotyledonous plants revealed that 77% of deciduous species represented very low to intermediate photosynthetic recovery meaning that highly acid rain impacted trees will be surviving less or none [DIATTA & al. 2021]. Acid rain of pH 3.0 inhibited plant 13C assimilation and the flow of fixed 13C to the soil. And reduces the photosynthesized C sequestration of maize soil system and soil microbial taxa interactions [LIU & al. 2023]. CHEN & al. (2013) reported photosynthetic and antioxidant responses of *Liquidambar formosana* and *Schima superba* seedlings to sulfuric rich and nitric rich simulated acid rain. *Acer amplum* subsp. *catalpifolium* is a critically endangered, native deciduous broad-leaved tree species mainly distributed in the rainy zone of west China. ZHANG & al. (2021) recorded the effects of acidity levels (pH 2.5, 3.5 and 4.5) on photosynthetic performance and stress status of *A. amplum* subsp. *catalpifolium* and conclude that simulated acid rain can enhance the peak photosynthetic rate and stomatal conductance. The significant degradation of natural ecosystem, photosynthetic performance, pigment composition, soil physiochemical and microbial properties due to pollutant stress reported [YAO & al. 2016; WEI & al. 2021]. In a study about the comparison of forest susceptibility to acid stress estimated a relative growth reduction in forest productivity in Sweden and north eastern United States [JONSSON & SUNDBERG, 1972a; JONSSON & SUNDBERG, 1972b].

The influence of different acidic mists (pH 5, 4, 2) treatment on height, biomass accumulation, leaf chlorophyll, net photosynthesis, and photosystem activities in *Vigna sinensis* L. and *Phaseolus mungo* L. were investigated [MUTHUCHELIAN & al. 1994]. The level of chlorophyll on a unit fresh weight basis showed progressive reduction upon increasing acidity of mists treatment; the reduction was due to the loss of Chl a and Chl b. The increased stomatal diffusive resistance and reduced photosynthetic pigments lowered the net photosynthetic rate. However, when various photosynthetic activities were followed in isolated chloroplast, a decrease in the rates was obtained in the seedlings exposed to pH 4 and 2. The impact of soil pH (2-6.4) on seed germination rates, plant growth, chlorophyll content, and the accumulation of phenolics on invasive weed *Phytolacca americana* (pokeweed – PaU) growing in industrially contaminated (Ulsan) and noncontaminated (Suwon-PaS) sites in South Korea were measured to assess the effects of industrial pollution and global warming related stresses on plants. The highest seed germination rate and chlorophyll content occurred at pH 2.0 for both PaU and PaS plants. Increased pH from 2-5 correlated to increased phenolic compounds and decreased chlorophyll content. However, at pH 6.4, a marked decrease in phenolic compounds, was observed and chlorophyll content increased. These results suggest that although plants from Ulsan and Suwon sites are the same species, they differ in the ability to deal with various stresses [KIM & al. 2008].

Acid rain is a frequent environmental issue in southern China that causes damage to the growth and photosystems of subtropical tree species. Arbuscular mycorrhizal fungi (AMF) can improve plant tolerance to acidic conditions [WANG & al. 2021]. In this study, the inoculated *Zelkova serrata,* an important economic tree species in China, with *Rhizophagus irregularis*, and *Diversispora versiformis*, alone and in combination, under three simulated acid rain regimes (pH 2.5, 4.0, and 5.6). The results revealed that acid rain sharply reduced photosynthetic ability and total biomass of non-mycorrhizal plants. Moreover, the acid tolerance of *Z. serrata* was positively correlated with net photosynthetic rate. Acid rain has progressively

become more problematic due to increasing concentrations of atmospheric pollution, particularly in China [LY & al. 2023]. *Mirabilis jalapa* L. is an important landscaping ground cover plant with significant resistance to multiple stressors and its tolerance to acid stress was reported. In this study, the effects of acid rain on the growth and numerous physiological indexes of *M. jalapa* at different growth stages such as plant height, leaf growth, chlorophyll content, and chlorophyll fluorescence were investigated under increasingly acidic conditions of pH 5.6 (control), pH 4.0, pH 3.0, and pH 2.0. The plant height, leaf length, and leaf area of *M. jalapa* showed significantly variable results. As the simulated acid rain pH decreased, the plant height, leaf length, and leaf area showed the trend of first increasing before decreasing. In the peak at pH 4.0 treatment, the plant height, leaf length, leaf area, and chlorophyll content were significantly higher than that of the control, pH 3.0 and pH 2.0 ($P < 0.05$).

Table 7. The effects of acid rain on photosynthetic pigments (chlorophyll a, b) in plant species

Name of Plant species	Acid rain pH 2.0 - 6.0		
	2.0 - 6.0	**Symptoms**	**Reference**
Pinus taeda L.	5.3, 4.0	Leaf pigment concentrations	EDWARDS & al. 1990
Red spruce (*Picea rubens* Sarg.)	3.0	Photosynthetic decline	BORER & al. 2005.
Liquidambar formosana Schima superba	3.0	Inhibited photosynthetic, soluble protein, proline content and antioxidant enzymes activities	CHEN & al. 2013
Tomato seedlings	2.5	Inhibition of photosynthesis, severity of oxidative damage were found at pH 2.5	DEBNATH & al. 2018
Tea (*Camellia sinensis*)	3.5, 2.5	Restrict photosynthesis, antioxidant defense system, and metabolic disorder	ZHANG & al. 2020 - China
13 deciduous tree and 10 dicotyledonous plants	3.0, 3.5 4.0, 4.5 5.0, 5.5	77% of deciduous species represented very low to intermediate photosynthetic recovery	DIATTA & al. 2021
Mirabilis jalapa L.	4.0, 3.0, 2.0	There are significant differences in chlorophyll fluorescence parameters under different treatments ($P < 0.05$)	LY & al. 2023

The effects of acid rain on plant physiology (water relation), metabolic disorder, mineral nutrients, microbial activities in plant species

Acid rain alters soil carbon cycling by influencing the soil microbial community structure and functions (Table 8). Previous studies have indicated that acid rain both indirectly by inducing nutrient leaching and increasing availability of toxic heavy metals [de VRIES & al. 2015]. The influence of simulated acid rain on photosynthetic pigment, proline, malondialdehyde, antioxidant enzyme activity, total nitrogen, caffeine, catechins, and free amino acids in seedlings of Tea (*Camellia sinensis*) showed that increase in acidity increased total nitrogen, certain amino acid content (theanine, cysteine), and decreased catechin and caffeine contents, resulting in an imbalance of the carbon and nitrogen metabolisms. These results further indicated that simulated acid rain at pH 3.5 and pH 2.5 could restrict photosynthesis and the antioxidant defense system, causing metabolic disorders and ultimately affecting plant development and growth [ZHANG & al. 2020]. The response of soil microbial communities to acid rain under acid rain (pH 5.0, pH 4.0, and pH 3.0) in an agricultural soil of southern China showed that the pH 3.0 acid rain increased the total, bacterial, gram positive bacterial, and actinomycetal [LIU & al. 2021].

At the same time, these effects of acid rain impact the total biomass of microorganisms and the structural distribution of different strains [WAKELIN & al. 2008; LIU & al. 2017], resulting in changes in microbial respiration. The research results showed acid rain changes soil respiration and forest type in China [FENG & al. 2002, 2017]. LIANG & al. (2016) also found that different types of forest soil have different responses to simulated acid rain, which may be caused by the differences in the acid buffering capacity of different forest stands and the original pH value of soil and litter layers [LIANG & al. 2013]. Response of soil microbial community, seed production, soil respiration and its components in a mixed coniferous broadleaved forest to simulated acid rain in the three gorges reservoir area reported [LI & al. 2011; LI & al. 2019; LI & al. 2021]. PIGNATTELLI & al. (2021) found reduction in physiology and growth of *Lepidium sativum* due to acid rain stress. All around the world, Europe, North America and Southeast Asia, especially central and southern China are affected by acidic deposition [MENZ & SEIP, 2004]. In another study, the effects of simulated acid rain pH 3.5-2.5 on the antioxidative system in *Cinnamomum philippinense* seedlings was recorded [LIU, 2011]. Plants ability depends on meteorological conditions and geochemical characteristics [AKSELSSON & al. 2013]. In Cina, the pH value of acid rain was below 5.6 is a severe environmental issue and affecting ecosystem health since 1970's [QU & HAN, 2021].

The plant water relations control the transport and loss of water evaporation from the soil. In a study, the pressure volume curves, day and night transpiration rates, needle drying curves, and shoot water potentials were determined for 2 year old red spruce trees by exposing for the three months to a range of acid mists (pH 2.5 to pH 5.0) containing equimolar $(NH_4)_2SO_4$ and HNO_3 [EAMUS & al. 1989]. Simulated acid rain has been reported to cause physiological changes in various plant species. SMITH & al. (1990) were conducted studies in 1983, 1984 and 1985 to determine the effect of acid rain on some physiological parameters in two corn (*Zea mays* L.) hybrids. Simulated rain of pH 3.0, 4.2, and 5.6 was applied throughout the growing season onto plots protected from ambient rain and grown on a Flanagan silt loam (fine, montmorillonitic, mesic Aquic Argiudoll). Individual plants were evaluated for change in leaf CO_2 fixation, water potentials, chlorophyll content, and in vitro pollen germination.

Significant decreases in maximum turgor, the relative water content associated with zero turgor, bulk volumetric elastic modulus occurred as the pH of the mist decreased from 5.0 to 2.5 and in result the shoot water potential was declined with a decrease in pH of the mist (Table 8). The effects of simulated acid rain pH 5.1 and 3.0 and ozone (ambient and twice ambient) on tissue water relations of mature clones of a fast growing genotype of ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) was investigated [MOMEN & HELMS, 1995]. The treatments showed little effect on the water relations of branches of mature trees. It was concluded that twice ambient ozone caused osmotic adjustment in seedlings, and the response was magnified by pH 3.0 rain. The low pH 4.5-6.5 reduced the propensity of *Acer rubrum* (L.) and *Quercus alba* L. to adjust leaf water relations and xylem anatomical traits in response to nutrient manipulations [MEDEIROS & al. 2016].

Table 8. The effects of acid rain on plant physiology (water relation), metabolic disorder, mineral nutrients, microbial activities in plant species

Name of Plant species	Acid rain pH 2.0 - 6.0		
	2.0 - 6.0	Symptoms	Reference
Red spruce	5.0, 2.5	Shoot water potential declined	EAMUS & al. 1989
Ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.)	3.0	Water relation of branches similar to drought conditions.	MOMEN & HELMS, 1995
The change in the soil C/N ratio would affect the release of nutrients during the decomposition of organic matter by microorganisms	4.0, 3.25, 2.5	Soil organic carbon content increased, inhibiting microbial respiration.	HESSEN & al. 2004
plant growth, litter, fungi	4.0, 3.25	Increased heavy metal content in soil, decomposition of litter by fungi increased.	ROUSK & al. 2009
Juvenile Japanese red pine tree *Pinus densiflora* Sieb. et Zucc.	3.0-2.0	The needle gas exchange, chlorophyll fluorescence, chemical contents and visual and physiological damage to needles.	OGUNTIMEHIN & al. 2013
Acer rubrum (L.) *Quercus alba* (L.)	4.5	The leaf nutrient content, water relations, leaf level and canopy level gas exchange, total biomass and allocation decreased.	MEDEIROS & al. 2016
Tea (*Camellia sinensis*)	3.5, 2.5	Proline, malondialdehyde, antioxidant enzyme activity, total nitrogen, caffeine, catechins, and free amino acids increase catechin and caffeine contents decreased	ZHANG & al. 2020

Conclusions

Many plant species have shown the harmful effects of acid mist or acid rain on plant growth. The published scientific results clearly illustrated that increase in simulated acid rain significantly decreased the germination and growth characteristics of plant. In addition, the decrease in the pH value of the simulated acid rain produced more negative impact on physiological and biochemical parameters in plants. The variable changes in the nutrient availability, photosynthetic activities and yield for plants mainly due to the low pH values available in the immediate environment. This review also highlighted the effects of acid rain on plant growth in the context of acid rain pollution as a key driving ecological indicator. Further literature research into the screening for better acid mist tolerant species is recommended. There is a need of coordination in multidisciplinary research and development programme leading to utilization of acid tolerant species for plantation at the industrial, urban centers and acid mist deposit areas. This article reviews recent developments in our knowledge of acid mist impact on plants growing in the different parts of the world.

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		BOSCAIU N. 1971. *Flora si vegetatia Muntilor Tarcu*, *Godeanu si Cernei*. Bucuresti: Edit. Acad. Române, 494 pp. HILLIER J. & COOMBES A. 2004. *The Hillier Manual of Trees & Shrubs*. Newton Abbot, Devon, England: David & Charles, 512 pp.
	□	**Chapters in books:** author(s), year, title, pages, a point sign, followed by "In": author (s) of the book, city, publishing house, number of pages. TUTIN T. G. 1996. *Helleborus* L. pp. 249-251. In: TUTIN T. G. & al. (eds). *Flora Europaea*. 2nd ed., 1993, reprinted 1996. Vol. **1**. *Psilotaceae to Platanaceae*. Cambridge: Cambridge University Press, XLVI, 581 pp.
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	□	Thesis: NTORE S. 2004. Contribution à la connaissance systématique du genre afrotropical *Pauridiantha* (Rubiaceae). PhD Thesis, K. U. Leuven, Leuven, Belgium.

CHECKLIST FOR *ILLUSTRATIONS*

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