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STUDIES ON GENETIC VARIABILITY AND TRAIT RELATIONSHIP IN CASTOR (*RICINUS COMMUNIS* L.)

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Abstract: Castor oil plant (*Ricinus communis* L.) is one of highly treasured industrial oil crop around the world. In the present study, genetic variability and trait relationships in some castor genotypes were examined to generate information for castor breeding purposes in Nigeria. Ten castor genotypes were evaluated in a Randomized Complete Block Design with three replications at the Research Farm of Niger State College of Agriculture, Mokwa, Nigeria. The analysis of variance results showed significant differences among the germplasm for all the traits studied, except leaf petiole length. The seed yield (kg/ha) ranged between 411.11 kg/ha and 1140.00 kg/ha with average of 852.19 kg/ha. High (> 20%) genotypic and phenotypic coefficient of variations were observed for days to 50% flowering, number of branches per plant, number of effective racemes per plant, effective raceme length, number of capsules per raceme and height at maturity. Positive and significant genotypic correlations were found between the seed yield, and number of effective racemes per plant (0.501**), effective raceme length (0.402**), number of capsules per raceme (0.361**) and 100 seed weight (0.560**). Positive direct effects on the seed yield were observed for nine out of thirteen traits studied. The cluster analysis grouped the genotypes into two main clusters with 4 and 6 cluster members. Based on these findings, it is showed that there is adequate genetic variability in the castor genotypes evaluated. The findings on the trait relationships revealed the raceme characters as important traits for indirect selection for increase in seed yield of castor.

Keywords: castor, correlation, path coefficient, trait relationships, variability.

Introduction

The castor oil plant (*Ricinus communis* L.) is non-edible oil crop which is widely acknowledged as an ideal industrial crop for tropical and sub-tropical regions, addressing the need for commercial crop with low input costs and valuable returns [SALIHU & al. 2014]. Castor is a hardy crop easy to establish on the field, resistance to drought, tolerates different types of soil even marginal soil and yield 350-900 kg oil per/ha [GANA, 2015]. The oil, which is extracted from castor seed, is used in the pharmaceutical, rubber/plastic, lubricants/biodiesel and food industries [MUTLU & MEIER, 2010]. A food condiment (Ogiri), custom to Southern part of Nigeria, is produced from castor seeds [GANA, 2015]. The residual meal of castor seed, after oil extraction, could be used as supplement feed in preparation of broiler finishing diets and also in sheep rations without any harmful effects [ANI & OKORIE, 2009].

Castor plant varies greatly in its growth and appearance [WEISS, 2000]. It varies in growth habit, seed size and colour, petiole length, branching pattern, capsule number, spike length, germination, flowering, oil content such that different cultivars often bear little resemblance to one another. Assessment of castor genotypic and phenotypic variability through several methodologies has been detailed by RAO & al. (2009) and

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ZHANG-XISHUN & YANG-JIAN (2006). Studies on partitioning of phenotypic variability into hereditary segment and non-hereditary (environment) parts have been proposed long ago [SHIVANNA, 2008]. SERVUGAPERUMAL & al. (2000), in a study on sixty castor cultivars, reported a high genotypic coefficient of variety for seed yield per plant, racemes per plant and plant height. RAO & al. (2006) detailed that heritable variability existed for the majority of the yield parameters and the traits are normally inherited in a quantitative manner. High heritability was estimated for earliness, seed weight and plant height by SOLANKI & JOSHI (2000). High genetic variation coupled with high heritability and expected genetic progress for branches per plant, capsules per raceme, length of raceme and seed per plant was documented by SHIVANNA (2008).

However, despite its economic importance, castor has not attained full commercial production in Nigeria due to little research attention. In the present study, studies on genetic variability and trait relationships in some castor genotypes was carried out, in an effort to generate basic information which could be useful to castor breeding programme in Nigeria.

Material and methods

The experiment was carried out in year 2018 wet season at the Research Farm of Niger State College of Agriculture, Mokwa, Nigeria. The genetic material used for the study comprised of five local and five exotic germplasm obtained from Castor Research Programme of National Cereals Research Institute (NCRI) Badeggi, Nigeria. Sources and some seed physical properties of the germplasm used are presented in the Table 1. The ten genotypes were grown in a Randomized Complete Block Design (RCBD) with three replications. Plot size of 3 m x 3 m with inter and intra rows spacing of 0.75 cm each was used. Two seeds were planted and latter thinned to one seedling per hole after three weeks after planting. Standard agronomic practices for castor were followed.

Data were taken on five plant samples per plot. Parameters considered include Days to 50% Seedling Sprouting, Days to 50% Flowering, Height at Flowering, Stem Girth, Leaf Area Index, Petiole Length, Height at Maturity, Days to Maturity, Number of Branches per Plant, Number of Effective Racemes per Plant, Number of Capsule per Spike, Length of Effective Spike, Seed yield per Hectare and 100 Seeds Weight. The data were subjected to analysis of variance (ANOVA). Multivariate analysis was carried out following the procedure of Statistical Tool for Agricultural Research (STAR 2.0.1). Path Analysis was done according to AKINTUNDE (2012).

Table 1. List and Seed physical characteristics of the Castor Genotypes used for the study

Acc. Number	Source	Type	Seed Shape	Seed Colour	Seed Mottle	Caruncle	Seed Size
Acc.001	Brazil	Exotic	Square	Black	Less conspicuous	Conspicuous	Large
Acc.005	Nigeria	Local	Oval	Brown	Less conspicuous	Less conspicuous	Small
Acc.006	Nigeria	Local	Oval	Brown	Less conspicuous	Less conspicuous	Small
Acc.036	Brazil	Exotic	Oval	Dark-chocolate	Conspicuous	Less conspicuous	Small
Acc.044	Nigeria	Local	Square	White	Less conspicuous	Conspicuous	Large
Acc.045	Nigeria	Local	Square	White	Less conspicuous	Less conspicuous	Large

Acc.052	Turkey	Exotic	Oval	Brown	Less conspicuous	Less conspicuous	Medium
Acc.082	India	Exotic	Oval	Dark-chocolate	Conspicuous	Less conspicuous	Medium
Acc.083	India	Exotic	Elongated	Brownish Red	Conspicuous	Conspicuous	Large
Acc.033	Nigeria	Local	Elongated	Brown	Conspicuous	Conspicuous	Medium

Results

The analysis of variance (ANOVA) showed significant differences for all the traits studied, except leaf petiole length, among the germplasm (Table 2). Days to 50% seedling sprouting ranged from 14 to 20 days with average of 16.23 days (Table 2). A range between 40 and 94 days, and average of 60.23 days were observed for days to 50% flowering. An average of 5.37, with minimum and maximum values of 2 and 14 respectively, was recorded for number of effective racemes per plant among the entries. Number of capsules per raceme varied from 24 to 68 with average of 43.07 capsules. Days to maturity was found to vary from 87 to 123 days with population mean value of 102.93 days. The seed yield (kg/ha), among the genotypes evaluated, was observed to range between 411.11 kg/ha and 1140.00 kg/ha with the average being 852.19 kg/ha. Estimates (Table 2) for coefficient of variations revealed high (> 20%) genotypic and phenotypic coefficient of variations for days to 50% flowering (GCV – 26.78 %; PCV – 27.20%), number of branches per plant (GCV – 50.99%; PCV – 78.62%), number of effective racemes per plant (GCV – 47.63%; PCV – 53.93%), effective raceme length (GCV – 22.59%; PCV – 29.00%), number of capsules per raceme (GCV – 22.30%; PCV – 27.77%) and height at maturity (GCV – 22.85%; PCV – 27.43%). Moderate genotypic coefficient of variation (10 < GCV > 20) was observed for height at flowering, leaf petiole length, stem girth and 100 seed weight. High heritability (H2 > 60%) coupled with high genetic advance as percentage of mean (GAM > 20%) were observed for seven traits out of thirteen traits examined (Table 2).

Table 3 presented genotypic correlation coefficients among all the traits studied. Positive and significant genotypic correlations were found between the seed yield, and height at flowering (0.309**), number of branches per plant (0.419**), number of effective racemes per plant (0.501**), effective raceme length (0.402**), number of capsules per raceme (0.361**), stem girth (0.408**), height at maturity (0.327**) and 100 seed weight (0.560**). Negative and significant correlations to the seed yield were observed for days to seedling sprouting and leaf petiole length. At a residual effect of 12.10%, positive direct effects on seed yield were recorded for all the traits; except height at flowering, leaf petiole length and height at maturity (Table 4). The highest positive direct effect on the seed yield was observed for number of effect racemes per plant and the highest negative direct effect was recorded for height at maturity. Height at flowering and height at maturity exerted positive indirect effects (0.423 and 0.337) on the seed yield through days to maturity and stem girth respectively. Days to seedling sprouting showed positive indirect effect on seed yield through number of effective racemes per plant and number of capsules per racemes. Number of branches per plant had positive indirect effect on seed yield through number of effective racemes per plant and effective raceme length.

Table 2. Genetic parameter estimates for thirteen agronomic traits among ten Castor Genotypes

Trait	Min	Max	Mean	S.E	MS	$\mathfrak{e}^2_g$	$\mathfrak{e}^2_p$	$\mathfrak{e}^2_e$	GCV	PCV	H^2 (%)	GAM (%)
DS	14.00	20.00	16.23	0.31	7.63**	2.31	3.00	0.69	9.37	10.68	77.06	16.95
DF	40.00	94.00	60.23	1.89	788.89**	260.10	268.69	8.58	26.78	27.22	96.81	54.27
HF	31.00	74.20	49.81	2.27	340.52**	93.25	154.02	60.78	19.39	24.92	60.54	31.07
LPL	6.60	21.00	13.23	0.59	18.09ns	3.44	11.21	7.77	14.02	25.31	30.68	16.00
BPP	2.00	8.00	3.47	0.16	2.46**	0.56	1.34	0.77	50.99	78.62	42.06	68.11
RPP	2.00	14.00	5.37	0.23	4.18**	1.27	1.63	0.36	47.63	53.93	78.00	86.65
ERL	11.80	32.80	21.53	1.11	86.27**	23.65	38.98	15.33	22.59	29.00	60.67	36.24
CPS	24.00	68.00	43.07	2.11	327.54**	92.25	143.04	50.80	22.30	27.77	64.49	36.89
SG	4.00	8.40	5.91	0.24	3.71**	0.98	1.76	0.79	16.73	22.46	55.45	25.66
DM	87.00	123.00	102.93	1.76	293.61**	96.70	100.22	3.53	9.55	9.73	96.48	19.33
HM	46.60	164.60	89.25	4.39	1431.48**	416.04	599.41	183.37	22.85	27.43	69.41	39.22
SY	411.11	1140.00	852.19	45.23	15932.18**	3666.78	8598.63	4931.85	7.11	10.88	42.64	9.56
SW	17.00	32.60	25.70	0.90	75.78**	24.98	25.83	0.85	19.45	19.77	96.72	39.40

Note: $\mathfrak{e}^2 g$ = genotypic variance, $\mathfrak{e}^2_P$ = phenotypic variance, $\mathfrak{e}^2_e$ = environmental variance, GCV = genotypic coefficient variance, PCV = phenotypic coefficient variance, H^2 = broad sense heritability, GA = genetic advance, GAM = genetic advance as percentage of mean; DS – Days to 50% Seedling Sprouting, DF – Days to 50% Flowering, HF – Height at Flowering, SG – Stem Girth, LPL – Leaf Petiole Length, HM – Height at Maturity, DM – Days to Maturity, BPP – Number of Branches per Plant, RPP – Number of Effective Racemes per Plant, CPS – Number of Capsule per Spike, ESL – Length of Effective Spike, SY – Seed yield per Hectare and SW – 100 Seeds Weight.

Table 3. Genotypic correlation coefficients of thirteen agronomic traits among ten Castor Genotypes

	DS	DF	HF	LPL	BPP	RPP	ESL	CPS	SG	DM	HM	SW	SY/Ha
DS	1	0.129	-0.405**	-0.240	-0.520**	0.158	-0.281*	0.177	-0.437**	-0.161	-0.461**	-0.622**	-0.509**
DF		1	0.870**	0.408**	-0.024	-0.044**	-0.436**	-0.392**	0.226	0.784**	-0.225	0.155	0.086
HF			1	0.646**	0.330**	-0.110	0.512**	-0.048	0.804**	0.494**	0.798**	0.601**	0.309**
LPL				1	-0.349**	-0.228	0.264*	0.070	0.741**	0.675**	0.203	0.424**	-0.256*
BPP					1	0.704**	0.054	-0.338**	-0.167	0.623**	0.374*	-0.131	0.419**
RPP						1	-0.113	-0.265*	-0.084	0.542**	0.090	-0.272*	0.501**
ESL							1	0.493**	0.432**	0.029	0.603**	0.479**	0.402**
CPS								1	0.111	0.102	0.270*	-0.138	0.361**
SG									1	0.577**	0.551**	0.584**	0.406**
DM										1	0.044	0.463**	0.119
HM											1	0.546**	0.327**
SW												1	0.560**

Note: DS – Days to 50% Seedling Sprouting, DF – Days to 50% Flowering, HF – Height at Flowering, SG – Stem Girth, LPL – Leaf Petiole Length, HM – Height at Maturity, DM – Days to Maturity, BPP – Number of Branches per Plant, RPP – Number of Effective Racemes per Plant, CPS – Number of Capsule per Spike, ESL – Length of Effective Spike, SY – Seed yield per Hectare and SW – 100 Seeds Weight.

Table 4. Genotypic path coefficients of thirteen agronomic traits among ten Castor Genotypes

	DS	DF	HF	LPL	BPP	RPP	ESL	CPS	SG	DM	HM	SW
DS	**0.198**	0.025	0.180	-0.047	-0.503	0.031	-0.057	0.037	-0.086	-0.032	-0.091	0.223
DF	0.016	**0.122**	-0.476	0.050	-0.003	-0.005	-0.553	-0.048	0.028	0.096	-0.027	0.029
HF	0.000	0.001	**-0.041**	0.001	0.260	0.000	0.001	0.023	0.001	0.000	0.012	0.001
LPL	0.260	-0.273	-0.432	**-0.178**	0.233	0.152	-0.376	-0.047	-0.495	-0.451	-0.136	-0.283
BPP	-0.709	-0.070	0.132	-0.140	**0.401**	0.282	0.422	-0.266	-0.067	-0.250	0.150	-0.053
RPP	0.397	-0.027	-0.098	-0.140	0.433	**0.616**	0.470	-0.363	-0.052	-0.334	0.055	-0.167
ESL	-0.367	-0.445	0.288	0.148	0.350	-0.074	**0.562**	0.277	0.243	0.016	0.339	0.269
CPS	0.522	-0.471	-0.433	0.048	-0.234	-0.183	0.341	**0.591**	0.177	0.071	0.187	-0.095
SG	-0.255	0.132	0.269	0.433	-0.097	-0.049	0.252	0.065	**0.504**	0.337	0.322	0.341
DM	-0.073	0.354	0.423	0.304	0.281	0.244	0.013	0.046	0.260	**0.451**	0.130	0.209
HM	0.433	0.602	0.135	-0.543	-0.600	-0.241	-0.913	0.426	-0.474	-0.118	**-0.675**	-0.461
SW	-0.901	0.075	0.291	0.205	-0.063	-0.232	0.232	-0.382	0.282	0.224	0.264	**0.484**

Note: DS – Days to 50% Seedling Sprouting, DF – Days to 50% Flowering, HF – Height at Flowering, SG – Stem Girth, LPL – Leaf Petiole Length, HM – Height at Maturity, DM – Days to Maturity, BPP – Number of Branches per Plant, RPP – Number of Effective Racemes per Plant, CPS – Number of Capsule per Spike, ESL – Length of Effective Spike, SY – Seed yield per Hectare and SW – 100 Seeds Weight. Residual effect = 0.121

Table 5. Membership performance summary of 10 Castor Genotypes in two Cluster Groups

Variable	Statistics	Cluster I	Cluster II
Days to 100% Sprouting	Min	14.00	17.00
	Max	17.00	19.00
	Mean	15.17	17.50
Days to 50% Flowering	Min	49.00	45.00
	Max	94.00	68.00
	Mean	69.83	55.00
Height at Flowering (cm)	Min	50.60	31.20
	Max	74.20	55.60
	Mean	60.97	41.30
Leaf Petiole Length (cm)	Min	12.00	8.40
	Max	17.20	15.60
	Mean	14.67	11.15
Branches per Plant	Min	3.00	3.00
	Max	6.00	7.00
	Mean	4.53	5.75
Effective Racemes per Plant	Min	4.00	4.00
	Max	8.00	10.00
	Mean	5.33	6.53
Effective Raceme Length (cm)	Min	14.40	12.00
	Max	32.80	21.80
	Mean	25.67	17.30
Capsules per Plant	Min	24.00	32.00
	Max	62.00	63.00
	Mean	40.17	45.75
Stem Girth (cm)	Min	6.00	4.60
	Max	8.40	5.60
	Mean	7.10	5.00
Days to Maturity	Min	92.00	88.00
	Max	123.00	102.00
	Mean	107.17	95.75
Height at Maturity (cm)	Min	85.80	46.60
	Max	164.60	92.60
	Mean	109.07	70.40
100 Seed Weight (g)	Min	26.00	18.00
	Max	30.60	27.00
	Mean	28.80	20.80
Seed Yield (kg/ha)	Min	726.67	445.56
	Max	1140.00	1035.56
	Mean	991.67	659.73
Number of Members		6	4
Membership (Genotypes Members)		Gen1 (Acc.001), Gen3 (Acc.006), Gen5 (Acc.044) Gen6 (Acc.045), Gen9 (Acc.083), Gen10 (Acc033)	Gen2 (Acc.005), Gen4 (Acc.036), Gen7 (Acc.052), Gen8 (Acc.082)

The result of cluster analysis delineated the ten genotypes evaluated into two main cluster groups (Figure 1). The cluster I had the higher (6) number of cluster members. The cluster pattern showed random distribution of the genotypes into the two groups irrespective of the sources of the genotypes (Table 5). The local genotypes Acc. 006, Acc.044, Acc.045 and Acc. 033 were clustered together with the exotic ones (Acc. 001, Acc. 082 and Acc.033) in cluster I. In the cluster II, the genotypes were exotic, except Acc. 005. Membership performance mean (Table 5) showed that cluster I comprised of high

yield members with average seed yield (991.67 kg/ha) greater than the average population mean (852.19 kg/ha). The cluster group II, though had low seed yield (659.73 kg/ha), contained genotypes with high number of capsules per plant, low days to maturity and shorter height at maturity.

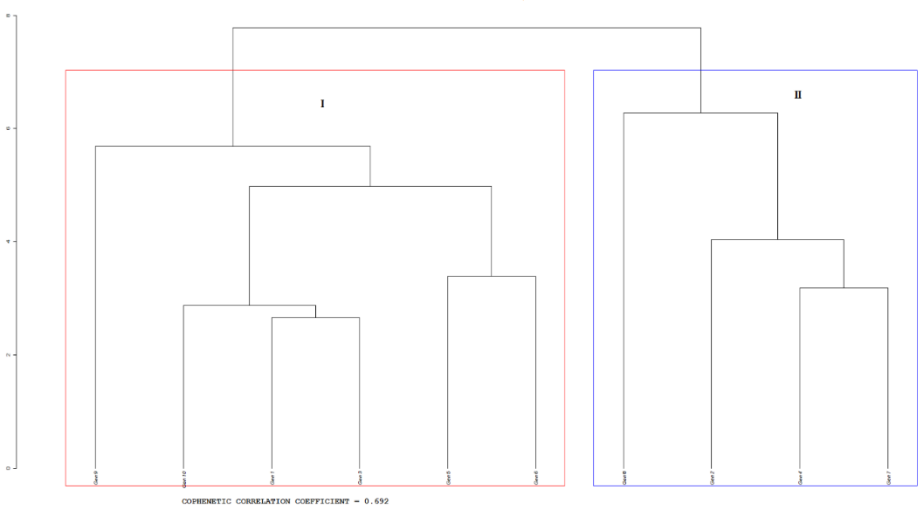

Figure 1. Dendrogram constructed through agglomerative cluster analysis of the ten Castor Genotypes evaluated

Discussion

The significant differences showed by ANOVA revealed adequate variability for most of the traits among the genotypes evaluated. The magnitude of the variability existed among the genotypes indicated ample scope for selection of most of the traits. SHIVANNA (2008) explained that genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (GCV) could be partitioned as high (> 20%), moderate (10-20%) or low (< 10%). Based on this, moderate to high GCV and PCV were observed for ten out of thirteen characters studied and in all the traits, the magnitude of differences between the GCV and PCV was low for all the traits except for the leaf petiole length branches per plant where it was moderate and high respectively. The low differences is an indication of low influence of environmental factors on the phenotypic expression of genotypes for the traits and as such there is high chance of improving these characters through selection based on the phenotypic data. Earlier researchers [RAO & al. 2009; ZHANG-XISHUN & YANG-JIAN, 2006] had reported similar magnitudes of variability in the works on castor. PATEL & JAIMINI (1988) also reported moderate to high coefficient of variation for most of the traits in castor irrespective of the environment. In a study on the genetic variation of 68 castor lines, LAKSHMAMMA & al. (2005) reported high genotypic and phenotypic coefficient of variability for capsule weight per plant, plant height, capsule number and leaf area index. GOLAKIA & al. (2007) observed high PCV for seed yield in his work on castor. This is in contrary to the present findings where low PCV was observed for seed yield.

The high heritability observed for most of the traits suggested that selection for the traits could be easy and their improvement would be fairly possible using selection breeding. This finding is in accordance with the findings of SHIVANNA (2008) who reported high heritability for all these traits in castor. However, for the best prediction of genetic progress in selection, heritability estimate is said to be along with genetic advance. The high genetic advance as percentage of mean (GAM) observed for most of the traits is an indication that selection at 5% selection differential could result in high (> 20%) expected genetic progress (gain) in a selection programme for the traits. Similar results on genetic advance as percentage of mean in castor were reported by LAKSHMAMMA & al. (2005).

The significant correlations and positive direct effects observed for number of branches per plant, effective number of racemes per plant, effective raceme length, number of capsules per raceme and seed weight indicated true relationship between the seed yield and the mentioned traits and as such direct selection for improvement of these traits would likely be effective as indirect selection for increase in seed yield of castor. The direct effects of effective number of racemes per plant, effective raceme length, number of capsules per raceme and seed weight on seed yield revealed the importance of raceme characters as yield contributors in castor. The results reported here is similar to findings in earlier studies of RAMESH & VENKATE (2001) who recorded strong correlation between seed yield and plant height to primary raceme, and length of raceme. TORRES & al. (2015) observed that direct selections of genotypes with tall plant height, wide stem girth, high branching per plant and seed weight are effective to select genotypes with high seed oil. ASWANI & al. (2003), in his work on castor, reported positive and significant genotypic relationships among seed yield, seed weight, days to flowering, days to maturity and plant height.

The result of the cluster analysis gave insight on possible parental combinations to get useful recombinants. The high yielding cluster group (Cluster I) are the potential parents for increased seed yield. The random distribution of the genotypes into the two cluster groups, irrespective of the geographical sources, revealed no geographic influence on the diversity observed and thus, suggesting other forces such as natural and artificial selection, and genetic drift as contributory factors to the observed genetic divergence among the genotypes. High genetic variability in segregating populations can only be generated by crossing genetically diverse individuals. It has been long theoretically demonstrated that the higher the divergence between the genotypes, the higher will be the heterosis [SHIVANNA, 2008]. Similar absence of correlation between genetic diversity and geographic diversity was also reported by COSTA & PEREIRA (2006) and ZHANG-XISHUN & YANG-JIAN (2006).

Conclusion

The results of the study revealed substantial genetic variability in the germplasm evaluated for most of the traits. High genotypic and phenotypic coefficient of variations were observed for days to 50% flowering, number of branches per plant, number of effective racemes per plant, effective raceme length, number of capsules per raceme and height at maturity. Positive and significant genotypic correlations were found between the seed yield, and height at flowering, number of branches per plant, number of effective racemes per plant, effective raceme length, and number of capsules per raceme, stem girth, height at maturity and 100 seed weight. Positive direct effects on seed yield were observed for all the traits; except height at flowering, leaf petiole length and height at maturity. Out

of all the studied traits, raceme characters are identified as most effective traits for indirect selection for seed yield increase in castor.

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Kasim Alhassan ALHAJI is a lecturer/botanist with research interest in crop protection. His research focuses on plant pathology.

Bolaji Zuluqurineen SALIHU is a plant geneticist/breeder with a special interest in plant population improvement. His research focuses on genetic improvement of castor oil plant.

Ann OLISEH is an Agricultural superintendent whose work is focused on crop (castor) husbandry.

Maryam Alfa KABARAINI is a researcher who has research interest in castor disease control and management.

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FOLIAR MICRO-MORPHOLOGY AND PHYTOCHEMICAL STATUS OF *LEEA GUINEENSIS* G. DON LEAVES

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Abstract: *Leea guineensis* G. Don leaves are known to contain some active compounds that certify its usage as a medicinal plant. The establishment of a comprehensive pharmacognostic profile of *L. guineensis* leaves will help in the standardization of quality and proper identification. Evaluation of the fresh and powdered leaves was carried out using standard methods to determine the macro-morphological, micro-morphological (both the qualitative and quantitative), chemo-microscopic and phytochemical profiles. The result obtained shows that macroscopically, the leaf was simple, opposite and entire in shape, having a cylindrical and undulating edge with a hard and smooth texture. The internodes are short with a spot of pink colour at the interval nodes (nodules). It has a simple trunk without thorns with a granular fracture surface. The colour is pale green when young and deep green at maturity. Microscopically, the stomata were paracytic on the abaxial and absent on the adaxial. The epidermal cells are irregular and rectangular, the epidermis possesses straight anticlinal walls and is slightly undulating both on the abaxial and adaxial epidermis. Trichomes are not present on both epidermises, while crystals are present on the abaxial epidermis. The stomata index (18.87%) was calculated for abaxial, the mean cell length (43.46 and 43.89) and width (30.50 and 31.61) was comparably similar for both abaxial and adaxial epidermis respectively. The number of stomata detected on the abaxial was 20. Chemo-microscopic characters present include Starch, Calcium carbonate crystals while lignin, fat, and mucilage were absent while phytochemical screening revealed that alkaloid, saponin, flavonoids, tannin, phenolics and anthraquinone were present. The foliar micro-morphological findings are of great importance in the proper and correct identification, standardization, and authentication of medicinal plants.

Keywords: chemo-microscopic, *Leea guineensis*, macroscopic, micromorphology, pharmacognostic.

Introduction

The foliar epidermal profile is an important taxonomic character that assists in understudying the leaf epidermis. The epidermal cells, trichomes, stomata, stomata frequency, cell length, cell sizes, distribution, and orientation are important in taxonomy [ALBERT & SHARMA, 2013]. Medicinal plants produce some secondary metabolites that are capable of being stored in different plant organs including leaves, stems, and roots. The outer cell layer of the plants also contains hairs that are functionally classified as glandular or non-glandular trichomes. *Leea guineensis* is a small tree or a shrub that belongs to the *Leeaceae* family. It grows up to 450-610 cm high [MUHAMMAD & AJIBOYE, 2010]. It originates from tropical Africa, distributed throughout Northern and Eastern Australia, New Guinea, South, and Southeast Asia and parts of Africa. It is mainly propagated through stem cutting or by seed. It germinates in 14-21 days at 21^{o}C. In Yoruba language, it is called Alugbokita, while in Twi language, it is called Okatakyi [MUHAMMAD & AJIBOYE, 2010]. It grows in the humid places found in the forest region, moister woodlands and forest area of tropical Africa. Traditionally, it is used in the treatment of diverse ailments. *L.*

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guineensis leaves are usually effective in treating toothache, skin ulcers, paralysis, vertigo, rheumatism and epileptic fits [MSHANA & al. 2000]. Also, they are used in pregnancy detection, purgative, general weakness, skin rash, gonorrhea, convulsions, stomach troubles, boils and swollen spleen in children [HASSAN & ABD EL-RAZEK, 2011; BURKILL, 1985; MSHANA & al. 2000; MOLINA, 2009]. The micro-morphological feature gives detailed information to assist in a proper investigation of varieties of species, solving problems in evolutionary relationships [SEGARRA & MATEU, 2002]. Micro-morphological characteristics of plants are usually expressed in leaves, stems, roots, and bark of plants and it is always important in plant taxonomy avoiding taxonomic conflict in different species of the plant [SONIBARE & al. 2014]. Hence, the dire need to explore the proper identification, pharmacognostic and phytochemical status of *Leea guineensis.*

Materials and methods

Plant collection

Fresh healthy leaves of *Leea guineensis* were collected from the arboretum of the Forestry Research Institute of Nigeria, Ibadan, Oyo State. The samples was identified at the taxonomy unit of the institute and a voucher specimen (FHI 112460) was deposited at the Forest Herbarium Ibadan. The leaves sample were air-dried, powdered and stored in an appropriate container until required for use.

Variable assessed

The fresh leaves were used for qualitative and quantitative micro-morphology evaluation using a light microscope [EVANS, 2005; BRAIN & TURNER, 1975], macroscopic features [BRAIN & TURNER, 1975], chemo-microscopic examination [EVANS, 1996] and phytochemical status [BOYE & al. 2012; OMORUYI & al. 2012] of *Leea guineensis.*

Microscopic evaluation

This involves the description of the different microscopic characters of the plant such as leaf content, cell length, and width, stomata, trichomes, etc. [RADFORD & al. 1974; KHATIJAH & ZAHARINA, 1998; ADEDEJI, 2004; METCALFE & CHALK, 2004].

Epidermal section (ES) preparations using a light microscope

The leaf samples were soaked in concentrated (HNO_3) after being cut into reasonable portions. They are soaked in well-covered Petri dishes for about two hours depending on the leaf, in order to macerate the mesophyll. Tissue disintegration was indicated by bubbles and the epidermal layers were carefully peeled off with forceps and a fine caramel hairbrush. The peeled layers were put into a clean Petri dishes containing distilled water and later put into another Petri dish containing 2 ml of ethanol for 1-2 minutes to allow the hardening of cells. Afterwards, the tissues were stained with safranin (red stain), then removed and dipped into another Petri dish containing distilled water to remove excess staining. The tissue is then mounted on a microscopic slide, after that, a drop of glycerol was dropped on the tissue, then it was covered with coverslips, nail varnish was used as a sealant to protect the edges from dehydration and damage. The microscopic slides were labeled appropriately and then viewed under a light microscope. [RADFORD & al. 1974; KHATIJAH & ZAHARINA, 1998; ADEDEJI, 2004; METCALFE & CHALK, 2004; EVANS, 2005; BRAIN & TURNER, 1975].

The stomata index was analyzed using the formula below:

$$I = \frac{S}{E+S} \times 100$$

Where I – Stomata Index
S – No of Stomata per unit area
E – No of epidermal cells in the same unit area [SALISBURY, 1927].

Transverse section (TS) preparations

Anatomical sections of the fresh leaf, stem, and root were prepared using standard laboratory techniques. The transverse sections were cut with the aid of a sledge micrometer. The leaf blade that was cut, was then stained in a staining jar for 5 minutes. Distilled water was used to rinse the outer cell layer removed, followed by ethanol and it was stained again, finally being washed with absolute ethanol. It was put into a container containing 50/50 alcohol/xylene and rinsed vigorously until it was clear. The sections were cleared with chloral hydrate solution and the tissue was mounted on a slide with a drop of dilute glycerin. [EVANS, 2005; BRAIN & TURNER, 1975].

Macroscopic evaluation

The macroscopic features of the leaf and organoleptic properties like taste, odour, and colour were described according to the standard botanical method of BRAIN & TURNER (1975).

Chemo microscopic evaluation

The powdered leaf sample was cleared in a solution of chloral hydrate to remove chlorophyll; then, the cleared powdered leaf sample was mounted on the microscopic slides and observed under a compound microscope to reveal chemical substances like lignin, starch, calcium oxalate, calcium carbonate, fats and oil and mucilage [EVANS, 1996]

Lignin test

The powdered plant was mounted in phloroglucinol followed by concentrated hydrochloric acid; a red coloration indicates lignifications.

Starch test

The powdered plant was mounted in N/50 iodine. Bluish coloration shows that starch is present.

Calcium oxalate crystals test

The powdered plant was cleared in a solution of chloral hydrate; the presence of calcium oxalate crystals reveals bright definite shapes and sizes. The addition of 80% hydrochloric acid and viewing under a microscope, the disappearance of calcium oxalate crystals confirms their presence.

Calcium carbonate test

The powdered plant containing the chloral hydrate solution was mounted on a microscopic slide, after which 1-2 drops of the acetic acid solution were added. The evolution of gas reveals the presence of calcium carbonate.

Test for oils (fats)

The powdered plant was mounted in Sudan IV reagent. Pinkish coloration is an indication of the presence of oils.

Mucilage test

The powdered leaf sample was placed on the slide and Ruthenium red (a drop) was added, a pink coloration shows the presence of mucilage.

Phytochemical screening

The phytochemical screening of *Leea guineensis* leaves was carried out as described by standard analytical methods of OMORUYI & al. (2012), BOYE & al. (2012). The phytochemicals to be detected include alkaloids, phenolics, saponin, flavonoid, cardiac glycosides, tannin, and anthraquinone.

Results

Microscopic examination

The results of the microscopic examination of *Leea guineensis* are expressed in Table 1. They show that the epidermal leaf shape is irregular, rectangular to polygonal and slightly undulating on both the lower and upper epidermis. It has a straight anticlinal wall. There is a presence of paracytic stomata on the lower epidermis and absent on the upper epidermis (Figure 2E). Trichomes are absent on both epidermises. Meanwhile, the cell length for the lower and upper epidermis is (43.46 and 43.89) respectively, while the cell width for the lower and upper epidermis is (30.50 and 31.61) respectively. The cell density for both epidermises is (86.0 and 64.0). Stomata length and width were calculated only for the lower epidermis alone, while the mean stomata number is 20. The stomata index is 18.87%, and it is calculated for the abaxial epidermis.

Table 1. Epidermal characters and their qualitative and quantitative descriptions

Epidermal features	Characters	
	Lower epidermis (abaxial)	**Upper epidermis (adaxial)**
Cells		
Shape	Rectangular to polygonal and slightly undulating	Rectangular to polygonal and slightly undulating
Anticlinal walls	Straight and irregular	Irregular
Cuticle	Present	Present
Mean length (μm)	43.46	43.89
Mean width (μm)	30.50	31.61
Density (μm)	86.0	64.0
Stomata		
Type	Paracytic	Absent
Frequency	Numerous	Absent
Stomata length (μm)	16.06	Absent
Stomata width (μm)	7.0	Absent
Mean stomata	20	Absent
Stomata index (%)	18.87%	Absent
Trichomes		
Type	Absent	Absent

Macroscopic examination

The macroscopic and organoleptic features of *Leea guineensis* fresh leaves are expressed in Table 2. The result shows that the leaf has a light green color when young and

deep green at maturity. It has a faint odor and a bitter taste. The leaf is small, cylindrical and has undulating edges. The arrangement of the leaf is in opposition. The fractured surface is non-glandular and the petiole is short. The leaf (Figure 1) is simple, opposite and entire. The venation is pinnate and its margins are smooth, apex of the leaf is acute, while the base is equal. It has a slightly hard and smooth texture with a smooth surface and spot of pink nodules. The internodes are short.

Figure 1. *Leea guineensis* leaves

Table 2. Macroscopic and organoleptic characters of the leaf of *Leea guineensis*

Features	Descriptions
Leaf shape	Small, cylindrical and undulating edges
Arrangement	Opposite
Fractured surface	Granular
Petiole	Short
Lamina	
Composition	Simple, opposite and entire
Venation	Pinnate
Margin	Smooth
Apex	Acute
Base	Equal
Texture	Slightly hard and smooth
Surface	Smooth with spot of pink nodules
Internode	Short
Organoleptic properties	
Color	Light green
Odor	Faint
Taste	Bitter

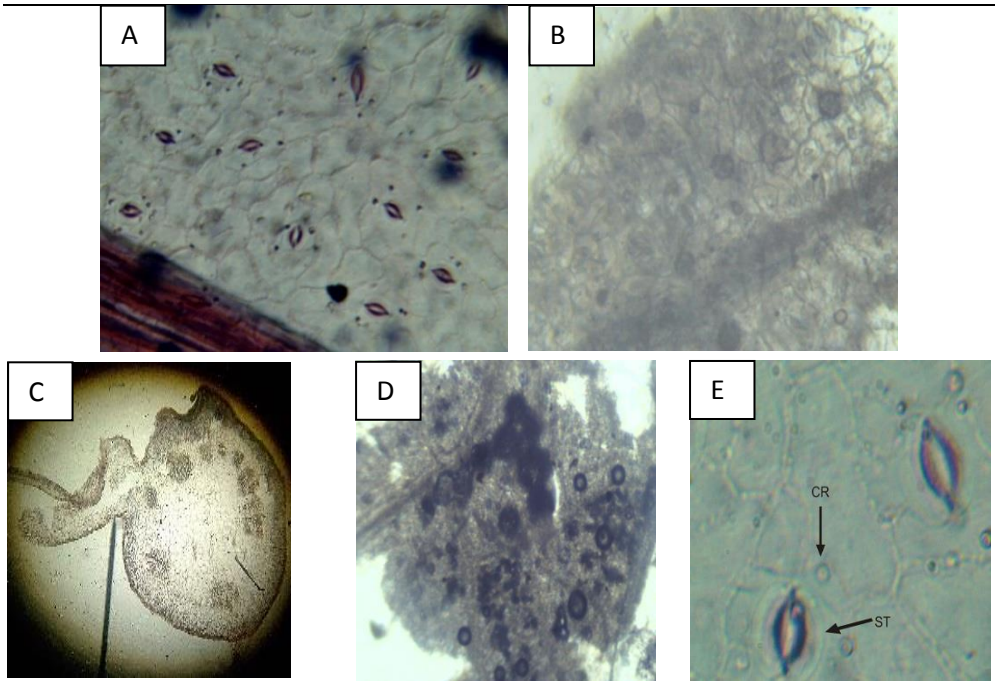

Figure 2. Leaf Micrograph of the leaves of *Leea guineensis.* **(A)** Leaf clearing showing secretory cavities and epidermal cells of the abaxial surface. **(B)** Stained Leaf clearing showing epidermal cells and starch grains. **(C)** Anatomical overview of the leaf blade. **(D)** Stained powdered leaf showing starch grains (sg). **(E)** Leaf clearing showing paracytic stomata and calcium carbonate crystals (cr) on the lower epidermis

Chemo-microscopic evaluation

The chemo-microscopic evaluation of the powdered leaves sample of *Leea guineensis* in Table 3. Shows that lignin, fats, mucilage and calcium oxalates are absent in the epidermal layer of the plant while starch, calcium carbonate and crystals are present.

Table 3. Chemo microscopic evaluation of the powdered leaves sample

Parameter	Observation	Result
Lignin	No red colouration observed	-
Starch grains	A blue colouration	+
Fats	No pink colouration	-
Calcium oxalate crystals	No effervescence	-
Calcium carbonate	Effervescence	+
Mucilage	No pink colouration	-
Crystals	A blue colouration observed	+

Phytochemical screening

Table 4 presents the results of the preliminary phytochemical screening of *L. guineensis* leaves. The results reveal that all the phytochemicals examined which are alkaloids, saponin, flavonoid, tannin, phenolics, anthraquinone and cardiac glycosides are present except steroids and

phlobatanins. The phytochemicals present in *L. guineensis* leaves suggests that the plant is of high medicinal value and could be used in the management and treatment of various diseases.

Table 4. Phytochemical screening of *Leea guineensis* leaves

Phytochemicals	*Leea guineensis*	Remarks
Alkaloids	+	Present
Saponin	+	Present
Flavonoid	+	Present
Tannin	+	Present
Phenolics	+	Present
Anthraquinone	+	Present
Cardiac glycosides	+	Present
Steroids	-	Absent
Phlobatanins	-	Absent

Discussions

Wrong identification of medicinal plants usually creates a big obstacle in the use and the misuse of herbal drugs and medicines. It is a fact that medicinal plants' therapeutic potency depends on its mode of identification. However, complete acceptance of herbal alternative medicines is still facing some obstacles as a result of the dearth of proper documentation as well as appropriate standardization and quality control processes. Thus, proper identification of the medicinal plant which is used for various medical implications is very imperative to ensure its chemical components and its pharmacognostic details.

Foliar microscopic description

The qualitative and quantitative leaf micro-morphological characteristics of the epidermal cells of *Leea guineensis* are summarized in Table 1. The photomicrographs of the light microscope of *Leea guineensis* leaf surfaces revealed a paracytic type of stomata. In paracytic (parallel-celled) type of stomata, the secretory cavity is always accompanied by one or more guard cells on both sides. They are widely distributed on the abaxial epidermis and are absent on the adaxial epidermis. The result obtained for stomata index on the abaxial is 18.87%. The stomata are arranged randomly on the lower epidermis and are very obvious. The stomata length and width could not be calculated because the lower epidermis are tightly closed and appear very small at magnification x10. Stomata are secretory cavities usually located on the lower epidermis which allow the exchange of water vapour, oxygen and carbon dioxide. The stomata opening increases rate of transpiration which increases water absorption and nutrient, mostly at night, the stomata are closed and often transpiration rate drops, consequently plant nutrient and water intake reduces. The stomata are surrounded by guard cells with some intercellular spaces. The stomata exist only on the lower epidermis. The outer layer cells are medium sized having an irregular cell on both surface of the plant epidermis. The leaf possesses straight anticlinal walls and are slightly undulating on both the epidermis. Trichomes are not evident both on the abaxial and adaxial epidermis of *Leea guineensis.* Micro-crystals are present on the lower epidermis and absent on the upper epidermis. This aligns with the result reported by METCALFE & CHALK (2004). Further epidermal research findings reveal that the mean average number of cells ranged from 64.0 μm on the adaxial epidermis to 86.0 μm on the abaxial epidermis. The stomata length and width are (16.06 μm and 7.0 μm) respectively. While the cell length (43.46 μm) for abaxial have almost the same value with the cell length (43.89 μm) on the adaxial and also the cell width (30.50

μm) on the abaxial epidermis is a bit lower than the cell width (31.61 μm) of the adaxial epidermis. The stomata number recorded for abaxial is 20.

Macroscopic evaluation

The macroscopic features of *Leea guineensis* showed that the leaves are opposite, simple and entire. The color is pale green when young and deep green at maturity with an odorless smell and bitter taste. The internodes are short, smooth with spot of pink at each node. The bark is rough and the texture is hard and smooth. The shape is cylindrical and are undulating at the edges. The fractured surface is granular, evident, trunk hard and brittle when dry.

Chemo microscopic evaluation

Crystal deposits were detected on the surface of the leaves Figure 1E. The crystals deposit on the leaf usually gives it anti-herbivory features, meanwhile, the crystals act as a defence mechanism on the epidermal surface of the leaves of many plant species [ASHAFA & al. 2008]. Crystals play important role in the cellular ion balance and the rigidity of the tissue. Also it helps in the detoxification of dangerous metals [OTANG & al. 2014].

Phytochemical composition

Medicinal plants always contain some active substances that have been widely reported to contribute their metabolic, physiologic and protective effects to humans [EDEOGA & ERIATA, 2001]. The result obtained in this study corroborates the one reported for *Leea guineensis* leaves by AWOTEDU & al. (2019), except for the absence of steroids and phlobatanins. It is also in consonance with the one reported for other plants [OMOTAYO & BOROKINI, 2012; OYEYEMI & al. 2014]. Saponin produced by plant always fight infections produced by parasites. When saponin is taken by human beings, it helps the body system to fight against viruses and bacteria. However, the occurence of saponin in this study suggest it for use in fighting against infections and recommending it for soap making properties because of its foamy abilities. Alkaloids have been known to have antihypertensive, anti-inflammatory, antifungal, antifibrogenic and microbiocidal effect [GHOSHAL & al. 1996]. Alkaloid present in this study also aligns with the work reported by AWOYINKA & al. (2007) who also reveals that alkaloid is present in *Cnidoscolus aconitifolius.* Alkaloids are beneficial chemicals to plants, serving as repellent to predators and parasites. Tannins is reported to serve as antidotes for many poisons [NORTON, 2000], antibacterial [AKIYAMA & al. 2001], anti-parasitic [KOLODYIEJ & KIDERLEN, 2005] and also it can help in the protection of the kidney. Hence, the presence of tannin in this study makes the plant a useful source of antidotes for poisons and serve as immediate relief for people with sore throat, diarrhea and dysentery and wounds [OKWU, 2004]. In this study, flavonoid is present in the leaves of *Leea guineensis*, this agrees with that reported by AKUBUGWO & al. (2007) for *A. hybridus.* Presence of flavonoids in plants generally serve as flavouring agents [KUJUMGIEV & al. 1999]. Availability of all the secondary metabolites suggest that *Leea guineensis* is an important herbal drug that can be used in folkloric medicine.

Conclusion

The information obtained from this study can serve as a proper guide for the exact identification and authentication. Besides identification and authentication, micro-morphological and macro-morphological evaluation of the leaves could provide a rich information about certain plants physiological performances. The quantitative determination

of some diagnostic features is useful for setting proper standard in comparing and differentiating closely related plant species.

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Notes on Contributors

Olamilekan Lanre AWOTEDU is a plant physiologist and biochemist, a PhD student and a senior research fellow with special interest in plant physiology, biochemistry, phytochemistry and ethnobotany.

Paul Oluwatimilehin OGUNBAMOWO is an environmental chemist and a biochemist, a PhD student and a senior research fellow with special interest in phyochemistry and analytical chemistry.

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SYMBIOTIC EFFECTIVENESS OF BRAZILIAN RHIZOBIAL STRAINS IN IMPROVING N-FIXATION AND PRODUCTIVITY OF COWPEA IN MINNA, SOUTHERN GUINEA SAVANNA OF NIGERIA

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Abstract: In a bid to evaluate the effectiveness of two Brazilian rhizobial strains in improving nitrogen fixation and productivity of cowpea (*Vigna unguiculata* (L.) Walp) in the southern Guinea savanna of Nigeria, field trials were conducted in 2016 and 2017. Four rhizobial inoculation levels (control, inoculation with BR 3262, BR 3267 and application of 90 Kg N ha^{-1}) and three varieties (IT93K-452-1, IT99K-573-1-1 and TVx 3236) were arranged in randomized complete block design. Inoculation with BR 3267 significantly increased nodule number by 27% over the control in 2017 and there was significant variation in the response of the varieties to inoculation in the two years in respect of nodule weight. Plants fertilized with 90 Kg N ha^{-1} consistently had the least number of nodules, weight and percentage of effective nodules in the two years. N-uptake and shoot biomass yield was however significantly higher in the N-fertilized and uninoculated plants than plants inoculated with the BR strains. Plants inoculated with both strains fixed significantly lower nitrogen than the uninoculated plants. However, the inoculated plants partitioned greater nitrogen to their seeds having significantly higher % protein in their seeds than the uninoculated plants. Plants fertilized with 90 kg Nha^{-1} had the highest grain yield (1761.2 kg ha^{-1}) which was at par with the value recorded in the uninoculated plants (1479.60 Kg ha^{-1}) but significantly higher than the values recorded in the inoculated plants (1320.1-1338.0 Kg N ha^{-1}). It could therefore be concluded that the BR strains tested in this study are not more effective than the indigenous strain in improving N-fixation and productivity of cowpea in the study area.

Keywords: BR strains, nodulation, n-fixation, biomass yield, grain yield.

Introduction

Over the years, food requirements have increased while land availability has become less due to increase in population and urban development. Thus, the only way to increase agricultural production is to increase the yield of individual crop per unit area of land. Low crop productivity is a general problem facing most farming systems in sub-Saharan Africa. These low yields are pronounced in grain legumes and are often associated with declining soil fertility and reduced N_2-fixation [MFILINGE & al. 2014]. Cowpea which is the most important legume in Nigeria is the most affected with an average yield of 450 kg ha^{-1} [OMOTOSO, 2014].

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Rhizobial inoculation has been reported by a number of workers to improve the yield of legumes and enhance soil fertility [SINGH & al. 1997; KENNEDY & al. 2004; MATIRU & DAKORA, 2004; HAN & LEE, 2005; ZHOU & al. 2006; ELKOCA & al. 2007; HUANG & ERICKSON, 2007; BEJANDI & al. 2011; SCHWEIGER & al. 2012]. Effective symbiotic relationship depends on the cultivar and the rhizobium strain [FALL & al. 2003]. Rhizobia strains differ in their ability to fix atmospheric nitrogen [FALL & al. 2003]. A rhizobium strain can only be considered effective if it can fix nitrogen in the nodules of the host plant. The symbiotic effectiveness of a rhizobium strain is an estimation of host growth promotion and is usually based on the enhancement of plant shoot dry weight upon inoculation [LARANJO & al. 2014]. Many soils are heavily infested with ineffective rhizobia capable of inducing nodulation without host benefit [FENING & DANSO, 2002]. Under such condition, inoculation with highly competitive and effective strain of rhizobia is needed to replace the ineffective native rhizobia. Inoculation is usually done to ensure the availability of the correct bacteria species and an effective strain of that species [BASHAN & al. 2014]. This study therefore aimed to evaluate the effectiveness of two Brazilian rhizobial strains in improving the nitrogen fixation and productivity of cowpea in Minna, southern Guinea savanna of Nigeria.

Material and methods

The study was conducted on three farmers' fields (09° 27.832' N 006° 25.375' E, 09° 31.203' N 006° 27.678' E and 09° 28.026' N 006° 25.325' E) (but researcher managed) in Minna southern Guinea savanna of Nigeria. The climate of Minna is sub humid with mean annual rainfall of about 1284 mm and a dry season of about 5 months duration occurring from November to March. The mean maximum temperature (about 33.5 °C) remains high throughout the year [OJANUGA, 2006]. The physiographic characteristics of Minna area consist of gently undulating high plains developed on basement complex rocks made up of granites, migmatites, gneisses and schists. Inselbergs of "older granites" and low hills of schists rise conspicuously above the plains. Beneath the plains, bedrock is deeply weathered and constitutes the major parent material (saprolites) [OJANUGA, 2006].

The treatments consisted of four levels of rhizobial inoculation (control, inoculation with BR 3262, BR 3267 and application of 90 kg N ha^{-1}) and three cowpea varieties (IT93K-452-1, IT99K-573-1-1 and TVx 3236). These were factorial combined to give twelve treatments combinations which were arranged in randomized complete block design. There were three replicates and each farmer's field served as a replicate. Gross plot size was 3.75 m × 4 m (15 m^2) consisting of five rows, and net plot size was 2.25 m × 4 m (9 m^2) consisting of three rows, with 75 cm spacing between rows. Intra and inter row spacing of 20 × 75 cm were maintained. Three seeds per hole were planted and the resultant seedlings were later thinned to two per stand at seven days after planting. Simultaneously, maize (the reference crop) was planted in between the cowpea plots and intra and inter row spacing of 75 cm × 75 cm was maintained.

All the plots received basal application of 45.8 kg P_2O_5 and 40 kg K_2O ha^{-1} at planting. Nitrogen was applied to the plots that received 90 kg N ha^{-1} in two split. Twenty five percent was applied at 3 weeks after planting and the remaining 75% was applied at flowering. The inoculants were applied at the rate of 5 g per kg seed using the slurry method which was done by first coating the seeds with a sticker (solution of 85 ml water and 15 g sugar) before applying the inoculant to the coated seeds [IITA and N_2Africa, 2014].

At 50% flowering, the shoot of cowpea plants within 1 m along the ridge were cut at the soil surface, put inside a paper envelope. The roots were carefully dug out with a spade and the soil mass around the roots were carefully washed off to recover the nodules. The nodules were counted and packed inside a paper envelope. Ten nodules were selected at random from each treatment and were dissected into halves. Nodules that appear pinkish, reddish or wine in colour are considered effective as they contain leghemoglobin which is an important enzyme for nitrogen fixation in rhizobia [TAJIMA & al. 2007]. At 50% tasseling, the shoot of the maize plants were also cut at soil surface and put inside a paper envelope. All the enveloped plant materials were then oven dried at 70 °C for 48 hours to obtain their dry weight and weighed on a Mettler balance.

The dried shoots (cowpea and maize) were then used for the determination of total N in shoot for N-uptake and N fixation estimation using the N-difference method. Micro Kjeldahl's oxidation method involving digestion and distillation was used for the determination of the N in the shoot of the cowpea plant and the reference crop. N fixed was calculated using the formula:

$$\text{Total N in plants} = \frac{\text{Dry weight} \times \%\ \text{N in plants}}{100}$$

N fixed (NDFA) = Total N in legume – Total N in reference crop [YAKUBU & al. 2010].
The total N in seeds were also determined using the Micro Kjeldahl's oxidation method and the % N was multiplied by a factor of 6.25 to obtain the protein content.

Data collected were subjected to analysis of variance using the General Linear Model procedure of Minitab software. Differences between the means were separated using Least Significant Difference (LSD) at 5% level of probability.

Results

Nodule number

The nodule number was significantly affected by rhizobial inoculation and year but the varieties were similar in response in respect of nodule number (Table 1). Figure 1 shows the interaction between year and inoculation on nodule number. In 2016, there was no significant difference between the number of nodules recorded in the inoculated and uninoculated plants. However, plants inoculated with BR 3262 produced significantly higher number of nodules than plants fertilized with 90 kg N ha^{-1}. In 2017 however, inoculation with BR 3267 significantly increased nodule number by 27% over the control. Plants fertilized with 90 kg N ha^{-1} had the least number of nodules in the two years.

Nodule dry weight

The nodule dry weight was significantly influenced by rhizobial inoculation ($P < 0.01$) and interaction between year, inoculation and variety ($P < 0.05$) (Table 1). Table 2 shows the interaction between year, inoculation and variety in respect of nodule dry weight. Inoculated and uninoculated IT93K-452-1 plants had similar nodule dry weight in both years. However, the uninoculated plants and plants inoculated with BR 3267 had the highest nodule dry weight which were significantly higher than the values recorded in plants fertilized with 90 kg N ha^{-1} in 2016 and 2017 respectively. Similarly, in IT99K-573-1-1 variety, there was no significant difference between the nodule dry weight of the inoculated and uninoculated plants in 2016 but in 2017, the uninoculated plants and plants inoculated with BR 3267 had significantly heavier nodules than the same variety inoculated with BR 3262. There was no

significant difference between nodule weight of the inoculated and uninoculated TVx 3236 plants in both years. Plants fertilized with 90 kg N ha^{-1} had the lightest nodules across the three varieties in the two years.

Nodule effectiveness

Highly significant difference ($P < 0.001$) exists between the inoculation treatments in respect of percentage of effective nodules and the interaction between year, inoculation and variety was equally significant (Table 1). The interaction between year, inoculation and variety in respect of nodule effectiveness is presented in Table 3. There was no significant difference between the percentage of effective nodules of inoculated, uninoculated and 90 kg N ha^{-1} fertilized IT93K-452-1 plants in 2016. Similarly in 2017, there was no significant difference between the % of effective nodules recorded in inoculated and uninoculated IT93K-452-1 plants but nodules obtained from plants fertilized with 90 kg N ha^{-1} had significantly lower % of effective nodules than the uninoculated plants and those inoculated with BR 3262.

IT99K-573-1-1 plants inoculated with BR 3262 had significantly higher percentage of effective nodules (90%) than plants inoculated with BR 3267 (40%) and plants fertilized with 90 kg N ha^{-1} (46.67%) in 2016. In 2017, the uninoculated plants produced significantly higher number of effective nodules than plants fertilized with 90 kg N ha^{-1}. Inoculated and uninoculated TVx 3236 plants had similar number of effective nodules which were significantly higher than the values recorded in plants fertilized with 90 kg N ha^{-1} in 2016. In 2017, the percentage of effective nodules recorded in the inoculated, uninoculated and 90 kg N ha^{-1} fertilized TVx 3236 plants were similar (Table 3).

Nitrogen fixation

Nitrogen fixation was significantly affected by rhizobial inoculation and the varieties also differ significantly in their ability to fix nitrogen ($P < 0.01$). Similar trend were observed in respect of N-fixation in the two years (Table 1). The uninoculated plants fixed significantly higher nitrogen than plants inoculated with BR 3262 and BR 3267 which fixed similar amount of N. (Figure 2a). IT93K-452-1 and IT99K-573-1-1 plants fixed similar amount of N which were significantly higher than the amount fixed by TVx 3236 plants (Figure 2b).

Nitrogen uptake

Highly significant difference exists ($P < 0.01$) between the inoculation treatments and varieties in respect of N-uptake but the trend observed in the two years were similar (Table 1). N-uptake was significantly higher in the uninoculated plants and plants fertilized with 90 kg N ha^{-1} than the inoculated plants (Table 4). IT93K-452-1 and IT99K-573-1-1 plants had similar N-uptake values which were significantly higher than the values recorded in TVx 3236 plants (Table 5).

Shoot biomass yield

The shoot biomass yield was significantly affected ($P < 0.01$) by rhizobial inoculation and variety and similar trends were observed in the two years (Table 1). The control plants and plants fertilized with 90 kg N ha^{-1} had similar shoot biomass yield which were significantly higher than the value recorded in plants inoculated with BR 3267 but at par with the biomass of plants inoculated with BR 3262 (Table 4). IT93K-452-1 and IT99K-

573-1-1 plants had similar shoot biomass yield values which were significantly higher than the values recorded in TVx 3236 plants (Table 5).

Seed protein

The seed protein content was significantly influenced ($P < 0.01$) by rhizobial inoculation and variety. The trend observed in the two years were similar (Table 1). The control plants partitioned significantly lesser quantity of N to the seeds as the percentage protein recorded in seeds of the control plants were significantly lesser than the value recorded in the inoculated plants and plants fertilized with 90 kg N ha^{-1}. IT93K-452-1 variety had the highest seed protein content and the values recorded in IT99K-573-1-1 and TVx 3236 were at par (Table 5).

Grain Yield

The grain yield was significantly influenced ($P < 0.05$) by rhizobial inoculation but the varieties were not significantly different in their grain yield (Table 1). Plants fertilized with 90 kg N ha^{-1} had the highest grain yield (1761.2 kg ha^{-1}) which was at par with the value recorded in the uninoculated plants (1479.60 kg ha^{-1}) but significantly higher than the values recorded in the inoculated plants (1320.1-1338.0 kg N ha^{-1}) (Figure 3).

Discussion

The significantly higher biomass and grain yield obtained in plants fertilized with 90 kg N ha^{-1} than the inoculated plants could be an indication that the symbiosis of the inoculant strains with the varieties were not fixing enough nitrogen that the plant need for optimum productivity. In the experiment carried out by FENING & al. (2001) to assess the potential of improving N fixation in cowpea in Ghananian soils, it was reported that all the 45 cowpea cultivars used showed significant response to increasing N fertilizer application to a particular level, indicating that N fixation was not providing the plants with sufficient N for maximum growth and yield.

The variation in the nodulation and effectiveness of the nodules of the varieties as a result of rhizobial inoculation as well as the variation in the N-fixation capability of the strains used agrees with the assertion of FALL & al. (2003) and who reported that effective symbiotic relationship depends on the cultivar and the rhizobium strain. PULE-MEULENBERG & al. (2010) similarly reported that cowpea genotypes differed significantly in growth, N content and N fixation and that the 18 bradyrhizobia strains the authors isolated differed significantly in their N_2 fixing efficiency. The effective nodules recovered from the inoculated plants in this study indicates that the strains used were equally effective and they can successfully form symbiosis with cowpea in the study area but they were not as effective as the indigenous strains resident in the soil of the study area considering the better performance recorded in the uninoculated plants.

The better performance exhibited by the uninoculated plants in respect of N-fixed, biomass and grain yield than the inoculated plants is in agreement with the report of ALIYU & al. (2013) who determined the response of grain legumes to rhizobial inoculation in two savanna soils of Nigeria (Eutric Cambisols and Rhodic Nitisols). The authors reported that cowpea did not respond to rhizobial inoculation and attributed the poor response to the high rhizobial population density in the experimental soils. FENING & DANSO (2002) reported that there has been low response of cowpea to inoculation probably due to high incidence of

cowpea bradyrhizobia in most tropical soils and the promiscuous nature of cowpea. Cowpea appears to be the most promiscuous legume which has been intensively studied, nodulating with a wide range of fast and slow growing rhizobia [MPEPEREKI & al. 2000].

Though the inoculated plants did not perform better than the uninoculated plants in respect of the productivity in this study, the inoculated plants partitioned significantly higher nitrogen to their seeds thereby having higher protein content in the seeds than the uninoculated plants. This concurs with the report of VOLLMANN & al. (2011) and BEJANDI & al. (2012) who reported an increase in seed protein content as a result of rhizobial inoculation.

The grain yield obtained in this study (1338 -1761 kg ha^{-1}) though not up to the potential yield of the varieties (2000-2600 kg ha^{-1}) (Nigerian seed portal, 2018) is a big improvement on the average of 350-450 kg ha^{-1} obtained on farmers' fields in Nigeria [KAMAI & al. 2014; OMOTOSO, 2014]. This could be a pointer to the fact that ineffectiveness of our indigenous rhizobial strains is not the major problem responsible for the low yield obtained on farmers' fields in Nigeria.

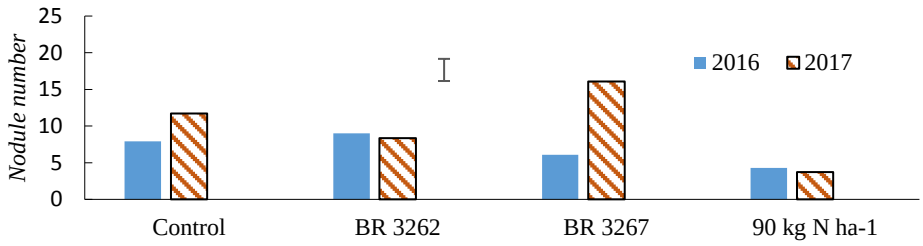

Figure 1. Nodule number of cowpea as affected by rhizobial inoculation across two years, I-LSD (P = 0.05)

Table 1. Mean square values for response of cowpea varieties to rhizobial inoculation

Source of variation	Nodule number (per plant)	Nodule dry weight (g/plant)	Nodule effectiveness (%)	N-fixed (kg ha^{-1})	N-uptake (mg/plant)	Shoot biomass yield (t ha^{-1})	Grain yield (t ha^{-1})	Seed protein content (%)
Year (Y)	177.00**	26.53	34.72	1174.09	118537.14	0.06	255477.26	16.52
Inoculation(I)	170.02**	6190.91***	2656***	7833.27**	248255.45**	2.32**	748178.86*	119.96**
Variety (V)	42.44	1464.97	816.67	8741.88**	212689.40**	3.08**	248133.24	47.17**
Y x I	113.37**	747.78	182.87	646.67	101792.81	0.37	385828.72	8.97
Y x V	30.50	874.67	372.22	1346.00	65193.67	0.49	2844856.43	13.12
V x I	5.09	57.57	577.78	1045.24	17723.06	0.29	326999.46	12.19
Y x V x I	45.59	2249.11*	1081.48**	748.83	42551.37	0.60	248870.94	6.08

*, **, ***- significant at 5, 1 and 0.1 percent respectively

Table 2. Nodule dry weight of cowpea as affected by rhizobial inoculation across two years

Rhizobial inoculation	Variety	Nodule dry weight (mg/plant)	
		2016	2017
Control	IT93K-452-1	60.93abc	49.31a-e
	IT99K-573-1-1	31.2b-f	73.07ab
	TVx 3236	52.11a-d	21.4c-f
BR 3262	IT93K-452-1	48.93a-e	37.46a-f
	IT99K-573-1-1	70.98ab	20.1c-f
	TVx 3236	35.85a-f	35.85a-f
BR 3267	IT93K-452-1	33.3b-f	76.17ab
	IT99K-573-1-1	36.74a-f	80.66a
	TVx 3236	65.52abc	23.31c-f
90 kg N ha^{-1}	IT93K-452-1	13.7def	22.8c-f
	IT99K-573-1-1	20.0c-f	1.82f
	TVx 3236	4.5ef	4.63ef
SE±		9.34	

Table 3. Percentage of effective nodules as affected by rhizobial inoculation across two years

Rhizobial inoculation	Variety	Effective nodules (%)	
		2016	2017
Control	IT93K-452-1	60.00b-f	66.67a-e
	IT99K-573-1-1	63.3a-f	66.7a-e
	TVx 3236	80ab	66.7a-e
BR 3262	IT93K-452-1	73.33a-d	70.00a-d
	IT99K-573-1-1	40.00e-h	56.67b-g
	TVx 3236	90.00a	70a-d
BR 3267	IT93K-452-1	56.67b-g	50.00c-g
	IT99K-573-1-1	90.00a	56.67b-g
	TVx 3236	63.3a-f	70.00a-d
90 kg N ha^{-1}	IT93K-452-1	50.00c-g	36.67fgh
	IT99K-573-1-1	46.67d-h	30.00gh
	TVx 3236	20.00h	70a-d
SE±		10.20	

Means followed by similar alphabets are not significantly different at P = 0.05 using LSD

Table 4. N-uptake, shoot biomass yield and seed protein content of cowpea as affected by rhizobial inoculation

Treatments Rhizobial inoculation	N-uptake (mg/plant)	Shoot biomass yield (t ha^{-1})	Seed protein content (%)
Control	617.45	2.35	22.05
BR 3262	475.77	1.95	23.80
BR 3267	406.74	1.64	24.00
90 kg N ha^{-1}	656.18	2.40	24.06
LSD(0.05)	136.87	0.45	1.75

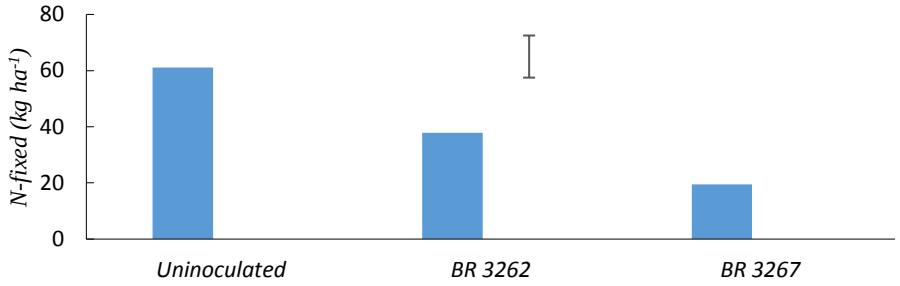

Figure 2a. Effect of rhizobial inoculation on N fixation of cowpea
I- LSD (P = 0.05)

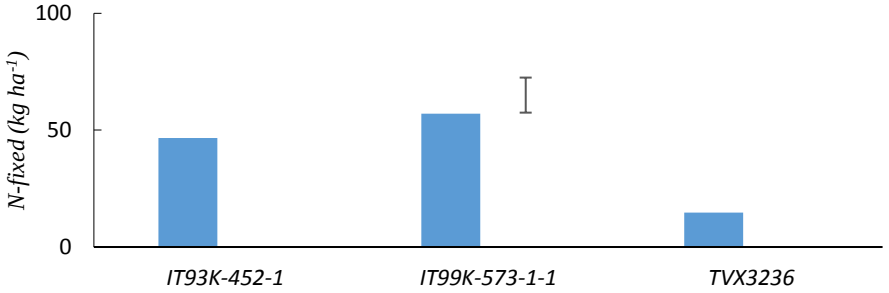

Figure 2b. Varietal effect of cowpea on N-fixation
I-LSD (P = 0.05)

Table 5. Varietal effect of cowpea on N-uptake, shoot biomass yield and seed protein content of cowpea

Variety	N-uptake (mg/plant)	Shoot biomass yield (t ha^{-1})	Seed Protein content (%)
IT93K-452-1	617.20	2.26	24.97
IT99K-573-1-1	565.37	2.32	23.27
TVx 3236	434.53	1.67	22.19
LSD(0.05)	118.53	0.39	1.51

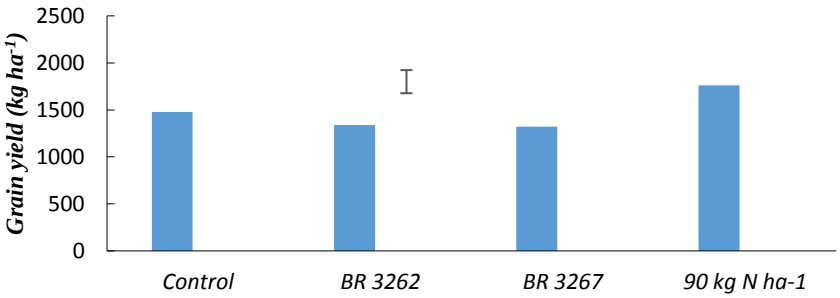

Figure 3. Effect of rhizobial inoculation on grain yield of cowpea
I- LSD (P = 0.05)

Conclusions

The BR strains tested in this study were not more effective than the indigenous strains resident in the soil of the study area in improving the N-fixation and productivity of cowpea. More cowpea rhizobia strains should be developed and tested for their effectiveness. Furthermore, other factors such as poor management practices, inappropriate planting date and the cropping system adopted, use of poor quality seeds and unimproved varieties which contribute to low yield of cowpea in the study area should be worked on.

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EFFECTS OF *GMELINA ARBOREA* BARK AND *AZADIRACHTA INDICA* LEAF POWDERS ON GERMINATION AND SEEDLING VIGOUR OF *CORCHORUS OLITORIUS* (JUTE MALLOW)

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Abstract: Effects of plants extract powders is the application of these extracts is to improved and increase the germination, emergence of the seedlings. Efficacy of *Gmelina arborea* bark and *Azadirachta indica* leaf powders on the germination and seedling vigour of *Corchorus olitorius* (jute mallow) was evaluated. Top loam soil was collected from Agriculture Research farmland of Ibrahim Badamasi Babangida University Lapai, Niger State, Nigeria and sterilized at 50 °C for an hour. *Gmelina arborea* bark and *Azadirachta indica* leaf were obtained from *Gmelina arborea* and *Azadirachta indica* leaves were also collected from the farmland and pulverized into powder form. Two kilograms (2 kg) of sterile soil samples in twenty one (21) polythene bags were amended with *Gmelina arborea* bark powder and *Azadirachta indica* leaf powders at different concentrations (100 g, 150 g, and 200 g) each in triplicates. Soil samples contained in three of the polyethylene bags were left un-amended to serve as control. *Corchorus olitorius* seeds was procured from the harvested farmland and were planted and raised in each polythene bag for a period of twelve (12) weeks. *Azadirachta indica* leaf powder had a significant impact on the growth of *Corchorus olitorius* (shoot length = 26.52 cm; root collar diameter = 0.33 cm, number of leaves = 21) at $p < 0.05$ compared to the control group (20.60 cm, 0.24 cm and 16 respectively) and its effects improved with increase in concentration. *Gmelina arborea* bark powder also had a less significant impact on the plant growth parameters (6.45 cm, 0.14 cm and 9 respectively) compared to the control. Its effect decreased with increase in concentration. *Azadirachta indica* leaf powder is a much better and more effective organic amendment than *Gmelina arborea* bark powder and could be employed by *Corchorus olitorius* farmers to improve soil fertility and crop yield.

Keywords: *Azadirachta indica*, *Corchorus olitorius*, germination, *Gmelina arborea*, seedling vigour.

Introduction

Jute mallow (*Corchorus olitorius* L.) is a popular vegetable in West Africa. *Corchorus olitorius* is commonly known as Long-fruited jute, Tossa jute, Jute mallow, Jew's mallow, Bush okra and West African sorrel [SEMRA & al. 2007], wild okra [NDLOVU & AFOLAYAN, 2008], Egyptian Spinach and Molokhiya [YOUSSEF & al. 2014]. The Yoruba of Nigeria call it "Ewedu", the Hausa people and their Fulbe neighbours call it "Rama". It is called "Ayoyo" in Nupe. It is dicotyledonous fiber-yielding plant of the genus *Corchorus* [ISLAM, 2013]. It is an annual herb with sparsely branching stems, often growing as tall as 4 m.

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Corchorus olitorius is rich nutritionally and serves as a source of nutrients for individuals that include it in their diet. It was observed in different literatures that this green, leafy vegetable is rich in beta-carotene for good eyesight, iron for healthy red blood cells, calcium for strong bones and teeth, and vitamin C for smooth, clear skin, strong immune cells, and fast wound-healing [ISLAM, 2013]. *Corchorus olitorius* possesses properties that can boost our immune system to help prevent diseases such as cancer, premature aging, osteoporosis, fatigue, high blood pressure and anemia.

Gmelina arborea Roxb. is a fast growing tree which grows on different localities and prefers moist fertile valleys with 750-4500 mm rainfall. Neem is a medium sized to large evergreen tree with straight trunk and dark brown to grey bark. It can grow up to 30 m in height and 70 cm in diameter. This tree is grown all over the northern states of Nigeria to provide shade along the major high- ways and the fruit is fed to livestock. *Gmelina arborea* fruits possess antibacterial activity. It has been found to be effective against some pathogenic bacteria involved in wounds and burns [AKYALA & al. 2013].

SHANKAR & al. (2014) analyzed allelochemicals effect of *Gmelina arborea* on *Vigna mungo* and *Vigna radiata*. The presence of allelopathic compounds such as polyphenols and terpenoids was analyzed. The extract inhibited the proteolytic enzyme important for seed germination. The extract inhibited the germination, seedling growth, and total protein content of both test crops. SHANKAR & al. (2014) analyzed the effect of allelochemicals from leaf leachates of *Gmelina arborea* on Inhibition of some essential seed germination enzymes in *Vigna mungo* (Green gram), *Vigna radiata* (Black gram), *Cajanus cajan* (Red gram), and *Cicer arietinum* (Chickpea).

Azadirachta indica A. Juss. is widely planted and naturalized in semiarid areas throughout Asia and Africa. It belongs to the family Meliaceae and is related with Chinaberry – *Melia azedarach* [NTALLI & al. 2010]. The leaves are glabrous and divided into leaflets with a sharp bitter taste. The bark is fairly thick, furrowed longitudinally or obliquely and is reddish brown inside and dark grey outside. The tree produces flowers throughout the year but fruits during the cold harmattan.

Adding neem leaves as organic matter increases the activity and number of soil organisms. Over time, a well-amended soil will supply more of the nutrients the plants require, which will reduce fertilizer requirements [KHAN & al. 2012]. The mode of action of organic amendments leading to plant disease control and stimulation of microorganisms is complex and dependent on the nature of the amendments [DIACONO & MONTEMURRO, 2011]. Azadirachtin, a phytochemical found in Neem repels more than 200 insect species, including such pests as locusts, gypsy moths, and cockroaches.

Poor crop plants germination and growth due to diseases, poor environmental conditions and poor soil conditions are of concern to every farmer. Jute (*Corchorus olitorius)* is a very important crop cultivated and consumed in Nigeria known for the sliminess of its leaves and tends to lose its value due to poor seed germination and seedling vigour. The research was carried out to determine the effects of *Gmelina arborea* bark and *Azadirachta indica* leaf amended soil on the germination and seedling vigour of jute mallow (*Corchorus olitorius).*

Materials and methods

Study area

The experiment was conducted at Department of Biological Science, Faculty of Natural Science, Ibrahim Badamasi Babangida University Lapai Niger State situated in the

middle belt (North Central) Zone of Nigeria and lies along latitude 80^0 and 110^0, 30^0 East [AKINTAYO & al. 2011]. The experimental area is under subtropical climate, characterized by dry/harmattan and rainy season; the rainy season begins from March/April and ends October, the dry/harmattan season occurs between November and March.

Samples collection

Seeds of jute mallow, Fresh *Gmelina arborea* bark and *Azadirachta indica* leave samples were collected from *G. arborea* and *A. indica* trees of the Agriculture Research Farm of Ibrahim Badamasi Babangida University Lapai, Niger State, Nigeria.

Preparation of powdered samples of *G. arborea* bark and *A. indica* leaves

Each fresh sample was rinsed separately with clean tap water to make it dust and debris free. Then the samples were separately spread evenly and dried in a shady condition for four-seven days until they became dry while still retaining their colour [NAHAK & SAHU, 2010]. Powders were prepared by pulverizing the dried bark and leaf samples with the help of a mortar and pestle. Then the powders were passed through a sieve to obtain finer and more uniform particles. The powders were preserved in airtight condition in plastic buckets tightly covered till their use in soil amendment [MAMUN & SHAHJAHAN, 2011].

Soil sterilization

Soil sample was freed from debris and sterilized in a dry oven at 50 °C for an hour in the laboratory. Sterilized soil sample was allowed to cool for some minutes.

Amendment of soil

Two kilograms of sterilized soil was measured into each of the 21 polythene bags containing bored holes at the bottom to allow for drainage during the course of the experiment. The polythene bags were randomly divided into seven groups each containing three polythene bags. The seven groups are described below:

I. The soil samples in the first group (*G. arborea* 100 g I, II and III) were mixed with 100 g *G. arborea* powder weighed with a weighing machine.
II. The second group (*G. arborea* 150 g I, II and III) were mixed with 150 g *G. arborea* powder.
III. The third group (*G. arborea* 200 g I, II and III) were mixed with 200 g *G. arborea* powder.
IV. The fourth group (*A. indica* 100g I, II and III) were mixed with 100 g *A. indica* powder.
V. The fifth group (*A. indica* 150 g I, II and III) were mixed with 150 g *A. indica* powder.
VI. The sixth group (*A. indica* 200g I, II and III) were mixed with 200 g *A. indica* powder.
VII. The seventh group (Control I, II and III) were left unmixed with any of the powdered samples.

The soil in all the three groups were supplied with water daily and allowed to stand for seven days [OLOGUNDUDU & al. 2013].

Raising of *Corchorus olitorius* seeds

Corchorus olitorius seeds were broadcast on soil samples contained in all twenty-one polythene bags on the eight day after amendment. Each polythene bag was supplied with water on a three day basis when rainfall became less frequent until the plants reached their fruiting stage. Seedlings were thinned to four (4) per bag ten days after planting.

Growth parameters

Seedling emergence test

Eight seeds of *Corchorus olitorius* were broadcast on each of the amended soil replicates as well as the control. All soil samples were watered regularly and number of seedlings that emerged each day from all polythene bags was recorded for a period of ten days. The percentage seedling emergence per amended soil was calculated using the formula of OKUNOMO (2010) below:

$$\%\ \text{Seedling emergence per soil sample} = \frac{\text{Number of emerged seedlings}}{\text{Total number of seeds planted}} \times \frac{100}{1}$$

Seedling morphology

The morphological characteristics of the seedlings were observed and recorded weekly for a period of twelve (12) weeks. The shoot length of seedlings was measured with a centimeter ruler and a tape. The root collar diameter was measured with the aid of a Vanier caliper and recorded in centimeters. Dickson quality index was calculated using one quality indices (slenderness index = shoot length/root collar diameter) given by DICKSON & al. (2000). The leaves, flowers and fruits were physically counted weekly.

Results

Seedling emergence

The result of the seedling emergence test carried out for ten (10) days showed that soil amended with 200 g *G. arborea* bark powder had the highest percentage of emerged seedlings (100%), soil amended with 200 g *A. indica* leaf powder had the second highest percentage of emerged seedlings (87.5%), soils amended with 100 g *G. arborea* bark, 150 g *G. arborea* bark, 100 g *A. indica* leaf, as well as the control had 75% emerged seedlings while 150 g *A. indica* had the least percentage seedling emergence (62.5%).

Table 1. Percentage Seedling Emergence

Soil sample	Percentage Seedling Emergence (%)
Control	75
100 g *G. arborea*	75
150 g *G. arborea*	75
200 g *G. arborea*	100
100 g *A. indica*	75
150 g *A. indica*	62.5
200 g *A. indica*	87.5

Effects of the organic amendments on shoot length, root collar diameter and Dickson quality index of *Corchorus olitorius*

The results showed that soil amended with 100 g *Gmelina arborea* bark powder produced plants with the highest shoot length (17.11 cm) compared to 150 g *G. arborea* which had the second highest shoot length (15.9 cm) and 200 g *G. arborea* which had the least shoot length (6.45 cm). When compared with the control, *G. arborea* bark amended soil at all concentrations yielded plants with lower plant shoot length than the control (20.60 cm).

However there was no significant difference at $p < 0.05$ between the different concentrations of *Gmelina arborea* bark as shown in Table 2.

The results indicated that soil amended with 100 g *Gmelina arborea* bark powder produced plants with the highest root collar diameter (0.23 cm) compared to 150 g *G. arborea* which had the second highest root collar diameter (0.21 cm) and 200 g *G. arborea* which had the least root collar diameter (0.14 cm). When compared with the control however, *G. arborea* bark amended soil at all concentrations yielded plants with lower plant root collar diameter than the control (0.24). There was no significant difference at $p < 0.05$ between the different concentrations of *Gmelina arborea* bark as shown in Table 2.

Also, the results showed that that soil amended with 100 g *Gmelina arborea* bark powder produced plants with the highest Dickson quality index (64.04 cm) compared to 150 g *G. arborea* which had the second highest Dickson quality index (59.88 cm) and 200 g *G. arborea* which had the least root collar diameter (39.85 cm). When compared with the control, *G. arborea* bark amended soil at all concentrations yielded plants with lower Dickson quality index than the control (72.45 cm). There was a significant difference at $p < 0.05$ between the different concentrations of *Gmelina arborea* as shown in Table 2.

The results showed that soil amended with 200 g *Azadirachta indica* leaf powder produced plants with the highest shoot length (26.52 cm) compared to 150 g *A. indica* which had the second highest shoot length (25.95 cm) and 100 g *A. indica* which had the least shoot length (23.18 cm). When compared with the control however, *Azadirachta indica* leaf amended soil at all concentrations yielded plants with higher plant shoot length than the control (20.60 cm). There was however no significant difference at $p < 0.05$ between the different concentrations of *Azadirachta indica.*

The results indicated that that soil amended with 200 g *Azadirachta indica* leaf powder produced plants with the highest root collar diameter (0.33 cm) compared to 150 g *A. indica* which had the second highest root collar diameter (0.31 cm) and 100 g *A. indica* which had the least root collar diameter (0.29 cm). When compared with the control however, *Azadirachta indica* leaf amended soil at all concentrations yielded plants with higher plant root collar diameter than the control (0.24). There was however no significant difference at $p < 0.05$ between the different concentrations of *Azadirachta indica*.

Also, the results showed that that soil amended with 150 g *Azadirachta indica* leaf powder produced plants with the highest Dickson quality index (73.40 cm) compared to 200 g *A. indica* which had the second highest Dickson quality index (72.48 cm) the next higher Dickson quality index recorded was in control (72.45 cm) while the least was in soil amended with 100 g *A. indica* (69.85 cm). There was however no significant difference at $p < 0.05$ between the different concentrations of *Azadirachta indica.* This is demonstrated in Table 2 below:

Table 2. Effects of different concentrations of *Gmelina arborea* bark and *Azadirachta indica* leaf powders on shoot length, root collar diameter and Dickson quality index of *Corchorus olitorius*

TREATMENT	SHOOT LENGTH	ROOT COLLAR DIAMETER	DICKSON QUALITY INDEX
CONTROL	20.60 ± 4.90[a]	0.24 ± 0.03[a]	72.45 ± 7.21[a]
100 g *G. arborea*	17.11 ± 4.31[a]	0.23 ± 0.03[a]	64.04 ± 7.45[a]
150 g *G. arborea*	15.9 ± 3.89[a]	0.21 ± 0.02[a]	59.88 ± 7.16[a]
200 g *G. arborea*	6.45 ± 1.13[a]	0.14 ± 0.01[a]	39.85 ± 4.14[b]
100 g *A. indica*	23.18 ± 4.37[a]	0.29 ± 0.04[a]	69.85 ± 5.72[a]
150 g *A. indica*	25.95 ± 5.16[a]	0.31 ± 0.04[a]	73.40 ± 6.19[a]
200 g *A. indica*	26.52 ± 5.32[a]	0.33 ± 0.05[a]	72.48 ± 5.74[a]

Values are MEAN ± SEM. Values with same superscript down the column are not statistically significant at ($p < 0.05$).

Effect of *Gmelina arborea* bark powder on number of leaves, flowers and fruits produced

The results showed that soil amended with 100 g *G. arborea* bark powder and control yielded the highest number of leaves (16), this was followed by 150 g *G. arborea* (14), least number of leaves was recorded in soil amended with 200 g *G. arborea* (9).

The control produced the highest number of flowers (2), soil amended with 100 g and 150 g *G. arborea* bark yielded equal number of flower (1), while 200 g *G. arborea* bark amended soil produced no flowers (0).

Highest number of pods produced was recorded in 100 g *G. arborea* bark amended soil and control (4), higher number of pods was recorded in 150 g *G. arborea* amended soil (3) and the least number of pod occurred in 200 g *G. arborea* amended soil (0). This is demonstrated in Table 3.

Effect of *Azadirachta indica* leaf powder on number of leaves, flowers and fruits produced

The results showed that soil amended with 200 g *Azadirachta indica* leaf powder yielded the highest number of leaves (21), this was followed by 150 g *A. indica* (20), and soil amended with 100 g *A. indica* (17). The least number of leaves was recorded in control (16).

The control produced the highest number of flowers (2), soils amended with 100 g 150 g and 200 g *A. indica* leaf powder yielded equal number of flower (1). Highest number of pods produced was recorded in 150 g *A. indica* leaf powder amended soil and control (4), higher number of pods was recorded in 200 g *A. indica* amended soil (3) and the least number of pods occurred in 100 g *A. indica* amended soil (2) as shown in Table 3.

Generally, results showed that *Corchorus olitorius* plants in soil samples amended with 200 g *Azadirachta indica* leaf powder had the highest number of leaves (21) with 200 g *G. arborea* bark powder amended soil having the lowest number of leaves (9). It was also observed that the control produced the highest number of flowers (2) as compared to soil samples amended with varied concentrations of *Gmelina arborea* bark and *Azadirachta indica* leaf powders. The lowest number of flower production was observed in soil amended with 200 g *Gmelina arborea* (0). Highest number of pods was produced by control, 100 g *Gmelina arborea*, and 150 g *Azadirachta indica* (4), while the lowest pod production was

observed in 200 g *Gmelina arborea* bark amended soil (0). Number of leaves, flowers and pod per plant in all soil samples is demonstrated in Table 4 below.

Table 3. Number of leaves, flowers and fruits produced after twelve weeks of planting

SOIL SAMPLE	NO. OF LEAVES	NO. OF FLOWERS	NO. OF PODS
100 g *G. arborea*	16	1	4
150 g *G. arborea*	14	1	3
200 g *G. arborea*	9	0	0
100 g *A. indica*	17	1	2
150 g *A. indica*	20	1	4
200 g *A. indica*	21	1	3
Control	16	2	4

Discussion

The seedling emergence test revealed that soil amended with 200 g *Gmelina arborea* bark had the highest germination percentage (100%). This finding disagrees with the finding of SHANKAR & al. (2014) that allelochemicals of *Gmelina arborea* are of inhibitory type to germination of *Vigna mungo* (Green gram), *Vigna radiata* (Black gram), *Cajanus cajan* (Red gram) and *Cicer arietinum* (Chickpea).

Azadirachta indica (200 g) amended soil produced plants with longest shoots (26.52 cm); this was followed by 150 g *A. indica* amended soil (25.95 cm) and lastly 100 g *A. indica* amended soil yielded plants with shortest shoot lengths (23.18 cm) when compared with the other two. This finding agrees with the finding of CHANNAL & al. (2000), study of allelopathic effect of leaf extracts from *Azadirachta indica*, *Acacia arabica*, *Eucalyptus tereticornis*, *Tamarindus indica*, *Tectona grandis*, *Samanea saman* and *Syzygium cumini*, all applied at 5 and 10% concentration, on seed germination, vigour index, seedling length, and seedling dry matter of sorghum and rice. Irrespective of concentration, all tree leaf extracts promoted germination in sorghum to (15-32% over the control), while only *Azadirachta indica* and *Acacia arabica* increased the germination in rice (3.50-3.81% over the control).

In contrast to this, 100 g *Gmelina arborea* amended soil yielded plants with highest shoot length (17.11 cm) followed by 150 g *G. arborea* amended soil (15.9 cm) and lastly, 200 g *G. arborea* amended soil yielded plants with the shortest length of shoot (6.45 cm). This agrees with the finding of SHANKAR & al. (2014) that *G. arborea* extracts inhibited seedling growth of *Vigna mungo* and *Vigna radiata*. It could be the result of the presence of allelochemicals in the amendment that were able to inhibit the synthesis of growth hormones which in turn prevented cell division and differentiation to increase the length of the shoot [ABUGRE & al. 2011]. Allelochemicals are not only species specific but organ specific [MARHAJAN & al. 2007].

Different allelochemicals have different sites of action in a plant. Thus, the sensitivity to allelochemicals and the extent of inhibition varies with species and organs. *Corchorus olitorius* plants in all replicates of soil amended with different concentrations of *Azadirachta indica* had longer shoots than the control and *G. arborea* amended soils. Plants from the control were generally longer than those in all *G. arborea* amended soils.

Also, the results indicated that 200 g *Azadirachta indica* amended soil produced plants with the highest number of leaves (21); this was followed by 150 g *A. indica* amended soil and lastly (20), 100 g *A. indica* amended soil yielded the lowest number of leaves compared to the other two (17). The results of *Gmelina arborea* on the other hand showed that soil amended with 100 g *G. arborea* yielded plants with the highest leaf number (16); followed by 150 g *G. arborea* amended soil (14) and 100 g *G. arborea* amended soil yielded plants with the least number of leaves (9).

The control produced the highest number of flowers (2); 100 g and 150 g *G. arborea* bark as well as 100 g, 150 g and 200 g *Azadirachta indica* leaf amended soils yielded equal number of flower (1) while 200 g *G. arborea* produced no flowers.

Highest number of pods produced was recorded in 100 g *G. arborea* amended soil, 150 g *A. indica* amended soil and control (4); higher number of pods was recorded in 150 g *G. arborea* amended soil and 150 g *A. indica* amended soil (3); this was followed by 100 g *A. indica* amended soil (2) and the least number of pod occurred in 200 g *G. arborea* amended soil (0).

Conclusions

Azadirachta indica leaf powder at all concentration has a significant effect on the germination and seedling vigour of *Corchorus olitorius* than control and *Gmelina arborea* bark powder. However *G. arborea* bark powder at lower concentrations (less than 100 g to 2 kg of soil) would yield more significant effect on germination and seedling vigour of *C. olitorius* than higher concentrations. Higher concentrations of *Azadirachta indica* leaf powder yields more significant effect on germination and seedling vigour of *Corchorus olitorius* when compared with lower concentrations, control and *Gmelina arborea* bark.

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SEED GERMINATION, SEEDLING ESTABLISHMENT AND REINTRODUCTION INTO THE WILD OF THE HEMIPARASITIC MEDICINAL PLANT *MONOCHASMA SAVATIERI*

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Abstract: The ecology, seed germination, seedling establishment, and reintroduction of *Monochasma savatieri* Franch. ex Maxim, a traditional Chinese hemiparasitic medicinal herb, were studied by focusing on the distribution, climate, soil type, flowering and seed production, co-occurring vegetation, and pollinators. The distribution range of *M. savatieri* lies between 25°03'-29°12' N to 113°39'-120°27' E, based on our sampling. *M. savatieri* requires vernalization to induce flowering. Seed germination was enhanced by gibberellic acid at an optimum concentration of 500 µM. Other plant growth regulators, including indole-3-acetic acid and 6-benzyladenine, did not enhance seed germination. Low temperature (5 °C) storage was favorable to keep seed viability, which was negatively influenced by increasingly long storage periods. We also examined seedling development and transplantation to the field. Seedlings were interplanted with a moss *Hypnum plumaeforme*, until they developed true leaves. Developed plants were then transplanted into the wild alongside a host, *Gardenia jasminoides*, and 23% of the transplanted plants survived after 8 months.

Key words: distribution, host, light, plant growth regulators, seed viability, transplanting, vernalization.

Introduction

Monochasma savatieri Franch. ex Maxim (Orobanchaceae, Rhinantheae tribe) is a perennial medicinal herb with widely documented medicinal properties [YAHARA & al. 1986; KOHDA & al. 1989; LI & al. 2012; LIU & al. 2013]. Its distribution is limited to a very narrow geographic belt, namely Southeast China and Kyushu, Japan [YAMAZAKI, 1993; HONG & al. 1998]. Phylogenetic studies indicate that *M. savatieri* is likely to have originated in East China and nearby regions [HONG, 1986; BENNETT & MATHEWS, 2006]. The flora of Japan also documents that *M. savatieri* is likely to have been introduced from China [YAMAZAKI, 1993]. Recent years have witnessed the disappearance of wild populations of *M. savatieri* due to overexploitation and habitat destruction, so much so that it has already been listed as an endangered and threatened species in Japan (Environment Agency of Japan, 2000). An additional reason for its sensitivity to ecological changes is that *M. savatieri* is a hemiparasitic plant that requires a suitable host plant [ZHANG & al. 2015]. To better understand why *M. savatieri* is only distributed in a narrow geographic belt, and to better explain its ecological sensitivity, an investigation into ecological aspects, including climate, soil type, flowering and seed production, co-occurring vegetation, and pollinators is required to assess whether these factors influence its natural growth and reproductive traits. To date, only one preliminary study exists on the germination of *M. savatieri* induced by gibberellic acid (GA_3) [YANG, 2009]. However, the conditions required for seed germination

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and seedling development in nature are not known, nor are the influence of light, plant growth regulators and seed storage time on seed germination and seed viability clear. It is also not known whether seeds can be used to establish seedlings for transplantation back into the wild to replenish natural stands. In a bid to shed light on these unknowns, the main objectives of our study were to develop a seed-to-site protocol that would allow for the successful germination of *M. savatieri* seeds, the subsequent growth of seedlings, and the effective transplantation of seedlings back into the wild following sexual propagation. This study, conducted in Southeast China, also aimed to investigate the ecology of existing wild *M. savatieri* resources, and understand its vernalization requirements.

Materials and methods

Distribution of wild resources

Wild populations of *M. savatieri* were investigated between 2010 and 2014. Plants were collected from 14 sites in five provinces of Southeast China (Zhejiang, Jiangxi, Fujian, Hunan and Guangdong) based on the advice and guidance of local elders in these areas (Figure 1). Geographic coordinates were estimated from Google Earth. These sites show relatively abundant populations with a density of 10-50 mature individuals per 100 m^2 (10 m × 10 m). We focused our investigation on Jiangxi province because it is near to South China Botanical Garden (SCBG) (Figure 1O), Guangzhou, in Guangdong province. Data on the climatic conditions and geographic features of the areas studied were obtained from the China Statistical Yearbook (1996-2012). For each collection site, several habitat features, including topography, sunlight and moisture regimes, soil and vegetation type, pollinators, growth and sexual (seed) reproduction were investigated and recorded. A previous study used the same collection sites to confirm the hemiparasitic nature of *M. savatieri* [ZHANG & al. 2015]. Collected plants were identified at SCBG.

Natural growth and vernalization test

To investigate flowering and sexual reproduction, living plants (including hosts) were brought back to SCBG, Guangzhou from Pan'an, Zhejiang province (Figure 1A) on October 25, 2010 and November 5, 2011, from Zherong, Fujian province (Figure 1E) on March 16, 2011, from Shihan, Ganxian county, Jiangxi province (Figure 1H) on December 20, 2012 and from Tengtian, Yongfeng county, Jiangxi province (Figure 1D) on November 29, 2013. All plants (100 individuals/collection) were cultivated separately in plastic pots (10 cm high and 10 cm in diameter) containing loess and placed under a net-covered shelter that reduced natural light incidence by 80%. Southeast China is the natural distribution zone of *M. savatieri* [HONG & al. 1998], and includes a total of five provinces: Jiangsu, Zhejing, Fujian, Jiangxi and Hunan. Among them, Jiangsu, Zhejing, and Fujian belong to a subtropical monsoon climate zone that is influenced by Pacific Ocean circulation. Hunan province belongs to a mainland subtropical monsoon climate, which is affected by both East Asia monsoon circulation and cold and dry air in winter. Jiangxi province is located between Fujiang province and Hunan province, which belong to a subtropical hill and mountainous moist monsoon climate. All these areas belong to a monsoon climate and experience abundant rain in spring and summer [ZHU & al. 2011]. Guangzhou belongs to a subtropical monsoon climate with an average temperature of 21-22 °C and usually no frost in winter and a historically extreme minimum of -2 °C. During our test period, Guangzhou's lowest temperature was always above 0 °C. In Jiangxi province, both Nanchang (Northern Jiangxi)

and Ganzhou (Southern Jiangxi) belong to a subtropical hill and mountainous moist monsoon climate. Nanchang's average temperature is 16-17 °C with a historically extreme minimum of -15 °C; Ganzhou's average temperature is 18-19 °C with a historically extreme minimum of -6 °C, and is the southernmost point of *M. savatieri*'s natural distribution in Jiangxi province (Figure 1N). In these areas, in winter, low temperatures (-6 to -10 °C) usually last for one or two months but seldom drop below -15 °C. In summer, high temperatures usually do not exceed 35 °C because *M. savatieri* is distributed in mountain areas.

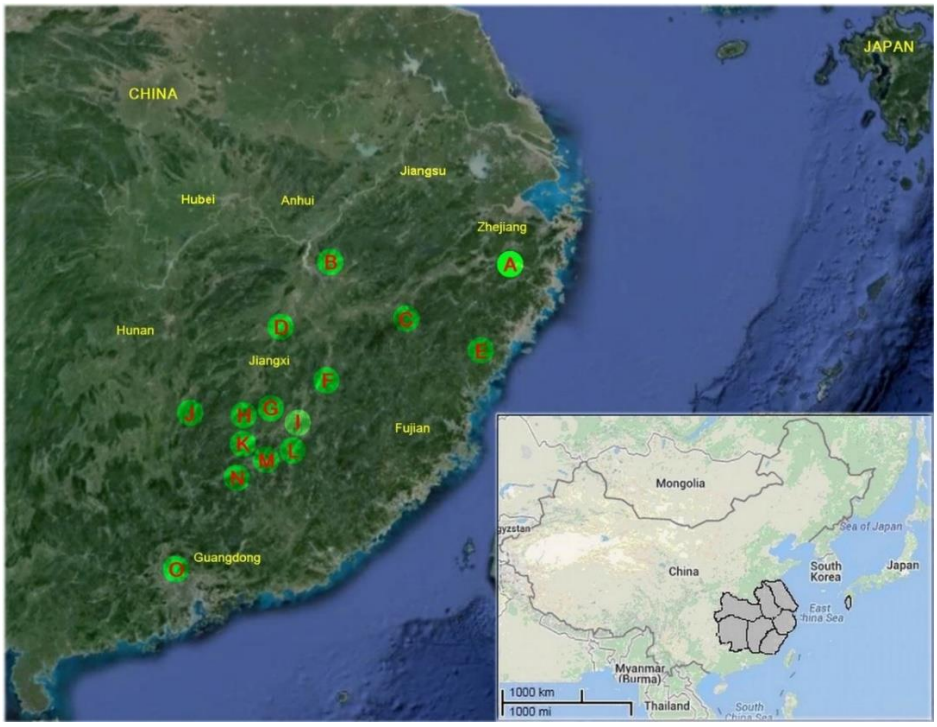

Figure 1. The distribution of *Monochasma savatieri* in East Asia. Areas in grey in the lower right side represent the *M. savatieri* distribution range according to YAMAZAKI (1993) and HONG & al. (1998). Green points with red uppercase letters show the distribution areas of *M. savatieri* in Southeast China based on this study. A: Pan'an (29°40'N 120°27'E); B: Boyang (29°10'N 116°42'E); C: Wuyishan (27°51'N 118°2'E); D: Yongfeng (27°25'N 115°27'E) (transplant location); E: Zherong (27°21'N 119°56'E); F: Shicheng (26°33'N 116°24'E); G: Yudu (25°57'N 115°24'E); H: Ganxian (25°54'N 114°59'E); I: Huichang (25°37'N 115°47'E); J: Rucheng (25°38'N 113°39'E); K: Anyuan (25°14'N 115°25'E); L: Wuping (25°11'N 116°8'E); M: Xunwu (25°30'N 115°38'E); N: Xinfeng (25°05'N 114°56E); O: South China Botanical Garden (23°10'N 113°21'E).

Seed germination tests

M. savatieri seeds for three germination tests were collected from Yongfeng County in Jiangxi province (Figure 1D) in May, 2011-2013. All seeds were surface sterilized with 0.5% mercuric chloride for 3 min then rinsed in distilled water three times. In test 1 (light and PGRs), seeds were stored in a refrigerator (5 °C) for two weeks, and then germinated in Petri dishes on filter paper soaked for 24 h in distilled water, 500 µM GA_3, 500 µM 6-

benzyladenine (BA) or 500 µM indole-3-acetic acid (IAA). Dishes were transferred to a light or dark culture room. In test 2 (storage temperature and duration), seeds were stored in a 25 ± 2 °C culture room or in a refrigerator (5 °C) for one month, six months, one year and two years, and then germinated on Petri dishes with moist filter paper in a culture room at 25 ± 2 °C in the dark by covering with a black cloth.

TTC test for assessing seed viability

The triphenyltetrazolium chloride (TTC) test was conducted to assess seed viability [PETERS, 2000]. Seeds were stored in a refrigerator (5 °C) for one week, one month, one year or two years, and then placed on Petri dishes (100 seeds/dish) with a single sheet of filter paper. Seeds were soaked in 0.5% TTC solution for 24 h and incubated in a growth chamber at 25 °C under a 12–h photoperiod at a photosynthetic photon flux density of 50 µmol m^{-2} s^{-1}. TTC staining was observed under a stereoscope (Olympus SZX16) at 10-20X magnification. Seeds stained red were considered to be viable while white seeds were unviable (Figure 2E). Each treatment was replicated four times in separate Petri dishes.

Statistical analyses

A completely randomized design was applied for seed germination experiments. Germination percentage was calculated as: (number of germinated seeds/total number of seeds) × 100%. Survival percentage was calculated as: (number of surviving plantlets/total number of transplanted seedlings) × 100%. The normal distribution of data was confirmed before performing analysis of variance (ANOVA). Two-way ANOVA was performed using SPSS version 13.0 (SPSS Inc., Chicago, USA) for Microsoft Windows, and means were considered to be significantly different from each other by the Least Significant Difference (LSD) test at $P \leq 0.05$.

Seedling development and field transplantation

A total of 2000 *M. savatieri* seeds were collected from Yongfeng county, Jiangxi province (Figure 1D) in May, 2013, stored in a refrigerator (5 °C), then germinated in December 2013. Seeds were divided into two parts spread evenly on one plate filled with vermiculite and one tray filled with a mixture of peat and sand (1:1, v/v), respectively. Plates and trays were kept in a moist environment in an open greenhouse, and wrapped in 4 m^2 of shade net, which reduced natural sunlight by 95%. After one month, seedlings in the cotyledonary stage about 1 cm tall were transferred to paper cups 4.5 cm high and 4.5 cm in diameter filled with loess. Moss (*Hypnum plumaeforme* Wilson), covering about one quarter of the paper cup's surface area and with the objective of retaining moisture, was placed on the surface of the loess with forceps while 1-4 seedlings were placed gently on top of the moss [ZHANG & al. 2015]. Cups were maintained in a moist and shaded environment (with a black net) for one month and then kept in an open greenhouse for three months at ambient temperature (10-20 °C). In April 2014, after seedlings grew to 2-3 cm in height and developed to the euphyllous stage with 3-4 leaves, 100 cups with about 200 seedlings were transported to Yongfeng county in Jiangxi province (Figure 1D), which is a natural habitat of *M. savatieri*. The four-month-old seedlings, including the loess from the cups, were transferred to small holes (5 cm deep and 5 cm wide) together with a suitable host plant *Gardenia jasminoides* J. Ellis (a good host shrub with an extensive root system) at a distance of 20-40 cm from the host plant stem [ZHANG & al. 2015]. Soil was initially moistened then watered occasionally. After 8 months, seedling growth and survival percentage were assessed.

Results

Distribution of wild resources

Most *M. savatieri* plant populations discovered were sporadically distributed in Jiangxi province but were also distributed in other provinces of Southeast China, albeit in fewer numbers. The existing distribution range of *M. savatieri* is from 25°03'-29°12' N to 113°39'-120°27' E, based on our survey. Growth locations were typified by hills, including in Jiangxi, Fujian, Zhejiang and Hunan. Our assessment indicates that *M. savatieri* is distributed between the northernmost points of Pan'an, Zhejiang province (29°40'N 120°27'E) and Boyang, Jiangxi province (29°10'N 116°42'E) (Figure 1A, 1B) and the southernmost point of Xinfeng County, Jiangxi province (25°05'N 114°56'E) (Figure 1N). The dominant soil types are loess or red earth. Vegetation consists mostly of sparse grasses and shrubs in the center of Jiangxi province (Table 1).

Table 1. Vegetation growing around *Monochasma savatieri*

Species	Family	Habit	Resource
Hypnum plumaeforme Will.	Hypnaceae	moss	relatively abundant
Funaria hygrometrica Sibth.	Funariaceae	moss	relatively abundant
Camellia oleifera Abel.	Theaceae	shrub	abundant
Symplocos chinensis (Lour.) Druce	Symplocaceae	shrub	relatively abundant
Polygala japonica Houtt.	Polygalaceae	forb	abundant
Vitex negundo var. *cannabifolia* Siebold & Zucc.	Verbenaceae	shrub	relatively abundant
Stimpsonia chamaedryoides Wright ex A. Gray	Primulaceae	forb	abundant
Toxicodendron succedaneum (Linn.) O. Kuntze	Anacardiaceae	shrub	infrequent
Berchemia floribunda (Wall.) Brongn.	Rhamnaceae	woody climber	abundant
Wikstroemia indica (L.) C. A. Mey	Thymelaeaceae	small shrub	abundant
Glochidion puberum (L.) Hutch.	Euphorbiaceae	small shrub	abundant
Lagerstroemia indica L.	Lythraceae	shrub	abundant
Lespedeza formosa (Vog.) Koehne	Fabaceae	shrub	relatively abundant
Galactia tenuiflora (Klein ex Willd.) Wight et Arn.	Fabaceae	herbaceous climber	infrequent
Gardenia jasminoides Ellis	Rubiaceae	shrub	abundant
Serissa serissoides (DC.) Druce	Rubiaceae	small shrub	relatively abundant
Hedyotis acutangula Champ. ex Benth.	Rubiaceae	forb	infrequent
Lindera aggregata (Sims) Kosterm.	Lauraceae	shrub	infrequent
Salvia plebeia R. Br.	Lamiaceae	forb	abundant
Scutellaria indica L.	Lamiaceae	forb	infrequent
Ixeridium chinense (Thunb.) Tzvel.	Asteraceae	forb	infrequent
Solidago decurrens Lour.	Asteraceae	forb	infrequent
Eupatorium chinense Linn.	Asteraceae	forb	relatively abundant
Gerbera anandria (Linn.) Sch.-Bip.	Asteraceae	forb	infrequent
Liquidambar formosana Hance	Hamamelidaceae	tree	infrequent
Loropetalum chinense (R. Br.) Oliv.	Hamamelidaceae	shrub	relatively abundant
Smilax glabra Roxb.	Smilacaceae	woody climber	infrequent
Smilax chinense L.	Smilacaceae	woody climber	infrequent
Crataegus cuneata Sieb. et Zucc.	Rosaceae	shrub	infrequent
Rubus parvifolius L.	Rosaceae	woody climber	abundant
Potentilla discolor Bunge	Rosaceae	herbaceous climber	abundant
Rosa cymosa Tratt.	Rosaceae	woody climber	relative abundant
Rhaphiolepis indica (L.) Lindl. ex Ker	Rosaceae	shrub	infrequent
Imperata cylindrica Linn. Beauv.	Poaceae	grass	relatively abundant
Pogonatherum crinitum (Thunb.) Kunth	Poaceae	grass	common

Natural growth and vernalization test

M. savatieri usually grows as a shoot cluster, which may arise from the simultaneous development of multiple seedlings from independent seeds arising from a single capsule, or from one seedling because roots can develop underground adventitious shoots that sprout. These shoots can grow to as high as 20-30 cm, one cluster usually needing 3-5 years to reach this height. In the southernmost point (Figure 1N), *M. savatieri* usually flowers annually in March to April and in the northernmost point (Figure 1A, 1B), it flowers annually in April to May. A single shoot can develop 3-9 flowers and one large shoot cluster can develop several dozen flowers, which flower acropetally on axillary stalks (Figure 2A). Flowers are white-pink and about 3 cm long (Figure 2A) and can be pollinated by both small insects and wind. A single shoot cluster can last for one month during flowering and fruit dehiscence stages. The fruits open upwardly and remain attached to the plant (Figure 2A). Thus, seeds are retained in the capsule, avoiding decay in soil or predation by insects, and tend to be discharged from the capsule after a storm. In normal conditions, capsules drop naturally to the ground usually between May and June. In east China, March to May is usually the wet and rainy season while storms are typical in May to August. These periods provide a wet environment for seed germination and seedling development when seedlings usually grow on moss (Figure 2B), which allows moisture to be retained. Seeds are spindle-shaped and very small, 0.7 mm long and 0.4 mm wide (Figure 2A, 2F), and 10,000-seed weight is only 0.8 g. Seed germination and development of 1-cm high plantlets needs half a year to complete. Seedlings 3-5 cm high growing in the wild in May were derived from the previous year's seed bank (Figure 2B). During the seedling stage, some shrubs and forbs were assumed as host plants (Table 1). The matured plants usually grow rapidly in spring (3-4 cm long each month) and autumn but slowly in summer and winter (0-1 cm long each month). In the south and centre of Jiangxi province, plants can survive year round. However, in winter in northern Jiangxi province, after above-ground shoots die-back while underground roots remain alive, new shoots sprout from its roots in the next spring. Plants that were brought back to Guangzhou (Figure 1O), Guangdong province where temperatures often exceed 35 °C in summer, could grow well in winter and spring, but not in summer. In Guangzhou, *M. savatieri* plants collected from Pan'an on October 25, 2010 and November 5, 2011 could not flower when planted in SCBG, Guangzhou (Figure 2C), while those collected from Zherong on March 16, 2011, from Shihan on December 20, 2012 and from Tengtian on November 29, 2012 were able to flower in February of the next year (Figure 2D).

Figure 2. Flowering, fruits, seedling investigation, vernalization test, seed germination tests, seedling cultivation and transplantation of *Monochasma savatieri*. (A) Both flowers grew acropetally where axillary mature fruits gathered on the same plant. Flowering was acropetal or axillary and fruit opened upwardly and remained attached to the plant; bar = 1 cm. (B) One-year-old plantlets found in the wild at Yudou in May 2013 (Figure 1G); bar = 2 cm. (C) A plant brought back from Pan'an (Zhejiang province) on October 25, 2010 and potted, but did not flower in February, 2011 in Guangzhou; bar = 5 cm. (D) A plant brought back from Tengtian (Jiangxi province) in December 20, 2012 and flowered in February, 2013 in Guangzhou; bar = 5 cm. (E) Seed germination test with 500 μM GA_3 in the light after culture on a Petri dish with a single sheet of filter paper for 28 days; bar = 5 cm. (F) Seed viability as assessed by the TTC staining test; black arrows indicate seeds stained red (viable) while white arrows indicate non-stained seeds (unviable); bar = 0.5 mm. (G) Seeds, after pretreatment with 600 μM GA_3, germinated in vermiculite and developed into seedlings at the euphyllous stage after 4 months; bar = 5 cm. (H) Seeds were pretreated with 800 μM GA_3 and germinated and developed into seedlings at the cotyledonary stage (white arrow) on peat and loess (1:1, v/v) after culture for one month; bar = 5 cm. (I) Seedlings at the cotyledonary stage were transferred to paper cups that contained loess and moss (*Hypnum plumaeforme*) after culture for 4 months; bar = 2.5 cm. (J) Seedlings grew well (white arrows) after transplanting with *Gardenia jasminoides*, a shrub, for 8 months; bar = 5 cm.

Effects of light and plant growth regulators on seed germination

When cultured in light, seed germination percentage following exposure to 500 µM GA_3 was about 80% within 28 days (Figure 3). However, after exposure to BA and IAA, seed germination percentage was only about 50% within 28 days. This was the same level as the control but significantly lower than the GA_3 treatment (Figure 3). In the dark, seed germination percentage after treatment with 500 µM GA_3 was only 38%, which was significantly lower than in light culture (80%). After exposure to BA and IAA, seed germination percentage was only 3-4%, which was the same level as the control (Figure 3), but significantly lower than the GA_3 treatment.

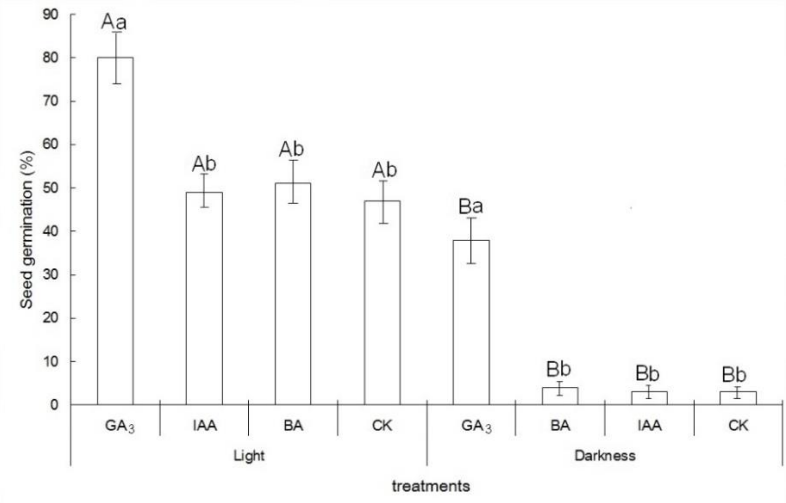

Figure 3. *Monochasma savatieri* seed germination in response to treatments with light and plant growth regulators. The same lowercase letters are not significantly different for the same light (or dark) treatment while the same uppercase letters are not significantly different for the same plant growth regulator treatment (LSD test; $P \leq 0.05$).

Effects of storage temperature and duration on seed germination

Both the germination percentage of *M. savatieri* seeds stored at 25 °C or at 5 °C was about 80%, respectively. The germination percentage of seeds stored for six months decreased to 45% at 25 °C and to 76% at 5 °C. The germination percentage of seeds stored for one year decreased to 18% at 25 °C and to 66% at 5 °C. After two years, seed germination percentage decreased to 1% at 25 °C and to 45% at 5 °C (Figure 4).

Tetrazolium tests to assess seed viability

TTC staining percentage was 86%, 47%, 17% and 3% for seeds stored at 25 °C for one month, six months, one year and two years, and 85%, 74%, 65% (Figure 2F) and 43% (Figure 5), respectively for seed stored at 5 °C. These results almost coincided with the seed germination values.

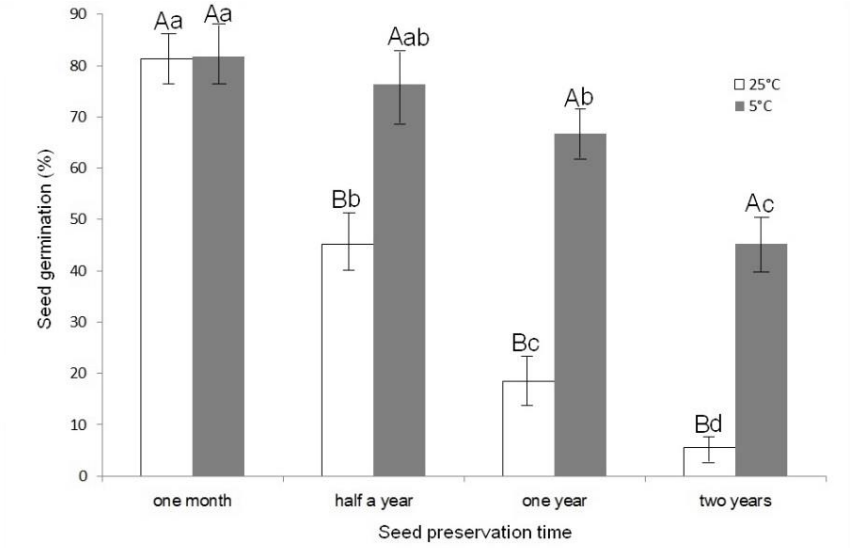

Figure 4. *Monochasma savatieri* seed germination at two temperatures and four storage periods. The same lowercase letters are not significantly different at the same storage temperature (5 °C or 25 °C) and the same uppercase letters are not significantly different at the same seed storage period (LSD test; $P \leq 0.05$).

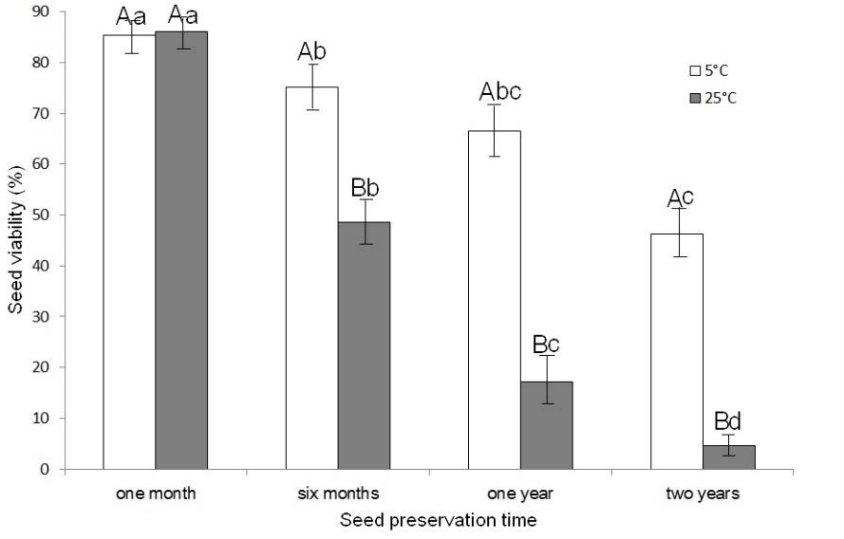

Figure 5. Effects of storage temperature and duration on *Monochasma savatieri* seed viability. The same lowercase letters are not significantly different at the same seed storage temperature and the same uppercase letters are not significantly different at the same storage period (LSD test; $P \leq 0.05$).

Seedling development and field transplantation

Seeds germinated on the surface of vermiculite, peat and loess within two weeks, exceeding 80% (Figure 2G, 2H) after treatment with 600 µM GA_3. It is interesting to note that seedlings without an attached host grew well up until four months of age. Under dim light and wet conditions, seedlings grew slowly and the cotyledonary stage lasted for at least 2-3 months before developing the first true leaf (Figure 2I). During the cotyledonary stage, plantlets grew very slowly, possibly due to the lack of a suitable host and nutrition. After culture for another four months, seedlings could grow to 4-5 cm in height. After interplanting with *G. jasminoides*, 33 *M. savatieri* plantlets survived (23.3%) to 8 months (Figure 2J).

Discussion

Southeast China and Kyushu (Amakusa Islands, Japan) have a subtropical monsoon climate and hilly landform that allows *M. savatieri* to grow. Special biological requirements of *M. savatieri* such as the requirement for low temperature vernalization, moderate temperature for growth or hemiparasitism on host plant roots presumably result in special habitat requirements, which may account for its limited distribution. Since *M. savatieri* is distributed naturally in east China, including Jiangxi, Fujian, and Zhejiang provinces but not in South China (Guangzhou, Guangdong province), we believe that vernalization temperature is likely to be at most -6 °C because the lowest temperature is -6 °C at the southernmost point (Xinfeng County, Jiangxi).

Under natural conditions, a single flowering shoot cluster lasts one month and seeds, which also take one month to mature, also survive for an additional month. In the center of Jiangxi province, *M. savatieri* flowers from April to May and produces seeds in May. The capsules dehisce when they mature, releasing seeds that may germinate if they encounter ideal conditions. During this period, it often rains, which favors seed germination and seedling development. From our results, seed germination was highest within one month but after one year, seed germination decreased to 20%. In the rainy season, the soil is moist and shaded and moss grows easily, providing a suitable environment for seed germination and seedling growth (Figure 1B). As seedlings mature, *M. savatieri* favors a sunny environment and higher terrain that does not easily become waterlogged. The growth conditions in the same area can change between seasons and may face biotic and abiotic stresses, which may explain the low natural sexual reproduction of *M. savatieri*. Artificial seed storage and proper sowing conditions thus need to be considered.

Our studies also showed that light and GA_3 pretreatment enhanced seed germination, which indicates that *M. savatieri* seeds favor light. Moreover, under natural conditions, after seeds drop to the ground and encounter suitable conditions, natural light can enhance germination. Light usually acts synergistically with GA_3 to enhance seed germination [TOYOMASU & al. 1998; YAMAGUCHI & KAMIYA, 2001; SEO & al. 2006]. Sometimes, seed remains dormant due to the presence of abscisic acid (ABA) while GA_3 can counter the effects of ABA [SHINOMURA & al. 1994; LOVEGROVE & HOOLEY, 2000; NAMBARA & MARION-POLL, 2005]. The physiological dormancy of *M. savatieri* seeds might be broken by GA_3 or low temperature [YANG, 2009]. Even in the absence of GA_3 treatment, seed germination was high (> 60%), indicating that seeds broke dormancy in the first two weeks. At this stage, GA_3 and light could enhance seed germination. After one month, most seeds had broken dormancy, and even in the absence of GA_3, seed germination was high (> 80%). Seed germination and seed viability declined after one month. This careful fine-scale

understanding of the balance between these two parameters is necessary to accurately time artificial seed germination.

In its natural environment, all *M. savatieri* seeds germinate on moss (*Hypnum plumaeforme* or *Funaria hygrometrica*) [ZHANG & al. 2015]. This moss might not only supply a moist environment and nutrients for seed germination and seedling growth of some species [REN & al. 2010], but might also secrete phytohormones to enhance seed germination [RESKI, 2006; VON SCHWARTZENBERG, 2006].

The wild habitats of *M. savatieri* are shrinking, and are under rapid threat and decline. By understanding the growth habits of this plant in the wild, including its complex hemiparasitism and the need for host plants to develop haustoria [ZHANG & al. 2015], as well as its vernalization and flowering requirements, seed can be effectively produced, allowing for artificial sexual reproduction of *M. savatieri*, thus providing a robust protocol that would buffer the extinction of this species in the face of rapid urbanization and habitat destruction in China. The protocol in this study is thus useful in practical terms and is also socially important. A transplantation test in Guangzhou indicated that *M. savatieri* plants need a period of vernalization, i.e., a period of low winter temperature, to induce floral bud differentiation in order to flower and produce seeds. Thus, this species is not suitably cultivated in South China where winters are short, not allowing plants to flower in spring. Is then culture of the species suitable in North China? Here, the answer is also negative since too low a temperature (< -20 °C) in winter may kill the whole plant including above - and below - ground parts. *M. savatieri* is confined to small parts of Southeast China and Japan [YAMAZAKI, 1993; HONG & al. 1998]. What confined its distribution, and how? This study shows that characteristic *M. savatieri* habitats include sunny slopes of small mountains and hills, rich with grasses and shrubs, that often contain red clay and loess soil. Thus, it can be concluded that *M. savatieri* is sun-loving, grows in acidic soil and prefers to parasitize small herbs and shrubs. Since *M. savatieri* has strict habitat conditions, it can only grow in limited areas of favorable climatic and geographic environments. Besides, this study also shows that the primary mode of reproduction of *M. savatieri* is by seeds, which is not very efficient because of seed dormancy and a requirement for the early establishment of seedling parasitism. After shedding from plants, seeds may take several months to germinate. This may cause a loss in seedling vigour. Seedlings that form successfully also risk untimely parasitism, which is vital to their survival, especially in dry soil. Thus, certain environmental conditions involving water, temperature, or host roots in the vicinity of seedlings must prevail.

Our study indicates that transplanted *M. savatieri* seedlings could survive in the field when planted with a suitable host. Thus, artificial propagation and reintroduction of this medicinal plant back into the wild is possible and serves as a feasible method for the ecorehabilitation of areas that have been depleted of natural stocks, or that lack genetic heterogeneity.

Conclusions

In this study, the distribution, climate, soil type, flowering and seed production, co-occurring vegetation, pollinators, ecology, seed germination, seedling establishment, and reintroduction of the traditional Chinese hemiparasitic medicinal herb, *Monochasma savatieri*, were investigated. Seed germination was enhanced by gibberellic acid and low temperature storage, which benefitted and sustained seed viability. Seedling development

and subsequent transplantation to the field resulted in a low survival percentage, indicating that much more work is needed on this species before cultivation.

Acknowledgments and conflicts of interest

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COLLECTION AND DOCUMENTATION OF MELON GERMPLASM IN NIGERIA

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Abstract: Members of family Cucurbitaceae are collectively called melon; they are mainly found in the warmer parts of all continents. A germplasm collection was undertaken in Nigeria between April and August, 2015 which corresponded to the periods of harvesting of the crop across major growing regions of the country. Eighteen states including Federal Capital Territory, which are the major growing areas of the crop, were visited. The collections were done in collaboration with Agricultural Development Projects (ADP) extension officers. Fifty five farmers were interviewed and a total of 60 accessions were collected. The accessions collected were identified to species level. All the 60 accessions fall into 5 genera and 7 species; *Colocynthis citrullus* (45), *Cucurbita moschata* (3), *Cucurbita maxima* (3), *Lagenaria siceraria* (2), *Lagenaria sphaerica* (2), *Cucumeropsis mannii* (4) and *Cucumis melo* (1). All the farmers interviewed preferred cultivation of *Colocynthis citrullus* due its high oil content, demand and more acceptance by consumers all over the regions. Niger state had highest number (8) of Egusi melon (*Colocynthis citrullus*) while Nasarawa and Kogi had same number (5) each. The high number of egusi melon encountered might be due to fact that it is the most cultivated member of Cucurbitaceae in the country. This collection had boasted the baseline information of diversity of family Cucurbitaceae. Also it has generated source of genetic variability for members like *Colocynthis citrullus* which can be aid in improvement of the crop.

Key words: Cucurbitaceae, *Colocynthis*, Egusi, *Lagenaria*, melon.

Introduction

Melon is the common name of members of family Cucurbitaceae. It is found mainly in the warmer parts of all continents. It consists of 119 genera with altogether 825 species [SCHIPPERS, 2002]. Fruits of Cucurbitaceae have a considerable economic values. One of the main uses of the cucurbits apart from their fruits, leaves, flowers and occasionally their root is that of its seeds. The seed kernels of the Cucurbitaceae family found in markets throughout West Africa are important source of edible oil. Those oil-rich seeds are found in a range of genera of which the most important are *Colocynthis* (Egusi melon), *Citrullus* (Watermelon), *Cucurbita* (Pumpkin), *Lagenaria* (Bottle gourd), *Cucumis* (Melon), *Telfairia* (Fluted pumpkin) and *Luffa* (Sponge gourd) [SCHIPPERS, 2002]. According to [OGBONNA, 2013], *Colocynthis citrullus* (Egusi melon), which is one of the most cultivated species of family Cucurbitaceae, has been wrongly referred to by different scientific names by different authors such as; *Citrullus vulgaris*, *Citrullus lanatus*, and *Citrullus colocynthis*.

It is not out of point to belief that these genera of the family Cucurbitaceae could have the same Centre of Origin or Centre of Diversity. Since it is believed by numerous authors that many members of this family are found in warmer parts of all continents; it is pertinent to collect the germplasm of these crops in Nigeria. This will enhance the study of

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genetic diversity among the various members of the family Cucurbitaceae in the country.

Germplasm is a term used to describe living genetic resources such as seeds or tissues, maintained for the purpose of breeding, preservation, and other research uses [MAHMUT, 2012]. It provides potential diversity-base in genetic resources of cultivated plants [MAHMUT, 2012]. Germplasm collections can range from collections of wild species to elite, domesticated breeding lines that have undergone extensive human selection.

According to MAHESH & RONNIE (2017) insufficient germplasm is a global problem and no single country can boast of self-adequacy in germplasm to meet its food obligation. Many African countries including Nigeria depend greatly on non-native crops and imported germplasm for food and agricultural development. Thus this research aimed at evaluating existing genetic diversity of melons, through its germplasm collection, in Nigeria.

Materials and methods

A germplasm collection was under taken from April to August 2015, in collaboration with Agricultural Development projects (ADP) extension officers to major growing areas of the crop across Nigeria. Five geopolitical zones (north central, North West, south east, south west and south south) formed the major growing areas. The states visited, were Niger, Kogi, Nassarawa, Kwara, Ondo, Osun, Benue, Plateau, Kaduna, Abia, Anambra, Rivers, Oyo, Ogun, Edo, Enugu, Imo and Federal Capital Territory (FCT). The exercise involved visits to farming villages in the states to collect available accessions of Cucurbitaceae. Each accessions collected were well-packed in an envelope and assigned an entry number and local name. The length of the seeds were taken using veneer callipers. Fifteen seeds were randomly selected, measured and the mean for each accession was taken.

Results and discussion

A total sixty accessions were collected which fall in to five genera and seven species; *Colocynthis*, *Cucurbita*, *Lagenaria*, *Cucumeropsis*, *Cucumis*. The genus *Colocynthis* had highest number (43) of accessions, followed by *Cucurbita* (6), *Lagenaria* (4), *Cucumeropsis* (4), *Cucumis* (1) (Table 1, Figure 1). The *Colocynthis* (egusi melon) recorded highest occurrence in almost all parts of the country while *Lagenaria* and *Cucumeropsis* were more in the south-western part of the country (Table 1). This implied that there was non-uniform distribution of the genera across the country.

The uneven distribution and confinement of some species to one particular area or region of the country might be due variation in availability of rainfall and or edaphic factors. This can corroborated by the report of ADOJUTELEGAN & al. (2015) who identified rainfall and soil factor among others that limit production of watermelon.

The occurrence of species like *Colocynthis citrullus* across the regions might not be unconnected with germplasm flow in and out of the regions. Such flows are probably facilitated by Agricultural Development Projects (ADPs). Similar suggestion was made by MAHESH & RONNIE (2017). They reported that one of the routes through which germplasms could get in to a region is donor-assisted projects involved in agricultural development. The number of accessions of 'Egusi' melon collected is higher than those from earlier reports of IDEHEN & al. (2007); this could be due to smaller number of areas / regions visited in their research. In addition, they wrongly addressed egusi melon (*Colocynthis citrullus*) as *Citrullus lanatus*. The high number of egusi melon accessions which were collected from Niger (8), Nassarawa (5) and Kogi (5), is an indication that these states have the greatest diversity of the crop genetic resources in Nigeria. It also showed that these

regions might be the secondary centre of origin of the crop. This can be buttressed by opinion of SCHIPPERS (2000), SCHAEFER & RENNER (2011), KOUAME & al. (2014), RAGHAMI & al. (2014). They all opined that the family Cucurbitaceae has its centre of origin in West Africa.

Conclusion and recommendations

Nigeria harbour appreciable diversity of members of family Cucurbitaceae especially egusi melon (*C. citrullus*) which is the most important in Nigerian economy. These collections which are now organised can provide baseline information of diversity of melon as well as raw materials for improvement of the crops. Evaluation of these germplasm for desirable traits is therefore recommended.

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Colocynthis citrullus

Cucumis melo

Cucurbita moschata

Lagenaria sphaerica

Lagenaria siceraria

Cucumeropsis mannii

Cucurbita maxima

Figure 1. Seeds of the different species of melon encountered

Table 1. Sources and description of Melon Germplasm in Nigeria

S/ No	Accession no.	Local name	Scientific Name	Place of collection	State	Zone/ region	Seed Coat colour	Rim/eye colour	Seed size (mm)	Seed texture	Seed shape
1.	NGR-NG-01	Eashi	*Colocynthis citrullus*	Gita/Paiko	Niger	NC	Brown	Black	1.7	Smooth/glabrous	Oval
2.	NGR-NG-02	Bologi	*Colocynthis citrullus*	Lenfa/Lapai	Niger	NC	Brown	White	1.7	Smooth/glabrous	Oval
3.	NGR-NG-03	Eashi	*Colocynthis citrullus*	Gidan Mangoro	Niger	NC	Brown	Rimless	1.2	Smooth/glabrous	Oval
4.	NGR-NG-04	Eashi	*Colocynthis citrullus*	Agaie	Niger	NC	Brown	Black	1.5	Smooth/glabrous	Oval
5.	NGR-NG-05	Eashi	*Colocynthis citrullus*	Paiko	Niger	NC	Brown	Rimless	1.1	Smooth/glabrous	Oval
6.	NGR-NG-06	Eashi	*Colocynthis citrullus*	Gidan Mangoro	Niger	NC	Brown	White	1.5	Smooth/glabrous	Oval
7.	NGR-NG-07	Eashi	*Colocynthis citrullus*	Bosso	Niger	NC	Brown	Rimless	1.5	Smooth/glabrous	Oval
8.	NGR-NG-08	Agushi	*Colocynthis citrullus*	Mashegu	Niger	NC	Brown	Black	1.6	Smooth/glabrous	Oval
9.	NGR-KG-09	Abaro	*Cucumeropsis mannii*	Dekina	Kogi	NC	White	Rimless	2.5	Smooth/glabrous	Spherical elongated
10.	NGR-KG-10	Epi	*Colocynthis citrullus*	Ankpa	Kogi	NC	Brown	Black	1.8	Smooth/glabrous	Oval
11.	NGR-KG-11	Epi Igala	*Colocynthis citrullus*	Idah	Kogi	NC	Brown	Rimless	1.4	Smooth/glabrous	Oval
12.	NGR-KG-12	Ito	*Cucumeropsis mannii*	Lokoja	Kogi	NC	White	Rimless	2.6	Smooth/glabrous	Spherical elongated
13.	NGR-KG-13	Epapara	*Colocynthis citrullus*	Adavi	Kogi	NC	Brown	Black	1.7	Smooth/glabrous	Oval
14.	NGR-FCT-14	Paper Babba	*Colocynthis citrullus*	Kwali	Fct	NC	Brown	Rimless	1.5	Smooth/glabrous	Oval
15.	NGR-FCT-15	Eshi Lala Eguain	*Colocynthis citrullus*	Gwagwalada	Fct	NC	Brown	Black	1.6	Smooth/glabrous	Oval
16.	NGR-FCT-16	Paper kanana	*Colocynthis citrullus*	Gosa	Fct	NC	Brown	Rimless	1.2	Smooth/glabrous	Oval
17.	NGR-FCT-17	Gunayi	*Colocynthis citrullus*	Garki	Fct	NC	Brown	Rimless	1.1	Smooth/glabrous	Oval
18.	NGR-NS-18	Eashi egi	*Colocynthis citrullus*	Kufan Gwari	Nasarawa	NC	Brown	Black	1.9	Smooth/glabrous	Oval
19.	NGR-NS-19	Eashi Letelete	*Colocynthis citrullus*	Kufan Gwari	Nasarawa	NC	Brown	Rimless	1.5	Smooth/glabrous	Oval

20.	NGR-NS-20	Tetele	*Colocynthis citrullus*	Wakama	Nasarawa	NC	Brown	Rimless	1.7	Smooth/glabrous	Oval
21.	NGR-NS-21	Kagala	*Colocynthis citrullus*	Guduma	Nasarawa	NC	Brown	Black	1.6	Smooth/glabrous	Oval
22.	NGR-NS-22	Tetele	*Colocynthis citrullus*	Jitata	Nasarawa	NC	Brown	White	1.6	Smooth/glabrous	Oval
23.	NGR-KN-23	Kabewa	*Cucurbita maxima*	Rano	Kano	NW	Cream	Cream	1.7	Smooth/glabrous	Spherical
24.	NGR-KD-24	Dan Keffi Babba	*Colocynthis citrullus*	Kaduna	Kaduna	NW	Brown	Rimless	1.5	Smooth/glabrous	Oval
25.	NGR-KD-25	Agushi	*Colocynthis citrullus*	Doka	Kaduna	NW	Brown	White	1.8	Smooth/glabrous	Oval
26.	NGR-KD-26	Agushi mai bakin kai	*Colocynthis citrullus*	Kaduna	Kaduna	NW	Brown	Black	1.8	Smooth/glabrous	Oval
27.	NGR-OY-27	Ntoh	*Cucumeropsis mannii*	Saki/Ibadan	Oyo	SW	White	Rimless	2.5	Smooth/glabrous	Spherical elongated
28.	NGR-OY-28	Papa	*Colocynthis citrullus*	Saki/Ibadan	Oyo	SW	Brown	Black	1.4	Smooth/glabrous	Oval
29.	NGR-OY-29	Bojuri	*Lagenaria sphaerica*	Ibadan	Oyo	SW	Brown	Cream	2.1	Smooth/glabrous	Spherical elongated
30.	NGR-OY-30	Igba	*Lagenaria siceraria*	Saki/Ibadan	Oyo	SW	Dark brown	Rimless	2.2	Smooth/glabrous	Triangular
31.	NGR-OS-31	Igba	*Lagenaria siceraria*	Egbebore	Osun	SW	Dark brown	Rimless	2.3	Smooth/glabrous	Triangular
32.	NGR-OS-32	Ntoh	*Cucumeropsis mannii*	Egbebore	Osun	SW	White	Rimless	2.0	Smooth/glabrous	Spherical elongated
33.	NGR-OS-33	Papa	*Colocynthis citrullus*	Oshogbo	Osun	SW	Brown	Black	1.7	Smooth/glabrous	Oval
34.	NGR-OS-34	Egusi	*Colocynthis citrullus*	Egbebore	Osun	SW	Brown	Rimless	1.7	Smooth/glabrous	Oval
35.	NGR-OD-35	Atogbo	*Cucumeropsis mannii*	Ilodaada	Ondo	SW	White	Rimless	2.1	Smooth/glabrous	Spherical elongated
36.	NGR-OD-36	Atan	*Lagenaria sphaerica*	Ilodaada	Ondo	SW	Brown	Cream	2.0	Smooth/glabrous	Spherical elongated
37.	NGR-OD-37	Epapara	*Colocynthis citrullus*	Agogoro	Ondo	SW	Brown	Rimless	1.5	Smooth/glabrous	Oval
38.	NGR-OD-38	Egusi	*Colocynthis citrullus*	Ifon	Ondo	SW	Brown	Rimless	1.8	Smooth/glabrous	Oval
39.	NGR-OG-39	Wewe	*Colocynthis citrullus*	Hunguru	Ogun	SW	Brown	Rimless	1.5	Smooth/glabrous	Oval
40.	NGR-OG-40	Serewe	*Colocynthis citrullus*	Ijebu	Ogun	SW	Brown	Rimless	1.4	Smooth/glabrous	Oval

41.	NGR-OG-41	Papa	*Colocynthis citrullus*	Ilara	Ogun	SW	Brown	Black	1.3	Smooth/glabrous	Oval
42.	NGR-KW-42	Egusi	*Colocynthis citrullus*	Illorin	Kwara	NC	Brown	Black	1.7	Smooth/glabrous	Oval
43.	NGR-KW-43	Papa	*Colocynthis citrullus*	Oke Oyi	Kwara	NC	Brown	Black	1.3	Smooth/glabrous	Oval
44.	NGR-BE-44	Cheghar	*Colocynthis citrullus*	Makurdi	Benue	NC	Brown	Black	1.7	Smooth/glabrous	Oval
45.	NGR-BE-45	Cheghar	*Colocynthis citrullus*	Makurdi	Benue	NC	Brown	Rimless	1.3	Smooth/glabrous	Oval
46.	NGR-RV-46	Ardogha	*Colocynthis citrullus*	Abua/Odual	Rivers	SS	Brown	White	1.6	Smooth/glabrous	Oval
47.	NGR-RV-47	Ardogha	*Colocynthis citrullus*	Andoni	Rivers	SS	Brown	Black	1.8	Smooth/glabrous	Oval
48.	NGR-RV-48	Ardogha	*Colocynthis citrullus*	Abua/Odual	Rivers	SS	Brown	Rimless	1.5	Smooth/glabrous	Oval
49.	NGR-ED-49	Irere	*Cucumis melo*	Etsako West	Edo	SS	Cream	Rimless	0.7	Smooth/glabrous	Elliptical
50.	NGR-ED-50	Eivo	*Colocynthis citrullus*	Etsako West	Edo	SS	Brown	Rimless	1.5	Smooth/glabrous	Oval
51.	NGR-ED-51	Eivo	*Colocynthis citrullus*	Etsako East Auchi	Edo	SS	Brown	Rimless	1.4	Smooth/glabrous	Oval
52.	NGR-EN-52	Ugboro	*Cucurbita moschata*	Emene/Enugueast	Enugu	SE	Brown	Rimless	1.3	Hairy	Oval
53.	NGR-EN-53	Enine	*Colocynthis citrullus*	Igboekiti	Enugu	SE	Brown	Rimless	1.5	Smooth/glabrous	Oval
54.	NGR-EN-54	Anyu	*Cucurbita maxima*	Nkanu	Enugu	SE	Tan	Cream	2.3	Smooth/glabrous	Spherical
55.	NGR-AB-55	Egwusi	*Colocynthis citrullus*	Ugbo/Arochukwu	Abia	SE	Brown	Rimless	1.4	Smooth/glabrous	Oval
56.	NGR-AB-56	Ugboyoro	*Cucurbita moschata*	Ugbo/Arochukwu	Abia	SE	Brown	Rimless	1.3	Hairy	Oval
57.	NGR-AN-57	Egwusi	*Colocynthis citrullus*	Orumba	Anambra	SE	Brown	Rimless	1.4	Smooth/glabrous	Oval
58.	NGR-IM-58	Anyu	*Cucurbita maxima*	Akuwanta	Imo	SE	Tan	Cream	2.3	Smooth/glabrous	Spherical
59.	NGR-IM-59	Egwusi	*Colocynthis citrullus*	Akuwanta	Imo	SE	Brown	Rimless	1.4	Smooth/glabrous	Oval
60.	NGR-IM-60	Ugboyoro	*Cucurbita moschata*	Akuwanta	Imo	SE	Brown	Rimless	1.3	Hairy	Oval

NC = North Central, NW = North West, SW = South West, SS = South South, SE = South East

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PROXIMATE AND MINERAL COMPOSITION OF SELECTED SOYBEAN GENOTYPES IN NIGERIA

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Abstract: An experiment was conducted at the Central Services Laboratory of the National Cereals Research Institute, Badeggi, Nigeria to evaluate proximate and mineral composition of eight soybean accessions (NG/MR/11/11/060, NG/SA/07/100, NG/SA/JAN/09/48, NGB00111, NGB00113, NGB00116, NGB01318) conserved in the National Genebank at National Centre for Genetic Resources and Biotechnology [NACGRAB), Ibadan, Nigeria and twelve varieties (TGX1019-2EB, TGX1019-2EN, TGX1440-1E, TGX1448-2E, TGX1485-1D, TGX1835-10E, TGX1904-6F, TGX1951-3F, TGX1987-62F, TGX1989-19F, TGX923-2E, TGX1987-10F) developed at National Cereals Research (NCRI) in collaboration with International Institute for Tropical Agriculture (IITA) and the result showed wide significant variations in the proximate and mineral contents. TGX1987-62F recorded the highest dry matter content of 98.34% while TGX 923-2E gave the highest moisture content (6.12%). Highest values for Crude ash (5.02%) and crude fibre (6.12%) were recorded for TGX1951-3F. NGB00116 had the significantly highest protein content of 41.92% and an appreciable oil content of 27.65%. Protein content was also high for TGX1987-10F (41.33%) and NG/MR/11/11/060 (41.31%). TGX1989-19F had the highest oil content of 30.45% and energy value (496.37Kcal/g). NGB00113 had the highest carbohydrate content of 52.74%. K. Na, Ca, Mg and P were highest in NG/SA/JAN/09/48 (1.99%), NGB00116 (0.57%), TGX1485-1D (1.60%) TGX1951-3F (0.84%) and NGB00113 (0.85%) respectively. The study provides valuable information on soybean genotypes with very high protein and oil content for recommendation to farmers and other end users and for breeders to select and utilize in soybean quality improvement programmes.

Keywords: accessions, oil content, protein content, proximate, soybean.

Introduction

Soybean (*Glycine max* (L.) Merr.) is a member of Leguminosae family, believed to have originated in Northeastern China and distributed in Asia, USA, Brazil, Argentina and other parts of the world. This crop is aptly called as "Miracle crop" of the 20th century due to its multiple uses. Soybean with an average protein content of 40% and oil content of 20% has the highest protein of all crops for food and feed [SHARMA & al. 2013; HOSSAIN & KOMATSU, 2014], including those of cowpea and common bean [NWADIKE & al. 2018],

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and second only to groundnut in terms of oil content among food legumes. Compared to other protein rich foods such as meat, fish, and eggs, it is by far the cheapest. Soybean seeds have been described as "protein hope" of the future [SATHE & al. 2009] and meat that grows on plant [LEHNER & GAWRISCH, 2000] owing to its high nutritive value. Soybean seeds besides being rich in proteins, contain unsaturated, cholesterol free fatty acids, minerals and vitamins A, B, C and D which meet the nutritional needs of humans and other animals [MALEK & al. 2014; GHOSH & al. 2014], and contain numerous antioxidants beneficial to human health, as they significantly reduce the risk of many diseases [KUMAR & al. 2014].

Nigeria is the largest producer in Africa. Though, soybean is grown in many states of Nigeria, the Northern and Southern guinea savanna regions record the highest soybean production. Production mainly in the southern Guinea savanna zone comprising Benue, Kaduna, Oyo, Ondo, Adamawa, Taraba and Plateau with Benue as the highest [SMITH & al. 1995]. According to ODELEYE & al. (2007), there is a wide margin between what is needed and what is currently produced.

Due to its great potentials in Nigeria for oil production and relatively cheap protein source for the vast resource poor populace, evaluation of collections released varieties and accessions is necessary to identify their individual nutritional composition. Information on the protein content of soybean varieties released over the years in Nigeria is non-existent at worst and at best rare to come by in literature. This may be due to the general assumption that soybean is rich in protein and oil. Thus most of the varieties released in Nigeria are based on seed yield, early maturity, low shattering and resistance to disease especially rust. Similarly, accessions held in the National Genebank have not been previously evaluated for nutritional content. Therefore, this pioneer attempt to unravel the proximate and mineral compositions of some released varieties as well as accessions will give depth knowledge to the wealth of nutrient in each studied genotype. This will also provide very useful information for breeders seeking to improve the nutritional quality of this crop while equipping farmers and the populace with information on the specific varieties to plant and consume for specific nutritional needs.

Materials and methods

Genotypes used for the study and evaluated parameters

Twenty (20) soybean genotypes (Table 1) comprising 11 varieties released in Nigeria and 9 accessions collected from different parts of Nigeria and held in trust of the National Genebank at National Centre for Genetic Resources and Biotechnology (NACRAB), Ibadan, Nigeria were evaluated for proximate and macro mineral nutrient contents. Percentage compositions in dry matter, moisture, ash, crude fat/oil, crude protein, crude fibre, carbohydrate and energy value (Kcal/g) were evaluated. Potassium (K), sodium (Na), calcium (Ca), magnesium (Mg) and phosphorus (P) contents of these genotypes were also evaluated. The experiment was conducted at the General Laboratory of National Cereals Research Institute, Badeggi, Nigeria.

Proximate Composition

Seed bean nitrogen (N) was determined by the thermal conductivity procedure that included the combustion of the sample to 40 ºC [SADZAWKA & al. 2007]. The N content of the seed was multiplied by 6.25 to obtain the protein content [GUZMÁN-MALDONADO & al. 2000]. Moisture content was determination using the air oven method. Crucibles were washed and dried in an oven. They were allowed to cool in the desiccator and weight was

noted. A known weight of samples were then transferred into the crucibles and dried at a temperature between 103-105 ºC. The dry samples were cooled in a desiccator and the weight noted. They were later returned to the oven and the process continued until constant weights were obtained. Moisture content was calculated using the formula:

$$\% \text{ moisture content} = \frac{\text{weight loss } \times \ 100}{\text{weight of smaple}}$$

Ash content was determined by weighing of finely ground sample into clean, dried previously weighed crucible with lid (W1). The sample was ignited over a low flame to char the organic matter with lid removed. The crucible was then placed in muffle furnace at 600 ºC for 6h until it ashed completely. It was then transferred directly to desiccators, cooled and weighed immediately (W2).

$$\% \text{ Ash content} = \frac{\text{weight loss } \times \ 100}{\text{weight of smaple}}$$

Crude fat was determined using Soxhlet apparatus. A known weight of sample was weighed into a weighed filter paper and folded neatly. This was put inside pre-weighed thimble (W1). The thimble with the sample (W2) was inserted into the Soxhlets apparatus and extraction under reflux was carried out with petroleum ether [40 ºC-60 ºC boiling range) for 6h. At the end of extraction, the thimble was dried in the oven for about 30 minutes at 100 ºC to evaporate off the solvent and the thimble was cooled in a desiccator and later weighed (W3). The fat extracted from a given quantity of sample was then calculated:

$$\% \text{ Fat } = \frac{\text{loss in weight of sample } \times \ 100}{\text{original weight of smaple}}$$

Crude fibre was determined by taking the fat free extract obtained after determining Ether Extract and weight part of it. This is then serially heated with dilute acid and dilute alkali to hydrolyze away the digestible portion. The residue is dried and weighed. This weight, minus the weight of ash represents the fibre content. The percentage carbohydrate content of seeds was determined by summing up the percentages of moisture, ash, crude protein, fat (ether extract) and subtracting from 100% CHO = 100 – (Sum of the percentages of moisture, ash, fat, protein and crude fibre). The difference in value was taken as the percentage total carbohydrate content of seed [A.O.A.C., 2006; AMEH, 2007].

Mineral composition analysis

Twenty (20) accessions of soybean genotypes were analyzed for Ca, Fe, B, Zn, K, Mg, Mn and P by flame atomic absorption spectrometry (Perkin-Elmer spectrophotometer, model 1100B, Phoenix, Arizona, USA), [SADZAWKA & al. 2007]. Bean seed samples were ground to a fine powder to ensure homogeneity before analysis of macro and micronutrients. The samples were concentrated by evaporating 100 ml of sample to about 20 ml. They were thereafter aspirated through the nebulizer into the air-acetylene flame where atomization took place. Using a source lamp for each element, the amount of energy absorbed in the flame is proportional to the concentration of the element in the sample over a limited concentration range.

Statistical Analysis

All data were subjected to an ANOVA. The least significant difference (LSD) was used to compare the means of the genotypes using the STAR (2014) statistical software. Significantly different means were separated using SNK at $P \leq 0.05$ probability level.

Table 1. List and sources of plant materials used in the experiment

S/N	Accessions	Source	Status
1	NG/AA/SEP/09/166	NACGRAB, Ibadan	Farmer's cultivar conserved in the genebank
2	NG/MR/11/11/060	"	"
3	NG/SA/07/100	"	"
4	NG/SA/JAN/09/48	"	"
5	NGB 00111	"	"
6	NGB 00113	"	"
7	NGB 00116	"	"
8	NGB 01318	"	"
9	TGX 1019-2EB	IITA, Ibadan	Improved registered variety
10	TGX 1019-2EN	"	"
11	TGX 1440-1E	"	"
12	TGX 1448-2E	"	"
13	TGX 1485-1D	"	"
14	TGX 1835-10E	"	"
15	TGX 1904-6F	"	"
16	TGX 1951-3F	"	"
17	TGX 1987-62F	"	"
18	TGX 1989-19F	"	"
19	TGX 923-2E	"	"
20	TGX1987-10F	"	"

Results

Results of the proximate and mineral compositions of twenty soybean genotypes (Table 2) showed significant differences for all the evaluated parameters. Total dry matter (%) value of 98.34% recorded for TGX1987-62F was significantly highest, followed by 97.27% for TGX1019-2EN. TGX923-2E had the least dry matter content of 93.48%. Moisture content varied significantly among the genotypes with TGX923-2E (6.12%) and TGX1989-19F (5.12%) having the highest moisture contents while, least moisture content of 1.88% was observed for TGX1987-62F. NGB00116 recorded the highest ash content of 5.36%, while BG/SA/JAN/09/48 had the lowest (3.34%).

Crude fat/oil content varied significantly with the highest value of 30.45% recorded for TGX1989-19F, followed by 28.44% and 28.11% recorded in NG/MR/11/11/060 and TGX1904-3F respectively. The least value of 21.12% recorded for NG/SA/JAN/09/48. Protein content was significantly highest at 41.92% for NG00116 followed by 41.33%, 41.31% and 40.32% in TGX1987-10F, NG/AA/SEP/09/166 and TGX1835-10E respectively. NG00113 (12.96%) had the lowest recorded crude protein.

Crude fibre (%) was observed to be highest in TGX1951-3F recording 6.12% followed by TGX1440-1E (6.09%), but the least value for this parameter observed in TGX923-2E was 3.75%. NGB00113 (52.74%) recorded significantly highest carbohydrate value followed by TGX1019-2EN (46.64%). The least value for carbohydrate (15.83%) was recorded for NGB00116. Energy value (Kcal/g) was significantly highest for TGX1989-19F

at 496.37 Kcal/g. The genotype with significantly lowest energy value of 459.97 Kcal/g was TGX1440-1E.

Mineral composition results (Table 3) showed that NG/SA/JAN/09/48 (1.99%) and TGX923-2E (1.09%) had the significantly highest and lowest potassium (K%) content respectively among the genotypes used in this study. Sodium (Na%) composition was significantly highest in NGB00116 (0.57%) and lowest in NG/MR/11/11/060 (0.10%). Similarly, NG/SA/JAN/09/48 (1.60%) and TGX 1485-1D (1.60%) had significantly highest calcium compositions. This value was not significantly different from 1.57% and 1.56% recorded for TGX1987-62F and NGB00111 respectively (Table 3).

Magnesium (Mg%) composition varied from 0.84% significantly highest in TGX1951-3F to 0.15% lowest in TGX 1019-2EB and TGX1987-62F (Table 3). NGB00113 (0.85%) was significantly highest in phosphorus (P%) followed by 0.78% in TGX 1019-2EN. TGX 1019-2EB (0.26%) and TGX1987-10F (0.27%) had the least phosphorus contents.

Table 3. Mineral composition of 20 soybean genotypes in Nigeria

Genotype	K (%)	Na (%)	Ca (%)	Mg (%)	P (%)
NG/AA/SEP/09/166	1.76	0.47	0.99	0.47	0.62
NG/MR/11/11/060	1.76	0.10	0.58	0.81	0.59
NG/SA/07/100	1.36	0.23	1.48	0.58	0.44
NG/SA/JAN/09/48	1.99	0.53	1.60	0.39	0.46
NGB 00111	1.72	0.20	1.56	0.82	0.45
NGB 00113	1.45	0.26	0.86	0.45	0.85
NGB 00116	1.72	0.57	1.24	0.45	0.72
NGB 01318	1.58	0.51	1.06	0.54	0.34
TGX 1019-2EB	1.68	0.46	1.09	0.15	0.26
TGX 1019-2EN	1.39	0.44	0.94	0.21	0.78
TGX 1440-1E	1.67	0.24	0.76	0.65	0.46
TGX 1448-2E	1.84	0.54	0.96	0.15	0.55
TGX 1485-1D	1.86	0.48	1.60	0.27	0.50
TGX 1835-10E	1.83	0.18	0.86	0.34	0.58
TGX 1904-6F	1.46	0.19	0.70	0.49	0.44
TGX 1951-3F	1.85	0.19	0.95	0.84	0.54
TGX 1987-62F	1.52	0.18	1.57	0.15	0.74
TGX 1989-19F	1.45	0.15	0.86	0.75	0.58
TGX 923-2E	1.09	0.10	0.66	0.55	0.50
TGX1987-10F	1.68	0.54	1.23	0.08	0.27
SE±	0.05	0.04	0.06	0.05	0.04
LSD (P = 0.05) SNK	0.10	0.09	0.13	0.099	0.09

Table 2. Proximate composition of 20 soybean genotypes in Nigeria

Genotype	Dry matter (%)	Moisture (%)	Ash (%)	Crude fat/oil (%)	Crude protein (%)	Crude fibre (%)	CHO (%)	Energy value Kcal/g
NG/AA/SEP/09/166	96.45	3.44	3.75	25.36	41.31	4.72	21.33	479.60
NG/MR/11/11/060	95.53	4.12	4.53	28.44	32.50	4.98	26.38	488.89
NG/SA/07/100	96.54	3.69	4.31	23.12	16.06	5.06	48.00	462.61
NG/SA/JAN/09/48	97.20	2.60	3.34	21.12	23.94	4.64	44.43	468.12
NGB 00111	95.30	4.74	5.12	27.65	20.48	5.11	36.63	479.39
NGB 00113	96.20	3.69	4.11	22.39	12.96	4.92	52.74	464.68
NGB 00116	96.42	3.66	5.36	26.93	41.92	5.32	15.83	477.17
NGB 01318	96.49	3.45	4.26	22.46	33.38	5.12	31.26	461.39
TGX 1019-2EB	96.52	3.55	3.97	26.07	22.39	5.32	38.55	478.92
TGX 1019-2EN	97.25	2.75	4.57	23.40	18.24	4.28	46.64	470.4
TGX 1440-1E	95.25	4.75	4.12	24.30	32.56	6.09	28.57	459.97
TGX 1448-2E	96.35	3.68	4.31	25.39	39.98	5.60	21.24	473.50
TGX 1485-1D	96.62	3.82	4.11	23.54	27.31	5.11	36.40	468.93
TGX 1835-10E	95.90	4.10	3.88	23.52	40.32	4.95	22.55	465.21
TGX 1904-6F	96.42	3.62	4.19	28.11	36.30	4.18	23.54	484.44
TGX 1951-3F	95.45	4.47	5.02	26.22	35.11	6.12	23.06	468.11
TGX 1987-62F	98.34	1.88	5.11	25.15	31.43	5.12	31.50	476.04
TGX 1989-19F	94.64	5.12	4.22	30.45	39.31	4.12	16.72	496.37
TGX 923-2E	93.48	6.12	4.54	24.82	37.06	3.75	23.83	464.48
TGX1987-10F	96.02	3.99	4.05	25.28	41.33	5.34	20.27	474.83
SE±	0.57	0.24	0.29	0.60	0.56	0.32	0.48	6.33
LSD (P = 0.05) SNK	1.15	0.48	0.58	1.21	1.12	0.64	0.97	12.78

Discussion

Proximate composition of soybean genotypes

The low moisture content observed in all the genotypes (Table 1), 1.88% in TGX1987-62F through 6.12% in TGX923-2E shows these genotypes have less moisture due to more concentrates of other nutrients. This is an indication that all the genotype can be stored for a very long time since moisture which is an important medium for multiplication of microorganisms is very low in the genotypes. These results are in agreement with DAVIES (2008) who reported lower moisture content in full-fat soybeans. ETIOSA & al. (2017) on the other hand reported higher moisture content in contrast to the findings of this experiment. The ash contents ranging from 3.34% to 5.02% for the genotypes under study is an indication that the genotypes could be important sources of minerals. SAULAWA & al. (2014); SIULAPWA & MWAMBUNGU (2014); EDEMA & al. (2005) all reported higher ash contents, but reports of ranges of 1.01-1.67% for ash content [ESHUN, 2012] was lower than those observed by the researchers. The high crude fat content observed in the genotypes NG/SA/JAN/09/48 (21.12%) through TGX1989-19F (30.45%) are in agreement with that reported by OGBEMUDIA & al. (2017). These values however, were much higher than those reported by ADIE & al. (2015). These authors had reported oil content range of 7.0-25% for all 78 soybean varieties registered by Ministry of Agriculture Republic of Indonesia. LIU (2000) also reported that soybean seed had 18-21% oil. This suggests that the genotypes used for this study may be a viable source of higher oil quantity, going by their crude fat contents. And not just for Nigeria alone but beyond. Soybean crude fat is reported to have cholesterol reducing ability thereby making it essential oil for obese people diet. BALASUBRAMANIYAN & PALANIAPPAN (2003) reported that the oil in soybean consists of 85% unsaturated fatty acid with two essential fatty acids (lenoleic and linolenic acid) which are not synthesized by the human body, thus highly desirable in human diet. Seeds from these exceptional genotypes could be used in weight-loss drinks, sport drinks, as low-fat substitute in hamburger, in commercial bakery industry to aid in dough conditioning and bleaching, as it possesses excellent moisture-holding qualities that help to retard staling in bakery products. Greater cultivation and consumption of these high oil containing genotypes will go a long way in improving diet and food security of the Nigerian populace.

The high protein content obtained in the following genotypes ranging from highest NGB 00116 TGX 1987-10F, NG/AA/SEP/09/166, TGX1835-10E, TGX1448-2E, TGX1989-19F are higher than the reported values 37.69% by SIULAPWA & MWAMBUNGU (2014), 36.27% by LIU (2000), 38.42% by STEIN & al. (2008). The above genotypes are of nutritional importance to sub-Sahara Africa where protein energy malnutrition is a menace. They can be incorporated into diet formulations for weaning foods in infant and as an alternative to animal proteins which has problem low density lipoprotein that have adverse health effect such coronary diseases. Moreover, the health benefits of soy protein related to the reduction of cholesterol levels, menopause symptoms and reduction in risk for several chronic disease i.e., cancer, heart disease and osteoporosis have been reported [POTTER, 1998]. The genotypes with lower protein contents can be channeled into oil production and animal feed stock. The crude fibre of the genotypes ranging from 3.75% to 6.009% is relatively low as compared to OSMAN (2004) but similar to that of OKOYE & al. (1980). The presence of fibre in foods is known to be beneficial. Fibre has some physiological effects in the gastrointestinal, tract. These effects include variation in fecal water, fecal bulk and transit time and elimination of bile acids and neutral steroids which lower the body

cholesterol pool.The high carbohydrate contents of the genotypes suggests that the genotypes could be used in managing protein-energy malnutrition since there is enough quantity of carbohydrate to derive energy from in order to spare protein so that protein can be used for its primary function of building the body and repairing worn out tissues rather than as a source of energy. Genotypes having carbohydrates percentage above 25% are in agreement with the report of OGBEMUDIA & al. (2017). This could be as result of the location or environment of cultivation and the type of nutrient supply in the course of production.

Mineral concentrations soybean genotypes

The minerals (calcium, potassium, sodium magnesium and phosphorus) contents of the genotypes are appreciably high, but they are consistent with the findings of OSMAN (2004) who found high level of these minerals. The above minerals are required in large quantity by humans thus processing is needed to be adopted in making them available for use. High calcium suggests that the genotypes could be used in complementary foods to help build the bones and teeth since calcium is one of the main components of teeth and bones. Calcium also plays a role in blood clotting. Magnesium is involved in making proteins and releasing energy and helps hold calcium in the enamel of the teeth. Phosphorus is closely linked with calcium. The two minerals combine to form calcium phosphate, which gives bones their rigid structure. Sodium is needed in the body in a small amount to help maintain normal blood pressure and normal function of muscles and nerves. Potassium helps in lipid metabolism and energy transduction in cellular membrane function [MEHAS & RODGERS, 2012].Though it is a fact that mineral nutrients of plant origin are not readily available to man as a result of the inability to be absorbed in the small intestine, but complement those from animal origin [AGBAIRE & EMOYAN, 2012].

Conclusion and recommendations

The studied genotypes have shown good nutritional qualities especially for protein and oil contents. The information contained in this paper will provide long needed bases for soybean quality improvement. The previously uncharacterized accessions NGB00116 and NG/AA/SEP/09/166 could be further evaluated by NACGRAB in collaboration with soybean breeding institutes for possible registration and release for exceptional quality of high protein content and NG/MR/11/11/060 for high oil content.

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HPLC ANALYSIS OF PHENOLIC COMPOUNDS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *AMORPHA FRUTICOSA* L. EXTRACTS

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Abstract: The present study focuses on the chemical and biological analysis of the ethanolic 70% v/v extracts obtained from *Amorpha fruticosa* leaves, branches and fruits. The total phenolic content determined by Folin-Ciocalteu method was the highest in leaves extract (159.5 mg/g). The HPLC-MS analysis indicated the presence of ferulic acid, luteolin and rutoside in all three extracts. The antioxidant activity was tested by the DPPH method and the results indicated a good scavenging activity (EC_{50} = 18.8 ± 0.2 µg/mL) for the fruit extract, followed by leaf extract (EC_{50} = 38.03 ± 0.75 µg/mL) and branch extract (EC_{50} = 221.16 ± 1.7 µg/mL). The antimicrobial activity evaluated by agar disc diffusion method indicated the inhibition of growth for *Staphylococcus aureus* and *Sarcina lutea* for all samples.

Keywords: *Amorpha fruticosa* L., antimicrobial, antioxidant, polyphenols.

Introduction

Amorpha fruticosa L. (indigo bush) is a perennial shrub in the Fabaceae family, order *Fabales*. It is native to south-western part of North America and it was introduced in Europe as an ornamental plant. The species could potentially be used for livestock forage, biomass energy, reclamation of degraded environments, or as insecticide [DE HAAN & al. 2006, PAPANASTASIS & al. 2008]. *Amorpha fruticosa* is considered invasive in Eastern Romania, because it is a naturalized plant that has capacity to spread over a large area [SÎRBU & al. 2012].

Indigo bush has been used as a Chinese folk medicine for hypertension, hematomas, and contusions. Some isoflavones, flavanones, and rotenoids along with their biological activities have been reported for this plant [LEE & al. 2006]. For instance, an isoflavone from *Amorpha fruticosa* can protect the liver from hepatotoxicity induced by acetaminophen [DIAO & al. 2009]. Also rotenoids and flavanones have been shown to possess antibacterial activity via neuraminidase inhibition [KIM & al. 2011].

The immunomodulatory and anticancer activity was also reported for *Amorpha fruticosa* compounds. For example, a prenylfavanone type of flavonoid, amoradicin, manifests tumour necrosis factor (TNF)-alpha inhibitory action [CHO & al. 2000] and phenolic constituents of *Amorpha fruticosa* can inhibit NF-kB activation and related gene

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expression [DAT & al. 2008]. Some rotenoid glycosides of *Amorpha fruticosa* have immune activation effects and could be developed into immunomodulatory agents [LEE & al. 2006].

The fruits contain volatile oil and glycosides of isoflavonoid type, specifically rotenones. One of the most important glycosides present in plant is amorphine. The compound was used in the former U.S.S.R. as a cardio-sedative drug in nervous complaints, vegetative neurosis of the cardiovascular system and paroxysmal tachycardia [KADYROVA & al. 1973a, KADYROVA & al. 1973b].

This study focuses on the chemical and biological analysis of alcoholic extracts from *Amorpha fruticosa* fruits, leaves and branches, collected from Romania.

Material and methods

Plant material

The fruits, branches and leaves of *Amorpha fruticosa* were harvested in October from Pietrarie area (Iași, Romania). The plant material was authenticated by specialists from Department of Pharmaceutical Botany, "Grigore T. Popa" University of Medicine and Pharmacy Iasi, Romania, and the voucher specimens were deposited in the Department Herbarium. The plant material was dried at room temperature, under shade.

Chemicals

Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteu's phenol reagent, methanol, ethanol, acetic acid and hydrochloric acid, Mueller Hinton broth and agar were obtained from Merck (Darmstadt, Germany), while Sabouraud 4% glucose agar was from Fluka Biochemika (Buchs, Switzerland). The antibiotics discs were purchased from Himedia (Mumbai, India).

Standards: caffeic acid, chlorogenic acid, *p*-coumaric acid, kaempferol, apigenin, rutoside, quercetin, quercitrin, isoquercitrin, fisetin, hyperoside, and myricetin from Sigma (Germany), ferulic acid, gentisic acid, sinapic acid, patuletin, and luteolin from Roth (Germany), and caftaric acid from Dalton (USA). Methanolic stock solutions (100 g/mL) of the above standards were prepared and stored at 4 °C, protected from daylight. Before being used as working solutions, they were appropriately diluted with double distilled water.

Preparation of extracts

50 g of pulverized plant material (leaves, fruits and branches) were extracted by percolation with 70% v/v ethanol according to the 10th edition of Romanian Pharmacopoeia (1993).

HPLC apparatus and chromatographic conditions

The analysis used an Agilent 1100 HPLC Series system, equipped with G1315A DAD detector and Agilent 1100 Ion Trap VL mass detector. The column used was Zorbax SB-C18 analytical column, with the following characteristics: 100 mm x 3.0 mm id, 3.5 μm particle, maintained at 48 °C. For preparation of the mobile phase was used a binary gradient made up of methanol:acetic acid 0.1 % (v/v). In the first 35 minutes, gradient elution started at 5% to 42% methanol, and in the next three minutes was isocratic elution with 42% methanol.

UV detection and quantification of polyphenol compounds was achieved at 330 nm and 370 nm for flavonoids. Electrospray ionization (ESI) interface for mass detector was used and the analysis mode was multiple reaction monitoring (MRM) and single ion monitoring (SIM). We optimized conditions: negative ionization, ion source temperature 350 °C, nebulizer nitrogen pressure at 60 psi, capillary voltage + 3000 V, nitrogen gas with a flow rate of 12/min.

The conditions of the methods like retention time, parameters of calibration curves, characteristic ions were described in our previous papers [IVĂNESCU & al. 2010].

Determination of total phenols

The content of total phenolic compounds in branches, leaves and fruits extracts of *Amorpha fruticosa* was assessed by a variant described by Singleton of the Folin-Ciocalteu colorimetric method [LUNGU & al. 2011]. The method is based on the reduction of Folin-Ciocalteu reagent to give a blue color, with visible spectrophotometric detection. For this purpose, for 2 mL of each extract in suitable dilution were added 10 mL of Folin-Ciocalteu reagent (1:10) and 8 mL solution of 7.5% Na_2CO_3. The absorbance was measured at a wavelength of 760 nm after a reaction time of 2 hours at 20 °C, vs a blank consisting of water and reagent, using a Jasco V530 UV-VIS Spectrophotometer. The total concentration of phenolic compounds was calculated using the regression equation obtained from a standard curve prepared with gallic acid solutions in ranging concentrations 0-500 μg/100 mL. The total content of phenolic compounds was expressed as gallic acid equivalents meaning milligrams of gallic acid per gram of the dried plant material.

DPPH Radical Scavenging Activity Assay

The assay of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was carried out by the method of MALTERUD & al. (1993). Each extract was dissolved and diluted in dimethylsulfoxide in different concentrations (0.75 mg/mL and 1.25 mg/mL fruit extract, 1.5 mg/mL and 3 mg/mL leaves extract, 9 mg/mL and 18 mg/mL branches extract). Each dilution of extracts (0.05 mL) was added to 2.95 mL solution of DPPH in methanol (A_{517}nm = 1.00 ± 0.05) and mixed vigorously. The absorbance of DPPH radical solution was measured spectrophotometrically at 517 nm before (A_0) and 5 minutes after mixing (A_1). The inhibition of free radicals from DPPH as a percentage was calculated with the following formula:

DPPH radical scavenging activity (%) = $100 \times (A_0 - A_1)/A_0$

For each sample the effective concentration (EC_{50}) was calculated. This value was defined as the concentration of 50% DPPH radical scavenging activity. Quercetin was used as a positive control and all tests were carried out in triplicate.

Microorganisms

The antimicrobial activity was studied using Gram positive bacteria (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Bacillus cereus* ATCC 14579, *Bacillus subtilis*), Gram negative bacteria (*Escherichia coli* ATCC 25922) and pathogenic yeasts (*Candida albicans* ATCC 10231, *Candida sake*, *Candida glabrata*). All these strains were obtained from the Culture Collection of the Department of Microbiology, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania.

Agar disc diffusion method

Antimicrobial activity was evaluated by agar disc diffusion method according to described protocols [NCCLS, 2012]. Sterile stainless steel cylinders (50 mm internal diameter; 100 mm height) were applied on the agar surface in Petri plates. 100 μL of each volatile oil sample were added to each cylinder. Commercial available discs containing ampicillin (25 μg/disc), chloramphenicol (30 μg/disc) and nystatin (100 μg/disc) were used. The plates were incubated at 37 °C for 24 h (bacteria) and at 24 °C for 48 h (yeasts). After incubation the diameters of inhibition zones were measured.

Statistical analysis

All assays were carried out in triplicate. Results are expressed as means ± SD. The EC_{50} values were calculated by linear interpolation between values above and below 50% activity.

Results and discussions

Total phenolic content

Table 1 shows the total phenolic content in *Amorpha fruticosa* extracts, determined by spectrophotometric method. The content of total phenolic compounds was highest in leaves extract followed by fruits extract and branches extract.

Table 1. Total phenolic content in *Amorpha fruticosa* extracts

Total phenolic content mg/g	***Amorpha fruticosa* extract**		
	branches	leaves	fruits
	14.08	159.5	122.10

The total polyphenols in leaf extract determined in our study was higher than the one determined in an ethanolic extract (54.7 mg GAE/g dried plant) by HOVANET & al. (2015). These results can be explained by the different harvest period (August vs. October), and also by the distinct environmental conditions.

HPLC analysis of polyphenols

Eighteen polyphenolic compounds have been investigated: one hydroxybenzoic acid, six cinnamic acid derivatives, four quercetin glycosides, and seven aglycones of flavonol and flavone type. The amounts of polyphenols found in *Amorpha* extracts are presented in Table 2, expressed in mg/g dried plant material. The polyphenolic compounds are shown in order of their retention time. Quantification of constituents was performed using UV detection at 330 nm for phenol carboxylic acids and 370 nm for flavonoids.

Table 2. Polyphenol compounds in *Amorpha fruticosa* extracts (mg/g)

Polyphenols	*Amorpha fruticosa* extract								
	branches			leaves			fruits		
	UV	MS	Conc. mg/g	UV	MS	Conc. mg/g	UV	MS	Conc. mg/g
Gentisic acid	-	*	-	-	*	-	-	*	-
Caffeic acid	-	*	-	-	*	-	-	*	-
Chlorogenic acid	-	*	-	-	*	-	-	*	-
p-coumaric acid	*	*	0.0232	*	*	-	*	*	0.0557
Ferulic acid	*	*	0.0081	*	*	0.0121	*	*	0.0233
Hyperoside	*	*	-	*	*	0.2402	*	*	0.0122
Isoquercitrin	*	*	-	*	*	9.4476	*	*	0.1765
Rutoside	*	*	0.5931	*	*	11.8603	*	*	0.2754
Quercitrin	*	*	0.0117	*	*	0.3139	-	-	-
Quercetin	*	*	0.0089	*	*	0.0641	-	-	-
Luteolin	*	*	0.0162	*	*	0.0093	*	*	0.0688
Kaempferol	-	-	-	*	*	0.0202	*	*	0.1595

*****only MS qualitative determination was done; UV signal < LoQ (limit of quantification) or interferences/peak overlapping from other compounds does not allow the quantitative determination of these substances
Not found: sinapic acid, caftaric acid, fisetin, patuletin, apigenin, myricetin

The results indicate the presence of ferulic acid, luteolin and rutoside in all samples in different concentrations. Gentisic acid, caffeic acid and chlorogenic acid could not be quantified in any of samples, but they were identified. Quercitrin and quercetin could not be founded in fruits extract and kaempferol in branches extract. Hyperoside and isoquercetrin from branches extract and p-coumaric acid from leaves extract were identified by UV and MS, but could not be determined quantitatively because the amount was below the limit of quantification. Rutoside was found in large quantities in all three extracts, and in leaves and fruits extracts was followed by isoquercitrin. The quantity of quercitrin and quercetin was higher in leaves extract than branches extract. Also, hyperoside and isoquercitrin were in higher quantities in leaves extract than fruits extract.

Our results correlated with those of CUI & al. (2017) who also have isolated and identified rutin, trans-p-coumaric acid, quercetin, apigenin and other compounds in leaf extracts harvested in different periods of the year (May to August).

Antioxidant activity

The free radical scavenging activity of the three extracts was measured by the DPPH method. This method has been widely used to evaluate free radical scavenging ability of different plant extracts. DPPH is a free radical stable at room temperature that possesses a characteristic absorption at 517 nm (purple in colour). It is reduced in the presence of an antioxidant to yellow-coloured methanol solutions. Table 3 shows the EC_{50} values for the antioxidant activity of *Amorpha fruticosa* extracts.

Table 3. DPPH radical scavenging activities (EC_{50}) of *Amorpha fruticosa* extracts

***Amorpha fruticosa* extracts/ Positive control**	**EC_{50}[a] (µg/mL)**
Leaves extract	38.03 ± 0.75
Fruits extract	18.8 ± 0.2
Branches extract	221.16 ± 1.7
Quercetin	2.2 ± 0.0

[a]values are mean ± SD of three determinations

The highest scavenging effect was observed in *Amorpha fruticosa* fruits extract (EC_{50} = 18.80 ± 0.20 µg/mL), followed by leaves extract (EC_{50} = 38.03 ± 0.75 µg/mL) and branches extract (EC_{50} = 221.16 ± 1.70 µg/mL). The EC_{50} value of quercetin was lower than those of *Amorpha fruticosa* extracts indicating higher DPPH scavenging activity. Quercetin is a known flavonol with high ability to scavenge free radicals. The presence of three hydroxyl groups, a 2,3-double bond and a 4-oxo function in the C-ring are important structural element for enhanced antioxidant activity [CAI & al. 2006].

The antioxidant activity of the ethanolic extracts of *Amorpha fruticosa* has not been yet investigated. Recently, ZHELEVA-DIMITROVA & al. (2013) reported that the 80% methanol (v/v) extract of *Amorpha fruticosa* fruits showed a stronger scavenging activity on DPPH radicals (EC_{50} = 9.83 µg/mL) than leaves extract (EC_{50} = 11.23 µg/mL). Different antiradical activities of the same species can be explained by differences in plant extraction, method of analysis used and geographical origin of plant.

Antimicrobial activity

The antimicrobial activity of the extracts was tested against four Gram-positive bacteria (*Staphylococcus aureus*, *Sarcina lutea*, *Bacillus cereus*, *Bacillus subtilis*), two Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) and against three fungi (*Candida albicans*, *Candida glabrata*, *Candida sake*). The test was carried out by a disc diffusion method, using ampicillin, chloramphenicol and nystatin as positive control. The effects of ethanol extracts from branches, leaves and fruits of *Amorpha fruticosa* on the tested strains are shown in Table 4.

According to results, the fruit extract was active against all Gram positive bacteria with a diameter of inhibition zone ranging between 15 and 25 mm. The branches and the leaves extracts showed similar activity against *Staphylococcus aureus* ATCC 25923 and *Sarcina lutea* ATCC 9341. No extract was active on the Gram negative strains and fungi.

Table 4. Antimicrobial activity of *Amorpha fruticosa* extracts

Microorganism	**Diameter of inhibition zone (mm)**					
	Branches extract	Leaves extract	Fruits extract	Ampicillin (25 µg/disc)	Chloram phenicol (30 µg/disc)	Nystatin (100 µg/disc)
Staphylococcus aureus ATCC 25923	16	21	19	26	24	nt
Sarcina lutea ATCC 9341	15	22	25	28	26	nt
Bacillus cereus ATCC	0	0	16	0	21	nt
Bacillus subtilis	0	0	17	26	29	nt
Escherichia coli ATCC 25922	0	0	0	21	29	nt
Pseudomonas aeruginosa ATCC 27853	0	0	0	0	16	nt
Candida albicans ATCC 10231	0	0	0	nt	nt	18
Candida glabrata ATCC MYA 2950	0	0	0	nt	nt	19
Candida sake	0	0	0	nt	nt	20

nt – not tested

For fruits, the results obtained in the antimicrobial test are similar to those reported by BORCHARDT & al. (2008) for *Amorpha fruticosa* fruit and seeds harvested from the Mississippi river basin (USA). The 50% methanol extract showed a 17 mm zone of inhibition against *Staphylococcus aureus* and demonstrated no effect on *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* [BORCHARDT & al. 2008].

For the leaf extract, our results can also be correlated with those of HOVANET & al. (2015). They determined that the extract was active on Gram positive bacteria (*Staphylococus aureus* ATCC 6538 and *Bacillus subtilis*), inactive on some of Gram negative strains (*Pseudomonas aeruginosa* 13202, *Escherichia coli* ATCC 13202) and on some *Candida* strains. There are no reported results in literature for the antimicrobial activity of the branch extract.

Conclusions

The content of total polyphenolic compounds within extracts is correlated with their antioxidant and antimicrobial activity. Overall, fruits extract of *Amorpha fruticosa* proved to have a good DPPH scavenging effect. These results indicate that the ethanol extract of fruits may be a good source of natural antioxidants. In addition, the antimicrobial test showed that Gram positive bacteria are moderately sensitive to the action of *Amorpha fruticosa* extracts.

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ASSESSMENT OF GENETIC VARIATION OF *TILIA TOMENTOSA* BY RAPD MARKERS

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Abstract: *Tilia tomentosa* varieties were analyzed using Random Amplified Polymorphic DNA (RAPD) primers to determine genetic diversity. In this study, six samples provided by the Botanical Garden "Anastasie Fătu" of Iasi, Romania were compared to a variety collected from an ecosystem in Giessen, Germany. A total of 91 bands were obtained from nine RAPD primers, 79 (86.8%) of which were polymorphic. Marker data was used for a principal component analysis that showed clear differentiation among Romanian and German samples. Furthermore, Romanian genotypes were clustered together in the principal component analysis. PCA analysis was followed by an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis which revealed two major groups. Samples from the Romanian ecosystem showed high genetic similarity. Generally, samples did not separate from each other in the UPGMA analysis, excepting the German sample.

Keywords: DNA, genetic diversity, principal component analysis, silver lime tree, UPGMA cluster analysis.

Introduction

Trees of the genus *Tilia* L. (*Malvaceae*) can be found in urban and forest ecosystems especially in the northern hemisphere in Europe, but also in Asia and Northeastern and Central America. Due to their polymorphism as well as a large number of transitional forms and hybrids, more than 60 species of *Tilia* are reported [WERYSZKO-CHMIELEWSKA & al. 2019]. The Plant List website reports more than 45 accepted *Tillia* species and a number of 477 lines and hybrids. This genus grows in temperate, subtropical and tropical climates and occurs from moist to dry regions [MUIR, 1984]. Linden trees could act as noise filters and purify the air through capturing the pollutants originating from traffic and different industrial activities. They reduce flooding and damage to urban property during stormwater runoff and also urban heat intensity [SJÖMAN & al. 2012]. As a result, representatives of these genera became increasingly important for local Romanian landscapes, in municipal parks in large cities or as avenue trees [SUKOPP & WURZEL, 2000].

In many European cities, growing damages of *Tillia tomentosa* Moench trees are caused by abiotic stress factors like salt and drought [Helsinki: TERHO & HALLAKSELA (2005), Warsaw: CHMIELEWSKI & al. (1998), Budapest: SINKO (2005)]. For this reason, in Europe since the 1960s *T. cordata* Mill. and *T. platyphyllos* Scop. were increasingly replaced by *T. tomentosa* Moench and *T.* × *euchlora* K. Koch originating from Southeastern Europe and the Caucasian region. These varieties are better adapted to urban conditions in Central Europe. Nowadays, breeding efforts should focus more on better adapted plant

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material to ever-changing climatic challenges as high drought stress resistance, salt-resistance and tolerance to soil compaction for urban areas occur. (KARNOSKY & al. 1982).

Material and methods

Plant material

Leaf material was collected from "Teiul lui Eminescu" ("Eminescu's Linden") – an emblematic 500-year old tree for Romanian culture, being set in Copou Park in Iaşi, Romania (P5 in Table 1), three putative clones of P5 (P1 and P2 – cultivated in the Botanical Garden "Anastasie Fătu" of Iaşi since 1981 – in Table 1), P3 – being a clone of P1, cultivated in the Botanical Garden "Anastasie Fătu" of Iaşi since 2012 and offered by the city officialities to the people of Wien and a *T. cordata* Mill. sample (P4 in Table 1) available at the Botanical Garden of Iaşi, Romania, a *T. tomentosa* Moench samples from USAMV-Iaşi, Romania (P6, in Table 1) and one extra sample of *T.* × *euchlora* K. Koch collected from Giessen, Germany (P7 in Table 1). All samples from Iasi are very close to *T. tomentosa* regarding morphological traits, while P4 was identified previously as *T. cordata* and P7 as *T.* × *euchlora* by morphological traits. *T.* × *euchlora* (Caucasian or Crimean linden) is a hybrid whose parentage is unclear but is considered *T. cordata* and *T. dasystyla*. The different species far distant from the putative relatives of the interesting clones were integrated as outgroups to test the reliability of the analytic methods.

Table 1. Plant material collection of genera *Tilia*, including clones

No.	Species	Abbreviation	Location
1.	*T. tomentosa*	P1 (Clone)	Botanic Garden "Anastasie Fătu", Iaşi, RO
2.	*T. tomentosa*	P2 (Clone - atypical appearance)	Botanic Garden "Anastasie Fătu", Iaşi, RO
3.	*T. tomentosa*	P3 (Clone)	Botanic Garden "Anastasie Fătu", Iaşi, RO
4.	*T. cordata*	P4	Botanic Garden "Anastasie Fătu", Iaşi, RO
5.	*T. tomentosa*	P5 (*Teiul lui Eminescu – Eminescu's Linden*)	Parcul "Mihai Eminescu", Iaşi, RO ("Mihai Eminescu" Park)
6.	*T. tomentosa*	P6	USAMV "Ion Ionescu de la Brad", Iaşi, RO
7.	*T. euchlora*	P7 (*Krimlinde*)	Lonystrasse, Giessen, DE

Random Amplified Polymorphic DNA (RAPD) analysis

Fresh tissues were harvested in September 2017 in Iasi and Giessen. Leaves were frozen in liquid nitrogen and homogenized. DNA was isolated following the Doyle and Doyle DNA extraction protocol [DOYLE & DOYLE, 1990] DNA extraction efficiency was evaluated using the Qubit™ Fluorometer (Thermo Fisher Scientific Inc., USA) and NanoDrop™ 2000 spectrophotometric assays (Thermo Fisher Scientific Inc., USA) for specifically quantitate DNA.

A standard protocol was used to carry out the RAPD analysis. As a preliminary study, 12 arbitrary primers (Operon Technologies Inc., USA) were used for the amplification (Table 2). From these 12 primers, nine were selected for Polymerase Chain Reaction (PCR). PCR was

performed in 20 µl reaction volume containing 10-20 ng template DNA, dNTP mix (200 µM each nucleotide), 0.25 µM primer, reaction buffer (including $MgCl_2$ to a final concentration of 2.5 mM) and 1 Unit *Taq* DNA polymerase (Thermo Fisher Scientific Inc., USA).

The amplification reactions were carried out in a Thermocycler (Bio-Rad Laboratories, Inc., USA) with the following program: Initial denaturation 3 minutes at 94 °C; 35 cycles with 1 min 94 °C denaturation, 1 min 35 °C annealing, 1 min 72 °C elongation; final extension 10 min 72 °C; hold 4 °C.

PCR products were separated in 1% agarose gel with 1×Tris-borate-Ethylenediaminetetraacetic acid (EDTA) (TBE) – Life Technologies buffer. The gels were previously run at 120V for 2 hours and afterward were digitally photographed under UV light. The used marker was a 100-bp DNA ladder (Thermo Fisher Scientific Inc., USA). Replications of DNA extraction and amplification were performed with relevant primers and a subset of samples to confirm reproducibility.

Table 2. Molecular markers used for RAPD analysis of *Tilia* specimens

No.	Primer code	Sequence (5'–3')
1.	R5	CTG GGG CTG A
2.	R8	GGT GGT GAT G
3.	R9	AAA GTG CGG C
4.	R12	AAT GCG GGA G
5.	R17	TGC TCT GCC C
6.	R18	GGT GAC GCA G
7.	L 12	GGG CGG TAC T
8.	OPA 03	AGT CAG CCA C
9.	OPB 10	CTG CTG GGA C
10.	OPE 20	AAC GGT GAC C
11.	OPG 14	GGA TGA GAC C
12.	OPD 11	AGC GCC ATT G

Data analysis

The RAPD bands were scored as present (1) or absent (0). A matrix of similarities between every pair of individuals was obtained by using the Euclidean distance index [GOWER, 1985]. Principal component analysis (PCA) was performed using the RAPD relationship between individuals using the R-code 3.3.3 (R Core Team, 2018) software and the "prcomp" function [VENABLES & RIPLEY, 2002]. Additionally, a dendrogram was generated based on Euclidean's similarity coefficients using the unweighted pair group method with calculating the arithmetic average (UPGMA) by R-code, "agnes{cluster}".

Results and discussions

A total of 12 RAPD primers were tested and nine of them produced suitable and reproducible banding patterns. We obtained 91 fragments that could be scored and used for further analysis. Out of the 91 fragments, 79 were polymorphic with an average of 8 polymorphic fragments per primer. The highest number of polymorphic bands (12) was obtained with primer L12 and R18, whereas the lowest number of bands was obtained with primers R5 (5) and OPG 14 (6).

Based on UPGMA analysis, two major groups were observed: P4 and P7 clustered together with a major group, whereas the other 6 genotypes were grouped together in another major group (Figure 1). The *T. tomentosa* clonal genotypes (P1, P2, P3 indicated in black color) were clustered together with the P5 ("Teiul lui Eminescu") genotypes in the first major group. Notably, the highest similarity value was found to be between genotype P1 and genotype P5. The dendrogram was in accordance with the geographic distributions of *Tilia* genotypes, by separating sample P4 and P7 from the group from Iaşi.

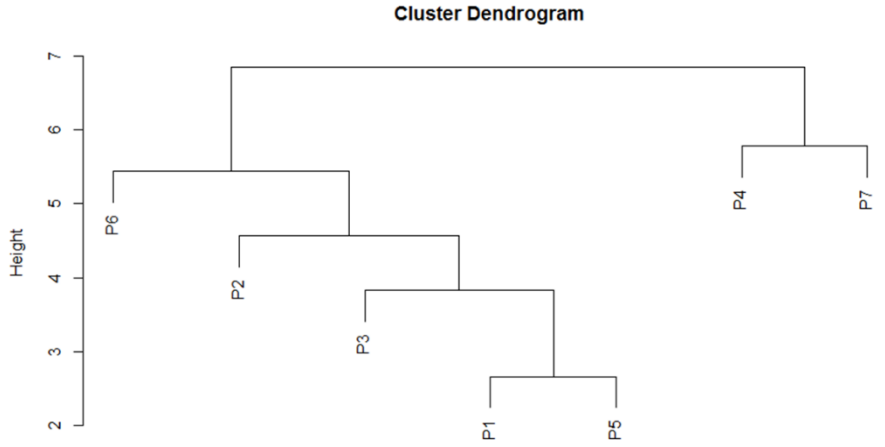

Figure 1. Dendrogram using the unweighted pair group method with calculating the arithmetic average (UPGMA) cluster analysis based on Euclidean similarities of 7 *Tilia* genotypes by using RAPDs (Abbreviations see Table 1).

PCA was performed to visualize the genetic relationship between *Tilia* genotypes (Figure 2). The first two principal components explain a large proportion of genotypic variance present in the data set, namely 46.2% (PC1) and 16.4% (PC2). A wide range distribution profile with two subplots with *Tilia* genotypes was obtained. Two subplots comprised Iasi genotypes (1), whereas the other one (2) comprised the genotypes from Giessen.

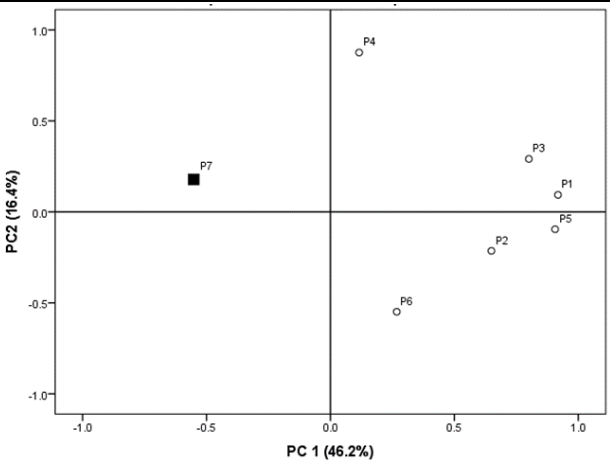

Figure 2. Principal component analysis plot of the RAPDs data among 7 genotypes of *Tilia*

In-plant sciences, biological diversity can be separated into three levels, such as genetic diversity, species diversity and ecosystem diversity [RAO & al. 2002]. Nowadays, tree biodiversity is altered by increased human intervention in natural habitats, fragmentation and loss of fertile soils, the introduction of new species in urban areas, climate change and many more [KOWARIK, 2011]. LOVELESS & HAMRICK (1984) suggested that genetic variations in plant populations are considered to be shaped by location and time. Trees resistance is supported by its lifespan, phenotypic plasticity, and gene diversity among populations. Up to date, the genetic studies realized with Tilia species have been limited [LIESEBACH & SINKO, 2008; FILIZ & al. 2015]. Thus, in this study, the genetic variations of *T. tomentosa* genotypes were investigated by using RAPD markers.

In this study, polymorphic bands and the average population diversity were found to be high in *T. tomentosa* by using nine different RAPD primers. In a similar study, conducted with *T. rubra* populations from the north of Iran, polymorphic bands and average population diversity were found to be 41.4% and 0.22, respectively, by using seven RAPD primers [COLAGAR & al. 2013]. In another study, *T. cordata*, *T. platyphyllos*, *T. dasystyla*, *T. euchlora*, *T. europaea*, *T. tomentosa*, *T. americana*, 36 genotypes were genotyped using 17 RAPD primers that yielded 403 polymorphic bands [LIESEBACH & SINKO, 2008]. Results were comparable with our findings, although higher polymorphism levels are generated by a wide gene pool of different *Tilia* taxa. Also, FILIZ & al. (2015) found 53 polymorphic fragments and average population diversity of 94.29% in *T. tomentosa* by using eight different RAPD primers. Our results were similar to *T. tomentosa* previous reports suggesting that eco-geographic characteristics and life history are important factors for genome structure in woody plant species.

The cluster analysis using the UPGMA dendrogram revealed a clear differentiation among genotypes based on geographical origin (Figure 1). The principal component analysis confirmed the UPGMA tree data and clustered P7 separately. As expected, P1 to P6 clustered together in the PCA. Clonal propagation results in the reduction of genotypic diversity and population differentiation, but, on the other hand, may increase heterozygosity [BALLOUX & al. 2003]. Moreover, asexual multiplication is critical for studying the effects of heterozygosity and genetic structure [HALKETT & al. 2005]. Most *Tilia* taxa are produced

through vegetative reproduction, summing approx. 77%-80% of the young trees in the southwest region of Russia and almost 100% in the north-east region of Europe [FILIZ & al. 2015].

Conclusions

The high rates of asexual reproduction of *T. tomentosa* could be clearly seen in the UPGMA tree and PCA results. Similar genetic structures could also explain the origin of *T. tomentosa* individuals as some are influenced by eco-geographical factors. A further breeding effort for *Tilia* trees should focus on crossings between parental lines with high-stress tolerance and adequacy to urban environments. Furthermore, the ability for vegetative multiplication should be included in the selection criterions for breeding purposes. Further studies are necessary for a good classification of reference material and further taxonomic investigation. New developments in the area of the molecular marker should be included used to obtain reliable results and sustainable outcomes, according to breeding objectives.

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DEVELOPMENT AND TESTING A NEW TECHNOLOGY FOR PRODUCTION OF CHRYSANTHEMUMS PLANTING MATERIAL (*CHRYSANTHEMUM* s.l.)

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Abstract: Chrysanthemums represent an important horticultural segment with uses in the autumnal to winter season, aesthetically extending the capitalization of urban open spaces. Propagation by cuttings at *Chrysanthemum* is the most convenient method of vegetative multiplication. There is, however, a great variability in the ability to form adventitious roots and regenerate new individuals by cuttings. Some cultivars are relatively easy to multiply by cuttings, and other species have a low capacity to rooting. In the present paper we focused on researches regarding the propagation of chrysanthemums (a_1 – *Chrysathemum indicum*, a_2 – *Chrysanthemum* × *grandiflorum*) by new type of cuttings (stem fragment with one leaf and axillary bud – SFLAB, b_2) and on the evaluation of the capacity of rooting, given the increasing demand for floral planting material as a result of the development of landscape design interest in public spaces but especially private one. The main objective was to establish the influence of the cuttings characteristics (type of cuttings, with two variants – b_1-control, top stem fragment – TSF; and b_2, stem fragment with one leaf and axillary bud – SFLAB) on the rooting process and the length of developed roots in four variants of rooting substrate (c_1-c_4) for both types of proposed *Chrysanthemum* cuttings (b_1-b_2).

Keywords: *Chrysathemum*, cuttings, rooting substrate, stem fragment with one leaf and axillary bud – SFLAB, top stem fragment – TSF.

Introduction

Chrysanthemum growers are obvious interested to use low cost techniques whenever possible in their crop production. Modern technologies does not reduce the risk of producing non-compliant planting material nor the risk of contamination by viral and bacterial diseases, if certain procedures are not strictly followed. Chrysanthemum growers need to know the key practices for alternative multiplication methods to make good choices in their production planning.

In the last years have been conducted studies at many species in order to speed up the process of rhizogenesis, especially by using the induction of rhizogenesis with different chemicals (growth or rooting regulators). There are a number of factors such as the type of cuttings, rooting substrate, season, pre-treatments with growth regulators and also environmental conditions (such as temperature, light and humidity) that have been reported to influence successful rooting of *Chrysanthemum* s.l.

In Romania, fewer studies have been conducted on chrysanthemums regarding alternative ways of multiplication and the conditions that influence multiplication, but quite

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number of studies refer to biometric, morpho-anatomic aspects, also to some modern directions aimed at *in vitro* propagation of chrysanthemums [LAZĂR & CACHIȚĂ-COSMA, 1982; LAZĂR & al. 1981; PETRUȘ-VANCEA & CACHIȚĂ-COSMA, 2013]. So far, there are a lot of published reviews related to the Chrysanthemum biotechnology [ROUT & DAS, 1997; TEIXEIRA DA SILVA, 2003, 2004; SHINOYAMA & al. 2006; TEIXEIRA DA SILVA & al. 2013]. However, many new studies are published every year. By better understanding of the ornamental chrysanthemum biotechnology (traditional propagation, mutation reproduction or transgenic technologies), new types of plants could be generated: with high resistance to biotic stress (diseases and pests) and abiotic (temperature, salinity, water stress, etc.), or plants for cut flowers with a longer life after harvesting and thus better storage capacity, new colors for flowers, new leaf shapes or architecture [TEIXEIRA DA SILVA & al. 2013].

At dicotyledonous species, adventitious roots can be defined as roots that can develop under specific conditions, from organs such as leaves and stems. The formation of adventitious roots (AR) in the cuttings from top stem fragments, is a physiological key process in the propagation of many species of ornamental plants. Despite the intensive control on environmental factors in the modern ornamental plant propagation industry, significant economic losses continue to occur as a result of insufficient rooting [LIU & al. 2013]. Chrysanthemums (*Chrysanthemum* s.l.) have significant ornamental value and thus have great economic importance, being also subjected to losses due to insufficient rooting of young shoots, the main method of vegetative propagation practiced in this culture. Insufficient understanding of the mechanisms that control adventitious root formation prevents the use of reliable technologies to improve the percentage of adventitious root development in ornamental plant cuttings in general.

Adventitious rooting is a quantitative trait of a genetic nature that is affected by several endogenous but also environmental (exogenous) factors. One of the endogenous factors with a key role in controlling AR formation is auxin. Many authors have shown that auxin has the ability to initiate AR formation. PAGNUSSAT & al. (2002) demonstrated that nitric oxide mediates the auxin response that results in AR formation in cucumber, and LIAO & al. (2010) studied the role of nitric oxide in the process of AR formation in *Chrysanthemum*. The progressive accumulation and local concentration of auxin at the base of the cuttings seems to be important for initiating the rooting process [ACOSTA & al. 2009]. There is growing evidence that AR formation also depends on the action of ethylene [CLARK & al. 1999; SHIBUYA & al. 2004], which occurs as a result of the injury of the vegetal tissue in the process of making the cuttings and has been studied in a large number of species of ornamental plants or of industrial value. The response of the plant to the injury produced by the cut applied to the stem is a necessary step in the process of forming AR [DA COSTA & al. 2013]. Once the stem has been excised from the mother plant, it must redistribute the remaining resources as soon as possible, to form adventitious roots and restore the physiological balance, which allows the transfer of resources between the different parts of the seedling, and some research has shown that the distribution of carbohydrates in detached cuttings may be more important than the content itself in such substances [RUEDELL & al. 2013].

Traditional methods of propagation are no longer able to respond to growing market demands and to meet new consumer demands, so new biotechnological methods must be applied. Biotechnology includes areas of study such as in vitro culture and micropropagation, cryopreservation, molecular technologies, genetic transformation, synthetic seed technology,

secondary metabolism and acclimatization, and so far many studies have been published on the biotechnology of ornamental chrysanthemums.

The main purpose of the research is to improve production technology of chrysanthemum cuttings, in order to increase the efficiency of planting material production by a higher percentage of rooted cuttings and by a new quality planting material. The new methods proposed in the research in order to obtain chrysanthemum seedlings by testing the economic yield in the field, could lead to a decrease in total production costs, but also a quantitative reduction of the plant stock material used for propagation, with the unaltered maintenance of the quantitative yield for the final plant material.

Material and methods

Plant materials and growing conditions. The experimental field was set at "Anastasie Fatu" Botanical Garden belonging to "Alexandru Ioan Cuza" University of Iasi, Romania, in the heated greenhouses compartments and were used two *Chrysanthemum* cultivars from institution Collection – *Chrysanthemum indicum* L. cv. Carmina, noted in our research as (I, a_1) and *Chrysanthemum* × *grandiflorum* Ramat. cv. Yellow Stardust (II, a_2) [The Plant List, 2014; The Plant List, 2014a]. The cuttings were set about February 15, collected from stock-plants – Figure 1, maintained in a vegetative stage, in cold greenhouses with a main temperature of 13.8 °C and with moderate irrigation once for a week.

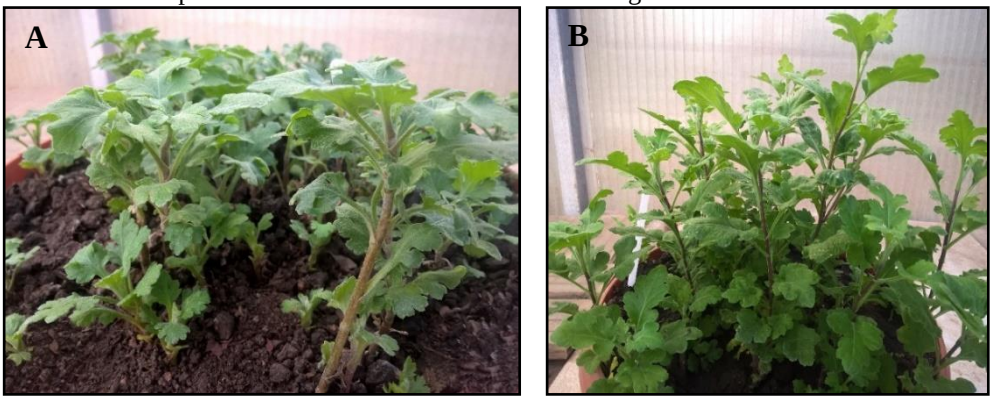

Figure 1. Stock-plants of *Chrysanthemum*, **A.** a_1 Cv. I – *Chrysanthemum indicum* 'Carmina', **B.** a_2 Cv. II – *Chrysanthemum* x *grandiflorum* 'Yellow Stardust' (details on vegetative shoots)

Experimental Model

For the proposed research, was organized a multifactorial experiment that included three factors (a, b, c). Multifactorial experiments are characterized by the fact that the same experiment studies the simultaneous influence of several factors and has the advantage of highlighting the interactions between such a different factors.

The type of chosen experiment was tri-factorial, type 2(a)x2(b)x4(c) – Figure 2. The studied factors were: factor A – the chrysanthemum cultivar, with two graduations, a_1: I – *Chrysanthemum indicum* 'Carmina' a_2: II – *Chrysanthemum* × *grandiflorum* 'Yellow Stardust'; Factor B – the type of cutting, with two graduations, b_1: top stem fragment – TSF, control and b_2: stem fragment with one leaf and axillary bud – SFLAB; Factor C – the type of rooting substrate with four graduations, c_1: pt - peat (100%), c_2: pt+p - peat+perlite (v/v),

c_3: pt+p+s - peat+perlite+sand (v/v/v), c_4: p - perlite (100%). Thus, from the combination of factors and their graduations, 16 experimental variants resulted, and for each experimental variant, 25 cuttings were made, with a total of 400 cuttings per experiment. The control variant (standard or control – b_1) is the variant that served as the basis of comparison for the other variants researched in the experiment.

a_1								a_2							
b_1				b_2				b_1				b_2			
c_1	c_2	c_3	c_4	c_1	c_2	c_3	c_4	c_1	c_2	c_3	c_4	c_1	c_2	c_3	c_4

Figure 2. Experimental scheme: factor A – the chrysanthemum cultivar, with two graduations, a_1 and a_2; factor B – the type of cutting, with two graduations, b_1 and b_2; factor C – the type of rooting substrate with four graduations, c_1, c_2, c_3 and c_4.

Sampling of chrysanthemum cuttings. In chrysanthemum crops, as a classical (control) manner, the cuttings are made from shoots that develop at the base of the stock-plants and are harvested when they are 8-10 cm long (Figure 3, A). Choosing healthy stock-plants is essential, and in order to reach the objectives of the present study, special attention was given to respecting their length at harvest (including the number of leaves), in order to maintain the experimental characteristics with at least 9 leaves on each vegetative shoot.

The two types of cuttings were made differently, according to the proposed experimental scheme:

TSF: represents the classic type of cuttings used in the vegetative propagation of chrysanthemums, respectively cuttings from the top of the shoots – top stem fragment (Figure 3, B). For the interpretations related to the influence of the type of cuttings on the rooting and obtaining of planting material, this type of cuttings represented the control and provided comparative data to establish the efficiency of the proposed new method for cuttings.

The TSF cuttings are made after a period when the stock-plants were forced, providing temperatures of 8-10-12 °C, normal aeration, lightning and watering, in order to start into vegetation. A shoot should be with 9-10 leaves, and 8-10 cm long (Figure 3, A). The first pairs of leaves are suppressed and only 3-4 leaves are left on the top, after which a cut with the scissors or the sharp knife under a node or oblique through the node is executed. The remaining leaves at the top are reduced by pinching, if the cuttings are very vigorous. In the end, a TSF cutting had a length of 5-6 cm, which provides at the base a minimum area of 2.5 cm for mounting in the rooting substrate, and at the top 3-4 cm for supporting the metabolic processes through the foliar apparatus (Figure 3, B).

SFLAB: represents a new type of proposed cutting for the experiment, made as a single-node stem fragment, with the corresponding leaf of the node, at least until the emergence of the new shoots developed from the axillary buds (Figure 4, A, B) – stem fragment with one leaf and axillary bud (SFLAB). The execution of such a cuttings is made by two cuts made on the stem, oblique, above and below the node (Figure 4, A), obtaining a fragment of the stem with a length of about 1 cm, having a corresponding node and leaf, with axillary buds intact.

As experimental sub-variants, we considered the achievement of 9 SFLAB cuttings from a single harvested shoot, marking three levels of leaf development, with different degree of stem maturation (Figure 4, B):

- Lower level (INF) – at the base of the shoot: 3 harvested SFLAB cuttings (1-3)
- Medium level (MED) – 3 harvested SFLAB cuttings (4-6)
- Upper level (SUP) – at the top of the stem: 3 harvested SFLAB cuttings (7-9).

The shaping of the SFLAB cuttings in both variants requires greater attention in terms of hygiene of the work, the working tools being periodically cleaned and disinfected, and the maneuvering space of the periodic vegetal material cleaned by removing the obtained vegetal debris.

The purpose of testing the second type of cuttings (SFLAB) is to verify the improvement of the yield of cuttings in chrysanthemums, in order to increase the number of cuttings that can be made from a single shoot: a single cuttings in the case of the control variant (TSF) and 9 cuttings for the proposed variant (SFLAB).

Observations, measurements and determinations

The parameterization of the qualitative and quantitative characteristics of the cuttings in the two varieties of chrysanthemums taken in the study consisted in making the following observations and measurements: observations on the degree of survival and the percentage of rooting of chrysanthemum cuttings; measurements on the aerial (vegetative) system of cuttings - height of cuttings (cm), measured at 4, 6, 8 weeks (W4, W6, W8), number of leaves developed per seedling, at 4, 6, 8 weeks (W4, W6, W8); measurements and observations on the root system of the cuttings – the total length of the developed roots/cuttings (cm), roots density (m/m^3), display and observations on the root architecture of chrysanthemum cuttings; observations to establish the days for development of new shoots formed from the axillary buds of the leaf, at the SFLAB cuttings.

Mathematical and statistical models for results interpretation

Within the experiment, for the determinations related to root length, the root system of chrysanthemum cuttings was taken from all 400 samples corresponding to the experimental variants. Only roots with a diameter below 2 mm were sorted by direct calibration, cleaned by water washing and examined in transparent Petri dishes on millimeter paper. The distribution of removed roots based on the exceeded diameter (> 2mm) was not quantitatively determined, but all the root samples examined included a typical root diameter range (0.1 to 2.0 mm). The root samples contained all types of roots, including suberized, unsubstituted, secondary thickened and lateral, as long as they respected the required diameter limit and were left as complete as possible [GOUBRAN & RICHARDS, 1979].

The roots were washed under water to remove adherent substrate particles and spread on laminated sheets of millimeter paper (A4 or A3 size). Thus, the roots were examined and the data were noted by evaluating the dimensions of the displaying zone and area determination, counting the vertical and horizontal lines taken into account, scoring the lengths of the background system lines, counting the intersections between the roots of the cuttings and the system of background lines. These data were inserted into tables to further facilitate the calculation of the total root length using Newman calculation formula.

The statistical methods were based on the experimental data obtained and which represent the numerical results of the experimental research. The data obtained during the experiment were noted in the centralization tables, on the basis of which several distribution functions were calculated. For the values obtained in the quantitative and qualitative biometric measurements (the height of the cuttings, the number of leaves, the length of the roots, the density of the roots) the average values were calculated based on the recordings for all 25 repetitions (cuttings) per experimental variant. Also, in order to appreciate the uniformity of the studied variants, the standard deviation (SD) of the obtained average was calculated. For roots density determination (D), the root length was reported at a given substrate volume, i.e. 60 ml (0.06 m^3), the results being expressed in m/m^3. Formula used in the calculation [ALI, 2010]: Root density (D) = total root length / substrate volume.

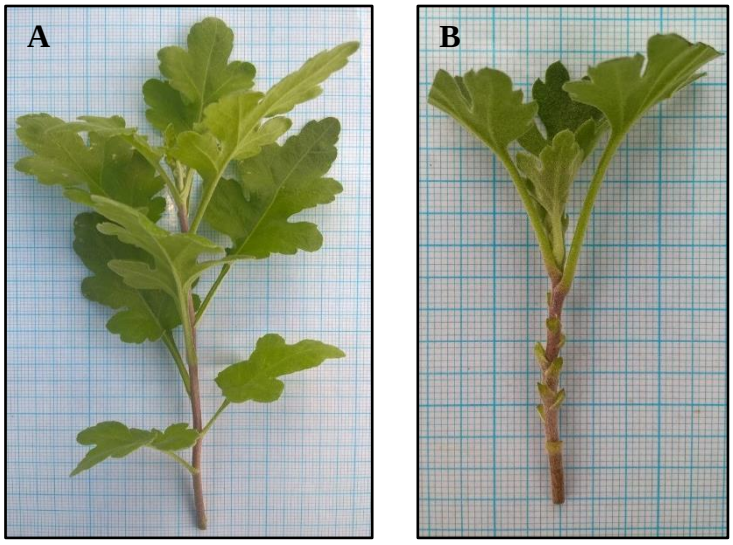

Figure 3. A – Shoot top fragment with minimum 9 leaves, sampled for *Chrysanthemum* cuttings, **B –** b_1 type of cuttings – control, *top stem fragment* – **TSF**

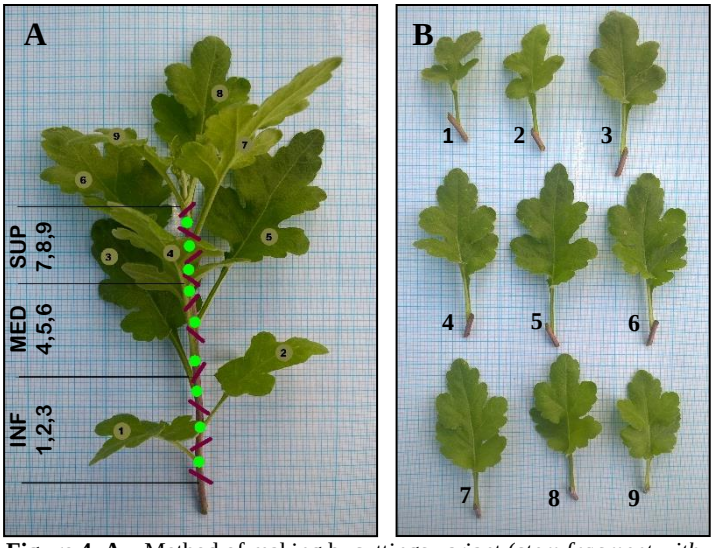

Figure 4. A – Method of making b_2 cuttings variant (*stem fragment with one leaf and axillary bud* – **SFLAB**), **B** – SFLAB cuttings, indicating sampling level, lower (1-3), medium (4-6) or higher (7-9)

Results and discussions

The observations were made during the experiment period (45 days) at equal time intervals, respectively at 7 days (W1-W6), following a series of elements such as: the number of rooted cuttings, the number of compromised cuttings, the percentage of losses, the total rooting percentage by type of cuttings (Table 1).

Table 1. Rooting percentage (W6) – chrysanthemum cuttings (TSF and SFLAB), by substrate type and cultivar

Total no. of cuttings / variant = 25	TSF								Total no. of cuttings
	pt		pt+p		pt+p+s		p		
	I	II	I	II	I	II	I	II	
rooted cuttings (no.)	25	25	25	25	24	25	25	25	200
rooting percent (%)	100	100	100	100	96	100	100	100	
compromised cuttings (no.)	0	0	0	0	1	0	0	0	
losses percent (%)	0	0	0	0	4	0	0	0	
total percentage rooted cuttings – TSF									**99,5 %**
	SFLAB								Total no. of cuttings
	pt		pt+p		pt+p+s		p		
	I	II	I	II	I	II	I	II	
rooted cuttings (no.)	25	21	23	23	25	25	25	24	200
rooting percent (%)	100	84	92	92	100	100	100	96	
compromised cuttings (no.)	0	4	2	2	0	0	0	1	
losses percent (%)	0	16	8	8	0	0	0	4	
total percentage rooted cuttings – SFLAB									**95,5 %**

Abbreviations: **I** – *Chrysanthemum indicum* 'Carmina', **II** – *Chrysanthemum* × *grandiflorum* 'Yellow Stardust', **TSF** – top stem fragment, **SFLAB** – stem fragment with one leaf and axillary bud, **pt** – peat (100%), **pt+p** – peat+perlite (v/v), **pt+p+s** – peat+perlite+sand (v/v/v), **p** – perlite (100%).

Given the fact that at mounting of cuttings into rooting substrate, has not been used substances with effect in rhizogenesis stimulation, the total losses percentage of chrysanthemum was only 5%. This insignificant percentage indicates that the genus *Chrysanthemum* s.l. has a very good capacity of rooting, even in the absence of hormonal stimulants, which are indicated for use in vegetative propagation, especially for the more difficult rooting species.

Data from the literature indicate a correlation between cultivar and rooting success of the cuttings, but with the use of IBA in different concentrations, the best results were obtained for *Chrysanthemum* cv. Crimson Robe, cv. Polario, cv. Escort, cv. Sterling and cv. Luysona at 50 ppm IBA, by spraying or immersing in the root stimulator, thus obtaining a more compact root system [PETTER, 1992]. Our results obtained for the varieties of *Chysanthemum indicum* (I) and *Chrysanthemum* × *grandiflorum* (II) indicate a correlation between the species and the rooting capacity, of the total losses of 5%, recorded 3.5% for cv. I (7 cuttings) and 1.5% for cv. II (7 cuttings). This difference, although insignificant could be explained by the fact that *Chrysanthemum* × *grandiflorum* – chrysanthemum for cut flower, is a complex hybrid species, less adapted to environmental variations, being cultivated only in protected spaces, while *Chrysanthemum indicum*, it is represented by rustic horticultural varieties with a better tolerance to variations of the environment, being successfully cultivated in open spaces.

Regarding the rooting capacity of cuttings according to the substrate, a lower rooting rate was observed for the peat and the peat+perlite substrates (96%), while for

peat+perlite+sand and perlite variants, the rate was 99%. The explanation of this difference could be given by the fact that peat is a substrate that becomes quite compact, which does not allow a good circulation of water and air at the root system level, while the perlite, through the porous structure allows this process. KHER (1976) reported the coarse sand as optimum rooting medium for chrysanthemums, also based on the high capacity of drainage and aeration at the root level.

During the experiment, observations were made regarding the influence of the type of cuttings on the obtaining of planting material, with the appreciation of the yield and the comparison of the classic variant (TSF) with the proposed new method (SFLAB), but also observations related to position of the harvested node from the stem (lower, medium or higher) and rooting rate. The best survival rate of cuttings (199 cuttings rooted out of the total 200 mounted in the rooting substrate) was recorded in the case of cuttings made according to the classical method (TSF) – 99.5%, so the losses in this case were insignificant. The cuttings made from the single-node stem fragment (SFLAB) have rooted in a proportion of 95.5%, which represents a very good percentage indicating a higher yield for this method.

For this new technique, in comparison with the classical method, evaluations were made regarding the yield, by relating the initial used material (number of shoots harvested) to the total number of rooted cuttings obtained (Table 2). The method of chrysanthemum cuttings made from the single-node stem fragment showed a much higher yield, over 8%, also appreciating that this yield could be even higher, up to 10-12% if the shoots of higher heights are used to make the cuttings, so also with a greater number of nodes on the stem. This technique could successfully answer the problem related to the amount of material from which it starts to multiply chrysanthemums, offering solutions to obtain a greater amount of planting material starting from a smaller number of stock-plants. For a production sector, the reduction of the number of stock-plants from which it starts could bring as an advantage a reduction of the storage space, respectively cold greenhouses for the rest period of the plants.

Table 2. Production efficiency of rooted cuttings at both types of cuttings

Cutting type	Number of shoots initially harvested	Total number of cuttings	Number of rooted cuttings	Yield (1:3)
	1	2	3	
TSF	200	200	199	1,005%
SFLAB	22	200	191	8,681%

For SFLAB cuttings we proceeded to harvest in equal proportion, from three different levels of the stem, starting from the base to the tip of the harvested shoots: lower level (INF), medium (MED) and higher (SUP). Three cuttings were harvested from each level on the same shoot, were scored accordingly and mounted on categories in the rooting substrate. The results regarding the number of cuttings rooted according to the level from which they were harvested, are presented in Table 3.

Table 3. Number of cuttings rooted according to the level from which they were collected

Harvest level	Number of cuttings	Number of rooted cuttings	Percent
INF	32	26	81,25%
MED	32	30	93,75%
SUP	32 *+4 (36)	31 *+4 (35)	97,22%

It is found that for the cuttings made of stem fragments located at the top of the shoots, the rooting percentage was over 97%, followed by the medium level with 93%, while

the cuttings made from the base of the shoots were rooted in proportion of 81%. These differences are explained by the theories regarding the presence and circulation of hormones in plants and those related to the apical dominance, being known that auxin, responsible for the formation of adventitious roots, is found in the highest concentration in the apical part of the plant, from where it has a downward path [ACOSTA & al. 2009]. At chrysanthemums, JOHTI & al. (1987) reported the influence of the stem type on the rooting, with reference to the origin, from basal shoots (95.85%) or from the stem tip of the vegetative growth period (82.5%), and thus reported a higher yield for cuttings and shoots grown at the base.

There are few bibliographic references regarding the type of cuttings and especially those made from the single-node stem fragment (SFLAB), in chrysanthemums, SINGH & CHETTRI (2013) conducting an experiment to test a new method of multiplication, using cuttings formed from the leaf with the axillary buds and the stem fragment, also using root stimulators (IBA and kinetin). Thus, they identified the method as effective, especially in terms of the complexity of the procedure, the time required and the efficiency of using the initial plant material.

In order to appreciate the interval required for the development of new shoots from the axillary buds at SFLAB cuttings, observations were made regarding the day when they became visible, starting with the second week of experiment. After the new shoots developed a minimum of 4 leaves, the leaf was removed from the initial stem to stimulate their independent development (Table 4, Figure 5).

Table 4. Duration of rooting at SFLAB cuttings of chrysanthemums, expressed in number of days to shoot emergence of axillary bud leaf – *Chrysanthemum indicum* 'Carmina' (I) and *Chrysanthemum* × *grandiflorum* 'Yellow Stardust' (II)

I	**pt**	**pt+p**	**pt+p+s**	**p**
	Number of days to shoot emergence of axillary bud leaf			
A	21 ± SD	16 ± SD	18 ± SD	15 ± SD
***SD**	*7,25	*2,94	*7,33	*5,19
II	**pt**	**pt+p**	**pt+p+s**	**p**
	Number of days to shoot emergence of axillary bud leaf			
A	21 ± SD	24 ± SD	27 ± SD	24 ± SD
***SD**	*4,47	*5,84	*5,99	*5,19

Abbreviations: **A** – Average, **SD** – Standard Deviation, **pt** – peat (100%), **pt+p** – peat+perlite (v/v), **pt+p+s** – peat+perlite+sand (v/v/v), **p** – perlite (100%).

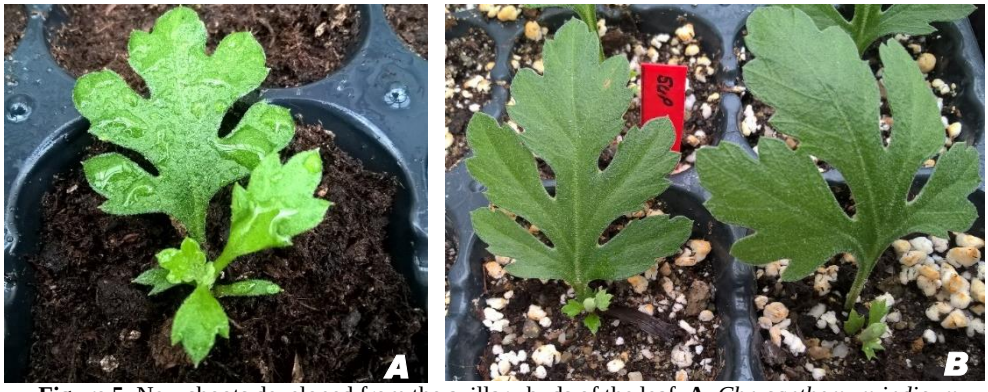

Figure 5. New shoots developed from the axillary buds of the leaf. **A.** *Chrysanthemum indicum* 'Carmina' (I), **B**. *Chrysanthemum* × *grandiflorum* 'Yellow Stardust' (II)

Observations related to the type of cuttings (Table 5), highlighted that by the classical method (control) with cuttings from the top of the stem, more vigorous cuttings are obtained, in both studied horticultural varieties, regarding both the number of developed leaves, as well as the height of the cuttings. This aspect is explained by the fact that the TSF cuttings start from an advanced stage, with vegetative part already developed and which supports the growth even to the adventitious root formation, while the SFLAB cuttings require a longer time until the new shoots are able to independently support the physiological processes of the rooted cuttings. However, in the case of SFLAB cuttings there is a greater uniformity of the development of cuttings, the standard deviation (SD) varying in the range 0.38-1.44, while for TSF cuttings in the range 0.55-2.18. At *Chrysanthemum indicum* 'Carmina', the highest average value of the number of leaves formed was recorded for TSF cuttings in peat substrate 100% – 9.12 leaves as average, and the lowest value was for perlite (average 7 leaves). In SFLAB cuttings, the minimum value recorded in TSF cuttings (7) was not exceeded, the average number of leaves being between 4.8 and 6.39. In height, the same tendency was maintained, for the TSF variant with the average height of the cuttings between 3.21 and 4.46 cm, while for the SFLAB cuttings the average values of the height of the cuttings were between 1.61 and 3.13 cm. Compared to the cv. Carmina, in *Chrysanthemum* × *grandiflorum* 'Yellow Stardust' there were higher values of the height of the cuttings, but smaller for the number of leaves formed. The TSF cuttings showed an advantage over the number of leaves formed (7.28-8.84) compared to the SFLAB cuttings (4.04-4.47), and as the height was registered the same tendency (TSF cuttings with height in the range 2.88-6.03 cm, and SFLAB cuttings in the range 1.64-2.52 cm.

Table 5. Number of developed leaves and height of TSF and SFLAB cuttings [W6] – *Chrysanthemum indicum* 'Carmina' (I) and *Chrysanthemum* × *grandiflorum* 'Yellow Stardust' (II)

I, TSF	**pt**	**pt+p**	**pt+p+s**	**p**
	Number of developed leaves [W6]			
A	9,12 ± SD	9,68 ± SD	9,24 ± SD	7 ± SD
***SD**	*1,76	*1,46	*2,18	*1,12
	Cutting height - cm [W6]			
A	4,22 ± SD	4,46 ± SD	4,2 ± SD	3,21 ± SD
***SD**	*1,03	*0,78	*0,55	*0,57
I, SFLAB	Number of developed leaves [W6]			
A	5,64 ± SD	6,39 ± SD	6,09 ± SD	4,8 ± SD
***SD**	*1,38	*1,30	*1,44	*0,87
	Cutting height - cm [W6]			
A	2,54 ± SD	3,04 ± SD	3,13 ± SD	1,61 ± SD
***SD**	*0,72	*0,88	*0,96	*0,65
II, TSF	Number of developed leaves [W6]			
A	8,84 ± SD	8,08 ± SD	7,96 ± SD	7,28 ± SD
***SD**	*1,21	*1,12	*1,14	*1,06
	Cutting height - cm [W6]			
A	6,03 ± SD	5,01 ± SD	5,25 ± SD	2,88 ± SD
***SD**	*0,97	*1,04	*0,85	*0,42
II, SFLAB	Number of developed leaves [W6]			
A	4,47 ± SD	4,39 ± SD	4,04 ± SD	4,20 ± SD
***SD**	*0,87	*0,89	*0,89	*0,78
	Cutting height - cm [W6]			
A	2,52 ± SD	1,89 ± SD	1,64 ± SD	1,97 ± SD
***SD**	*0,73	*0,59	*0,38	*0,44

Abbreviations: **A** – Average, **SD** – Standard Deviation, **W6** – week 6, **pt** – peat (100%), **pt+p** – peat+perlite (v/v), **pt+p+s** – peat+perlite+sand (v/v/v), **p** – perlite (100%).

A proposed step in the production of planting material was the transplanting of rooted cuttings to 9 cm diameter pots (Figure 6, A), using another soil mix, in equal proportions peat and leaf soil (1:1). This step was required to analyze the growth rate of chrysanthemum cuttings after rooting, in order to obtain high quality plant material, but also to observe the reaction of cuttings made by the two methods (TSF and SFLAB) in order to evaluate the final production of cuttings, both quantitatively and especially qualitatively.

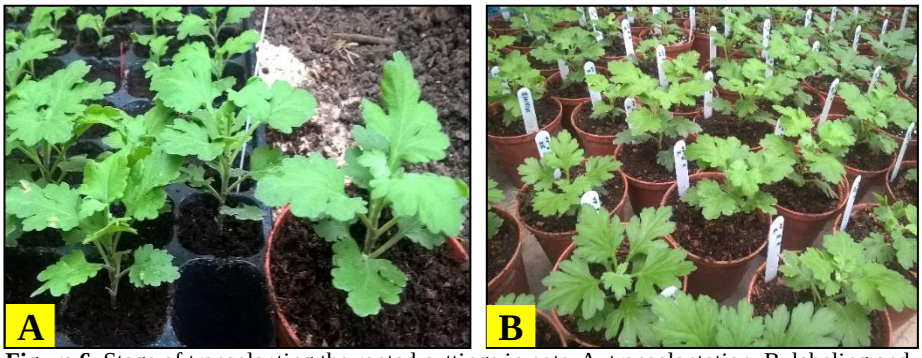

Figure 6. Stage of transplanting the rooted cuttings in pots, A. transplantation, B. labeling and the general aspect of the cuttings after transplanting

In the case of the SFLAB cuttings (Table 6), it is noticed the improvement of the growth rate, by accelerating the formation of new leaves and elongating the transplanted cuttings in increased volume of the substrate and improved composition. The standard deviation indicates the uniformity of the lots (values in the range 0.41-1.08) – Figure 6, B, which also shows that the cuttings obtained in different variants of substrate, including those rooted in variants of inorganic substrate (100% perlite), can recover the growth rate after transplanting them into a mixture of peat with leaf soil.

Table 6. The growth rate of the cuttings in pots after transplantation (SFLAB), the number of leaves and the height of the cuttings [difference W8-W6] – *Chrysanthemum indicum* 'Carmina' (I) and *Chrysanthemum* × *grandiflorum* 'Yellow Stardust' (II)

	pt	**pt+p**	**pt+p+s**	**p**
I	number of leaves [DIF. W8-W6]			
A	4,52 ± SD	4,52 ± SD	4,92 ± SD	2,8 ± SD
***SD**	*0,91	*1,08	*0,95	*0,82
	height of the cuttings [DIF. W8-W6]			
A	5,19 ± SD	3,91 ± SD	3,97 ± SD	3,66 ± SD
***SD**	*0,60	*0,46	*0,60	*0,41
II	number of leaves [DIF. W8-W6]			
M	3,71 ± SD	3,82 ± SD	2,88 ± SD	3,20 ± SD
***SD**	*0,56	*0,72	*0,72	*0,72
	height of the cuttings [DIF. W8-W6]			
M	4 ± SD	3,15 ± SD	4,21 ± SD	3,56 ± SD
***SD**	*0,57	*0,49	*0,67	*0,42

Abbreviations: **A** – Average, **SD** – Standard Deviation, **pt** – peat (100%), **pt+p** – peat+perlite (v/v), **pt+p+s** – peat+perlite+sand (v/v/v), **p** – perlite (100%); **W**-week, **DIF** – difference.

Observations made in experimental plots of chrysanthemum cuttings transplanted was continued until planting them in permanent place (May, 2018), in the field for the first variety – *Chrysanthemum indicum* 'Carmina', or protected greenhouses for the second variety – *Chrysanthemum* × *grandiflorum* 'Yellow Stardust'. Overall, the plants obtained as planting material showed a clear uniformity, regardless of the type of cuttings from which they were obtained (TSF or SFLAB) – Figure 7, 8. These observations allowed to recommend the use of the method with SFLAB cuttings, because vigorous plants can be obtained, a production yield of more than 8%, with the mention that it is possible to qualitatively increase the material produced by introducing the intermediate stage of transplanting to larger pots after rooting.

Figure 7. General aspect of experimental field, two weeks after transplantation (W8)

Figure 8. Form of plants obtained after transplanting, before planting in the field

In addition to morphological observations and measurements made on the aerial parts of the cuttings (number of leaves formed, height), some measurements are carried out at the level of the root system formed at chrysanthemum cuttings (Table 7, Figure 9).

Figure 9. The root system of *Chrysanthemum* cuttings developed in the substrate (substrate volume – 60 ml)

The total length of the roots of the cuttings was calculated using the intersected lines method, the root length of a sample being estimated using the intersection counting techniques of a given line system [NEWMAN, 1966; TENNANT, 1975]. The method proposed by Newman is based on the relationship between the root length and the number of

intersections between the roots as a system of lines spread over a well-defined surface, and arranged randomly with the known length lines in the given area. With greater attention, one can obtain coefficients of variation of the estimation of the root length of 5% or even less [TENNANT, 1975], this being the minimum possible error.

Table 7. The total length of the developed roots at chrysanthemum cuttings (SFLAB), expressed in cm – *Chrysanthemum indicum* 'Carmina' (I) and *Chrysanthemum* × *grandiflorum* 'Yellow Stardust' (II)

I	**pt**	**pt+p**	**pt+p+s**	**p**
	Root length / cutting - cm (Newman formula)			
A (cm)	34,01 ± SD	35,20 ± SD	35,72 ± SD	31,71 ± SD
D (m/m^3)	5,67	5,86	5,95	5,28
*SD	*3,71	*3,09	*3,28	*3,37
II	**pt**	**pt+p**	**pt+p+s**	**p**
	Root length / cutting - cm (Newman formula)			
A (cm)	33,44 ± SD	31,19 ± SD	30,53 ± SD	32,28 ± SD
D (m/m^3)	5,57	5,20	5,09	5,38
*SD	*2,99	*3,54	*3,57	*3,18
Abbreviations: **A** – Average, **SD** – Standard Deviation, **D** – roots density, **pt** – peat (100%), **pt+p** – peat+perlite (v/v), **pt+p+s** – peat+perlite+sand (v/v/v), **p** – perlite (100%)				

The root system of plants is in contact with a variety of abiotic factors, including soil water and nutrient availability, which influence both primary root growth and lateral root formation [INGRAM & MALAMY, 2010]. These factors are constantly changing, and the ability of a plant to respond at these changes and effectively acquire the necessary water and nutrients has a direct impact on reproductive health and success.

Conclusions

The growing demand for competitive floriculture planting material, both on the national and international markets, has led to the adoption of new, modern and efficient technologies for the production of planting material.

The obtained data indicated differences according to variety, in the sense that the cuttings of *Chrysanthemum indicum* 'Carmina' formed new shoots much faster (average range of 15-21 days), compared with those of *Chrysanthemum* × *grandiflorum* 'Yellow Stardust' (average 21-27 days). The 100% perlite as a substrate variant has shown the best efficiency regarding the emergence of new shoots in SFLAB cuttings. Based on the observations it can be concluded that the new method is recommended to obtain the planting material faster, but in order to obtain the lots more efficient and uniform, it is recommended mixture of perlite with peat (p+pt). According to the stem level for cuttings sampling, in the case of the top of the shoots (SUP), the rooting percentage was over 97%, followed by the medium level with 93%, while the cuttings made from the base of the shoots have rooted only in a proportion of 81% (INF).

By the new tested technique, for the manufacture of chrysanthemum cuttings using stem fragment with one leaf and axillary bud – SFLAB, 8-9 new plants can be obtained from a single basal shoots up to 10 cm in length, as opposed to the traditional method of propagation, which would produce only one plant per shoot, so an improved cuttings yield of over 8%. The new method for obtaining rooted cuttings is new, simple, fast, and efficient in terms of time required but also economically. This method is especially recommended

when there are small quantities of propagating material (stock-plant) and when the variety has a low capacity to develop shoots, which leads to obtaining more rooted cuttings, as a planting material for chrysanthemums.

At studied chrysanthemums varieties, the cuttings methods showed very good results, and the obtained planting material is of good quality and uniformity after the stage of transplantation into pots. As a general recommendation, it is concluded that the method of cutting using stem fragment with one leaf and axillary bud – SFLAB is very efficient, increasing the yield of planting material, by relating the number of rooted cuttings to the number of shoots collected from the mother plant.

Notes on contributors

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DEVELOPMENTAL CELL DEATH IN WHEAT FLAG LEAF TIPS IN TWO WHEAT CULTIVARS

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Abstract: Wheat stripe rust pandemics have been recorded across all cereal growing regions. *Lr34* provides an adult plant resistance and flag leaves of many wheat cultivars containing *Lr34* develop a necrotic flag leaf tip. We studied cell death process in progressive necrotic and non-necrotic tissues of flag leaves in wheat cultivars Frontana (resistant to stripe rust) and Fielder (highly susceptible to stripe rust). Cleavage of the poly(ADP-ribose) polymerase (PARP) was detected in necrotic tissues of Frontana flag leaves but not in the non-necrotic tissues or in the corresponding leaf sections in Fielder flag leaves. DNA repairing genes were also studied but their expression was similar in the two different leaf sections for both cultivars. Our work may indicate that protein cleavage is involved in the cell death of flag leaf tips in Frontana.

Keywords: cell death, DNA repair, Lr34, necrosis, stripe rust, wheat.

Introduction

Cereals are a major source for protein and energy for a growing global population. To secure a successful yield, these plants must continuously defend themselves against attack from pathogens. Stripe rust is not the most prevalent diseases to affects cereal crops, and is only detected sporadically, but the damage caused can result in significant yield loss ranging from 1% to 10%. [WELLINGS, 2011]. Stripe rust is caused by the pathogen *Puccinia striiformis*, specifically *P. striiformis* f. sp. *tritici* (*Pst*) strain in wheat.

The resistant traits in wheat either confer race specific (*R* gene) or non-race specific resistance. The former resistant types are dependent on the presence of effectors for infection recognition and activation of disease resistance pathways, which is often referred to Flor's gene-for-gene model [KAMOUN, 2001; XING, 2007]. Pathogens can overcome resistance by *R* genes, as is often the case with new *Pst* races. The emergency of the Ug99 race of stem rust, *Puccinia graminis* f. sp. *tritici*, which is virulent to all varieties of wheat that were once resistant to stem rust Sr24 illustrates the ability of this pathogen to overcoming *R* gene traits [AYLIFFE & al. 2008]. One of the *R* gene products in race-specific resistance is typically characterized by the presence of NBS-LRR motif, which mediates the regulation of several downstream plant defense responses including gene expression, protein modification and apoptosis [SPIELMEYER & al. 2003]. *Yr10* is one of the most populous *R* gene trait employed in the wheat varieties grown in Canada.

Adult-plant resistance (APR) is a more durable form of pathogen resistance. The expression ranges from the wheat tillering stage and continues past the booting stage, which parallels the periods when wheat is susceptible to *Pst* infection. APR is also positively correlated to increased resistance during plant maturity [ZHANG & al. 2012]. *Yr18*, the most predominant APR gene conferring partial *Pst* incompatibility, was thought to be located in the same qualitative loci trait (QLT) site on the short arm of chromosome 7D for leaf rust

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resistance gene, *Lr34* [MCINTOSH, 1992; MCCALLUM & al. 2012]. The QLT was also identified as a putative loci of powdery mildew, *Blumeria graminis*, resistant gene, *Pm38* [SPEILMEYER & al. 2005; LAGUDAH & al. 2009]. Gene-specific marker based mapping and mutation work by KRATTINGER & al. (2009) confirmed that it is a single gene, *Lr34/Yr18/Pm38*, confers partial resistance to leaf rust, stripe (yellow) rust and powdery mildew [LAGUDAH & al. 2009]. The presence of an ATP-binding cassette (ABC) transporter motif within the coding region of *Lr34/Yr18/Pm38* [KRATTIGER & al. 2009] was also confirmed and *Lr34* functions as a transporter of the ABCG subfamily [KRATTIGER & al. 2011].

Lr34 is predominantly expressed in adult foliar tissues, particularly of the flag leaf, and the highest transcript levels were found in the leaf tip, corresponding to the tissues that exhibit the phenotypic difference between the resistant and susceptible wheat lines [KRATTIGER & al. 2009]. Wheat cultivars with functional *Lr34* alleles can be distinguished phenotypically by the development of leaf tip necrosis in adult flag leaves [KRATTIGER & al. 2009; KANG & al. 2011]. Despite its resistance-conferring properties, *Lr34* is not responsive to pathogen inoculation, suggesting that it has constitutive rather than induced functions [KANG & al. 2011]. In spite of the significant contribution of flag leaves to the yield [DING & al. 2018; GAJU & al. 2011; KICHEY & al. 2007] and as a phenotypic indicator of stripe rust resistance in different cultivars of wheat, the underlying difference in flag leaf tips of stripe rust resistant and susceptible cultivars is still unclear. In this work, we examined cell death process and the activity of DNA repairing genes in necrotic and non-necrotic sections of flag leaf tips in a stripe rust resistant and a stripe rust susceptible cultivar.

Material and methods

Plant growth

Wheat seeds were sterilized in a solution of 70% ethanol for 2 min, then transferred to a bleach solution of 25 mL of bleach, 25 mL of distilled water and 10 µL of Triton extract. The seeds were then rinsed 10 times in distilled water. After drying 5-6 seeds were potted in autoclaved Pro-mix BX soil fertilized with 7-9 granular of slow release NPK fertilizer (14:14:14). The seeds were then placed in a growth chamber (Enconnair Technologies Inc., Winnipeg, MB, Canada) set for 16hr at 22 °C in the light and 8hr at 18 °C in the dark. Plants were watered every second day. For protein and RNA extraction, leaf materials were collected and placed in Falcon tubes and snap frozen in liquid nitrogen. The materials were then stored at -80 °C.

Trypan blue staining

Cells of wheat leaves undergoing cell death were photographed using an Axioplan 2 microscope (Carl Zeiss, Germany). Methods described by TANG & al. (1999) and STONE & al. (2000) were used with slight modifications. Leaf tissues were immersed in 10 mL of ethanol-lactophenol (2 volumes of ethanol and 1 volume of phenol-glycerol-lactic acid-water (1:1:1:1)) that contained 0.05% trypan blue. The leaves were placed in 15 mL Falcon tubes and covered with ethanol-lactophenol-trypan blue. The samples were incubated at 95 °C for 4 min and then kept at room temperature for 20 min. The staining solution was removed and 1.5 mL chloral hydrate destaining solution (2.5 g/mL of nano pure water) was added to each tube. The leaves were cleared for 2 days by replacing the destaining solution twice. After

destaining, leaves were suspended in 50% glycerol and examined under microscope with white light.

Protein extraction and determination of protein concentration

Wheat protein was extracted from leaves (100 mg) either in extraction buffer (20 mM Tris–HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM $NaVO_3$, 10 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 0.5% Nonidet P-40, and 1% Triton X-100), or using TRIzol Reagent kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Protein concentration in tissue extracts were determined using Coomassie blue dye binding method with the Bradford reagent and bovine serum albumin (BSA) as the standard.

SDS-PAGE and immunoblotting

Detailed protocol was described previously [GAO & al. 2011]. After SDS-PAGE and protein transfer onto nitrocellulose membranes. For antibody detection, the primary antibody used was cleaved PARP 1:1000 v:v (Cell Signaling Technology, Danvers, MA, USA). After overnight incubation with the primary antibody, blots were washed with TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) (5 min x 3) and then incubated at room temperature for 1 h with 1:2000 v:v dilution of the secondary antibody (anti-rabbit IgG, horse radish peroxidase-linked) (Cell Signaling Technology, Danvers, MA, USA). The target protein on the PVDF membrane was detected using an enhanced chemiluminescence (ECL) system containing 1 x LumiGLO Reagent and 1x peroxide (Cell Signaling Technology, Danvers, MA, USA). The membrane was scanned using FluorChem Q imaging system (Alpha Innotech Cooperation, Santa Clara, CA, USA).

RNA extraction and RT-PCR

Total RNA was extracted from wheat leaf tissues (100 mg) using TRIzol Reagent kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. After TRIzol extraction, DNase I kit (amplification grade, Invitrogen, Carlsbad, CA, USA) was used to eliminate genomic DNA contamination in the sample, and the cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis according to the manufacturer's protocol.

The primers for RAG50 were 5'-CAGGGACACATTGACTGGTG-3' (forward) and 5'-TTTCCTCGGCAAAATGTACC-3' (reverse). The following conditions were used for RT-PCR: 94 °C for 1 min; 94 °C for 1 min, 67 °C for 1 min, 72 °C for 30 seconds for 28 cycles, and then 72 °C for 10 min. The primers for RAD51 were 5'-CAGAAGGCACATTCAGACCA-3' (forward) and 5'-GCAAACCTTGTCTCCACCAT-3' (reverse). The following conditions were used for RT-PCR: 94 °C for 1 min; 94 °C for 1 min, 71 °C for 1 min, 72 °C for 30 second for 28 cycles, and then 72 °C for 10 min. GAPDH (glyceraldehyde-3-phosphate dehydrogenase, GenBank accession number EU022331.1) gene was used as an internal standard [LLOYD & al. 2007]. For RT-PCR, the primers were 5'-GTGAGGCTGGTGCTGATTACG-3' (forward) and 5'-TGGTGCAGCTAGCATTTGAGAC-3' (reverse). The following conditions were used for RT-PCR: 94 °C for 1 min; 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 30 seconds for 28 cycles, and then 10 min at 72 °C.

Results

Cell death in flag leaf tips

Wheat cultivars with functional *Lr34* alleles are phenotypically different from cultivars carrying no *Lr34*, and have a typical leaf tip necrosis in adult flag leaves [KRATTIGER & al. 2009; KANG & al. 2011]. Frontana (stripe rust resistant) and Fielder (stripe rust susceptible) [RANDHAWA & al. 2012] were selected for this work. Both cultivars were grown under identical conditions, and no significant differences were observed in development. The flag leaf tips of both cultivars were harvested at the beginning of the grain-filling stage. Cell death was examined on leaves detached from Frontana and Fielder. Trypan blue is commonly used to selectively stain dead tissues or cells blue, and under white light the dead cells appeared to be much darker compared to living cells. These blue dead cells scattered on leaves as clusters without defined margins and the cell death did not seem to occur in the whole leaf. The heavily blue staining areas represent cell death and Frontana showed much more significant cell death than Fielder (Figure 1).

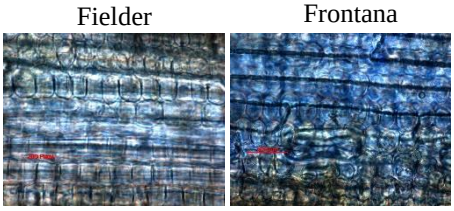

Figure 1. Microscopic images of flag leaves from Fielder and Frontana. Leaf tips of Fielder (left) and Frontana (right) 1 cm from the tip were stained with trypan blue.
Repeat experiment showed a similar result.

Cleavage of poly(ADP-ribose) polymerase (PARP)

Cleavage of key proteins by caspases is often taken as an indicator of cell death activity. Poly(ADP-ribose) polymerase (PARP) is among the first target proteins shown to be specifically cleaved by caspases [FAN & XING, 2004]. PARP is involved in the regulation of repairing DNA strand breaks and in cell recovery from DNA damage, so the physiological function of PARP includes DNA repair, DNA replication and maintenance of genome integrity [DE BLOCK & al. 2005]. To evaluate the possible involvement of caspases in cell death of flag leaf tips, we examined the integrity of PARP proteins in the leaf sections showing cell death progress (the first cm from the tip) and green sections (the second cm from the tip). Cleavage of PARP was detected in flag leaf tips of Frontana but not the corresponding tip tissue of Fielder (Figure 2). The cleavage was not detected in the green tissues below the flag leaf tips (Figure 2).

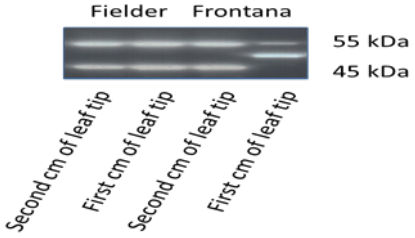

Figure 2. PARP status in flag leaf tips and in tissues below the tips detected by the antibody against cleaved PARP. Proteins were extracted from flag leaf tips of Frontana and Fielder the first 1 cm from the tip and the second 1 cm from the tip. Three experiments were carried out with similar results.

Expression of Radiation Sensitive 50 (RAD 50) and RAD 51

Plants are equipped with mechanisms to detect and repair multiple types of DNA lesions . It is possible that the cell death in flag leaf tips of Frontana and Fielder is regulated by DNA repairing capacity. DNA repairing genes including RAD50 and RAD51 have been identified in yeast, animals and plants and they are involved in various processes such as DNA damage repairing, DNA replication, meiosis, and telomere maintenance (BLEUYARD & al. 2005; LLOYD & al. 2007; SONG & al. 2011]. To examine whether RAD50 and RAD51 contribute to the difference of the leaf tip cell death between Fielder and Frontana, their expression levels was examined by RT-PCR (Figure 3). There was no significant difference in the expression levels of either RAD50 or RAD51 in the two cultivars.

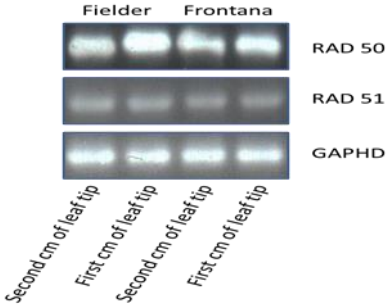

Figure 3. Expression of DNA repairing genes in flag leaf tips of Frontana and Fielder. GAPDH gene was used as an internal standard. Three experiments were carried out with similar results.

Discussion

Stripe rust can cause significant crop damage resulting in yield lose ranging from 1% to 10% [WELLINGS, 2011]. Wheat is specifically affected by *P. striiformis* f. sp. *tritici* (PST) strain. Resistance gene *Lr34* is predominantly expressed in the flag leaf and the expression correlates to cell death difference between the resistant and susceptible wheat cultivars [KRATTIGER & al. 2009; KANG & al. 2011]. This difference was confirmed in Frontana (resistant) and Fielder (susceptible) (Figure 1).

Cell death is mediated by the activities of protein cleavage enzymes. Activities displaying caspase cleavage specificity have been shown in cell death in various plant species including wheat [XING & al. 2005; FAN & al. 2016]. Genomic and molecular genetic approaches have supported the existence of caspase-like proteases in plants [VARTAPETIAN & al. 2011]. When we examined the caspase-like activity in flag leaf tips, cleavage of PARP was detected in flag leaf tips of Frontana but was undetectable in the corresponding tip tissue of Fielder. No cleavage was detected in green tissues below the flag leaf tips (Figure 2). Early work indicated PARP activity in wheat embryo cells [WHITBY & al. 1977, 1978, 1979]. However, our previous work showed no success in identifying TGYMFGKG, a PARP signature sequence, in wheat [XING & al. 2004]. On the other hand, a non-canonical PARP domain proteins was shown to act in stress responses in wheat [LIU & al. 2014].

As plant cells are subject to high levels of DNA damage resulting from dependence on sunlight for energy and the concomitant exposure to environmental stresses, mechanisms developed to sense the damage and to activate the DNA repair machinery to preserve the genome content [CIMPRICH & CORTEZ, 2008; DEVISETTY & al. 2010]. The expression levels of two DNA repair genes RAD50 and RAD51 in flag leaf tips of Frontana and Fielder were determined by semi-quantitative RT-PCR and no difference was found (Figure 3). However, since many other DNA repairing genes could be involved, it is reasonable to assume that its expression may not necessarily be altered as indicated in our previous study [ALBARAKY, 2008].

Our current work has suggested the involvement of a PARP-like protein in the phenotypic difference in cell death in wheat flag leaf tips between Frontana and Fielder. Questions still remain such as whether localized ability to regulate cell death may prime the stripe rust resistant cultivars against potential attack of stripe rust and how the cell death in flag leaf tips is integrated in *Lr34*-mediated stripe rust resistance.

Notes on contributors

Kipkios TUBEI is a MSc. student supported by a research grant from Bayer Crop Science Inc.

Lucas CHURCH is an undergraduate student.

Tim XING is an associate professor and a plant molecular biologist with a special interest in cell signaling and plant-microbe interactions. He teaches plant physiology, molecular plant development, and cell signaling.

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CONSERVATION STATUS AND THREATS TO ENDEMIC PLANT SPECIES OF GRIQUALAND WEST OF SOUTH AFRICA

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Abstract: Endemic species are important to biodiversity of any region they are situated. They are to be protected from over exploitation and population decline. This study evaluated the conservation status and threats to endemic plant species of Griqualand West in South Africa. The SANBI (South African National Biodiversity Institute) Red List data base was used in determining the conservation status and threats to these endemic species. The result of this study showed that all the endemic species of Griqualand West of South Africa are of conservation concern meaning they need to be given attention in terms of conservation. Also human induced threats were discovered in this study. This study thereby recommend that conservation priority should be given to the endemic species of this region that are of conservation concern, and also efforts should be made to control the human induced threats identified in this study.

Keywords: biodiversity loss, conservation, extinction risk, species endemism, threats.

Introduction

Many species are going extinct at an unprecedented rate with the current extinction crisis seen as the sixth mass extinction the world has ever witnessed [DANA & al. 2012]. The effect of these biodiversity loss is resulting into great loss of ecosystem services [KHAN & al. 2013; TALI & al. 2015]. Factors causing species extinction includes human population growth, anthropogenic activities, climate change, invasive species and unsustainable resource utilization [HOFFMANN & al. 2010].

Endemic species are critically important to biodiversity and their loss translates into significant biodiversity loss [ANDERSON, 2002]. These species are exploited for several human uses and some of them that are protected by authorities are illegally collected to meet certain human demands [WILLIAMS & al. 2013; BAMIGBOYE & al. 2017]. Several regions of plant endemism have been identified in South Africa and some of these regions are being faced with threats to these species hence increasing their risk of extinction [van WYK, 1996; SIEBERT & al. 2002; BIGGS & al. 2008; CLARK & al. 2009; WILLIAMSON & BALKWIL, 2015; HAHN, 2017; MORASWI & al. 2019].

This study evaluated the conservation status and threats to the endemic plant species of Griqualand West in South Africa, a region where the endemic species list have recently been updated.

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Materials and methods

Griqualand West is primarily situated in the Northern Cape Province but extend to the Northwest Province of South Africa with a coverage of 40,000 km^2 [COON & HUNT, 1965]. It is a semi-arid region with average rainfall of 262 to 411m annually [FRISBY & al. 2019]. The mean annual temperature of this region is between 17 to 18 °C [MUCINA & RUTHERFORD, 2006]. The vegetation type is savannah biome which is dominated by grasses and herbaceous ground layers [MUCINA & RUTHERFORD, 2006].

The list of the 25 endemic plant species was gotten from FRISBY & al. (2019). The Red List status of these species was gotten from SANBI Red List web page (http://redlist.sanbi.org/redcat.php) 2017 version. The threats for each of the species were also gotten from the SANBI Red List data base 2017 version. Percentages of different threat categories of SANBI Red List were calculated and the percentage of the species facing different kind of threats were also calculated.

Results and discussion

The result of the SANBI Red List categorical percentages of endemic plant species of Griqualand West of South Africa are as follows: 8% are Vulnerable, 8% are Near Threatened, 12% are Data Deficient Taxonomically Problematic, 4% are Rare, 4% are Critically Rare, 4% are not found on SANBI Red List and 60% are of Least Concern. 36% of all the endemic plant species of Griqualand West of South Africa are of conservation concern (Vulnerable, Near Threatened, Critically Rare, Rare, and Data Deficient Taxonomically Problematic). 12% are facing threats due to habitat degradation, while 4% facing threat due to pollution.

Irrespective of their conservation status, all endemic species that are within a very small geographic range in a country (e.g a state, a province, local districts or municipalities) deserves to be protected. But more attention should be drawn to threatened endemic species in terms conservation. It is recommended that high priority be given to species that are of conservation concern listed in this study (Table 1) in terms of conservation management. Endemic plant species that are Data Deficient Taxonomically Problematic could possibly be facing threats [MORASWI & al. 2019]. Effort should be made to reassess the Data Deficient Taxonomically Problematic species found in this study (Table 1) to determine if some of them are threatened and also unravel factors that might be posing threats to them.

A species whose conservation status showed not threatened (*Glossochilus burchellii* Nees) in this study was discovered to be facing threat due to habitat degradation by reason of mining activities and overgrazing (Table 1). This showed that species that are not threatened in this study needs to still be further evaluated for threats and potential threats. The human induced threats already identified in this study (Table 1) should be controlled to reduce their impact on the endemic plant species of Griqualand West of South Africa.

New species description are sometimes setback in determining the overall conservation status of endemic species in a certain location [RIEMANN & EZCURRA, 2005]. Because new species that are newly described may not have been assessed for conservation purpose and this results in shortfalls in quantifying overall conservation status of endemic species in certain regions [RIEMANN & EZCURRA, 2005; MORASWI & al. 2019]. A new species (*Deverra rapaletsa* Magee & Zietsman) described by VAN MUNSTER & al. (2019) found in this study is not yet on SANBI Red List and has not yet

been assessed for conservation. It is recommended that this species should be assessed for conservation purpose to further contribute to the overall conservation of endemic plant species of Griqualand West of South Africa.

There is need to continuously evaluate endemic species to keep track their population trend and also determine how utilization of these species are posing threats to their existence [BAMIGBOYE & al. 2017; MORASWI & al. 2019]. This will enhance the monitoring of these species and also give direction to how initiatives can be generated to further protect and preserve these species. This means that studies on population size, structure and distribution of these species should be carried out continuously at least a decade interval on endemic plant species.

Table 1. List of plant species endemic to Griqualand West of South Africa, their SANBI Red List Status and their threats on SANBI Red List

Family	Species	SANBI Red List Status	Threats on SANBI Red List
Acanthaceae	*Barleria media* C. B. Clarke	Vulnerable	Habitat degradation due to overgrazing
Acanthaceae	*Blepharis marginata* (Nees) C. B. Clarke	Least Concern	No threat found
Acanthaceae	*Glossochilus burchellii* Nees	Least Concern	Habitat degradation due to mining and overgrazing
Acanthaceae	*Justicia puberula* Immelman, FSA	Least Concern	No threat found
Acanthaceae	*Justicia thymifolia* (Nees) C. B. Clarke	Least Concern	No threat found
Aizoaceae	*Antimima lawsonii* (L. Bolus) H. E. K. Hartmann	Rare	No threat found
Aizoaceae	*Hereroa wilmaniae* L. Bolus	Data Deficient Taxonomically Problematic	No threat found
Aizoaceae	*Lithops aucampiae* L. Bolus subsp. *euniceae* (de Boer) D. T. Cole	Vulnerable	Pollution which is affecting both the habitat and the species
Aizoaceae	*Lithops bromfieldii* L. Bolus	Least Concern	No threat found
Aizoaceae	*Lithops lesliei* (N. E. Br.) N. E. Br. subsp. *burchellii* D. T. Cole	Near Threatened	No threat found
Aizoaceae	*Prepodesma orpenii* (N. E. Br.) N. E. Br.	Least Concern	No threat found
Anarcadiaceae	*Searsia tridactyla* (Burch.) Moffett	Least Concern	No threat found
Apiaceae	*Deverra rapaletsa* Magee & Zietsman	Not on Red List	No threat found
Asteraceae	*Amphiglossa tecta* (Brusse) Koek.	Critically Rare	No threat found
Asteraceae	*Cineraria exilis* DC.	Data Deficient Taxonomically Problematic	No threat found

Asteraceae	*Dicoma kurumanii* S. Ortiz & Netnou	Least Concern	No threat found
Asteraceae	*Eriocephalus ericoides* (L. f.) Druce subsp. *griquensis* M. A. N. Müll.	Least Concern	No threat found
Asteraceae	*Gnaphalium englerianum* (O. Hoffm.) Hilliard & B. L. Burtt	Least Concern	No threat found
Asteraceae	*Pentzia stellata* (P. P. J. Herman) Magee	Near Threatened	Habitat degradation due to trampling and overgrazing
Asteraceae	*Tarchonanthus obovatus* DC.	Least Concern	No Threat found
Celastraceae	*Maytenus ilicina* (Burch.) Loes.	Least Concern	No threat found
Celastraceae	*Putterlickia saxatilis* (Burch.) Jordaan	Least Concern	No threat found
Fabaceae	*Calobota cuspidosa* (Burch.) Boatwr. & B.-E. Van Wyk	Least Concern	No threat found
Poaceae	*Brachiaria dura* Stapf. var. *pilosa* J. G. Anderson	Data Deficient Taxonomically Problematic	No threat found
Stilbaceae	*Nuxia gracilis* Engl.	Least Concern	No threat found

Conclusions

This study recommend protection of all the endemic plant species of Griqualand West of South Africa. But beyond protection, there is a need to come up with initiatives on how threats and potential threats to these species can be controlled. Further studies of recent population biology of these species is recommended to further unravel their risk of extinction and their persistence amidst ecological forces posing threats to their existence.

Notes on contributors

Samuel Oloruntoba BAMIGBOYE is a plant biologist with specialization in plant ecology and conservation biology. His research interest is studying patterns of extinction risk in African flora with special interest in endemic species. He is currently focusing on threatened species and endemic species in South Africa.

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NEW DATA ABOUT *FRITILLARIA MELEAGROIDES* IN ROMANIA

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Abstract: *Fritillaria meleagroides*, a rare species recently registered in the Romania's vascular flora, has been identified in two other new localities from the north-eastern part of the country. The average density of reproductive individuals of *F. meleagroides* in the area of Cotu Morii has been estimated at 3.7 individuals per m^2. The paper reveals the structure and some ecological peculiarities of plant communities in which *F. meleagroides* grows at Cotu Morii (Iaşi county), and Ştefăneşti (Botoşani county). The protection of this species in Romania by declaring a natural reserve at Cotu Morii (Iaşi county) is supported in the paper. At Cotu Morii, besides *F. meleagroides*, other rare and threatened plant species have been identified, including *Bulbocodium versicolor*, which is reported here for the first time from the Iaşi county.

Key words: Jijia and Başeu river basins, Liliaceae, plant communities, population density, salty meadows, vascular flora.

Introduction

In our previous paper [OPREA & al. 2015], we reported for the first time in the vascular flora of Romania the species *Fritillaria meleagroides* Patrin ex Schult. & Schult. f. (Liliaceae), identified in the northern part of the Cotu Morii village (Popricani commune, Iaşi county). This place is located at the western limit of natural area of *F. meleagroides*, which stretches from the North-West China and Central Asia to the Eastern Europe (European Russia, Ukraine and Bulgaria) [CHEN & MORDAK, 2000; KOROTCHENKO & ORLOV, 2009; LOZINA-LOZINSKAYA, 1935; YANEV, 1964].

Since *F. meleagroides* is an endangered species in a large part of its natural range, including eastern Europe [IVANOVA, 2015; KOROTCHENKO & ORLOV, 2009; PETROVA & VLADIMIROV, 2009], we expressed the view that urgent actions to protect its populations from Romania should be introduced [OPREA & al. 2015].

Some preliminary data on the habitat and plant communities which host *F. meleagroides* in north-eastern Romania were previously presented by us [OPREA & al. 2015].

The present study is a continuation of our previous work and was undertaken to obtain additional information on the population structure, ecology and phytosociology of this rare species, in order to support its *in situ* conservation.

Another aim of this paper is to report *F. meleagroides* from other new localities in North-Eastern Romania, not mentioned in our previous paper, or by other authors until now (although we have some reason to assume that the species has been met in at least one of those new localities some decades ago, but its presence has been overlooked, due to possible confusion with a much better known species *F. meleagris*).

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Material and methods

Floristic research was performed on the itinerary, during our field work (April 2016, 2019), in the North-East Romania. Specimens collected on the field were deposited in the herbarium of the University of Agricultural Sciences and Veterinary Medicine (IASI) in Iaşi. The geographic coordinates were recorded using an eTrex Legend HCx GPS system.

To estimate population size and density of *F. meleagroides*, a survey was performed in April 2016, in the Northern part of the Cotu Morii village (Popricani commune, Iaşi county), in the same area described in our previous work [OPREA & al. 2015]. The sampling area covers approx. 63 ha (excluding the plowed lands and abandoned crops). This territory was divided on map into squares of 100 × 100 m, and sampling plots of 2 × 2 m were established in the center of each 100 × 100 square, in which individuals of *F. meleagroides* were counted. The population density was afterwards expressed as number of individuals per square meter. Only reproductive individuals (at the flowering stage) were considered.

Phytosociological survey was made in April 2016, according to the standard Central European phytosociological methodology [BORZA & BOŞCAIU, 1965; BRAUN-BLANQUET, 1964; CRISTEA & al. 2004].

The nomenclature of the plant taxa follows SÂRBU & al. (2013).

Results and discussion

Figure 1. *Fritillaria meleagroides*, North of the Ştefăneşti town, Botoşani county

a) New localities with *Fritillaria meleagroides* in North-Eastern Romania

On April, the 10th, 2016, we identified *F. meleagroides* (Figure 1) in a new locality, in North-Eastern part of Romania (at a distance of approx. 80 Km, North – Northwest to the first reported locality), namely North of the Ştefăneşti town, Botoşani county (N47º48'24.50", E27º11'39.60"; alt. 62 m; N47º48'22.59", E27º11'44.12"; alt. 62.5 m), on the hay meadow from the right side of the Başeu river and the left side of the road between the Ştefăneşti town and Stânca village (Figure 2). This population was not very rich, consisting of several hundred individuals scattered on the river meadow. Upstream, toward the Murguţa village, the meadow is grazed by sheep and no individual of *F. meleagroides* was found on the grazed area. The habitat of the species in the new place is very similar to that from the Cotu Morii village (Iaşi county) [OPREA & al. 2015], namely a river meadow with a vegetation specific to the order *Potentillo-Polygonetalia* R. Tx. 1947 (see below a more detailed description of plant communities), on soils which, according to VASILINIUC & SECU (2007), are represented by fluvisols in association with gleysols, more or less salinized.

In addition, recent investigations on the field (April, the 12th, 2019), carried out first by the second author, at the suggestion of Mr. G. Davideanu, from the Museum of Natural Sciences in Iași (to whom we are very grateful), led us to identify a new, very rich population of *F. melagroides* between Larga Jijia, Mihail Kogălniceanu and Țigănași villages, Iaşi county, on the Jijia river meadow (N47º19'35.20", E27º25'55.17"; N47º19'39.71", E27º25'55.14"; N47º19'35.25", E27º25'53.68). This place is not far away from Cotu Morii (approx. 10 Km to the West, following the course of the Jijia River) (Figure 3, Figure 4).

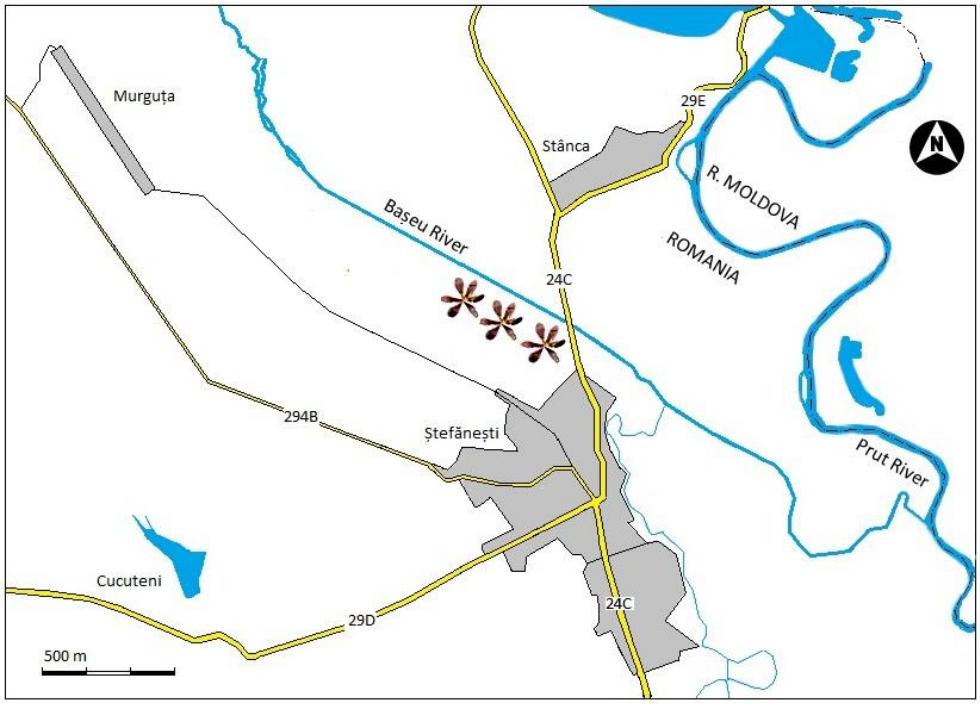

Figure 2. Location of *Fritillaria meleagroides* in the surroundings of Ştefăneşti town, Botoșani county, on the lower meadow of the Bașeu River

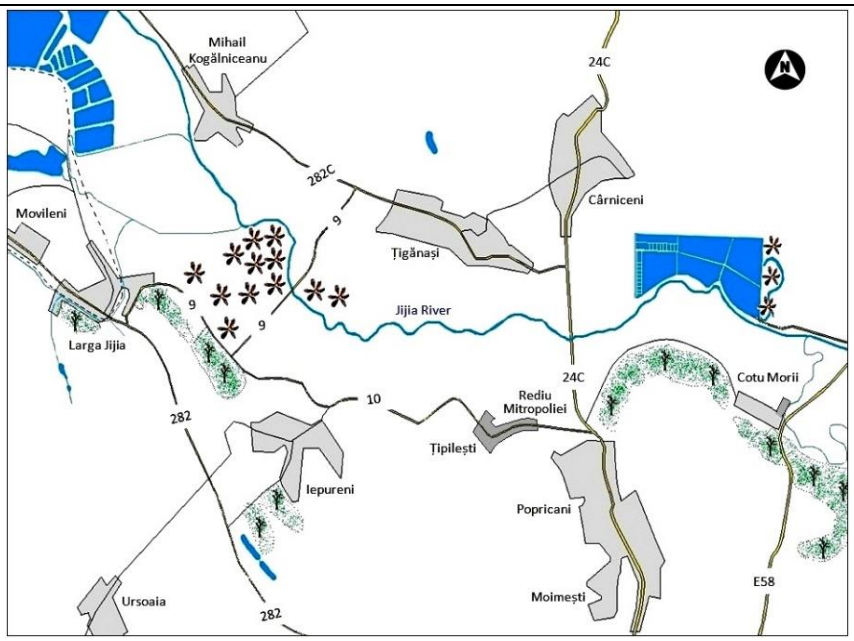

Figure 3. Location of *Fritillaria meleagroides* on the lower meadow of the Jijia River, Iași county. Both the previously known population (on the right), and the recently discovered one (on the left) are marked on the map

Figure 4. Population of *Fritillaria meleagroides* between the Țigănași, Larga Jijia and M. Kogălniceanu villages (Iași county)

b) An estimation of the population density (at Cotu Morii, Iaşi county)

According to our current data, *F. meleagroides* has very rich populations in Cotu Morii, far exceeding our previous estimate [OPREA & al. 2015]. The number of reproductive individuals of *F. meleagroides* in the sampling area at Cotu Morii (Iaşi county) varied widely among sample plots, from 0 to 16.25 per m^2, with a mean (± standard deviation) of 3.78 ± 3.73 per m^2. In 93.6% of the sample plots, *F. meleagroides* was represented by at least one reproductive individual. The distribution of the mean number of reproductive individuals per m^2 in the study area is shown in Figure 5.

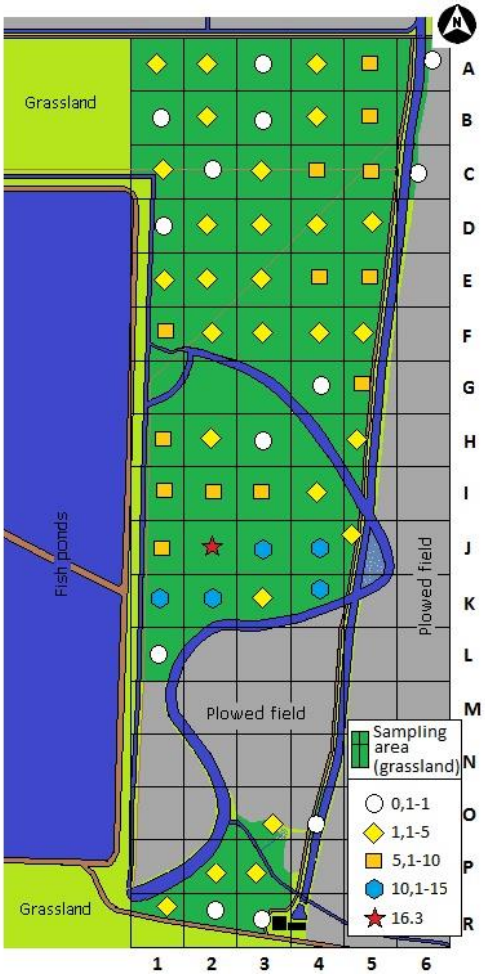

Figure 5. Sampling area to estimate the number of reproductive individuals of *Fritillaria meleagroides* at Cotu Morii (Iaşi county)

The number of individuals was smaller in the peripheral areas of the studied territory. In the more humid places, usually invaded by *Phragmites australis* (e.g., G1-G3, H4, Figure 5), either no individual was recorded, or those were very rare. Rare, solitary individuals have also been found on plowed fields, in abandoned arable areas, or on the edges of the channels, but in those places we did not made any registrations.

According to the literature, in the related species *F. meleagris*, the ratio between the reproductive (flowering) and vegetative (juvenile, subadults) individuals vary considerably, depending on the site, between 0.056 and 0.128 [CSERGŐ & FRINK, 2003] or between 0.016 and 0.667 [ZHANG, 1983]. As mentioned above, in our survey on *F. meleagroides* population at Cotu Morii, only individuals in the reproductive stage were registered, but it would be expected this ratio to vary greatly in this species, too.

c) Phytosociological data, with some historical considerations

According to our field data, both at Cotu Morii, in the lower basin of the Jijia river (Iaşi county), and to the North of Ştefăneşti town, in the basin of Başeu river (Botoşani county), *F. meleagroides* enters into the structure of the association *Rorippo austriacae-Agropyretum repentis* [Timár, 1947] R. Tx. 1950, alliance *Potentillion anserinae* R. Tx. 1947, order *Potentillo-Polygonetalia* R. Tx. 1947, class *Molinio-Arrhenatheretea* R. Tx. 1937 (Table 1).

The association *Rorippo austriacae-Agropyretum repentis* has been previously cited (by a single relevé) from Cotu Morii (Iași county) by CHIFU & al. (1998), along with two other plant communities, all from the same alliance (*Potentillion anserinae*), namely: *Rumici crispi-Agrostietum stoloniferae* Moor 1958 (one relevé) and *Dactylido-Festucetum arundinaceae* Tx. 1950 (two relevés). However, no species of *Fritillaria* was listed in any of the phytocoenoses reported by the above mentioned authors.

Table 1. Species composition of the plant communities with *Fritillaria meleagroides* (association *Rorippo austriacae-Agropyretum repentis* (Timár 1947) R. Tx. 1950)

Date and place of relevés:	**rel. 1-6: 05.apr.2016, Cotu Morii, Iași county**						**rel. 7-12: 11.apr.2016, Ștefănești, Botoșani county**						
Relevé area (m^2)	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	
General coverage (%)	**90**	**100**	**100**	**95**	**100**	**100**	**100**	**100**	**100**	**95**	**95**	**100**	**K**
Relevé no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
Number of species	**35**	**34**	**34**	**43**	**33**	**33**	**18**	**28**	**30**	**27**	**26**	**27**	
Fritillaria meleagroides	1	1	+	1	+	+	+	+	+	+	+	+	V
Charact. association													
Elymus repens ssp. repens	3	4	4	4	4	4	5	5	4	4	3	3	V
Rorippa austriaca	·	+	·	+	·	·	·	·	·	·	+	+	II
Potentillion anserinae & Potentillo-Polygonetalia													
Oenanthe silaifolia	+	+	·	+	+	·	+	+	·	+	·	+	IV
Rumex crispus	·	+	+	+	·	+	+	+	+	+	·	+	IV
Serratula coronata	+	1	1	+	1	+	·	·	·	·	·	·	III
Silaum silaus	+	·	·	+	+	·	·	·	·	+	+	·	III
Carex melanostachya	1	·	+	+	+	+	·	+	·	·	·	·	III
Rorippa sylvestris ssp. sylvestris	·	+	·	+	·	·	+	·	+	+	+	+	III
Potentilla reptans	·	+	·	·	+	·	·	·	+	1	+	c	III
Bromus commutatus	·	·	+	+	·	·	·	+	+	+	·	+	III
Agrostis stolonifera ssp. stolonifera	1	·	+	+	·	+	·	·	·	·	·	·	II
Inula britannica	·	·	·	·	·	+	·	+	·	·	·	·	I
Rumex confertus	+	·	·	·	·	·	·	·	·	+	·	·	I
Lythrum virgatum	+	·	·	·	·	·	·	·	·	·	·	·	I
Carex hirta	+	·	·	·	·	·	·	·	·	·	·	·	I
Ranunculus repens	·	+	·	·	·	·	·	·	·	·	·	·	I
Plantago major ssp. major	·	·	+	·	·	·	·	·	·	·	+	·	I
Scutellaria hastifolia	·	·	·	·	+	+	·	·	·	·	·	·	I
Potentilla anserina ssp. anserina	·	·	·	·	+	·	·	·	·	·	·	·	I
Juncus compressus	·	·	·	·	·	·	·	·	·	+	·	·	I
Trifolium hybridum ssp. elegans	·	·	·	·	·	·	·	·	·	·	+	·	I
Molinio-Arrhenatheretea													
Alopecurus pratensis ssp. pratensis	2	2	2	1	2	2	1	+	2	·	3	3	V
Poa pratensis ssp. pratensis	·	+	+	1	+	+	+	+	1	1	1	+	V
Taraxacum officinale	·	·	·	·	·	·	+	+	+	+	+	·	III
Vicia cracca	·	·	·	·	·	·	·	+	+	+	+	+	III
Lotus corniculatus	+	·	·	·	·	·	·	·	·	·	+	+	II
Plantago lanceolata ssp. lanceolata	·	·	·	+	·	·	·	+	+	·	+	·	II
Symphytum officinale ssp. officinale	·	·	·	+	+	+	·	·	·	·	·	·	II
Rhinanthus rumelicus	·	·	·	·	·	·	·	+	+	·	·	+	II
Lathyrus pratensis	·	+	·	·	·	·	·	·	·	·	·	·	I

Dactylis glomerata ssp. glomerata	·	·	·	·	+	·	·	·	·	·	·	·	I
Thalictrum lucidum	·	·	·	·	·	+	·	·	·	·	·	·	I
Achillea millefolium ssp. millefolium	·	·	·	·	·	·	+	·	·	·	·	+	I
Trifolium repens ssp. repens	·	·	·	·	·	·	·	·	·	+	·	+	I
Stellaria graminea	·	·	·	·	·	·	·	·	·	·	+	·	I
Trifolium pratense ssp. pratense	·	·	·	·	·	·	·	·	·	·	·	+	I
Phragmitetea													
Carex acutiformis	+	.	.	+	.	.	.	.	.	.	.	.	I
Iris pseudacorus	+	.	.	.	.	.	.	.	.	.	.	.	I
Lythrum salicaria	+	.	.	.	.	.	.	.	.	.	.	.	I
Phragmites australis ssp. australis	+	.	.	+	.	+	.	.	.	+	.	.	I
Galium palustre ssp. palustre	.	.	.	.	.	+	.	.	.	.	.	.	I
Carex vulpina	.	.	.	.	.	.	+	.	.	.	.	.	I
Eleocharis palustris ssp. palustris	.	.	.	.	.	.	.	.	.	.	.	+	I
Festuco-Puccinellietea													
Scorzonera cana	+	·	+	+	+	·	·	+	+	1	+	+	IV
Limonium gmelinii	+	+	+	+	·	+	·	·	·	·	·	·	III
Iris halophila	1	+	+	+	+	+	·	·	·	·	·	·	III
Cerastium dubium	·	+	+	+	·	·	+	·	+	·	·	+	III
Beckmannia eruciformis	+	·	·	·	·	·	+	·	·	·	·	+	II
Rhaponticum serratuloides	·	+	+	·	+	·	·	·	·	·	·	·	II
Dianthus pratensis ssp. racovitzae	·	+	+	·	·	+	·	·	·	·	·	·	II
Ranunculus sardous	·	·	·	·	·	·	·	·	+	·	+	+	II
Juncus gerardii	+	·	·	·	·	·	·	·	·	+	·	·	I
Peucedanum latifolium	·	·	·	+	·	+	·	·	·	·	·	·	I
Ranunculus pedatus	·	·	·	+	·	·	·	·	·	·	·	·	I
Iris brandzae	·	·	·	+	·	·	·	·	·	·	·	·	I
Carex distans	·	·	·	·	·	+	·	·	·	·	·	·	I
Rorippa sylvestris ssp. kerneri	·	·	·	·	·	+	·	·	·	·	·	·	I
Trifolium fragiferum ssp. fragiferum	·	·	·	·	·	+	·	·	·	·	·	·	I
Lotus tenuis	·	·	·	·	·	·	·	+	·	·	·	·	I
Matricaria chamomilla	·	·	·	·	·	·	·	·	+	·	·	·	I
Puccinellia distans ssp. limosa	·	·	·	·	·	·	·	·	·	+	·	·	I
Festuco-Brometea													
Galium verum	+	+	+	+	+	+	·	+	·	+	+	·	IV
Achillea setacea	+	+	+	+	+	+	·	+	+	·	+	·	IV
Filipendula vulgaris	+	+	+	+	+	+	·	·	·	·	·	·	III
Carex praecox ssp. praecox	·	+	+	+	·	·	+		1	1	+	·	III
Fragaria viridis ssp. viridis	+	·	·	·	·	·	·	·	·	·	·	·	I
Medicago falcata	·	·	·	·	+	·	·	·	·	·	·	·	I
Arenaria serpyllifolia	·	·	·	·	·	·	·	·	+	·	·	·	I
Festuca valesiaca	·	·	·	·	·	·	·	·	+	·	·	·	I
Artemisietea vulgaris													
Euphorbia virgata ssp. virgata	·	+	+	+	+	+	·	·	·	·	·	·	III
Cardaria draba	·	·	+	·	·	·	·	+	+	+	+	·	III
Cichorium intybus	·	·	·	·	+	·	·	+	·	·	+	·	II
Picris hieracioides ssp. hieracioides	·	·	+	·	+	·	·	·	·	·	·	·	I
Salvia nemorosa ssp. nemorosa	·	·	·	+	·	+	·	·	·	·	·	·	I
Daucus carota ssp. carota	·	·	·	·	+		·	·	·	·	·	·	I

Convolvulus arvensis	·	·	·	·	·	·	·	+	+	·	·	·	I
Crepis setosa	·	·	·	·	·	·	·	+	·	·	·	·	I
Medicago lupulina	·	·	·	·	·	·	·	·	·	·	+	·	I
Stellarietea mediae													
Lathyrus hirsutus	+	+	+	+	+	+	+	+	+	+	·	·	V
Vicia tetrasperma	+	+	+	·	·	+	+	+	+	+	+	+	V
Trigonella caerulea	+	+	·	+	+	·	+	1	+	1	·	+	IV
Vicia sativa ssp. sativa	+	·	+	+	+	·	+	·	+	+	+	+	IV
Cirsium arvense	+	+	+	+	+	+	·	·	·	·	·	·	III
Lathyrus tuberosus	+	+		+	+	+	·	·	+	·	·	·	III
Vicia pannonica ssp. pannonica	+	+	+	+	+	·	·	·	+	·	·	·	III
Vicia hirsuta	·	+	+	+	·	·	·	·	+	+	·	·	III
Veronica arvensis	·	·	+	·	·	·	·	+	+	·	+	·	II
Allium rotundum ssp. rotundum	·	·	+	·	+	·	·	+	·	·	·	·	II
Vicia villosa	·	·	·	+	·	·	+	·	·	·	·	+	II
Capsella bursa-pastoris	·	+	·	·	·	·	·	·	·	·	·	+	I
Tripleurospermum inodorum	·	+	·	·	·	·	·	·	·	·	·	·	I
Lathyrus nissolia ssp. nissolia	·	·	·	+	·	·	·	·	·	·	·	·	I
Lactuca serriola	·	·	·	+	·	·	·	·	·	·	·	·	I
Erysimum repandum	·	·	·	·	·	·	·	·	·	·	+	+	I
Variae													
Allium scorodoprasum	+	+	+	+	+	+	·	+	+	+	·	·	IV
Trifolium campestre	+	+	+	+	+	·	+	+	·	+	·	+	IV
Asparagus officinalis ssp. officinalis	+	+	+	+	+	+	·	·	·	·	·	·	III
Galium rubioides ssp. rubioides	+	+	·	·	·	+	·	·	·	·	·	·	II
Bulbocodium versicolor	·	·	+	·	·	·	·	·	·	·	·	·	I
Myosotis arvensis ssp. arvensis	·	·	·	+	·	·	·	·	·	·	·	·	I
Ranunculus polyanthemos ssp. polyanthemoides	·	·	·	·	·	+	·	+	·	·	·	·	I

Previously, a rather extensive description of the grasslands in the lower basin of the Jijia river was published by RĂVĂRUȚ & al. (1968). The association "*Agropyretum repentis*" was marked on a map, north of Victoria village (not very far from Cotu Morii village), but within its plant structure (and to all other plant communities described by the cited authors in the lower basin of Jijia river) no species of *Fritillaria* was recorded.

On the other hand, the Basin of Başeu River has been extensively studied from botanic point of view by Professor Gh. MIHAI, in the past. In his PhD thesis [MIHAI, 1969], as well as in a subsequent paper [MIHAI, 1971a], he reported from the Başeu river basin, among other rare plants, *F. meleagris* L. from Ștefănești, "on salty marshy meadows". The same author [MIHAI, 1971b] reported *F. meleagris* (AD +), in a phytocoenosis of the association *Alopecuretum pratensis* Nowinski 1928, subassociation *poëtosum pratensis* Soó 1957 (although *Poa pratensis* was missing there), classified in the alliance "*Agrostion albae* Soó 1957"– a name currently [COLDEA, 2012] considered as a synonym for the alliance *Deschampsion caespitosae* Horvatić 1930. This phytocenosis, including some facultative or obligate halophytes (*e.g. Beckmannia eruciformis*, *Ranunculus sardous*, *Rorippa sylvestris* ssp. *kerneri*, *Juncus gerardii*, *Limonium gmelinii*), was recorded on a strongly grazed meadow in the surroundings of the Murguța village, approx. 5 Km upstream of Ștefănești town (see map in Figure 1), on salty soils (solonetz), with *p*H=8 (according to the cited author). As a matter of fact, according to recent literature [COLDEA, 2012], those stational conditions are not suitable

for the alliance *Deschampsion caespitosae* Horvatić 1930 (syn.: *Agrostion albae* Soó 1957; *Agrostion stoloniferae* Soó (1943) 1971), but for alliance *Potentillion anserinae* R. Tx. 1947 (syn. *Agrostion stoloniferae* Görs 1966 in Oberd. & al. 1967).

In our fieldwork on the surveyed area for this paper, we have not found at all *F. meleagris*, but only *F. meleagroides*. Therefore, we assume that the mention of *F. meleagris* in this locality was due to misidentification. If this assumption is correct (although it cannot be proven by voucher specimens collected at that time), it follows that *F. meleagroides* was found in Romania about 5 decades ago, but it remained unknown until recently due to the confusion with *F. meleagris*.

MITITELU & BARABAŞ (1975) also indicated *F. meleagris* (K=I) from the Prut river meadow, within the association "*Agrostetum stoloniferae* (Ujvarosi 1941) Arvat 1939", classified in the alliance*Agrostion stoloniferae* Soó 1933, order *Molinietalia* W. Koch 1926. Unfortunately, one cannot know in which of the listed localities (Berezeni, Vetrişoaia, Stânca-Ştefăneşti, Râşeşti, Cristeşti, Bosia, Oancea, Probota, Vlădeşti, Trifeşti, Brăneşti, Galaţi) *Fritillaria* was identifed, since the table given by the authors in that paper is a synthetic one. It is not excluded, however, that *Fritillaria* was present in the relevé registered at Stânca-Ştefăneşti (Botoşani county), a place near Ştefăneşti town, where we identified *Fritillaria meleagroides*. Regardless of the accurate location of that phytocenosis with *Fritillaria* along the Prut river meadow, the presence of other more or less halophilous species (such as *Trifolium fragiferum* ssp. *fragiferum*, *Oenanthe silaifolia*, *Ranunculus sardous*, *Mentha pulegium*, *Inula britannica*), in the structure of the association given by the above mentioned authors indicates rather the presence of *F. meleagroides* than *F. meleagris*.

Due to its linear leaves, easily confused with those of the dominant *Poaceae*, the individuals of *F. meleagroides* is easily distinguishable within the phytocoenosis structure only during the flowering time (April), when its stems having nodding flowers, are easily noticeable in the relatively low vegetation. With the full development of the dominant or subdominant *Poaceae* (*Elymus repens*, *Alopecurus pratensis*, etc.), *F. meleagroides* can only be hardly noticed. This may explain its lack on some of the previous phytosociological records [RĂVĂRUŢ & al. 1968; CHIFU & al. 1998].

The phytocoenoses of *Rorippo austriacae-Agropyretum repentis*, in which *F. meleagroides* was identified by us, have a wide distribution, both along the Jijia meadow river, at Cotu Morii, Ţigănaşi, Larga Jijia, Mihail Kogălniceanu (Iaşi county), as well as along the Başeu plain, on the river meadow, north of Ştefăneşti town (Botoşani county). They occupy flat, meadow land, with aluviosols (fluvisols), more or less salinized, in some places associated with saline soils, with phreatic water at shallow depth, and temporary stagnation of the rainfall waters in the spring season.

The vegetation is dominated by *Elymus repens*, with *Alopecurus pratensis* ssp. *pratensis* as a subdominant (rarely codominant), while the species *F. meleagroides*, although present in all the recorded relevées, has a reduced coverage, up to 10%.

Besides *F. meleagroides*, other rare and threatening species, according to IUCN criteria [DIHORU & NEGREAN, 2009; OLTEAN & al. 1994], are also included in the plant structure of phytocoenoses, as: *Bulbocodium versicolor* (VU), *Dianthus pratensis* ssp. *racovitzae* (CR), *Iris brandzae* (LR), *I. halophila* (R), *Serratula coronata* (R), *Rhaponticum serratuloides* (R). To mention, *Bulbocodium versicolor* (Figure 6) is reported here for the first time in Iaşi county. It should be noted, however, that all of these rare species were recorded only along the Jijia meadow river, at Cotu Morii (Iaşi county), but not in the phytocoenoses investigated in Başeu river basin (Botoşani county).

The phytocoenoses analyzed by us have a characteristic structure for the plant association (*Rorippo austriacae-Agropyretum repentis*), with a weight of approx. 40% of the diagnostic species for the higher syntaxa (alliance, order, and class). Approximately a quarter of the recorded species belong to the ruderal vegetation (20% *Stellarietea mediae*, 6% *Artemisietea vulgaris*), reflecting the high anthropo-zoogenic impact in the communities containing *F. meleagroides*, impact which is manifested both by grazing with sheep or cattle and by rebuilding the land on some surfaces (by plowing, digging ditches or channels, etc.).

Phytocoenoses are dominated by hemicriptophytes (51%), with an important weight of therophytes (20%) and geophytes (19%). Helohidatophytes, in small numbers (about 4%), are generally settled down in some microdepressions, where the water stagnates for a longer time.

Phytogeographically, the Eurasian elements predominate (54.3%), followed at a long distance by the European (14.3%) and the general Pontic ones (11.4%). The endemic and subendemic elements deserve also a special attention. Although they are represented by one taxon each (*Dianthus pratensis* ssp. *racovitzae* and *Iris brandzae*, respectively), they contribute, besides *F. meleagroides* and other rare species above mentioned, to increase the conservative value of the meadows nearby the Cotu Morii village.

Figure 6. *Bulbocodium versicolor*, North of the Cotu Morii village

Another important feature of these plant communities is a high presence of the obligatory halophilous species (*e.g. Scorzonera cana*, *Iris halophila*, *Cerastium dubium*, *Limonium gmelinii*, etc.), characteristic for the class *Festuco-Puccinellietea*, representing between 6.9% and 20.7% (13.1% in average) of the total number of species per relevé. In addition, many other salinity-tolerant plant species (*e.g. Oenanthe silaifolia*, *Silaum silaus*, *Inula britannica*, etc.) are present, too.

The important participation of the halophilous and ruderal species within the floristic structure of these communities is consistent with the literature data for the *Potentillion anserinae* alliance [BURDUJA & al. 1956; CHIFU & al. 1998; CHIFU & al. 2006; COLDEA, 2012; RĂVĂRUȚ & al. 1956].

The plant communities with *F. meleagroides* registered by us are generally heliophilous, meso-thermophilous, (meso-) hygrophilous, light alkalinophilous, moderately nitrophilous and light- to moderately salty tolerant. The alternation between an excess moisture in spring, and land drought during the summer, makes possible the presence of both hygrophilous and xerophilous species within these plant communities (the latter, however, much less numerous).

The tolerance to the soil salinity of *F. meleagroides*, proven by a significant presence of halophilous plants which accompany it in the analyzed communities, is also consistent with the literature data [ARTIUSHENKO, 1979; GOLUB, 1994; GOLUB & SAVELJEVA, 1991; KOROTCHENKO & ORLOV, 2009; LOZINA-LOZINSKAYA, 1935; PROKUDIN & al. 1987]. By this feature, *F. meleagroides* obviously differs from *F. meleagris*, which actually is a halophobous plant [BORHIDI, 1995; ELLENBERG & al. 1992], although the two species resemble in that both are hygrophilous.

As a result of ecological differences, the two species develop in different phytocoenotic environments. Thus, *F. meleagroides* has been identified, as shown in this paper, in the structure of some communities within *Potentillion anserinae* alliance, whereas *F. meleagris* is known in the structure of plant communities from various alliances, such as: *Deschampsion caespitosae* Horvatić 1930 [SĂMĂRGHIŢAN & OROIAN, 2011], *Molinion* W. Koch 1926, *Calthion* Tx. 1937 and *Magno-Caricion elatae* W. Koch 1926 [CSERGŐ & FRINK, 2003], *Alnion incanae* Pawłowski, Sokołowski *et* Wallisch 1928 [BUJOREAN & GRIGORE, 1965], *Salicion cinereae* Müller *et* Görs *ex* Passarge 1961 [BUJOREAN & GRIGORE, 1965; ULARU & PARASCAN, 1970] or even in some meso-hygrophilous plant communities in *Lathyro hallersteinii-Carpinion* (Soó 1964) Boşcaiu 1979 [SEGHEDIN & al. 1979].

Consequently, *F. meleagris* should not be indicated as a characteristic species for the alliance *Potentillion anserinae* [CHIFU & al. 2006; SÂRBU & al. 2013], since this was due, in our opinion, to the fact that the individuals of *Fritillaria* in the Plain of Moldavia were previously [MIHAI, 1969, 1971a, 1971b; MITITELU & BARABAŞ, 1975] misidentified with *F. meleagris* (instead of *F. meleagroides*).

d) The need of conservation

Taking into account the botanical importance of the wet meadow near Cotu Morii village (Iaşi county), from the inferior basin of the Jijia river, supported by our present and previous studies, we reinforce our proposal [OPREA & al. 2015] to establish a nature reserve within this area. The territory is included in the perimeter of ROSCI0222 Sărăturile Jijia Inferioară-Prut. A rational exploitation of meadows through mowing or non-aggressive grazing, without major interventions (such as plowing the ground, digging other drainage channels, or the excessive collecting) would allow both the long-term survival of the rare species here (and on the other places with *F. meleagroides* above mentioned), as well as a good economic valorizing of the meadows.

Conclusions

Fritillaria meleagroides, a rare species recently reported in the Romania's vascular flora, was identified in other localities, as nearby of the Ştefăneşti town (Botoşani county), as well as between Ţigănaşi, Mihail Kogălniceanu and Larga Jijia villages (Iaşi county). The number of reproductive individuals of *F. meleagroides* at Cotu Morii (Iaşi county) has been estimated. In the sample area, the population has an average density of 3.7 individuals per m^2. The paper reveals the structure and some ecological peculiarities of plant communities in which *F. meleagroides* grows at Cotu Morii (Iaşi county), and Ştefăneşti (Botoşani county). At Cotu Morii, besides *F. meleagroides*, other rare and threatened plant species have been identified (e.g. *Dianthus pratensis* ssp. *racovitzae*, *Iris brandzae* and *Bulbocodium versicolor*, the last species being a floristic novelty for the Iaşi county). The authors

recommend the protection of all these species and their habitat by declaring a natural reserve at Cotu Morii (Iași county).

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EPIPACTIS × SCHMALHAUSENII K. RICHT. (*ORCHIDACEAE*), A NEWLY IDENTIFED TAXON IN ROMANIAN FLORA

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Abstract: The authors reports the presence in the Romanian wild flora of the nothospecies *Epipactis* × *schmalhausenii*. This taxon of hybrid origin (*Epipactis atrorubens* × *E. helleborine* subsp. *helleborine*) was recently identified, together with the two genitors, on some limestone mountains in the Romanan Eastern Carpathians (Bistriţei Mountains; Ceahlău Mountains; Hăşmaş Mountains), as well as in Southern Carpathians (Bucegi Mountains). Although, this one is relatively widespread in other European countries, even in Kazakhstan (Central Asia), nothosp. *Epipactis* × *schmalhausenii* has not been reported in botanical literature of Romania, until now.

Keywords: Carpathian Mountains, *Epipactis* × *schmalhausenii*, newly identified, *Orchidaceae*, Romania.

Introduction

The genus *Epipactis* Zinn includes approximately 20 species, distributed in the temperate zone of the boreal hemisphere [PAUCĂ & al. 1972]. In *Flora Europaea*, 9 species were mentioned only (to which was added another species, *Epipactis confusa* D. P. Young, from Denmark and South of Sweden, but not recognized at a species level) [MOORE, 1980].

In the scientific work *Flora României* [PAUCĂ & al. 1972], as well as in the field identification book of the vascular plants in Romania [BELDIE, 1979], 5 species were reported only.

The most recent field identification books of vascular plants in Romania [CIOCÂRLAN, 2009; SÂRBU & al. 2013], registered 6 and 7 species of *Epipactis* Zinn, respectively.

In recent years, new species of the genus *Epipactis* have been described. Thus, through the recent floristic researches [ARDELEAN, 2011; MOLNÁR & SRAMKÓ, 2012; ARDELEAN & al. 2018a, b] the number of reported species in Romania has doubled compared to previous evaluations.

In this paper we report another taxon within the *Epipactis* genus, previously unknown in the botanical literature of Romania, namely *Epipactis* × *schmalhausenii* K. Richt. nothosubsp. *schmalhausenii*.

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Material and method

The species has been identified during our field surveys in several mountains in the Eastern and Southern Carpathians, over the last two years (2018 and 2019).

Some important regional or local floras were used in order to identify or confirming the authencity of the samples collected in Eastern Carpathians (Romania), as: *Flora Europaea* [MOORE, 1980], *Flora of Romania* [PAUCĂ & al. 1972], *Flora of ex-USSR* [KOMAROV, 1968], web databases [PRIDGEON & al. 2006; emonocots, 2015; tropicos, 2012; IPNI, 2012; arnsidesilverdale, 2012; europlusmed, 2019; species.wikimedia, 2019; science.kew, 2019], etc. Besides, there have been seen other scientifical operas in order to check up the presence of this hybrid in Romania [GRECESCU, 1898; PANȚU, 1915]. Some of the international databases on the *Orchidaceae* Juss. have been seen [GOVAERTS & al. 2012; RICH & JERMY, 2019].

The local coordinations (northern latitude, eastern longitude, altitude) have been taken using a GPSMAP Garmin 60CSx.

The collected specimens were included in public herbaria (IASI, IAGB).

Results and discussions

The genus name, *Epipactis*, is a name still found in Dioscoride [PAUCĂ & al. 1972], being taken as such in the botanical nomenclature by the German anatomist and botanist Johann Gottfried Zinn, starting with 1757.

The epithet of this nothotaxon was given by the Austrian botanist Karl Richter (1855-1891) in his work *Plantae Europae* (t. I, p. 284) in honor of the Ukrainian botanist Johannes Theodor Schmalhausen (1849-1894), known for his studies on East European plant species [RICHTER, 1890]. But, the name of the nothospecies appears in Richter's work only in its binomial form, in the enumeration of the species of the genus *Epipactis*, as being the 5th of the 7 species counted by the author, without any description of this nothotaxon in the cited work.

Later on, to this name were given other names, which became synonymous, such as:

- homotypic synonyms: *Helleborine* × *schmalhausenii* (K. Richt.) Vollm. – this name was given by the German botanist Franz Vollmann (1858-1917), in his work *Flora von Bayern* [VOLLMANN, 1914], on page 169, thus emending the original name of the nothospecies;
- heterotypic synonyms: *Epipactis* × *trikalana* B. Baumann & H. Baumann – this name was given by the German botanists Brigitte Baumann and Helmut Baumann [BAUMANN & BAUMANN, 1988].

It is admitted that *Epipactis* × *schmalhausenii* includes 4 nothosubspecies, namely:

- nothosubsp. *capellonensis* (B. Baumann & H. Baumann) R. Govaerts (*E. atrorubens* (Hoffm.) Besser × *E. helleborine* (L.) Crantz subsp. *latina* W. Rossi & E. Klein) [GOVAERTS & al. 2012];
- nothosubsp. *fleischmannii* (Heimerl) R. Govaerts (*E. atrorubens* × *E. helleborine* subsp. *orbicularis* (K. Richt.) E. Klein) [GOVAERTS & al. 2012];
- nothosubsp. *zaisii* Riech. (*E. atrorubens* × *E. helleborine* subsp. *minor* (R. Engel) R. Engel) [RIECHELMANN, 2013; https://www.ipni.org/n/77135233-1];
- nothosubsp. *schmalhausenii* (*E. atrorubens* × *E. helleborine* subsp. *helleborine*) (cf. https://species.wikimedia.org/wiki/Epipactis_schmalhausenii).

During the field surveys of the last two authors conducted in the summer of 2019 in the Eastern Carpathians, near the town of Tulgheș, Harghita county, on Piatra Comarnicului Mountain (Bistriței Mountains), at the southern end, within the area of the rocks called "Pietrele Roșii", an *Epipactis* population with intermediate characters between *E. atrorubens* (Hoffm.) Besser and *E. helleborine* (L.) Crantz subsp. *helleborine* has been identified. It has been found that this population belongs to *E.* × *schmalhausenii* nothosubsp. *schmalhausenii*, a taxon unknown so far in the Romanian botanical literature. Information on the presence of this taxon in the Bucegi Massif was previously disseminated, in the online environment (facebook), by the first two authors of this paper. The review of some previously collected herbarium specimens led us to find that, besides the two locations above mentioned, *E.* × *schmalhausenii* nothosubsp. *schmalhausenii* is also present in the Ceahlău and Hășmaș mountain ranges, in the Eastern Carpathians.

Description

Epipactis* × *schmalhausenii K. Richt. [Pl. Eur. **1**: 284 (1890)] nothosubsp. ***schmalhausenii***

Genitors: *Epipactis atrorubens* × *E. helleborine* subsp. *helleborine*

Perennial, geophyte, with stems of 20-75 (-80) cm, rigid, erect, short and whitish pubescent, reddish-purple tinted. The leaves are alternate, arranged until under inflorescence, 4-10 (-15), 7-21 cm long and (3-) 4-8 cm wide, longer than the internodes, semi-amplexicaul, without wavy edges; on the edges and on the ribs on the back are minutely scabrious. The leaves that accompany the flowers (= the bracts) are lanceolate, the lower ones longer than the flowers, the upper ones shorter. The flowers are arranged in elongated inflorescences, more or less unilateral, many flowered, with (10-) 15-60 (-80) flowers, 7-9 mm long, wide open, with the labellum longer than the other tepals (Figures 1-4).

Figure 1. *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* – habitus (Pietrele Roșii, Tulgheș, Bistriței Mountains, Romania)

Period of de anthesis: July-August.

The morpho-anatomical diagnostic features of the nothospecies *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* against its genitors, *E. helleborine* subsp. *helleborine* and *E. atrorubens* (according to data in: PANȚU, 1915; MOORE, 1980; PAUCĂ & al. 1972; JAKUBSKA-BUSSE & GOLA 2010; SÂRBU & al. 2013, as well as to our own researches) are shown in the next table (Table 1).

Figure 2. *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* – habitus (Zănoagei Gorges, Bucegi Mountains, Romania)

Figure 3. *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* – inflorescences (Pietrele Roşii, Tulgheş, Romania)

Figure 4. *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* – flower details (Pietrele Roşii, Tulgheş, Romania)

Table 1. Distinctive morpho-anatomical traits of nothospecies *Epipactis* × *schmalhausenii* and its genitors

	Species		
	E. helleborine subsp. ***helleborine***	***E.* × *schmalhausenii***	***E. atrorubens***
Stem	(20-) 40-80 (-130) cm	20-75 (-80) cm	10-60 (-100) cm
Leaves shape	medium leaves elliptic-broad-ovate, with a thin, relatively soft lamina	middle leaves ovate to ovate-lanceolate, the upper lanceolate (>*E. helleborine*), thicker and more rigid lamina; basal leaf blade obtuse, patent	middle leaves ovat-lanceolate to lanceolate, with thicker and stiffer lamina
Leaves colour and edges	green leaves, usually with non-undulating edges, with pale green to whitish sheaths (basal sheaths are rarely light purple)	leaves green to blue-green, with edges, ribs and basal part of sheaths pale-violet or pink-purple (>*E. atrorubens*)	lamina green, but the edges, the tip, the base, the ribs and sheaths are red-purple
Leaves epidermis	margins and main ribs with papillary cells on 2-3 rows, elongated, tapered and slightly inclined (at the median row), shorter and rounded (the lateral rows)	papillae on the median row of variable length (in the form of those of *E. helleborine*); those on the lateral rows are shorter and flattened (in the form of those of *E. atrorubens*)	lamina edges always papillose, with papillae shorter and flattened than in *E. helleborine*
Rachis of inflorescenses/flowers	inflorescences rachis and flower pedicels glabrous to scabrous	rachis with hairs; pedicels weakly hairy, with hairs	rachis densely-pubescent, gray-green
Flowers number	(10-) 15-40	10-80	10-15
Flowers fragrance	of *Valeriana* sp. (Schulze in Panțu 1915)	weak smelling	with a vanilla fragrance, but at the end with a fragrance of *Eugenia caryophyllata* (PANȚU, 1915)
Outer tepals	elliptic-ovate, green on the outside and pink or greenish-purple on the inside, 10-13 mm long	ovate-lanceolate to lanceolate, 7-9 mm long	ovate, acuminate, dark purple to purple-brown, 6-7 mm long, slightly pubescent on the outside
Inner tepals	shorter and wider than the outer ones, greenish, pinkish-purple towards the base	pink-purple	dark purple, elliptical, 6-7 mm long (rarely shorter), glabrous, wavy-curled at the edges
Labellum length	9-11 mm	9-11 mm	5,5-6,5 mm
Hypochil	concave, dark brown inside, with nectariferous glands	evidently concave, reddish-brown	oblong, concave, dark purple-red
Epichil shape, size & colour	triangular-cordate to broad-ovate, ± recurved, l> L → l = L, greenish-whitish to pinkish-purple, finely crenated on the edges	cordate to reniform, 6-7 mm long, pinkish-purple to green, 1.2 times longer than wide	cordate-reniform, l> L, dark reddish-purple, acuminated, often crenated on the edges
Epichil protuberances	at the base of the epichil, relatively small, elongated, smooth or slightly rough	half of the hpichil width, triangular (Rich & Jermy 1998)	at the base of the epichil, large, rough (crenated-wrinkled), darker colored, confluent at the tip

Ovar	green, glabrous to rarely hairy, with 6 ribs, elongated	green to pinkish, slightly hairy	± pinkish, densely gray pubescent, with 6 ribs [VLČKO & al. 2003]
Capsula	(8-) 10-15 mm long, 5-8 mm wide, oblong-obovate, glabrous or glabrescent	9-10 mm long, 6-8 mm wide, oblong-ovate, sparsely and soft pubescent (>*E. atrorubens*)	ovate to oblong-ovate, obtuse at the tip, base sharply narrowed in a stalk, soft pubescent
2n	36, 38, 40, 44	?	40
Ecology	meso-hygrophyillous and in shady sites	on dry andf moderately dry, sunny sites	on dry to moderately humid, heliophyllous sites
Frequency, habitat	frequent, from the oak area to the spruce floor, through forests, bushes, forest edges, parks and gardens, through shady places, in coniferous or mixed forests - up to 2000 m alt. (Jakubska-Busse et Gola 2010)	very rare to rare	sporadically, from the floor of the *Quercus petraea* to the floor of the Norway spruce (even in the subalpine floor), in rocky meadows, bushes, forest edges and ridges, grassy rocks, on limestone soils - up to 2400 m alt (JAKUBSKA-BUSSE & GOLA, 2010)
Coenology	*Epipactido-Fagenion*, *Fagetalia*, *Abieti-Piceion*	*Piceion excelsae*, *Epipactido-Fagenion*	*Epipactido-Fagenion*, *Pinetalia*, *Abieti-Piceion*
World distribution	Eurasian (from the Mediterranean area to the Boreal area), being identified also in North America and northern Africa (Jakubska-Busse et Gola 2010)	Eurasian (Europe: from the British Isles to the W Russia, including Crimea and Caucasus), Iran	Eurasian (Europe to Siberia, including the Caucasus and N Iran) (JAKUBSKA-BUSSE & GOLA, 2010)

Location of the nothospecies in Romania

Until now, the nothospecies of *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* has been identified in:

– Eastern Carpathians: Bistriței Mountains (in the neighborhood of Tulgheș town, South of the rocks "Pietrele Roșii" (GPS coordinations: N46º97849/E25º77029/altitude circa 1000 m s.l.), Ceahlău Mountains (near the rock called "Căciula Dorobanțului"), Hășmaș Mountains (near the rock called "Piatra Singuratică");
– Southern Carpathians: Bucegi Mountains (in Zănoagei Gorges, in the lower half, towards the lake Scropoasa) (Figure 5).

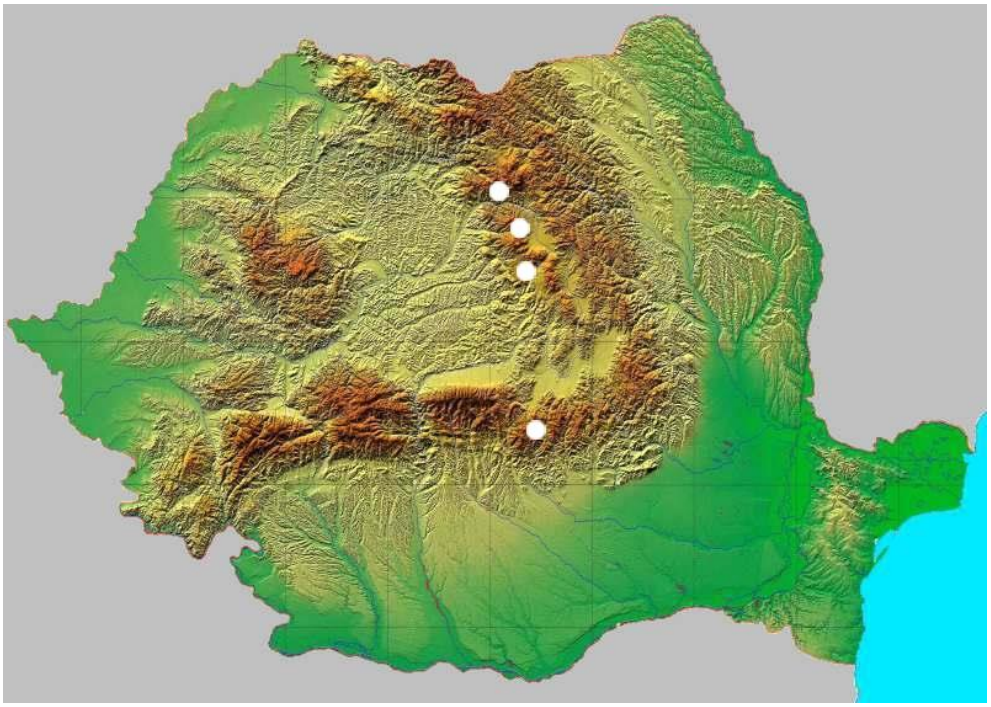

Figure 5. Map distribution of *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* in Romania (https://www.welcometoromania.eu/Romania/Romania_Harta_Geografica_e.htm)

The general distribution of nothosp. *Epipactis* × *schmalhausenii* is as follows:

- Central Europe: Austria, Belgium, former Czechoslovakia, Germany, Poland, and Switzerland;
- West and North of Europe: France (in the subalpine vegetation of the Alps), Spain, Great Britain, Sweden, South of Norway, the european part of North-West Russia;
- South and East of Europe: Greece, Italy, former Yugoslavia;
- Asia: East Kazakhstan, in steppe vegetation areas (ADAMOVSKI, 1995).

But, the general distribution of *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* is restricted to the European continent only, as follows: Sweden, Great Britain, France, Belgium, Germany, Poland, Switzerland, Austria, former Czechoslovakia, Greece, and the european part of North-West Russia, and Romania.

In herbaria: the collected individuals of *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* have been deposited in the public herbaria in Romania, thus:

- herbarium of the Botanic Garden "Anastasie Fătu" (IAGB**)**: sheet no. 47.704 - collected from nearby of Tulgheş, in Bistriţei Mountains, Eastern Carpathians, Romania, leg. A. Oprea, at 13th of July, 2019;
- herbarium of the University of Agronomical Studies and Veterinary Medicine "Ion Ionescu de la Brad" in Iaşi (IASI): sheet no. 17960 - collected from nearby of Tulgheş, in Bistriţei Mountains, Eastern Carpathians, Romania, leg. C. Sîrbu, at 13th of July, 2019.

Ecology of the nothospecies *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* in Romania: it grows in shady and half shady places, on moist soils, on calcareous substrates.

Coenology: in Norway spruce forests (*Piceion* excelsae Pawł. în Pawł. et al. 1928) or in beech forests (*Epipactido-Fagenion* Boşcaiu et al. 1982). Besides the genitors (*Epipactis helleborine* subsp. *helleborine* and *E. atrorubens*), other accompanying plant species on the place, are: *Picea abies* (L.) H. Karst., *Fagus sylvatica* L., *Betula pendula* Roth, *Hieracium pojoritense* Woł. subsp. *pojoritense*, *Campanula carpatica* Jacq., *Aconitum anthora* L., *Arabis hirsuta* (L.) Scop., *Clinopodium vulgare* L. subsp. *vulgare*, *Erysimum witmannii* Zaw. subsp. *witmannii*, *Gnaphalium sylvaticum* L., *Silene nutans* L. subsp. *dubia* (Herbich) Zapał., *Hepatica transsilvanica* Fuss etc.

Sozology: in the Romanian Red Lists/Red Book, the parents of the nothospecies are listed as follows:

- in a red list on the vascular plant species of Romania [OLTEAN & al. 1994], *Epipactis helleborine* and *E. atrorubens* are considered to be rare (R) plant species;
- other authors [BOŞCAIU & al. 1994] does not consider the genitors as to be at risk in Romania;
- other authors [DIHORU & DIHORU, 1994] also does not consider the genitors as to be at risk in Romania;
- other authors [SÂRBU & CHIFU, 2003] consider both genitors as to be rare (R) species within the flora of the Moldavian province (the eastern part of Romania);
- the *Red Book* of plant species in Romania [DIHORU & NEGREAN, 2009] does not include any of the genitors in it.

Our proposal is: *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* could be considered as a vulnerable species in Romanian flora (VU, under the IUCN risk cathegories).

Conclusions

A new taxon of genus *Epipactis* was identified in Romanian vascular flora, namely: *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii.*

The distribution of this newly identified taxon in the wild flora of Romania is: mountains of Bistriţei, Ceahlău, Hăşmaş, and Bucegi.

Epipactis × *schmalhausenii* nothosubsp. *schmalhausenii* could be considered as a vulnerable (VU) plant species into the Romanian flora.

The world distribution of nothosp. *Epipactis* × *schmalhausenii* is in Central Europe, West and North of Europe, South and East of Europe, and East Kazakhstan, but the distribution of *Epipactis* × *schmalhausenii* nothosubsp. *schmalhausenii* is restricted to Europe, only.

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GERANIO PRATENSI-CIRSIETUM CANI ASS. NOVA PÎNZARU, IONIȚA & JARDAN (*FILIPENDULION* SEGAL EX WESTHAFF ET DEN HELD 1969) IN THE REPUBLIC OF MOLDOVA

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Abstract: The phytocoenoses of *Cirsium canum* (L.) All. with *Geranium pratense* L., occurring on the Central Moldavian Plateau, are described in this article. Based on 26 relevés, the authors propose another association for science – *Geranio pratensi-Cirsietum cani* ass. nova Pînzaru, Ioniță et Jardan of the alliance *Filipendulion ulmariae* Segal ex Westhoff et Den Held 1969, order *Molinietalia caeruleae* Koch 1926, class MOLINIO-ARRHENATHERETEA Tx. 1937.

Keywords: *Geranio pratensi-Cirsietum cani* ass. nova, characteristics plant ofspecies, ecology, range, Republic of Moldova.

Introduction

Spear thistle or Queen Anne's thistle (*Cirsium canum* (L.) All., Figure 1) – a perennial species, geophyte, East-European, meso-hygrophilic, is characteristic of wet meadows from lowland to mountainous areas, included in the order *Molinietalia caeruleae* W. Koch 1926 [AESCHIMANN & al. 2004; SÂRBU & al. 2013]. The Central European plant communities with *Cirsium canum* are included in the associations: *Cirsio cani-Festucetum pratensis* Májovsky et Ruzicková 1973 of the alliance *Deschampsion cespitosae* Horvatić 1930 (= *Alopecurion pratensis* Passarge 1964), *Scirpo sylvatici- Cirsietum cani* Bálátová-Tulačová 1973 of the alliance *Calthion palustris* R.Tx. 1937, *Angelico sylvestris-Cirsietum cani* P. Burescu 1998 corr. Chifu et Zamfirescu 2014 of the alliance *Calthion palustris* R. Tx. 1937 [BĂDĂRĂU & ALEC-FARCAS, 2010; CHIFU & al. 2014; COLDEA & al. 2012; HÁJKOVÁ, 2010].

Figure 1. *Cirsium canum* (L.) All.

The vegetation of floodplain grasslands in the Republic of Moldova has been described in more detail in the monograph of the botanist Ștefan Lazu, where he has also mentioned an association with *Cirsium canum* (L.) All. – *Cirsietum cani* Tx. 1951, with a short characterization based on 4 relevés, grouped

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in the alliance *Agrostion stoloniferae* Soó 1933 [LAZU, 2014]. At that time, in 2014, the association *Cirsietum cani* R.Tx. et Preising 1951 was considered as a synonym of the association *Angelico sylvestris-Cirsietum cani* P. Burescu 1998 corr. Chifu et Zamfirescu 2014 of the alliance *Calthion palustris* R.Tx. 1937 [CHIFU & al. 2014]. The new classification of the vegetation in Europe [MUCINA & al. 2016] does not indicate the alliance *Agrostion stoloniferae* Soó (1933) 1971 [BĂDĂRĂU & ALEC-FARCAS, 2010], but only *Agrostion stoloniferae* Görs 1966, which is synonymous with the alliance *Potentillion anserinae* Tx. 1947.

The phytocoenoses of *Cirsium canum* (L.) All. with *Geranium pratense* L. recorded in the floodplains of rivers on the Central Moldavian Plateau are described in this article.

Materials and methods

The phytocoenological research was carried out in 2018-2019. The research methodology adopted is that of the better write "The Zürich-Montpellier School" founded by Braun-Blanquet [BRAUN-BLANQUET, 1964]. The area of a relevé was 100 m [CRISTEA, 2004]. The plant species nomenclature is presented in accordance with recent publications [PÎNZARU & SÎRBU, 2016]. Air temperature and atmospheric precipitation – according to the Atlas of Climate Resources of the Republic of Moldova [NEDEALCOV & al. 2013].

Results and discussions

The plant communities of *Cirsium canum*, *Geranium pratense*, *Inula helenium* and other accompanying species, which occur on slightly alkaline stratified alluvial soils, in the floodplains of rivers on the Central Moldavian Plateau, are tall (the upper layer is about 140-180 cm in height) and contain a group of species that is characteristic of the alliance *Filipendulion ulmariae* Segal ex Westhoff et Den Held 1969, which is the reason why we include them in this alliance.

The alliance *Filipendulion ulmariae* Segal ex Westhoff et Den Held 1969 consists of herbaceous, meso-hygrophilic, tall plants, which occur in river valleys and valleys between hills or between mountains, on alluvial soils, which are moist and rich in nutrients. The characteristic plant species of the alliance are: *Filipendula ulmaria*, *Geranium palustre*, *Valeriana officinalis*, *Calystegia sepium*, *Lysimachia vulgaris*, *Lythrum salicaria*, *Mentha longifolia*, *Euphorbia palustris*, *Epilobium hirsutum*, *E. parviflorum*, *Petasites hybridus Stachys palustris*, *Symphytum officinale*, *Poa palustris*, *Hypericum tetrapterum* [CHIFU & al. 2014; COLDEA & al. 2012; HÁJKOVÁ, 2010; PÎNZARU, 1996; SÂRBU & al. 2013].

The described associations, occurring in Moldova, *Petasitetum hybridi* (Dostal 1933) Soó 1940 [LAZU, 2014; PÎNZARU, 1996] and *Filipendulo-Geranietum palustris* W. Koch 1926 [LAZU, 2014], previously included in the alliance *Filipendulo-Petasition* Br.-Bl. 1947, are now grouped in the alliance *Filipendulion ulmariae* Segal ex Westhoff et Den Held 1969. The alliance *Filipendulo-Petasition* Br.-Bl. ex Duvigneaud 1949 contains a group of associations of the submontane-montane layer in Western and Central Europe [MUCINA, 2016]. In Romania it hasn't been detected [CHIFU & al. 2014; COLDEA & al. 2012].

The description of the association of *Cirsietum cani* Tx. 1951 made by LAZU (2014, tab. 20, 4 relevés) is incomplete. The constancy has to be calculated on the basis of at least 5 relevés [HÁJKOVÁ & al. 2010], but the author indicates the constancy of the species based on 4 relevés, and when describing the given association, he lists *Festuca pratensis*, *Poa*

pratensis, *Juncus articulatus*, *Symphytum officinale*, *Ranunculus acris* and *Lythrum salicaria* as frequent species, but, in fact, they are absent in the 4 described relevés. The phytocoenoses included by LAZU (2014) in the association *Cirsietum cani* Tx. 1951, we include in the new association *Geranio pratensi-Cisietum cani*, containing the following common species: *Cisium canum*, *Geranium pratense*, *Inula helenium*, *Filipendula ulmaria*, *Taraxacum camylodes* (= *T. officinale*), *Althaea officinalis*, *Lathyrus pratensis*, *Valeriana officinalis*, *Trifolium pratense*, *Angelica sylvestris*. Unfortunately, the localities from where these relevés were made are not indicated, being indicated only the Central Codrii area.

The new association is described below.

Ass. *Geranio pratensi-Cirsietum cani*

Pînzaru, Ioniţa et Jardan, ass. nova, h. l., Figure 2, 3, 4

Syn.: *Cirsietum cani* Tx. 1951: Lazu, 2014

Relevé type h. l.: Table 1, rel. 22.

Table synthetic h. l.: Table 1, 26 relevés

The total area of the association in the described locations is about 30 ha.

Locations: Altitude 135-150 m. Relief: Central Moldavian Plateau, in floodplains of rivers. Soils – alluvial, stratified, slightly alkaline. Climate – temperate-continental, the average annual temperature is 10.0-10.5 °C, the average annual precipitation varies between 650 mm and 700 mm.

Figure 2. Ass. *Geranio pratensi-Cirisetum cani* – 29 May 2018, Corneşti commune

Figure 3. *Geranio pratensi-Cirsietum cani* ass. nova (type) – 12 July 2019, "Codru" Scientific Reserve

Figure 4. Ass. *Geranio pratensi-Cirsietum cani* – 12 July 2019, "Codru" Scientific Reserve

Characteristic species: *Cirsium canum*, *Geranium pratense*, *Inula helenium*.

Constant species: *Valeriana officinalis*, *Symphytum officinale*, *Veronica longifolia*, *Thalictrum lucidum*, *Taraxacum camylodes*, *Lathyrus pratense*, *Achillea pannonica*.

Rare species: *Anacamptis palustris* (= *Orchis palustris*) [Endangered (EN)], included in the Red Book of R. Moldova, *Dactylorhiza incarnata* (= *D. majalis* auct.mold. non (Rchb.) P. F. Hunt et Summ.) [Critically Endangered (CR)], *Ophioglossum vulgatum* [Critically Endangered (CR)], included in the Red Book of R. Moldova, *Thelypteris confluens* (= *T. palustris*) [Endangered (EN)], included in the Red Book of R. Moldova, *Epipactis helleborine* [Vulnerable (VU)], *Ranunculus binatus* [Vulnerable (VU)], *Silene flos-cuculi* [Endangered (EN)], *Senecio sarracenicus* (= *S. fluviatilis*) [Critically Endangered (CR)], *Galium rivale* (Sibth. & Sm.) Giseb. [Critically Endangered (CR)] [12-14].

Structure: the herbaceous layer has 100 % coverage (Figure 2-4). Vertically, three layers are distinguished in phytocoenoses:

1. The upper layer, about 140-180 cm in height, consists of the species: *Cirsium canum*, *Inula helenium*, *Thalictrum lucidum*, *Lysimachia vulgaris*, *Heracleum sibiricum*, *Filipendula ulmaria*, *Veronica longifolia*, *Dactylis glomerata*, *Valeriana officinalis*, *Sium sisarum*, *Angelica sylvestris*, *Senecio erucifolius*, *Phleum pratense*, *Elymus repens*, *Festuca arundinacea*.
2. The second, middle layer, 35-110 cm in height, consists of *Geranium pratense*, *Serratula tinctoria*, *Lathyrus pratensis*, *Ranunculus acris*, *Carex riparia*, *Equisetum telmateia*, *Lythrum salicaria*, *Poa pratense*, *Symphytum officinale*, *Carex hirta*, *Bromus arvensis*, *Achillea pannonica*, *Vicia tenuifolia*, *Galium aparine*, *Erigeron annuus*.

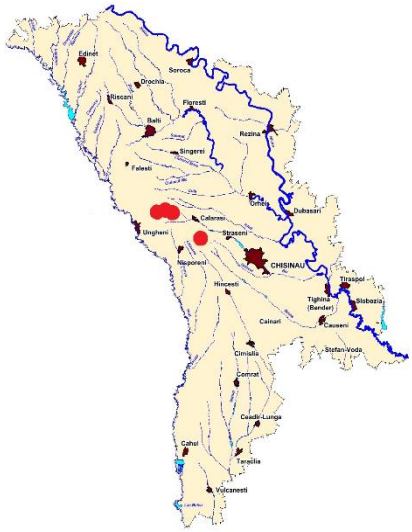

Figure 5. Locations of the ass. *Geranio pratensi - Cirsietum cani* in the Republic of Moldova

3. The third, lower layer, which reaches up to 30 cm in height, is represented by *Taraxacum camylodes*, *Ranunculus repens* L., *Potentilla reptans*, *Lysimachia nummularia*, *Glechoma hederacea*, *Veronica chamaedrys*, *Medicago lupulina*.

Range. The plant communities of *Cirsium canum* with *Geranium pratense* occur on the Central Moldavian Plateau in the districts: Ungheni (Pojarna, Cornești), Călărași (Sipoteni), Strășeni (Lozova) (Figure 5).

Territorial protection. The phytocoenoses of this association are protected in "Codru" Scientific Reserve.

Conservation value. The given association includes phytocenoses of great value and should be protected in all the identified locations.

Conclusions

The association *Geranio pratensi-Cirsietum cani* Pînzaru, Ioniţa et Jardan ass. nova includes phytocoenoses of tall herbaceous plants, meso-hygrophilic, which occur on slightly alkaline, stratified, alluvial soils, which are moist and rich in nutrients.

The association *Geranio pratensi-Cirsietum cani* Pînzaru, Ioniţa et Jardan ass. nova is included in the alliance *Filipendulion ulmariae* Segal ex Westhoff et Den Held 1969, ord. *Molinietalia caeruleae* Koch 1926, cl. MOLINIO-ARRHENATHERETEA Tx. 1937.

The phytocoenoses of the given association are particularly interesting from a botanical point of view. They include some very rare species in R. of Moldova, such as: *Anacamptis palustris* (Jacq.) R.M.Boteman, Pridegon & M. W. Chax, *Dactylorhiza incarnata* (L.) Soó, *Ophioglossum vulgatum* L., *Thelypteris confluens* (Thunb.) C. V. Morton, *Senecio sarracenicus* L., *Silene flos-cuculi* (L.) Clairv., and the species *Galium rivale* (Sibth. & Sm.) Giseb. and *Ranunculus binatus* Kit. ex Rchb. have been found only in these phytocoenoses.

We suggest to include the as. *Geranio pratensi-Cirisetum cani* in the List of Rare plant communities of the Republic of Moldova, with a high conservation status, and to include the sites near the communes Sipoteni (d. Călăraşi) and Cornești (d. Ungheni) in the network of protected areas of the Republic of Moldova.

Table 1. Ass. Geranio pratensi-Cirsietum cani ass. nov.

Relevé no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	*22	23	24	25	26	K
Altitude (m)	150	150	150	150	150	150	150	150	135	135	135	135	135	140	140	140	140	140	140	140	140	140	140	140	140	140	
General coverage (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
Surface of relevé (m²)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
Number of species	21	24	34	32	47	52	38	25	27	47	27	23	25	33	25	28	27	43	40	37	32	27	37	34	29	33	
Characteristic species																											
Cirsium canum	3	3	1	2	3	2	4	4	2	3	1	3	2	3	3	2	2	2	1	1	2	3	3	2	4	4	V
Geranium pratense	2	2	1	1	2	3	3	2	2	2	3	2	1	2	+	+	1	2	2	2	2	3	2	2	3	3	V
Inula helenium	-	-	+	-	1	2	+	+	3	+	2	+	-	+	+	-	3	3	2	4	3	1	1	+	-	+	IV
Filipendulion																											
Valeriana officinalis	-	-	+	+	+	+	-	-	1	+	+	+	-	-	+	-	+	1	+	1	+	+	+	+	+	+	IV
Veronica longifolia	1	1	+	1	+	+	1	+	2	2	2	2	-	+	-	-	-	-	-	-	-	-	-	-	-	-	III
Thalictrum lucidum	-	-	+	+	+	+	1	+	-	+	+	+	-	+	-	-	-	+	-	+	-	+	-	-	-	1	III
Filipendula ulmaria	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	+	2	-	-	+	1	+	-	-	II
Lysimachia vulgaris	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	+	+	-	-	II
Lythrum salicaria	-	-	-	-	-	+	+	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	II
Equisetum telmateia	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	1	3	3	2	2	-	2	1	-	-	II
Eupatorium cannabinum	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	1	+	+	-	-	-	+	+	-	-	II
Mentha longifolia	-	-	-	-	-	1	-	-	+	-	-	-	+	-	+	+	+	1	+	-	+	+	1	-	1	1	II
Epilobium hirsutum	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Calystegia sepium	-	-	2	-	-	-	+	-	-	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Stachys palustris	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	I
Silene baccifera	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	I
Euphorbia lucida	-	-	-	-	-	+	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Epilobium tetragonum	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Elymus caninus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	I
Deschampsion caespitosae																											
Glechoma hederacea	2	2	-	1	-	-	-	-	-	2	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	-	II

Phleum pratense	-	-	1	-	1	-	-	-	1	1	2	-	1	-	-	-	-	-	-	2	1	-	-	1	1	-	II
Festuca arundinacea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	2	1	1	-	+	1	+	II
Alopecurus pratensis	-	-	-	-	-	-	-	-	1	-	-	1	1	-	-	-	-	-	-	-	1	+	-	-	-	-	1
Scutellarria hastifolia	-	-	-	1	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Lythrum virgatum	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	I
Agrostis stolonifera	-	-	-	-	-	-	-	2	-	-	2	-	1	-	-	-	-	-	-	+	-	-	-	1	-	-	I
Festuca pratensis	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	2	-	-	-	-	-	-	-	-	-	-	I
Molinion																											
Stachys officinalis	-	-	-	+	1	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	-	II
Serratula tinctoria	+	-	-	2	-	-	-	-	-	1	1	1	-	-	-	-	-	+	1	-	+	-	-	-	-	-	II
Anacamptis palustris	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Dactylorhiza incarnata	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	-	-	-	-	-	-	r	-	-	r	I
Ophioglossum vulgatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	I
Molinietalia																											
Symphytum officinale	+	+	1	+	+	1	1	1	+	1	-	-	-	1	+	-	-	+	+	-	+	+	+	+	+	+	IV
Ranunculus repens	1	-	2	1	-	-	-	2	3	2	-	-	-	-	2	-	-	1	2	2	-	-	2	-	-	2	III
Angelica sylvestris	-	-	-	-	+	+	+	-	1	+	+	+	-	-	+	-	+	+	+	1	+	+	-	-	-	+	III
Silene flos-cuculi	-	+	3	2	-	-	-	-	-	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	I
Equisetum palustre	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	2	2	I
Juncus articulatus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	I
Potentillo-Polygonetalia																											
Potentilla reptans	1	2	2	1	2	2	2	2	2	-	-	-	2	2	-	-	-	2	-	-	-	2	2	-	2	2	IV
Elymus repens	-	-	-	-	-	2	2	2	-	2	2	-	2	-	-	2	2	-	2	1	3	1	2	-	-	-	III
Althaea officinalis	-	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-	-	r	-	-	-	-	-	-	+	+	II
Carex hirta	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	-	2	3	2	2	2	II
Rumex crispus	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	+	+	+	+	+	+	-	+	-	-	-	II
Dipsacus laciniatus	-	-	+	-	+	+	+	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	II
Potentilla anserina	-	-	-	-	-	1	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	1	-	2	2	I
Rorippa sylvestris	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	I

Mentha pulegium	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	I
Galega officinalis	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	I
Rumex conglomeratus	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	I
Senecio erucifolius	-	-	-	-	-	-	-	-	-	-	-	-	r	-	-	-	+	-	-	-	-	-	-	-	+	-	I
Trifolium elegans	-	-	-	-	2	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Juncus inflexus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	1	1	-	1	I
Cerastium dubium	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Arrhenatheretalia																											
Taraxacum campylodes	1	1	1	2	-	+	1	-	2	1	-	-	2	1	3	2	-	1	1	1	-	1	2	+	-	+	IV
Peucedanum carvifolia	+	+	+	1	-	-	+	+	1	+	-	-	2	1	1	+	-	+	-	-	-	+	-	-	-	-	III
Heracleum sibiricum	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	-	-	-	III
Dactylis glomerata	-	-	-	-	-	1	-	-	-	1	-	-	-	1	-	-	1	+	-	1	1	+	+	1	-	-	II
Veronica chamaedrys	-	-	+	+	-	-	-	-	-	-	-	-	-	1	1	1	-	-	+	+	-	1	-	-	-	-	II
Senecio jacobaea	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	II
Galium mollugo	1	-	-	-	1	-	1	-	-	+	-	-	-	-	-	1	-	-	-	-	-	1	-	-	-	-	II
Carex polyphylla	-	-	1	-	-	2	-	-	-	-	-	-	-	1	-	+	-	-	+	-	-	-	-	-	1	+	II
Rumex obtusifolius	-	+	+	+	-	1	+	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	II
Carex tomentosa	-	-	2	-	-	2	2	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	2	2	-	-	II
Lotus corniculatus	-	-	-	-	+	-	1	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Vicia sepium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-	I
Leontodon hispidus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	I
Equisetum arvense	-	-	-	-	-	-	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Medicago lupulina	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	1	-	-	-	+	-	+	-	-	-	-	I
Ononis spinosa	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	I
Pastinaca sativa var. sylvestris	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	-	-	I
Leucanthemum vulgare	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Rhinanthus alectorolophus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	I
Molinio-Arrhenatheretea																											
Lathyrus pratensis	1	2	-	1	2	2	2	2	1	2	-	-	-	2	2	-	2	2	1	1	2	2	-	2	-	2	IV

Achillea pannonica	1	1	-	-	1	-	1	-	-	-	1	+	-	1	1	1	1	1	1	1	1	1	1	+	1	1	IV
Ranunculus acris	+	2	-	2	-	1	2	3	1	-	1	-	1	3	1	1	2	-	+	-	-	-	-	-	-	-	III
Lysimachia nummularia	-	3	2	-	3	-	-	-	-	3	-	-	-	-	-	2	-	2	2	2	2	2	2	2	-	2	III
Centaurea jacea	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	1	1	1	-	1	1	-	+	-	+	III
Poa pratensis	-	-	2	2		2	-	-	-	-	-	-	-	2	-	-	-	-	2	-	-	-	2	-	-	-	II
Allium oleraceum	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	+	-	+	II
Prunella vulgaris	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	I
Trifolium pratense	-	-	-	-	-	-	2	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	1	1	-	-	I
Cerastium holosteoides	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	I
Myosotis arvensis	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	I
Trifolium repens	1	-	-	-	-	-	-	-	-	-	-	-	-	-	2	3	-	-	-	-	-	-	-	-	2	1	I
Ranunculus binatus	1	2	-	2	-	-	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	I
Ranunculus stevenii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	I
Phragmitetea																											
Iris pseudacorus	+	+	+	+	+	2	1	1	1	1	1	+	-	1	-	-	-	-	-	-	-	-	-	-	-	-	III
Carex riparia	-	-	2	1	2	2	-	2	2	+	2	2	-	-	-	-	3	2	2	2	-	-	-	-	1	2	III
Phalaris arundinacea	-	-	-	2	1	2	1	1	1	1	-	2	-	-	-	-	-	1	2	1	-	1	-	1	1	+	III
Sium sisarum	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	1	III
Phragmites australis	-	-	-	1	-	-	-	-	-	+	-	-	-	+	-	-	-	1	-	-	-	-	2	-	-	-	I
Thelypteris confluens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	I
Lycopus exaltatus	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Senecio saracenicus	-	-	-	-	-	r	-	-	r	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Glyceria arundinacea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	I
Carduus crispus	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	I
Variae syntaxa																											
Erigeron annuus	-	-	+	-	-	+	+	-	+	-	+	+	-	-	+	-	-	+	+	+	-	-	-	+	+	-	III
Galium verum	-	1	-	+	1	+	-	+	1	+	+	-	+	-	-	+	-	+	-	-	-	-	-	1	-	-	III
Galium aparine	1	-	2	1	-	-	-	-	-	-	-	-	-	1	-	1	2	2	2	2	2	-	1	2	-	-	III
Daucus carota	-	-	+		+	-	+	+	+	+	-	-	+	-	-	-	-	+	-	-	+	+	-	-	+	-	II

Arctium tomentosum	-	-	-	+	+	+	+	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	II
Veronica arvensis	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	II
Lactuca serriola	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	II
Agrimonia eupatoria	-	-	-	-	+	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	II
Dipsacus fullonum	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Chaerophyllum aromaticum	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	I
Chaerophyllum bulbosum	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	I
Torilis japonica	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	I
Ambrosia artemisiifolia	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	I
Inula germanica	-	-	-	-	2	-	+	1	-	+	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Xanthium strumarium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	I
Tripleurospermum inodorum	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	I
Senecio vernalis	-	-	-	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Cirsium vulgare	-	-	-	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Cirsium arvense	-	-	-	-	1	1	1	-	-	+	-	-	-	1	-	-	+	-	-	-	-	-	-	-	-	-	I
Picris hieracioides	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Tragopogon dubius	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	r	-	-	-	-	+	-	-	-	I
Cichorium intybus	-	-	-	-	-	-	+	-	-	1	-	-	-	-	-	-	-	r	r	-	-	-	-	-	-	-	I
Lactuca saligna	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Thlaspi perfoliatum	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	I
Capsella bursa-pastoris	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	I
Valerianella locusta	1	+	1	2	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	I
Humulus lupulus	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	I
Cerastium semidecandrum	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Convolvulus arvense	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Medicago sativa	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Melilotus albus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	I
Vicia sativa	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	I
Vicia hybrida	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	-	I

Vicia tetrasperma	-	-	-	-	-	-	-	-	-	1	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	I
Vicia tenuifolia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	I
Lathyrus tuberosus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	I
Lathyrus sylvestris	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	I
Clinopodium vulgare	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	I
Lamium purpureum	1	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	I
Lamium amplexicaule	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Epilobium lamyi	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Plantago major	-	-	1	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	I
Veronica hederifolia	-	-	-	-	-	-	-	-	-	-	-	-	+	1	-	1	-	-	-	-	-	-	-	-	-	-	I
Veronica polita	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	I
Verbascum blattaria	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Linaria vulgaris	-	-	-	-	1	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Ficaria verna	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	I
Rosa canina	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	I
Rubus caesius	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Filipendula vulgaris	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I
Galium humifusum	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	I
Cruciata pedemontana	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	I
Galium rivale	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	I
Urtica dioica	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	I
Epipactis helleborine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	r	-	-	I
Carex praecox	-	2	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	I
Aristolochia clematitis	-	-	-	-	1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Campanula trachelium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	r	-	-	-	-	-	-	-	I
Bromus arvensis	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	I

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DEAD WOOD, FOREST FRAGMENTATION AND ELEVATION INFLUENCES MACROFUNGAL DIVERSITY ON DOWNED COARSE WOODY DEBRIS IN BEECH AND OAK OLD FOREST ECOSYSTEMS FROM NORTHEASTERN ROMANIA

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Abstract: Coarse woody debris is often highlighted as the most important microhabitat for numerous saproxylic species, including macrofungi. Providing valuable nutrients, stable microclimatic conditions and development space, logs and large branches are considered of great ecological value for macrofungal diversity conservation. Old forests are especially rich in downed coarse dead wood both at quantity and quality level. Unfortunately, these forests are also affected by human interventions, through wood extraction and forest fragmentation. The main objective of this study was to find the factors that best explain the macrofungal diversity on downed coarse woody debris (DCWD). For this, we sampled 21 plots in forests dominated by beech or oak from Northeastern Romania, where we collected data about fungi, forest structure, and dead wood. We completed the variables set with forest fragmentation and topographic indices. In order to find the best models and predictors, we used generalized linear models (GLM). We found 163 taxa, polypores and agarics being the most frequent. The two most important predictors had a positive effect, increasing macrofungal diversity: 2'nd and 3'rd decay stages DCWD volume and elevation while the third one had a hump-shape effect on diversity. In old forests, downed dead wood quality and quantity is a vital component for numerous species of fungi to survive and develop. Elevation is a known proxy of macroclimatic conditions, furthermore creating new rich-resources niches because increasing humidity and taxonomic diversification by conifers occurrence. Patch shape can have divergent effects on fungi, as increasing perimeter is associated from one point on, with human deforestation and accessibility. Overall, we believe that Northeastern Romania's old forests hosts a great lignicolous macrofungal richness, which will be protected through silvicultural practices such as keeping valuable dead wood on site.

Keywords: broadleaved dominated forest, forest structure, lignicolous fungal richness, logs and large branches, shape index, topography.

Introduction

Coarse woody debris (CWD) is one of the most important types of microhabitats found in forest ecosystems [HEILMANN-CLAUSEN & CHRISTENSEN, 2003]. Numerous groups of organisms depend on food and/or shelter provided by this type of wood, including lichens, bryophytes, insects and fungi [ABREGO & SALCEDO, 2011; GOIA & GAFTA, 2018; HEILMANN-CLAUSEN & CHRISTENSEN, 2003].

The most important forest types characterized by high dead wood volume and high-decay wood volume are the old forests [MORRISSEY & al. 2014]. Numerous studies carried out in these forests across the Northern hemisphere highlighted the importance of those habitats for lignicolous fungal high diversity conservation [RUOKOLAINEN & al. 2018;

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RUNNEL & LÕHMUS, 2017; SAAR & al. 2007]. Furthermore, this importance was sustained by studies comparing natural/old-growth forests with managed ones, silvicultural practices usually changing lignicolous fungal composition and reducing diversity [ABREGO & al. 2014; JUUTILAINEN & al. 2014; KEBLI & al. 2012]. Unfortunately, old forests take a long time to develop, period during which human intervention should be restricted and driven by special conservative measures focused on retaining large dead wood (including both DCWD and dead trees) and large living trees [SIITONEN & al. 2000].

Dead wood characteristics were studied in lignicolous fungal diversity or composition-related researches in forest habitats, either at plot-level: volume, dead wood species diversity [RUNNEL & LÕHMUS, 2017] or substrate-level: species, diameter, decay class, bark cover, moss cover, age, complexity, microclimatic conditions [HEILMANN-CLAUSEN, 2001; HEILMANN-CLAUSEN & CHRISTENSEN, 2003; HEILMANN-CLAUSEN & al. 2005; HEILMANN-CLAUSEN & al. 2014; ABREGO & al. 2017; RUOKOLAINEN & al. 2018]. Different forest characteristics have been studied in relation to fungal diversity and composition of lignicolous fungi in Europe: tree richness, relative volume of dominant, codominant or non-dominant trees [KUTSEGI & al. 2015]; or structure: tree density, large tree density, tree basal area, mean tree DBH, tree DBH coefficient of variation, snags volume, cover of understorey vegetation [KUTSEGI & al. 2015]. Also, forest fragmentation was included in lignicolous fungal-related studies, using characteristics such as forest area, broadleaved or coniferous forest area [RUNNEL & LÕHMUS, 2017], forest connectivity at different spatial scales, reserve size [ABREGO & al. 2015].

This study tries to respond to the following questions: i) does forest structure influence lignicolous fungal diversity? ii) is the forest fragmentation important in beech/oak old forests from Northeastern Romania for wood-inhabiting fungi? iii) does dead wood high quality and quantity increases the number of lignicolous fungal species?

Material and methods

Study area

The study was conducted in beech- and oak-dominated forests from Northeastern Romania (Figure 1). These forests are situated in the Moldavian Plateau, Sub-Carpathians Hills and the Eastern part of Eastern Carpathians. The main soils are cambisoils and luvisoils. The climate is temperate continental, with an increasing continentality towards the eastern-most located studied forests. The studied forests are dominated either by *Fagus sylvatica* L. (European beech) or species of *Quercus* (oak), ones of the most important tree genera in Europe [PETRITAN & al. 2012] and Romania [MILESCU & al. 1967]. Region's forests are prone to deforestation and illegal logging [ANDRONACHE & al. 2017]. Also, even if there measures for old-growth forests protection were taken, logging, invasive species, and climate change are still important threats [KNORN & al. 2012].

Datasets

In beech and oak forests we chose circular plots of 1,000 m for forest and dead wood data inventory. The plots were chosen using the 50% threshold in terms of tree basal area of beech/oak from the total basal area. Each plot had at least one big branch or log (diameter at larger end > 10 cm). Within each plot, all living and dead trees with a diameter at breast height (DBH) > 10 cm were measured for diameter. Old or large-sized trees were considered those trees with DBH > 50 cm [LOMBARDI & al. 2012]. For old-forests delineation, we

chose only plots in which old trees basal area proportion was at least 50% of the total tree basal area.

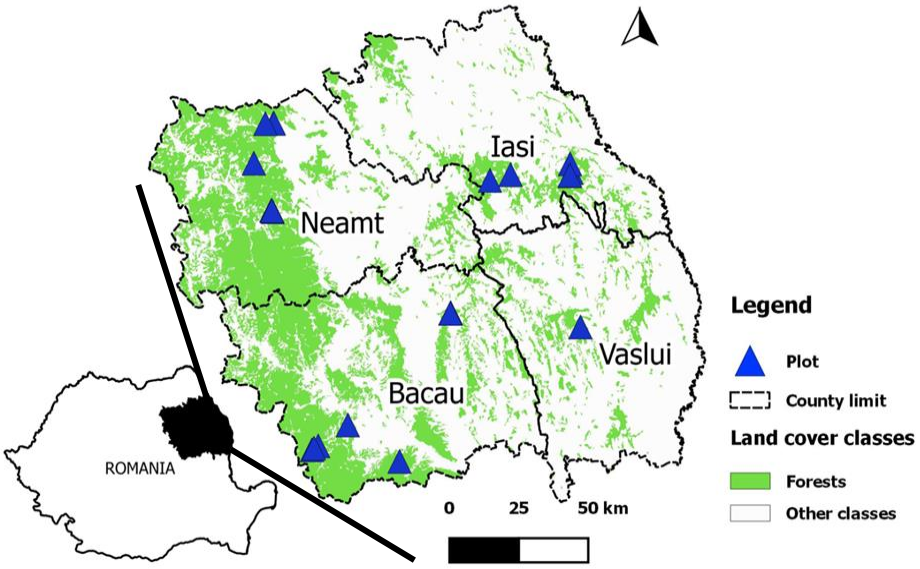

Figure 1. Plots location in Northeastern Romania

In each plot, we measured all logs and large branches with the diameter at large end > 10 cm, the diameter of both ends, length and decay stage using 3 classes. The decay classes are an adaptation of the classification proposed by HEILMANN-CLAUSEN & CHRISTENSEN (2003): (i) incipient stage – intact bark, twigs presence, hard texture, original color of wood, the knife penetrates less than 1 cm into the wood; (ii) intermediary stage – absent bark, absent twigs, hard and soft texture, changed wood color, the knife penetrates up to 6 cm into the wood; (iii) advanced stage: absent bark, absent twigs, completely change wood color, wood crashes in hand when humid, the knife penetrates fully into the wood.

For each plot, forest structure-specific variables were assessed (Table 1): tree richness, tree density, young tree density, tree basal area, tree diameter coefficient of variation, snags basal area, snags density, beech basal area proportion, and oak basal area proportion.

Dead wood characteristics were also calculated (Table 1): DCWD volume, DCWD taxonomic diversity, DCWD decay diversity, DCWD volume in middle and late decay stages. DCWD volume was calculated using frustum of cone formula. DCWD decay and taxonomic diversity were calculated following RUNNEL & LÕHMUS (2017).

Forest fragmentation was based on Corine Land Cover 2018 raster with 100 m resolution (https://land.copernicus.eu). The classes were reclassified such that CLC forest classes were grouped in 'forest class' and the others in 'other classes'. In circular areas of 2,000

m radius having in the center the forest plot, we calculated indices at class level: patch area mean index and edge density index, or at patch level: shape index.

Main topographic variables were obtained for each plot: slope, positive openness, aspect index, and elevation. The derived topographic indices were calculated based on SRTM at 30 m resolution, downloaded from the official database of United States Geological Survey's EarthExplorer [www.earthexplorer.usgs.gov].

Table 1. Variables considered in the analysis

Abbreviation	Name	Range	Mean	Units	GLM
Forest structure					
TREE_RICH	tree richness	1 – 7	4	None	1
TREE_N	tree density	160 - 410	285	trees ha^{-1}	0
TREE_U50_N	young tree density	50 – 320	190	trees ha^{-1}	1
TREE_DBH_CV	tree diameter coefficient of variation	0.4 – 1.9	0.9	None	1
TREE_BA	tree basal area	22.7 – 68.2	50.5	m^2	1
TREE_BA_MN	mean tree basal area	0.1 – 0.3	0.2	m^2	0
SNAG_BA	snags basal area	0 – 14.2	0.6	m^2 ha^{-1}	1
SNAG_N	snag density	0 – 60	10	snags ha^{-1}	1
BEECH	beech trees basal area proportion	0 – 100	51.7	%	0
OAK	oak trees basal area proportion	0 – 82.1	7.2	%	0
Downed dead wood					
DCWD_DIV	DCWD taxonomic diversity	0 – 0.9	0.2	None	1
DCWD_DECAY	DCWD decay diversity	0 – 0.9	0.6	None	1
DCWD_VOL	DCWD volume	0.1 – 148.8	2.8	m^3	1
DCWD_VOL23	DCWD volume in 2'nd and 3'rd decay stages	0 – 68.1	1.1	m^3	1
Forest fragmentation					
AREA_MN	mean of patch area index	54.6 – 1600.0	388.7	ha	1
SHAPE	shape index	5.9 – 33.4	15.6	None	1
ED	edge density index	0 – 23.3	13.5	m ha^{-1}	0
Topography					
ALT	elevation	186 – 706	450	m	1
SLOPE	slope	0.7 – 30.4	7.7	°	0
ASPI	aspect index	6.7 – 156.0	56.3	°	1
PO	positive openness index	1.2 – 1.5	1.4	None	1

In a concentric plot of 2,000 m^2, each macrofungal species found on downed coarse woody debris was identified at species or genera level. Those which couldn't be determined *in situ* were identified in the laboratory using mycological literature [BERNICCHIA, 2005; BREITENBACH & KRÄNZLIN, 1986; COURTECUISSE & DUHEM, 2013; GERHARDT, 1999; RYVARDEN, 1991; SĂLĂGEANU & SĂLĂGEANU, 1985; TĂNASE & al. 2009]. Index Fungorum [http://www.indexfungorum.org/Names/Names.asp] was used for fungal nomenclature.

Data and analysis

We used R software version 3.5.1 (R Core Team 2012) in data exploration and statistical analysis. The R packages used were: landscapemetrics, MuMIn, MASS, reshape, rsq. Packages ggplot2 and RColorBrewer were used for graphical representation of relationships. For topographical and CLC processing, we used QGIS [QGIS Development Team (2018). QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org] and SAGA GIS software [CONRAD & al. 2015].

We tested the 20 variables for collinearity with Pearson correlation and removed the variables from collinear pairs so that only one remained using the correlation coefficient threshold of 0.7. Using 15 non-correlated variables, we made GLM (Generalised Linear Models) with Poisson probability distribution and tested them for overdispersion. The resulted models were selected based on AIKc (Akaike Information Criterion corrected) value, using the method proposed by BURHNHAM & ANDERSON (2002). These models were used to select the most important variables that explained the lignicolous macrofungal diversity.

Results and discussions

Taxonomic diversity

We found 163 species in approximately 500 records, on 249 logs and large branches. The diversity was higher than other studies made in similar habitats – 44 [BÎRSAN & al. 2014], 72 [ŽUPANIC & al. 2009]. In a study made in beech forests in the same region, the authors [COPOȚ & al. 2018] found 110 species, but they only gathered data from fine woody debris (FWD), with diameter less than 10 cm. In similar studies, like those from Danish deciduous forests, the authors [HEILMANN-CLAUSEN, 2001; HEILMANN-CLAUSEN & CHRISTENSEN, 2003] found a higher number of species on beech logs. Still, the logs had more than 70 cm in diameter and consequently, the higher richness could be explained, besides other factors, by the log diameter, as it is known that diameter and associated-big log characteristics (e.g. better microclimate for mycelial development) is highly associated with lignicolous fungal diversity [HEILMANN-CLAUSEN & CHRISTENSEN, 2003].

The species belong to 113 genera, of which *Mycena* and *Pluteus* were the richest. The majority of the species (159) belongs to 58 families (4 species were included in *Incertae Sedis*), from which the most important in diversity were: Polyporaceae and Mycenaceae, each of them with 15 taxa. 162 species belong to 22 orders and one to *Incertae Sedis*, from which Agaricales (36.2% of total), Polyporales (19.0%), Xylariales (8.5%) and Hymenochaetales (7.3%) where the richest. Basidiomycota phylum comprises 79.1% of total diversity, while Ascomycota, 20.9%.

Half of the species (88) were identified in a single plot and another 24 in two plots. The most frequent found taxa in terms of plots presences were: (a) saproparasites: *Fomes fomentarius*, *Fomitopsis pinicola*; (b) saprotrophes of first decay stages: *Jackrogersella*

cohaerens, *Schizophyllum commune*, *Stereum hirsutum*, *Trametes versicolor*; or (c) middle and late decay stages: *Chlorociboria* sp., *Mollisia* sp., *Orbilia* sp., *Scutellinia scutellata*, *Pluteus cervinus*. Those species were also frequently found growing on logs, especially beech [ABREGO & SALCEDO, 2011; HEILMANN-CLAUSEN, 2001], but can also grow on other tree genera [BREITENBACH & KRÄNZLIN, 1986; TĂNASE & al. 2009]. Among the most frequently found species on fresh dead wood, *F. fomentarius* is a common sight on stem/large branches, of living, dying or fallen trees [BAUM & al. 2003], as it is one of the main decay fungi of beech [SCHWARZE, 1994].

Models that explain macrofungal diversity on DCWD

Following model selection, we obtained two models that best explain the macrofungal diversity on downed coarse woody debris in beech or oak-dominated forests (Table 2).

Table 2. Best explaining models of macrofungal diversity found on downed coarse woody debris in beech or oak-dominated forests

Intercept	ALT	DCWD_VOL	DCWD_VOL23	SHAPE	R^2	R^2 adj.	AICc	delta	weight
1.784	0.003	-	0.015	-0.031	0.99	0.99	140.04	0	0.67
1.775	0.003	0.003	0.012	-0.036	0.99	0.99	141.48	1.44	0.33

The variables found important were: SHAPE (Shape Index), DCWD_VOL_D23 (DCWD volume in 2'nd and 3'rd decay stages) and ALT (elevation), (Table 3). Together, they explained 76% of the fungal richness variation.

Table 3. Linear models highlighting effect of the most important variables on macrofungal richness

Variable	Estimate	Standard Error	Explains (%)	p-value (< 0.05)	Sign	Importance
Elevation	0.032	0.014	17	< 0.05	↑	0.90
2'nd and 3'rd decay DCWD volume	0.484	0.121	42	< 0.001	↑	0.63
Shape Index	5.713 -0.137	1.227 0.028	50	< 0.0005 < 0.0005	∩	0.62

Predictors that influence macrofungal diversity on DCWD

Elevation

In this study, as the elevation is increasing, the macrofungal richness is higher (Figure 2.A). The elevation is considered as a proxy for macroclimatic conditions, especially mean annual precipitations and mean annual temperatures [VAN GILS & al. 2012]. As a consequence, it is possible that the high richness associated with higher altitudes is in fact an effect of precipitations and temperature regimes. Indeed, the plots associated with higher diversity are found in mountainous forests, at elevations above 600 m. Because of the mountain encampment, higher precipitations and lower temperatures create more humid conditions in the forests, which enhance and keep for long periods of time, the deadwood humidity. This can be a decisive factor, especially in carpophore-based studies, when fungi produce a large amount of carpophores during rich-pluvial seasons [BRAZEE & al. 2014; RUDOLPH & al. 2018]. Linked to the next found predictor is the fact that in temperate forests, the regions with colder summers (like mountains) are associated with higher dead

wood quantities [WOODALL & LIKNES, 2008]. Thus, the higher elevation plots can host a higher lignicolous macrofungal richness due to higher dead wood volumes.

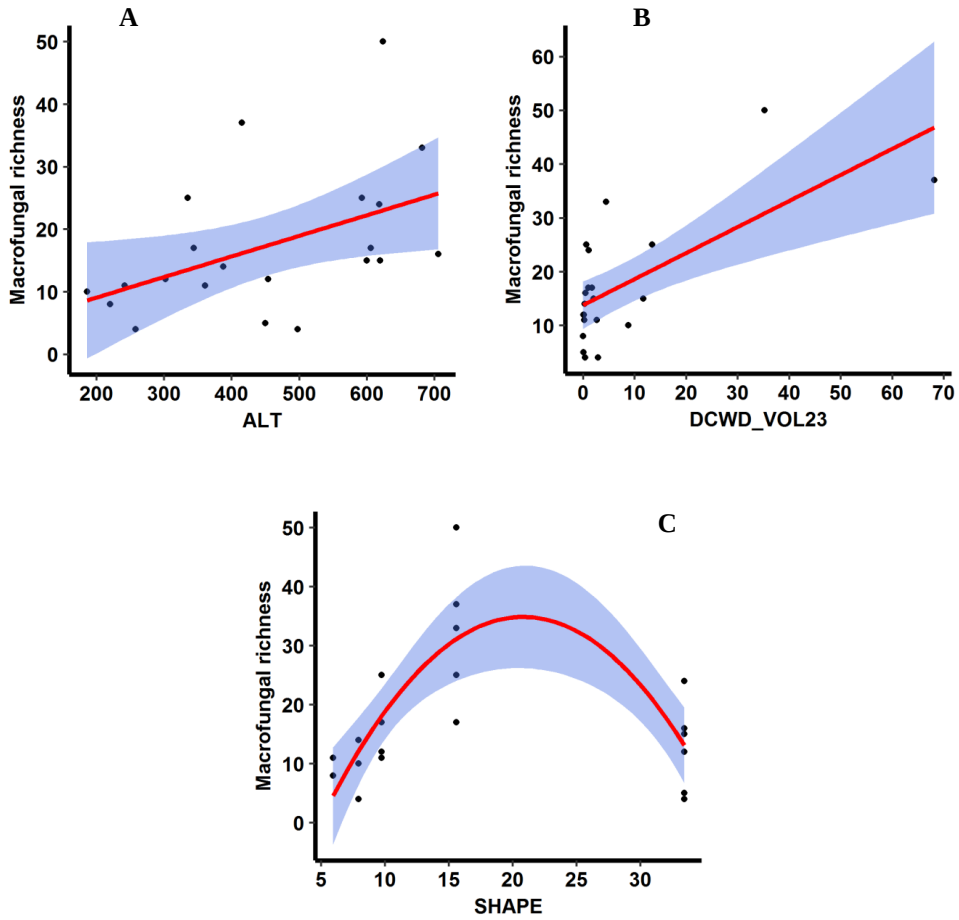

Figure 2. Relationship between macrofungal diversity and the most important variables: A. elevation (m); B. 2'nd and 3'rd decay stage of downed coarse woody debris (m^3); C. Shape Index

Another effect of elevation on forest plots composition differentiation is the occurrence of conifers in Romanian forests. Thus, at higher altitudes, the presence of coniferous dead wood creates important ecological niches which do not exist in forests dominated by pedunculate oaks (*Quercus robur*), like in Moldavian Plateau. Those niches are colonized both by some broadleaved-associated fungi and especially by coniferous-specific fungi, thus increasing total diversity on DCWD.

Middle and late stage decayed DCWD volume

In our study, the DCWD volume in the middle and last decay stages was positively and significantly influencing the macrofungal diversity (Figure 2.B). This variable was calculated taking into account two assumptions: (i) one of the main old-growth forests characteristics is the presence of dead wood in large volumes [BRUNET & al. 2010; DVOŘÁK & al. 2017]; the main reason behind this is represented by the protective status of those forests, as silvicultural measures tend to keep dead trees on-site [ÇOLAK & al. 2010] – the source of downed dead wood [DOMKE & al. 2013]; thus, the presence of large quantities of middle and late decayed wood is a measure of continuous forest protection; pro-conservative forest management proved to positively influence lignicolous fungal diversity in old-growth hardwood forests [BRAZEE & al. 2014]; (ii) middle and last decay stage of wood hosts a diverse variety of macrofungi, many of them decay-stage specialists [HEILMANN-CLAUSEN & CHRISTENSEN, 2003; HEILMANN-CLAUSEN & al. 2014].

Here, this volume alone explains approximately 40% of total macrofungal richness. The species-richest plots are found in Nemira mountains (Eastern Carpathians, Bacău county), in a beech-silver fir forest. This habitat is rich in downed deadwood of both beech and coniferous trees, thus completing the previous predictor explanation.

Shape Index

In our study, the macrofungal richness follows a hump-shaped relationship with the Shape Index (Table 2). Initially, as the index is increasing, so is the diversity, until it approaches a mid-value of approximately 20, after which the diversity is decreasing as the index is increasing (Figure 2.C). It is the variable that explains the most of macrofungal diversity, counting approximately 50% of the total variation.

According to authors [HESSELBARTH & al. 2019], the Shape Index is calculated as the ratio of patch perimeter and the minimum perimeter. Consequently, it takes values from 1 beyond, rising as the patch shape becomes more complex. Through reducing patch complexity, there are created rectangular patches, characterized by regularity and straight borders [MOSER & al. 2002].

In order for the macrofungal diversity-shape index complex relationship to be understood, patch shape values variation causes must be known. There are three main groups of factors that influence patch complexity: (i) historical human intervention; (ii) topography; (iii) forest composition [DORNER & al. 2002; SAURA & CARBALLAL, 2004]. Shape Index can be used as a direct measure of human interventions, complexity usually decreasing as human activity increased [SAURA & CARBALLAL, 2004]. On the other side, topographical factors (slope, aspect, terrain rugosity) where found to increase forest patch shape irregularity in Galicia (Spain) [SAURA & CARBALLAL, 2004]. Also, mountainous forest habitats are characterized by increasing elevation and steep slopes, which hinder human accessibility [SAURA & CARBALLAL, 2004], which in turn translates through less extracted dead wood and consequently more available wood resources to macrofungi. Thus, increasing shape complexity results in macrofungal diversity increasing, in the up-section of hump-shaped relationship (Figure 2). Irregular shapes and consequently higher Shape Index values can also result from increasing tree diversity [SAURA & CARBALLAL, 2004]. In our case, the oak-dominated forests from mountainous areas in Neamț County are characterized by higher tree diversity, even if they have high shape index values. In those plots, the macrofungal diversity is lower than in less-rich beech-fir forests from Nemira Mountains. This can explain the second down-section of hump-shaped relationship (Figure 2). Still, this phenomenon might raise a question because high tree diversity is often

associated with increasing niche variation, because of macrofungal association with particular genera, thus rising fungal diversity [BÎRSAN & al. 2014]. But forest stands with a higher number of species is less vulnerable to climatic hazards [JACTEL & al. 2017], represented in the Carpathians by strong winter winds and long snow periods [ANM, 2008]. Therefore, there are more chances for climatic hazards to manifest in mountain beech-dominated stands, represented here in the middle of the hump-shaped relationship. This will increase downed dead wood volume in beech-dominated stands, but less in oak-dominated ones, a situation which confirms in our study.

Conclusions

This is the first study in which diferent types of predictors explain the macrofungal richness found on dead wood in Northeastern Romania. Dead wood quality, elevation and forest fragmentation proved to be key factors in explaining the differences between the total number of fungi in broadleaved-dominated forests. It is the first study that shows the importance of patch shape to macrofungal diversity conservation. Also, dead wood-associated silvicultural practices proved to be of high importance in macrofungal preservation success. As a consequence, it is important to properly manage old forests, keeping high quality downed dead wood, especially in regular forest patches.

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NEW LOCALITIES AND HABITAT CONDITIONS FOR *CHOIROMYCES MEANDRIFORMIS* VITT. (ASCOMYCOTA, FUNGI) IN NORTHERN ROMANIA

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Abstract: Although *Choiromyces meandriformis* is considered as a wide-spread fungus species in forest habitats of Europe, still it is listed as rare, endangered and even critically threatened in some countries. In Romania the species is sporadically spread throughout the Carpathian Mountains and surrounding areas, in soils under both deciduous and coniferous species. In this study there are presented two new localities for *Choiromyces meandriformis* in northern part of Romania, where the species was identified in spruce communities. The specimens were macroscopically and microscopically characterized and soil analyses were performed. Also, a phytosociological investigation of the forest habitat was performed. Soil analysis highlighted the species preference for acidic soils, with a medium content of humus, total nitrogen, potassium and total phosphorus, and a reduced amount of organic matter. The forest community (*Hieracio transsivanici-Piceetum*) was characterized by a uniform and species-poor floristic composition.

Keywords: *Choiromyces meandriformis*, ecology, habitat, soil characteristics, hypogeous fungi.

Introduction

Choiromyces meandriformis is a hypogeous fungal species, wide-spread in Europe, from the UK in the western part of the continent to the extreme east of Russia, from Sweden in the north, to Spain, Italy and Greece to the south. Etymology of name derives from the Greek words *choiros* = pig + *mykes* = mushroom (porcine truffle) and Latin *maeander* = meander, sinuous river + *formis* [BRESSON, 1996]. The genus *Choiromyces* is characterized by hypogeal, sub-globular or irregular shaped sporocarps. Their gleba is solid and has visible veins. The asci are indehiscent, clavated, with 8 spores, and no reactivity to the Melzer reagent. The spores are usually globose, hyaline or yellow-brown and ornamented with thorns, pits, or alveolated cross-links. The species *Choiromyces meandriformis* is collected and consumed as a delicacy in Sweden [WEDEN & al. 2009]. Despite its economic importance, limited information on this fungal genus diversity and distribution is available worldwide. Based on the morphologic similarity, some species within the *Choiromyces* genus may be confused with species of the *Tuber* genus on the market. Thus, some species of *Choiromyces* (e.g. *C. meandriformis*) may be sold as *T. magnatum* Picco, a highly edible species of the *Tuber* genus on European markets for the uninitiated people [MORENO & al. 2012].

Choiromyces meandriformis occasionally occurs under duglas, pines and under *Tsuga* at low altitudes, being slightly widespread in North America (and common in Europe)

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[BEUG & al. 2014]. It is apparently a relative common species of truffle and appears to be widespread in the United Kingdom [HOBART, 2005]. In Italy, collected data over the last decade regarding the ecology and distribution of hypogean and semi-hypogean fungi allowed the assessment of their status, and species within *Choiromyces* sp. were considered rare [VENTURELLA & al. 2011]. Also, in Poland, it is a considered a rare species [HALAMA & PANEK, 2000], that produces sporocarps at the end of summer and in autumn and occur in mixed oak and beech forests. In France, it is also encountered during the summer season, being one of the largest hypogean mushrooms in the country, which often produces cracks in the soil, thus being easily identified [FOURRÉ, 2000]. In Spain, it has been identified in the Basque Country [CALONGE & al. 1977] and in Navarra at higher elevation (c.a. 1200 m a.s.l.) under *Picea abies* trees [SIERRA & al. 1991]. In Lithuania, the species was found in the vicinity of hardwoods (oaks, birch, alder and willow), in mixed and coniferous forests, from August to October [KUTORGA & KATARŽYTĖ, 2008]. Some localities (Izmir, Uşak, Bolu, and Samsun) have been reported from Turkey [TÜRKOĞLU & CASTELLANO, 2014]. In Bulgaria is included in the Red List of Fungi [GYOSHEVA & al. 2006] as endangered (EN) species. It is also included in the Red Book of Lithuania (2007) as endangered [KUTORGA & KATARŽYTĖ, 2008].

In Romania, *Choiromyces meandriformis* is encountered from July to September [SĂLĂGEANU & SĂLĂGEANU, 1985]. The species is widespread throughout the Carpathian Mountains and surrounding regions, and can be identified in soils under both deciduous and coniferous species. Comprehensive information on distribution of hypogeal fungi in Transylvania (and Harghita County in particular) was published in the work of PAP & al. (1983). Also, according to PAZMANY (1990) *Choiromyces meandriformis* is found in Alba County (Zlatna), Caras-Severin County (Anina, Băile-Herculane and Moldova Nouă), Cluj County (Cluj-Napoca and surrounding areas), Covasna County (Sânzieni and Ojdola), Harghita County (Gheorgheni, in forests dominated by fir, Borsec, Ditrău, Mihăileni, Topliţa, Rugăneşti, Cristuru-Secuiesc), Maramureş County (Ocna-Şugatag, in areas dominated by oak and Maramureş depression), Salaj County (Romanaşi). It was also identified in Sibiu County, in Tufa Mohului Forest [NEGREAN & DRĂGULESCU, 2005].

The main objective of this article was to present new localities where the species was identified and to characterize the habitat in respect to soil properties and forest characteristics.

Material and methods

The specimens were identified based on the analysis of macroscopic and microscopic characters, using literature: GERHARDT (1999), SĂLĂGEANU (1985), ASTIER (1998), COURTECUISSE & DUHEM (2013), TRAPPE & CASTELLANO (2007). The nomenclature followed Index Fungorum [http://www.indexfungorum.org/Names/Names.asp]. The soil analyses were performed at The Offices of Pedological and Agrochemical Studies (OSPA) Iaşi. The trees age was estimated according to the diameter and the forest documents. In order to characterize the floristic composition of habitats, phytosociological relevés have been performed according to the method of the Central European School for the study of vegetation [BRAUN-BLANQUET, 1964; BORZA & BOŞCAIU, 1965].

Results and discussions

In this study are presented two new localities for *Choiromyces meandriformis* from northern part of Romania (Suceava County), where the species was identified in spruce young forests (*Picea abies*), in the first half of July 2013 and 2014 (Figure 1). The first spruce community was situated near Capu Câmpului village (47°30'48.42''N, 25°57'18.73"E) at approximately 500 m a.s.l., on a terrain with an inclination of 3-5°, eastern aspect, and had trees cover of c.a. 90%. The second point was near Izvoarele Sucevei locality (47°43'53.52''N and 25°08'49.16"E), at 1050 m a.s.l., in a terrain of 7-10° inclination with northwestern aspect. The specimens of porcine truffle have been identified close/under to the spruce trees. The investigated truffle species has an important scientific and practically role because of their importance in mycorrhizal associations with different tree species. At the establishment of spruce plantation, seedlings were brought together with this porcine truffle. Later, after a certain number of years, it produced sporocarps which emerged to soil surface through the soil cracks under favorable conditions of development.

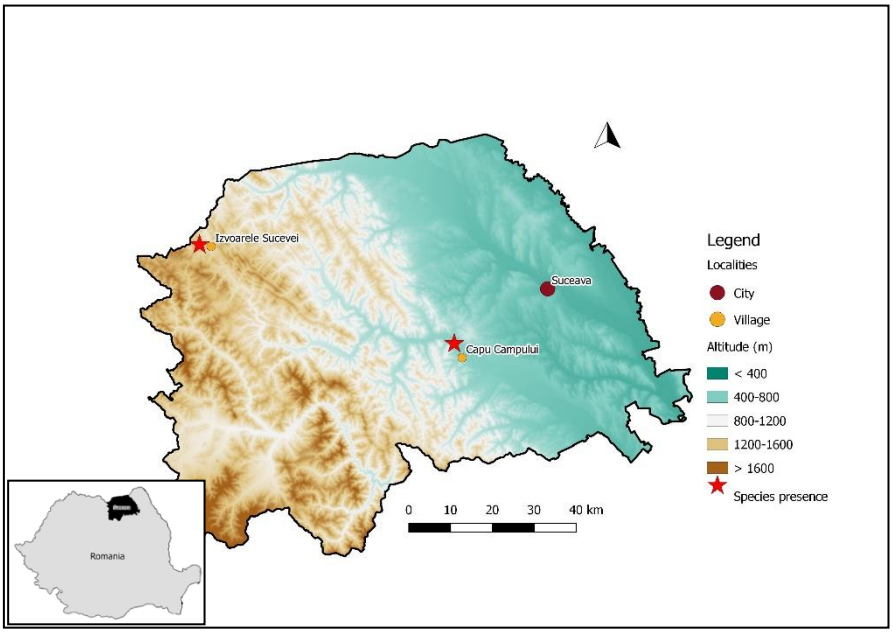

Figure 1. Map indicating the new localities for *Choiromyces meandriformis* in Northern region of Romania

The spruce forest from Capu Câmpului was about 40 years old, while the spruce forest at Izvoarele Sucevei area was approx. 50 years old.

The sporocarp was white at the beginning, then its colour became light yellow-brown, with a scented smell. Peridia was thin, smooth, often cracked. The gleba was fleshy, soft, white at first, and then marbled in yellow-brown by sinuous bands of pedunculated axes arranged in palisades (Figure 2). They were found in soil, under coniferous trees, in July-September period. In Bulgaria it was also found at the beginning of October, in soil, in habitats with *Picea abies* and *Abies alba* in the Rhodope Mountains [LACHEVA, 2012].

The favourable conditions for this species in Northern Romania were represented by the temperate climate, more humid during all seasons, soil characteristics and vegetation type (forest vegetation with *Picea abies* in different age stages). Soil analysis (Table 1) highlighted the species' preference for acidic soils (pH 5.06), with a medium content of humus, total nitrogen, potassium and total phosphorus, and a reduced amount of organic matter. These results in agreement (acid soil, wet site, spruce forest) with RODRIGEZ'S (2008) findings who identified the species (in Spain) also in acid and clay soils, in areas with increased rainfall, forming mycorrhizae with common oak (*Quercus robur* L.) and spruce (*Picea abies*).

Figure 2. Macroscopic aspect of analyzed specimens of *Choiromyces meandriformis* (Photo C. Bîrsan)

Table 1. Results of chemical analysis of soil samples collected in plots with *Choiromyces meandriformis*

	Soil characteristic	Value	U/M	Evaluation
1.	pH	5,06	-	Acid soil
2.	Humus	2,89	%	Middle
3.	Total Nitrogen	0,126	%	Middle
4.	Phosphorus	46,0	ppm	Good
5.	Accessible Potassium	83,0	ppm	Middle
6.	Total Phosphorus (P_2O_5)	0,205	%	Middle

7.	Total Potassium (K_2O)	0,187	%	Middle
8.	Organic Matter	5,70	%	Reduced
9.	Total Zinc	52,0	mg/kg	Max. val. 2000
10.	Total Copper	19,15	mg/kg	Max.val. 500
11.	Total Lead	26,0	mg/kg	Max.val. 300
12.	Total Iron	956,0	mg/kg	-

The forest habitat (Figure 3) was characterized by a uniform and species-poor floristic composition, typical to *Hieracio transsivanici-Piceetum* Pawl. et Br.-Bl. 1939 community (Table 2). Trees layer presented high cover (90%) and was dominated by *Picea abies* while *Sorbus aucuparia*, *Fagus sylvatica*, *Betula pendula*, *Acer pseudoplatanus* were sporadically identified. The shrubs layer (with *Corylus avellana* and *Spiraea chamaedryfolia*) had very low cover (3-5%). Herbs layer was also species-poor, and had low cover (2-10%) with *Luzula luzuloides*, *Salvia glutinosa*, *Geranium robertianum*, *Euphorbia amygdaloides*, *Veronica officinalis* etc. among the most frequent species.

Figure 3. General aspect of the habitat (young *Picea abies* forest) where *Choiromyces meandriformis* was identified (Photo C. Bîrsan)

Table 2. Phytosociological table of *Hieracio transsivanici-Piceetum* Pawl. et Br.-Bl. 1939 forest communities where the specimens of *Choiromyces meandriformis* were identified

No. of relevé	1	2	3	4	5	6	7	
Altitude (m a.s.l.)	1050	1040	1000	1050	1040	500	510	
Aspect	NW	NW	NW	NW	NW	E	E	
Slope (˚)	7	10	7	8	10	5	3	**K**
Trees cover (%)	90	90	85	90	90	95	90	
Shrubs cover (%)	5	3	3	5	3	5	3	
Herbs cover (%)	2	5	10	10	5	5	10	
Plot area (m^2)	500	500	500	500	500	1000	1000	
			Car. ass.					
Picea abies	5	5	5	5	5	5	5	V
Picea abies (juv.)	1	-	+	+	+	+	-	IV
Hieracium transsilvanicum	-	+	-	-	+	-	-	II
			Vaccinio-Piceetea					
Luzula luzuloides	+	+	-	+	+	-	-	IV
Oxalis acetosella	+	+	+	+	+	-	-	IV
Melampyrum sylvaticum	-	+	+	-	+	-	-	III
Luzula sylvatica	-	-	+	+	+	-	-	III
Calamagrostis arundinacea	-	+	+	-	-	-	-	II
Sorbus aucuparia	-	+	-	-	+	-	-	II
Sorbus aucuparia (juv.)	-	-	-	-	+	-	-	I
Juniperus communis	-	+	-	-	-	-	-	I
Campanula abietina	-	-	+	-	-	-	-	I
			Querco-Fagetea					
Fagus sylvatica	-	+	+	-	-	+	+	IV
Fagus sylvatica (juv.)	+	-	+	+	-	+	+	IV
Geranium robertianum	-	-	+	+	-	+	+	IV
Spiraea chamaedryfolia	+	-	+	+	-	+	-	IV
Daphne mezereum	-	+	+	-	+	+	-	IV
Euphorbia amygdaloides	-	+	-	-	+	+	+	IV
Pulmonaria rubra	-	+	+	-	-	-	+	III
Salvia glutinosa	-	-	-	+	-	+	+	III
Veronica urticifolia	-	-	+	+	+	-	-	III
Corylus avellana	-	+	-	-	-	+	+	III
Dryopteris filix-mas	-	-	+	+	+	-	-	III
Mycelis muralis	-	-	-	+	-	+	+	III
Poa nemoralis	-	-	-	-	-	+	+	II
Acer platanoides (juv.)	-	-	-	-	-	+	+	II
Epilobium montanum	-	-	-	+	-	-	-	I
Lonicera xylosteum	-	-	-	+	-	-	-	I
Brachypodium sylvaticum	-	-	-	-	-	-	+	I
Glechoma hirsuta	-	-	-	-	-	-	+	I
Carex sylvatica	-	-	-	-	-	+	-	I
			Epilobietea angustifolii					
Fragaria vesca	-	+	-	+	-	+	+	IV
Galeopsis speciosa	-	-	+	-	-	-	-	I
Betula pendula	-	-	+	-	-	-	-	I
Silene dioica	-	-	-	+	-	-	-	I
Tussilago farfara	-	-	-	-	-	-	+	I

Digitalis grandiflora	-	-	+	-	-	-	-	I
Variae syntaxa								
Veronica officinalis	+	-	-	-	+	+	+	IV
Urtica dioica	-	-	-	+	+	-	-	II
Acer pseudoplatanus	-	-	-	+	-	+	-	II
Geum urbanum	-	-	+	-	-	+	-	II
Tilia cordata (juv.)	-	-	-	-	+	-	-	I
Populus tremula (juv.)	+	-	-	-	-	-	-	I
Prunella vulgaris	-	-	-	-	-	-	+	I
Pulmonaria officinalis	-	-	-	-	-	-	+	I

Place and date of relevés: Izvoarele Sucevei: 10.07.2014 (rel. 1-5);
Capu Câmpului: 12.07.2013 (rel. 6-7).

In a phytosociological perspective, increased constancy presented the diagnostic species for *Piceion* alliance, *Piceetalia* order and *Vaccinio – Piceetea* class (*Melampyrum sylvaticum*, *Luzula luzuloides*, *Oxalis acetosella*, *Sorbus aucuparia* etc.). Also, the floristic composition included some diagnostic species for *Querco – Fagetea* (*Daphne mezereum*, *Corylus avellana*, *Dryopteris filix-mas* etc.) and *Epilobietea angustifolii* (*Galeopsis speciosa*, *Fragaria vesca* etc.) classes. Most of the plant species were sciophytes, preferring the cool mountain areas or temperate sub-mountain areas with moderate humid, acid and nutrient poor soils. Canopy closure in investigated areas was high, maintaining soil moisture by not directly exposing it to sunlight. Rainfall and soil moisture were very important in investigated habitats for development of porcine truffle sporocarps.

Conclusions

This study presented new localities and highlighted similarities with other findings regarding the ecological requirements of the species *Choiromyces meandriformis*. This species produced hypogeous sporocarps in summer and autumn, in moderate acid and moist soils in some spruce forests from northern part of Romania. Unlike other truffle species, *Choiromyces meandriformis* was much more easily to be identified because a part of their sporocarps came to the surface without the need for dogs or other animals specially trained to find them. According to the Romanian literature, this species is edible with caution, while in other countries it is considered as toxic species, producing gastrointestinal disorders.

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THE QUALITY OF SILAGE FROM *FESTUCA ARUNDINACEA* AND *MISCANTHUS GIGANTEUS* AS FEEDSTOCK FOR BIOMETHANE PRODUCTION IN REPUBLIC OF MOLDOVA

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Abstract: Biomethane production is environmentally friendly and rapidly expanding in the latest years. Energy crops can be a suitable feedstock and if ensiled it can be supplied to biogas plants continuously throughout the year. The aim of the current work was to evaluate quality and biochemical methane production potential of silage prepared from *Poaceae* plant species: *Festuca arundinacea* and *Miscanthus giganteus* grown in experimental land of the National Botanical Garden (Institute), Chişinău. The samples were collected from the 3-year-old *Miscanthus giganteus* (June 16, August 17, October 2) and *Festuca arundinacea* (June 16). The biochemical methane production potential of *Miscanthus giganteus* silage prepared from first mowing in June reached 355 L/kg, but second mowing in October – 318 L/kg, single mowing regime in August – 290 L/kg; *Festuca arundinacea* silage – 340 L/kg, respectively.

Keywords: biochemical methane production potential, *Festuca arundinacea*, *Miscanthus giganteus*, silage.

Introduction

A continuous increase in energy demand, in the light of running low its conventional carriers, forces the mankind to produce energy from renewable sources. At the present time, renewable energy accounts for 19.3% of the global final energy consumption. Biomass plays a key role in the considerations how to secure enough amount of energy for the next generations, while biomass is a source of energy which is largely available; it provides 63.7% of the global renewable energy supply [REN21, 2017]. Sustainable bio energy represents a huge potential for making a significant contribution to rural and economic development, enhancing energy security and reducing environmental impact. The utilization of plant biomass for energy purposes allows the consumption of air CO_2 during photosynthesis, while its release back into the atmosphere is closed in a relatively short time. The ideal energy crop has to have good capacity for energy transformation from solar to harvestable biomass with maximum efficiency, minimal input requirements and favourable environmental influence [ROMAN & al. 2016].

The technology of biomass conversion through anaerobic digestion is a quite promising option, as biomethane production represents the source of energy with great potential, environmentally friendly and rapidly expanding in the latest years [AMON & al. 2007; KLIMIUK & al. 2010; VINTILĂ & al. 2012; DANDIKAS & al. 2014]. The digestate serves as an excellent fertilizer and soil improver of high quality, replacing mineral fertilizer [BORSO & al. 2018]. The use of biogas for the needs of the transport sector has increased significantly in the USA and has continued to increase its share in the fuel mix in European Union [REN21, 2017]. The profitability of many biogas investments depends on the substrate costs and certificate price. In Europe, maize is the most commonly used energy crop as biogas

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feedstock [AMON & al. 2007], its cultivation, harvest and mineral fertilization require high financial and fossil fuel inputs. However, the cultivation in fertile agricultural land with high input crop-management techniques made maize, as energy crop, responsible of elevated environmental impact, increment in food price volatility and in associated risks for food security. Latterly, much attention has been focused on identifying suitable non-food biomass species. Perennial grasses are promising candidates as bioenergy crops. The mobilization and selection of new perennial species, as well as the elaboration of specific agro-technical measures cultivation of plants as bioenergy crops are an important priority to meet the need of biomass production [VALENTINE & al. 2012; ROMAN & al. 2016].

Currently, grasses from the genus *Miscanthus,* which includes about 16-25 species with C_4 photosynthetic pathway, native to the south-eastern Asia, from China, Japan to Polynesia and few species originating from Africa, are considered to be key renewable raw materials for industrial processing and transformation into energy, which can play an important role in the biorefining industry and energy production. The natural hybrid *Miscanthus* × *giganteus*, discovered in Japan in 1935, the most commonly planted miscanthus type, has very high photosynthetic capacity and growth rate at low temperature. The exceptionally vigorous growth and remarkable adaptability of *Miscanthus* × *giganteus* to different environments make this novel crop suitable for cultivation and distribution under a range of European and North American climatic conditions *Miscanthus* is a high-yielding lignocellulosic crop providing up to 40 t/ha/year of dry matter [KIESEL & LEWANDOWSKI, 2016; FARRAR & al. 2018].

The tall fescue, *Festuca arundinacea* Schreb. (syn. *Schedonorus arundinaceus* and *Lolium arundinaceum*), is a cool season perennial grass species, C_3 photosynthetic pathway, native to Europe, common in the spontaneous flora of the Republic of Moldova. Tall fescue has been cultivated since the beginning of the 20th century. It has been investigated in many scientific centres and implemented as crop in different regions of the Earth, not only as a source of fodder and a phytoremediation plant, but also as feedstock for bioenergy production. The selected forms and new cultivars have a productivity of 50-65 tons/ha of fresh mass or 15-17 tons/ha of hay [JANČÍK & al. 2011; MARUȘCA & al. 2011; BAHCIVANJI & al. 2012].

Ensiling is one of the most effective methods of storage and conservation of harvested green mass, playing an essential role in livestock feeding, but in recent decades, it has also been used as substrate in biogas production. Energy crops can be a suitable feedstock for anaerobic digestion and if ensiled it can be supplied to biogas plants continuously throughout the year [TRULEA & al. 2013; FRANCO & al. 2016; WHITTAKER & al. 2016; BORSO & al. 2018].

The aim of the current study was to evaluate the quality and the biochemical methane production potential of the silage prepared from *Festuca arundinacea* and *Miscanthus giganteus*, grown under the conditions of the Republic of Moldova.

Material and methods

Perennial plants of the *Poaceae* family, the local ecotype of *Festuca arundinacea* and cv. *Titan* of *Miscanthus giganteus*, which was cultivated in the experimental plot of the National Botanical Garden (Institute) Chișinău, latitude 46°58′25.7″N and longitude N28°52′57.8″E, served as subjects of this study.

The green mass of 3-year-old perennial grasses was harvested manually. The samples of *Miscanthus giganteus* were collected under different harvest regimes and on different dates: single mowing regime (June 16, August 17) and double mowing regime – 1st mowing (June 16) and 2nd mowing (October 2), but *Festuca arundinacea* – under single mowing regime in the full flowering (June 16). The green mass was shredded and compressed in well-sealed glass containers. After 30 days, the containers were opened, the organoleptic characteristics were analyzed and the biochemical composition of the silage was determined in accordance with the Moldavian standard SM 108. Dry matter or total solid content was detected by drying samples up to constant weight at 105 °C. Organic dry matter or volatile solids, was calculated through differentiation, the crude ash being subtracted from dry matter. The content of neutral detergent fibre, acid detergent fibre and acid detergent lignin, was evaluated using the near infrared spectroscopy (NIRS) technique PERTEN DA 7200 of the Research-Development Institute for Grassland Brașov, România. The biochemical biogas potential (Yb) and methane potential (Ym) were calculated according to the equations of DANDIKAS & al. (2014), based on the chemical compounds – acid detergent lignin and hemicelluloses values:

biogas potential Yb=727+0.25 HC-3.93 ADL

methane potential Ym=371+0.13HC-2.00ADL

Results and discussions

It is known that the growth and development rates of plants influence biomass accumulation, dry matter content and biochemical composition. In our previous papers [TELEUȚĂ & ȚÎȚEI, 2013; ȚÎȚEI, 2015, 2016], we mentioned that studied grasses species were characterized by a different growth and development rates. Thus, in the first year of vegetation, *Miscanthus giganteus* was distinguished by faster growth, developing shoots, which reached 152-183 cm, while *Festuca arundinacea* did not develop shoots. In the following years, the regrowing season for *Festuca arundinacea* started in the first half of March, when the average soil temperature was above 3-5 ºC and for *Miscanthus giganteus* – in April, when the temperature was above 10-12 ºC. Thus, by late April, *Festuca arundinacea* plants grew over 70 cm, *Miscanthus giganteus* was 8 cm tall at that time and, by full flowering, the studied plants reached 131 cm and 324 cm, respectively.

Some biological peculiarities of *Festuca arundinacea* and *Miscanthus giganteus* in the third growing season are described in Table 1. It was determined that *Festuca arundinacea* resumed growth in the first days of March, but *Miscanthus giganteus* – in April. The studied perennial grasses were characterized by faster growth rates. The peak growth of *Festuca arundinacea* occurred during the period of reproductive growth (middle May – June), when shoots were over 135 cm tall. *Miscanthus giganteus* plants developed shoots that reached a height of 157 cm in mid-June, 260 cm in mid-August and in the period when the panicle development started, the first days of October – 385 cm.

Analyzing the results of the study on the influence of the harvest time on the leaf : stem ratio of *Miscanthus giganteus*, we found that stem dry matter increased from 10.16 to 25.53 g, but the leaf mass – from 10.94 to 16.36 g, which caused a decrease in the leaf content in the harvested biomass from 53.50 to 39.05%. The studied perennial grasses were distinguished by different dry matter content in harvested green mass.

We may mention that after mowing in June, the plants of the cultivar *Titan* of *Miscanthus giganteus* were characterized by a moderate rate of revival and, in early October,

the stems reached 193 cm, but *Festuca arundinacea* was characterized by slow growth, the secondary peak of vegetative growth occurs in autumn, this species did not develop shoots.

Table 1. Some biological peculiarities of *Festuca arundinacea* and *Miscanthus giganteus*

Harvesting period	Plant height, cm	Stem, g		Leaf, g		Leaves content, %
		green mass	dry matter	green mass	dry matter	
Festuca arundinacea						
16 June (1st mowing)	137	6.95	2.28	7.16	2.40	40.73
Miscanthus giganteus						
16 June (1st mowing)	157	60.18	10.16	42.83	10.94	53.50
17 August (1st mowing)	260	65.95	25.53	43.42	16.36	39.05
2 October (2nd mowing)	193	30.11	11.76	26.54	10.30	46.69

The investigated silage from studied perennial grasses was distinguished by a different dry matter content and organoleptic characteristics. When opening the glass vessels with silage made from tall fescue *Festuca arundinacea*, there was a pungent, unspecific odour, somehow similar to the smell of fresh pine wood, but it disappeared later. The silage made from green mass of *Miscanthus giganteus* harvested under double mowing regime (June and October) had a pleasant smell, specific to pickled vegetable, but the silage made from green mass harvested in August, after opening the glass vessels, intensively eliminated carbon dioxide – a by-product of fermentation, the smell was unpleasant but, about 5 minutes after opening, it changed and became specific, like corn silage.

During the organoleptic assessment, it was found that the colour of the *Festuca arundinacea* silage was dark green leaves and yellow stems; the silage made from *Miscanthus giganteus* green mass harvested under double mowing regime in June and October, it was similar – homogeneous green-olive, but the silage from green mass harvested in August – yellow-green leaves and stems. The consistency of the silage from perennial grass species was retained, in comparison with the initial green mass, without mould and mucus.

It is known that the microflora in the harvested green mass is totally different from that of the future silage. During the process of ensiling, epiphytic bacteria produce organic acids. Optimal ensiling results in rapid lactic acid and acetic acid fermentation, causing a decrease of the pH to 4-4.5 within several days.

The fermentation quality of silage prepared from the studied grass species are shown in Table 2. As a result of the performed analysis, it was determined that the pH index of the prepared silage varied from 4.02 to 5.45. The pH index of the silage prepared from *Miscanthus giganteus* green mass in double mowing regime met the standard SM 108.

Analyzing the data regarding the overall content of organic acids, we conclude that the concentration of organic acids was higher in *Festuca arundinacea* silage and lower in *Miscanthus giganteus* silage prepared from green mass harvested in August. The lactic acid concentration in *Festuca arundinacea* silage – 2.01%, but *Miscanthus giganteus* silage was characterised by lower concentration (1.12-1.66%). The *Miscanthus giganteus* silage prepared from green mass harvested in June was characterized by high acetic acid concentration (10.1 g/kg), but the silage prepared from green mass harvested in August – by lower acetic acid concentration (0.9 g/kg). Butyric acid was not found in the silage prepared from *Miscanthus giganteus* harvested under double mowing regime, but it was present, in very high amounts in *Festuca arundinacea* (11.6 g/kg) and *Miscanthus giganteus* harvested

in August (4.6 g/kg). As previously mentioned, butyric acid had a share of 27.63 - 33.72%, which caused the pH level to rise and the fermentation quality to worsen in these silages. A high content of butyric acid indicated large production of CO_2 and ammonia as well, which was observed while opening the glass vessels.

According to WHITTAKER & al. (2016), *Miscanthus giganteus* silage, made from biomass harvested in October, indicated pH higher than 5 and 7% ethanol content, but lower lactic and acetic acid, sugar and starch contents.

Table 2. The fermentation quality of *Festuca arundinacea* and *Miscanthus giganteus* silages

Indices	*Festuca arundinacea* 16 June	*Miscanthus giganteus* 16 June	*Miscanthus giganteus* 17 August	*Miscanthus giganteus* 2 October
pH index	5.04	4.15	5.45	4.02
content of organic acids, g/kg	34.4	26.7	16.7	28.1
free acetic acid, g/kg	0.1	4.4	0.4	2.3
free butyric acid, g/kg	2.9	0	0.2	0
free lactic acid, g/kg	1.4	6.8	1.8	8.8
fixed acetic acid, g/kg	2.6	5.7	0.5	4.6
fixed butyric acid, g/kg	8.7	0	4.4	0
fixed lactic acid, g/kg	18.7	9.8	9.4	12.4
total acetic acid, g/kg	2.7	10.1	0.9	6.9
total butyric acid, g/kg	11.6	0	4.6	0
total lactic acid, g/kg	20.1	16.6	11.2	21.2
acetic acid, % of organic acids	7.85	37.83	1.38	24.55
butyric acid, % of organic acids	33.72	0	27.63	0
lactic acid, % of organic acids	58.43	62.17	66.99	75.44

The quality of feedstock for biogas production depends on how accessible the biomass is to enzymes and microbes. To measure the quality, wet chemical analyses are needed, analyses that are laborious and time consuming. Near infrared reflectance spectroscopy has been used in agricultural research for years, as a robust method, low cost and doing non-destructive measurements with limited sample preparation, providing quantitative and qualitative information [VIDICAN & al. 2000; MAYER, 2015; VANCE & al. 2016].

The Near infrared reflectance spectroscopy study revealed the compositional content of carbohydrates and biochemical methane potential of prepared grass silage, and the results are presented in Table 3. The obtained data showed that the concentrations of carbohydrates and their compositional content in silage differed significantly, depending on the species and harvesting period. The prepared grass silage was characterized by the highest concentrations of structural carbohydrates and low concentrations of soluble sugars and this fact affected the quality of silage fermentation. The total soluble sugars content it is important to create favourable conditions for the development of lactic acid bacteria responsible for a successful ensilage process. In the silage from *Festuca arundinacea* and *Miscanthus giganteus* harvested in August, there was a significant decrease in soluble sugars (8-19 g/kg DM) and an increase in cellulose (452-489 g/kg DM), which also affected the quality of silage fermentation. The hemicellulose content was approximately at the same level in the prepared grass silage (308-328 g/kg DM).

Lignification of cell walls during plant development was identified as the major factor limiting nutrient digestibility, degradation of feedstock for anaerobic digestion and

concomitantly biomethane productivity [KLIMIUK & al. 2010; TRIOLO & al. 2011; DANDIKAS & al. 2014]. The concentrations of acid detergent lignin in the investigated silage varied from 28 g/kg to 61 g/kg. In the silage from *Miscanthus giganteus* harvested in August, the acid detergent lignin content increased to 61 g/kg, probably because from late July the lower leaves started to dry and soluble nutrients moved back to the rhizome.

The differences in the concentrations of carbohydrates affected the potential of biogas and methane production of silage substrate. The biochemical gas forming potential of obtained grasses silages varied from 567 to 694 L/kg VS. The biochemical methane production potential based on the chemical compounds – acid detergent lignin and hemicelluloses of *Miscanthus giganteus* silage made after the first mowing in June reached 355 L/kg, but after the second mowing in October – 318 L/kg, under single mowing regime in August – 290 L/kg; *Festuca arundinacea* silage – 340 L/kg, respectively.

Table 3. The concentrations of carbohydrates in *Festuca arundinacea* and *Miscanthus giganteus* silages and potential of biochemical methane production

Indices	*Festuca arundinacea* 16 June	*Miscanthus giganteus* 16 June	*Miscanthus giganteus* 17 August	*Miscanthus giganteus* 2 October
Acid detergent fibre, g/kg DM	489	452	550	485
Neutral detergent fibre, g/kg DM	817	760	869	804
Acid detergent lignin, g/kg DM	37	28	61	47
Total soluble sugars, g/kg DM	8	32	19	24
Cellulose, g/kg DM	452	424	489	438
Hemicelluloses , g/kg DM	328	308	319	319
Bio biogas potential, L/kg VS	664	694	567	622
Bio methane potential, L/kg VS	340	355	290	318

Some authors mentioned various findings about the quality of grasses silage and specific methane yield. According to JANCIK & al. (2011), the chemical composition of of silage dry matter prepared in May, in Czech Republic, from *Festuca arundinacea* was: 17.80% protein, 2.76% fat, 8.59% ash, 51.20% NDF, 31.10% ADF and 2.66% ADL, but from *Dactylis glomerata* – 14.90%, 3.08%, 4.66%, 54.10%, 33.30% and 3.12%, respectively. BALDINI & al. (2016), reported that dried biomass of *Miscanthus giganteus* silage prepared from 1st mowing consists of 4.27% raw protein, 1.07% fat, 43.0% cellulose, 28.0% hemicellulose and 7.33% acid detergent lignin, but 2nd mowing – 2.77% raw protein, 1.04% fat, 41.9% cellulose, 28.8% hemicellulose and 7.97% acid detergent lignin. AMON & al. (2007), clearly demonstrated the grass grown at the valley site produced 190–392 L/kg VS, the highest specific methane yield was measured for the biomass from the second cut from the "four-cuts variant". HERRMANN & al. (2016), reported that *Miscanthus* silages contained 30-40% dry matter, 5-7% crude ash and methane yield 200-260 L/kg VS, but meadow fescue (late 1st cut) silages 27-44% dry matter, 5-7% crude ash and methane yield 277-342 L/kg VS. Based on the batch experiments DANDIKAS & al. (2014), published that average methane yield of grassland silage varied from 177 to 371 L/kg VS, but maize silage – from 327 to 401 L/kg VS. WHITTAKER & al. (2016), remarked that methane yield averaging 186 L/kg VS from untreated *Miscanthus giganteus* silage prepared in October, in contrast, KLIMIUK & al. (2010) observed lower yields, 100 L/kg VS in *Miscanthus giganteus* silages prepared in autumn. BORSO & al. (2018) reported that in Mediterranean climate *Miscanthus* harvested in August showed 171.4 L/kg VS methane yield, but harvested in winter period 120.5 L/kg VS.

Conclusions

The obtained results showed that fermentation quality, dry matter content, concentrations of neutral detergent fiber, acid detergent fiber, lignin and cellulose in silage from studied grass species differed significantly depending on the species and harvesting period, which have influenced the methane yield.

The silage obtained from *Miscanthus giganteus* harvested in double mowing regime, by organoleptic characteristics and biochemical indices (pH, content and correlation of organic acids, chemical composition of the dry matter), largely, met the standards.

The biochemical methane production potential of *Miscanthus giganteus* silage made as a result of the first mowing in June reached 355 L/kg, but second mowing in October – 318 L/kg, single mowing regime in August – 290 L/kg; *Festuca arundinacea* silage – 340 L/kg, respectively.

Preliminary scientific researches allow mentioning that the local ecotype of *Festuca arundinacea* and cv. *Titan* of *Miscanthus giganteus* can be used to produce silage and possibility of its use as feedstock for biogas production.

Notes on contributor

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THE PRODUCTIVITY AND THE QUALITY OF GREEN MASS AND HAY FROM ROMANIAN CULTIVARS OF *FESTUCA ARUNDINACEA*, GROWN IN THE REPUBLIC OF MOLDOVA

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Abstract: Grasses are considered as one of the most important sources in low-cost feed for for animals, but also a substrate for the production of renewable energy. We studied the quality of the freshly harvested biomass and hay of Romanian cultivars of tall fescue *Festuca arundinacea*: 'Adela', 'Brio', 'Măgurele 5', created at the Research and Development Institute for Grasslands, Braşov, Romania and cultivated in the experimental plot of the National Botanical Garden (Institute) "Alexandru Ciubotaru", Chisinau, Republic of Moldova. The samples for assessment were taken in the 2nd year of growth, when the plants were cut for the 1st time. The amount of dry matter (DM), crude protein (CP), crude ash (CA), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose (Cel), hemicellulose (HC), digestible dry matter (DDM), organic matter digestibility (OMD), relative feed value (RFV), the metabolizable energy (ME), the net energy for lactation (NEl), biomethane production potential (BMP) were analyzed. It has been determined that the green mass productivity of the tall fescue cultivars achieved 30.83-36.96 t/ha, the dry matter contained 114-136 g/kg CP, 74-89 g/kg CA, 582-593 g/kg NDF, 392-396 g/kg ADF, 34-41 g/kg ADL, 322- 329 g/kg Cel, 226-229 g/kg HC, 60.3-63.8% DDM and 57.2-62.2% OMD, 9.12-9.62 MJ/kg ME and 5.69-5.86 MJ/kg NEl; the hay dry matter contained 99-117 g/kg CP, 80-86 g/kg CA, 582-593 g/kg NDF, 356-366 g/kg ADF, 34-37 g/kg ADL, 351-356 g/kg Cel, 236-260 g/kg HC, 55.8-57.2% DDM and 51.4-53.3% OMD, 9.51-9.59 MJ/kg ME and 5.53-5.59 MJ/kg NEl, this fact indicates a optimal quality of the roughage feed for ruminants. The substrate for the anaerobic digestion, consisting of fresh mass of tall fescue, had a biomethane production potential of 349-354 L/kg of organic matter. The studied cultivars of tall fescue could be used in the Republic of Moldova for the restoration of degraded permanent grasslands, as a component of the mix of grasses for the creation of temporary grasslands and can be planted between rows in vineyards and orchards. Besides, the obtained biomass can be used as feed for animals or as substrate at biomethane production plants.

Keywords: biomethane production, cv. *Adela*, cv. *Brio*, cv. *Măgurele 5*, feed value, *Festuca arundinacea*, productivity.

Introduction

Grasslands are important as habitat for many plant species, including species at risk, provide soil and water conservation, nutrient recycling, pollination, habitat for livestock grazing, genetic material for crops, recreation, climate regulation, and storage for about 34% of the terrestrial global carbon stock. Grasses are considered as one of the most important sources in low-cost feed for domestic herbivores animals [COTIGĂ, 2010; MARUŞCA & al. 2011; BAHCIVANJI & al. 2014], but also a feedstock for the production of renewable energy [ENIRY & O'KIELY, 2014; ROMAN & al. 2015; KANDEL & al. 2017].

An important component of the land resources of Romania is the area of 4.9 million hectares of permanent grasslands, considered, quite rightly, a national treasure because it represents 33% of the agricultural land, occupying, in Europe, the 5th place, 68% of which

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are pastures and 32% – hayfields [MARUȘCA & al. 2014]. Permanent grassland in the Republic of Moldova constitutes 10.1% of the territory, represented by 339000 ha of pastures and 2000 ha hayfields. The productivity of natural grasslands on slopes is low and of poor quality, reaching 400-600 kg/ha of hay, and that of floodplain grasslands is higher – 2000-2600 kg/ha of hay. Uncontrolled grazing has diminished the abundance and dominance of typical grassland species, and thus they have been replaced by adventitious, segetal, quarantine weeds and other non-fodder and poisonous species [BAHCIVANJI & al. 2014; LAZU, 2014; LEAH, 2016]. In the vegetation of the permanent grasslands, the most common plant species with high fodder value are representatives from *Poaceae* and *Fabaceae* families.

The Plant List includes 1741 scientific plant names of species rank for the genus *Festuca*, Family *Poaceae*, of these 646 are accepted species names. Globally, the species of the genus *Festuca* L. are common in the floristic composition of permanent and temporary grasslands.

Tall fescue *Festuca arundinacea* Schreber. (syn. *Lolium arundinaceum* (Schreb.) Darbysh.; *Schedonorus arundinaceus* (Schreb.) Dumort.) is a long-lived perennial grass, native to Europe, C_3 photosynthetic group, with vigorous and erect culms 60 to 200 cm tall. Leaves form basal tufts, blades are 20-70 cm long and 3-12 mm wide. A tuft produces 10 to 30 flowerstalks. The inflorescence is an open to narrow branched panicle 12 to 35 cm long. Spikelets are three to nine flowered. Lemmas are awnless to short-awned. The fruit is a caryopsis with adherent pericarp, brownish-yellow, glabrous, oblong to ellipsoid, 6-9 mm long, the weight of 1000 seeds 1.8-2.6 g. Tall fescue develops an extensive and robust root system which reaches up to 150 cm deep, is characterised by secretions that contribute to the mobilization and use of nutrients from the soil, adapts well to conditions of excess moisture as well as drought, at the same time, it develops normally on soils with pH 5.5-8.0, on eroded hills and slightly salty soil. Tall fescue reproduces by seed and increases vegetatively, produces new tillers from the root crown. It is perennial and is favored by the development of short stolons, thus, in periods with excess humidity, they provide the whole root system with oxygen. Tall fescue is common in wet, alluvial grasslands, and in various areas – from plains to mountains, it has medium feed value, is resistant to grazing, being an effective solution in preventing the problems related to rumen acidosis in cows [WALSH, 1995; COTIGĂ, 2010; MARUȘCA & al. 2011]. *Festuca arundinacea* is among the perennial species with the highest frequency in the floristic composition of temporary grasslands, buffer strips and lawns in green spaces. This species is almost always present in the mixtures of grassland species used in Switzerland, France and the Netherlands, as well as in the scientific recommendations developed and implemented by the Research and Development Institute for Grasslands Braşov, Romania [MARUȘCA & al. 2014].

The valorification of renewable energy is a very topical subject at global and local level, and, the production and use of phytomass to obtain different types of fuel is very promising in our region [ROMAN & al. 2015]. The research carried out has helped us find out that the solid biofuel from the dried biomass of *Festuca arundinacea* has specific density of 600-660 kg/m3, calorific value of 16.82-17.34 MJ/kg and ash content of 2.3-3.6% [ȚÎȚEI, 2015].

The aim of the current study consisted in determining the productivity of some Romanian cultivars of *Festuca arundinacea* grown under the conditions of the Republic of Moldova, the quality of the green mass and hay as roughage feed for ruminant animals, as well as substrate for the production of biomethane by anaerobic digestion.

Material and methods

The Romanian cultivars of tall fescue, *Festuca arundinacea*: 'Adela', 'Brio', 'Măgurele 5', created at the Research-Development Institute for Grasslands Braşov, România and cultivated in the experimental plot of the National Botanical Garden (Institute) "Alexandru Ciubotaru", Chişinău, latitude 46°58′25.7″N and longitude N28°52′57.8″E, served as subjects of the research.

The green mass was harvested manually. The samples were collected after cutting the plants for the 1st time in the second growing season, in the pre-anthesis period, in 2018. The leaf/stem ratio was determined by separating the leaves from the stem, weighing them separately and establishing the ratios for these quantities (leaves/stems). The prepared hay was dried directly in the field. The dry matter content was detected by drying the samples to a constant weight at 105 °C. Some assessments of the main biochemical parameters: protein, ash, acid detergent fibre (ADF), neutral detergent fibre (NDF), acid detergent lignin (ADL), digestible dry matter (DDM), organic matter digestibility (OMD) have been evaluated using the near infrared spectroscopy (NIRS) technique PERTEN DA 7200 of the Research and Development Institute for Grassland Braşov, România. The concentration of hemicellulose (HC) and cellulose (Cel), the relative feed value (RFV), the digestible energy (DE), the metabolizable energy (ME) and the net energy for lactation (NEl) were calculated according to standard procedures.

The carbon content of the substrates was obtained from data on volatile solids, using an empirical equation reported by BADGER & al. (1979). The biochemical biogas potential (Yb) and the methane potential (Ym) were calculated according to the equations elaborated by DANDIKAS & al. (2015), based on the chemical compounds – protein, acid detergent lignin (ADL) and hemicellulose (HC) indices:

biogas Yb=670+0.44PB+ 0.16HC-3.02ADL

metan Ym=370+0.21PB+0.05HC-1.61ADL

Results and discussions

The leaf/stem ratio essentially influences the chemical composition of the harvested phytomass to be used as feed for animals or as feedstock for biorefinery and obtaining different industrial products. The results regarding some agrobiological peculiarities of the studied cultivars of tall fescue, *Festuca arundinacea*, and the structure of the harvested biomass are presented in Table 1. We would like to mention that the studied cultivars of tall fescue, at the time of harvest, in the middle of May 2018, reached the height of 68.3-75.0 cm, the plants of the cv. 'Brio' being the highest. In the tall fescue harvested biomass the leaves content was 53.9-57.1%, the amount of dry matter – 23.4-25.8%. The green mass productivity did not differ essentially of the cv. 'Adela' and cv. 'Brio' (36.34-36.96 t/ha) being higher in comparison with the cv. 'Măgurele 5' (30.83 t/ha).

Table 1. Some agrobiological peculiarities and the structure of the harvested biomass of the studied cultivars of tall fescue *Festuca arundinacea*

Cultivars	Plant height, cm	Leaf, g		Stem, g		Productivity, t/ha	
		green mass	dry matter	green mass	dry matter	green mass	dry matter
Adela	73.8	1.63	0.36	1.71	0.42	36.34	8.51
Brio	75.0	1.59	0.36	1.83	0.48	36.96	8.98
Măgurele 5	68.3	1.48	0.34	1.62	0.42	30.83	7.96

Because feeding costs can account for over 50% of the cost of livestock production, knowing forage quality and the needs of animals can have a significant impact on profitability. Analyzing the results of the evaluation of the biochemical composition of the dry matter from the harvested mass of the studied cultivars of tall fescue, Table 2, we found that the cultivar 'Adela' was characterized by a higher content of proteins (13.6%), as compared with the cultivar 'Brio' (11.4%). The cultivars 'Adela' and 'Măgurele 5' did not differ essentially in the amount of ADF and NDF, cellulose and ash in the dry matter. The cultivar 'Brio' had a lower concentration of ADF, NDF, ADL, cellulose and ash, which had a positive effect on digestibility. The cultivars 'Brio' and 'Măgurele 5' had similar concentrations of hemicelluloses in the dry matter, but lower concentrations compared with the cultivar 'Adela'.

The amount of nutrients in fodder and their digestibility influence the health of animals and the amount and quality of animal products. The digestibility of dry matter and organic matter in the cultivar 'Brio' reached 63.8% and 62.0% respectively, being higher in comparison with other cultivars. We found that the cultivar 'Măgurele 5' was characterized by lower digestibility, determined, among other things, by the increased content of acid detergent lignin.

Table 2. Biochemical composition and nutritive value of the green mass of the studied cultivars of tall fescue *Festuca arundinacea*

Indices	Cultivar *Adela*	Cultivar *Brio*	Cultivar *Măgurele 5*
Crude protein, g/kg DM	136	114	128
Acid detergent fibre, g/kg DM	364	356	366
Neutral detergent fibre, g/kg DM	593	582	592
Acid detergent lignin, g/kg DM	35	34	37
Cellulose, g/kg DM	329	322	329
Hemicellulose, g/kg DM	229	226	226
Crude ash, g/kg DM	89	74	89
Digestible dry matter, %	62.2	63.8	60.3
Organic matter digestibility, %	59.1	62.0	57.2
Digestible energy, MJ/ kg	11.46	11.75	11.09
Metabolizable energy, MJ/ kg	9.37	9.62	9.12
Net energy for lactation, MJ/ kg	5.86	6.02	5.69
Relative feed value	95	98	95
Potential crude protein, kg/ha	1157	1024	1020
Potential metabolizable energy, GJ/ ha	79.74	86.39	72.60

The relative feed value (RFV) is an index that characterizes the quality of the feed by the potential capacity of the animals' body to assimilate the digestible dry matter from the feed. The studied cultivars of tall fescue had a relative feed value of 95-98 points, being of moderate to high quality in comparison with alfalfa. The experimental data indicated above reveal that the metabolizable energy of the tall fescue feed was 9.12-9.67 MJ/kg, and the net energy for lactation reached 5.69-6.02 MJ/kg. The highest potential for accumulation of crude protein was found in the cultivar 'Adela' (1157 kg/ha) and the highest metabolizable energy – in the cultivar 'Brio' (86.39 GJ/ha).

Hay is a very popular form of preserved fodder and valuable feed for farm animals, a rich source of protein, vitamins and minerals, both in winter and throughout the year, especially for the young animals, pregnant females and breeding males. Feeding high quality hay can also reduce the level of grain supplementation needed during winter. To most livestock farmers, crude protein (CP) and relative feed values (RFV) are the basis on how much hay to buy or feed livestock [ANGIMA & KALLENBACH, 2008]. The quality of the hay prepared from studied cultivars of tall fescue *Festuca arundinacea*, are presented in Table 3. The prepared hay prepared contained 99-117 g/kg CP, 80-86 g/kg CA, 582-593 g/kg NDF, 356-366 g/kg ADF, 34-37 g/kg ADL, 351-356 g/kg Cel, 236-260 g/kg HC, 55.8-57.2% DDM and 51.4-53.3% OMD, 9.51-9.59 MJ/kg ME and 5.53-5.59 MJ/kg NEl Thus, the preparation of the hay resulted in a decrease in the content of crude protein and an essential increase in the content of structural carbohydrates as compared with the freshly harvested mass, and fact had a negative impact on the net energy for lactation and relative feed values.

Table 3. Biochemical composition and nutritive value of the hay prepared from of the studied cultivars of tall fescue, *Festuca arundinacea*

Indices	Cultivar *Adela*	Cultivar *Brio*	Cultivar *Măgurele 5*
Crude protein, g/kg DM	117	99	108
Acid detergent fibre, g/kg DM	392	386	392
Neutral detergent fibre, g/kg DM	638	646	628
Acid detergent lignin, g/kg DM	36	34	41
Cellulose, g/kg DM	356	352	351
Hemicellulose, g/kg DM	246	260	236
Crude ash, g/kg DM	86	99	108
Digestible dry matter, %	57.2	56.3	55.8
Organic matter digestibility, %	52.0	53.3	51.4
Digestible energy, MJ/ kg	11.59	11.66	11.59
Metabolizable energy, MJ/ kg	9.51	9.59	9.51
Net energy for lactation, MJ/ kg	5.53	5.59	5.53
Relative feed value	85	87	86

Different results regarding the biochemical composition and the nutritive value of the green mass and hay from *Festuca arundinacea* are given in the specialized literature. Thus, the research conducted in Argentina by SCHENEITER & al. (2014), revealed that, depending on the harvest time, the yield increased from 0.64 to 2.82 t/ha DM, the NDF content increased from 503 to 604 g/kg and the digestibility decreased from 684 to 558 g/kg. FLORES & al. (2017), mentioned that, in the USA, tall fescue contained 56.5-67.8% NDF, 27.7-34.9% ADF, 28.8-34.0% hemicellulose, 25.0-28.1% cellulose, 3.61-10.05% lignin. ENIRY & O'KIELY (2014), mentioned that, in Ireland, the biomass of *Festuca arundinacea*,

harvested on 12 May, contained 15.2% protein, 8.6% ash, 52.9% NDF, 26.7% ADF, 16.1% soluble carbohydrates, and the biomass harvested on 9 June contained 11.2% protein, 9.0% ash, 62.3% NDF, 37.2% ADF, 9.2% soluble carbohydrates. POCIENĖ & KADŽIULIENĖ (2016), found that the biomass of tall fescue, depending on the amount and type of applied fertilizers, contained 14-20% hemicellulose, 34-36% cellulose and 6-9% lignin. In Turkey the hay yield in pure *Festuca arundinacea* stands varied from 3.7 to 11.6 t/ha and concentration of crude protein from 10.0 to 10.9% depending on the dose nitrogen fertiliser (KOC & al. 2004), in USA hay prepared from tall fescue cv. Kentucky 31 contained 6.37-7.85% crude protein with RFV 96-98 [ANGIMA & KALLENBACH, 2008].

Table 4. Chemical composition and biomethane production potential of green mass substrates from the studied cultivars of tall fescue, *Festuca arundinacea*

Indices	Cultivar *Adela*	Cultivar *Brio*	Cultivar *Măgurele 5*
Crude protein, g/kg DM	136	114	128
Minerals, g/kg DM	89	74	89
Carbon, g/kg DM	506.1	514.4	506.1
Nitrogen, g/kg DM	21.8	18.2	20.5
Carbon/nitrogen ratio	23	28	25
Acid detergent lignin, g/kg DM	35	34	37
Hemicellulose, g/kg DM	229	226	226
Biogas potential, L/kg VS	661	654	651
Biomethane potential, L/kg VS	354	351	349
Methane productivity, m^3/ha	3013	3152	2778

The biomass is converted into biogas by anaerobic digestion in special devices, called anaerobic digesters, by a wide variety of microorganisms, and this process results in fuel gas, which consists of methane and carbon dioxide, and digestate, which is rich in macro- and micronutrients and can be used as fertilizer in organic farming. The carbon nitrogen ratio (C/N) of biomass plays a crucial role in the process of decomposition of organic matter by microorganisms. DOBRE & al. (2014) mentioned that the optimal C/N ratio was expected to be in the range 15-25, when the anaerobic digestion process was carried out in a single stage and for the situation when the process developed in two steps, the optimal C/N ratio ranged between 10-45 for step I and 20-30 for step II. The results regarding the quality of the substrate and the potential for obtaining biogas and biomethane from the freshly harvested mass of the studied cultivars are shown in Table 4. We found that the substrate of *Festuca arundinacea*, according to the C/N ratio, which constituted 23-28, the amount of acid detergent lignin (34-37 g/kg) and hemicellulose (226-229 g/kg) met the established standards. No essential differences were observed between the studied cultivars, in the potential for biogas (651-661 l/kg organic matter) and biomethane production (349-354 l/kg organic matter). Methane productivity ranged from 2778 to 3152 m^3/ha. The cultivars 'Brio' and 'Adela' were characterised by higher indices due to a higher yield of biomass. KANDEL & al. (2017), indicated a potential methane productivity of tall fescue biomass, from the 1st harvest, of 401-428 l/kg and an annual productivity, from three harvests per growing season, of 6871 m^3/ha, in Denmark. ENIRY & O'KIELY (2014), mentioned that the potential methane productivity of tall fescue biomass was 216-268 l/kg, varying depending on the harvest time.

Conclusions

Tall fescue, *Festuca arundinacea*, is a perennial grass with economic and social interest, and the Romanian cultivars 'Brio' and 'Adela' can be used in the Republic of Moldova to restore degraded permanent grasslands, as a component of the mixtures of plants sown to create temporary grasslands, grass strips in vineyards and orchards, and the harvested biomass can be used as fodder for animals and as substrate for the production of biomethane in anaerobic digesters.

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Professor Tatiana Eugenia ŞESAN at her 75th anniversary

Tatiana Eugenia ŞESAN (born at 12th January 1944), corresponding member of the Academy of Agricultural Sciences and Forestry in Romania, is a Professor at the Faculty of Biology - University of Bucharest since 2004, PhD in Biology, specialized in Mycology / Plant Pathology since 1985, PhD promoter in the field of Life Sciences, Biology domain (since 2000).

The first eight years of her professional activity have been developed in the Pedagogical Institute Bacău, teaching Botany (Morphology, Anatomy, and Taxonomy), Plant Protection and Technics of Preparing Didactical Materials for Biology a.o. For the next 30 years she worked at the Research Institute for Plant Protection in Bucharest, coordinated by the Academy of Agricultural Sciences and Forestry, as a scientist/senior scientist in the field of Plant Pathology, Mycology, Sustainable Development, Biotechnologies and, especially, Biological Control of Plant Diseases and Weeds.

All this activity includes a high volume of original, personal scientific results. The scientific contributions were materialized until now in: 26 books (1 chapter in an international volume), more than 430 articles, from which, 16 papers in ISI indexed journals, 10 inventions and 4 new requests for patent. In 2019, Patent no. 13177/2018 – Bio-stimulated strain of *Trichoderma asperellum* and composition based on it for use in conservative agriculture system was awarded.

In order to celebrate hers 75th birthday and 53 years of activity, the volume "Professor PhD Tatiana-Eugenia Șesan – a tireless life for the plant's health" was published at the Bucharest University Press.

The national recognition is materialized in the Prize "Traian Săvulescu" of the Romanian Academy (for Agriculture), obtained for the books *Diseases of industrial plants. Prevention and control* (1988) and *White rot of cropped plants. Prevention and control* (1998) as first author. Together with Professor PhD C. Tănase she has been rewarded with "Emanoil Teodorescu" Prize by the Romanian Academy (Biology) for the book *Present concepts in fungal taxonomy* (2008) and with "Iuliu Prodan" Prize by the Academy of Agricultural Sciences and Forestry (2010) for the volume *Fungi with applications in agriculture, medicine and patrimony*. In 2013, Ana Tomescu and T. E. Șesan have received "Ernest Grinţescu" Prize by the Romanian Society of Horticulture at its Centenary for the book *The known and less known plant world. Fabaceae family*. In 1999 she has been rewarded with "M. Constantineanu" Prize by the Romanian Association for Ecosanogenesis. She has won during her activity research fellowships in Russia, France, Poland, The Netherlands and has collaborated for long periods in the frame of international research projects in The Netherlands in the field of biological control of plant diseases (1993, 1995, 2000), mycorrhizas (COST 870, manager for Romania), *Erwinia amylovora* (COST 674), organic horticulture (COST FA 1105), strigolactones (COST FA 1206, manager for Romania). In Romania, she has worked in different national research projects RELANSIN,

BIOTECH, CEEX, PN II, CNCSIS Ideas a.o. She is one of the authors of 4 patents homologated by OSIM and of an application for an European patent, all of these being the results of the research projects successfully finished. She is a member of national scientific and *In Honorem* international organizations, invited at many international congresses in 20 countries: Denmark (1991), The Netherlands (1992, 1993, 1995, 1996, 2000, 2003), Belgium (2000, 2011, 2013), Turkey (1993, 2002, 2008), Poland (1994, 1997, 1998, 1999, 2005, 2012), Great Britain (1994), Czech Republic (1997), Greece (1997, 2011), Switzerland (1997), Austria (1998, 1999), Israel (1998, 2009, 2013), Hungary (1999), France (1987, 2000), Spain (2004), Portugal (2010), Taiwan (2000), South Africa (1999), Russia (1979, 2007), Italy (2017); Bosnia & Herzegovina (2018) a.o. She is since 2006 the vice-president of the Romanian Mycological Society (SMR), being one of the founding members of this association and one important contributor in the development of SMR. Since 2011, she has been elected in the board of the *European Mycological Association* (EMA) as executive editor for *EMA Newsletter*.

She has been member of the organizing committee of some international events in Plant Pathology and Mycology in Romania and Poland. Mentioned in *Who's Who*, *Personenenzyklopädien* AG, Switzerland (2009), *Dictionary of the Personalities in Romania*, *Romanian Who's Who* (2010), *Dictionary of the Fungi* (2001, 2008), *Contemporary feminine personalities in Romania* (2013), and *Meronia Publishing House*.

In 2014, at the celebration of 70 years of life and 48 years of activity, during the *Annual Session of Scientific Communications* she was given by the "Dimitrie Brândză" Botanical Garden of Bucharest an Excellence Diploma; a similar Excellence Award was received from the "Anastasie Fătu" Botanical Garden of "Alexandru Ioan Cuza" University of Iași; with the same occasion she received a series of homage and friendship letters from collaborators, colleagues and friends from Romanian and foreign (the Netherlands, Poland, Bulgaria, Egypt, etc.) universities and research institutions.

The longest international activity as member of an editorial board starting with 2006 was carried out by Professor PhD Tatiana Eugenia Șesan at *Phytopatologia Polonica – Phytopathologia* (after 2009), honoring her membership, since 1999, within the Polish Phytopathological Society.

From 2014 until 2019, Professor PhD Tatiana Eugenia Șesan had a rich volunteering activity in reviewing several articles for multiple international journals, such as: *Electronic Journal of Polish Agricultural Universities*, *American Journal of Experimental Agriculture*, *Asian Journal of Research in Crop Science*, *Journal of Experimental Agriculture International*, *Asian Journal of Agricultural and Horticultural Research*, *Journal of Advances in Medical and Pharmaceutical Sciences*. Through letters and excellence certificates each of these publications appreciated her contributions, comments and academic suggestions which kept these journals high standard.

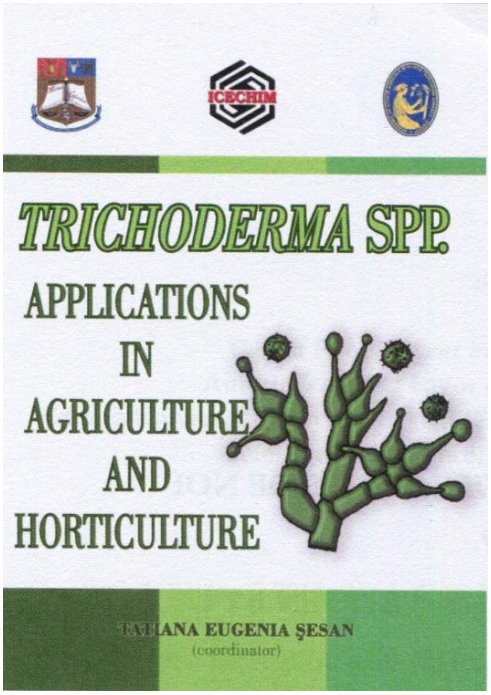
Thus, the journal *Acta Scientiarum Polonorum. Hortorum Cultus* from Poland, asked Professor PhD Tatiana Eugenia Șesan to accept the position as member of the Scientific Committee, while the *Asian Journal of Research Crop Science* offered her the position of Academic Editor, professional endeavor with remarkable results.

She is Scientific Referee for renowned journals in the fields of mycology, phytopathology, environment and plant protection: *Plant Pathology*, *Mycological Research* (Great Britain), *Phytoparasitica* (Israel), *Phytopathologia*, the former *Phytopathologia Polonica* (Poland), *Mycologia Balcanica* (Bulgaria), *Biologia* (Slovakia), *Acta Scientiarum Polonorum – Hortorum Cultus* (Poland), ISI indexed; *African Journal of Agricultural Research*, *African Journal of Microbiology Research*; *Romanian Biotechnological Letters*, *Electronic Journal of Polish Agricultural Universities*, *Biocontrol Science Technology*,

Journal of Ocular Pharmacology and Therapeutics, *Applied Ecology and Environmental Research Hungary*, *Asian Journal of Agricultural and Horticultural Research*, *Journal of Advances in Pharmaceutical Sciences*, and *Asian Journal of Research in Crop Science*.

The celebration of the 75th birthday represents a special moment for Professor PhD Tatiana Eugenia ȘESAN, but also for the entire academic community, who wishes her many happy years, with health and remarkable achievements!

Constantin TOMA
"Alexandru Ioan Cuza" University of Iași, Faculty of Biology
Cătălin TĂNASE
"Alexandru Ioan Cuza" University of Iași, Faculty of Biology



Participants at the 1st Congress on Strigolactones, Wageningen, The Netherlands, 2015

The 75th anniversary of the biologist Angela TONIUC

Angela Toniuc (daughter of Iosif and Elisabeta Bișoc) was born in the village of Ciumbrud (part of the city of Aiud, Alba county) on July 24, 1944. She attended pre-university studies in the city of Roman (Neamț county), and in 1962 she graduated the "Roman – Voda" High School with the baccalaureate diploma. Between 1964 and 1969, she attended the Department of Biology-Botany within the Faculty of Biology-Geography (since 1990, the Faculty of Biology). In 1979-1980 she completes her studies by attending postgraduate courses in English within the Faculty of Philology at the "Alexandru Ioan Cuza" University of Iași.

In the second year of her university studies she became an active member of the scientific circle of students, involved in the study of the corn (*Cornus mas*) from the Moldavian Plateau, which was also the subject of her diploma paper. The full recognition of the competences in the field of botanical research came with the title of doctor in biology of the University "Al. I. Cuza" Iași, in 1998, with the thesis "Embryological researches in some *Campanulaceae* both spontaneous and cultivated in Romania".

Immediately after graduation, she joined the team of the Botanical Garden of Iași, recently (1962-1963) transferred on the site of the Copou hill. The main responsibilities within the institution were permanently related to the Globe Flora section. Initially Angela Toniuc coordinated the *Alpine* sub-section, for which she elaborated and transferred to the field the scientific topic. Since 1993 she has taken over the surface of the entire sector, acting permanently in the spirit of preserving and enriching the botanical material. One of the most important achievements of the biologist Angela Toniuc within the Globe Flora section is the establishment of a bonsai collection, which over the past 25 years has come to contain hundreds of specimens of indisputable value. It is probably still one of the most valuable collections of the type in the whole country.

Her passion for bonsai art was complemented by her interest in other aspects of the Japanese artistic life (Japanese gardens, tea art etc.). They were all inspirational for the exhibitions organized in the Botanical Garden and, especially after 1990, the space organized under the inspired guidance of Angela Toniuc was highly appreciated by the visitors initiated in the Japanese culture.

Between 1985 and 2001 she coordinated the activity of the section "International seed exchange", which annually publishes the journal "*Delectus Seminum et Sporarum"*, as a base for exchanges of botanical material with our national and international partners.

As coordinator of these sections, botanist Angela Toniuc collaborated in the publication of four issues of the "Botanical Garden of Iași. Guide" journal, in 1983, 1988, 1994 and 2003, respectively. The experience regarding the activities specific to the botanical gardens and her talent to put on paper the vast information that she scrupulously accumulated were very useful in writing and publishing the book "File de istorie" (2006, in collaboration with Prof. M. Mititiuc), a true chronicle of the institution she has permanently promoted.

Botanist Angela Toniuc had the opportunity to promote the Botanical Garden of Iasi during various exchanges with similar institutions in Switzerland (1990), France (1990), Italy (1990, 1995), Belgium (1998), Republic of Moldova (1992, 1994, 1995, 1996, 1998) and Sweden (2000).

The valuable experience regarding the specificity of the botanical gardens resulted in her becoming a member of the scientific council of the Botanical Gardens of Iași and Galați, (after 1990). The specialists of the latter benefited from the guidance and the botanical material generously offered by the Botanist Angela Toniuc.

Between 1990 and 2000, she was associated professor of "Plant anatomy", "Plant embryology" and "Ecological biogeography" at the Faculty of Biology of the University "Alexandru Ioan Cuza" of Iasi. In this period, she generously shared with the students her valuable botanical knowledge with real pedagogical talent, collaborating in the laboratory and specialized practice, but also supervising their graduation and dissertation papers.

She devoted an important part of her time to research, the results of this activity being found in the over 130/110 works published, alone or in collaboration, four of them being published in international journals (Italy, Sweden). She also published in collaboration "Adaptation to the environment in the world of plants" (1991) and "Flora spontaneous and cultivated in Romania" (4 volumes). For this reference work (extremely useful to specialists), in 2006, Mrs. Angela Toniuc received the "EMANOIL TEODORESCU" prize from the Romanian Academy (along with Dr. Ion Sârbu). She was also among the collaborators of the work "The illustrated flora of vascular plants from Eastern Romania" (vol. 1, 2001). She was involved in 6 research contracts on rare plants, with ornamental value, medicinal and tincture, affected by polluting factors etc.

After retirement (in 2001), she continued to be interested in the botanical field, so that in 2014 she published in collaboration the book "Common names of Romanian Plants - expression of their morphological and ecological characteristics".

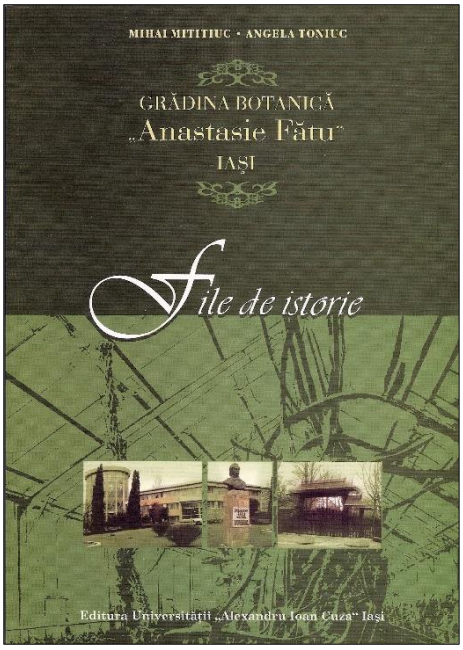
All the achievements of Mrs. Angela Toniuc, whether it was scientific work, obtaining exotic taxonomy, setting up the bonsai collection, participating in exhibitions etc., were marked by the seriousness, tenacity, refinement and remarkable energy that characterized her during the 35 years of activity. She was and remains a true mentor to the young biologists who took over the specific work of a botanical garden. The authors of these lines are grateful for the opportunity to collaborate for several years with Mrs. Angela Toniuc, a model of erudition, perseverance, fairness, creative but also critical spirit; a multitude of qualities from which we want to highlight the altruism with which she supported, encouraged and guided all the young people who had the chance to collaborate with her: graduates, doctoral, master and high school students or just nature lovers.

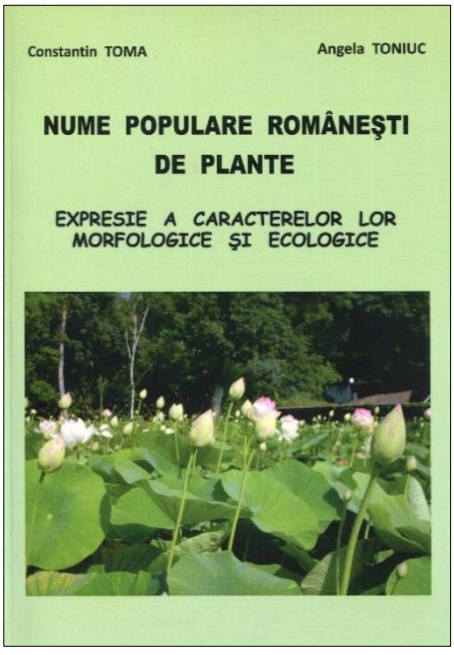
On the occasion of her 75th anniversary, we wish her the best of health, innumerable happy moments with her beloved ones, and also energy to complete the botanical projects at which she still works with dedication and that are dearly expected by those who know and admire her.

Many happy returns of the day!

Camelia IFRIM
"Anastasie Fătu" Botanic Garden, "Alexandru Ioan Cuza" University of Iași
Lidia ADUMITRESEI
"Anastasie Fătu" Botanic Garden, "Alexandru Ioan Cuza" University of Iași

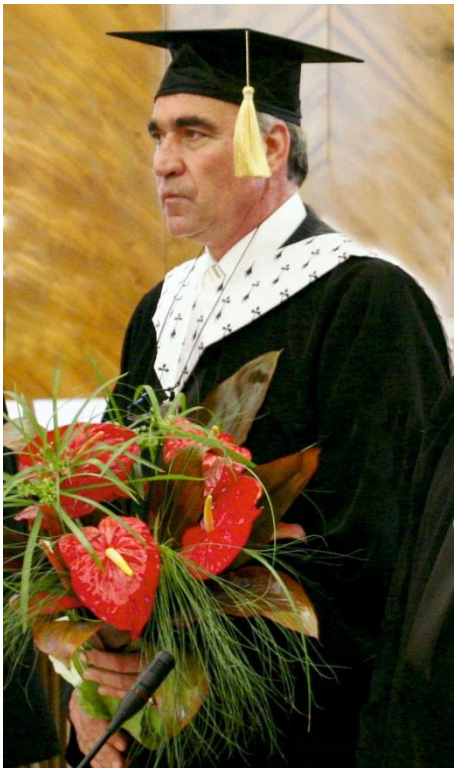

VALERIU ZANOSCHI ❖ ION SÂRBU ❖ ANGELA TONIUC
FLORA LEMNOASĂ SPONTANĂ ŞI CULTIVATĂ DIN ROMÂNIA
IV
EDITURA UNIVERSITĂŢII „ALEXANDRU IOAN CUZA" IAŞI


MIHAI MITITIUC • ANGELA TONIUC
GRĂDINA BOTANICĂ
„Anastasie Fătu"
IAŞI
File de istorie
Editura Universităţii „Alexandru Ioan Cuza" Iaşi


Constantin TOMA
Angela TONIUC
NUME POPULARE ROMÂNEŞTI DE PLANTE
EXPRESIE A CARACTERELOR LOR MORFOLOGICE ŞI ECOLOGICE


VALERIU ZANOSCHI
ADRIAN IONEL
ANGELA TONIUC
ADAPTAREA LA MEDIU ÎN LUMEA PLANTELOR

Professor Vasile CRISTEA at his 70th anniversary



On 5th of January 2019 we celebrated the 70th anniversary of Professor Vasile Cristea, a prestigious personality of Babeş-Bolyai University and Romanian phytosociology, a Professor with a great scientific career and a model for his students and colleagues. It was an occasion for the entire community of botanists to recognize his scientific activity with significant contribution to development of biological sciences in Romania.

Remarkable personality in the field of botany, phytosociology and biodiversity conservation, Professor Vasile Cristea was born on 5th January 1949 in Sibiu. He dedicated his entire scientific and teaching activity to Department of Taxonomy and Ecology within Faculty of Biology and Geology of the "Babeş-Bolyai" University from Cluj-Napoca. He has a degree in *Biology* (1971) and a doctorate in *Geobotany* (1981) for the thesis "*Flora and Vegetation of Secaşelor Plateau*". He is considered a disciple of Professor Ştefan Csürös and of the academician Nicolae Boşcaiu, a continuer of the tradition of the School of botany and nature conservation founded by Alexandru Borza. Honoring his magisters, he developed this School by inserting new and modern approaches in phytosociological studies and giving it international recognition. In this respect, we highlight the originality of the research on the diversity of vascular flora and vegetation in Transylvania, but also the studies on population ecology of some threatened species or ecological reconstruction and importance of conservation of plant diversity within scientific reserves.

The scientific activity was materialized by publishing more than 130 scientific papers presenting the results of his prolific work on botany, phytosociology and nature conservation. Also Professor Cristea is author or co-author of 12 books (3 were published in Italy), 5 manuals for students and 2 monographies. We emphasize the high scientific value of some monographies and reference volumes synthesizing his enormous experience in botany, vegetation classification and plant communities ecology, as "*The flora and vegetation of the Zarand Mountains*" (1978), distinguished with the Emanoil Teodorescu Award, granted by the Romanian Academy, "*Phytosociology and Romanian Vegetation*" (1993), "*Nature Conservation in Romania*" (1995), "*From biodiversity to GMOs?*" (2004), "*Phytosociology*" (2004), "*Vascular Plants: diversity, systematics, ecology and importance*" (2014) – one of the most comprehensive and actualized books in field of vegetal biology in Romania. He has also focused on some aspects of bioethics and cultural biodiversity in which

particular attention was paid to anthropology and sociology issues correlated with economic problems and with the principles of ecological ethics.

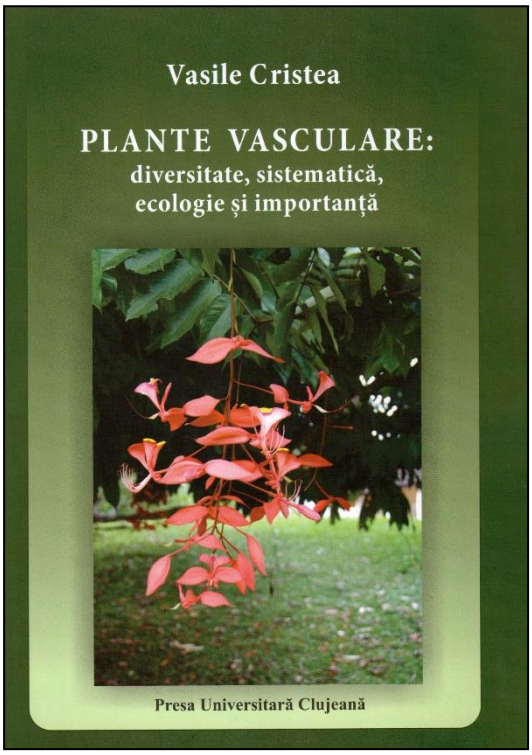
As a recognition of professional experience and of the value of his research he was awarded *Honorary Professor at* "*Alexandru Ioan Cuza*" *University from Iaşi* (2010) and *Doctor Honoris Causa of Banat University of Agricultural Sciences and Veterinary Medicine in Timişoara*. Also, he was invited to hold courses and lectures in the University of Camerino (where he held the course of Conservation of nature and resources), and invited professor at the University of Catania, Université Libre de Bruxelles, University of Alicante, etc. He collaborated in many national and international projects with numerous research institutions from Romania, Belgium, France, Germany, Italy, etc. By his rigor and tenacity Professor Vasile Cristea gained the respect of his colleagues from the academic community by the results obtained as Head of the Department of Botany, Dean of the Faculty of Biology and Geology, General Chancellor and Vice-Rector of the "Babeş-Bolyai" University. He was Director of the "Alexandru Borza" Botanical Garden in Cluj-Napoca, being actively involved in *ex situ* conservation of plant species and diversification of the plant collections. In this position he initiated projects of exchanges with plant material and good practices with other similar institutions and collaborated in order to organize the *International Scientific Symposium Conservation of Plant Diversity in situ and ex situ*, in Iaşi and Cluj-Napoca. Besides this, he also organized numerous national and international conferences, ones of the most prestigious being "*The 5th European Conference on the Conservation of Wild Plants in Europe*" (2007) in collaboration with Plantlife International and Planta Europa and the International Symposium "*Floristic patterns at different organisation and distribution levels*" (2014).

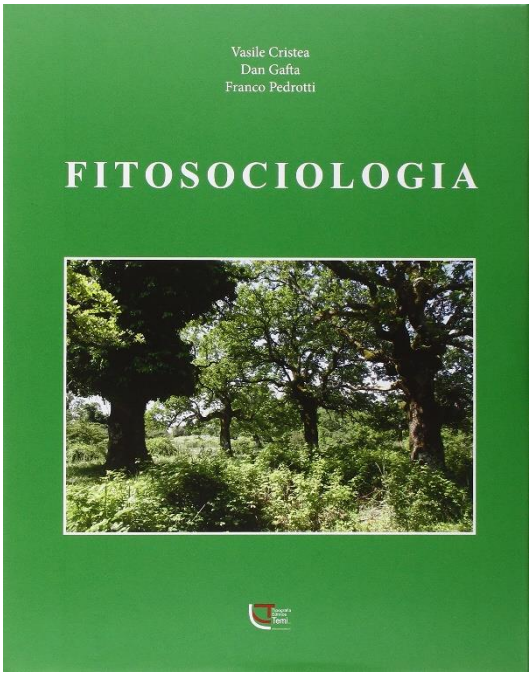
His professional prestige and competence are widely recognized and, consequently, he is a valuable member in the *Association Amicale Internationale de Phytosociologie* (Bailleul – France), member of the *Academia degli Accesi*, (Trento – Italy) and founding member of the *International Academy of Environmental Sciences* (Venice – Italy). He is the president of the *Romanian Society of Phytosociology*, ex vice-president of the *Association of Botanical Gardens from Romania*, member in the *Society of Biological Sciences of Romania*, member in the *Romanian Committee of History and Philosophy of Science*, member in the *Ecology Commission* and member in the *Commission of Nature Monuments* within Romanian Academy. Also Professor Cristea was for a significant time period the president of the *Biology and Biochemistry Commission* within the National Council for Attestation of Academic Titles, Diplomas and Certificates.

The whole didactic and scientific activity made Professor Vasile Cristea, at his 70th anniversary, a prestigious model for the entire academic community, students and specialists. On the behalf of all the colleagues from the Botanical Garden "Anastasie Fătu" of Iaşi, we wish Professor Vasile Cristea a long life, good health, new accomplishments and all the best for many years to come!

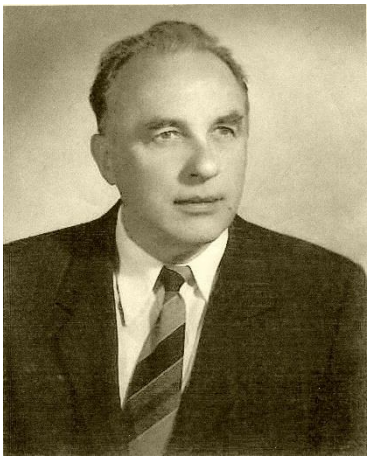
Happy Anniversary!

Constantin MARDARI
"Anastasie Fătu" Botanic Garden, "Alexandru Ioan Cuza" University of Iaşi
Cătălin TĂNASE
"Alexandru Ioan Cuza" University of Iaşi, Faculty of Biology


Vasile Cristea
PLANTE VASCULARE:
diversitate, sistematică,
ecologie și importanță
Presa Universitară Clujeană


Vasile Cristea
Dan Gafta
Franco Pedrotti
FITOSOCIOLOGIA

PROFESSOR DR. DUMITRU MITITELU
(1929-2005)



One of the outstanding professors of the Faculty of Biology of "Alexandru Ioan Cuza" University of Iaşi, was Dumitru Mititelu. He has marked many series of students and graduates of bachelor's, master's or doctoral studies, specializations in biology, biology-geography or natural and agricultural sciences, for more than four decades.

Of course, Professor Dumitru Mititelu was honored during his life by the Faculty of Biology members, by his doctoral students and doctors, but also by the students he guided, all with a rare competence and a deep attachment to quality of their scientific works, done together or under his direct guidance.

Thus, on October 19, 1929, the only son of Constantine and Euphrosine Mititelu, came to the world in Vaslui town. It is known that him graduated the primary school in Vaslui. The high school studies begin at the "Vasile Alecsandri" humanist high school in Galaţi, but the last two high school classes he followed at the "Mihail Kogălniceanu" Vaslui high school, which he graduated in 1948. After a short career as a teacher at a school in Dăneşti, Vaslui, he attended his military training (1950-1951).

He is admitted on a base of an examination to the Faculty of Natural Sciences of Iaşi, in 1951, the Biology-Botany section, which he graduated in 1955. Later on, he also graduated the courses of the Faculty of Philosophy, the evening section (in 1963), and of Faculty of Agronomy, the section with no regular frequency (in 1969), both in Iaşi. He completed and supported his doctoral thesis entitled "Flora and vegetation from Depression and Elan hills (Vaslui county)" in 1973, under the guidance of Professor Constantin Burduja, obtaining the title of the Doctor in Biology (Geobotanical specialty).

On a professional level, after graduating his bachelor's degree in Biology-Botany, he was appointed as a Preparator at the Botany Department of the Agronomic Institute of Iaşi, where he advanced to the position of Head of Laboratory in 1957, becoming an University Assistant in 1959.

In 1961 he transferred to the Pedagogical Institute in Iaşi, advancing to the position of University Lecturer, and in 1964 he advanced to the position of the University Reader. Also, in the same year he became Head of the Department, so that from 1965 he became Prodean of the Faculty of Natural Sciences. Here he teaches the disciplines of morphology, systematics and plant physiology, of microbiology and technique of preparation of biological material, and others. As a result of the integration, in 1968, of the Pedagogical Institute into the structures of the "Alexandru Ioan Cuza" University, he taught courses in systematic botany, general biology, phytocoenology and the vegetation of Romania to the students of the Faculty of Biology-Geography-Geology. For a short period of time, starting in 1990, he taught the Theoretical Biology course to the students of the Faculty of Philosophy of the

University “Alexandru Ioan Cuza”. In the same year, he was promoted to the position of University Professor.

In 1975 he acted as a Professor-Cooperating of the University of Kisangani (Republic of Zaire), teaching, in french language, the courses of Tropical Taxonomy and Synecology.

Due to his scientific probity, Professor Dumitru Mititelu has fulfilled many professional-scientific functions, such as: member of the Professional Council of the Faculty of Biology, member of the University Senate, member of the Scientific Council of the Botanic Garden, scientific secretary of the Professional Council of the Pedagogic Institute, secretary of the Subcommittee of the Romanian Academy for the Protection of Nature in Moldova, member of the Group of the Romanian Academy for the mapping of the Carpathian flora and vegetation, editorial secretary of the publication “Scientific Works” of the Pedagogic Institute, and member of the editorial board of the *Biology* series of the *Scientific Annals of the University* “Alexandru Ioan Cuza”, Iaşi.

Being an acknowledged specialist, he was appointed as Doctoral supervisor of Faculty of Biology, “Alexandru Ioan Cuza” University in Iaşi, starting with 1992, in the Systematic Botany and Phytocoenology specialties.

During his life, Professor Dumitru Mititelu has been a scientific reviewer in many commissions for the award of doctoral degrees in biology, university teaching degrees or scientific degrees in various research institutes. He has led and guided many methodological-scientific works for granting the first degree in pre-university education and many graduate or master's degree works of biology graduates.

As students, we were enchanted both in the classrooms, but also in the botanical trips, by the vast scholarship of Professor Dumitru Mititelu, also by his botanical knowledge and didactic mastery.

The festive graduation courses of the students of Faculty of Biology, whenever he was invited to lecture, were very lively and attractive, leading the audience in many fields of Romanian and universal science and culture.

As a result of his professional activity and his numerous botanical incursions in the field, he left a very rich didactic material, represented by large color plates, includng all the plant species studied by students in the disciplines of Systematic Botany or Phytocoenology, a representative herbar used in the classrooms at the practical works by students, a personal collection of over 8,000 color slides with plants photographed by himself in their natural environment (slides presented to students in their classrooms) or with various aspects of the protected natural areas of Romania and visited countries.

He wrote, alone or in collaboration, a lot of scientifical papers in which he brought to the attention the personal biographies and scientific contributions of over 150 Romanian botanists, thus contributing decisively to the knowledge of the history of Romanian botany. Professor Dumitru Mititelu elaborated, in collaboration, floristic and geobotanical bibliographies on the historical provinces of Romania, as Moldavia, Bucovina, Maramureş, as well as Dobrogea, works that synthesized all botanical knowledges (up to the time of their appearance), from many Romanian or foreign scientific publications.

In the 43 years of professional activity (in 1998 he retired) he published, alone or with more than 80 collaborators, over 230 scientific, bibliographic works, reviews, etc., summing over 5,000 pages. The scientific fields addressed in these works are: flora and vegetation of Romania or Zaire, taxonomy and phytogeography of vascular plants, vegetal sozology, inventory of decorative woody plants, ecology and productivity of terrestrial ecosystems, phytocoenology, forestry and praticole typology, mapping of Romanian vegetation, etc.

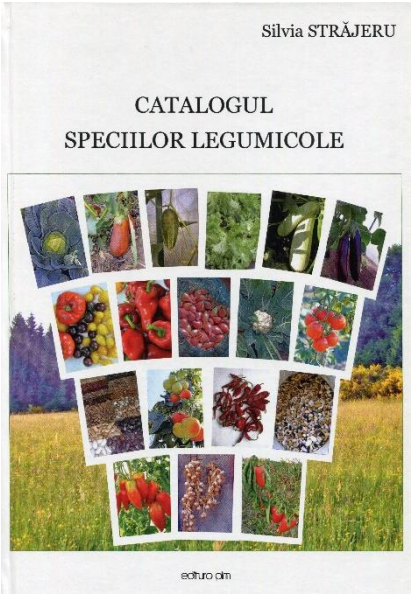
In recognition of his scientific probity, Professor Dumitru Mititelu was one of the co-authors of the monograph "Vegetation of Romania", published in 1992 (under the editorship of Professor Doina Ivan, University of Bucharest). Subsequently, the synthetic paper "Vegetation potentielle de la Roumanie", in which Professor Dumitru Mititelu was one of the collaborators, was published in *Braun-Blanquetia* scientific magazine, in Camerino, Italy, in 1993.

Among the scientific contributions of Professor Dumitru Mititelu is the description, as new for science (alone or in collaboration with other botanists), of a number of 28 plant communities (among which two plant communities of tropical epiphytic ferns, identified on the oil palm trees in Zaire) and two subassociations, identification of a significant number of new vascular plant species for the flora of Romania or Moldavia, elaboration of botanical and phytocoenotic monographs for all the counties of Moldavia, as well as monographs for many botanical reserves in Moldavia, Muntenia, Transylvania, Dobrogea and Maramureş, of some botanical field guides on some areas of Moldavia or Dobrogea, etc.

For all his scientific contributions, for his qualities as a teacher, for his honesty and humanity, Professor Dumitru Mititelu will remain forever in the memories of those who were close to him. For the community of Romanian scientists he will remain a landmark of competence and hard work in the field of Romanian botany.

Adrian OPREA
"Anastasie Fătu" Botanic Garden, "Alexandru Ioan Cuza" University of Iaşi

SILVIA STRĂJERU, ***Catalogul speciilor legumicole [Catalogue of vegetable species]***, 2018, PIM Publishing House, Iași, 180 p, ISBN 978-606-13-4639-4.



The preservation of agro-biodiversity genetic resources, of the wild genitors and regionally and locally cultivated varieties is of great importance for humankind, both in terms of their role as sources of food, condiments, fodder, fuel, textile fibres, bioactive compounds for the medical and pharmaceutical industries, traditional medicine, but also for their potential as genetic material for developing new and improved varieties with higher productivity and adapted to various climatic conditions, being at the same time a crucial component of social and cultural values.

One of the most efficient and safest way to preserve these resources is using *ex situ* conservation methods of genotypes with different origins within Gene Banks, under strict and controlled conditions. In Romania, the activities regarding this aspect started with the first breeding attempts, but is not until 1990 that a real gene bank was founded by PhD engineer Mihai Cristea in Suceava. The extensive collections of this institution, which include traditional varieties and national/international new created cultivars, represent an important source of material for scientific research in various fields: agriculture, plant breeding, biotechnology etc.

With this volume, the author, Silvia Străjeru, Scientific Researcher and Director of the Suceava Plant Genetic Resources Bank "Mihai Cristea" presents the valuable inventory of the plant species and cultivars managed by three major Romanian entities involved in the preservation of cultivated vegetal germplasm: Suceava Plant Genetic Resources Bank "Mihai Cristea", Bacău Vegetable Research and Development Station and Dăbuleni Research and Development Station for Plants Grown on Sandy Soils.

The volume is divided into three different parts. The first part presents the significance of passport descriptors that characterize every entry, the FAO codes for the

country/institute of origin and a theoretical presentation regarding the definition and classification of the vegetable species, their importance and, for every species, the main vegetal organ used for alimentary purposes.

The second part represents the actual inventory of the 97 species, subspecies and varieties of vegetables (Dicotyledonatae and Monocotyledonatae), with 6.828 entries, alphabetically ordered and accompanied by relevant and specific data regarding the popular name (in Romanian and English), origin and unique identification code. The highest number of cultivars belong to *Phaseolus* sp. (beans, kidney beans, dwarf beans, runner beans,) 3.357 entries, followed by *Vicia* sp. (faba beans, horse beans, tick beans) with 806 entries, *Capsicum* sp. (peppers, round peppers, long peppers, bell peppers, hot peppers) with 406 entries and *Solanum lycopersicon* (tomatoes, cherry tomatoes) with 345 varieties.

The third part includes 17 maps illustrating the origin of the main landraces described in the catalogue and a selection of 147 photographs of the fruits and seeds of cultivars listed in the Catalogue.

Through this volume, the author presents to the public the extensive and valuable collections of vegetable species preserved in three major Romanian institutions that activate in the field of *ex situ* conservation and development of cultivated plant varieties, which represent an important source for further research activities and also an essential part of our national cultural and historical legacy.

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"Anastasie Fătu" Botanic Garden, "Alexandru Ioan Cuza" University of Iași
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JOURNAL OF PLANT DEVELOPMENT
GUIDE TO AUTHORS

AIMS AND SCOPE OF THE JOURNAL

Journal of Plant Development is the official scientific journal of the "Anastasie Fătu" Botanical Garden, which belongs to "Alexandru Ioan Cuza" University from IAŞI, ROMANIA. It was first published in 1979 (at that time as "Culegere de Studii şi Articole de Biologie"). The new series begins in 1993 under the name "Buletinul Grădinii Botanice Iaşi". From 2008 on, it has been published under its present name "**Journal of Plant Development**". It appears in one volume, with one or two issues per year.

Journal of Plant Development (JPD) is an international journal that acts as a medium for the exchange of ideas and provides publication (yearly) of articles in all areas of Plant Science and Botany (of all 'plant' groups in the traditional sense - including algae, cyanobacteria, fungi, myxomycetes). It covers topics in plant development field, as well as the plant ecology. The Journal also covers related fields such as: plant conservation, plant taxonomy, plant embryology, phytosociology, ecology, plant morpho-anatomy and histology, comparative and developmental morphology, physiology, ecophysiology, plant distribution, natural and artificial habitats, ornamental plants, pharmaceuticals uses of plants, plant molecular biology, plant cell, tissue and organ culture etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. All articles published in JPD are peer-reviewed.

TYPES OF MANUSCRIPTS AND LANGUAGE

The journal publishes original research articles, short communications and reviews in English. Journal of Plant Development also publishes book reviews and conference reports. Manuscripts may be of any length, but must be clearly and concisely written.

Three main *types* of manuscripts may be submitted:

Original research articles: should reports results of a substantial, completed and original work, and describe new and carefully confirmed findings. Experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short communications: are suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods or techniques. The style of main sections need not conform to that of original research articles. Short communications are 2 to 4 pages in length.

Review articles. Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Review articles are critical evaluations of material that has already been published. By organizing, integrating, and evaluating previously published material, the author considers the progress of current research toward clarifying a problem. A review article is a tutorial in that the author defines and clarifies a problem, summarizes previous investigations in order to inform about the state of current research, identifies relations, contradictions, gaps, and inconsistencies in the literature, suggests the next step or steps in solving the problem. Reviews should be concise and no longer than 14-16 printed pages. Reviews are also peer-reviewed.

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Journal of Plant Development publishes fully open access articles, which means that all articles are available on the internet to all users immediately upon publication. Non-commercial use and distribution in any medium is permitted, without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles in this journal without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of open access.

REVIEWING POLICY

All contributions are subject to a double-blind reviewing process. Acceptance of papers is supervised by an international Editorial Board. Manuscripts considered suitable for peer review by the Editorial Board are sent to at least two referees (members of the Board or external). The journal gives referees a target of four weeks for the return of their reports. The review process takes between three weeks and two months.

PROOFS AND REPRINTS

Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage. Because JPD will be published freely online to attract a wide audience, authors will have free electronic access to the full text (in both HTML and PDF) of the article. Authors can freely download the PDF file from which they can print unlimited copies of their articles.

SUBMISSION

Manuscripts should be submitted electronically by sending a message to gbot.is@uaic.ro or ana.cojocariu@uaic.ro. The message should include:

(1) a cover letter, that should include the corresponding author's full name, address and telephone/fax numbers and should be in an e-mail message sent to the Editor. A Cover Letter is to be made upon submission, sending a revision or re-submission.

(2) a text file with the entire text, as an attachment, whose name should begin with the first author's surname.

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Submission of a paper implies that it has not been published before (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors, and that, if accepted, will not be published elsewhere in the same form, in English or in any other language, without the written consent of the publisher.

JPD Formatting and template: http://www.plant-journal.uaic.ro/instructions.htm

Journal of Plant Development have its own specific formatting. This defines how an article will look when it is published online or in print. In order to format your article ready for submission to JPD, **Word Template** are available on the website, ready for you to download and apply to your document. Read the instructions for the author/s, download and save the word template file to your computer and apply these styles to your paper as appropriate.

Authors should consult the **checklists** given here on **how to prepare the files**. Authors are expected to have their papers well checked for content and correctness in presentation of text and illustrations. Manuscripts not conforming to the guidelines will be returned to the author until satisfactory files are provided.

The corresponding author receives by e-mail an acknowledgment of receipt of the manuscript, mentioning the communicating editor and a manuscript reference number (Article ID). The manuscript number will be mailed to the corresponding author same day or within 72 hours. If you do not receive an acknowledgement you should inquire to be sure it was received.

Details on types of contributions

1. Original research articles

The papers will be published only in a foreign language (English), structured as follows: title, authors, affiliation of the authors, abstract, keywords, introduction, material and method, results & discussions, conclusions, acknowledgements, references, tables, figure captions.

Title should be a brief phrase describing the contents of the paper.

Authors names would not be abbreviated, capitals for surname (family name) and no capitals for first name (except initial letter). Each author name would be accompanied by a complete address, as a footnote on the first page. The affiliation should be provided in the following order: university (institution) name; faculty/department name; number and street name; city; country and email address. One of the authors should be designated as the corresponding author.

Abstract should be concise informative and completely self-explanatory, briefly present the topic, state the purpose of the research, indicate significant data, and point out major findings and conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. References should therefore be avoided, but if essential, they must be cited in full, without reference to the reference list. Non-standard or uncommon abbreviations should be avoided but, if essential, they should be defined at their first mention in the abstract itself.

Key Words: five to seven words, the most important ones, in alphabetical order, after someone could discover your paper on the internet engines. Key words should not repeat the title of the manuscript.

The **Introduction** should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines. The introduction should conclude with a brief statement of the overall aim of the experiments and a comment about whether that aim was achieved.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely

without referring to the literature. Discussion and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The **Discussion** should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

Conclusions should spell out the major findings of the work and may include some explanation of the significance of these conclusions and what further research should be done. Authors should include a general interpretation of the results in the context of current evidence, and not restricted to that which supports the findings of the present study.

Notes on contributors in maximum 65 words, provide short biographical notes on all contributors.

The **Acknowledgments** of people, grants, funds, etc. should be brief. People who contributed to the work but do not fit criteria for authorship should be listed in the Acknowledgments, along with their contributions. It is the authors' responsibility to ensure that anyone named in the acknowledgments agrees to being so named.

References should be listed at the end of the paper in alphabetical order. In the text, a reference identified by means of an author's name should be followed by the date of the reference in parentheses. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text. Authors are fully responsible for the accuracy of the references.

2. Short communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. Short Communications follow the same format as for the original research papers, with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion sections should be combined into a single section.

3. Review articles

Review articles are critical evaluations of material that has already been published. By organizing, integrating, and evaluating previously published material, the author considers the progress of current research toward clarifying a problem. Reviews should be concise and no longer than 14-16 printed pages. Reviews are also peer-reviewed.

4. Book reviews and conference reports

These types of contributions would not exceed an A4 format page.

5. Special Issues

Proposals for Special Issues of full research papers that focus on a specific topic or theme will be considered.

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	☐ **Abbreviations** are used for units of measurement, molecular terminology, common statistical terms (e.g. ANOVA, *t*-test and r^2), names of chemicals (e.g. ATP, Mes, Hepes, NaCl, O_2), and procedures (e.g. PCR, PAGE, RFLP). Other abbreviations are spelled out at first mention and all terms are written out in full when used to start a sentence.

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