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ANATOMICAL-HISTOLOGICAL OBSERVATIONS CONDUCTED ON AQUATIC FERNS IN THE DANUBE DELTA

Anca SÂRBU[1*], Daniela SMARANDACHE[1], Antonia Teona MARINESCU[2], Anca Monica PARASCHIV[3], Clara MIHAI[1], Andreea Maria VELICU[4]

Abstract: This paper analyses aquatic ferns from the genera *Azolla* Lam., *Marsilea* L. and *Salvinia* Séguier, which occur in the Danube Delta, Romania, and comprises a series of anatomical and histological observations of taxonomical, chorological and eco-morphological importance. The research conducted on specimens collected between 2005-2013 from the natural habitats of the Danube Delta, but also from the extra-deltaic artificial habitats have enabled: i) a reconsideration of some chorological aspects regarding the species of the genus *Azolla* in Romania; ii) a greater understanding of the adaptive plasticity relative to the factor water for the taxon *Marsilea quadrifolia* L. collected from natural and artificial habitats; iii) the enrichment of the data regarding the structural characteristics of the taxon *Salvinia natans* (L.) All., particularly around the adaptive elements associated with living on the surface of the water.

Keywords: adaptability, anatomy, aquatic ferns, chorology, taxonomy.

Introduction

This paper discusses the aquatic ferns in the Danube Delta, namely the species of the genera *Azolla* Lam., *Salvinia natans* (L.) All. and *Marsilea quadrifolia* L.

The representatives of the genus *Azolla* in Romania are *Azolla filiculoides* Lam., *A. caroliniana* Willd. and *A. mexicana* C. Presl., aquatic-natant adventive hydrophytes [SÂRBU & al. 2013]. As regards the presence and the distribution of these taxa in the flora of Romania, numerous bibliographical references have been made over time. Initially, only *Azolla filiculoides* and *A. caroliniana* were mentioned in the flora of Romania [ANTONESCU, 1951; ȚOPA, 1952; CIOCÂRLAN, 1994]. In the year 2000, in the paper The Illustrated Flora of Romania *Pteridophyta* et *Spermatophyta* [CIOCÂRLAN, 2000] the species *Azolla filiculoides* and *A. mexicana* are noted, the latter with unconfirmed presence. In subsequent papers [OPREA, 2005; SÎRBU & OPREA, 2011; SÂRBU & al. 2013] all three species of the genus *Azolla* (*filiculoides*, *caroliniana*, *mexicana*) are mentioned in Romania, but for the Danube Delta only the presence of the taxon *Azolla filiculoides* is recognised [OPREA, 2005; CIOCÂRLAN, 2011; SÂRBU & al. 2013].

Salvinia natans, an aquatic-natant, annual hydrophyte, protected at European level [Council Directive 92/43/EEC, 1992 – Habitat Directive] is mentioned at being presented in

[1] University of Bucharest, Faculty of Biology, Department of Botany-Microbiology, Intrarea Portocalelor no. 1-3, Sector 6, Bucharest – Romania.

[2] Graduate of the Faculty of Biology in 2010, University of Bucharest – Romania.

[3] University of Bucharest, Botanical Garden "D. Brandza" of Bucharest, Sos. Cotroceni no. 32, Sector 6, Bucharest – Romania.

[4] Graduate of the Faculty of Biology in 2016, University of Bucharest – Romania.

[*] Corresponding author. E-mail: anchusa24@yahoo.com

the Danube Delta [ȚOPA, 1952; CIOCÂRLAN, 1994; OPREA, 2005, SÂRBU & al. 2005; SÂRBU & al. 2011; SÂRBU & al. 2013], particularly in the habitat type 3150 – Natural eutrophic lakes with vegetation of *Magnopotamion* or *Hydrocharition* [GAFTA & MOUNTFORD, 2005], in lakes, ponds, moors, shallow closed canals, spread across the deltaic complex [SÂRBU, 2003; SÂRBU, 2006; SÂRBU & al. 2013].

Marsilea quadrifolia, a perennial hydro-hygrophyte plant, vulnerable in Romania [OLTEAN & al. 1994] and protected at European level [Council Directive 92/43/EEC, 1992 – Habitat Directive], is present in the Danube Delta through two forms of growth, namely f. *terrestris* and f. *natans* [ȚOPA, 1952; CIOCÂRLAN, 1994; SÂRBU, 2002; SÂRBU, 2015]. The species is mentioned in the Natura 2000 habitats in the Danube Delta, belonging to the habitat type 3150 - Natural eutrophic lakes with vegetation of *Magnopotamion* or *Hydrocharition*, in shallow stagnant waters and swampy areas, which offers them an enhanced conservation value [SÂRBU & al. 2013]. In 2013, the presence of this taxon was reported in the specialty literature also for the artificial aquatic habitats in Romania [SÂRBU & al. 2014]. The plant was identified in a pre-decanter of the Arcuda Station for Treating and Drinking Water Production.

This paper presents a series of anatomical and histological observations regarding the taxa mentioned above. These observations are of taxonomical and chorological importance in the case of the *Azolla* species, while new structural information of eco-morphological importance is provided for *Marsilea quadrifolia.* Using optical microscopy images, the paper also documents a series of data from the literature regarding *Salvinia natans.*

Materials and methods

For the representatives of the genus *Azolla*, vegetal material was collected during the period 2005-2010 from several aquatic ecosystems of the Danube Delta: Mila 23 Zone, Șontea Canal and Înfundata Canal (Fig. 1). The specimens of *Salvinia natans* were collected during the period 2005-2008, from shallow waters connected to the Magearu Canal (Fig. 2). *Marsilea quadrifolia* was collected from two types of habitat: i) natural habitats from the Danube Delta, where specimens of f. *natans* and f. *terrestris* were collected between 2004 and 2005 (Fig. 3, Fig. 4) and ii) an artificial habitat represented by the pre-decanter of the Arcuda Station for Treating and Drinking Water Production, where three samples of the f. *natans* were collected in 2013 (Fig. 5, Fig. 6).

The vegetative organs were analysed morphologically, anatomically and histologically: i) the leaf of the specimens of *Azolla*, with an emphasis on the characteristics of the epidermal trichomes which are important in the identification of the species, ii) the floating leaf, the rhizophyll (root-like leaf submerged) and the stem of *Salvinia natans*, and iii) the rhizome, petiole and lamina of the two forms of growth of the taxon *Marsilea quadrifolia*. The vegetal material was analysed as required, eitherun-sectioned and observed in apical image (e.g. *Azolla*), or sectioned (cross-section, paradermal section), processed according to the double coloration technique (Carmine alum and Iodine green) or treated with specific identification substances such as IIK and Sudan III [ȘERBĂNESCU-JITARIU & al. 1983]. The cross sections were carried out, through the median area of the vegetative organs.

The dimensional measurements were made with the micrometer kit and the microphotographs were taken using the optic microscope DOCUVAL, equipped with a photographic camera NIKON 90. All the optical microscopy images are original: Anca Sârbu (Fig. 1-25, 27-30, 32-54), Daniela Smarandache (Fig. 26, 31, 54 – the schema).

Fig. 1. *Azolla filiculoides*, Danube Delta.

Fig. 2. *Salvinia natans*, Danube Delta.

Fig. 3. *Marsilea quadrifolia*, f. *natans*, Danube Delta.

Fig. 4. *Marsilea quadrifolia*, f. *terrestris*, Danube Delta.

Fig. 5. *Marsilea quadrifolia*, f. *natans*, Arcuda Station.

Fig. 6. *Marsilea quadrifolia*, f. *natans*, Arcuda Station.

Results and discussion

***Azolla* species.** According to the data in the literature [LUMPKIN, 1993; SÂRBU & al. 2013] the three species of the genus *Azolla* (*filiculoides*, *caroliniana*, *mexicana*) mentioned in the flora of Romania are thermophile plants, alien to Romania and native to North America. Of these three species, only *Azolla filiculoides* has been confirmed for the Danube Delta.

These taxa differ among themselves through several types of characteristics such as the size of the plants and the leaves, the morphology of the megaspores and the aspect of the glochidia [TRYON & TRYON, 1982; CODY & BRITTON, 1989; STRASBURGER & al. 1990], but also through the characteristics of the epidermal trichomes [LUMPKIN, 1993; SÂRBU & al. 2013]. The species of the genus *Azolla* are often much more difficult to differentiate on the basis of the morphology of the vegetative body or the characteristics of the megaspores, because often the sporocarps are absent.

Given these difficulties, this paper aims to highlight the characteristics of the epidermal trichomes using microscopy images, and to use these images as an initial tool for the differentiation of the *Azolla* species recorded in Romania.

According to the description provided in the Flora of the North America [LUMPKIN, 1993], the characteristics of the epidermal trichomes are the following:

- *Azolla filiculoides* has strictly only unicellular trichomes, located on the superior lobes of the leaves;
- *Azolla caroliniana* has the longest trichomes, formed of two or more cells, located on the superior lobe of the leaf, close to the stem; the apical cell is often curved;
- *Azolla mexicana* has the longest trichomes formed of 2(-3) cells, located on the superior lobe of the leaf, close to the stem; the apical cell is often curved.

Based on the characteristics of the epidermal trichomes, the *Azolla* plants collected in the Danube Delta can be grouped in three categories: *Azolla filiculoides* – collected on the Şontea Canal, *Azolla caroliniana* – collected in the Mila 23 area, and probably *Azolla mexicana* – collected on the Înfundata Canal. All the taxa have small, sessile, bi-lobed, imbricate leaves with a membranous margin. In 1972, OGURA defined these lobes as 'natant' – for the superior lobe, and 'submersed' – for the inferior lobe. Subsequently KAUL (1976) introduced the terms 'aerial lobe' for the superior lobe, and 'submersed lobe' for the inferior one.

Regardless of their position, all the foliar lobes have a membranous margin, which becomes narrower towards the top. The term 'membranous margin' is somewhat inadequate because this part of the leaf is in fact a multi-cellular structure, unistratified, formed by hetero-dimensional cells, which contain chloroplasts. The remaining part of the leaf, has a pluristratified structure, with prominent and ramified nervures. The two epidermises, superior and inferior, are organised differently and they differentiate stomata. Below the superior epidermis there is a layer of assimilating cells, rich in chloroplasts, which offers the dark green colour to the foliar lobes.

At *Azolla filiculoides* (Fig. 7, Fig. 8) the superior epidermis of the foliar lobes features stomata and strictly unicellular trichomes, displayed relatively uniformly (Fig. 9). These have a papilliform appearance (Fig. 10) and are 50-60 μm long. The papilliform formations that are differentiated by the superior epidermis of the foliar lobes were described

by OGURA as early as 1972. Subsequently, in the identifying keys these have been considered unicellular trichomes. The stomatic cells have a relatively triangular form (Fig. 11). The inferior epidermis has elongated cells with a sinuous outline in apical image. These cells contain chloroplasts (Fig. 12).

At *Azolla caroliniana* (Fig. 13) the superior epidermis of the foliar lobes differentiates unicellular, papilliform trichomes (Fig. 14), ~50 µm long, similar to the ones observed at *Azolla filiculoides*. The epidermises also differentiate stomata, with relatively triangular shaped stomatic cells, which in some cases coalesce, forming a circular stomatic cell approximately 100 µm long (Fig. 15). The inferior epidermis of the foliar lobes is formed of cells with a sinuous outline (~80-100 µm long; ~30-50 µm wide), rich in chloroplasts (Fig. 16) and differentiate long, uniseriate, multicellular trichomes of 3-12 cells, with a long basal cell and the apical cell acuminate curved (Fig. 17, Fig. 18).

Azolla mexicana differs from the previous two taxa through the characteristics of the epidermal trichomes. At this species, the superior epidermis of the foliar lobe differentiates papilliform unicellular trichomes (~60 µm long), but also bi-cellular trichomes (Fig. 19, Fig. 20).

The three groups of plants included in the genus *Azolla* and collected in the Danube Delta, differ among themselves through the characteristics of the epidermal trichomes, as suggested by the specialty literature [LUMPKIN, 1993].

The epidermal trichomes can be considered an initial anatomical clue towards the differentiation of the three species of *Azolla* recorded in Romania. According to this criterion, it can be argued that in the Danube Delta there exist not only *Azolla filiculoides* and *A. caroliniana*, but most likely also *Azolla mexicana*. Further morphological and anatomical research on the latter taxon is required.

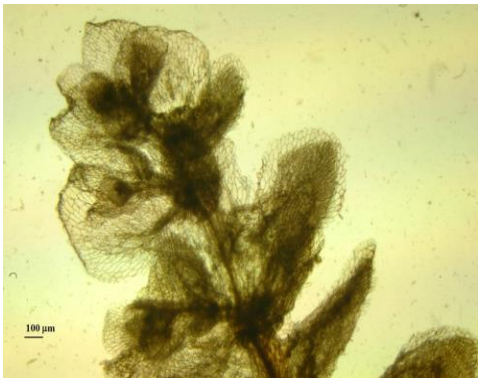

Fig. 7. *Azolla filiculoides* – a fragment.

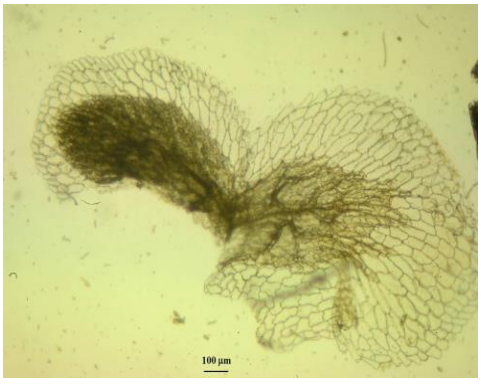

Fig. 8. *Azolla filiculoides* – foliar lobes.

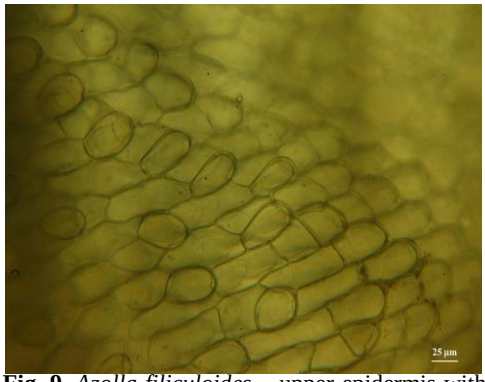

Fig. 9. *Azolla filiculoides* – upper epidermis with unicellular, papiliform trichomes (in front side view).

Fig. 10. *Azolla filiculoides* – upper epidermis with unicellular, papiliform trichomes (lateral view).

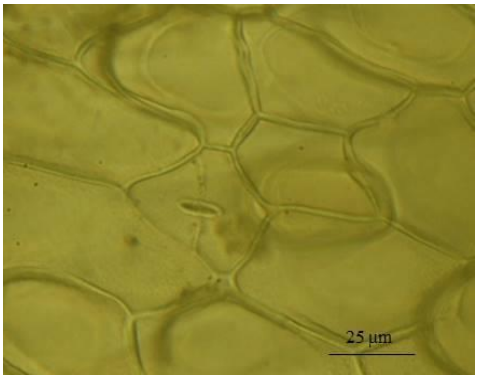

Fig. 11. *Azolla filiculoides* – upper epidermis with stomata (in front side view).

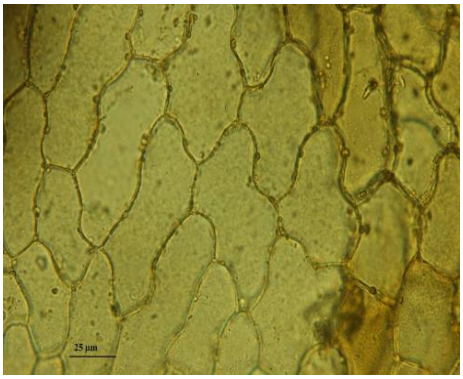

Fig. 12. *Azolla filiculoides* – lower epidermis (in front side view).

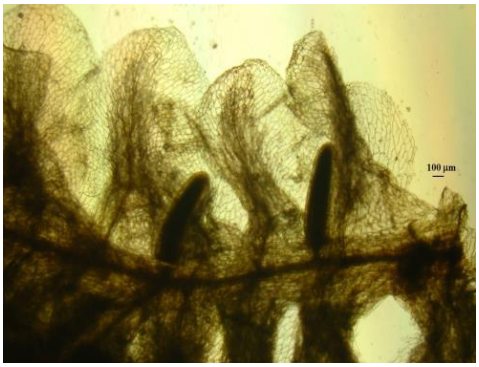

Fig. 13. *Azolla caroliniana* – a fragment.

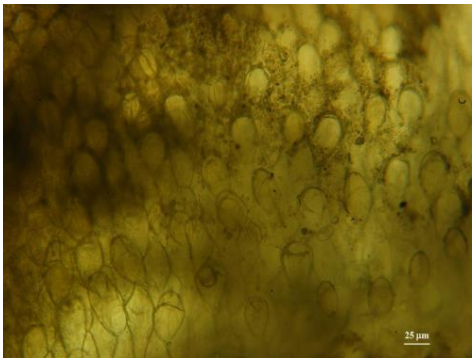

Fig. 14. *Azolla caroliniana* – upper epidermis with unicellular, papiliform trichomes (in front side view).

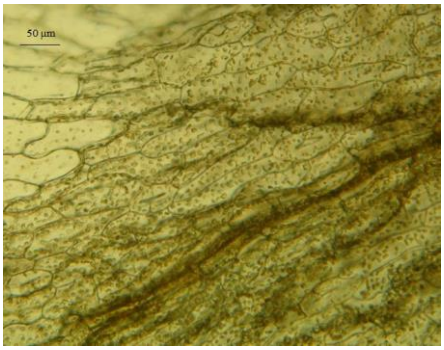

Fig. 15. *Azolla caroliniana* – lower epidermis with stomata (in front side view).

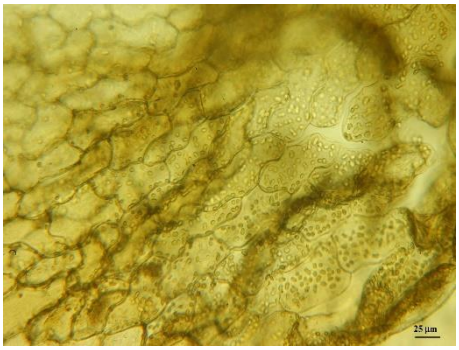

Fig. 16. *Azolla caroliniana* – lower epidermis (in front side view).

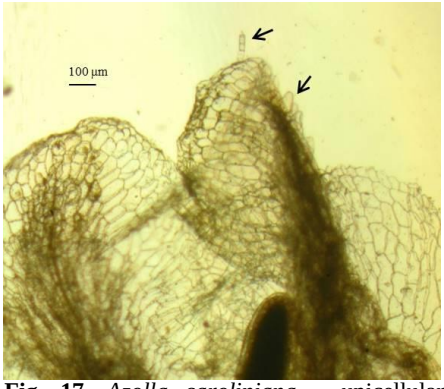

Fig. 17. *Azolla caroliniana* – unicellular papiliform on the upper epidermis and multicellular trichomes on the lower leaf epidermis.

Fig. 18. *Azolla caroliniana* – multicellular trichomes, on the lower leaf epidermis.

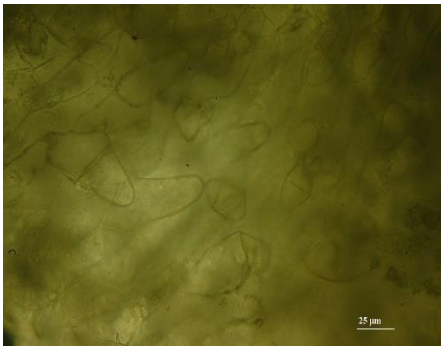

Fig. 19. *Azolla mexicana* – upper epidermis with unicellular and bicellular trichomes (in front side view).

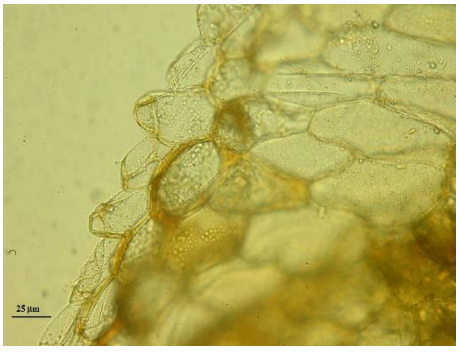

Fig. 20. *Azolla mexicana* – detail of bicellular trichomes.

The observations conducted on the specimen of *Azolla* collected in the Danube Delta highlighted the following aspects:

i. *Azolla filiculoides* features only papilliform unicellular trichomes, which are only differentiated at the level of the superior epidermis, while the inferior epidermis does not differentiate any trichomes,
ii. *Azolla caroliniana* features at the level of the superior epidermis papilliform unicellular trichomes and at the level of the inferior epidermis long and rare multicellular trichomes, with an acicular-curved apical cell,
iii. for *Azolla mexicana* at the level of the superior epidermis there have been observed both papilliform unicellular epidermal trichomes and bi-cellular trichomes, and
iv. at all the taxa analysed, the membranous margin of the foliar lobes is represented by a unistratified and photosynthesising multicellular structure.

Salvinia natans. This fern, which floats on the surface of the water, has a vegetative body formed of a stem and leaves. The stem is short and ramified. The leaves are dimorphic, displayed in trimerous whorls. Two leaves are floating, whole, oval and oval-elliptical, papillated, pubescent and greenish. The third leaf in the whorl is submerged metamorphosed, finely divided and root-like, multi-trichomic and pendent in the water.

The floating leaf

The floating leaf is differentiated in the lamina and the short petiole. The lamina is bifacial, uni-nerve, with a mesophyll of 0.7-0.8 mm width, partitioned, formed of two overlaid layers of polygonal chambers (Fig. 21). The adaxial side of the lamina is concave and papillate. At the level of the papillae, the superior epidermis differentiates moniliform trichomes (shaped like a string of beads), up to 0.7mm long, grouped in bunches of 3-4. The abaxial side of the lamina, which is in contact with the water, is convex and differentiates multicellular trichomes (4-5 cells), uniseriate, ~0.5 mm long, with an acicular apical cell (Fig. 21, Fig. 22).

The two epidermises of the lamina have irregularly-shaped cells. The epidermises are kept distanced from each other by a network of polygonal spaces delimited by, elongated parenchymatic cells arranged uniseriat (Fig. 23). From this structure there start uni-stratified multicellular trabeculae which partition the mesophyll into two levels of hexagonal chambers (Fig. 24). The chambers of the mesophyll host septate, assimilating cells (Fig. 25). In the adaxial zone of the mesophyll, the assimilating cells are smaller (~120 µm long) and rich in chloroplasts. In the abaxial zone, the assimilating cells are bigger (~250 µm long) and have less chloroplasts. As KAUL mentioned in 1976, all the parenchymatic cells of the floating leaf contain chloroplasts, with the exception of the trichomes. It should be added here that these cells with a role in photosynthesis also accumulate starch (Fig. 24, Fig. 25).

The nervure, with a median position, is delimitated by a uni-stratified endodermis with cells that are 30-50 µm long. The cells of the endodermis have thickened and suberified walls (Sudan III). The pericycle is uni-stratified, and the vascular bundle from the structure of the nervure is hadrocentric (Fig. 26).

The root-like leaf (rhizophyll)

The rhizophylles are filamentous, cylindrical, with a circular outline in cross section (Fig. 27). The epidermis differentiates numerous multicellular trichomes (10-15 cells), uniseriated, with a sharp apical cell. The apical cells of the rhizophyllic trichomes often appear pluri-nucleate (Fig 28). The cortex is a well-represented aerenchym, formed of a ring

of eight aeriferous canals. The endodermis is present. The stele, delimitated by the pericycle, contains a hadrocentric vascular bundle.

The stem

The stem is short, cylindrical, with a diameter of 1.5-2 mm, with a circular outline in cross section (Fig. 29). The epidermis differentiates numerous trichomes 0.5-2.0 mm long, multicellular (up to 15 cells), uniseriated, with a sharp apical cell. The cortex is an aerenchym with a single ring of large aeriferous canals (8-9 canals), delimitated by uni-stratified multicellular walls (Fig. 30). The uni-stratified endodermis feature unequal cells (~25-60 µm long) with thickened and suberified cellular walls (Sudan III). The stele (~150 µm diameter) can be considered a protostele, with few phloem elements (Fig. 31). The disorganisation of the xylem from the central zone of the stele can often be observed, with a forming of lacuna (Fig. 32).

As regards the structure of the floating leaf, the rhizophyll and the stem of *Salvinia natans*, there are numerous relevant data in the specialty literature [OGURA, 1972; KAUL, 1976; CROXDALE, 1981; SEO & KIM, 2008; JAMPEETONG & BRIX, 2009]. In this context, our observations completed the existing data with optical microscopy images, highlighting the architecture of the mesophyll of the floating leave, the particularities of the endodermis, the formation of the medullary lacune, and the characteristics of trichomes, structures which ensure the efficient floating of the plant on the surface of the water and prevent the plant from sinking.

On the two types of leaves at *Salvinia natans* there are three types of trichomes: i) groups of moniliform trichomes at the level of the superior epidermis of the floating leaf, which due to their relatively spherical cells prevent the adaxial zone of the lamina from becoming wet, ii) solitary, uniseriated, multicellular trichomes, with a sharp apical cell, differentiated at the level of the inferior epidermis of the floating leaf, which contribute alongside the cameral mesophyll to the stability of the floating leaf on the surface of the water, and iii) submersed multicellular rhizophyllic trichomes, long and numerous, involved in absorption.

The presence of moniliform trichomes on the adaxial side of the lamina provides to it hydrophobe properties (water repellent surface). Drops of water remain on the top of the trichomes, while the stomata can continue their exchange of respiratory gases [CERMAN & al. 2009]. This characteristic offers the plant remarkable ecological advantages.

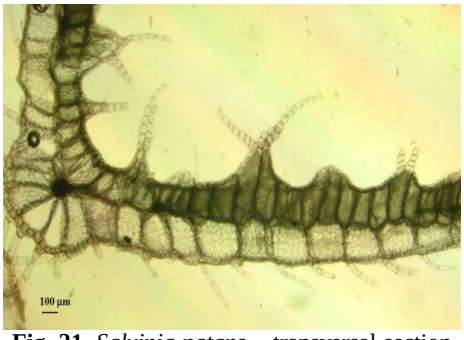

Fig. 21. *Salvinia natans* – transversal section throught the floating leaf (Iodine green, Carmine alum).

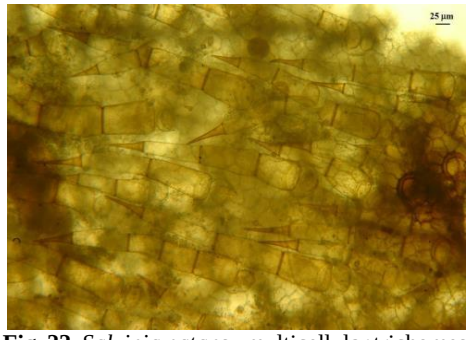

Fig. 22. *Salvinia natans* –multicellular trichomes on the lower epidermis (in front side view).

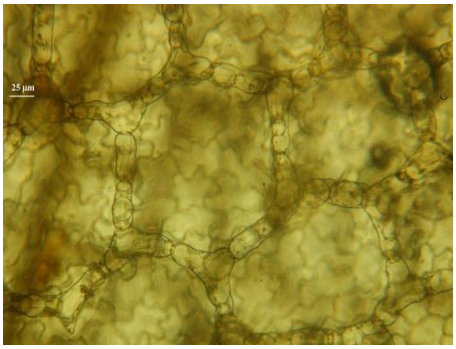

Fig. 23. *Salvinia natans* – subepidermal frame of the floating leaf (in front side view).

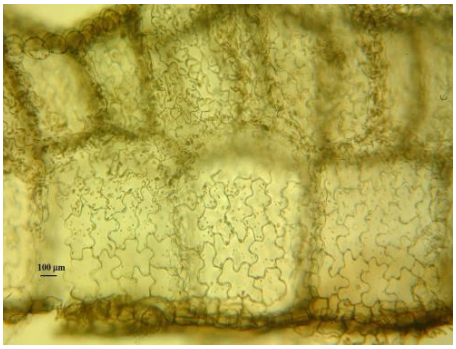

Fig. 24. *Salvinia natans* – pluricameral mesophyll of the floating leaf, in transversal section (IIK).

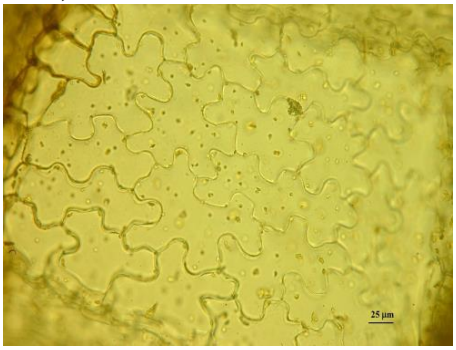

Fig. 25. *Salvinia natans* – cells from the adaxial chamber floor of the mesophyll (IIK).

Fig. 26. *Salvinia natans* – transversal section through the floating leaf (Sudan III).

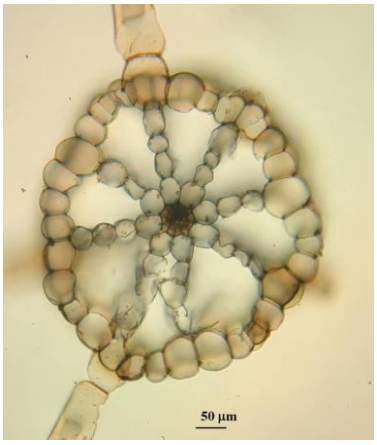

Fig. 27. *Salvinia natans* – transversal section through the rizophylle (Iodine green, Carmine alum).

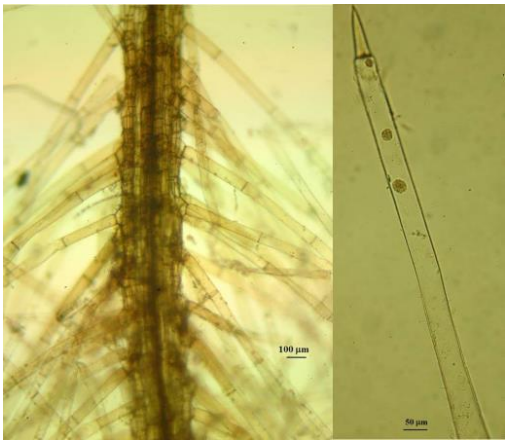

Fig. 28. *Salvinia natans* – the rizophylle and the rizophylle trichomes.

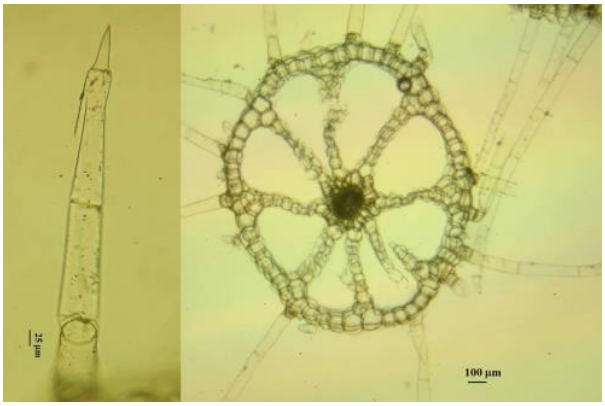

Fig. 29. *Salvinia natans* – transversal section through the stem, emphasizing structure and trichomes.

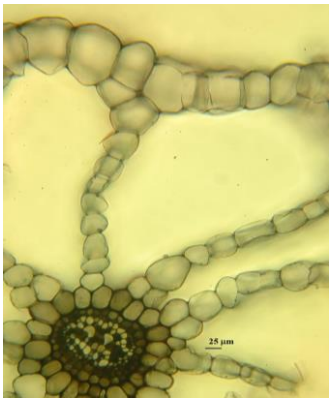

Fig. 30. *Salvinia natans* – transversal section through the stem (Iodine green, Carmine alum).

Fig. 31. *Salvinia natans* – the stele structure, in transversal section through the stem (Sudan III).

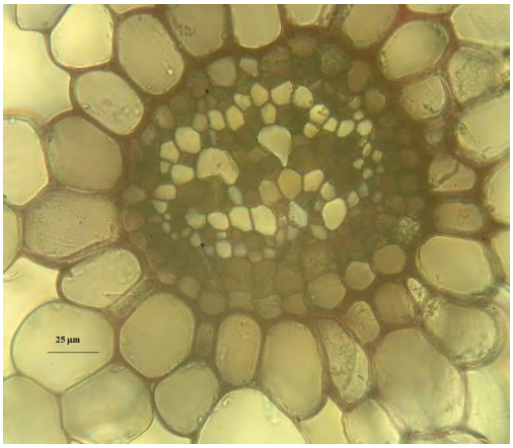

Fig. 32. *Salvinia natans* – transversal section through the stem highlighting the stele's gaps (Iodine green, Carmine alum).

Marsilea quadrifolia. This perennial hygro-hydrophyte, has a long rhizome (0.5-1 m), thin, crawling, on which there grow fixating, adventive roots and petiolate leaves with 4 obovate leaflets, with a whole edge.

The rhizome

For all the growth forms analysed, the rhizome has a circular outline in cross section (Fig. 33, Fig. 34, Fig. 35). The uni-stratified epidermis is formed of small cells, relatively isodiametrical.

The cortex is voluminous, reaching up to 70-80% of the diameter of the rhizome. The external cortex is represented by an aerenchym, with 20-22 large aeriferous canals, separated by uni-stratified trabeculae and containing diaphragmatic tissue (Fig. 36). The internal cortex, peristelic, is formed of 6-7 layers of parenchymatic cells at f. *terrestris* and f. *natans* collected in the Danube Delta, and 4-5 layers of cells with slightly and uniformly thickened walls at f. *natans* collected in Arcuda (Fig. 37). The last layer of the cortex is a

uni-stratified endodermis, formed of small cells (~10-12 µm long; ~6-10 µm wide). Caspary thickenings are small and lenticular in cross section. The cortical cells, except for the endodermis, accumulate starch (Fig. 38).

The stele is an amphiphloic siphonostele, with a dense parenchymatic pith at f. *terrestris* and meatic at f. *natans* (Fig. 39, Fig. 40, Fig. 41). At f. *terrestris* the stele has the largest diameter. This is about 20% smaller at the f. *natans* from the Danube Delta and about 50% smaller at f. *natans* from Arcuda, for which a reduction of 60% in the number of xylem vessels has been observed as compared to f. *terrestris*. Only at the f. *natans* from the Danube Delta a pith lacuna was observed (Fig. 40).

The petiole

The petiole is cylindrical, more or less long, depending on the form of growth and the type of habitat (Tab. 1).

At f. *terrestris*, the petiole is short and thick.

At f. *natans* the petiole is longer, but its diameter is smaller. The longest (35-40 cm) and thinnest (1.0 mm diameter) petiole was observed at f. *natans*, collected from Arcuda (Fig. 42).

Tab. 1. *Marsilea quadrifolia* – petiole, morphology data.

Growth form	Petiole length (cm)	Petiole diameter (mm)
f. *terrestris* – Danube Delta	8-10	2.5
f. *natans* – Danube Delta	20-25	1.3
f. *natans* – Arcuda Station	35-40	1.0

In the cross sections conducted through the median zone of the petiole, this features a circular outline (Fig. 43, Fig. 44, Fig. 45). The epidermis is uni-stratified. At f. *terrestris* it is formed of relatively isodiametrical cells in cross section and it differentiated stomata (Fig. 46). At f. *natans* from the Danube Delta, the epidermal cells are heterogeneous, small, and the stomata are absent (Fig. 47). At f. *natans* collected from Arcuda the epidermal cells are rectangular and have uniformly thickened walls (Fig. 47).

The cortex is voluminous, representing 80% of the diameter of the petiole at f. *terestris* and 85% at f. *natans*. The external cortex is an aerenchym with 10-16 large aeriferous canals, separated from the uni-stratified trabeculae and containing a diaphragmatic tissue (Fig. 43, Fig. 44, Fig. 45). The internal cortex is parenchymatic and meatic at f. *natans* collected in the Danube Delta and at Arcuda (Fig. 44, Fig. 45).

At f. *terrestris* there is a median zone of the cortex formed of 1-2 layers of cells with slightly and uniformly thickened walls (sclerenchymatous cells) and a parenchymatic slightly meatic internal zone (3-4 layers of cells) (Fig. 43). The last layer of the cortex is an endodermis, uni-stratified, of primary type.

The stele has a roughly semi-circular outline in cross section. It is delimitated by a uni-stratified pericycle and has a protostelic structure. The xylem is well represented and is surrounded by phloem. The protoxylem has an exarch disposition, and the metaxylem formed of several large vessels is endarch. The protoxylematic lacuna is only present at f. *natans* (Fig. 48, Fig. 49, Fig. 50).

The lamina

The diameter and thickness of the lamina vary according to the form of growth and the type of habitat (Tab. 2). The natant forms have a wider and thinner lamina than f.

terrestris. At f. *natans* from Arcuda the lamina is ~40% wider and ~35% thinner than f. *terrestris*.

Tab. 2. *Marsilea quadrifolia* – lamina, morphology data.

Growth form	Lamina diameter (cm)	Lamina thickness (mm)
f. *terrestris* – Danube Delta	2.5	0.24
f. *natans* – Danube Delta	3.2	0.21
f. *natans* – Arcuda Station	4.0	0.16

The lamina is epistomatic at the floating forms and amphistomatic at f. *terrestris*. Analysed at the level of the leaflets, the lamina features at all three types of plants a dorsi-ventral heterogeneous structure, characterised by the presence of a zone of palisadic tissue, positioned adaxially, and a zone of lacunous tissue, displayed abaxially (Fig. 51, Fig. 52, Fig. 53). The nervures each contain a vascular bundle of hadrocentric concentric type, surrounded by a uni-stratified endodermis.

At the floating forms, the palisadic tissue is form of a single layer of long palisadic cells (~50-80 µm), and at f. *terrestris* from 1-2 layers of shorter palisadic cells (~30-40 µm long), but very rich in chloroplasts (Fig. 51, Fig. 52, Fig. 53). In this latter case, as the specialty literature mentions [ESAU, 1965], there is a transition zone between the palisadic tissue and the lacunous tissue. The lacunous tissue is more developed at the natant forms, where it forms aeriferous canals (Fig. 52, Fig. 53). At f. *terrestris*, the chloroplasts are abundant both in the palisadic cells and in those of the lacunous tissue (Fig. 51).

At f. *natans* collected from the artificial habitat at Arcuda (Fig. 54), where the lamina is wide (~4 cm) and thin (~0.16 cm), between the level of palisadic cells and the zone of lacunous tissue there is a continuous layer of short mechanical cells, represented by isodiametrical and slightly elongated sclereids, which contribute to the rigidity of the lamina.

The general organisation of the vegetative body of the species *Marsilea quadrifolia* analysed from a morphological and anatomical point of view generally fits in with the characteristics mentioned for this plant in the literature [OGURA, 1938; TRYON & TRYON, 1982; GRINȚESCU, 1985; LERSTEN, 1997; TOMA & GOSTIN, 2000; BERCU, 2004].

However, differences have been noted as regards the forms of growth, reflecting the adaptation of the species to life in palustre habitats with reduced humidity, but also in natural and artificial aquatic habitats of different depths. As such, the transition from the aquatic environment to the terrestrial one is associated with a series of morphological and structural changes:

i. changes which enhance the resistance of the vegetative body, such as the reduction in the dimensions of the petiole and the lamina; the increase in the share of the xylem and the mechanical elements in the structure of the vegetative organs; the reduction of the intercellular spaces, and
ii. changes which support the respiratory and photosynthesis processes, such as the transition from epistomatic to amphistomatic leaves, the organisation of the mesophyll and the increase in the number of chloroplasts in the cells.

At *Marsilea quadrifolia* f. *natans* collected from Arcuda, the greater depth of the water accentuated some of the morphological changes, such as the elongation of the petiole, the enhancement of the foliar surface and the reduction of the thickness of the lamina, generating at the same time associated structural changes which help improve the resistance of the plant, such as the formation of sclereids in mesophyll, the thickening of the cellular

walls of the epidermis and cortical cells from the structure of the petiole, the lack of the pith lacuna in the rhizome.

In the literature, the maximum length of the petiole of the f. *natans* is 20-25 cm [ȚOPA, 1952; CODY & BRITTON, 1989]. Our observations have highlighted a greater adaptive plasticity of this parameter, and respectively this plant, in relation to the depth of the water, which points to the ability of this plant to populate a wider range of aquatic habitats.

Phytohormones are thought to be some of the most important factors responsible for the morphological and structural adaptive changes that occur in plants with the transition from one life environment to the other.

For example, the literature highlights that for *Marsilea quadrifolia* the transitioning from an aquatic environment to a terrestrial one involved the abscisic acid phytohormone (ABA) which, as it has been demonstrated, influences the length of the petiole, the morphology of the lamina and the structure of the internodes [LIN & al. 2005].

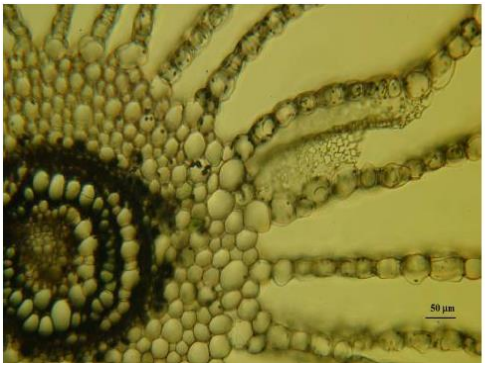

Fig. 33. *Marsilea quadrifolia*, f. *terrestris* – transversal section throught the rhizome (Iodine green, Carmine alum, IIK).

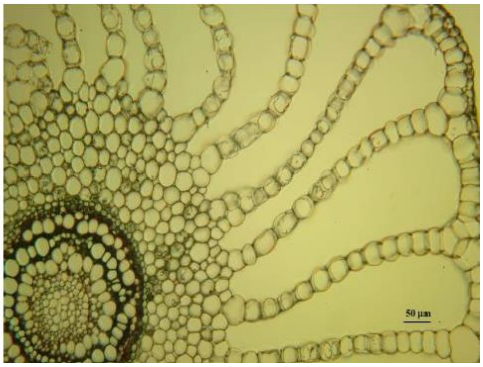

Fig. 34. *Marsilea quadrifolia*, f. *natans* (Danube Delta) – transversal section through the rhizome (Iodine green, Carmine alum).

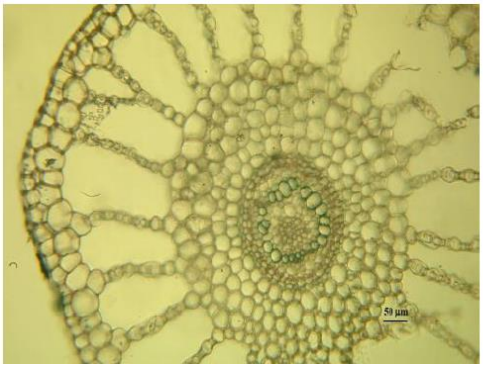

Fig. 35. *Marsilea quadrifolia*, f. *natans* (Arcuda Station) – transversal section through the rhizome (Iodine green, Carmine alum).

Fig. 36. *Marsilea quadrifolia*, f. *terrestris* – rhizome in transversal section; diaphragmatic tissue (Iodine green, Carmine alum).

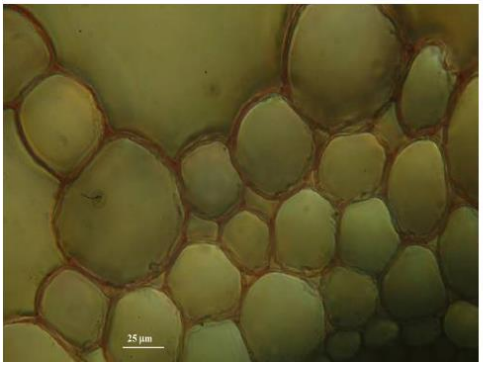

Fig. 37. *Marsilea quadrifolia*, f. *natans* (Arcuda Station) – rhizome in transversal section; peristelic area (Iodine green, Carmine alum).

Fig. 38. *Marsilea quadrifolia*, f. *natans* (Arcuda Station) – rhizome in transversal section; starch granules (IIK).

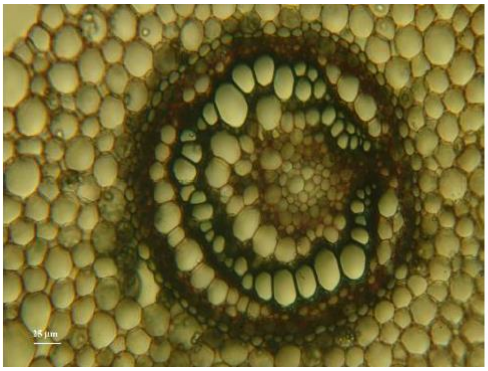

Fig. 39. *Marsilea quadrifolia*, f. *terrestris* – rhizome in transversal section; the stele (Iodine green, Carmine alum).

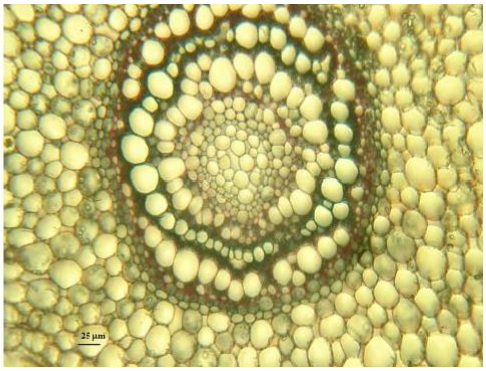

Fig. 40. *Marsilea quadrifolia*, f. *natans* (Danube Delta) – rhizome in transversal section; the stele (Iodine green, Carmine alum).

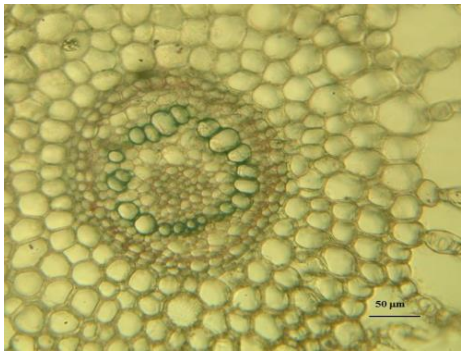

Fig. 41. *Marsilea quadrifolia*, f. *natans* (Arcuda Station) – rhizome in transversal section; the stele (Iodine green, Carmine alum).

Fig. 42. *Marsilea quadrifolia*, f. *natans* (Arcuda Station) – habitus.

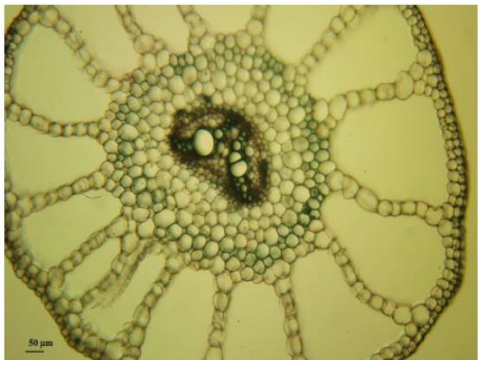

Fig. 43. *Marsilea quadrifolia*, f. *terrestris* – petiole in transversal section (Iodine green, Carmine alum).

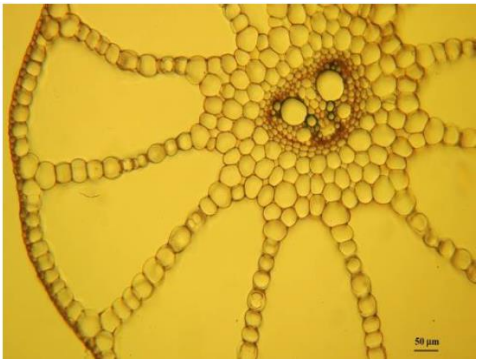

Fig. 44. *Marsilea quadrifolia*, f. *natans* (Danube Delta) – petiole in transversal section (Iodine green, Carmine alum).

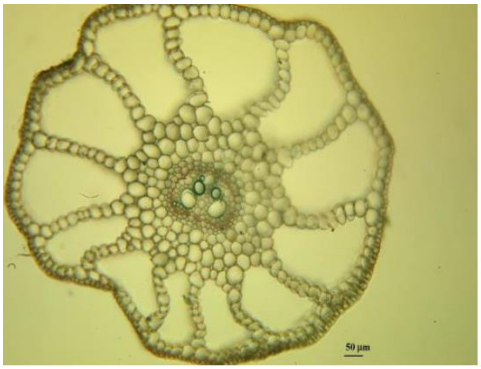

Fig. 45. *Marsilea quadrifolia*, f. *natans* (Arcuda Station) – petiole in transversal section (Iodine green, Carmine alum).

Fig. 46. *Marsilea quadrifolia*, f. *terrestris* – petiole in transversal section; the epidermis (Iodine green, Carmine alum).

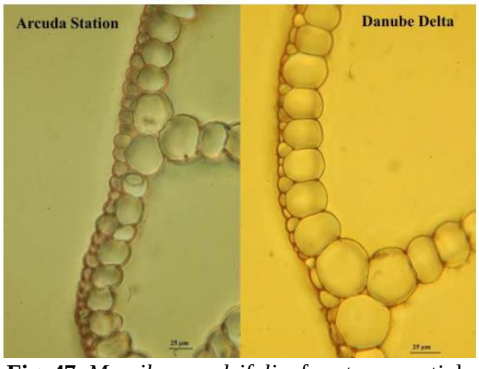

Fig. 47. *Marsilea quadrifolia*, f. *natans* – petiole in transversal section; the epidermis (Iodine green, Carmine alum).

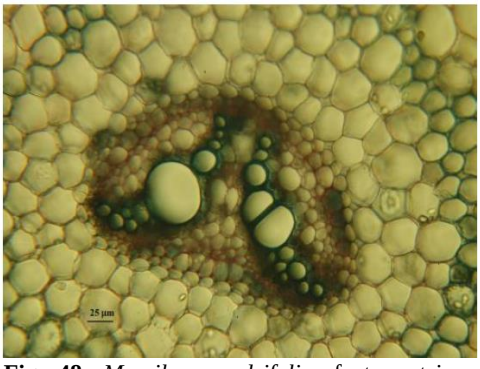

Fig. 48. *Marsilea quadrifolia*, f. *terrestris* – petiole in transversal section; the stele (Iodine green, Carmine alum).

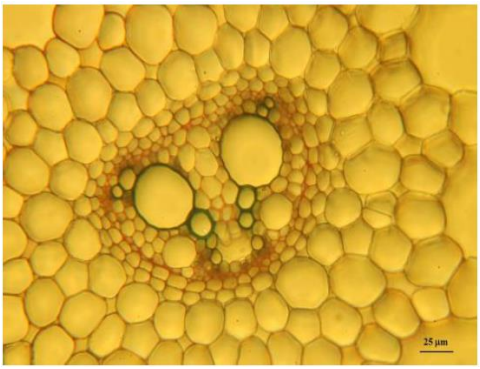

Fig. 49. *Marsilea quadrifolia*, f. *natans* (Danube Delta) – petiole in transversal section; the stele (Iodine green, Carmine alum).

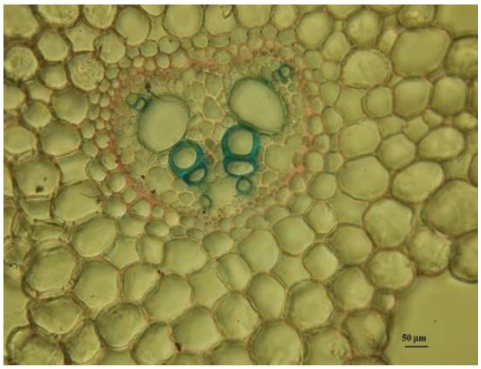

Fig. 50. *Marsilea quadrifolia*, f. *natans* (Arcuda Station) – petiole in transversal section; the stele (Iodine green, Carmine alum).

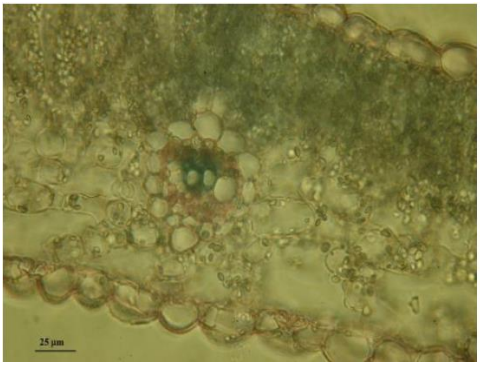

Fig. 51. *Marsilea quadrifolia*, f. *terrestris* – transversal section through the foliole (Iodine green, Carmine alum).

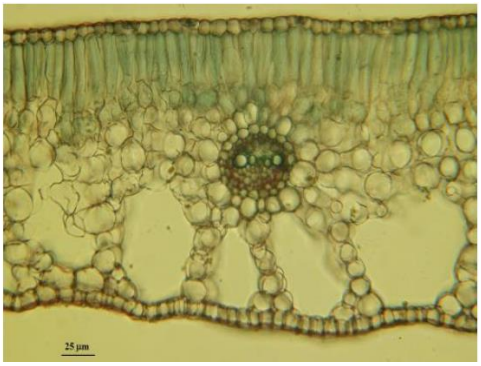

Fig. 52. *Marsilea quadrifolia*, f. *natans* (Danube Delta) - transversal section through the foliole (Iodine green, Carmine alum).

Fig. 53. *Marsilea quadrifolia*, f. *natans* (Arcuda Station) – transversal section through the foliole (Iodine green, Carmine alum).

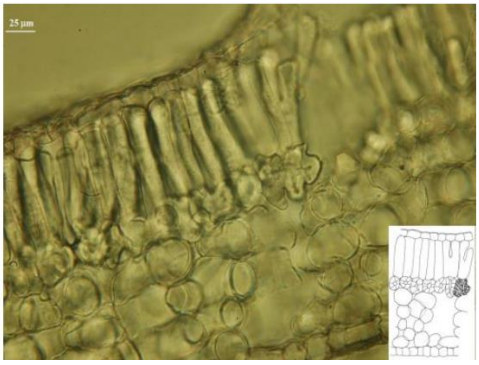

Fig. 54. *Marsilea quadrifolia*, f. *natans* (Arcuda Station) – foliole in transversal section; the sclereides (Iodine green, Carmine alum).

Conclusions

The species of the genus *Azolla* are difficult to identify based on the appearance of the vegetative body such as the shape, dimension and colour of leaves, and the sporocarps are not always present. Nonetheless they can be differentiated through the micro-morphological characteristics of the epidermal trichomes, which can be utilized as an initial, more accessible criterion for identifying these taxa. Based on this type of character, we can demonstrate the presence of the taxon *Azolla caroliniana* in the Danube Delta, while the presence of the taxon *Azolla mexicana* can be considered likely in the habitats of the Danube Delta.

Salvinia natans, a natant plant, shows numerous adaptations to life on the surface of the water. Among these adaptations it is worth noting the architecture of the mesophyll and the presence of the two types of epidermal trichomes. These adaptive elements contribute to enabling the plant to float and maintain its balance on the surface of the water, thus preventing it from sinking under the direct impact of the water. The hydrophobe structure of the foliar surface ensures an unhindered functioning of the stomata.

At *Marsilea quadrifolia*, which was analyzed both in natural and artificial habitats, the organization of the vegetative body reveals morpho-structural adaptations induced by the life in aquatic habitats of varying shallows and by the transition from an aquatic to a terrestrial environment. These observations support the adaptive plasticity of this protected species.

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This paper was possible thanks to the activities conducted as part of several research studies such as *Macrophyte Inventory Danube – Corridor and Catchment – MIDCC*, *Adaptive management of Climate-Induce Changes of Habitat Diversity in Protected Areas*, *The inventory and the assessment of biodiversity within the Arcuda Station for Treating and Drinking Water Production*, complemented by some observations conducted within the BA and MA programmes at the University of Bucharest.

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KNOCKOUT OF *ATMKK1* REDUCES *ARABIDOPSIS* RESPONSE TO 2, 3, 5-TRIIODOBENZOIC ACID IN LEAVES

Mahta HEMMATI[1], Hamta HEMMATI[1], Chad CONROY[1], Tim XING[1*]

Abstract: Mitogen activated protein kinase (MAPK) pathways are crucial for plant growth and development. The most commonly identified pathways that AtMKK1 has been connected to are wounding, bacterial pathogen response, cold, drought, salt stress, reactive oxygen species stress, touch, and abscisic acid. There is also evidence that AtMKK1 regulates development. In leaf development, auxin can modulate both cell division and expansion and has a key role in both initiation and elaboration of final morphology of both leaves and vascular networks. Distribution of auxin to different tissues and organs relies on auxin transport systems. In our study, it was found that there was reduced response in *atmkk1*, the *AtMKK1* knockout mutant, to 2,3,5-triiodobenzoic acid, an auxin polar transport inhibitor. Analysis of protein-protein interactions has suggested that AtMKK1 may interact with the downstream AtMPK12, which is a negative regulator of auxin signaling. Our results indicate that AtMKK1 may play a role in leaf development.

Keywords: *Arabidopsis*, auxin, leaf, PIN1, TIBA.

Introduction

Mitogen-activated protein kinase (MAPK) pathways play an important role in regulation of plant growth and development. These pathways consist of at least three core enzymes: a MAPK (MPK), activated by a MAPK kinase (MAPKK, MKK or MEK), which is in turn activated by a MAPK kinase kinase (MAPKKK, or MEKK). MAPKKs all have a common activation motif, S/TXXXXXS/T, as well as a high specificity for the downstream MAPKs [MAPK Group., 2002; XING & al. 2002]. In *Arabidopsis* there are approximately 20 MAPKs, 10 MAPKKs, and about 12-60 MAPKKKs [XU & ZHANG, 2015]. The functions of most of them are unknown. There are several completed pathways that have been identified, but the relationships between many MAPK cascade proteins are still not clear. While all components in a MAPK cascade have the ability to phosphorylate and activate their direct downstream targets, MAPK cascade proteins can also be involved in pathways without direct interaction through activating other pathways or by acting as scaffold proteins [MESZAROS & al. 2006]. The cross talk and wide ranging interactions of MAPK pathways also mean that the isolation of a single point of effect from the removal or overexpression of a gene is extremely difficult.

Some fully identified pathways are as follows. AtMEKK1-AtMEK1/AtMEK2-MPK4, involved in a variety of stresses ranging from pathogen response, to wounding, to environmental stresses such as salt or temperature [PITZSCHKE & al. 2009; QIU & al. 2008; TEIGE & al. 2004]. The most convincing and representative work in the identification of a complete signaling path was from the analysis of *Arabidopsis* response to flagellin, a highly conserved component of bacterial flagella that functions as a pathogen-associated molecular

[1] Carleton University, Department of Biology and Institute of Biochemistry, Ottawa ON – Canada K1S 5B6.
[*] Corresponding author. E-mail: tim.xing@carleton.ca

pattern (PAMP) in plants and mammals [ASAI & al. 2002]. The signaling components include a complete plant MAP kinase cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) and WRKY22/WRKY29 transcription factors that function downstream of the flagellin receptor FLS2, a leucine-rich-repeat (LRR) receptor kinase in *Arabidopsis* [ASAI & al. 2002]. The majority of study on MAPK cascades in *Arabidopsis* has been carried out in stress response analysis [COLCOMBET & HIRT, 2008], but there are examples of MAPK pathways in *Arabidopsis* playing roles in developmental cascades, such as dwarfism seen in *mpk4* and *mekk1* mutants [QIU & al. 2008], or changes in stomata patterning or pollen development [COLCOMBET & HIRT, 2008].

A complete pathway for AtMKK1 has been identified, AtMEKK1 was shown to phosphorylate AtMKK1 [HADIARTO & al. 2006], and activate AtMKK1 in a yeast two hybrid system. AtMPK4 was shown to be connected to AtMKK1 in the same yeast two hybrid system [MIZOGUCHI & al. 1998]. Since the initial identification of the AtMEKK1-AtMKK1-AtMPK4 pathway a multitude of other interactors have been identified. AtMKK1 plays a role in the regulation of not only AtMPK4, but also AtMPK3 and AtMPK6 [MESZAROS & al. 2006; XING & al. 2008]. The upstream AtMEKK1 can also activate numerous downstream proteins such as AtMKK2, AtMKK4, AtMKK5, and the downstream targets of AtMKK1 can also be activated by AtMKK2, AtMKK4, and AtMKK5 [COLCOMBET & HIRT, 2008]. The most commonly identified pathways that AtMKK1 has been connected to are wounding, bacterial pathogen response, cold, drought, salt stress, reactive oxygen species (ROS) stress, touch, and abscisic acid (ABA) [CONROY & al. 2013; HADIARTO & al. 2006; MATSUOKA & al. 2002; MESZAROS & al. 2006; PITZSCHKE & al. 2009; TEIGE & al. 2004; XING & al. 2008]. Some of the stresses such as wounding have been accepted with little in the way of conflicting data, but other stresses such as salt have met with mixed responses. There are studies linking AtMKK1 with the activation of MPK4 in salt stress [MATSUOKA & al. 2002], but there are other studies that have refuted that claim and stated that AtMKK1 is not involved in salt response [TEIGE & al. 2004]. Our previous work has indicated that AtMKK1 knockout mutant, *atmkk1*, could germinate in highly saline environments to a level far above that of the wild type, and the adult plants could resist the effect of high salinity, indicating that AtMKK1 is likely a negative regulator of salt stress response [CONROY & al. 2013]. Studies have indicated that that AtMKK7 controls plant architecture through the negative regulation of polar auxin transport (PAT) [DAI & al. 2006] and AtMPK12 is a negative regulator auxin signaling [LEE & al. 2009]. However, little is known about the involvement of AtMKK1 in auxin signaling pathways [XU & ZHANG, 2015].

Auxin can modulate both cell division and expansion, and has a key role in both initiation and elaboration of final morphology of both leaves and vascular networks [SCARPELLA & al. 2010]. A key feature of auxin action is the existence of feedback loops through which auxin regulates its own transport [BAYER & al. 2009; GUENOT & al. 2012; HEISLER & al. 2005; WISNIEWSKA & al. 2006]. AtMPK12 acts as a negative regulator of auxin signaling, with IBR5, a protein phosphatase that targets AtMPK12 [LEE & al. 2009]. Some MAP kinase cascade components are also shown to function in rosette leaf expansion (e.g. MAP3Ke1/MAP3Ke2) [CHAIWONGSAR & al. 2012] and cell plate expansion (AtMPK4 and AtMPK11) [KOSETSU & al. 2010]. More recently, a genome-wide search of the rice genome database and a yeast two-hybrid assay identified OsAux/LAX1, an auxin influx carrier, as a potential target protein of OsMPK3, OsMPK4 and OsMPK6, suggesting

a direct involvement of MPKs in the auxin transport and signaling pathway [MOHANTA & al. 2015]. Highly specific and transient expression of AtMPK10 determines auxin-induced leaf venation patterns in *Arabidopsis* and the AtMKK2-AtMPK10 module regulates venation complexity by altering polar auxin transport efficiency [STANKO & al. 2014]. Here, leaf phenotypic changes were analyzed after *atmkk1* plants were treated with 2, 3, 5-triiodobenzoic acid (TIBA), an auxin transport inhibitor.

Materials and methods

Mutant plants and plant growth conditions

T-DNA insertion mutant was obtained from the ARBC (The *Arabidopsis* Resource Centre; http://www.*Arabidopsis*.org). Details on its locus identification, insertion confirmation and selection on selective media were described previously [CONROY & al. 2013]. Seeds were surface sterilized, stratified for 4 days and then plated on MS plates containing kanamycin. Wild type (WT) seeds were not plated on plates containing kanamycin. Mutant (line A51) and wild type *Arabidopsis* (Col-0) were grown in a growth chamber (ENCONAIR Technologies Inc.) under a 16 h light and 8 h darkness cycle at 22 °C. After ten days the seedlings were transferred into autoclaved soil and allowed to grow for a further fourteen days before experimental treatments began.

Response to TIBA

Treatments was carried out using 2, 3, 5-triiodbenzoic acid (TIBA) at a concentration of 0.1 mM (first dissolved in ethanol and then made to the concentration with H_2O). The solution of TIBA was spread over leaves using a cotton swab with a water control. Leaf width and length were measured at 0 and 6 days. Leaves from both time points were also frozen in liquid N_2 and stored at -80 °C till further use.

RT-PCR

RT-PCR was used for the evaluation of PIN1 expression with primers 5'-TGCAGGTCTAGGCATGGCTA-3' and 5'-TTTAACGCCATGAACAACCCA-3'. Actin2 primers used are 5'-CCTCATGCCATCCTCCGTCTTG-3' and 5'-TTCCATCTCCTGCTCGTAGTCAAC-3'. PCR was carried out under the following conditions: 94 °C for 3 min; 30 s at 94 °C, 30 s at 59 °C, and 30 s at 72 °C for 25 cycles; and then 10 min at 72 °C.

Bioinformatics analysis

Protein-protein interactions were predicted using STRING 9.0 (http://string-db.org/) databases.

Results

Effect of TIBA on leaf growth

The effect of TIBA, an auxin transport inhibitor, on leaf growth was examined. Leaf length and width were inhibited in wild type but not in *atmkk1* plants (A51) (Fig. 1). The ability to grow in the wild type leaves is significantly suppressed after the addition of the TIBA, contrast that to the unchanged parameters of the *atmkk1* plants (Fig. 1). The *atmkk1* plants appeared to be capable of resisting the effect of the TIBA more dramatically than the wild type.

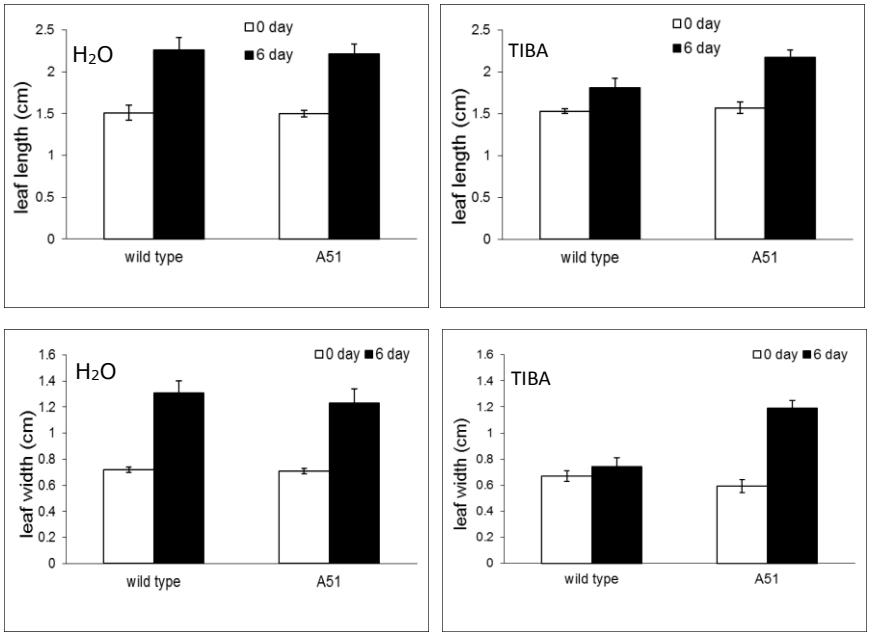

Fig. 1. Leaf length and width after TIBA treatment. Leaves were treated with TIBA for 6 days. This experiment was carried out three times.

Expression of PIN1 upon TIBA treatment

Alterations of auxin transport could induce changes in PIN gene expression [BLAKESLEE & al. 2004]. We examined the expression level of PIN1 after TIBA treatment. While in wild type the expression level was increased by about 8 fold, PIN1 expression was slightly inhibited in *atmkk1* (A51) leaves (Fig. 2). However, the knockout mutant seems to have a background PIN1 level that is 5-6 fold higher than that in the wild type (Fig. 2). It suggests that the knockout of *AtMKK1* may have enhanced PIN1 expression.

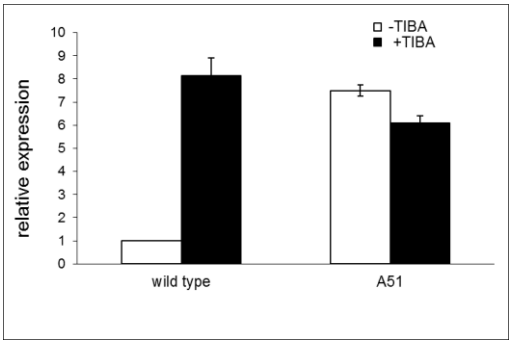

Fig. 2. Expression of PIN1 after TIBA treatment. This experiment was carried out three times.

Prediction of interactive partners of AtMKK1

Protein-protein interaction analysis with STRING indicates that AtMKK1 interacts with AtMPK4, 11, and 12 (Fig. 3 and Tab. 1). AtMPK12 is a negative regulator of the auxin transduction signaling pathway [LEE & al. 2009].

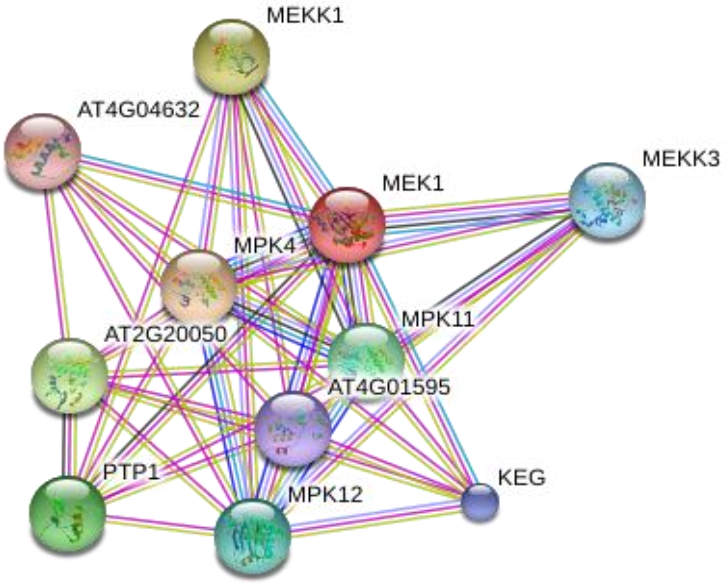

Fig. 3. Prediction of interactive partners of AtMKK1.

Tab. 1. Possible functional partners of MAP kinase kinase 1. The prediction tool STRING 9.0 (http://string-db.org/) used different modes of prediction to determine which proteins interact with MAPKK1 and described most of their associated functions.

Protein ID/ Name	Mode of prediction	Function
MPK4 MAP kinase 4	Experiments, textmining, homology, co-occurrence, co-expression, databases	Involved in cortical microtubules organization and stabilization. Involved in root hair development process. Negative regulator of systemic acquired resistance (SAR) and salicylic acid- (SA) mediated defense response. Required for jasmonic acid- (JA) mediated defense gene expression. May regulate activity of transcription factor controlling pathogenesis-related (PR) gene expression. Seems to act independently of the SAR regulatory protein NPR1 (Nonexpresser of PR1).
MEKK1 MAPK/ERK kinase kinase 1	Experiments, textmining, homology, databases	Involved in the innate immune MAP kinase signaling cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) downstream of bacterial flagellin receptor FLS2. May be involved in the cold and salinity stress-mediated MAP kinase signaling cascade (MEKK1, MEK1/MKK2 and MPK4/MPK6). Activates downstream MKK2, MKK4 and MKK5
AT2G20050	Experiments, textmining	protein phosphatase 2C and cyclic nucleotide-binding/kinase domain-containing protein

PTP1 tyrosine phosphatase 1	Experiments, textmining, co-expression	Protein-tyrosine-phosphatase that dephosphorylates and probably inhibits MPK6 in non-oxidative stress conditions. In association with MKP1, represses salicylic acid (SA) and camalexin biosynthesis, thus modulating defense response. May also repress MPK3. Dephosphorylates and inactivates MPK4 in vitro
MPK11 MAP kinase 11	Experiments, textmining, homology, co-occurrence, co-expression	
MPK12 mitogen-activated protein kinase 12	Experiments, textmining, homology, co-occurrence	Negative regulator of the auxin transduction signaling pathway
MEKK3 MAPK/ERK kinase kinase 3	Experiments, textmining, homology, co-expression, databases	
KEG- KEEP ON GOING	Experiments, textmining, databases	Mediates E2-dependent protein ubiquitination. Acts as a negative regulator of abscisic acid signaling. Required for ABI5 degradation, by mediating its ubiquitination. Together with EDR1, may regulate endocytic trafficking and/or the formation of signaling complexes on trans-Golgi network (TGN)/ early endosome (EE) vesicles during stress responses
AT4G01595 *protein kinase-like protein*	Experiments, textmining	
AT4G04632- protein kinase family protein	Experiments, textmining, databases	

Discussion

Experiments carried out using TIBA may indicate that AtMKK1 could be involved in auxin response pathways. The data show a trend towards there being less impact upon the AtMKK1 mutation following TIBA treatment. TIBA had a different effect upon the growth of the leaves. While there was a noticeable difference in the leaf growth of the wilt type leaves, there was little change in the response of the AtMKK1 mutation. The *atmkk1*plants appeared to have the same growth patterns regardless of whether TIBA was applied to the leaves or not. The treatment showed that alerting the auxin transport in the leaves of wild type plants can have a significant impact upon the growth patterns. But the inhibition of polar auxin movement had little effect on *atmkk1* plants. The *atmkk1*plants were phenotypically indifferent to TIBA (data not shown). Whether AtMKK1 mutations allow for *Arabidopsis* plants to resist the changes of auxin transport, or just better regulate the movement of auxin in the leaf blades is unknown.

ABP1 had been considered a membrane-bound auxin receptor until recent re-analysis of new *abp1* null alleles generated by CRISPR/Cas9 [GAO & al. 2015]. The loss of ABP1 resulted in no obvious defects in auxin response and *Arabidopsis* morphology [GAO & al. 2015]. It is suggested that ABP1 is likely not the auxin receptor for auxin-mediated non-genomic effects and it is worth revisiting the hypothesis that auxin efflux carriers may serve as auxin receptors [GAO & al. 2015; STRADER & ZHAO, 2016]. Root, young leaf and seedling growth are found to be regulated by PAT. PIN (PIN-FORMED) proteins are well-characterized auxin efflux carriers, playing essential roles in many developmental processes [GALWEILER & al. 1998; PETRASEK & al. 2006]. Genetic studies suggest that

members of several gene families such as PID (PINOID), NPY (NAKED PINS IN YUC MUTANTS), and ARF (AUXIN RESPONSE FACTOR) can potentially function downstream of PIN1. PIN and PID may form a plasma membrane localized auxin receptor complex important for auxin-mediated *Arabidopsis* organogenesis. As PID lacks a receptor domain, it is hypothesized that PIN proteins may function as an auxin receptor based on genetic evidences. Previous studies focused on the capacity and directionality of PIN-mediated auxin transport. This new model suggests that auxin transport may be coupled with a signal transduction pathway, which can reasonably account for the observed pin-like phenotypes in various *Arabidopsis* mutants [STRADER & ZHAO, 2016]. TIBA was also shown to have only slight effects on PIN1 localization on their own and leave PIN1 accumulation at the plasma membrane unaffected [GELDNER & al. 2001]. Genevestigator data mining also indicated that TIBA treatment (3 hours) did not have effect on AtMKK1 or PIN1 expression in *Arabidopsis* seedlings (data not shown), while TIBA treatment for 6 days in our work enhanced PIN1 expression level in wild type *Arabidopsis*.

In the attempt to find possible mechanisms, we examined if AtMKK1 knockout may lead to disconnection of components in MAP kinase cascade. Search for potential AtMKK1 interactive proteins has indicated that the expression of AtMPK4, 11, and 12 co-occur and co-expressed with AtMKK1, and particularly each of these three AtMPKs interacted with AtMKK1experimentally (Tab. 1). The analysis also indicates that AtMKK1 experimentally interacted with upstream MEKK1 and MEKK3. AtMKK7 was shown to mediate the regulation of auxin polar transport [DAI & al. 2006] and at the MPK level, there is evidence that AtMPK12 is a negative regulator auxin signaling [LEE & al. 2009]. MAPK cascade involvement in PAT is also shown in previous studies. Increased expression of AtMKK7 caused deficiency in polar auxin transport and leads to plant architectural abnormality in *Arabidopsis* [DAI & al. 2006]. It was thus suggested that AtMKK7 negatively regulates PAT. It is possible that loss of connection of AtMKK1 to AtMPK12 in *atmkk1* plants may be attributed to reduced response to auxin fluctuation upon TIBA treatment.

Conclusions

AtMKK1 is a protein involved in *Arabidopsis* defense and developmental pathways. While the literature has presented numerous, often contradictory roles for AtMKK1, this project has provided some clarification of the uncertainties surrounding AtMKK1, as well as providing new potential areas for research. The fact that *atmkk1*plants were unaffected by TIBA while the wild type did show signs of impact allows for the conclusion that AtMKK1 may play a role in auxin signaling. The possible targets for AtMKK1 in auxin signaling remain unclear and the precise role of AtMKK1 in auxin signaling should be further studied. Among these, it would be interesting to study if and how AtMKK1, PIN1, and the regulation of auxin responsive genes are connected, and if PIN1 protein is modified at post-translational level when connection of AtMKK1 and AtMPK12 is lost due to the knockout of *AtMKK1*.

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EFFECTS OF CRUDE OIL POLLUTED SOIL ON THE SEEDLING GROWTH OF *PENNISETUM GLAUCUM* (L.) R. BR.

Huda SHAHID[1], Muhammad Zafar IQBAL[1], Muhammad SHAFIQ[*1], Mohammad ATHAR[2]

Abstract: Pollution by crude oil is an important environmental issue all around the world. Increase in oil pollution level in the environment produce toxic effects on flora and fauna of the region. The effects of different levels (0%, 5%, 10%, 15%, and 20%) of crude oil polluted soil on the growth of pearl millet (*Pennisetum glaucum*) were studied. The polluted soil affected the root, shoot length, seedling size, number of leaves and leaf area of *P. glaucum*. The significant ($p<0.05$) effects of polluted soil on fresh and dry weight of root, stem, leaves, and seedling of *P. glaucum* were also recorded. Leaf area, leaf number and total seedling dry weight were noticeably reduced in 10 and 15% polluted soil than control soil treatment. Principally, 20% crude oil polluted soil treatment exhibited highest percentage of decrease in most of the seedling growth parameters of *P. glaucum* than control. Hence, the effects on seedling growth parameters were increased with increasing levels of polluted soil. For most of the growth parameters, the mean values obtained were found higher for the control soil and progressively decreased from 5-20% crude oil polluted soils. The seedlings of *P. glaucum* were also tested for tolerance to polluted soil treatment. The results showed that the seedlings of *P. glaucum* showed high percentage of tolerance to low concentration (5%) of polluted soil treatment as compared to control soil treatment (0%).

Keywords: Contamination, crude oil, growth, *Pennisetum glaucum*, soil.

Introduction

Crude oil contains a wide variety of elements combined in various forms [ABB, 1997]. The petroleum products are strongly enriched with hydrocarbons, leaving most crude based inorganic materials and other types of organic compounds [POTTER & SIMMONS, 1998]. The effect of crude oil pollution from an accidental blowout of an oil well on soil pH, temperature, crude oil content and its flora was studied [DEBOJIT, 2006]. The oil pollution significantly affected the soil environment and reduced the number of plant species and vegetation productivity. Perennials are less affected then the annuals. About 84% of the plant species were reported wiped out in the highly oil contaminated site. Crude oil is a naturally occurring hydrocarbon compound used by humans in a variety of ways: fuelling of cars, lorries and trucks; heating of homes, cooking and other fractions utilized in the manufacture of synthetic products [EDEMA & al. 2009].

The industrial revolution of the past century has resulted in significant damage to environmental resources such as air, water and soil [ABEDI-KOUPA & al. 2007]. Soil, which is the result of interaction between climate, organisms and mother rock and formed under specific topographic conditions within a certain time, is an essential physical component that

[1] Department of Botany, University of Karachi, Karachi – 75270 – Pakistan.
[2] California Department of Food and Agriculture, 3288 Meadowview Road, Sacramento, CA 95832 – U.S.A.
[*] Corresponding author. E-mail: shafiqeco@yahoo.com

covers extensive areas above the lithosphere. The conservation of soil which is the base of plant growth became a national demand. Soil profiles are subjected to a continuous exhaustion in several forms, such as: soil erosion, depletion of nutrients and pollution [ODAT & ALSHAMMARI, 2011]. Petroleum and its products are of specific concern in pollution studies due to their structural complexity, slow biodegradability, bio-magnification potential and above all the serious health hazards associated with their release into the environment and their transport across the world frequently result in oil spillage, contaminating the soil and water alike [KATHI & KHAN, 2011]. Spent engine oil is the hydrocarbon product of crude oil and it is unsatisfactory for growth of *Azadirachta indica* A. Juss due to insufficient reaction of the soil and the microbes because of the displacement of air from spaces between soil particles [ALAMU, 2012]. It has been observed by various researchers also that oil spills have significant effects on agricultural lands, crop, trees, forest, and their seedlings [UDO & FAYEMI, 1975; BARTHA, 1977; UDO & OPARA, 1984]. Seed germination, percentage of seedling emergence, number of root nodules/plant, total soluble sugars, total soluble proteins, free amino acids, total chlorophyll and carotenoids and nucleic acids of the leaves of *Vigna mungo* (L.) Hepper grown in the oil polluted soil decreased significantly due to persistence of hydrocarbon [ILANGOVAN & VIVEKANANDAN, 1992]. The study of plant behavior in petroleum contaminated soils allows the identification and selection of oil pollution indicating species [MARANHO & al. 2009].

The continuous growth of environmental pollution and anthropological disturbances to ecosystems has made the study of abiotic stress responses in plants [ALKIO & al. 2005]. Effects of waste engine oil pollution on physical and chemical properties of soil have been observed [ATUANYA, 1987; EKUNDAYO & OBUEKWE, 1994; BENKA-COKER & EKUNDAYO, 1995]. Effects of crude oil pollution on the growth of some plant species viz. *Zea mays*, *Abelmoschus esculentus*, *Capsicum frutescens*, *Capsicum annuum*, *Lycopersicon esculentum* were reported by AMAKITRI & ONAFEGHARA, 1983; ANOLIEFO & VWIOKO, 1994. The effects of crude oil contaminated soils on seedling growth of six agronomic crop species observed and concluded that *Zea mays* and *Glycine max* seedlings show the greatest potential to enhance remediation compared to *Medicago sativa*, *Lolium perenne*, *Triticum aestivum* and *Vicia villosa* [ISSOUFI & al. 2006].

Oil pollution is an important problem in many parts of the world. The disposal of used lubricating oil into the immediate environment is an important environmental issue affecting on plant growth. Therefore, the present study was carried out with the aim to study the effects of crude oil polluted soil on the growth of an important annual grass pearl millet (*Pennisetum glaucum* (L.) R. Br.).

Materials and methods

This study was conducted at the Department of Botany, University of Karachi, Pakistan. Seeds of *Pennisetum glaucum* were obtained from the local market. Spent crude oil was obtained from Motor Transport Workshop located at University of Karachi Campus. Spent oil was used rather than fresh one because the former would more closely mimic in nature. Seeds of *P. glaucum* were sown in garden soil at 1 cm depth in large earthen pots placed in natural environmental conditions of Department of Botany, University of Karachi. The pot was kept moist by adding water when necessary. Initially the experiment was

conducted with high level of polluted soil but all the plants died. Later 20% crude oil polluted soil was chosen as the highest level of polluted soil other levels were 5%, 10%, 15% polluted oil soil. The experiment was lasted for 35 days. Seedlings were allowed to grow in the pots for 7-8 days to enable a reasonable height of 3 cm. Seedlings of equal height were selected and transplanted into small pots of 7.3 cm in diameter and 9.6 cm in depth having different ratios of crude oil contaminated soil (5% crude oil contaminated soil + 95% garden soil; 10% crude oil contaminated soil + 90% garden soil; 15% crude oil contaminated soil + 85% garden soil; 20% crude oil contaminated soil + 80% garden soil) and garden soil without crude oil contamination used as a control. One seedling of *P. glaucum* was planted in a small pot representing each treatment and this was replicated five times and the experiment was completely randomized. Normal water was added to all the samples when necessary in order to keep the soil moist and no nutrient solution was provided. The height of the plants was measured from the soil level to the terminal bud using a steel scale. This was done at a regular interval of seven days. The numbers of leaves were counted as the plant grew. This was done by visual counting of the leaves at regular intervals of seven days. The plants were carefully uprooted after 35 days and the root part rinsed with clean water. The fresh weight of the root, stem and leaves was then determined separately and kept separated part of the plant in marked paper envelope and finally place in an oven at 80 degree centigrade for 24 hours to obtain the dry weight. Fresh and oven dried weights for roots, shoot, leaves and total plant weight was recorded. Leaf area were determined by multiplying length and breadth and multiply 2/3. While the tolerance indices was determined by the following formulae:

Mean root length in oil polluted soil concentration / Mean root length in without oil polluted soil concentration x 100

The means as well as standard errors were calculated. Data collected were subject to one-way analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) using personal computer software packages COSTAT version 3.00. Level of significance for these tests was at $P < 0.05$.

Results

The effect of different levels (0%, 5%, 10%, 15%, 20%) of crude oil polluted soil on the seedling growth performances of pearl millet *Pennisetum glaucum* was recorded (Figs. 1-3). The oil pollution contamination affected root, shoot length, seedling size, number of leaves and leaf area significantly ($p<0.05$) as compared to control (Fig. 1). The mean root, shoot, seedling length, number of leaves and leaf area of *P. glaucum* was found high in control soil treatment. The treatment of 5% and 10% oil polluted soil decreased root, shoot and seedling length of *P. glaucum* as compared to control soil. Number of leaves and leaf area also decreased at similar contamination of crude oil. The mean values obtained for root length (11.74 cm), shoot length (9.10 cm), seedling length (20.84 cm), number of leaves (7.40), leaf area (3.71 sq. cm) were highest in control soil. The seedling length (18.10 cm), root length (10.04 cm), number of leaves (6.80), leaf area (3.77 sq. cm) were found significantly low when treated with 05% crude oil soils. Further increase in treatment of crude oil polluted soil treatment at 10% significantly reduced seedling growth (17.74 cm), root length (9.48 cm), number of leaves (5.80) and leaf area (2.87 sq. cm) of *P. glaucum* as compared to control. The lower mean values obtained for seedling length (13.86 cm), root

length (6.28 cm), number of leaves (3.40) and leaf area (1.95 sq. cm) were found for 15% crude oil treated soils. A slight increase in seedling length (14.32 cm) and root length (7.90 cm) were found for the 20% crude oil treated soils as compared to 15% oil polluted soil treatment. The significant effects of crude oil polluted soil on root, shoot, leaves and seedling fresh weight were observed (Fig. 2). The fresh biomass production of *P. glaucum* in terms of total seedling fresh weight (1.09 g), root weight (0.38 g), shoot weight (0.389 g) was recorded in control soil. The lowest seedling fresh weight (0.14 g), root weight (0.03 g) and leaf weight (0.05 g) was recorded in 20% polluted soil treatment. The effects of oil polluted soil on root, shoot, leaves and seedling dry weight were also observed (Fig. 3). A significant ($p<0.05$) relationship was found to exist between the inhibitory effects of oil polluted soil on seedling dry weight with the increase in polluted soil treatment concentrations as compared to control. 20% crude oil polluted soil exhibited more reduction in seedling dry weight of *P. glaucum* as compared to control. Similarly biomass production of *P. glaucum* in terms of total seedling dry weight (0.06 g), root dry weight (0.01 g), shoot dry weight (0.02 g) and leaf dry weight (0.02 g) was recorded in 20% oil polluted soil as compared to control soil treatment.

Oil polluted soil treatment at different concentration (5, 10, 15, 20%) decreased high percentage of root, shoot, seedling length, number of leaves, leaf area and seedling fresh and dry weight of *P. glaucum* as compared to control treatment (Tab. 1). Oil polluted soil treatment of 5% concentration was found responsible for high percentage of decrease in seedling length (13.14%), root length (10.87%), shoot length (11.42%), number of leaves (8.10%) and leaf area (0.27%) of *P. glaucum* as compared to control, while polluted soil at 10% and 15% concentration was found responsible for further decrease in seedling length, root length, number of leaves and leaf area *P. glaucum* as compared to control. A slight increase in root and seedling enhanced at 20% concentration of oil polluted soil as compared to 15% polluted oil soil. Similarly, oil polluted soil treatment at 5-20% concentration was found responsible for decrease in seedling fresh and dry weight of *P. glaucum* as compared to control (Tab. 1).

The seedlings of *P. glaucum* were also tested for percentage of tolerance to polluted oil soil treatment as compared to control (Tab. 2). The results showed that seedlings of *P. glaucum* have greater tolerance to low concentration of oil polluted soil treatment as compared to control. Increase in oil polluted soil treatment decreased the low percentage of tolerance indices in seedlings of *P. glaucum* as compared to control. According to tolerance test it was observed that seedlings of *P. glaucum* showed high percentage of tolerance (85.51%) with the treatment of 5% crude oil polluted soil as compared to control soil treatment. The treatment of 10% crude oil contaminated soil showed low percentage of tolerance (80.74%) in seedlings of *P. glaucum* as compared to garden soil treatment. Similarly, seedling growth of *P. glaucum* showed lowest percentage of tolerance (53.49%) in 15 % crude oil contaminated soil treatment.

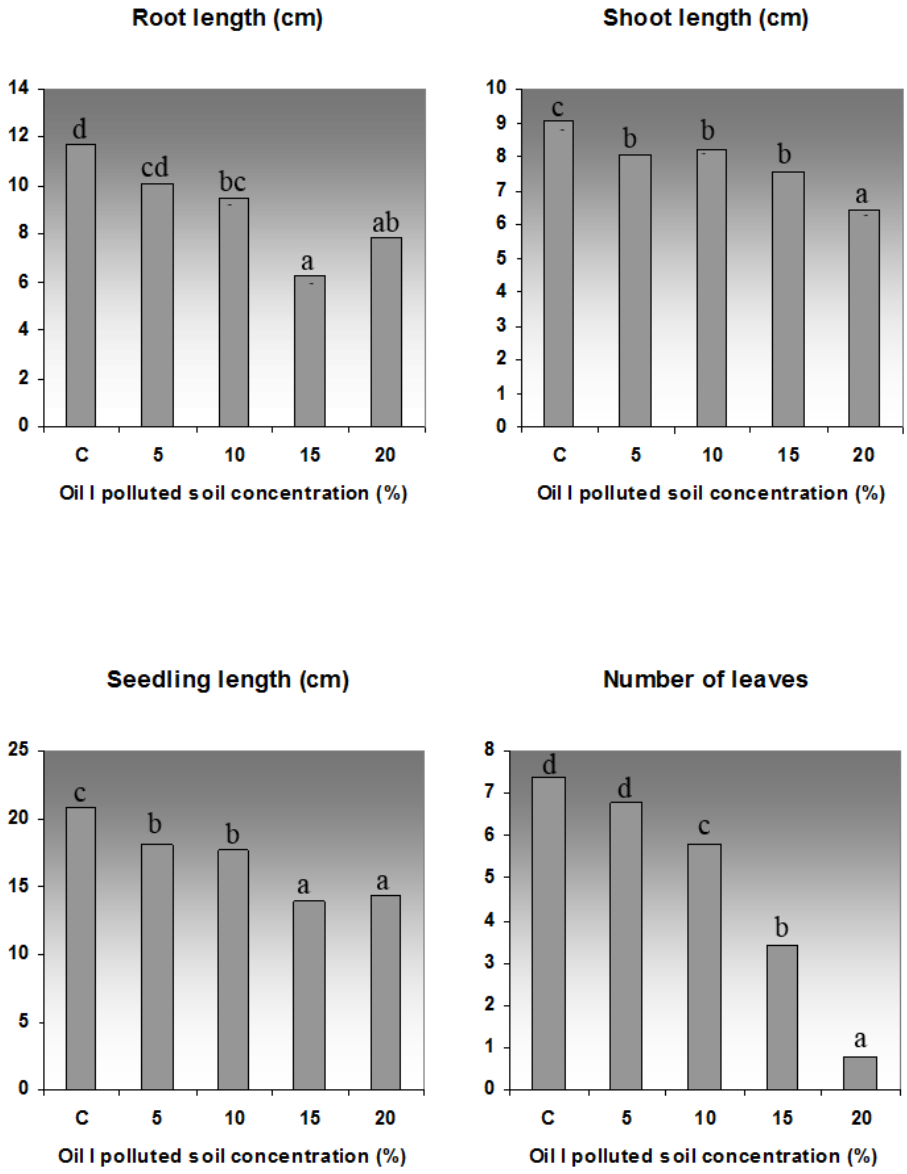

Fig. 1. Effects of different concentration of polluted soil on root, shoot, seedling length (cm) and number of leaves of P. *glaucum* as compared to control.

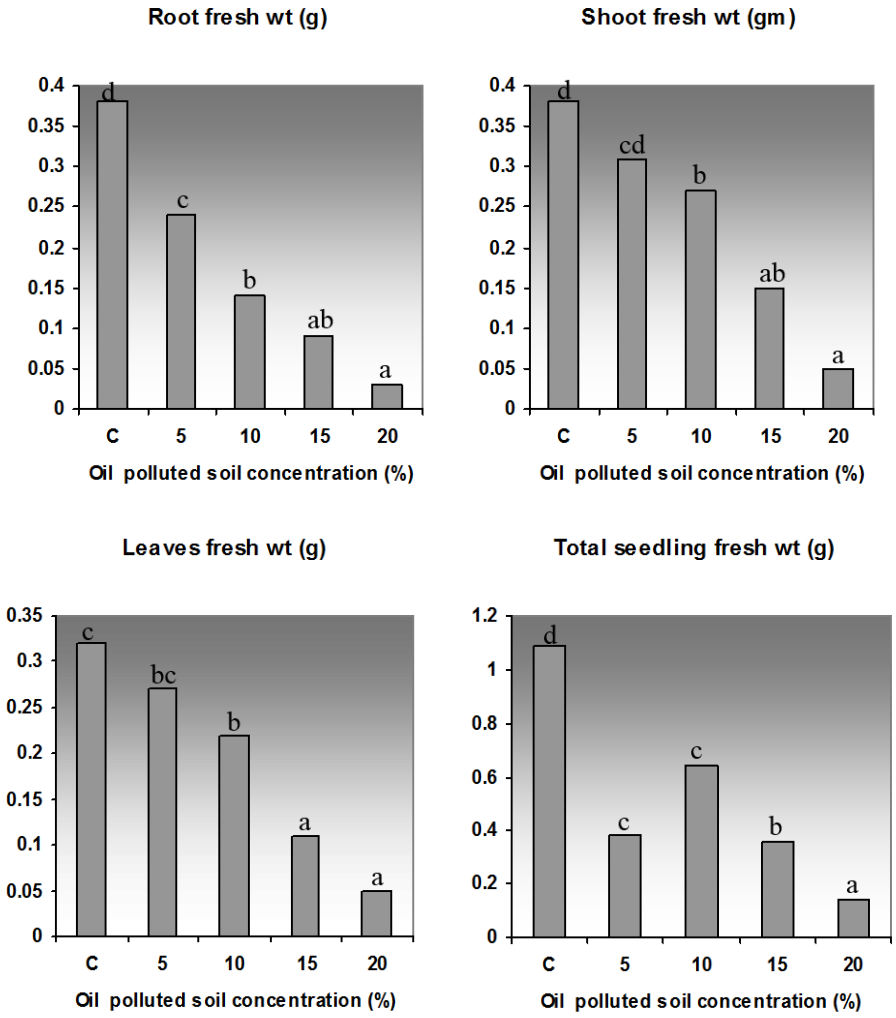

Fig. 2. Effects of different concentration of polluted soil on root, shoot, leaves and total seedling fresh weight (g) of P. *glaucum* as compared to control.

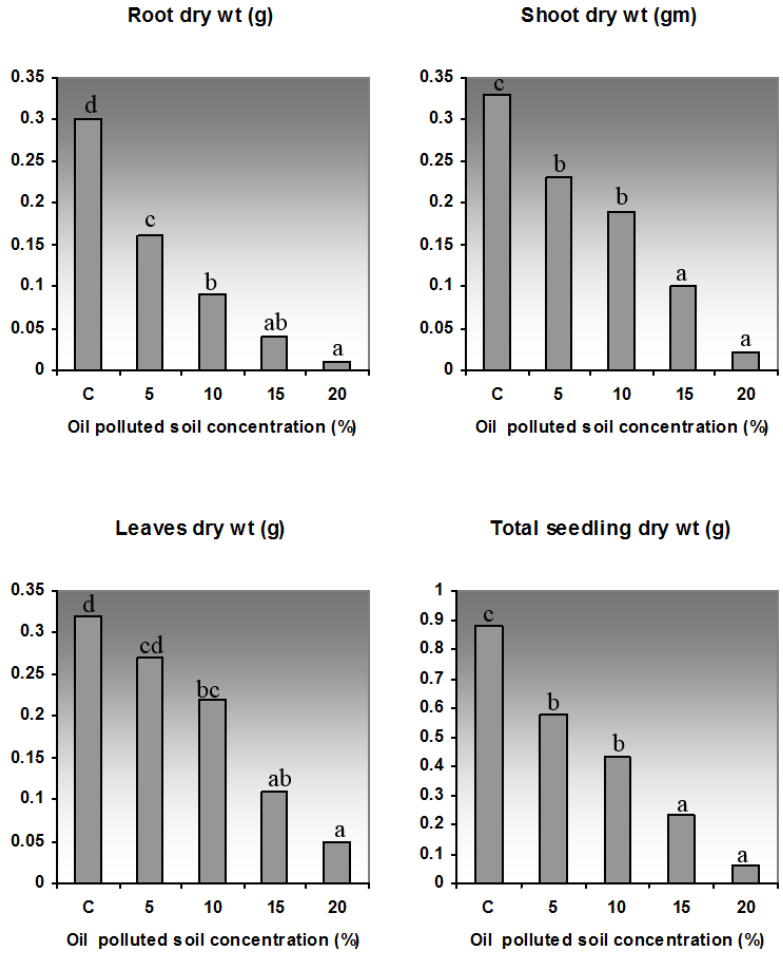

Fig. 3. Effects of different concentration of polluted soil on root, shoot, leaves and total seedling dry weight (g) of P. *glaucum* as compared to control.

Tab. 1. Percentage decrease in seedling growth parameter and biomass production of *Pennisetum glaucum* under different concentration (05, 10, 15, 20%) of crude oil polluted soil as compared to control soil (0%).

Growth parameters	Soil concentration (%)			
	05	10	15	20
Seedling length	13.14	15.06	33.49	31.28
Root length	14.48	19.25	46.50	32.70
Shoot length	11.42	9.89	16.70	29.45
Number of leaves	8.10	21.62	54.05	89.18
Leaf area	0.27	22.64	47.43	59.56
Root fresh weight	36.84	63.15	76.31	92.10
Shoot fresh weight	20.51	64.10	76.92	92.30
Leaf fresh weight	12.82	31.25	65.62	77.14
Seedling fresh weight	65.13	41.28	66.97	87.15
Root dry weight	46.66	53.84	86.66	96.66
Shoot dry weight	30.30	42.42	69.69	93.93
Leaf dry weight	20.83	41.66	66.66	91.66
Seedling dry weight	34.09	51.13	73.86	93.18

Tab. 2. Percentage of tolerance in seedlings of *P. glaucum* against different (05, 10, 15, 20%) concentrations of crude oil polluted soil treatment as compared to control.

	Oil polluted soil concentration (%)			
	5	10	15	20
Tolerance indices	85.51	80.74	53.49	67.29

Discussion

Pollution caused by petroleum and its derivatives is the most prevalent problem in the environment. The release of crude oil into the environment by oil spills is receiving worldwide attention [MILLIOLI & al. 2009]. The results of the present study showed variability in seedling growth performance of *Pennisetum glaucum* as compared to control soil treatment. In the present studies the treatment of different level of oil polluted soil showed a clear variation in seedling growth performance of *P. glaucum* as compared to control soil treatment. Seedling length of *P. glaucum* was variable and appeared to be driven mostly by the treatment of different level of oil polluted soil treatment. The toxic effects of spent engine oil on chlorophyll and protein levels of *Amaranthus hybridus* and germination of perennial rye grass and maize growth performance was reported by some workers [ISIRIMAH & al. 1989; ODJEGBA & SADIQ, 2002; SIDDIQUE & ADAMS, 2002]. This study demonstrated that crude oil application at high concentration in soil has significant effect on the seedling growth performance of *P. glaucum.* These results are in conformity with the findings of other researcher's recording the effects of crude oil on the growth of few plant species [ANOLIEFO & al. 2003; VWIOKO & FASHEMI, 2005]. Reduction in the number of leaves of *P. glaucum* recorded when treated with different level of crude oil soil as compared to control. Similar trend of decline in leaf growth was recorded by ANOLIEFO & EDEGBAI (2001). The negative effects of oil contamination on the reduction of the total biomass and the length of the roots in *Avena sativa*, *Secale cereale* and *Hordeum vulgare* was recorded and suggested that these plants could be used as test organisms in analyzing the

toxicity of pollutants in soil and water [PETUKHOV & al. 2000]. In another investigation, MARANHO & al. (2006) investigated the effect of petroleum pollution on the leaf structure of *Podocarpus lambertii* Klotzsch ex Endl. (Podocarpaceae), and concluded that the leaf anatomy revealed a large variability related to pollution. Our data also showed the negative influence of oil polluted soil on leaf area of *P. glaucum* as compared to control soil treatment. The relatively low leaf area was observed in oil polluted soil at the level of 5, 10, 15 and 20% polluted soil. The availability of crude oil in soil make unsuitable environment for the development of root development for *P. glaucum*. Crude oil is phytotoxic because it creates unsatisfactory conditions for plant growth ranging from heavy metal toxicity to inhibited aeration of the soil [EDEMA & al. 2009].

Crude oil contamination at higher level (10-20%) affected root growth performance of *P. glaucum* due to development of unsuitable growth condition by oil pollution. Oil in soil creates unsatisfactory conditions for plant growth [DE JONG, 1980] probably due to insufficient aeration of the soil [ROWELL, 1977]. The contamination with petroleum affects the development of plants due to different physical effects. The oil film that covers the roots, modifying water absorption and nutrients considered as the main physical effect [XU & JOHNSON, 1995; HESTER & MENDELSSOHN, 2000; PEZESHKI & al. 2000]. According to BONA & SANTOS (2003) oil diminishes the soil capacity for retaining water, thus interfering with plant growth. The seedlings biomass of *P. glaucum* was least productive in oil contaminated soil. The continuous decrease in the seedling growth of growth parameter of *P. glaucum* in this study revealed that it is due to abiotic stress. Increase in the crude oil contamination which contains a wide variety of elements such as carbon, hydrogen, sulphur, nitrogen and oxygen [ABB, 1997; POTTER & SIMMONS, 1998; MARANHO & al. 2009] could be an important cause of decline in average shoot length of *P. glaucum*. There was a similar trend of negative effects on the productivity of *P. glaucum* recorded. The addition of different level of crude oil can leads to some physical and chemical changes in the soil resulting reduction in seedling growth performance of *P. glaucum*. The presence of the toxic pollutants from the crude oil in soil can be an important cause of decrease in seedling growth and ultimately resulted in reduction of biomass production performance for *P. glaucum*. Crude oil contains components that are toxic to plants [JESSUP & LEIGHTON, 1996].

Conclusions

It is concluded that the significant reduction in the seedling growth and production of *P. glaucum* grown in the crude oil contaminated soil can be served as good pollutant indicator of crude oil pollution. The results also showed that seedlings growth performance of *P. glaucum* decreased with the increase in oil polluted soil treatment as compared to control. Similar types of studies are suggested for other plant species to ascertain their possible use of plantation in oil polluted areas.

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NUTRIENT CONTENT IN DURIAN (*DURIO ZIBETHINUS* L.) BRANCH BARK

SAKHIDIN[1*], Jaime A. TEIXEIRA DA SILVA[2], Slamet Rohadi SUPARTO[1]

Abstract: Durian (*Durio zibethinus* L.) fruit form on the bark of branches. The aim of our research was to assess whether branches bearing different number of fruits have different nutrient contents in their bark. We determined the nitrogen (N), phosphorous (P), potassium (K), and carbon (C) content in branch bark 30 days after fruit set using branches bearing different number of fruits per panicle (0, 1, 2 or >2) of two varieties ('Otong' and 'Kani'). Bark was cut into 0.03 m long and 0.005 m wide segments with an average thickness of 0.00085 m. The bark of branches bearing a different number of fruits had the same N, P, K, and C content but different ratios of C/N, C/P, C/K, N/K, and P/K. The bark of 'Otong' branches had a higher N content but a lower C/N ratio than 'Kani' bark.

Keywords: durian, bark, nutrient, 'Otong', 'Kani'.

Introduction

Durian (*Durio zibethinus* L.) flowers are not terminal but form directly on the bark of branches. Consequently, to assess the link between nutrient content and fruiting quality, this study aimed to assess the content of key nutrients in the bark of durian branches. SAKHIDIN (2008) noted that the N and C content in the bark of durian branches, rather than leaves, influenced flower induction.

'Otong' and 'Kani' are two of the most important durian varieties in Central Java, Indonesia [DIRECTORATE OF FRUIT CROPS, 2008]. Flowering induction, fruit set, and fruit growth need a certain content of nutrients. VEMMOS (1995) stated that the carbon to nitrogen ratio (C/N) affects flowering and fruiting. A low carbohydrate concentration in trees caused by shading reduced flower number in *Lantana camara* L. [MATSOUKIS & al. 2003]. WU & al. (2013) stated that a high starch concentration in branches before flower formation and a high soluble sugar concentration during flower bud formation might benefit flower bud formation in carambola (*Averrhoa carambola* L.), which yields star fruit.

An insufficient supply of assimilates causes fruit abscission [BANGERTH, 2000]. In durian leaves and the bark of branches, nutrient content is relevant for the identification of nutritional deficiencies, imbalances or excesses. In durian, the highest C/N ratio was observed in a treatment that induced off-season flowering [SAKHIDIN, 2008].

Materials and methods

This research was conducted in a durian orchard that belongs to a farmer located in Pageralang Village, Kemranjen District, Banyumas Regency, Central Java, Indonesia from 15 September until 15 December 2013, at 45 m above sea level. The research

[1] Faculty of Agriculture, Universitas Jenderal Soedirman, Jl. Dr. Soeparno, Purwokerto 51123, Central Java – Indonesia.

[2] P. O. Box 7, Miki-cho post office, Ikenobe 3011-2, Kagawa-ken, 761-0799 – Japan.

[*] Corresponding author. e-mail: sakhidin1207@yahoo.com

materials were 12-year-old durian trees of two varieties, namely 'Otong' and 'Kani'. The study was conducted as a completely randomized design in which the first factor was variety while the second factor was the number of fruits per panicle (0, 1, 2, and >2). There were eight treatment combinations with four replications, so a total of 32 durian trees.

To analyze the N, P, K, and C content, branch bark was removed from the 32 trees when the fruits were 30 days old after fruit set. Analysis of N content was conducted by the KJELDAHL (1983) method. P and K content were determined by spectrophotometry, P content with a UV mini-1240 UV-Vis spectrophotometer at $\lambda = 430$ nm (Shimadzu, Kyoto, Japan), and K content with a Polarized Zeeman Automatic Absorption spectrophotometer at $\lambda = 768$ nm (Hitachi, Tokyo, Japan). C content was determined by the WALKLEY & BLACK (1934) method with a UV mini-1240 UV-Vis spectrophotometer at $\lambda = 560$ nm.

Bark at the bottom of each horizontal branch located between fruit and the leaf, at about 1 m from the main trunk and about 3 m above ground level, was cut into a segment 0.03 m long and 0.005 m wide (average thickness was 0.00085 m). Bark was dried at 60°C for 48 h in an oven. Data were analyzed using analysis of variance (ANOVA) with the statistical program SAS version 9. Following ANOVA, means were separated using Duncan's multiple range test and the *t*-test at $p<0.05$.

Results and discussion

The N, P, K, and C content in the bark of branches was statistically similar for all of the number of fruits per panicle (Tab. 1). Both varieties showed variation in N content in the bark, but no variation in P, K, and C content. Table 2 indicates that the branches bearing different numbers of fruit per panicle showed variation in the C/N, C/P, C/K, N/K, and P/K ratios, but not the N/P ratio. The C/N ratio of both varieties was significantly different (Tab. 2).

N, P, K, and C content

Table 1 shows that branches bearing different number of fruits per panicle had the same N, P, K, and C content in the bark. This result is in contrast with a claim made by HUETT (2000) that the productivity of horticultural crops is dependent on an adequate N status because photosynthetic capacity is dependent on leaf N content per unit area. URBAN & al. (2004) reported that fruit set was associated with a decrease in leaf carbohydrate concentration, probably as a consequence of an increased demand for both N and energy of the developing fruits.

TAHIR & HAMID (2012) showed the highest content of N, P, and K (1.67%) in the leaves of guava (*Psidium guajava* L.) plants when all fruits were removed, i.e., fully thinned. UPRETI & al. (2013) and DAVENPORT (2009) stated that a high C/N ratio was required for floral initiation in mango. As reproductive growth is a developmental event that requires high energy, flowering requires a large supply of carbohydrates [SANDIP & al. 2015]. In mango, the accumulation of carbohydrates and N in leaves is positively associated with flower bud initiation and differentiation [KUMAR & al. 2013].

Ratio of C/N, C/P, C/K, N/P, N/K and P/K

Table 2 shows that the highest C/N ratio (60.18) was achieved when there was one fruit per panicle, but this ratio was not significantly different to the C/N ratio when there were no fruits or more than two fruits per panicle, which shows that C in the bark of

branches is needed for fruit development. A lower – but insignificant – C content in branch bark was due to the absence of fruits or the presence of too many fruits. The highest C/P ratio was achieved when there were >2 fruits per panicle, but this was not significantly different to the C/P ratio when there were no or one fruit per panicle. ELKHISHEN (2015) reported that an increase in the C/N ratio led to improved fruit retention.

THAMRIN & al. (2009) showed that increasing C/N ratio by bark strangulation increased fruit set of pummelo (*Citrus grandis* (L.) Osbeck). A high C/N ratio in durian bark can increase fruit set in response to water stress in off-season production [SAKHIDIN, 2008]. The highest C/K, N/K, and P/K ratios occurred when there were no fruits on the panicle (Tab. 2). This implies that there was a relatively lower K content in the bark of branches without fruit and a relatively higher C, N, and P content than other fruit densities, although differences were not significant (Tab. 1). LOVATT & al. (1988) also proposed that in citrus, N and carbohydrates serve as substrates for the synthesis of key metabolites that act alone or work with plant hormones to initiate the flowering process.

'Otong' showed a higher N content and lower C/N ratio in the bark of branches than 'Kani', leading to a 5.50% and 11.94% fruit set, respectively (Tab. 1). A higher C/N ratio in 'Kani' was attributed to higher fruit set [PEBRIYANTI, 2014]. HIMAWAN (2014) showed that these two durian varieties show differences in fruit drop, 66.67% in 'Kani' but 70.89% in 'Otong', possibly as a result of this difference in the C/N ratio of the branch bark. SANDIP & al. (2015) and KUMAR & al. (2013) showed that the C/N ratio differed during shoot growth in mango varieties, which reveals its dependence on environmental conditions and prevailing metabolic balance.

Tab. 1. N, P, K, and C content (%) in bark of branches with different numbers of fruit per panicle of two durian varieties

		N	P	K	C
Number of fruits per panicle*	0	0.763 a	0.908 a	0.704 a	40.437 a
	1	0.729 a	0.874 a	0.818 a	41.240 a
	2	0.891 a	0.987 a	1.044 a	38.752 a
	>2	0.720 a	0.806 a	1.016 a	37.370 a
Varieties**	'Otong'	0.879 a	0.878 a	0.927 a	40.405 a
	'Kani'	0.672 b	0.910 a	0.864 a	38.494 a

*Means followed the same letter within each treatment are not significantly different (DMRT; 5%)
** Means followed the same letter within each treatment and variety are not significantly different (*t*-test; 5%)

Tab. 2. Ratio of C/N, C/P, C/K, N/P, N/K and P/K in bark of branches with different numbers of fruit per panicle of two durian varieties

		C/N	C/P	C/K	N/P	N/K	P/K
Number of fruits per panicle*	0	54.62 ab	49.19 ab	80.37 a	0.89 a	1.50 a	1.64 a
	1	60.18 a	49.94 ab	62.02 b	0.86 a	1.07 b	1.19 b
	2	49.37 b	41.72 b	43.84 c	0.89 a	0.89 b	1.07 b
	>2	54.19 ab	51.40 a	40.42 c	0.95 a	0.76 b	0.84 b
Varieties**	'Otong'	49.51 b	50.72 a	57.24 a	1.03 a	1.15 a	1.12 a
	'Kani'	59.68 a	45.41 a	56.09 a	0.76 a	0.96 a	1.25 a

Explanations as for Tab. 1.

Conclusions

The durian (*Durio zibethinus* L.) tree is unique in that it forms fruit directly on the bark of branches. This study found that there were no differences in the N, P, K, and C content of bark when there was no fruit, or even when more than two fruits per panicle, although C/N, C/P, C/K, N/K, and P/K ratios differed. Two varieties of durian were tested, and 'Otong' bark had more N than 'Kani' bark. The ability to develop fruit in durian might not depend on the levels of N, P, K, and C content, but rather on other factors.

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ESTIMATION OF HERITABILITY AND PREDICTED GENOTYPE MEAN FOR SEED YIELD OF CASTOR (*RICINUS COMMUNIS* L.) USING BEST LINEAR UNBIASED PREDICTION (BLUP)

Bolaji Zuluqurineen SALIHU[*1], Ahmed Olamide FALUSI[2], Andrew Saba GANA[3], Matthew Omoniyi ADEBOLA[2], Oladipupo Yusuf DAUDU[2]

Abstract: Eighty six castor genotypes were evaluated to estimate heritability and predicted genotype mean for seed yield and its component traits. The genotypes were planted at three locations using incomplete block experimental design with three replications. The results revealed significant effects of genotypes for most of the traits evaluated. Significant interactions of genotypes by locations were observed for six traits including 100 seed weight and seed yield. Genotypic coefficient of variation and phenotypic coefficient of variation were high for number of branches per plant and 100 seed weight. Heritability for the studied traits ranged from 0.21 to 0.92. High genetic advance as percentage of mean coupled with high heritability were observed for most of the traits. Thirty six out of eighty six genotypes evaluated presented predicted genotypic seed yield mean above the general mean. These results revealed moderate to high possibility for improvement of five out of ten traits evaluated.

Keywords: Badeggi, component traits, genetic advance, Minna, Phenotypic variation.

Introduction

Castor oil plant (*Ricinus communis* L.) is one of the most versatile oil crops with high socio-economic values around the world [GANA & al. 2013]. The crop has been demonstrating its economic potentials by earning notable foreign exchange credits to many countries like India, China and Brazil [OGBEH, 2014]. The castor oil, which is extracted from castor seed, is very critical to many industrial applications because of its unique ability to withstand high and low temperatures, and to form many valuable derivatives; commanding huge amount of demand at the international market [MUTLU & MEIER, 2010; OGUNNIYI, 2006]. Annual world castor production is estimated at 1,314,193 MT, produced on a total area of 1,369,720 hectare with estimated seed yields of 1,200 kg per hectare [FAOSTAT, 2008]. This was produced majorly in India, China and Brazil. In Nigeria, hectarage was estimated at about 6000 ha, and production at about 3000–4000 MT in 2004. The major producers then were Cross River and Ebonyi states [Raw Material Research and Development Council – RMRDC, 2009]. However in 2013, Kogi, Enugu, Oyo and Osun states were identified as some of major castor producers in Nigeria [GANA, 2015].

The castor production in Nigeria is limited majorly by low average yield among the farmers [AMOSUN & al. 2013]. The low productivity is associated with many biotic (insects, diseases & weeds) factors, abiotic (drought, low soil fertility, etc.) factors and lack of improved varieties [SALIHU & al. 2014]. Therefore, developing improved varieties is one

[1] National Cereals Research Institute, Castor Research Programme, P.M.B. 8, Badeggi, Bida – Nigeria
[2] Federal University of Technology, Department of Biological Sciences, P.M.B. 65, Gidan Kwanu, Minna – Nigeria
[3] Federal University of Technology, Department of Crop Production, P.M.B. 65, Gidan Kwanu, Minna – Nigeria
[*] Corresponding author. E-mail: mobolajialabi2007@gmail.com

of important measures to curb the castor production constraints in Nigeria. In this respect, estimation of heritability for important agronomic traits is the primary precondition for any improvement programme. Precise estimation of heritable variance component and accurate selection are of great importance in this regard. This can be achieved through the use of optimal estimation/prediction procedures, which will lead to the maximization of genetic gain from selection. For unbalanced data sets (e.g. data from incomplete block design), the optimum estimation and prediction procedure could be achieved by likelihood ratio test and Best Linear Unbiased Prediction (BLUP). For balanced data sets, the estimation of variance components by the least squares method (analysis of variance) produces similar estimates [RESENDE & HIGA, 1994]. In this present study, 86 castor genotypes were evaluated in an incomplete block design across three locations. The objective of the study was to estimate the heritability, genetic advance and predicted genotype means with the purpose of starting a castor breeding programme within the study area.

Materials and methods

The research was carried out at three sites in Niger State. The state lies in the northern region of Nigeria with savanna vegetation. The state enjoys six month rainfall season (May to October) and six month dry season (November to April). The average rainfall ranges between 865 mm and 1139 mm, and temperature ranges between 24.3 °C and 33.9 °C. The trial sites within the state were Mokwa (Lat. 9°12`N, Long. 5°20`E), NCRI Badeggi (Lat. 9°45`N, Long. 6°07`E) and Minna (Lat. 9°36`50``N, Long. 6°33`25``E).

The planting material used for the present research comprised of 86 castor accessions obtained from National Cereals Research Institute (NCRI), Badeggi, Nigeria. The eighty six castor genotypes were evaluated at three locations mentioned above. The treatments were laid out on an incomplete block design with three replications. The plot size was 3 m by 1.5 m with Inter-row and intra-row spaces of 75 cm by 75 cm. Two seeds per hole were sown and later thinned to one seedling per hole at three to four weeks after planting. Planting at all the locations was done within a week (12–18th of June, 2015). Fractional fertilizer requirement [OGUNLADE, 1993] for individual plant stand was estimated and applied by band placement. Insecticide (Cepermithrin at 100ml/15L) and Fungicide (Mancozeb at 20g/15L) were applied three times before flowering. Morphological data were taken according to standard castor descriptor [INDIA, 2004]. The parameters considered include: Establishment counts (%), days to first spike flowering, days to first spike maturity, branches per plant, spike per plant, plant height at first raceme maturity, seed yield (Kg/Ha) and 100 Seed weight (g). Data were analyzed using random model procedure of Plant Breeding Tools [PBTOOLS 1.3, 2014]. Estimates of heritability and predicted genotype means were derived using the Best Linear Unbiased Prediction (BLUP). The models for testing the significant effects of each variance component are as follow.

Genotypic effect:

Model 1: Trait ~ 1 + (1|Treatment) + (1|Trial) + (1|Rep:Trial) + (1|Rep:Block:Trial) + (1|Treatment:Trial)

Model 2: Trait ~ 1 + (1|Trial) + (1|Rep:Trial) + (1|Rep:Block:Trial) + (1|Treatment:Trial)

Environment effect:

Model 1: Trait ~ 1 + (1|Treatment) + (1|Trial) + (1|Rep:Trial) + (1|Rep:Block:Trial) + (1|Treatment:Trial)

Model 2: Trait ~ 1 + (1|Treatment) + (1|Rep:Trial) + (1|Rep:Block:Trial) + (1|Treatment:Trial)

Genotype by Environment Effect:

Model 1: Trait ~ 1 + (1|Treatment) + (1|Trial) + (1|Rep:Trial) + (1|Rep:Block:Trial) + (1|Treatment:Trial)

Model 2: Trait ~ 1 + (1|Treatment) + (1|Trial) + (1|Rep:Trial) + (1|Rep:Block:Trial)

The magnitude of the effects was determined using ROBERT & RAFTERY (1995) procedure.

Genotypic variance ($\mathfrak{e}^2_g$) = $\mathfrak{e}^2_e$ + r $\mathfrak{e}^2_g$l+ r$\mathfrak{e}^2_g$

G x L ($\mathfrak{e}^2ge$) = $\mathfrak{e}^2_e$ + r $\mathfrak{e}^2_g$l

Phenotypic variance ($\mathfrak{e}^2_p$) = $\mathfrak{e}^2_g$ + $\mathfrak{e}^2ge$ / m_h + $\mathfrak{e}^2$/ P_h [PIEPHO & MÖHRING, 2007] - for incomplete block design)

$$m_h = \frac{n}{\sum_{i=1}^{n} 1/m_i} \qquad P_h = \frac{n}{\sum_{i=1}^{n} 1/P_i}$$

H^2 = 1─$\overline{\upsilon}_{BLUP}$ /2 $\mathfrak{e}^2_g$ [PIEPHO & MÖHRING, 2007]

GA = H^2 x I x $\mathfrak{e}_p$

GAM (%) = {GA/Mean} x 100

$\mathfrak{e}^2_p$ = phenotypic variance

$\mathfrak{e}^2_g$ = genotypic variance

$\mathfrak{e}^2_{ge}$ = GxE variance

$\mathfrak{e}^2$ = residual variance

m_i = number of environments for *ith* genotype

P_i = number of plots for *ith* genotype

n = number of genotypes

$\overline{\upsilon}_{BLUP}$ = mean variance of a difference of the BLUP of g_i

H^2 = broad sense heritability

GA = genetic advance

$\mathfrak{e}_p$ = phenotypic standard deviation

I = selection differential (at 5% = 2.06)

GAM = genetic advance as percentage of mean (expected genetic gain)

Results and discussion

Significance tests for effects of all sources of variation

The results of tests for the effects of all the sources of variation are showed in Tab. 1. The tests revealed significant effects of genotypes for all the studied traits except spikes per plant and plant height at raceme maturity. This is an indication for the existence of considerable genetic variability among the genotypes for most of the traits and as such there is ample scope of selection for different quantitative traits for improvement of the crop. The results revealed significant interactions of genotypes by locations for height at first spike flowering, spikes per plant, days to first raceme maturity, 100 seed weight and seed yield, indicating the possibility of exploiting different environments for development of location specific castor varieties from the genotypes. According to ROBERT & RAFTERY (1995) the magnitude of genotypic effects was found to be more in most of the traits ranging from

very strong effects (BIC2–BIC1 > 10) to strong (BIC2–BIC1; 6-10). The magnitude of GxL effects was only more (very strong) for spikes per plant and seed yield among the ten traits evaluated. The high magnitude of GxL effects indicated the need for considerable multi-environments testing for the ranking of the genotypes for the superior seed yield performance [GOMEZ & GOMEZ, 1984]. Similar GxL interactions in castor were reported by LAURETI (1988).

Estimates of variance components

Variability in all the studied traits was estimated through phenotypic and genotypic coefficient of variations. According to DESHMUKH & al. (1986), genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (GCV) can be partitioned as high (>20%), moderate (10-20%) or low (< 10%). On this basis, moderate to low GCV and PCV were observed for most of the traits studied (Tab. 2). However, high GCV and PCV were recorded for branches per plant and 100 seed weight. In all the traits, PCV was considerably higher than GCV. However, the magnitude of differences was low for all the traits except in spikes per plant where it was moderate. This is an evidence 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. These results are in agreement with the reports of ALLAN & al. (2008), RAO & al. (2009) and ZHENG & al. (2010). But the result is in disagreement with the findings of PATEL & JAIMINI (1988) who reported moderate to high coefficient of variation for most of the traits in castor irrespective of the environment.

Estimates of heritability in broad sense

Heritability of a character may be categorized to be high (> 0.6), moderate (0.3–0.6) and low when it is less than 0.3 [SHIVANNA, 2008]. High heritability was observed for seedling establishment, days to first spike flowering, spike length, branches per plant, days to first raceme maturity and 100 seed weight (Tab. 2). Low heritability was recorded for height at first spike flowering and spikes per plant, while seed yield showed moderate heritability. 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 reports of SEVUGAPERUMAL & al. (2000) and GOLAKIA & al. (2007). SHIVANNA (2008) reported high heritability for all these traits in castor in contrary to the present study where low heritability was recorded for height at first spike flowering and spikes per plant.

Estimates of Expected genetic advance

Heritability value alone does not imply the actual amount of genetic gain in selection programme [SHIVANNA, 2008]. Heritability estimates along with genetic advance gives best prediction of genetic progress in selection. According to DESHMUKH & al. (1986), genetic advance as percentage of mean (GAM) could be classified as low (<10%), moderate (10–20%) and high (>20%). Based on this classification, plant height at first spike flowering, spikes per plant, height at first raceme maturity and days to first raceme maturity recorded low genetic advance as percentage of mean. High GAM was observed for seedling establishment, days to first spike flowering, branches per plant, 100 seed weight and seed yield. The results showed that selecting the top 5% of the genotypes could result in a genetic improvement of 36.84% for seedling establishment, 52.30% for increased number of

branches, 69.92% for 100 seed weight and 11.33% of seed yield per hectare. The low GAM observed in some of the traits implies that there would be no rewarding selection process for the referenced traits in any new population after one cycle of selection. Similar results on genetic advance as percentage of mean in castor were reported by LAKSHMAMMA & al. (2005). SHIVANNA (2008) reported high GAM for plant at first spike flowering, days to 50% flowering, days to maturity, spike length and spikes per plant.

Mean performances and predicted genotype seed yield mean

The mean performances and predicted genotype seed yield mean of the genotypes evaluated (Tab. 3) showed a range of 40% (in Acc.041) to 93% (Acc.001) seedling establishment with average mean of 72.14%. Highest number of days (111.78 days) to first spike flowering and number of days (134.44 days) to first raceme maturity were recorded in Acc. 045. The least days to maturity (96.62 days) was observed in Acc.005. Plant height at maturity varied between 91.62 cm (Acc.36M1) and 141.77 cm (Acc.099) with average of 113.70 cm. Length of spike ranged from 13.33 cm (Acc. 036M1) to 29.56 cm (Acc.010). Hundred (100) seed weight ranged from 10.74 g (Acc. 005) to 50.36 g (Acc.045) with average of 24.91 g. Average value of 646.04 kg/ha and a range of 334.76 kg/ha (Acc.067) to 1348.84 kg/ha (Acc.001) was observed for Seed yield (kg/ha). SHIVANNA (2008) and GOLAKIA & al. (2007) reported similar mean performance for various traits in castor.

However, among the 86 genotypes evaluated, thirty six genotypes presented predicted genotype yield means above the general mean (Tab. 3). The genotype with the highest predicted genotypic value was Acc.001 while Acc.067 presented the least predicted genotypic value among all the entries. According to SIMEAO & al. (2002), selection of superior genotypes should be based on the predicted mean components. In this respect, the first best five genotypes with high predicted yield means were Acc.001, Acc.036M1, Acc.036, Acc.010 and Acc.045 among all the genotypes evaluated. The lowest genotypic value prediction observed in genotype Acc.067 may be partly due to its high susceptibility to leaf spot fungi infections incidence in the study area. Similar reports on the uses of BLUP for genetic prediction in annual crops were given by CARBONELL & al. (2004) in their work on 18 common bean genotypes and REIS & al. (2005) reported the estimation of variance components, prediction of breeding values of maize genotypes using REML/BLUP procedures.

Tab. 1. Effects of all components of variance for ten agronomic traits in castor at three locations

Parameters	Genotypic Effect		Environmental Effect		Genotype X Environ.	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
Establishment (%)						
AIC	7832.20	7894.12	7832.20	7830.20	7832.20	7830.21
BIC	7865.53	7922.69	7865.53	7858.77	7865.53	7858.78
LogLik.	-3909.10	-3941.06	-3909.10	-3909.10	-3909.10	-3909.11
Chisq.	63.92		0.001		0.01	
Df	1		1		1	
Pr (>Chisq)	0.00		0.98		0.92	
$BIC_2 – BIC_1$	57.16		-6.76		-6.75	
Days to Flowering						
AIC	6749.18	6833.60	6749.18	6754.74	6749.18	6747.31
BIC	6782.52	6862.18	6782.52	6783.31	6782.52	6775.88
LogLik.	-3367.59	-3410.80	-3367.59	-3371.37	-3367.59	-3367.65
Chisq.	86.42		7.55		0.12	
Df	1		1		1	
Pr (>Chisq)	0.00		0.01		0.73	
$BIC_2 – BIC_1$	79.66		0.79		-6.64	
Height at Flowering (cm)						
AIC	7647.90	7659.34	7647.90	7654.52	7647.90	7659.26
BIC	7681.24	7687.92	7681.24	7683.09	7681.24	7687.84
LogLik.	-3816.95	-3823.67	-3816.95	-3821.26	-3816.95	-3823.63
Chisq.	13.44		8.62		13.36	
Df	1		1		1	
Pr (>Chisq)	0.00		0.00		0.00	
$BIC_2 – BIC_1$	6.68		1.85		6.60	

Spikes per Plant						
AIC	3017.58	3016.54	3017.58	3042.61	3017.58	3058.38
BIC	3050.93	3045.12	3050.93	3071.19	3050.93	3086.97
LogLik.	-1501.79	-1502.27	-1501.79	-1515.30	-1501.79	-1523.19
Chisq.	0.96		27.03		42.80	
Df	1		1		1	
Pr (>Chisq)	0.33		0.00		0.00	
$BIC_2 - BIC_1$	-5.81		20.26		36.04	
Spike Length (cm)						
AIC	5584.31	5596.32	5584.31	5591.28	5584.31	5582.72
BIC	5617.65	5624.90	5617.65	5619.87	5617.65	5611.30
LogLik.	-2785.15	-2792.16	-2785.15	-2789.64	-2785.15	-2785.36
Chisq.	14.02		8.98		0.41	
Df	1		1		1	
Pr (>Chisq)	0.00		0.00		0.52	
$BIC_2 - BIC_1$	7.25		2.22		-6.35	
Branches per Plant						
AIC	3160.82	3192.03	3160.82	3158.82	3160.82	3158.86
BIC	3194.15	3220.60	3194.15	3187.39	3194.15	3187.43
LogLik.	-1573.41	-1590.02	-1573.41	-1573.41	-1573.41	-1573.43
Chisq.	33.22		0.00		0.05	
Df	1		1		1	
Pr (>Chisq)	0.00		0.98		0.83	
$BIC_2 - BIC_1$	26.45		-6.76		-6.72	
Height at Maturity (cm)						
AIC	8012.37	8014.09	8012.37	8020.26	8012.37	8011.48
BIC	8045.68	8042.65	8045.68	8048.82	8045.68	8040.04
LogLik.	-3999.19	-4001.05	-3999.18	-4004.13	-3999.19	-3999.74

Chisq.	3.73		9.89		1.11	
Df	1		1		1	
Pr (>Chisq)	0.05		0.00		0.29	
$BIC_2 - BIC_1$	-3.03		3.14		-5.28	
Days to Maturity						
AIC	6757.06	6780.94	6757.06	6755.71	6757.06	6762.77
BIC	6790.37	6809.50	6790.37	6784.26	6790.37	6791.33
LogLik.	-3371.53	-3384.47	-3371.53	-3371.85	-3371.53	-3375.39
Chisq.	25.88		0.65		7.71	
Df	1		1		1	
Pr (>Chisq)	0.00		0.42		0.00	
$BIC_2 - BIC_1$	19.13		-6.11		0.96	
Seed Weight (g)						
AIC	6287.97	6306.50	6287.97	6298.86	6287.97	6306.50
BIC	6321.28	6335.06	6321.28	6327.42	6321.28	6331.06
LogLik.	-3136.98	-3147.25	-3136.98	-3143.43	-3136.98	-3147.25
Chisq.	20.54		12.90		20.54	
Df	1		1		1	
Pr (>Chisq)	0.00		0.00		0.00	
$BIC_2 - BIC_1$	13.78		6.14		9.78	
Seed Yield (kg/ha)						
AIC	11121.66	11125.35	11121.66	11124.09	11121.66	11218.89
BIC	11154.98	11154.99	11154.98	11152.65	11154.98	11247.45
LogLik.	-5553.83	-5556.67	-5553.82	-5556.05	-5553.83	-5603.45
Chisq.	5.69		4.43		99.23	
Df	1		1		1	
Pr (>Chisq)	0.02		0.04		0.00	
$BIC_2 - BIC_1$	0.01		-2.33		94.47	

AIC – Akaike's Information Criterion, BIC – Bayesian Information Criterion, LogLik. – loglikelihood, Df – degree of freedom between the models

Tab. 2. Combined mean values, variance components, heritability and genetic advance for all the traits studied

Parameters	Mean	$\epsilon^2 g$	$\overline{\upsilon}_{BLUP}$	$\epsilon^2 gxe$	ϵ^2_P	GCV	PCV	H^2	GA	GAM
ESTAB	71.35	154.23	30.85	1.66	200.99	17.41	19.87	0.90	26.28	36.84
DF	69.21	51.73	9.31	1.62	64.88	10.39	11.64	0.91	15.10	21.82
HF	71.79	46.34	73.22	54.48	99.35	9.48	13.88	0.21	4.31	6.01
SL	19.03	3.5	2.17	0.82	7.37	9.83	14.27	0.69	3.86	20.28
BPP	4.98	0.37	0.13	0.02	0.59	24.43	30.96	0.82	1.30	26.11
SPP	5.92	0.06	0.10	0.51	0.39	8.30	21.11	0.21	0.27	4.56
HM	113.7	28.92	29.50	24.67	99.93	4.73	8.79	0.49	10.09	8.88
DM	109.81	24.91	13.45	14.77	42.56	4.55	5.94	0.73	9.81	8.93
SW	24.91	74.28	11.89	12.74	84.47	34.60	36.90	0.92	17.42	69.92
SY	646.04	2607.67	3129.20	10224.27	7656.79	7.90	13.55	0.40	72.10	11.33

$\epsilon^2 g$ = genotypic variance, υ_{BLUP} = mean variance of a difference of the BLUP of g_i, $\epsilon^2 gxe$ = variance due to interaction of genotype and environment, ϵ^2_P = phenotypic variance, GCV = genotypic coefficient variance, PCV = phenotypic coefficient variance, H^2 = broad sense heritability, GA = genetic advance, GAM = genetic advance as percentage of mean;

Parameters: ESTAB = Seedling establishment (%), DF = days to flowering, HF = height at first spike flowering (cm), SL = spike length (cm), BPP = branches per plant, SPP = spikes per plant, HM = height at first raceme maturity (cm), DM = days to first raceme maturity, SW = 100 seeds weight, SY = seed yield (kg/ha).

Tab. 3. Combined means for ten agronomic traits of castor across three locations

Genotypes	ESTAB	DF	HF (cm)	SPP	SL (cm)	BPP	HM (cm)	DF	SW (g)	SY (Kg/Ha)	Predicted SY(Kg/Ha)
Acc.001	93.44	95.67	74.76	5.12	22.33	4.66	117.57	128.44	49.89	1349.84	1083.24
Acc.036M1	88.33	70.89	49.15	10.44	13.33	12.56	91.62	122.00	13.18	1191.04	936.64
Acc.036	81.11	73.67	79.35	5.56	28.59	7.12	130.93	106.89	20.58	918.86	829.74
Acc.010	74.44	66.78	63.24	4.88	29.56	3.78	107.77	105.44	11.31	995.96	762.26
Acc.045	79.67	111.78	77.55	3.56	20.96	4.00	120.56	134.44	50.38	985.32	760.04
Acc.005	85.88	58.38	40.17	7.24	26.83	6.24	104.31	96.62	10.74	971.66	755.82
Acc.026	83.33	69.33	59.56	4.66	18.30	3.56	102.65	112.11	23.16	688.82	751.06
Acc.053	80.00	64.22	84.23	7.34	18.48	5.56	137.33	101.78	25.96	941.70	734.96
Acc.099	82.22	70.56	103.55	6.00	22.07	5.12	141.77	118.67	26.71	884.92	725.90
Acc.040	77.78	73.89	84.83	4.00	16.70	3.56	118.20	114.67	42.29	844.94	711.88
Acc.048	81.25	67.38	69.11	5.76	16.33	4.24	112.28	108.75	15.92	840.88	709.16
Acc.003	82.07	63.22	60.87	6.22	19.44	5.12	118.88	104.00	15.88	609.42	704.48
Acc.046	60.00	76.50	63.16	6.00	17.83	4.00	100.92	115.70	16.25	811.90	700.80
Acc.009	76.67	76.22	80.38	3.12	16.63	3.78	117.66	115.44	44.98	995.02	696.78
Acc.022	56.67	72.11	62.14	6.22	20.85	5.56	97.57	114.00	15.06	593.14	695.08
Acc.019	81.11	70.11	72.68	5.78	20.04	4.00	120.72	108.25	15.12	799.44	685.78
Acc.042	60.00	74.44	75.73	4.22	18.56	4.44	99.36	109.22	32.23	741.62	676.46
Acc.012	66.67	68.56	56.62	6.44	17.89	5.78	101.82	110.67	12.87	642.62	676.12
Acc.002	88.89	77.44	83.21	5.56	20.96	4.00	115.44	114.00	27.61	721.72	669.08
Acc.091	76.67	65.11	65.54	7.12	18.44	5.34	103.14	104.11	28.87	717.92	668.68
Acc.072	75.56	65.11	72.87	7.12	19.67	5.34	118.34	109.33	20.17	715.98	666.64
Acc.016	85.56	78.00	87.58	4.44	15.89	3.34	117.92	115.78	42.80	514.36	664.24

Acc.006	70.00	66.89	75.58	6.66	17.74	4.88	116.31	96.78	15.65	538.88	662.86
Acc.044	68.89	75.89	81.81	3.78	15.63	4.00	114.12	116.50	32.65	656.12	661.40
Acc.027	80.00	64.89	82.20	5.78	21.37	4.22	130.76	109.67	24.07	562.74	660.44
Acc.097	86.25	60.62	80.30	6.00	18.25	4.24	116.79	104.88	25.62	682.64	659.12
Acc.103	65.56	71.67	57.65	5.56	16.44	4.00	94.57	107.56	12.20	682.06	656.92
Acc.073	70.44	69.89	74.07	7.56	18.67	5.12	111.24	113.11	20.00	676.66	654.96
Acc.061	84.44	71.22	71.81	5.12	20.18	4.88	104.36	103.89	19.09	677.46	654.76
Acc.062	87.78	68.33	77.96	6.00	19.96	5.78	124.66	104.11	21.14	666.02	651.98
Acc.047	70.33	74.44	74.04	5.34	20.78	4.22	106.72	111.33	14.28	665.44	650.16
Acc.051	46.25	62.25	52.87	4.00	13.50	4.00	89.66	101.25	26.05	681.90	649.44
Acc.100	62.22	63.75	66.52	6.66	16.85	4.88	102.24	105.62	25.46	656.68	648.62
Acc.095	82.00	61.50	61.34	6.80	20.23	6.00	101.23	104.60	24.50	658.10	647.82
Acc.070	83.00	64.20	69.80	5.60	18.97	4.80	116.02	103.60	17.32	699.22	647.10
Acc.035	54.44	77.44	80.40	3.34	20.37	4.00	125.98	117.67	28.30	1002.88	646.58
Acc.039	75.00	75.33	72.26	4.44	18.37	4.44	111.46	117.22	15.83	642.40	643.06
Acc.015	62.22	69.89	82.43	4.00	16.26	4.00	123.64	110.67	46.14	706.54	642.56
Acc.031	42.50	70.00	56.80	4.00	16.00	4.66	112.83	108.83	14.34	559.30	642.48
Acc.056	81.11	68.33	82.62	8.66	21.67	8.44	126.44	110.56	26.84	633.74	640.50
Acc.096	81.11	54.78	62.13	7.34	19.19	6.22	108.01	101.78	27.20	631.76	639.88
Acc.050	57.78	77.33	61.71	4.88	22.00	4.66	101.56	108.89	15.24	619.66	634.70
Acc.089	85.56	66.11	71.14	6.00	15.89	4.88	109.56	106.44	26.10	612.14	631.82
Acc.004	86.67	66.67	62.51	6.00	20.41	3.78	106.79	108.78	15.03	971.44	631.00
Acc.018	84.44	72.78	75.87	6.00	19.59	3.78	111.68	109.67	22.16	718.32	629.04
Acc.033	28.33	72.78	61.88	6.00	23.78	4.44	102.88	114.56	25.04	542.10	628.92
Acc.093	71.11	56.78	64.55	5.78	20.18	6.22	121.23	100.67	32.54	597.86	627.96
Acc.063	70.00	71.57	67.37	5.14	18.86	4.58	110.00	107.86	20.07	608.84	627.92

Acc.024	82.22	75.56	82.25	4.66	15.30	4.22	120.93	114.89	31.83	961.16	625.54
Acc.057	80.00	57.73	93.67	8.72	19.67	4.90	131.81	100.09	22.23	565.32	623.08
Acc.098	58.89	67.22	66.26	6.00	17.30	4.22	109.72	110.00	23.50	583.24	622.36
Acc.090	54.44	69.22	83.52	7.34	19.85	6.22	114.42	109.11	29.87	565.08	620.14
Acc.054	77.78	70.22	73.58	5.12	21.69	4.66	124.58	110.33	31.59	569.38	620.00
Acc.064	87.78	66.89	74.93	6.88	15.85	5.34	108.78	112.89	25.61	566.98	618.70
Acc.081	80.00	75.33	84.84	5.78	19.37	5.12	124.04	114.67	25.12	565.60	616.78
Acc.028	81.11	72.89	67.35	6.22	18.70	5.12	110.63	117.89	23.64	531.44	616.12
Acc.032	78.38	79.11	69.52	4.00	17.93	4.44	104.22	116.67	34.07	597.26	613.42
Acc.007	74.44	63.56	66.19	5.78	18.22	3.78	113.73	110.44	30.00	541.34	608.76
Acc.034	70.00	70.00	72.23	6.00	21.52	4.22	112.31	110.89	17.58	654.30	608.72
Acc.008	73.33	73.89	67.99	4.88	16.26	4.66	120.12	107.56	29.38	797.60	608.34
Acc.043	38.89	80.00	69.56	3.78	18.63	3.56	102.26	120.22	48.24	536.60	606.14
Acc.041	40.44	74.22	78.89	5.56	19.04	5.12	110.50	112.78	38.64	533.94	605.24
Acc.029	85.56	71.22	58.47	6.44	19.07	4.44	107.01	111.22	14.12	646.96	604.76
Acc.066	56.67	67.00	74.20	6.88	19.15	5.78	116.91	99.56	23.49	526.10	604.32
Acc.065	69.90	66.38	78.25	8.00	21.83	5.24	126.65	107.00	18.89	505.86	604.00
Acc.087	79.78	66.00	73.17	6.00	15.30	5.56	112.89	103.67	32.85	519.58	601.38
Acc.017	52.22	71.44	62.31	5.56	18.30	5.78	109.07	122.89	17.81	601.08	600.26
Acc.080	76.67	69.78	68.56	6.44	20.81	5.12	122.60	109.11	25.45	516.30	599.68
Acc.077	62.00	68.80	79.10	6.00	21.17	4.80	119.63	110.30	19.21	511.12	592.44
Acc.068	83.33	64.22	77.16	5.56	17.00	4.66	126.12	101.89	23.18	496.28	592.14
Acc.052	66.67	60.00	67.11	7.12	17.85	4.66	107.23	101.67	23.09	489.64	591.28
Acc.102	55.56	75.00	62.82	5.34	18.11	4.44	97.69	115.62	14.57	488.46	590.50
Acc.058	68.00	64.30	69.91	5.60	17.63	4.60	114.30	106.50	16.10	455.36	582.30
Acc.094	70.56	65.00	64.80	6.00	16.70	4.66	104.06	109.11	30.04	450.42	577.68

Acc.085	66.67	71.00	85.79	5.78	19.30	4.66	130.53	120.33	25.60	450.54	577.08
Acc.060	78.89	66.44	82.55	5.34	19.15	5.34	120.62	111.22	25.68	442.70	575.30
Acc.071	73.33	79.11	73.23	4.44	18.93	4.00	108.71	123.78	16.88	443.70	574.16
Acc.075	82.22	66.56	73.57	5.56	19.81	4.66	115.91	109.44	24.85	441.60	571.72
Acc.088	80.00	57.44	63.83	9.12	17.52	5.78	108.98	104.11	27.01	423.82	568.06
Acc.059	75.00	58.00	70.60	6.76	16.38	6.24	112.31	105.88	37.00	430.26	567.26
Acc.076	83.33	59.11	114.78	6.66	19.00	4.66	137.71	105.11	17.34	414.30	567.14
Acc.055	73.33	69.11	67.06	6.00	19.56	4.88	118.87	110.22	25.06	412.52	565.40
Acc.069	66.67	61.44	65.04	6.44	15.52	4.66	108.04	109.00	17.11	397.58	560.16
Acc.101	62.00	65.30	78.13	6.60	28.57	5.20	110.63	106.70	25.25	367.30	553.66
Acc.083	83.33	74.67	76.93	6.88	17.56	5.34	135.58	113.22	26.84	363.92	548.20
Acc.067	72.00	64.75	65.74	6.76	17.92	5.50	112.62	107.38	35.95	334.76	540.76
Pooled Mean	**72.14**	**69.21**	**71.79**	**5.92**	**19.04**	**4.98**	**113.70**	**109.81**	**24.91**	**645.66**	**646.04**

ESTB – Seedling Establishment (%); DF – Days to first spike flowering; HF – Height at Flowering (cm); DM – Days to First Spike Maturity; HM – Height at Maturity (cm); BPP – Branches per Plant; SPP – Spikes per Plant; SL – Spike Length (cm); CPS – Capsules per Spike; SW – 100 Seed Weight (g); SY – Seed Yield (kg/ha)

Conclusions

The results of the study demonstrated moderate to high possibilities for improvement of five out of ten traits evaluated. This information is very critical for commencement of a castor breeding programme in the study area with the evaluated genotypes. It could be concluded that the genotypes evaluated presented potentials for generating superior population in an improvement programme. The use of BLUP provided higher selection accuracy and so permits the identification of potential genotypes for improvement exercise. According to the results, the first best two genotypes (Acc.001 and Acc.036M1) could be recommended for cultivation in the study area. Beside this direct selection for cultivation under short-term breeding plan, the genotypes with predicted values above the general mean could be used to initiate hybridization scheme and/or used to develop potential base breeding populations for medium and long term breeding programme.

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PRELIMINARY STUDIES ON EFFECTS OF GAMMA RAY ON SEED RETENTION INDICES OF THREE NIGERIAN SESAME (*SESAMUM INDICUM* L.) VARIETIES

Muhammad Liman MUHAMMAD[1*], Olamide Ahmed FALUSI[1], Mathew Omoniyi ADEBOLA[1], Onyedi David OYEDUM[2], Oladipopu Abdulazeez Yusuf DAUDU[1], Gado Adamu AISHATU[1]

Abstract: In an attempt to improve sesame for reduction of seed loss due to shattering of capsule at harvest, the seeds of three varieties of sesame (NCRIBEN-04E, NCRIBEN-01M and NCRIBEN-03L) were treated with five different doses (0, 250, 350, 450 and 550 Gy) of gamma irradiation; 0 Gy being the control. All the treatments including the controls, were grown and monitored till maturity. The seed retention power of the treatments were assessed. From the results, the treatment due to 550 Gy had highest score (6) for both NCRIBEN-04E and NCRIBEN-01M and was categorized as non-shattering (NSH) type. All other treatments were categorized as shattering (SHA) type. Similarly all the treatments from NCRIBEN-03L were categorized as SHA with treatment due to 250 Gy having highest score (5). The three varieties showed positive correlations between the seed/capsule in upright (U) and seed/capsule in inverted (I) position but NCRIBEN-01M was not significant (r = 0.650). The NCRIBEN-04E showed negative correlation between U and length of suture (L) and I and L (r = -0.570 and -0.358 respectively). This result has shown that the dose 550 Gy seems to be promising in generating mutants with high resistance to capsule shattering in sesame. There is need to advance these mutants to M_2 and M_3 generations to ascertain this seed retention capacity.

Keywords: Gamma Irradiation, inverted, non-shattering, resistance, shattering, upright.

Introduction

Sesame (*Sesamum indicum* L.) is a very ancient crop and one of the earliest domesticated oil crops in the world [ASHRI, 2007]. According to KUMAR & YADAV (2010), some archeological findings have supported that sesame is one of the most important crops in the world. It is known in Vietnam as the king of oil seeds due to the high oil content of its seed which ranged from 50-60% [TOAN & al. 2010]. Sesame is an important source of cheap vegetable oil and proteins, good source of natural oxidants (sesamin and sesamolin) which are unique for sesame and present in the oil [ASHRI, 2007].

The majority of the world's sesame (probably over 99%) have shattering capsule, and most of the harvest is manual [LANGHAM, 2001]. Shattering of capsules at maturity has posed serious problem in sesame production worldwide [ASHRI, 1994] and can account for up to 50% seed loss during harvest. Indehiscent capsules and superior architecture are amongst the basic objectives laid down for sesame breeding and the degree of dehiscence is a cultivar characteristic and is of great importance for mechanized harvesting [YADAVA & al. 2012; VAN ZANTEN, 2001]. This is achievable through mutation breeding [VAN

[1] Department of Biological Sciences, Federal University of Technology, Minna – Nigeria.
[2] Department of Physics, Federal University of Technology, Minna – Nigeria.
[*] Corresponding author. E-mail: ml.muhd@futminna.edu.ng

ZANTEN, 2001]. Developing mutants with shattering resistance will help curtail seed loss at harvest.

Irradiations have been routinely used in developing plant varieties with agriculturally and economically important traits [BOUREIMA & al. 2009; VAIZOĞULLAR & KARA, 2016]. Gamma rays has been reported to be very effective and economical among all the physical mutagens for crop improvement [VAIZOĞULLAR & KARA, 2016]. Thus this study designed to assess the effects of gamma irradiation on seed retention power of three varieties of Sesame is indeed a strategy in its improvement through mutation breeding programmes.

Higher yields, improved plant architecture, adapted crop duration, resistance to diseases and pests and indehiscent capsules are the major objectives in this crop. In an attempt to test the hypothesis that "closed capsule mutants are inducible with efficient mutagenesis and screening large populations", DIOUF & al. (2010) treated three varieties of sesame with two different doses (300 and 400 Gy) of gamma irradiation. They reported in M_2 and M_3 that at least one closed capsule mutant could be induced from each of the three genetic backgrounds of sesame tested. Also ÇAĞIRGAN (2007), irradiated four varieties of sesame with gamma rays (150-750 Gy) and evaluated the M_1 and M_2 populations for some breeding objectives of sesame. Mutants with closed capsule, determinate growth habit, wilting tolerance, chlorophyll deficiency, hairy capsule and multicarpelate, sterility as well as quantitative traits such as flowering time, capsule size, and plant height were reported. According to VAN ZANTEN (2001), 142 mutants from different national sesame improvement programmes have been registered all of which possessed agronomically useful characters. Most mutants (76) were selected for capsule related characters such as, 3-capsules-per-leaf-axil, shape, size, non/semi shattering, and capsule density on the stem.

Materials and methods

Collection of Sesame seeds and irradiation of the seeds

The seed collection and irradiation were done following the protocol of MUHAMMAD & al. (2013) as described by FALUSI & al. (2015). The seeds of three varieties of sesame (NCRIBEN-04E, NCRIBEN-01M and NCRIBEN-03L) were obtained from the National Cereal Research Institute (NCRI) Baddegi, Niger State, Nigeria. Seeds of each variety were divided into 5 groups. Group I was not exposed to gamma rays and served as the control. The remaining four groups were irradiated with gamma rays (from Co-60 source) at 250, 350, 450 and 550 Gy at the Centre for Energy and Research Training (CERT), Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

Seed viability and experimental design

The seed viability test was done before and after irradiation using germination test method as described by STEPHEN (2009).

The pot experiments were conducted during the 2016 rainy season (August-November) at the Biological Garden, Federal University of Technology, Minna, Niger State, Nigeria. A randomized block design with 60 pots/block was used. The experiment was replicated three (3) times, with a total of 180 pots. Five seeds were planted per pot. Three weeks after planting, each pot was thinned to four plants/pot and 12 pots/treatment combination were used.

Assessment of Capsule Shattering

Assessment of shattering resistance was done following the pattern of MANEEKAO & al. (2001) with little modification. For each treatment, the number of seeds per mature capsule (brown capsule when it is upright), the number of seeds left per capsule when it is inverted and the size of opening of the capsule were determined as described in Tab. 1. To get number of seeds per capsule in upright position, a mature capsule was plucked gently and then placed in an inverted position with a sheet of paper underneath. The sheet was to hold any seed that escaped from the capsule when inverted. The number of seeds per capsule in inverted position was calculated as the number of seeds left in the capsule after inverting the capsule. The number of seeds per capsule in upright position was number of seeds in inverted position plus the seeds that fall on the sheet as indicated by the formula below.

$$U = I + E$$

Where U = number of seeds in upright position; I = number of seeds in an inverted position; E = seed that escaped during inversion of the capsule

The shatter resistance was classified by using the concept of LANGHAM as described by WONGYAI & al. (2001) with modifications. The method for measuring shatter resistance on a 0–8 scale is shown in Tab. 1. The modified scale of WONGYAI & al. (2001) used for rating the plants is shown in Tab. 2.

Tab. 1. Capsule Shattering resistance scale for sesame

Scale	U (%)	I (%)	L (%)
0	< 10	< 10	0.0–10.0
1	10–20	10–20	11.0–20.0
2	21–30	21–30	21.0–30.0
3	31–40	31–40	31.0–40.0
4	41–50	41–50	41.0–50.0
5	51–60	51–60	51.0–60.0
6	61–70	61–70	61.0–70.0
7	71–80	71–80	71.0–80.0
8	> 80	> 80	80.0–100.0

U = the amount of the seed in the capsule in upright (as % of total seeds set)
I = the amount of seed in the capsule in inverted position (as % of total seeds set)
L = Length of the capsule opening (as % total pod length)

Tab. 2. Characteristics and Scale rating for Seed retention

Seed retained in inverted position (I)	Name of category	Abbreviation
Less than 10%	Super shattering	SUS
10%–50%	Shattering	SHA
50%–70%	Non-shattering	NSH
70%–90%	Direct combine	DC

Data analysis

The length of capsule opening, seed/capsule in upright and inverted position were converted to simple percentages of the total capsule length and total seed set respectively. All the parameters in percentages were transformed using arcsine transformation and then subjected to linear correlation to show if the relationship exist between I, U and L.

Results

Mutants with closed capsules and with shattering capsule are presented in Plate I (A and B respectively). All the treatments from Variety NCRIBEN-04E retained more than 50% of the total when in upright position (U), except the control which retained less than 50% (42.71%) of total seeds (Tab. 3). When the capsules were inverted (I), only the treatment with highest dose (550 Gy) retained more than 50% (63.99%) of the total seeds and was scored 6 and classified as non-shattering type, the other treatments retained less than 50% and were classified as shattering type (Tab. 3). Similarly, all the treatments from NCRIBEN-01M and NCRIBEN-03L, retained more than 50% of total seeds when in upright position. However, only 3 treatments from 01M (0, 350, and 550 Gy) and one (250 Gy) retained more than 50% of the total seeds in inverted position (Tab. 3). In terms of ranking of NCRIBEN-01M, the treatment due to 550 Gy scored highest (6) and those due to 250 and 350 Gy were least scored (4). In contrary, for 03L, the highest score was due to 250 Gy, and 350 Gy was the least (3) (Tab. 3). The control, 350 and 550 Gy treatments were classified as non-shattering (NSH), while others were shattering (SHA) (Tab. 3). All treatments of NCRIBEN-03L were classified as shattering type, except the treatment due to 250 Gy which was non-shattering (Tab. 3). The three varieties showed positive correlations (between U and I) but that of NCRIBEN-01M was not significant ($r = 0.650$), where NCRIBEN-03L was significantly correlated, NCRIBEN-04E was highly significantly correlated (Tab. 4). The NCRIBEN-04E showed negative correlation between U and length of suture (L) and I and L ($r = -0.570$ and -0.358 respectively); although they are not significantly correlated.

Discussion

The dose range 350-550 Gy generated mutants which were categorized as non-shattering type, for NCRIBEN-04E and NCRIBEN-01M. This implies that the dose range is capable of inducing mutants with capsule shattering resistance. This is similar to the report of ÇAĞIRGAN (2007) and DIOUF & al. (2010) on sesame. They opined that 300-400 Gy dose range of gamma rays is effective enough to induce closed capsule mutants from any sesame background. VAN ZANTEN (2001) had also reported that for gamma rays, doses ranging from 150-800 Gy proved successful in inducing useful mutations and that gamma ray (300-750 Gy) induced mutants with indehiscent (closed) capsules in sesame. The ability of gamma irradiation to induce indehiscent capsule trait could be due to the bombardments caused by the irradiation, thereby changing the genetic make-up of the crop.

The non-significant correlation of (U&L) and (I&L) for the three varieties implies that the seed retention in upright and inverted positions do not necessarily depend on the length of suture. This might be due to presence of membrane sheet which covers the locules thereby preventing direct exposure of the seeds.

Tab. 3. Assessment of seed retention power of the treatments

Variety	Seed in Upright position (U) (%)	Seed in inverted position (I) (%)	Length of split (%)	Score	Category
NCRIBEN-04E					
0 Gy	42.71	20.97	32.97	2	SHA
250	52.07	30.06	34.78	3	SHA
350	60.31	28.25	29.56	2	SHA
450	60.28	42.59	29.40	4	SHA
550	73.15	63.99	30.95	6	NSH
NCRIBEN-01M					
0 (Gy)	69.12	50.54	32.11	5	NSH
250	69.57	47.60	33.11	4	SHA
350	74.37	51.96	36.26	5	NSH
450	68.70	41.48	28.76	4	SHA
550	72.62	62.18	31.68	6	NSH
NCRIBEN-03L					
0 (Gy)	80.19	49.93	33.92	4	SHA
250	78.73	57.84	34.50	5	NSH
350	66.20	36.68	32.21	3	SHA
450	74.15	46.16	31.87	4	SHA
550	72.16	45.78	31.55	4	SHA

*SHA = Shattering, NSH = Non-shattering

Tab. 4. Linear correlation among the certain parameters of the capsules

Varietal name	U&I	U&L	I& L
NCRIBEN-04E	0.903**	-0.570	-0.358
NCRIBEN-01M	0.650	0.733	0.343
NCRIBEN-03L	0.887*	0.740	0.747

*Significant difference at P< 0.05
**Highly significant difference at P< 0.05

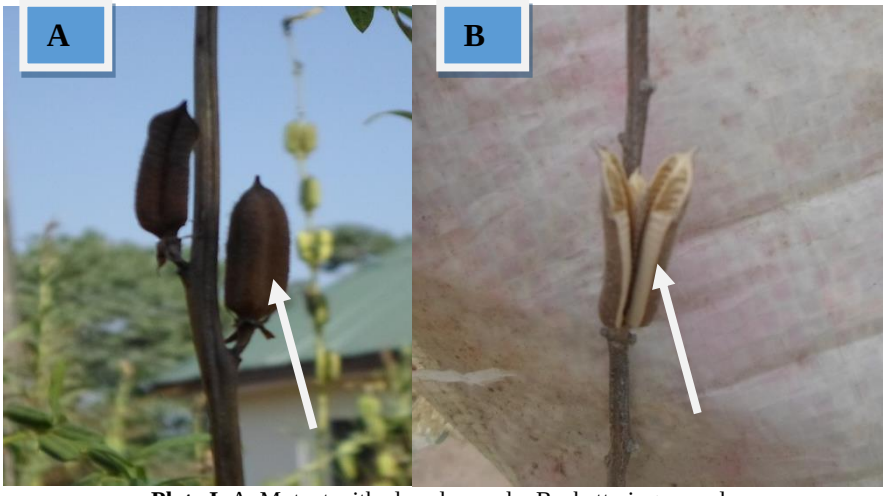

Plate I. A. Mutant with closed capsule; B. shattering capsule

Conclusions

The dose 550 Gy seems to be effective for NCRIBEN-04E and NCRIBEN-01M in generating mutants with shattering resistance. Thus the treatments with high scores should be advanced to M_2 and M_3 for further investigation of the seeds retention capability.

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VOLATILE COMPOUNDS IN THE AROMA OF THREE SPECIES OF WOOD-ROTTING BASIDIOMYCETES AND THEIR ANTIFUNGAL POTENTIAL

Cristiana Virginia PETRE[1*], Alin Constantin DÎRȚU[2], Marius NICULAUA[3], Cătălin TĂNASE[4]

Abstract: This study aims to determine the volatile organic compounds synthesized by three species of wood-rotting basidiomycetes: *Coriolopsis gallica*, *Megacollybia platyphylla* and *Lentinus arcularius* and test their antifungal potential. The species were cultivated on liquid media and kept for 25 days at 25 °C. The surface cultures were then homogenized, filtrated and extracted using solid-phase extraction and analyzed by GC-MS. The volatile compounds identified were mainly alcohols, ketones, aldehydes and terpenes. The most common volatiles identified in the experiment are: 1-octen-3-ol, 3-hexanol, 3-methyl-1-butanol, 3-octanone, 2-hexanone, benzaldehyde, and limonene. The volatiles metabolites of these species were tested for their antifungal activity using the bi-compartmented Petri dishes method against two species of plant pathogenic fungi: *Fusarium solani* and *Sclerotinia sclerotiorum*, on three media. The volatiles produced by *Coriolopsis gallica* showed the highest antifungal potential against the phytopathogens. The results revealed the importance of media composition in the synthesis of antifungal volatile compounds.

Key words: antifungal compounds, *Coriolopsis gallica*, *Lentinus arcularius*, *Megacollybia platyphylla*, volatile metabolites.

Introduction

In their ecological niches, fungi interact with different organisms and during these interactions cooperation or competition relations are established. Fungi communicate intra- and inter-specifically using several chemical signals that can spread in the substrate and act in the near vicinity of the mycelia or are propagated through the air, generating a signal that can be perceived at longer distances [WHEATLEY, 2002].

Responsible for the long distance communication are the volatile molecules, secondary metabolites synthesized by fungi as mixtures of chemical compounds such as alcohols, ketones, aldehydes and aromatic compounds that have specific smells and can be perceived by other organisms. Within the ecosystems, the fungal volatile compounds are involved in various processes: intra- and inter-specific communication [MORATH & al. 2012], defense [HANSON, 2008] and signaling [FÄLDT & al. 1999; DRILLING & DETTNER, 2009].

[1] "Alexandru Ioan Cuza" University of Iași, "Anastasie Fătu" Botanical Garden, 7-9 Dumbrava Roșie, 700487, Iași – Romania.

[2] Romanian Academy – Iași Branch, Oenological Research Center, 8 Carol I., 700506, Iași – Romania.

[3] "Alexandru Ioan Cuza" University of Iași, Faculty of Chemistry, 11 Carol I, 700506, Iași – Romania.

[4] "Alexandru Ioan Cuza" University of Iași, Faculty of Biology, 20A Carol I, 700505, Iași – Romania.

[*] Corresponding author. E-mail: criss_petre@yahoo.com

Many studies proved the role of these volatile molecules as insect attractants or repellents [BORG-KARLSON & al. 1994; JONSELL & NORDLANDER, 1995; WOOD & al. 2001; DE BRUYNE & BAKER, 2008]. LARSEN & FRISVALD [1995] demonstrated that specific types of volatile compounds are sometimes produced by certain phylogenetic lines, in certain concentration and therefore can be used as taxonomical criteria. The quality and quantity of these volatile molecules vary depending on several factors such as: media composition and pH [BRUCE & al. 2000; WHEATLEY, 2002; EWEN & al. 2004], temperature and water content [TRONSMO & DENNIS, 1978; JELEŃ, 2002], genera, species and presence of other organisms [GRIFFITH & al. 1994; DE JONG & FIELD, 1997; HYNES & al. 2007], age and development stage of the mycelia [JELEŃ, 2002; WU & al. 2005]. FÄLDT & al. (1999) mention that during sporulation, *Fomes fomentarius* and *Fomitopsis pinicola* synthesize higher quantities of volatiles and when the water content is low and the temperature is high the concentration of these molecules is low. RÖSECKE & KÖNIG (2000) demonstrated that compared to the rest of the fruiting body, the mycelia from crust produces high quantities of terpenes.

The volatile compounds synthesized by the fruiting bodies differ from the ones produced by the *in vitro* cultures. Moreover, *in vitro* observations revealed that depending on the media composition, the volatiles produced by wood-rotting fungi vary both as aroma and as intensity [PETRE & TĂNASE, 2013a, 2013b], fact that underlines the importance of nutrients in the synthesis of volatile molecules [KAHLOS & al. 1994; BJURMAN, 1999].

Both *in vivo* and *in vitro* through volatile metabolites, the mycelia that colonize the same substrate influence each other, inducing morphological and physiological changes [WHEATLEY & al. 1997; HEILMANN-CLAUSEN & BODDY, 2005]. These molecules act either as inhibitors towards certain organisms (affecting the growth, biomass development and respiration process) or as metabolic stimulators [HUMPHRIES & al. 2001].

The specific properties of these secondary metabolites are used in various biotechnological processes, in the pharmaceutical industry as antibiotics, antivirals, antioxidants, immunostimulants [MUSILEK & al. 1969; ZHONG, 2004; LINDEQUIST & al. 2005; LUO & al. 2005; SMÂNIA & al. 2007; MORATH & al. 2012], cosmetic industry and perfumery - the volatiles with pleasant aromas [FRAATZ & ZORN, 2010; MORATH & al. 2012] or agriculture as antimicrobial compounds that can be used in the production of biopesticides [CLOUGH, 1993; LORENZEN & ANKE, 1998; PARK & al. 2003; MORATH & al. 2012].

This study aims to identify the volatile organic compounds synthesized by three species of wood-rotting fungi and to test their inhibitory activity towards two species of plant pathogenic.

Materials and methods

Fungal strains. The species *Coriolopsis gallica* (Fr.) Ryvarden, *Megacollybia platyphylla* (Pers.) Kotl. & Pouzar, *Lentinus arcularius* (Batsch) Zmitr. were collected from broadleaf forests from Iași county, Romania, and isolated on malt extract agar (MEA) within the Research Laboratory for Fungi with application in ecological reconstruction, Faculty of Biology, "Alexandru Ioan Cuza" University of Iași. The plant pathogenic species *Fusarium solani* (Mart.) Sacc. and *Sclerotinia sclerotiorum* (Lib.) de Bary were isolated from potato tubercles (*Solanum tuberosum*) and carrot roots (*Daucus carota* subsp. *sativus*). All fungi are maintained on MEA at a temperature of 4 ºC.

Solid-Phase Extraction. The wood-rotting basidiomycetes were cultivated on a liquid medium (KM), rich in macro- and microelements in order to stimulate the synthesis of volatile compounds, as described by KAWABE & MORITA (1993): 20 g×l^{-1} glucose, 2 g×l^{-1} peptone, 2 g×l^{-1} yeast extract, 0.25 g×l^{-1} KH_2PO_4; 0.25 g×l^{-1} $MgSO_4$×7 H_2O. After 25 days of incubation in the dark at 25 °C, the surface cultures were homogenized; 10 ml of homogenate were filtered and mixed with 20 ml of pure water and 1 µl of 4-hydroxy-4-methyl-2-pentanone was added as internal standard. The mixture was extracted on LiChrolut cartridges (Merck Millipore): EN (40-120 µm) 100 mg (bottom); RP-18 (40-63 µm) 200 mg (top). The cartridges were previously conditioned with 2×6 ml *n*-hexane, 2×6 ml dichloromethane, 2×6 ml acetone, 2×6 ml methanol and 2×6 ml pure water, allowing each solvent to pass completely before adding the next conditioning solvent. On the same filter, 10 ml of pure water mixed with 1 g NaCl, 1 g Na_2SO_4 and 1 g of KH_2PO_4 were passed to increase the ionic strength, in order to facilitate the extraction of the remaining compounds from the biomass. The filtrate was later passed over the same cartridges. The SPE cartridges were completely dried using compressed air and placed in a desiccator at 600 mbar for 24 h. Next, the cartridges were eluted with 1.5 ml of *n*-hexane, dichloromethane and acetone respectively and the eluate was dried on anhydrous sodium sulfate. Each extraction was performed in duplicate for every species. The eluents were collected in separate vials and analyzed by gas-chromatography with mass spectrometer detection (GC-MS).

GC-MS analysis. The GC-MS analysis of the samples was performed on a Shimadzu GC-MS 2010 equipped with a ZB WAXplus capillary column (10 m × 0.1 mm × 0.1 µm) operated in split mode injection (split ratio 1/10). The GC oven temperature was set from 35 ºC for 5 minutes, with an increase of 5 ºC/min to 220 ºC and hold for 5 minutes, with a total analysis time of 47 minutes. Helium was used as carried gas, with a total flow of 15.9 ml/min, column flow of 0.9 ml/min and a purge flow of 6 ml/min. The MS ionization source was operated in electron impact mode (EI) with the EI source temperature set at 200 ºC. The full scan mass-spectrums were acquired at every 0.1 seconds (equivalent with 5000 a.m.u), between 30-500 Da (m/z). The volatile and semi-volatile compounds from the extracts were identified by comparison with the NIST 2.0 database (applying a > 85% match as acceptance criteria), mass spectra and retention times from literature [KAWABE & MORITA, 1993; BREHERET & al. 1997; STROBEL & al. 2001].

Antifungal screening. The antifungal potential of the volatiles synthesized by the species of wood-rotting basidiomycetes was evaluated against *F. solani* and *S. sclerotiorum* using the bi-compartmented Petri dish method used by STROBEL & al. (2001) and SCHALCHLI & al. (2011, 2015), in such way that the two mycelia didn't come into contact and the inhibitory activity was only due to the volatile compounds.

The screening activity was carried out on three media with different compositions in order to test if and how various concentrations and sources of carbon, nitrogen and microelements influence the synthesis of antifungal volatiles: potato flakes malt extract agar (PFMEA): 20 g×l^{-1} potato flakes, 5 g×l^{-1} malt extract, 5 g×l^{-1} glucose, 15 g×l^{-1} agar (Merck); malt extract agar (MEA): 30 g×l^{-1} malt extract, 5 g×l^{-1} glucose and 15 g×l^{-1} agar (Merck) and KM medium: 20 g×l^{-1} glucose, 2 g×l^{-1} peptone, 2 g×l^{-1} yeast extract, 0.25 g×l^{-1} KH_2PO_4; 0.25 g×l^{-1} $MgSO_4$ × 7 H_2O and 15 g×l^{-1} agar.

The species of wood-rotting basidiomycetes were inoculated in one compartment of the plate and the plant pathogen in the other. The Petri dishes were wrapped in two layers of Parafilm and incubated in the dark at 25 ºC. Four replicates were made for every combination. The control plate contained only the plant pathogenic species inoculated in one of the

compartments. Measurements of the test and target colonies were made daily, until the pathogen's colony from the control plate completely covered the compartment. The inhibitory percentage was calculated for every plate: P = [C-T] × 100/C, where C represents the diameter of the control colony and T represents the diameter of the pathogen's colony exposed to the VOC synthesized by the test fungi [NIDIRY & BABU, 2005]. The medium inhibitory percentage (P_{med}) was calculated as the average value of all four replicates inhibitory percentages.

Results and discussion

The mass-chromatograms obtained for the three species of wood-rotting fungi contained large numbers of peaks and therefore it was necessary to establish several acceptance criteria. In order to identify the volatile compounds from the samples we compared the obtained results to data from NIST 2.0 library and data from literature regarding the retention times and mass spectra. Because for each species the sample was done in two replicates we subtracted the results obtained for the control sample from the chromatograms of each replicate. Also, the variability between the two replicates regarding the volatile compounds after the blank subtraction was expressed as relative standard deviation (RSD%) of the results from the two replicates and the results with the lowest variability (<30%) were accepted as positively identified compounds in the samples.

Tab. 1, 2 and 3 contain data regarding some of the volatile compounds identified in the *n*-hexane, dichloromethane (DCM) and acetone fractions and their retention times (RT, minutes).

Tab. 1. Volatile compounds identified in *Coriolopsis gallica* extracts

Solvent	Retention times (min)	Average area	Contribution to the area	Compound
n-hexane	2.41	27,247.5	1.46	5-(2-methylpropyl)-nonane
	2.581	155,506	8.35	4,6-dimethyl-dodecane
	2.654	18,388.5	0.99	3-ethyl-3-methyl-heptane
	3.301	31,787	1.71	5-methyl-5-propyl-nonane
	5.259	118,889	6.39	limonene
	5.984	28,323	1.52	3-hexanol
	6.233	30,715	1.65	3-methyl-1-butanol
	7.064	36,246	1.94	2-heptanone
	9.309	80,301	4.31	4,6-dimethyl-dodecane
	10.115	46,800.5	2.51	2,6,11,15-tetramethyl-hexadecane
DCM	2.173	55,489	0.02	3-hexanone
	2.478	144,381	0.04	5-methyl-undecane
	2.533	98,938	0.03	2-hexanone
	3.156	23,808	0.01	2-methyl-2-pentanol
	3.502	38,824	0.01	4,4-dimethyl undecane
	7.074	21,810	0.01	4,6-dimethyl-2-heptanone
	9.101	121,688	0.04	4,6-dimethyl-dodecane
	16.633	85,612.5	0.02	4-hidroxy-3-methyl-2-butanone
Acetone	2.699	7,078.5	0.12	4-hidroxy-3-propyl-2-hexanone
	3.408	23,004	0.38	3-penten-2-one
	4.958	21,576	0.36	2,6-dimethyl-4-heptanone
	5.224	5,065,362	83.66	4-methyl-2-pentanol

Concerning the general aroma of the wood-rotting basidiomycetes, when cultivated on liquid KM media, *Megacollybia platyphylla* presented a sweet-almond, strong aroma that lasted for 8 weeks at 25 °C, the smell of *Coriolopsis gallica* culture was of rotting wood and lasted for 6 weeks, while for *Lentinus arcularius* we detected a mushroom-like odor that intensified with time and lasted for 8 weeks.

The mushroom-like aroma is given by 1-octen-3-ol, eight-carbon volatile produced *Megacollybia platyphylla* and *Lentinus arcularius* which is one of the most common volatile compounds synthesized by fungi [HANSON, 2008] and was mentioned as secondary metabolite in case of many species of basidiomycetes [BERGER & al. 1986a; GROSS & al. 1989; RAPIOR & al. 1996; GUEVARA & al. 2000a, 2000b; RÖSECKE & al. 2000; THAKEOW & al. 2008; ZIEGENBEIN & al. 2010].

Responsible for the almond aroma of *Megacollybia platyphylla in vitro* culture is the benzaldehyde also identified in the *Lentinus arcularius* samples, recorded by several authors in the volatile profile of other basidiomycetes [MAGA & al. 1976; BERGER & al. 1986a, 1987; GROSS & al. 1989; KAWABE & MORITA, 1993; FÄLDT & al. 1999; RÖSECKE & al. 2000; ZIEGENBEIN & al. 2006], volatile with different applications in biotechnology [MORATH & al. 2012]. Moreover, the sweet and fruity aroma of this species is also attributed to 3-octanone [BERGER & al. 1986b; COMBET & al. 2006] compounds identified in all three fractions.

Tab. 2. Volatile compounds identified in *Megacollybia platyphylla* extracts

Solvent	Retention times (min)	Average area	Contribution to the area	Compound
n-hexane	5.28	84,761	18.19	limonene
	6.19	72,511.5	15.56	2-methyl-1-butanol
	7.07	6,870.5	1.47	4,6-dimethyl-2-heptanone
	7.28	119,694.5	25.69	3-octanone
	8.69	34,925.5	7.50	1-octen-3-one
	10.37	23,267.5	4.99	2,9-dimethyl-undecane
	14.20	57,599	12.36	benzaldehyde
DCM	2.83	38,091	9.62	2-methyl-1-propanol
	7.27	90,251	22.79	3-octanone
	8.70	37,740.5	9.53	1-octen-3-one
	13.41	130,521	32.96	1-octen-3-ol
	14.21	27,020.5	6.82	benzaldehyde
	15.79	27,499	6.94	linalool
	16.23	22,850.5	5.77	dihydro-4-methyl-2(3H)-furanone
Acetone	4.95	25,367	6.4	2-methyl-4-octanone
	6.88	26,613	6.72	2-hexanol
	7.33	25,952	6.55	3-octanone
	13.31	23,350	5.89	1-octen-3-ol

The ketone 3-penten-2-one from the extracts of *Lentinus arcularius* was mentioned by BERGER & al. (1986a, 1986b) in the volatile profile of *Bjerkandera adusta* and *Polyporus durus*. Also *Lentinus arcularius* synthesizes *in vitro* pantolactone, chemical

compound mentioned by GUEDES DE PINHO & al. (2008) as secondary metabolite produced by several edible fungi.

Few monoterpenes were identified in the samples, as BJURMANN (1999) noticed that on rich media fungi synthesize higher quantities of alcohols, while terpenes are produced on poor media.

Limonene, a monoterpene with a fresh, sweet-citrusy aroma [BREHERET & al. 1997] was produced by all three tested species and the literature mentions this compound as a metabolite synthesized by other species of wood-rotting basidiomycetes [RAPIOR & al. 1996; FÄLDT & al. 1999; RÖSECKE & al. 2000; ZIEGENBEIN & al. 2006, 2010].

Also linalool, another monoterpene was identified in the *Megacollybia platyphylla* samples this compounds contributing to the characteristic *in vitro* fruity aroma of this species.

Tab. 3. Volatile compounds identified in *Lentinus arcularius* extracts

Solvent	Retention times (min)	Average area	Contribution to the area	Compound
n-hexane	2.42	233,241	3.4	5-(2-methylpropyl)-nonane
	3.29	12,071.5	3.22	5-methyl-5-propyl-nonane
	5.28	183,381	2.68	limonene
	6.24	25,147	0.36	3-methyl-1-butanol
	6.82	13,582	3.62	2-hexanol
	7.07	33,384	0.48	2-heptanone
	11.4	21,045	0.3	nonanal
	12.7	15,929	4.25	2,6,10,15-tetramethyl-heptadecane
	21.9	30,505	8.13	hexadecane
DCM	2.45	61,971	0.02	3,7-dimethyl-decane
	2.49	56,280	0.02	2-hexanone
	5.39	30,955,358	12.43	4-methyl-2-pentanol
	5.99	249,271	3.63	3-hexanol
	6.23	27,627	0.4	3-methyl-1-butanol
	7.06	12,808.5	0.01	4,6-dimethyl-2-heptanone
	13.40	24,336	0.35	1-octen-3-ol
	14.2	33,293.5	0.01	benzaldehyde
	25.3	24,154	0.01	pantolactone
	30.94	22,039.5	0.01	phenol
	33.96	26,869.5	0.01	lactone
Acetone	2.61	9,555	0.12	4,6-dimethyl-dodecane
	2.7	26,350.5	0.34	2-hexanone
	3.4	19,056.5	0.25	3-penten-2-one
	3.54	87,211.5	1.12	4-methyl-3-penten-2-one
	5.24	7,632,185	98.17	4-methyl-2-pentanol
	6.88	31,781	0.46	2-hexanol
	26	116,622	1.7	3-buten-2-ol

Regarding *Megacollybia platyphylla*, STADLER & STERNER (1998) tested the antimicrobian activity of the fruiting bodies belonging to this species, obtaining positive results only against *Bacillus brevis*. PUJOL & al. (1990) successfully tested the antifungal

potential of the culture fluid against *Aspergillus fumigatus*, *Candida albicans* and *Candida tropicalis*. In our study, the antifungal activity of the volatiles emitted by this species was low, which means that the results obtained by PUJOL & al. (1990) were attributed to compounds other than volatiles.

As far as know there is no study that regarding the volatile profile of *Coriolopsis gallica* and no study that evaluates the antifungal potential of these secondary metabolites.

The antimicrobial potential of *Lentinus arcularius* extracts was successfully tested against several genera of pathogenic bacteria [SUAY & al. 2000; YAMAÇ & BILGILI, 2006] and fungi: *Aspergillus niger*, *Botryodiplodia theobroma*, *Alternaria brassica* and *Penicillium digitatus* [SRIVASTANA & SHARMA, 2011]. Regarding the metabolites produced by this species FLECK & al. (1996) isolated isodrimenediol, compound that showed a moderate antimicrobial activity, inhibiting the growth and development of several bacterial strains and yeasts, CABRERA & al. (2002) discovered the criptoporic and isocriptoporic acids, that tested negative for antimicrobial and antibiotic activities and OTAKA & ARAYA (2013) isolated two sesquiterpenes, but without testing their antimicrobial activity.

Tab. 4. Inhibitory percentages of the volatiles synthesized by the wood-rotting basidiomycetes against *Sclerotinia sclerotiorum*

Species	IP (%) on PFMEA	IP (%) on MEA	IP (%) on KM
Coriolopsis gallica	3.52%	4.41%	6.17%
Megacollybia platyphylla	2.94%	3.23%	3.52%
Lentinus arcularius	4.41%	5%	5.29%

The results obtained after the antifungal screening against *Fusarium solani* (Tab. 4) and *Sclerotinia sclerotiorum* (Tab. 5) showed that the higher inhibitory percentages were calculated on KM medium for all three species of wood-rotting basidiomycetes. The higher percentages against *Fusarium solani* makes this species more susceptible to the activity of the volatiles synthesized by the basidiomycetes.

Tab. 5. Inhibitory percentages of the volatiles synthesized by the wood-rotting basidiomycetes against *Fusarium solani*

Species	IP (%) on PFMEA	IP (%) on MEA	IP (%) on KM
Coriolopsis gallica	5.29%	6.76%	7.94%
Megacollybia platyphylla	4.11%	4.41%	4.7%
Lentinus arcularius	6.76%	6.76%	6.47%

The inhibitory activity of *Coriolopsis gallica* and *Lentinus arcularius* can be partially attributed to 3-methyl-1-butanol, alcohol characterized by a pungent alcoholic odor [GROSS & al. 1989; ABRAHAM & BERGER, 1994] which was identified in the volatile profile of several species of wood-rotting basidiomycetes [BERGER & al. 1986a; KAWABE & MORITA 1993; SCHLACHLI & al. 2011], but also in species such as *Saccharomyces cerevisiae* [FIALHO & al. 2010], *Muscodor albus* [STROBEL & al. 2001; EZRA & al. 2004] and *Trichoderma* spp. [WHEATLEY & al. 1997] known for their antifungal potential.

The antifungal screening was performed on three media, with different carbon and nitrogen sources, with or without microelements in order to determine the effect of media composition on the antifungal activity of the volatiles synthesized by the wood-rotting

basidiomycetes. The highest antifungal activity was measured on KM medium which is more complex than the other two media, having glucose as carbon source (20 g/l), peptone (2 g/l) and yeast extract (2 g/l) as nitrogen sources and several microelements such as: potassium and phosphorous (0.25 g/l KH_2PO_4), magnesium and sulphur (0.25 g/l $MgSO_4 \times 7H_2O$).

NORRMAN (1971) investigated the effect that different carbon sources have on the production of volatile compounds in yeasts, determining that glucose stimulated the synthesis of these molecules compared with fructose and glycerol. An increase in the glucose concentration also determined an increase in the quantity of volatiles produced by fungi. The results obtained in the case of the three species of wood-rotting fungi demonstrate that these conditions are valid also for these species of wood-rotting basidiomycetes.

WHEATLEY & al. (1997) monitored the influence of media composition on the synthesis of volatile compounds by species of *Trichoderma* and discovered a higher antifungal activity of these molecules on media with malt extracts (with the exact same composition as the one used in this study) compared with a minimal medium with 5 g/l glucose, asparagine and K, P, Mg, Cl, Fe, S, Mn, Zn, Cu and Ca mineral salts. Contrary, in our study, we recorded the highest inhibitory percentages against the two phytopathogens on the KM medium that had a higher concentration of glucose and mineral compounds (K, P, Mg and S).

Conclusions

The GC-MS analysis of the samples revealed the presence of VOCs such as alcohols, ketones, aldehydes, terpenes and other compounds. Some of the molecules are common fungal metabolites such as: 1-octen-3-ol, 3-octanone, 3-hexanone, 3-hexanol, 2-hexanol, 2-hexanone, 4,6-dimethyl-2-heptanone, 2-methyl-1-butanol, 3-methyl-1-butanol, benzaldehyde, linalool, limonene. These molecules are responsible for the aroma of the *in vitro* culture of the wood-rotting basidiomycetes and have biotechnological potential.

From our knowledge this is the first study that focuses on the volatile profile of *Megacollybia platyphylla* and *Coriolopsis gallica* grown on liquid media using SPE as the extraction method.

The volatile compounds synthesized by these species were tested for their antifungal potential against two plant pathogens: *Fusarium solani* and *Sclerotinia sclerotiorum*. The results of the screening revealed that the volatile metabolites synthesized by *Coriolopsis gallica* had the highest antifungal activity against the phytopathogenic fungi, fact that makes this basidiomycete a potential resource of bioactive compounds. Also the influence of the media composition was evaluated during this screening, highlighting the importance of the quantity and quality of the macro- and micro-elements in the synthesis of bioactive metabolites.

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PHYSIOLOGICAL REACTION OF THE SPECIES *BRASSICA JUNCEA* (L.) CZERN. ON SALINIZED SOILS AMELIORATED WITH ZEOLITIC TUFF, PEAT AND PERLITE

Maria Cătălina PASTIA[1], Lidia ADUMITRESEI[2*],
Carmen Doina JITĂREANU[3], Cristina SLABU[3]

Abstract: The physiological reaction of saline stress which *Brassica juncea* (L.) Czern. plants undergo shows a greater growth and fresh substance gain process on previously cultivated soils that were fined with 20% zeolitic tuff and 5.09 g of neutral peat than the ones that had a substrate which hasn't been cultivated on before that was fined with 5% zeolitic tuff and 1.39 g of perlite. The dry substance values obtained present a positive correlation with the values of fresh substance. Analysis of stomatal conductance enhances the hydric stress of plants which respond to saline stress with osmotic adjustment, accumulating high quantities of water comparing to the witness plant, which induces lower values of stomatal conductance and implicitly values are decreasing for photosynthesis, determining a low productivity. Higher values of stomatal conductance are reached at plants grown on previously cultivated soils fined with 20% zeolitic tuff and peat, and also at the ones grown on uncultivated soils fined with peat (29.45, respectively 30.05 mmol/m^2/s).

Key words: amended soil affected by salinity, oriental mustard, peat, perlite, zeolitic tuff.

Introduction

Soil salinity creates a great environmental issue with economic and social consequences worldwide [SCHUBERT, 2011; SIDIKE & al. 2014]. Globally, estimations show that approximately one third of irrigated fields are affected by salinity issues. Moreover, a half of the field in semi-arid and coastal regions are being affected [MUNNS, 2011; FARHANA & al. 2014].

This issue is one of the processes leading to desertification [KASSAS, 1987], as well as one of the most important land degradation processes [THOMAS & MIDDLETON, 1993]. Salt accumulation in soli has a negative effect on the growth of most crops, these soils being associated with poor fertility [TANJI, 2002]. This also is one of the main causes of low productivity in agriculture throughout the world.

The reduction of productivity contradicts with the rising need of food for the population. The situation becomes even more alarming due to the expectation of population rising to 8-10 billion people by 2050 [LUTZ & SAMIR, 2010]. This problem represents a matter of concern for many teams of researchers for it to be found best solutions for improvement. Current methods of irrigation and cultivation practices contribute little to the rehabilitation of these soils [QADIR & OSTER, 2002], which requires the testing and

[1] Technical University "Gheorghe Asachi" of Iaşi, Faculty of Hydrotechnics, Geodesy and Environmental Engineering, Iaşi – Romania.

[2] University "Alexandru Ioan Cuza", Botanic Garden "Anastasie Fătu", 7-9, Dumbrava Roşie St., Iaşi – Romania.

[3] Agricultural Faculty of the USAMV "Ion Ionescu de la Brad" of Iaşi, Iaşi – Romania.

[*] Corresponding author. E-mail: lidia.adumitresei@yahoo.com

implementing other techniques. Population growth rate and global economic development inevitably lead to an increase in the consumption of materials and minerals.

Therefore the present research has focused on finding technical solutions that will show a smaller energy and fuel consumption. That led to research methods and technologies that can promote the principles of sustainable development. Thus it has been chosen the amelioration with zeolitic tuff, peat and perlite on soils affected by salinity. After COCHEME & al. (2003), volcanic tuffs in Romania originate in the explosive activities of volcanic materials that have accumulated in inclusions and benches in the Miocene and Pliocene ages. Previous analysis have shown that the effect of providing the radish crops, *Raphanus sativus*, with zeolitic tuff had a positive outcome with rising efficiency, improving the quality of the crop, by retaining salt in the soil, thus preventing root absorption [NOORI, 2006]. Analysis on *Raphanus sativus*, cultivated in salinity conditions, where it has been added zeolitic tuff and sand to the soil have proven that the tuff acts like a buffer system and although halophilic vegetation was used, it wasn't immune to the stress produced by the salinity that appeared during the fifth and the sixth months of growth [QIAH & al. 2001].

Materials and method

For testing, it was used the oriental mustard, *Brassica juncea* (L.) Czern., from *Brassicaceae* Family, *Brassica* genus, closely related to *Brassica oleracea* subsp. *oleracea* (cultivated cabbage). The leaves, the seeds and the stalk of this genus are edible. Encountered in Africa, Bangladesh, China, Japan, Korea, Italy and India, the varieties of *B. juncea* are grown both for the green plant and for the production of oilseeds. In Russia, it is the main cultivated variety for the production of mustard oil. *B. juncea* is used a lot, in the preservation, baking and margarine production in Russia, and most of the table mustard is made from the same species of mustard plant. Because of the content of erucic acid with toxic potential, mustard seed oil is restricted as vegetable oil. *Brassica juncea* is a plant whose tolerance to salinity had been researched and demonstrated in studies made by WRIGH & al. (1997); NORTON & al. (2004); ASHRAF & al. (2001); KUMAR & ABROL (1984), KUMAR & al. (2009). The soil samples used for the research were taken from the common meadow of Prut and Jijia in Prisacani, Iasi County, on a cultivated land. The soil of both sites is a clay soil characteristic of former marine basins formed by deposition and sedimentation [PASTIA & al. 2017; STĂTESCU & PAVEl, 2011].

The determinations were made in the Analytical Chemistry Laboratory of the Chemical Engineering and Environmental Protection Faculty of "Gheorghe Asachi" University of Iasi. The main features of the two soil categories are presented in Tab. 1 [LUCHIAN, 2016].

Tab. 1. Characteristics of soils used for amelioration

	Uncultivated Land (M)	Cultivated Land (S)
Sand (%)	4.25	8.17
Dust (%)	3.92	3.21
Clay (%)	56.55	48.62
pH	6.83	8.07
Cl^- (mg/100 g soil)	317.22	35.36
SO^{4-} (mg/100 g soil)	21.89	-
Ca^{2+} (mg/100 g soil)	2.54	18.08
Mg^{2+} (mg/100 g soil)	45.63	8.66
K^+ (mg/100 g soil)	230.75	465.65
Na^+ (mg/100 g soil)	589.0	40.68

Soil samples from the cultivated area (S) and those from the uncultivated area (M) were given 5%, 10%, 15%, 20% and 30% zeolitic tuff. In order to improve the porosity and hydraulic conductivity of the soil, it went in parallel with a 1:1 (v / v) soil / peat mixture and another 1:1 (v / v) perlite. The material used for the research was purchased from S.C. BIOSOLARIS S.R.L., data for chemical characterization of the material from the supplier are presented in Tab. 2.

Tab. 2. The chemical and mineralogical composition of zeolite tuff used in the research

Chemical composition	Mineralogic composition
SiO_2 - 68.75 %	Clinoptilolite 71% - 83.3%
Al_2O_3 - 11.35 %	Vocanic glass: 4.1% - 9.7%
Fe_2O_3 - 2.10 %	Plagioclase: 6.6% - 6.67%
CaO - 2.86 %	SiO_2: 2.25% - 2.6%
MgO - 1.18 %	Other minerals: 3% - 4%
$Na_2O + K_2O$ - 3.99 %	
P.C - 9.77 %	

Other features provided by the supplier are: apparent dry weight specific gravity of 1.65 - 1.75 gf/cm³, cation exchange capacity (CEC) of 1.51 me/100g, natural moisture (BET) of 23.4 m²/g, micronized product, pore diameter of 3.82 Å, total porosity of 33.08%, water absorption of 16.21%, specific mass of 2.15-2.25 g/cm³, bulk density 0.88 kg/dm3.

The peat used for research is TS3 Standard, produced by Klassmann, partially decomposed, pearly peat grains of 0-25 mm with the addition of microelements: Phosphorus, Nitrogen and Potassium of 1 g / l and pH 6.

The pearlite is a natural, inorganic, granular material containing silicon dioxide, in percent of approx. 75% and aluminum oxide, ca. 15%, is perfectly dry, sterile, environmental friendly, extremely light, very chemically stable, non-degradable over time.

The plants were grown under green conditions in the Botanical Garden of Iasi, in vegetation vessels. The samples were watered with distilled water in order not to modify the salinity of the substrate.

Harvesting: plants reached biological maturity, meaning flowering phenophase, technical maturity (stage of harvesting plants), which may occur earlier or later depending on use.

The plants were harvested and weighed individually. Then it was arithmetically calculated the average of the weight of the vegetal mass, expressed in grams (fresh substance)/subject, plant.

The water and dry matter content of the leaves was determined by the gravimetric method.

Chloride was extracted in hot water and measured coulometric by titrating with AgCl, using a Sherwood Chloride analyzer, model 926.

The plant material was dried until constant weight after inactivation of the enzymes for one hour in the oven at 105 °C in the laboratory of Plant Physiology of the Agricultural Faculty of the Agronomic University and Veterinary Medicine "Ion Ionescu de la Brad" of Iasi.

Results and discussions

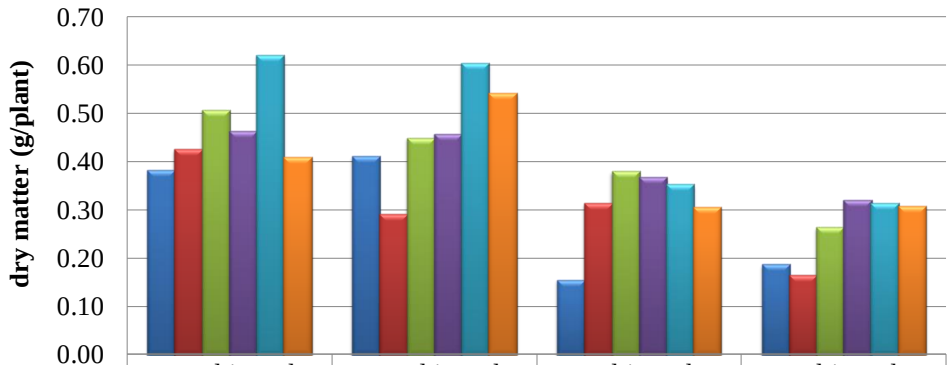
Effect of amendments on the plant growth process

The data presented in Tab. 3 shows the variations between the peat cultivated plants compared to the crops grown on perlite. Thus the average values of the vegetal mass per individual are higher for the plants cultivated on the peat and tufted soil, compared to the peat and tufted soil. The average value of the vegetal mass for the samples from the cultivated soil and fined with peat and pearlite ranges between 3.42 g / subject and 5.09 g, the maximum value being met with the 20% zeolitic pitch. The values for perlite and tuff fined cultivated soil are much smaller, varying between 1.77 g and 2.88 g / subject, the maximum value is reached at the 15% zeolitic tuff fine. The average value of the vegetal mass for the samples from the peat fined uncultivated soil, one series, and with another pearlite series, ranges between 3.8 g /subject and 5.44 g, the maximum value is reached at 20% zeolitic tuff. Values for perlite and tuff fined uncultivated soil are much lower, varying between 2.85 g and 3.9 g, the maximum value is reached at 30% zeolitic tuff.

Tab. 3. The average of the vegetal mass harvested at the end of the growing season, expressed in grams of fresh substance

	Witness (doesn't contain zeolitic tuff)	**Zeolitic tuff**				
		5%	**10%**	**15%**	**20%**	**30%**
Cultivated soil - S (peat + tuff)	3.98	3.42	3.94	3.96	5.09	4.65
Cultivated soil - S (perlite + tuff)	1.83	1.77	2.69	2.88	2.41	2.66
Uncultivated soil - M (peat + tuff)	3.20	3.8	4.06	3.90	5.44	4.13
Uncultivated soil - M (perlite + tuff)	1.39	2.85	3.35	3.13	3.11	3.9

After analyzing the dry matter content, the plants grown on the arable soil and peat or tuff present a slightly higher dry substance values than the dry substance values of plants originated on perlite and tuff fined cultivated soil (Fig. 2).



	necultivated soil+peat	necultivated soil+perlite	cultivated soil+peat	cultivated soil+perlite
control	0.38	0.41	0.15	0.19
tuff 5%	0.42	0.29	0.31	0.16
tuff 10%	0.51	0.45	0.38	0.26
tuff 15%	0.46	0.46	0.37	0.32
tuff 20%	0.62	0.60	0.35	0.31
tuff 30%	0.41	0.54	0.30	0.31

Fig. 1. Effect of changes in the dry matter

Regarding the dry matter content percentage, the data shown in Tab. 4 stands that the values of the witness samples of both samples that were amended with peat and perlite aren't showing really big differences. So for peat fined cultivated soil the dry substance value is 10.31%, and for perlite fined cultivated soil is 10.15%.

For samples from uncultivated soil fined with peat and zeolitic tuff, the minimum value is reached at sample ST 5% = 8.46% (cultivated soil + 5% zeolitic tuff), and the maximum value of the dry substance is reached at sample ST 20% = 11.86 (cultivated soil + peat + 20% zeolitic tuff).

For samples from cultivated soil fined with perlite and zeolitic tuff, the minimum value is reached at sample SP 5% = 9.29% (cultivated soil + perlite + 5% zeolitic tuff), and the maximum value is reached at sample SP 20% = 12.96 (cultivated soil + perlite + 20% zeolitic tuff).

Analyzing the data in Tab. 4, 5 it can be discovered that the plants grown on uncultivated soils that were fined with peat and tuff show values of the dry substance that are slightly higher that the values of dry substance from the plants grown on uncultivated soils that were fined with perlite and zeolitic tuff.

The values of witness samples that were fined with peat and perlite aren't showing major differences. The dry substance value for uncultivated soil fined with peat is 11.93%, while for the uncultivated soil fined with perlite it is 10.11%.

For samples from uncultivated soil fined with peat and zeolitic tuff, the minimum value is reached at sample MT 30% = 9.88% (uncultivated soil + peat + 30% zeolitic tuff) and the maximum value is reached at sample MT 10% = 12.5 (uncultivated soil + peat + 10% zeolitic tuff).

For samples from uncultivated soil fined with perlite and zeolitic tuff the minimum value is reached at sample MP 30% = 7.8% (uncultivated soil + perlite + 30%

zeolitic tuff) and the maximum dry substance value is reached at sample MP 15% = 11.7 (uncultivated soil + perlite + 15% zeolitic tuff).

Analyzing the results obtained on the two soil types (cultivated and uncultivated), we can see that for 4 out of 6 samples (blank, 5% tuff, 10% tuff, 15% tuff) the dry matter values are higher for the uncultivated soil, although for samples fined with 20 and 30% tuff, dry matter values are lower.

After further analysis of the plants regarding the vegetal, dry substance and water mass it can be concluded that although uncultivated soil was initially presenting less favorable physical or chemical conditions for the growth, with values of higher apparent density compared to cultivated soil, values of total porosity and aeration less than arable soil, values of approx. 100 times higher in Sodium. Following experiments have shown that crops similar to those from arable soil samples were obtained on uncultivated soil. The results confirm that by taking suitable hydro- and soil ameliorative measures, the soil can be cultivated to give positive results.

The effect of soil fining on stomatal conductance and photosynthetic index of cultivated plants

An analysis that helps in understanding the stress to which plants grown on these saline soils are submitted to is the stomatal conductance of plants. This procedure highlights the hydric stress that communicates indirectly data on the impairment of the photosynthesis process, as can be seen from the analysis of the data presented in Tab. 5.

Adjustment of stomatal conductance by plants is done to improve the ratio between carbon capture and water loss. If plants suffer from water deficiency, the plants will survive by completely closing the stomata.

A high level of conductance means open stomata, leading to a high level of photosynthesis and, of course, good plant productivity. Smaller conduction, although reducing the risk of dehydration of the plant will have an effect on productivity by reducing it.

Stomatal conductance depends not only on the species but also on the cultivar. The cultivars that under salt stress condition exhibiting higher chlorophyll concentrations and higher stomatal conductance have a good Photosynthetic capacity [BOLOGA & al. 2016].

Tab. 4. Data on dry substance, stomatal conductivity and the photosynthesis index for the investigated samples

Sample	Dry substance (%)	Stomatal conductance (mmol/m^2/s)	Photosyntesis index
STm	10.31	17.76	13.05
ST 5%	8.46	12.6	5.04
ST 10%	11.38	12.35	3.6
ST 15%	11.55	17.2	9.85
ST 20%	11.86	29.45	7.6
ST 30%	11.71	12.8	2.75
SPm	10.15	5.8	2.05
SP 5%	9.29	6.05	9
SP 10%	9.79	14.5	2.05
SP 15%	11.13	18.7	2.25
SP 20%	12.96	28.1	2.5
SP 30%	11.49	21.8	2.15
MTm	11.93	12.9	6.55

MT 5%	11.18	14.8	3.46
MT 10%	12.5	30.05	3.25
MT 15%	11.98	12.8	1.4
MT 20%	11.41	14.5	2
MT 30%	9.88	13.15	7.55
MPm	11.14	21.65	1.65
MP 5%	10.98	12.95	2.05
MP 10%	11.33	26.05	5.75
MP 15%	11.7	9.5	2.35
MP 20%	11.29	20.45	2
MP 30%	7.8	11.8	1.43

It can be noticed that the values of stomatal conductance are lower for the plants from the cultures obtained on the samples investigated before fertilization. After fertilization (1g/L Nitrogen, Phosphorus, Potassium) it can be observed a double increase of the stomatal conductance values for the plants on STm (cultivated soil + peat with no addition of zeolitic tuff), ST 10% (cultivated soil + peat + 10% zeolitic tuff), ST 15% (cultivated soil + peat + 15% zeolitic tuff), ST 15% (cultivated soil + perlite+ 15% zeolitic tuff), ST 30% (cultivated soil + perlite + 30% zeoltic tuff). A triple increase of stomatal conductance values can be observed for ST 5% (cultivated soil + peat + 5% zeolitic tuff), ST 30% (cultivated soil + peat + 30% zeolitic tuff) and for the SPm (cultivated soil + perlite, with no addition of tuff) and SP 5% (cultivated soil + peat + 5% zeolitic tuff) we obtained an increase of five or six times.

In Tab. 5 there are shown average values of stomatal conductance of plants grown on soils investigated in two moments: a reading of stomatal conductance on unfertilized plants was done and a second one after the fertilization with 1g/L of Nitrogen, Phosphorus and Potassium.

Tab. 5. Stomatal conductance plant before and after fertilization with 1 g / L of Nitrogen, Phosphorus and Potassium

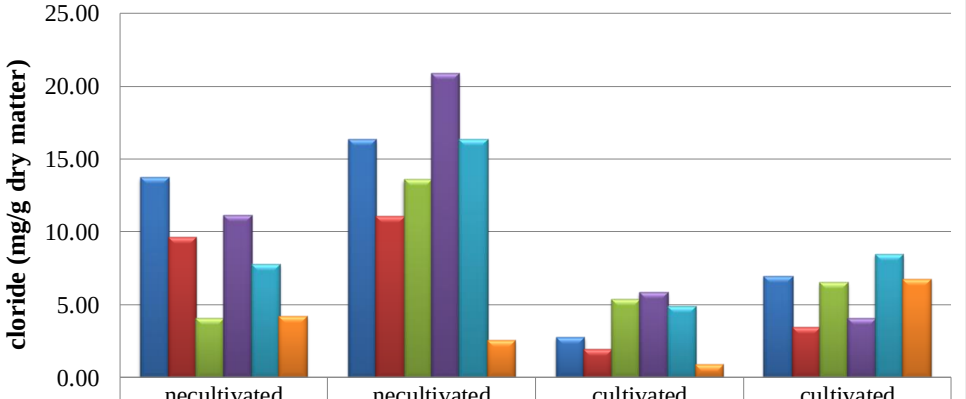
Sample	Stomatal conductance before fertilization (mmol/m^2/s)	Stomatal conductance after fertilization (mmol/m^2/s)	Sample	Stomatal conductance before fertilization (0mmol/m^2/s)	Stomatal conductance before fertilization (mmol/m^2/s)
STm	17.76	36	**MTm**	12.9	32.25
ST 5%	12.6	41.4	**MT 5%**	14.8	6
ST 10%	12.35	28.7	**MT 10%**	30.05	40.25
ST 15%	17.2	37.9	**MT 15%**	12.8	24.45
ST 20%	29.45	33.2	**MT 20%**	14.5	46.25
ST 30%	12.8	39.9	**MT 30%**	13.15	48.45
SPm	5.8	39.15	**MPm**	21.65	13.85
SP 5%	6.05	30.75	**MP 5%**	12.95	43.43
SP 10%	14.5	27.45	**MP 10%**	26.05	55.85
SP 15%	18.7	44.25	**MP 15%**	9.5	39.55
SP 20%	28.1	41.75	**MP 20%**	20.45	48.05
SP 30%	21.8	43.65	**MP 30%**	11.8	45.3

For samples coming from uncultivated soil fined with peat and perlite we obtained a double increase of stomatal conductance values for MTm (uncultivated soil + peat, with no addition of zeolitic tuff), MT 15% (uncultivated soil + peat + 15% zeolitic tuff), MP 10% (uncultivated soil + perlite + 10% zeolitic tuff) and MP 20% (uncultivated soil + perlite + 20% zeolitic tuff). Triple increase was noticed at MT 20% (uncultivated soil + peat + 20% zeolitic tuff), MT 30% (uncultivated soil + peat + 30% zeolitic tuff), MP 5% (uncultivated soil + perlite + 5% zeolitic tuff), MP 30% (uncultivated soil + perlite + 30% zeolitic tuff), cultivated soil + peat, and for the other plants that came from sample MP 15% (uncultivated soil + perlite + 15% zeolitic tuff) we have a 4 time-increase. For MT 5% (uncultivated soil + peat + 5% zeolitic tuff) and MPm (uncultivated soil + perlite, with no addition of zeolitic tuff) we registered a decrease in stomatal conductance values.

Analyzing the results obtained, it can be concluded that lower stomatal conductance values before fertilization show the degree of water stress of the plants, which, in order to protect themselves against dehydration, close their stomata, which has a negative effect on plant photosynthesis and productivity. The nutrient intake brought by fertilization helps plants increase the stomatal conductance, photosynthesis process and crop productivity.

Effect of soil fining on the accumulation of chlorine ions in plant tissues

Because the soils studied contain significant amounts of chlorine (Tab. 1), the research has also been aimed at the effect of soil improvement methods on the accumulation of chlorine ions in tissues. The determination of the chlorine content, expressed in mg/g of dry matter make able to observe the different effect of treatments on the accumulation of chlorine in tissues (Fig. 2).



	necultivated soil+peat	necultivated soil+perlite	cultivated soil+peat	cultivated soil+perlite
control	13.71	16.33	2.70	6.94
tuff 5%	9.65	11.05	1.95	3.45
tuff 10%	4.05	13.61	5.38	6.52
tuff 15%	11.14	20.88	5.79	4.04
tuff 20%	7.76	16.32	4.88	8.46
tuff 30%	4.17	2.53	0.88	6.76

Fig. 2. Effect of changes in chlorine concentration in plant tissues

In plants that were grown on soil from an uncultivated land was observed a significant decrease of chlorine concentration than from the witness, even after treatments with peat on all samples. On cultivated soil, the decrease only took place on the 5% and 30% samples. The perlite added on uncultivated soil determines reduction of chlorine accumulation in tissues only in MT 30% and on the uncultivated soil combined with 5% and 25% zeolitic tuff.

Conclusions

The investigation of amendment effect with zeolitic tuff on salinized soils, followed by a peat and respectively a perlite addition, have generated the following conclusions:

Plant growth, represented by biomass accumulation was positively influenced by the amendments application, the most efficient amendment variant was the 20% zeolitic tuff in a peat combination.

Stomatal conductivity and photosynthetic activity are not directly influenced by the applied amendments, in all cases the values of these parameters being reduced. Only after the phased plant fertilization with 1 g/l of nitrogen, phosphorus and potassium, an improvement in stomatal conductivity was observed in all research variants.

The concentration of chloride ions at the foliar level was influenced by the all soil treatments, the lowest values was recorded in the variants: 30% zeolitic tuff, in perlite and respectively peat combination, applied on the uncultivated soil, and at the cultivated soil, at the 30% zeolite tuff plus peat combination and 5% zeolite tuft and perlite combination.

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PHYSIOLOGICAL REACTION OF *BRASSICA RAPA* L. VAR. *PERVIRIDIS* L. H. BAILEY PLANTS CULTIVATED ON SALINIZED SOIL WITH ZEOLITIC TUFF AND PEAT

Carmen Doina JITĂREANU[1], Cristina SLABU[1],
Lidia ADUMITRESEI[2*], Maria Cătălina PASTIA[3]

Abstract: Our observations target the physiological response of plants with a short vegetation period like *Brassica rapa* var. *perviridis* L. H. Bailey to saline stress. The experiment uses white alkali soil amended with zeolitic tuff and peat as a substrate. There has been noticed a better behavior of the plants grown on cultivated soil amended with 15% zeolitic tuff and neutral peat. The chlorophyll content index was higher in plants grown on cultivated soil amended with 15% zeolitic tuff both when combined with neutral peat (14.0 and 30.6) or acidic peat (18.9 and 26.2) than in plants grown in soils that were amended with 20% zeolitic tuff. Regarding uncultivated soils previously, they are less favorable to plants than soils which had been included in the agricultural circuit. This proves once again that the non-use of salty lands accentuates the salinization process.

Key words: Komatsuna Torasan, peat, salinized soils, stomatal conductance, zeolitic tuff.

Introduction

Excessive accumulation of sodium in a soil causes numerous adverse phenomena, such as changes in the exchangeable ions in the soil solution and soil pH, destabilization of the soil structure, deterioration of the hydraulic properties of the soil, increased susceptibility to the formation of crusts, erosion and aeration as well as increased osmotic pressures [CORWIN & al. 1989, 2007]. These pressures have negative effects on the physiological processes of plants. In addition, there are serious imbalances in the plant nutrition process, these soils with nutrient deficiencies, structural changes and nutrient intake, all of which ultimately affect crop growth and decreasing efficiency [DE COSTA & al. 2007; IACOB & al. 1996; SCHUBERT, 2006; TOMEMORI & al. 2002, BOLOGA & al. 2015].

These problems of saline soils, together with the increase in the use of poor water quality for crop production, can lead to an increase in the problems that arise in the future [LUCHIAN, 2016].

This paper includes research on the growth of plants that tolerate the salinity of degraded soils using the physico-mechanical, chemical and microbiological factors of these soils from the Osoi-Moreni research base, Prisăcani, Iaşi County, as well as experiments regarding the improvement of their quality by fining with zeolitic tuff, neutral and acidic

[1] Agricultural Faculty of the USAMV "Ion Ionescu de la Brad" of Iaşi, Iaşi – Romania.
[2] University "Alexandru Ioan Cuza", Botanic Garden "Anastasie Fătu", 7-9, Dumbrava Roşie St., Iaşi – Romania.
[3] Technical University "Gheorghe Asachi" of Iaşi, Faculty of Hydrotechnics, Geodesy and Environmental Engineering, Iaşi – Romania.
[*] Corresponding author. E-mail: lidia.adumitresei@yahoo.com

peat, as well as the response of *Brassica rapa* var. *perviridis* plants to these additions [LUCHIAN, 2016].

The substrate used is the material transported and redeposited: carbonate, heavy clay and clays. The underlying roof is represented by fine, layered alluvial deposits. The soil that is the subject of this work is a white alkali soil affected by salinity, the samples used are from the layer 0-25 cm, where the plants develop their roots.

Material and method

The biologic material taken into study is represented by the seeds of *Brassica rapa* L. var. *perviridis* L. H. Bailey called the oriental mustard-spinach (Komatsuna Torasan) produced by Johnsons seed. Like other Brassicaceae, it exhibits salinity tolerance and is considered one of the plants of the future, the young leaves being used for salad. The plants were grown in small pots of green vegetation in the "Anastasie Fătu" Botanical Garden of Iaşi.

The substrate used is the material transported and redeposited: carbonate, heavy clay and clays. The underlying roof is represented by fine, layered alluvial deposits. The soil that is the subject of this work is a white alkali soil affected by salinity, the samples used are from the 0-25 cm layer where the plants develop their roots and come from cultivated lands (samples marked with S) and uncultivated (samples marked with M). For this reason, it has been chosen to investigate the zeolitic, neutral or acidic peat amendment of soils affected by salinity [TEJADA, 2006; TEJEDOR, 2007; TURAN, 2008; QIAH & al. 2001]. For the experiment, soil from the 0-25 cm layer was mixed with 15 and 20% zeolite tuff by mass and then 1 : 1 volumetric mixed with neutral or acidic peat.

The main physio-chemical features of amended soils are included in the table below (Tab. 1). The analysis of soil samples was done in the Pedology Laboratory within the Faculty of Hydrotechnics, Geodesy and Environmental Engineering of Technical University "Gheorghe Asachi" of Iasi.

Tab. 1. The white alkali soil used in our study has the following characteristics [LUCHIAN, 2016]

Characteristics	Cultivated soil (S)	Uncultivated soil (M)
Granulometric density (g/cm3)	1.79	1.69
Permeability coefficient (cm/s)	7.19×10^{-8}	4.97×10^{-7}
pH	6.83	8.07
Total exchange acidity (me/100 g soil)	0.13	0.14
Total content in cation exchangeable (me/100 g soil)	1,918.96	1,429.19
Total conductometric content of salts (mg/100 g soil)	317.22	29.78
Bicarbonate anion content (me/100g soil)	57.95	57.95
Value of the Cl^- (anion/100 g soil)	0.99	0.61
The presence of the anion SO_4^{2-} (me/100 g of soil)	0.026	-
Concentration of Ca^{2+} cation (me/100g soil)	67.58	18.08
The amount of Mg^{2+} cation (me/100 g soil)	45.63	8.66
The amount of K^+ (me/100 g of soil)	5.9	11.9
The amount of Na^+ (me/100 g of soil)	25.63	1.77

Zeolitic tuff has this mineralogical composition: clinoptilolite between 71-83.3%, volcanic glass between 4.1-9.7%, plagioclase 6.6-6.67%, SiO_2 3-4%, other minerals 3-4%.

Its chemical composition is: 68.75% SiO_2, 11.5% Al_2O_3, Fe_2O_3, CaO 2.86, Na_2O + K_2O 3.99%.

Other characteristics are: dry specific dry weight of 1.65-1.75 gf/cm³, cationic exchange capacity of 1.51 me/100g, natural humidity of 11-18%, specific surface area of 23.4 m²/g, micronized product, pore diameter of 3.82 Å, total porosity of 33.08%, water absorption of 16.21%, specific mass of 2.15-2.25 g/cm³, bulk density of 0.88 kg/dm³. After COCHEME & al. (2003), volcanic tuffs in Romania originate in the explosive activities of volcanic materials that have accumulated in inclusions and benches in the Miocene and Pliocene ages. The zeolitization took place in maritime environment, alkaline medium at 9.5-9.8.

To improve the physical and chemical properties of the substrate, Klassmann TS3 standard peat from Lithuania was used. It mainly contains partially decomposed blonde peat, the largest proportion of which has *Sphagnum* muscle with buffered pH 6 and, respectively, acidic Baltic peat from the same manufacturer with a pH of 4.2. These are enriched N, P, K with 1 g/l. Although peat naturally does not contain nutrients, it has the property of absorbing them and gradually giving it up to the soil. Peat has a role in loosening and airing the soils, has a high water absorption capacity, protects the soil from hardening, firms and increases the concentration of organic soil.

Stomatal conductance (mmol/m²/s) was measured using a leaf porometer (SC-1 model, Decagon Devices, Inc., Pullman, WA, USA). Chlorophyll content was determined using the CCM 200 Plus (Chlorophyll Content Meter Opti-Sciences), which uses a non-destructive method for assessing chlorophyll content. Determinations and data analysis were done within the Laboratory of Plant Physiology of the USAMV "Ion Ionescu de la Brad" of Iasi.

Results and discussions

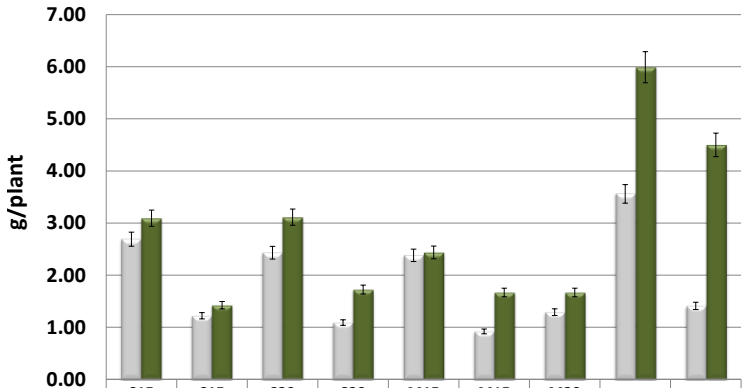
The relationships between the chemical composition of salinized soils, the way this salinity affects the physical, chemical and microbiological properties of soils, as well as the interaction between salinity and the mineral nutrition of crops are very complex.

Plant growth

It was influenced both by the type of nutrient substrate and its salinity (Fig. 1, Fig. 7). Most vegetative mass (6.0 g of fresh substance / plant) was recorded using neutral peat variant at 21 days post-emergence, and the lowest (1.7 g of fresh substance / plant) in the S15TA variant. Compared to acid peat, neutral peat has positively influenced plant growth, regardless of the experimental variant.

Plants obtained on the substrate that had not previously been grown showed a 6-8 day delay in emergence and other phenophases and were less developed.

Soil that was grown previously proved to be more favorable for plant growth and development compared to the uncultivated one.



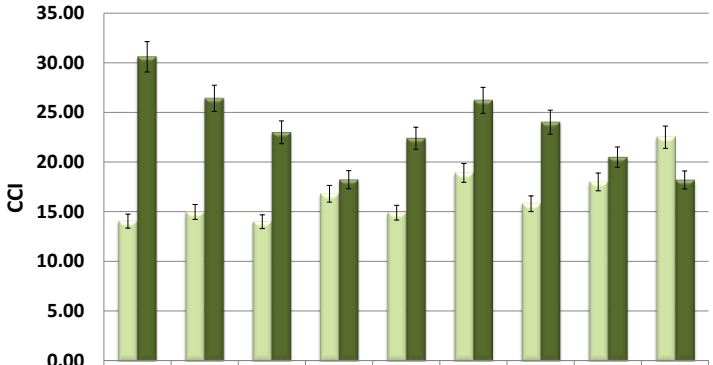
	S15 neutral peat	S15 acidic peat	S20 neutral peat	S20 acidic peat	M15 neutral peat	M15 acidic peat	M20 acidic peat	neutral peat	acidic peat
T1	2.69	1.22	2.43	1.09	2.38	0.92	1.29	3.56	1.41
T2	3.09	1.42	3.11	1.72	2.44	1.67	1.67	5.99	4.50

Fig. 1. Effect of zeolite tuff and peat on the growth of aerial parts of plants (g fresh substance / plant; bars represent the standard error n = 20)

Plant chlorophyll content

The chlorophyll content index (CCI) has varied both as a result of the phenological development of plants and under the effect of treatments (Fig. 2).

Noteworthy is that, unlike neutral peat, acid peat has negatively influenced chlorophyll content. Although we would have expected the acidic peat to further improve the chemical properties of the substrate, the effect was the opposite, negatively correlated with the specific consumption of *Brassicaceae* in general, which is high in alkaline ions [AMBE & al. 1999].



	S15 neutral peat	S15 acidic peat	S20 neutral peat	S20 acidic peat	M15 neutral peat	M15 acidic peat	M20 acidic peat	neutral peat	acidic peat
T1	14.05	14.97	13.99	16.80	14.90	18.90	15.80	18.00	22.50
T2	30.60	26.43	23.00	18.22	22.40	26.22	24.02	20.50	18.20

Fig. 2. Effect of zeolite tuff and peat on the chlorophyll content index (bars represent the standard error n = 20)

Between the growth of the aerial part of the plant and the chlorophyll content index was found a negative correlation (Fig. 2, 3): r = -0.58 at the first determination and r = -0.36 at the second determination.

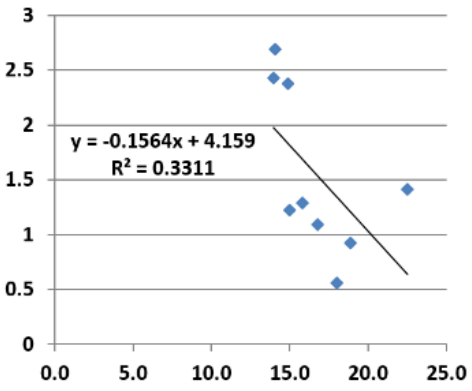


Fig. 3. The correlation between growth and content of chlorophyll of the aerial part at 20 day from the emergence

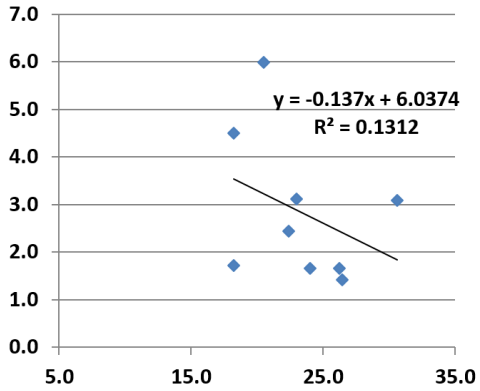


Fig. 4. The correlation between growth and content of chlorophyll of the aerial part at 30 day from the emergence

This negative correlation between plant mass biosynthesis and CCI value can be explained by the biphasic reaction of plants to saline stress, model explored by MUNNS (1993). According to this model, foliar growth is influenced, in a first phase, by the osmotic stress generated by the presence of salts. Plants have a low habitus and a dark green leaf color without being wilted [MENGEL, quoted by SCHUBERT, 2006, DE COSTA & al. 2007].

Another aspect that needs to be mentioned is that the same number of chloroplasts is formed as in normal conditions, which they have in a narrower space as a result of the reduction of cell growth by elongation. The high density of chloroplasts gives the leaves this intense green color. In the first phase of stress, the plant attempts to adapt to high concentrations of the external environment and accumulates ions, especially Na^+ and Cl^- in the root cells. These ions are then trained in the plant and reach various organs, especially in the leaves. In concentrations above certain tolerable limits by the plant, these ions trigger the second phase of stress- the appearance of some toxicity symptoms. Ionic toxicity is manifested, in particular by chlorosis or foliar necrosis.

It has been demonstrated that excess Na^+ causes disruption of stomata closure, which leads to an uncontrolled loss of foliar water and subsequent occurrence of imbalances and later to necrosis.

Stomatal conductance

It recorded values between 6.2 mmol $m^{-2}s^{-1}$ and 19.7 mmol $m^{-2}s^{-1}$ (Fig. 5) 20 days after emergence, there was a very close correlation (Fig. 6) between stomatal conductance and biomass synthesis expressed in dry matter (r = 0.80), which shows that plants exhibit normal photosynthetic activity. 10 days after the first determination, there is no correlation between the two parameters, which shows that in some of the samples, the plants start to pass into the second phase of saline stress, the high values of stomatal conductance being a proof of uncontrolled water loss as a result of disturbance of the stoma closure mechanism.

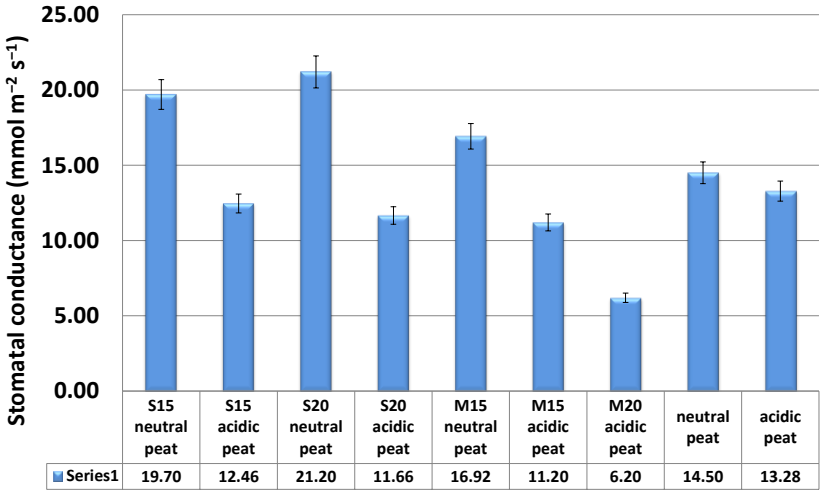


Fig. 5. Effect of zeolite tuff and peat on the stomatal conductance index (bars represent the standard error n = 20)

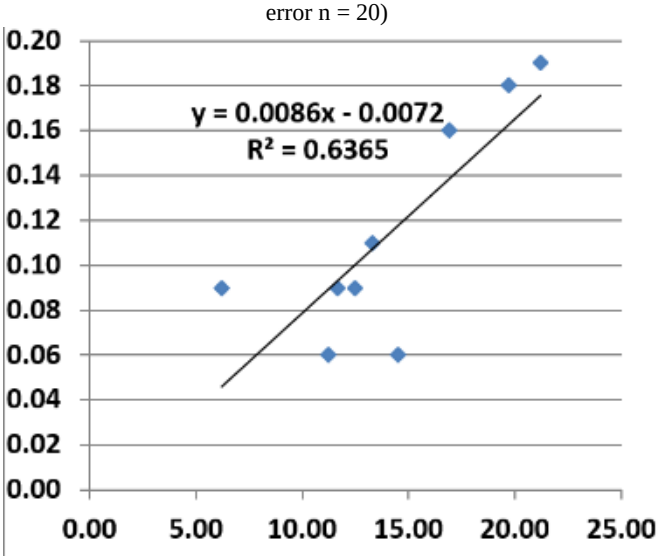


Fig. 6. Correlation between stomatal conductance and biomass synthesis at 30 days from the emergence

Control plant growth in neutral peat

Plant growth in acidic peat

S15 neutral peat

S15 acidic peat

M15 neutral peat

M15 acidic peat

S20 neutral peat

S20 acidic peat

Fig 7. Aspects of test plants.

Conclusions

On the degraded soils of the clay illuvial type can be cultivated a series of salinity-tolerant plants only after the improvement of the physical properties, but also by chemical ones by fining zeolitic tuff in percent of 15-20% and buffered or acidic peat, correlated with the requirements of the plant to be cultivated.

Brassic rapa var. *perviridis*, plants with a short vegetation period, manage to avoid saline stress and compensate for investment in culture to a certain extent.

Soils that have been grown on previously are more favorable to plant growth and development compared to uncultivated ones, which confirms that they are degraded more severely.

On previously uncultivated soils there is a delay of emergence, which is accentuated in the following phenophases, but also a decrease in the percentage of germination and viability of the plants.

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HIERACIO UMBROSI-QUERCETUM PETRAEAE PÎNZARU, CANTEMIR, MANIC & POPESCU – ASS. NOVA, FROM THE CENTRAL MOLDOVAN PLATEAU OF THE REPUBLIC OF MOLDOVA

Pavel PÎNZARU[1*], Valentina CANTEMIR[1], Ştefan MANIC[1], Andrei POPESCU[2]

Abstract: The forests of sessile oak (*Quercus petraea* (Matt.) Liebl.) with *Hieracium umbrosum* Jord., found on the Central Moldovan Plateau, are described in this article. Based on 12 relevés, the authors have grouped these forests in an plant community that is new for science – *Hieracio umbrosi-Quercetum petraeae* Pînzaru, Cantemir, Manic et Popescu, included in the alliance *Quercion pubescenti-petraeae* Br.-Bl. 1932, the order *Quercetalia pubescenti-petraeae* Klika 1933, cl. *QUERCO-FAGETEA* Br.-Bl. et Vlieger in Vlieger 1937.

Keywords: *Hieracio umbrosi-Quercetum petraeae* ass. nova, characteristics of phytocoenoses, ecology, range, R. Moldova.

Introduction

Hieracium umbrosum Jord. (*Asteraceae*) [incl. *H. umbrosum* Jord. ssp. *pseudofastigiatum* (Degen et Zahn) Zahn] is a xeromesophilic hemicryptophyte, found in the Mediterranean Basin, Central Europe, Ukraine (north-west) and Denmark [SELL & WEST, 1976; SHLJAKOV, 1989; CONTI & al. 2005; SÎRBU & al. 2013; TEOFILOVSKI, 2016].

In the Republic of Moldova, the species *Hieracium umbrosum* Jord. was collected, for the first time, in the early 70s of the last century, by the collaborators of the Botanical Garden of the ASM: Afanasie Istrati, Vasile Chirtoacă, Ksenia Vitko and Aglaia Railean, in the forests of the central area of the country (Logăneşti and Mereşeni villages), and by Dumitru Gociu, in the forest near Dondușeni town [NICOLAEV, 1994]. Unfortunately, no exsiccata has been preserved in the Herbarium of the Botanical Garden. This article presents new information on the chorology and phytocoenotic belonging of the species *H. umbrosum* Jord.

Materials and methods

The floristic and phytocenotic studies have been carried out in 2016-2017, in the forests of sessile oak (*Quercus petraea* (Matt.) Liebl.) found on the Central Moldovan Plateau. Twelve relevés have been described according to the methods of the Central European School [BRAUN-BLANQUET, 1964]. The area of a relevé is 600 m^2, according to the school of Cluj [CRISTEA & al. 2004]. The list of species is presented in accordance with recent publications [APG – III, 2009; PÎNZARU & SÎRBU, 2016]. Air temperature and

[1] Botanical Garden (I) of the Academy of Sciences of Moldova, Chişinău – Republic of Moldova.
[2] Hîncești Forest Management Unit, Republic of Moldova.
[*] Corresponding author. E-mail: p_panzaru@yahoo.it

atmospheric precipitation – according to the Atlas of Climate Resources of the Republic of Moldova [NEDAELCOV & al. 2013].

Results and discussion

During the floristic studies conducted on the Central Moldovan Plateau, the species *Hieracium umbrosum* Jord. was found in the forests of sessile oak in the forest divisions "Mereşeni" (Mereşeni commune, plots 57, 75), "Logăneşti" (Lăpuşna commune, plot 23, and Logăneşti commune, plots 28, 36, 41) and "Bobeica" (Văsieni commune, plots 27, 34) of the "Hînceşti-Silva" Forest Management Unit.

Under the conditions of the Republic of Moldova, the individualis of the species grow from 30 cm to 100 cm tall. The stems are erect, hairy (long, soft hairs), branched in the upper part, leafy up to the tip. The leaves are wide-ovate, wide-rhomboidal, the lower leaves are petiolate, the upper ones – short-petiolate to slightly amplexicaul, shortly-acute at the tip, on the margins – distantly denticulate, ciliate, with sparse hairs on both sides, on the dorsal side – the midrib with long, soft hairs, with no glandular hairs. Involucral hypsophiles with glandular, blackish, dense hairs and numerous stellate hairs. Yellow flowers. An inflorescence contains up to 21 erect anthodia; an anthodium produces 10-45 achenes. The plants flower in June-July (first half); at the end of July, the fruits are already mature. The achenes are cylindrical, blackish, ±3 mm long and 0.6-0.7 mm wide, longitudinally ribbed, covered with short, flat-lying hairs. The pappus is pale-yellow, with unequal, short, pinnate setae. The plants grow sporadically or in groups, in thermo-xeromesophilic forests of sessile oak, on hills with loamy soil.

Fig. 1. *Hieracium umbrosum* Jord.

The plant communities of sessile oak (*Quercus petraea* (Matt.) Liebl.) with *Hieracium umbrosum* Jord., accompanied by xeromesophilic species: *Cotinus coggygria* Scop., *Euonymus verrucosus* Scop., *Poa nemoralis* L., *Securigera elegans* (Pančić) Lassen, *Vicia cassubica* L., *Lathyrus niger* (L.) Bernh. etc., reveal a specific habitat of loamy hills,

of 210-360 m in height, differing from other sessile oak forests in the floristic composition and ecotope. These coenoses, having a unique character, are proposed to be included in a new association of the alliance *Quercion pubescenti-petraeae* Br.-Bl. 1932.

The description of the new association is presented below.

Ass. *Hieracio umbrosi-Quercetum petraeae*
Pînzaru, Cantemir, Manic et Popescu, *ass. nova, h. l.*

T y p u s h. l.: Tab. 1, rel. 7.
Table synthetic h. l.: Tab. 1, 12 relevés

Locations: Altitude 210-360 m. Relief: Central Moldovan Plateau, on hills with North-Western aspect, the inclination of the slopes varies between 7° and 25°. Soil: sandy clay. Climate – temperate-continental, the average annual temperature is 10.0-10.5°C, the average annual precipitation varies between 650 mm and 700 mm.

Characteristic species: *Quercus petraea*, *Hieracium umbrosum*.

Constant species: *Tilia tomentosa*, *Sorbus torminalis*, *S. aucuparia*, *Acer tataricum*, *A. campestre*, *Cotinus coggygria*, *Euonymus verrucosus*, *Poa nemoralis*, *Hieracium sabaudum*, *Securigera elegans*, *Vicia cassubica*, *Lathyrus niger*, *Galium schultesii*, *Convallaria majalis*, *Tanacetum corymbosum.*

Rare species protected by the state: *Securigera elegans* [Vulnerable (VU), included in the Red Book of R. Moldova], *Dryopteris filix-mas* [Vulnerable (VU), included in the Red Book of R. Moldova], *Genista elata* [Vulnerable (VU)], *Cystopteris fragilis* [Near threatened (NT)], *Sorbus torminalis* [Near threatened (NT)], *S. aucuparia* [Near threatened (NT)], *Asparagus tenuifolius* [Near threatened (NT)].

Fig. 2. Ass. *Hieracio umbrosi-Quercetum petraeae*

Structure: Vertically, three layers are distinguished in phytocoenoses:

1. The tree layer (A), with a height of about 20 m, the coverage of the canopy is about 70-80%. This layer consists of the dominant species *Quercus petraea*, with the cover-abundance (AD) of 5 points, the diameter of the stems varies between 25 cm and 45 cm. Accompanying species: *Tilia tomentosa*, *Cerasus avium*, *Carpinus betulus*, *Acer campestre*, rarely *Tilia cordata*, *Fraxinus excelsior*, *Acer platanoides*. The species of small trees, 7-8 m tall, *Sorbus torminalis* and *Sorbus aucuparia*, are found sporadically and aren't distinguished as a separate layer.

2. The shrub layer (B), is 1.5-3 m high, unevenly developed, with coverage of 15-50 (70)%, in some places is absent, sometimes it is removed during sanitation cuts. Constant species: *Cotinus coggygria*, *Euonymus verrucosus*, *Cornus mas*, *Crataegus monogyna*, *Viburnum lantana*. In the shrub layer, there are also young trees.

3. The herbaceous layer (C), with a height of 20-100 cm, is uneven. The spring synusia includes few species and is poorly defined, in large areas, there are no ephemeroidal and ephemeral plants, the following species have been found: *Corydalis solida*, *Anemonoides ranunculoides*, *Scilla bifolia* and *Cardamine bulbifera*. The summer synusia is richer, with the general coverage varying from 30% to 80%, and in the groups of European smoketree (*Cotinus coggygria* Scop.), the presence of herbaceous plants can be considered insignificant. The summer synusia is dominated by *Poa nemoralis* (AD = 2-4), *Galium schultesii* (AD = 2-3), *Vicia cassubica* (AD = 2-3), in some places, there are abundant clusters of *Convallaria majalis*, *Buglossoides purpurocaerulea*, *Stellaria holostea* and *Carex pilosa*. Constant species, but occurring sporadically, are *Hieracium umbrosum* (rarely forms small groups with AD = 2), *Tanacetum corymbosum*, *Sedum maximum*, *Securigera elegans*, *Lathyrus niger*, *L. vernus*, *L. aureus*, *Scutellaria altissima*. At the level of the herbaceous layer, there are abundant clusters of *Cotinus coggygria.* Young trees and shrubs are also found sporadically in this layer.

In the phytocoenoses of this association, 21 species of fungi have been found: *Agaricus altipes* Peck, *Agaricus bohusii* Bon, *Amanita crocea* (Quél.) Singer, *Boletus aereus* Bull., *Boletus reticulatus* Schaeff., *Boletus subtomentosus* L., *Daedaleopsis tricolor* (Bull.) Bondartsev & Singer, *Gymnopus fusipes* (Bull.) Gray, *Ganoderma lucidum* (Curtis) P. Karst., *Gymnopus peronatus* (Bolton) Gray, *Macrolepiota excoriata* (Schaeff.) Wasser, *Mycena pura* (Pers.) P. Kumm, *Neoboletus erythropus* (Pers.) C. Hahn, *Phellinus igniarius* (L.) Quel., *Russula amarissima* Romagn. & E.-J. Gilbert, *Russula delica* Fr., *Russula grisea* Fr., *Russula rubroalba* (Singer) Romagn., *Russula seperina* Dupain, *Stereum hirsutum* (Willd.) Pers., *Xerocomellus chrysenteron* (Bull.) Šutara.

Range. The plant communities of sessile oak (*Quercus petraea* (Matt.) Liebl.) with *Hieracium umbrosum* Jord., have been recorded in Hînceşti (in the vicinity of Mereşeni, Logăneşti and Lăpuşna communes) and Ialoveni districts (in the vicinity of Văsieni commune).

Conservation value. The conservation value has been assessed as high. In addition to the rare species – *Hieracium umbrosum*, there are other seven plant species protected by the state [Law on the Fund of Natural Areas Protected by the State, 1998; Red Book of the Republic of Moldova, 2015].

Conservation status. The phytocoenoses of this association are protected in the Logăneşti Nature Reserve of Medicinal Plants, plot 36 (24), and in the "Hînceşti Forest" Landscape Reserve, plot 41.

Protection measures. The plant communities of sessile oak with European smoketree are rare and it has been proposed to protect them on their entire range. The species

Hieracium umbrosum Jord., present in these rare plant communities, is found at the eastern limit of the range and it has been proposed to be included in the Red Book of the Republic of Moldova, in the Vulnerable (VU) category.

During the floristic field research, a new species, for the flora of the Republic of Moldova, was identified – *Chamaecytisus polytrichus* M.Bieb.

Conclusions

The association *Hieracio umbrosi-Quercetum petraeae* Pînzaru, Cantemir, Manic et Popescu is characterised by thermo-xeromesophilic, rare phytocoenoses, found on high hills with clayey and sandy soils of the Central Moldovan Plateau. It has been proposed to include this association in the List of Rare Plant Associations of the Republic of Moldova.

The association *Hieracio umbrosi-Quercetum petraeae* Pînzaru, Cantemir, Manic et Popescu ass. nova to the alliance *Quercion pubescenti-petraeae* Br.-Bl. 1932, the order *Quercetalia pubescenti-petraeae* Klika 1933, cl. *QUERCO-FAGETEA* Br.-Bl. et Vlieger in Vlieger 1937.

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Tab. 1. Ass. *Hieracio umbrosi-Quercetum petraeae* ass.nov.

Relevé no.	1	2	3	4	5	6	*7	8	9	10	11	12	K
Altitude (m)	240	210	250	270	275	265	230	250	300	340	360	305	
Aspect	E	E	N	SW	NW	NW	NW	NW	NW	NW	SW	SW	
Slope (°)	20	10	25	8	14	20	14	25	27	7	15	25	
Tree layer coverage (%)	75	75	70	70	70	75	75	80	75	80	75	80	
Shrub layer coverage (%)	30-50	40	55	20-70	35-60	30	15-20	0-35	0-30	0-45	0-50	0-60	
Spring herbaceous layer coverage (%)	15-30	30	50	15	10	-	-	-	-	15	10	-	
Summer herbaceous layer coverage (%)	20-80	30-80	80	15/85	20-65	75	60-80	50-70	40-75	60	40-80	30-70	
Relevé surface (m^2)	600	600	600	600	600	600	600	600	600	600	600	600	
Number of species	74	56	63	70	52	60	53	66	55	66	52	51	
Plots no.	57	57	75	23	36	36	41	41	41	27	34	28	
Characteristic species													
Quercus petraea	4	4	5	5	5	5	5	5	5	5	5	4	V
Hieracium umbrosum	1	+	+	+	+	1	2	2	1	+	1	+	V
Quercion et Quercetalia pubescenti - petraeae													
Sorbus torminalis	+	+	r	+	+	+	+	+	+	+	r	+	V
Sorbus aucuparia	+	r	r	r	r	r	r	+	r	r	r	-	V

Cotinus coggygria	2	2	3	3	3	1	1	2	2	2	3	4	V
Euonymus verrucosus	2	+	1	1	1	+	1	+	+	+	+	+	V
Poa nemoralis	2	2	-	2	2	4	3	4	3	2	3	2	V
Lathyrus niger	1	1	+	+	1	+	1	1	1	+	+	+	V
Vicia cassubica	3	2	3	1	+	1	2	3	3	2	3	3	V
Hieracium sabaudum	1	+	+	+	-	-	+	1	+	+	+	+	V
Melica picta	2	-	1	1	1	+	1	1	1	1	+	+	V
Tanacetum corymbosum	+	+	+	+	1	+	+	+	+	+	+	+	V
Campanula persicifolia	-	-	+	+	+	+	+	+	+	+	+	+	V
Cornus mas	1	2	+	r	+	+	-	r	r	+	r	1	V
Vincetoxicum hirundinaria	+	-	+	-	+	+	+	+	+	+	r	+	V
Galium schultesii	+	-	-	1	1	1	2	3	3	1	2	2	V
Acer tataricum	+	+	1	1	1	+	-	-	-	+	+	+	IV
Securigera elegans	+	+	+	-	-	-	2	2	1	1	1	+	IV
Vicia pisiformis	1	+	+	-	+	-	+	+	-	+	+	+	IV
Viburnum lantana	-	+	1	1	-	+	-	-	-	+	+	1	III
Digitalis grandiflora	r	+	-	r	-	-	+	1	+	-	-	-	III
Silene nutans s.l.	-	-	-	r	+	+	r	r	-	-	-	+	III
Asparagus tenuifolius	-	r	-	r	-	-	-	-	-	r	-	+	II

Prunus spinosa var. spinosa	1	-	-	-	+	-	-	-	-	-	r	-	II
Laser trilobum	+	+	-	-	-	-	-	-	-	-	+	-	II
Buglossoides purpurocaerulea	2	-	-	2	-	-	-	-	1	2	-	-	II
Silene noctiflora	-	-	-	+	-	-	-	-	-	r	r	+	II
Mercurialis ovata	1	-	1	1	+	-	-	-	-	-	-	-	II
Genista elata	-	-	-	r	-	-	+	+	-	-	-	-	II
Rubus canescens	-	+	-	-	-	-	-	-	-	-	-	-	I
Clematis recta	r	-	-	-	-	-	-	-	-	-	-	-	I
Veronica orchidea	-	-	-	-	-	r	-	-	-	-	-	-	I
Chamaecytisus polytrichus	-	-	-	r	-	-	-	-	-	-	-	-	I
Querco-Fagetea													
Convallaria majalis	2	2	2	2	2	1	2	2	1	2	1	-	V
Glechoma hirsuta	1	1	1	1	1	1	1	2	1	2	+	2	V
Scutellaria altissima	1	+	1	+	-	1	+	+	1	1	+	1	V
Crataegus monogyna	1	+	+	1	+	+	1	+	1	1	+	1	V
Geum urbanum	+	+	+	+	+	+	+	+	+	+	+	+	V
Campanula rapunculoides	+	+	+	+	+	+	1	2	1	1	+	+	V
Cerasus avium	+	+	r	+	+	-	+	+	r	+	-	+	V
Sedum maximum	+	+	+	+	+	+	+	+	+	+	r	+	V

Dactylis glomerata	+	-	-	+	+	+	+	+	+	+	+	1	V
Acer campestre	+	+	+	+	+	+	-	-	-	1	+	1	IV
Lathyrus vernus	1	+	1	-	-	+	+	+	-	+	+	+	IV
Rosa canina	+	r	r	r	r	-	r	r	r	-	-	-	IV
Lapsana communis	+	-	-	+	+	+	+	+	+	+	-	+	IV
Solidago virgaurea	r	-	-	+	+	+	+	+	+	+	-	-	IV
Polygonatum latifolium	1	-	1	2	1	-	1	1	1	-	-	1	IV
Veronica officinalis	-	-	+	-	-	1	+	+	+	-	-	-	III
Chaerophyllum temulum	-	-	+	+	-	+	-	-	-	-	-	-	III
Lactuca quercina	r	r	-	r	-	r	-	-	-	+	-	+	III
Hypericum hirsutum	-	-	+	-	-	-	-	+	+	+	+	+	III
Melampyrum nemorosum	-	-	-	1	-	-	1	-	-	+	-	-	II
Carex contigua	+	+	+	-	-	+	-	+	-	+	-	+	II
Cornus sanguinea	-	+	+	+	-	-	-	-	-	+	-	1	II
Euonymus europaeus	-	+	+	+	-	-	-	-	-	1	-	-	II
Geranium robertianum	+	+	-	-	-	+	-	-	-	+	-	-	II
Lactuca muralis	-	-	+	-	-	-	+	+	-	+	-	+	II
Polygonatum multiflorum	1	-	-	-	-	-	-	-	-	-	-	-	I
Melica nutans	-	-	1	-	-	-	-	-	-	-	-	-	I

Pyrus pyraster	r	-	-	-	-	-	-	-	-	-	r	-	I
Cruciata glabra	+	-	-	-	-	-	-	-	-	-	-	-	I
Pseudolysimachion longifolium	r	-	-	-	-	-	-	-	-	-	-	-	I
Fraxinus excelsior	+	-	-	+	-	-	-	-	-	-	-	-	I
Veronica hederifolia	2	1	-	-	-	-	-	-	-	-	-	-	I
Ulmus glabra	-	-	-	-	+	-	-	-	-	-	+	-	I
Campanula trachelium	-	-	+	-	-	-	+	-	-	-	-	-	I
Dryopteris filix-mas	-	-	-	-	-	-	-	r	-	-	-	-	I
Ulmus minor	-	-	-	-	-	-	-	-	-	+	-	-	I
Carpinion et Fagetalia sylvaticae													
Tilia tomentosa	1	+	r	+	+	+	+	+	+	1	1	2	V
Carex pilosa	2	3	1	3	2	1	2	2	1	1	1	3	V
Carex brevicollis	1	1	-	2	1	-	1	1	1	1	+	1	V
Stellaria holostea	1	1	2	1	1	1	2	2	2	1	2	3	V
Scrophularia nodosa	r	r	-	r	r	r	+	+	r	r	r	+	V
Carex digitata	-	-	+	-	+	+	+	+	+	-	+	+	IV
Carpinus betulus	-	1	-	+	+	r	+	+	+	1	1	1	IV
Viola suavis	+	+	+	1	-	1	-	-	-	1	-	+	III
Pulmonaria officinalis	+	+	+	+	-	-	+	-	-	+	-	+	III

Corydalis solida	1	2	2	2	1	-	-	-	-	1	1	-	III
Scilla bifolia	1	1	1	1	1	-	-	-	-	+	+	-	III
Anemonoides ranunculoides	+	1	+	+	+	-	-	-	-	1	-	-	III
Tilia cordata	+	+	-	-	+	+	-	-	-	-	-	-	II
Acer platanoides	+	-	-	+	-	+	-	-	-	-	-	-	II
Cardamine bulbifera	1	2	1	-	-	-	-	-	-	-	-	-	II
Lamium galeobdolon	-	-	1	-	-	-	-	-	-	-	-	-	I
Ranunculus auricomus	-	-	+	-	+	-	-	-	-	-	-	-	I
Galium odoratum	-	-	-	-	-	-	1	2	-	-	-	-	I
Cystopteris fragilis	-	-	-	-	-	-	-	+	-	-	-	-	I
Viola mirabilis	-	-	-	-	-	-	-	-	-	+	-	-	I
Lathyrus aureus	-	-	+	-	-	-	-	-	-	+	1	-	I
Asarum europaeum	-	-	-	-	-	-	-	-	-	+	-	-	I
Trifolio-Geranietea													
Hypericum perforatum	r	r	r	r	r	r	r	r	r	r	-	-	V
Astragalus glycyphyllos	+	r	r	-	r	-	+	+	+	-	+	-	IV
Brachypodium sylvaticum	+	+	-	-	1	1	-	1	+	1	-	-	IV
Veronica chamaedrys s.l.	-	-	-	+	+	+	+	+	+	+	+	+	IV
Clinopodium vulgare	+	r	-	r	-	-	+	+	+	+	+	+	IV

Pilosella bauhinii	-	-	-	-	+	+	+	+	+	-	-	-	III
Trifolium montanum	-	-	-	-	+	-	-	1	1	+	-	+	II
Agrimonia eupatoria s.l.	-	-	r	-	r	-	-	-	+	-	-	-	II
Campanula bononiensis	-	-	-	r	-	-	+	r	-	-	-	-	II
Stachys officinalis	r	-	r	-	-	r	-	-	-	-	-	-	II
Trifolium alpestre	1	+	+	-	-	-	-	-	-	-	-	-	II
Centaurea jacea s.l.	r	-	-	-	-	-	-	r	r	+	-	-	II
Fragaria viridis	-	-	-	-	-	-	-	1	1	+	-	-	II
Securigera varia	-	-	+	-	+	-	-	+	+	-	-	-	II
Trifolium medium	-	-	-	+	-	-	-	-	-	-	-	-	I
Hieracium virosum	+	-	+	-	-	-	-	-	-	-	-	-	I
Filipendula vulgaris	-	-	-	r	-	-	-	-	-	-	-	-	I
Dianthus membranaceus	-	-	-	r	-	r	-	-	-	-	-	-	I
Origanum vulgare s.l.	-	-	-	+	-	-	-	-	-	-	-	-	I
Hieracium umbellatum	-	-	-	r	+	-	-	-	-	-	-	-	I
Iris variegata L.	-	-	-	r	-	-	-	-	-	-	-	-	I
Inula britannica	-	-	-	-	-	-	-	-	-	1	-	-	I
Medicago falcata	-	-	-	-	-	-	-	-	-	+	-	-	I
Inula salicina	-	-	-	-	-	-	-	-	-	-	1	-	I

Aliae													
Fallopia convolvulus	r	-	-	r	-	r	+	+	+	r	+	-	IV
Galium mollugo	+	+	+	-	-	-	+	1	-	-	+	-	III
Torilis ucrainica	-	-	-	-	-	-	-	+	+	-	r	-	III
Prunella vulgaris	-	-	-	r	r	-	-	-	-	-	r	-	II
Torilis arvensis s.l.	+	-	-	-	-	-	-	-	-	-	-	-	I
Robinia pseudacacia	-	-	-	1	-	-	-	-	-	-	+	-	I
Alliaria petiolata	+	-	-	+	-	-	-	-	-	-	-	-	I
Galium aparine	+	-	-	+	-	-	-	-	-	-	-	-	I
Silene alba	-	-	-	-	-	-	r	+	-	-	-	-	I
Aristolochia clematitis	-	-	1	-	-	-	-	-	-	-	-	-	I
Silene vulgaris s.l.	-	-	-	-	-	-	-	-	-	+	+	-	I

Place and date of the relevés: 1-3, Mereşeni commune, Hînceşti district, 4-5.VIII.2016, 11.V.2017; 4, Lăpuşna commune, Hînceşti district, 46°54'31" lt. North, 28°28'24" lg. East, 05.IV.2017, 27.VI.2017; 5-6, Logăneşti commune, Hînceşti district, 46°54'24" lt. North, 28°51'05" lg. East and 46°54'28" lt. North, 28°52'30" lg. East, 05.IV.2017, 27.VI.2017; *7 (typus)-9, Logăneşti commune, Hînceşti district, 46°53'55" lt. North, 28°30'54" lg. East and 46°53'55" lt. North, 28°30'57" lg. East, 05.IV.2017, 14.VII.2017; 10-11, Văsieni commune, Ialoveni district, 46°57'39" lt.North, 28°31'35" lg. East and 46°57'14" lt. North, 28°32'52" lg. East,06.IV.2017, 03.VIII.2017; 12 - Logăneşti commune, Hînceşti district, SW, 05.IV.2017, 19.IX.2017.

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THREATENED MEDICINAL PLANTS OF UKRAINE: AN ASSESSMENT OF THE CURRENT PROTECTION STATUS

Valentyna M. MINARCHENKO[1]

Abstract: More than 2 200 vascular species of Ukraine were investigated as medicinal plants, containing biologically active substances which are used at present or can be used in the future for medicinal purposes, including production of commercial drugs. 1 975 from this number are wild plants and 537 are threatened in Ukraine and/or in Europe. For many medicinal plant currently highly threatened by direct or indirect human activities, which lead to transformation and loss of habitat. The overall purpose of the paper is to show the threats, current population trend and protection status of threatened species of medicinal plants. It has been established that the future of wild resource more than half (52%) of analyzed medicinal plants in Ukraine is at risk as are currently highly threatened the loss of its habitat. The current population trend of these species is mainly declining on assessment to Ukraine. The population trend near of 43.5% accessed species considered as stable. The state of only 3.7% of analyzed medicinal plants in Ukraine may be assessed as increasing.

Keywords: threatened medicinal plants, Ukraine, population trend.

Introduction

Ukraine is characterized by a considerable diversity of plants species because its location in different natural zones. There are more than 6 000 species of vascular plants in Ukraine (native, naturalized, occasionally introduced, and most common cultivated taxa) [MOSYAKIN & FEDORONCHUK, 1999]; more than 2 200 species were investigated to some extent as medicinal plants containing biologically active substances which are used at present or can be used in the future for medicinal purposes, including production of commercial drugs [MINARCHENKO, 2005, 2011]. About 10% of these (244 species, according to our estimates) are cultivated and introduced plants, while the remaining taxa are native or naturalized species growing in the wild. Among 1975 species of wild medicinal plants of Ukraine, 537 species are officially considered as threatened in Ukraine and/or at the European level [IUCN, 2015].

The *State Pharmacopoeia of Ukraine* (*SPU*) provides information on raw or processed products of 197 species of medicinal plants and lichens from 97 genus. Among them, 90 species are wild plants, including 16 taxa both: wild and cultivated (occurring in natural communities and also cultivated to obtain raw materials, or imported from other countries); 35 species are present in Ukraine only as cultivated plants. *SPU* also lists 72 species of medicinal plants, raw materials or substances of which are imported to Ukraine from other countries.

Ukrainian folk medicine uses raw materials of about 1 000 species of plants, more than 80% of which are wild-growing ones. Many of them have limited distribution and scarce resources, and thus are in need of various protection or conservation actions. Under the

[1] M. G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 2 Tereshchenkivska Str., Kyiv-1, 01601 – Ukraine. E-mail: valminar@ukr.net

present-day anthropic influence, populations of many species are shrinking (both in terms of the area of occupancy and numbers of individual plants), and quite often such populations degrade to the level when they are not able anymore to restore or reproduce normally.

Harvesting amounts of wild medicinal plants is regulated by executive governmental authorities at the local and state levels. The Ukrainian legal system of environmental protection and management includes laws, by-laws and other regulatory documents which regulate the specific types of activities in the field of the sustainable use and conservation of plants [MINARCHENKO, 2011].

The present article summarizes the results of our analysis of selected wild-growing medicinal plants of Ukraine, which are listed in the IUCN Red List of Threatened Species (version 3.1) and some species with the national protection status in Ukraine, especially those listed in the *Red Data Book of Ukraine* [DIDUKH, 2009]. This article focuses on the analysis of the causes and factors leading to degradation of populations of medicinal plants, as well as pathways of their conservation and restoration.

The conservation status and current population trend of analyzed species on European regional level was assessed using the IUCN Red List Categories and Criteria (IUCN 2015-4 (http://www.iucnredlist.org/). A wide range of literature and other sources were reviewed and consulted to identify endangered medicinal plants of Ukraine and their present conservation status.

Results and discussion

Among 537 analyzed the threatened wild medicinal vascular plants, to 477 species assigned some protection status (including Data Deficient) at the European level (on regional or/and global assessment), according to data of the European Red List of Threatened Species (IUCN 3.1). Three of them belong to the category Endangered (EN), five are Vulnerable (VU), 11 – Near Threatened (NT), 434 – Least Concern (LC), and 24 – Data Deficient (DD), however only 61 species from this number are protected at the state (national) level and included in the Red Data Book of Ukraine [DIDUKH, 2009]. Further 141 species of medicinal plants have protection status as regionally rare in Ukraine.

Furthermore, 63 species of medicinal plant are considered as threatened only for Ukraine and absent in the IUCN Red List, because of insufficient data available to assess these taxa against the IUCN Red List criteria. Their populations threatened by depletion in Ukraine as a result of the negative impact of complex natural and anthropogenic factors. They are few in number and mostly present in a few localities each. It is our belief that if a taxon is more threatened in Ukraine as in the whole of Europe (it has a higher conservation category in Ukraine than in the European Red List), its populations must still be regarded as important components for protection with appropriate conservation measures in Ukraine.

Threatened species of medicinal plants listed on the European Red List of Threatened Species

Only three species are endangered on a global, regional or both levels: *Daphne sophia*, *Aldrovanda vesiculosa* and *Neottianthe cucullata.*

Daphne sophia (EN) is restricted endemic to the Central Russian Upland, including Russia and Ukraine. Area of occupancy of *Daphne sophia* is severely fragmented, estimated

to be less than 500 Km^2; area, extent and quality of habitat continuing decline, therefore the state of this plant species was estimated as endangered on regional and global levels [MELNYK, 2013].

Detailed investigations of biologically active substances of this species are unknown, but there is information that the plant of *Daphne sophia* contain coumarins and catechins, which have antibacterial and anti-tumor activity. For medical purposes in Ukraine this species is unused.

There are known only four localities in Ukraine and in the Red Book is listed as Endangered [DIDUKH, 2009]. The populations are very small, the number of individual are 175 shoots near Ochrimivka village, 770 shoots near Mala Vovcha village, 600 shoots near Zovtneve Druge and 1,500 shoots near Kolodjazne village in Kharkiv Region. The main threat to the habitats is changes of ecotopes due to exploitation felling, forest planting on slopes, and chalk mining. An important threat to *Daphne sophia* is also harvesting of flowering shoots for bouquets.

Aldrovanda vesiculosa is assessed at the European level as Endangered B2ab (iii, v), since there is evidence of its disappearance in many countries [CROSS, 2012] due to irreversible changes in the environment. To assess the status of *Aldrovanda vesiculosa* at the global level, it is have been not enough specific data on localities and population dynamics in time, so now it was assessed as Data Deficient at the moment with an urgent need for further research [BILZ & LANSDOWN, 2013].

The data about its use for medical purposes are not available, but it is known that *Aldrovanda vesiculosa* contains carotenoids and naphthoquinones [MINARCHENKO, 2005]. In Ukraine *Aldrovanda vesiculosa* is listed in the Red Data Book as a rare species with a disjunctive spread in freshwater flat territory [DIDUKH, 2009].There are more than 20 confirmed localities of this species mainly in the catchment areas of the rivers Pripyat, Dnieper, and Danube. In general, the state of its population can be characterized as digressive, although the number of individuals of different populations can change dramatically within a few years, depending mainly on the water temperature. The main threats to *Aldrovanda vesiculosa* in Ukraine are the draining of wetlands and water pollution from agriculture (fertilizers, herbicides, wastewaters, etc.).

Neottianthe cucullata has been listed as Endangered on the European Red List according to European regional assessment and EU 27 regional assessment [RANKOU, 2011]. It is a Eurasian species with the total range from the Baltic to Japan, with quite numerous populations in Russia. *N. cucullata* is included to herbal drugs of a traditional Chinese medicine for treating a post-hepatitis syndrome. It is not used, however, in officinal and traditional medicine of Ukraine.

In Ukraine *Neottianthe cucullata* occurs at the southern limits of its total range. Populations of its are few in number and have a pronounced tendency to decrease due to anthropogenic transformation of habitats, primarily wetland drainage, general ecosystem dehumidification, habitat fragmentation, and livestock grazing. It is found only in a few localities in Kiev Region. Therefore, this species is listed as endangered at the national level and included in the Red Data Book of Ukraine [DIDUKH, 2009]. It is known that the trend of its population is decreasing throughout its range due to numerous threats, especially woodland management, causing an increase in the amount of light reaching the forest floor, the use of heavy machinery in forestry operation, and weather conditions [IUCN, 2015].

At the pan Europe level, the status of five species of medicinal plants was identified as Vulnerable: *Chimaphila umbellata*, *Dianthus hypanicus*, *Moehringia hypanica*, *Crambe*

aspera and *Onosma polyphylla.* The current population trend this species is mainly decreasing on assessment to Ukraine due to narrow ecologo-coenotic amplitude and low adaptive properties to a destruction of the ecological balance of the environment.

Chimaphila umbellata have different protection rank in separate countries of Europe [ALLEN & al. 2014]. It is not enough use from wildlife for medicine or pharmacy, but the population of this species is strongly fragmented, have the weak adaptive properties and high sensitivity to changes of the ecological balance of their habitat, which are the main threats to their exhaustion. The population has declined sharply in many countries and on a set of IUCN criteria at the European level currently this species is categorized as Vulnerable A2ac [CHADBURN, 2014].

Chimaphila umbellata in Ukraine grows on the southern boundary of the spread. Therefore, its populations are fragmented, small and few in numbers. The main regions of its distribution are Polissya and Carpathians. Sporadically occur in the forest-steppe and the Crimea. Due to the scattered nature of populations of *Chimaphila umbellata* in Ukraine, there are potential future threats, but these are not considered significant at present. However, a tendency to reduce the number of individuals and populations in all regions as a result of the economic use of forests was identified, so this species is under regional protection in all areas. *Chimaphila umbellata* belongs to the list of species that are not listed in the Red Book of Ukraine, but are permanently or temporarily endangered at the territory of different regions (oblasts) and in the near future can be classified as "endangered" if will be extended the effect of negative factors.

The main biological substances of wintergreen are: flavonoids (quercetin, kaempferol, dihydroquercetin, avikulyarin and giperin), phenolic glycosides, triterpenoids and steroids which have anti-inflammatory effect mainly on organs of the urogenital system. With medical purpose is mainly used in homeopathy and traditional medicine of many countries. The wild plants of *Chimaphila umbellata* for medicinal purposes in Ukraine are used in very rare cases. In order to give a better understanding of its possibility of rehabilitation the further studies of biology, population structure and threats is needed.

Moehringia hypanica is endemic to the Ukraine where it is restricted to the southern part of the Pridnjeprovian upland in the Mykolaiv region. Mostly it grows in the clefts of the rocks on the northern slopes along the rivers. Only five localities are known in a restricted area of the canyons of the Yuzhniy Bug and Mertvovod rivers [DIDUCH, 2009]. This species is listed under Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention), the IUCN Red list of threatened species as Vulnerable D2 [MELNYK, 2013].

The populations are very small with a low number of individuals (50-300 individuals in local populations). It therefore assumed that the whole population counts less than 1,000 individuals and the species is therefore assessed as Vulnerable. *Moehringia hypanica* is included in the Red Book of Ukraine (2009) as Rare narrow local endemic and is protected in the National Nature park "Buskij Gard" and in the protected natural boundary (landmark) "Labirint" (Mykolaivska oblast).

Populations of *M. hypanica* characterized by narrow ecological range, are sensitive to reduce of the moisture of substrate and illumination. *M. hypanica* usually grow in the cracks of granite rocks with a small amount of soil. In dry years a sharp decline in the number of individuals of different age groups, especially on the slopes of the south-eastern exposure was revealed [SOLOMACHA & al. 2006]. Therefore, the prolonged absence of rainfall and high temperatures in summer often lead to die of plants. The main threat is changes of

ecological conditions of habitats; consequently, the current population trend is decreasing. Information about the possibility of cultivation of this species is absent. The state of populations is worsens, therefore continuous monitoring of the number of plants in some localities and control of threats are carried out.

Dianthus hypanicus is a South-Bug endemic, adapted to the growth on granite outcrops and rocks. It is common in the southern part of the Dnieper Upland (the area between the Southern Bug and Ingul).

This species is listed under Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention), the IUCN Red list of threatened species as vulnerable D2 with stable current population trend [MELNYK, 2013], in the Red Data Book of Ukraine [DIDUCH, 2009] as Vulnerable narrow local endemic. In Ukraine is protected in the National Nature Park "Buskij Gard" and at the protected areas of local significance in the Kirovograd and Mykolaiv regions.

The main habitats of *Dianthus hypanicus* are granite and gneiss outcrops, crack rocks (composed of rock plant communities). It is known the local populations where individuals density is near of 60 units per 100 sqm [BARMAK, 2006]. The reducing the number of habitats for this species is due to the construction of hydraulic structures, granite quarrying (mining), range firing, collecting plants for ornamental purposes (collection of flowers and digging of plants) and recreation.

Dianthus hypanicus successfully introduced and it is grown in many botanical gardens of Ukraine and domesticated for decorative purposes by people; under conditions *ex situ* the plants are better blossom and fructify than in the wild [GONCHARUK, 2014]. There are no known information about medicinal use of this species but it potentially can be used as source of saponines [MINARCHENKO, 2005].

The species has no known widespread threats at present, although many researchers [SOLOMACHA & al. 2006; BARMAK, 2006; DIDUCH, 2009; MELNYK, 2013] express some concerns about possible population decrease due to economic, tourism, recreational activities and collection plants from the wild. Its protection is not very secure at the moment and the loss of the protection status could may to lead to a rapid decline number of populations. In Ukraine, there is a successful experience of the reintroduction of this species in nature, so there is hope that it will manage to keep.

Crambe aspera has been assessed as Vulnerable (B2ab (ii, iii, iv) in the European Red List of Threatened Species according to European regional assessment [SMEKALOVA & al. 2013]. It does not occur within the EU 27.

It is an Eurasian species with disjunctive area. In Ukraine is listed in the Red Book as Vulnerable with sporadic distribution in the forest-steppe and steppe zone, including on the Crimean peninsula. It is found in Chersonska, Donecka, Dnipropetrovska, Kharkivska, Luganska, Odeska, Vinnicka regions and in Crimea. There are known more than 20 confirmed localities of *Crambe aspera* with low number (low numerically) of individuals at the territory of Ukraine [DIDUCH, 2009]. The data on population structure of this species is missing; the plants were found single or in separate groups. It may be ultimately affect to their genetic diversity and reduce their viability.

There is a little documented data of medicinal or other use for this species, only known that it contains vitamins, flavonoids and fatty oil [MINARCHENKO, 2005). This species have else potentially value as wild crop relatives. Overall in Ukraine there is a trend decline of populations due to anthropogenic alteration (agriculture, fires of grazing) and

fragmentation of *Crambe aspera* habitats. There is need to establish the current protection range and population status by monitoring and quantify. Order to determine the status of protection of *Crambe aspera* is needed the further study of stress-adaptive properties, the possibility of introduction and reintroduction.

Onosma polyphylla is a Crimeo-Caucasian endemic, the main habitats of which associate with petrophyte communities in the Crimean Mountains [DIDUCH, 2009]. It is listed under Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). The species is classed as Vulnerable B1ab (iii) on European regional assessment and as Not Evaluated on EU 27 regional assessment (http://www.iucnredlist.org/details/165240/1).

In Ukraine listed in the Red Book as Vulnerable with sporadic distribution [DIDUCH, 2009].The increased intensive tourism and recreation may be a threat to its habitats.

In Ukraine are known four species of medicinal plants of the genus *Onosma*, two of which, including *Onosma polyphylla,* was found only in the Crimea. There is little published data on the medicinal properties and use in medicine these plants. It is only known that the main biological active ingredients of these species are naphthoquinones.

The main habitats of this species are confined to a particular spot on rocky slopes and rubbly talus with weakly developed grass cover. Now the clearly estimated threats for this species are not identified. The structure of the populations, the main current threats, as well as the possibility of the introduction have been poorly investigated. To clarify the protection status and the development of measures to restore this species in nature the further monitoring research is necessary.

Conservation status Near Threatened (NT) on both the European and EU 27 levels has nine medicinal plants of Ukraine: *Cypripedium calceolus*, *Drosera anglica*, *Drosera intermedia*, *Galanthus nivalis*, *Gypsophila perfoliata*, *Helichrysum arenarium*, *Iris sibirica*, *Trapa natans* and *Marrubium vulgare. Aster amellus* listed as Near Threatened according to data of EU 27 regional assessment and as Least Concern on European regional assessment. *Himantoglossum caprinum* is considered Near Threatened and in the EU27 member states is assessed as Data Deficient for the European regional assessment [RANKOU, 2011].

This species have different extinction risk; differ in distribution and their potential for adaptation to changing environmental conditions. However, most of them have weak adaptive abilities. These species mostly are associated with the specific conditions of habitat, disturbance of the ecological balance of which can lead to oppression and death of populations. So, the threat of depletion, considering the negative impact of various outside limiting factors, is rather high. Differences between species in such characteristics can be used to develop the measures of their protection.

The use of these species as a medicinal plant for most of these are little, but some of them have high decorative properties and people collect them for bouquets or digging for planting flower beds and gardens.

Cypripedium calceolus are included under the IUCN Red List of Threatened Species as Near Threatened on the European and EU 27 regional assessment [BILZ, 2013]. It listed in Annex B of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). It is also listed on Annex II of the Habitats Directive and under Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). This orchid is included in several national red lists as threatened.

The main threat to this species is collecting which led to major declines in the past and inappropriate forest management, but the current populations trend is assessed as stable.

Cypripedium calceolus belongs to the rare plants of Ukraine, having a considerable distribution at the main part of territory. This orchid is included in national Red Book as vulnerable [DIDUCH, 2009]. It has been well studied as a medicinal plant [MINARCHENKO, 2005], but it is more popular as an ornamental and it is a real threat to the populations of *C. calceolus* in the future, although collection plants are forbidden now by law.

There are known more than 50 sites localized mostly in the north-western regions and the Crimea. Number of individuals are very variable, from a few dozen up to a few hundreds.

The populations are commonly fragmented, sharply declines throughout its range as a result of anthropogenic transformation habitats and little control on the collection of plants for ornamental purposes. The species grows often as separate groups and the populations are decreasing in many locations. The rate of the decline has not been investigated at the whole territory, but many location have not been confirmed in the last decades [DMYTRASH & SHUMSKA, 2014; KUZMISHYNA & al. 2014]. However, it have been revealed some new localities for the species [DAVYDOV & al. 2014; MELNYK & LOGVYNENKO, 2013; TOKARYUK & CHORNEJ, 2009].

It has been introduced in many botanical gardens in Ukraine. Currently management of habitats and population monitoring is carried out.

Three species of genus *Drosera* spp., growing in Ukraine, there are known as medicinal plants: *Drosera anglica*, *Drosera intermedia* and *Drosera rotundifolia* [MINARCHENKO, 2005]. The first two species listed in the Red Book of Ukraine as Vulnerable; according to European regional assessment and EU 27 regional assessment as Near Threatened. *Drosera rotundifolia* has status as a regional protected species and it is classified as Least Concern in the IUCN Red List Threatened Species (Regional assessment) [KHELA, 2012].

This species has been shown by scientific research that is effective when used as an anti-inflammatory drug for the treatment of ailments involving the throat or chest [MINARCHENKO, 2005]. The most common usage of the plants is as a component of the complex homeopatic drugs for the treatment of bronchopulmonary diseases. The collection of wild species of genus *Drosera* for medicinal or other purposes is forbidden by law in Ukraine.

It have very narrow ecological amplitude and increased anthropopressure (mostly wetland amelioration) to the habitat of these species is a serious threat to their existence around the area, all of them are under the legal protection in many countries [KHELA, 2012].

The population data indicate that the populations of *Drosera anglica*, *Drosera intermedia* and *Drosera rotundifolia* are large at the Ukrainian Polissya, on the decline, due to changes in land management. The existing threats, especially dehumification of habitats, may cause the decline of species' populations to severely decline in the near future. Negative trends have been observed for their habitat and number of individuals in populations. However, the further research, conservation measures and monitoring are necessary.

Galanthus nivalis has been estimated as Near Threatened, Vulnerable or even Critically Endangered in several European countries and is included on nearly every country's Red Lists, including Ukraine. A rank of Near Threatened at the global level is suggested here due to all the above factors threatening the population as a whole, and the possibility of *G. nivalis* qualifying for a threat category in the near future (VU A3cd) [CROOK & DAVIS,

2013]. It is listed under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), under Annex B of the EU Wildlife Trade Regulation 318-2008 and on Annex V of the EC Habitats Directive 92/43.

Biologically active compounds of *G. nivalis* were studied enough, but the use in medicine is limited by the toxicity of compounds [MINARCHENKO, 2005]. The plant is very popular, primarily, due to its decorative properties. Major threats for this species are illegal collection of plants for decorative purposes, habitat loss due to forestry measures, infringement of the ecological balance of the habitats as a result of agricultural activities (soil eutrophication), as well as tourism.

In Ukraine, this species is a significant spread over most of the territory. The number of individuals in the populations is high, but there is a tendency of reduction of number of flowering individuals as a result of picking its flowers for bouquets, digging out bulbs and habitat destruction [DIDUCH, 2009]. The plant was successfully introduced in many botanical gardens and nature reserves. Further measures for the protection and repatriation of species in natural habitats are necessary.

Gypsophila perfoliata is considered to be Near Threatened in the EU27 and Europe as a whole [CHADBURN, 2014]. Several research have of the opinion that further surveys are needed to confirm the current area of occupancy and monitoring is necessary to detect and enumerate declines or indeed any extension of range, if the spread of this species is enabled by man-made communication corridors in the form of railway line habitat [ALLEN & al. 2014].

In Ukraine this species occurs sporadically along the coast of the Black and Azov Seas. There are typical littoral species, occupying the halophytic-meadow ecotops. It is considered as an adventive species of a technogenic ecotopes [GLUKHOV & al. 2012]. The structure and state of its populations have been little studied. The threats to this species are unknown currently and there are no conservation measures in place.

Further research is needed to give a better understanding of its biology, regenerative capacity, population dynamics, threats and adaptation to changing environmental conditions. Further surveys are needed to establish the current population size of this species and monitoring is needed to detect and enumerate declines. Such information may help to establish a need for protection early on.

Helichrysum arenarium listed on the IUCN Red List of Threatened species as Near Threatened approaching criterion A2cd on both the European and EU 27 levels due to although widespread across Europe, it is widely collected and in many places is considered as rare and threatened plant [KHELA, 2013]. However, it is observed that more specific information on the current population size, trend and the overall rate of decline is needed, especially from countries in Eastern Europe, to verify whether the species needs to be included in a higher threat category.

Helichrysum arenarium has a large area of occupancy in Ukraine, occurs in a wide range of habitats and the populations are assumed to be more or less stable in whole. Its natural resources are large. A few tons of raw materials (inflorescence) for pharmaceutical purposes are harvested every year. Managements the volume of harvesting wild plants is carried out at the local (regional) level by setting limits. The limits are set on the basis of the resource assessment in each area. In addition, this medicinal plant is cultivated in plant-specialty agricultural community. It is known some of cultivars with a high content of biologically active compounds. The threats to this species are unknown, but in Ternopilska and Odeska oblast it listed in some regional Red Lists.

There has been a slight increase of the resources of this species at Polissya in recent decades due to an exclusion of sandy arable fields from the agricultural use. Some subpopulations cover an area of tens hectares with an estimated coverage through to 10%. The trend and the overall rate of decline are unavowed at present time. Further research may to verify whether needs to be included in define a threatened category this species.

It is an old medicinal herb used mainly to treat of digestive system, biliary dyskinesia and other liver diseases. There are known nine pharmaceuticals drugs from the *H. arenarium* in Ukraine (State register of Medicinal Products of Ukraine).

Iris sibirica is classified on both EU27 and Europe levels as Near Threatened approaching criteria A2ce due, as it could likely have declined in many countries across its range [KHELA, 2013].

In Ukraine, the protection status of *Iris sibirica* is estimated as Vulnerable and it include in the Red Book as a rare species resided on the southern border of area [DIDUCH, 2009]. It has a significant distribution mainly in the northern and north-western part of the territory. The overall population decline has not been quantified, but the reduction of the area of local populations and reducing the number of generative individuals in many localities was noted [PODOROJNY, 2013].

This species is threatened by habitat loss and degradation caused by wetland amelioration, habitat transformation and fragmentation, pasture load and wild plant collection. Although it is protected on state level in Ukraine, it is facing many threats and is decreasing across much of its range. The rehabilitation of its habitat is highly unlikely. Furthermore, the species has highly fragmented distribution, it is therefore necessary to collect the detailed data about structure and dynamic of population from each locality for development of protection measures.

This is an ornamental and medicinal plant, but there is few information on its use [MINARCHENKO, 2005]. *Iris sibirica* have been successfully introduced in many botanical gardens of Ukraine, which allows to make an estimate of possibilities of its repatriation (reintroduction) in protected areas.

Trapa natans listed as Near Threatened according to data of EU 27 and European regional assessment due to declining everywhere throughout its range [LANSDOWN, 2013]. *Trapa natans* is included under Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention).

It is considered as Uncertain on the Red Book of Ukraine. There are known many locations of this species on the plain territory of Ukraine, but many of them are not supported by the latest studies. In separate water bodies it forms large populations at an area of several hectares with a number of individuals 1,5-2,5 thousand per 100 m^2 [DIDUCH, 2009]. The major treats to the populations of *Trapa natans* in Ukraine are: wetland amelioration, habitat transformation (especially industrial and agriculture pollution of waterbodies). It has been lost from many sites in the Ukraine through habitat loss due to contamination of water bodies.

The data about its use for medical purposes is little, but seeds have a high nutritional value [MINARCHENKO, 2005]. Successfully propagated in a culture, it has been introduced in many botanical gardens and protected in the reserves.

Marrubium vulgare is listed as Near Threatened approaching criteria A2cd on EU 27 and European regional assessment, because it is showing strong declines in at least a quarter of its European range, due primarily to changes in land use practices in the agricultural and pastoral sectors [KHELA, 2013].

In Ukraine, this species is sporadically distributed throughout the flat territory, but its natural resources are negligible due to of the use of its habitats for agricultural purposes (plowing land and pastures). Therefore *M. vulgare* was successfully domesticated and now the selective breeding programs are developed. This plant is in extensive cultivation in home plots as an aromatic plant.

The species is harvested as an aromatic plant and for medicinal purposes. It has been well studied as a medicinal plant, however it is a mild remedy that is little used in modern medicine (phytotherapy), but it is sometimes employed by traditional medicine in treating of the chronic bronchitis [MINARCHENKO, 2005]. Also used in homeopathy.

It is not protected in Ukraine and currently does not face any major threats. The most effective measure to protect it can be cultivation and reintroduction into natural habitats.

Aster amellus has a wide distribution in Forest-Steppe some of Ukraine, but the area of suitable habitat is limited due to use it on agriculture purposes (mostly as grazing) and today the species has become rarer. It is not included on the Ukrainian Red Book, but is under regional protection in some oblasts, as the number of sites where is found is decreasing and the number of individuals are decline over the last decades.

Aster amellus classed as Near Threatened according to data of EU 27 regional assessment and as Least Concern on European regional assessment [KHELA, 2013]. Across Europe, *Aster amellus* is assessed as Least Concern as it occupies a wider range of habitats and there are few documented declines across its southeastern and eastern range. This species has been listed on many national red lists in Europe, but protection rank is differ in separate countries.

The loss of habitats is a significant threat to this species; the lesser danger is the wild collection of plants for ornamental and medicinal purposes. In order to estimate the trend and rate of decline of populations in Ukraine there is a need for further information on the threats to population in the concrete localities. More studies are needed to monitor the decline of the *Aster amellus* on the central regions of Ukraine.

Himantoglossum caprinum is classed as Near Threatened according to data of EU 27 regional assessment and as Data Deficient on European regional assessment [RANKOU, 2011]. It is also listed on Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention); on Annex II of the Habitats Directive. All orchids are included under Annex B of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

In Ukraine the *H. caprinum* is considered as Vulnerable and listed in Red Book [DIDUCH, 2009]. It is endemic species, occasionally founded in Mountain Crimea and the southern coast of Crimea. The main threats are: afforestation and forestry maintenance, habitat dehumidification and fragmentation, recreation, tourism and collection. The underground part of the plant (bulbs) are harvested to prepare salep.

In a protected area the number of individuals and populations increase, so under the conditions of protection the population is stable, but there is an overall decline. The further monitoring and repatriation in ecologically optimal habitats are necessary.

Besides the groups of threatened species described above, the largest number of species (81%) has the status Least Concern. This species are characterized by different distribution in Ukraine, threats and adaptation possibilities to change of environment. Therefore, they have different protection status. For example, *Colchicum autumnale*, *Dictamnus albus*, *Gentiana punctata* s.l., *Gladiolus italicus*, *Leucojum vernum*, *Lilium martagon*, *Narcissus poeticus*, *Pulsatilla pratensis* are suffer through excessive use as

ornamental plants as well as through a violation of the environment. To *Adonis vernalis*, *Allium ursinum*, *Glycyrrhiza glabra*, *Rhodiola rosea*, *Scopolia carniolica* the collection for medicinal purposes and habitat destruction are threatens. A raw materials of many species, as *Betula humilis*, *Dactylorhiza incarnata*, *Huperzia selago*, *Nymphoides peltata*, *Iris sibirica* almost never used for medicinal purposes but the violations of ecological balance of their habitat leads to irreversible changes in the structure of populations, depression of resources and their gradual loss. The populations of all listed above species are decreasing in Ukraine and it are included in the Red Data Book [DIDUCH, 2009].

Some of the medicinal plants from the category LC are protected in Ukraine at the regional level, since their resources in certain areas or throughout are limited. For example, *Arctostaphylos uva-ursi*, *Centaurium erythraea*, *Centaurium pulchellum*, *Digitalis grandiflora*, *Ephedra distachya*, *Helleborus purpurascens*, *Lycopodium clavatum*, *Veronica officinalis* [Official …, 2012]. For these species a number of common features are peculiar: a small number of individuals in the population, negative reaction to the change of habitat, weak competitive and adaptive properties. Therefore, the harvesting of its resources can lead to a total depletion of resources and extinction. Some of them are protected only in certain areas (administrative units-oblast), where there is a threat of depletion of their populations, and in other regions they can be still harvested; for example, *Alnus incana*, *Convallaria majalis*, *Persicaria bistorta*, *Potentilla erecta*, *Sanguisorba officinalis*, *Vaccinium myrtillus*, *Veronica officinalis*, *Viburnum opulus* etc.

Several species from the category LC are popular medicinal herbs in Ukraine, their resources is large and raw materials for pharmaceutical purposes or phytotherapie used from environment. There are: *Alnus glutinosa*, *Artemisia absinthium*, *Bidens tripartita*, *Cichorium intybus*, *Equisetum arvense*, *Hypericum perforatum*, *Matricaria chamomilla*, *Mentha longifolia*, *Origanum vulgare*, *Plantago major*, *Symphytum officinale*, *Taraxacum officinale*, *Thymus serpyllum*, *Tussilago farfara* and others. The threats to their populations are small or not identified (unknown) and the current population trend are mostly stable**.** The use of resources shall be regulated by legislation at the local or national levels.

As a whole for most of the medicinal plants with the protection status Least Concern the current threats caused to reduction of population is undefined.

Special emphasis is known needs to be placed on Data Deficient species, especially as some are suspected to be in a critical state of decline at the national level in some parts of the EU and within pan Europe, but the lack of information from across the whole range or part of the range of these species meant that a threat category could not be assigned [ALLEN & al. 2014]. These species was recommended to further research across the region.

Among the analyzed medicinal plants only 23 species are assessed as Data Deficient. They have different protection status in Ukraine: nine species included in the Red Book, six are regionally rare; eight species there is insufficient data to determine it protected status. For example, *Paeonia tenuifolia* is considered as vulnerable due to steppe loss as the result of tilling, grazing and collecting for ornamental and medicinal purposes. This species, as has been founded, have a very high vulnerability to disturbance of habitats and low recoverability [DIDUCH, 2009].

Allium albidum, *Allium obliquum*, according to the data of Red Book, have been classified as endangered species; *Diplotaxis cretacea*, *Silene cretacea*, *Saxifraga hirculus*, *Prunus klokovii* – vulnerable; *Crambe koktebelica* – rare [DIDUCH, 2009]. *Hyssopus cretaceous* is classed as unknown due to the lack of reliable information that remains to be determined it protection status. Common characteristics of these species are their local or

limited distribution, weak adaptive capacity to change of the environment and lack of land for optimal growth and reproduction.

Glechoma hirsuta, *Beckmannia eruciformis*, *Crataegus pentagyna* are classified as Regional protected species due to their restricted range (distribution), but have stable populations and no major threats. The current population trend of *Iris pumila*, *Prunus fruticosa*, *Sisymbrium confertum* is decrease for a variety of reasons. *Iris pumila* has a widespread distribution; however it is vulnerable to collecting (digging) for landscape gardening, grazing, trampling and igniting of dry grass. The species are known to be endangered locally by habitat transformation and collection of the plant. *Prunus fruticosa* is sensitive to agricultural activities. *Sisymbrium confertum* has quite restricted distribution and is undergoing some local declines. Described above species are not considered to be of particularly important for medicinal use.

Lepidium syvaschicum, *Malus sylvestris*, *Potamogeton compresus*, *Pyrus elaeagrifolia*, *Rosa villosa*, *Rorippa brachycarpa*, *Vitex agnus-castus* are characterized as sporadically distributed species with stable trend of populations and the lack of known significant threats.

Armoracia macrocarpa is discribed in several countries as rare or very rare and is nationally threatened or Near Threatened throughout its range [STEVANOVIĆ & al. 2013]. It is currently assessed as Data Deficient on IUCN Red List due to the lack of reliable information that remains to be determined it protection status. The genus *Armoracia* is listed in Annex I of the International Treaty on Plant Genetic Resources for Food and Agriculture as part of the *Brassica* spp. complex and *A. macrocarpa* is listed in Annex I of the Convention on the conservation of European wildlife and natural habitats (Bern Convention). Currently it is unprotected at the state or regional level in Ukraine, as the data of its distribution, population structure and threats are being elaborated and an assessment is made.

In recent years, interest to *A. macrocarpa* significantly increased due to its rarity and reduction of habitats across human transformation of ecotopes and prospects for use in breeding practice as potential gene donor to cultivars.

Armoracia macrocarpa generally is rare throughout of the Ukrainian part of the valley of Danube - from the border (Reni district, Odessa region) to the sea. Most plants grow near streams or in their area of influence. It is known in the lower reaches of the Danube Kiliya it occurs in coastal areas of the islands. Also it is listed for Tisa river basin. The overall decline of *A. macrocarpa* at Ukrainian part of area is mainly attributed to secondary succession of grassland in the results of general ecosystem dehumidification; it is also threatened by afforestation, abandonment of livestock grazing on pastures and agricultural use of the habitat.

Proposals for inclusion of this species in the next edition of the Red Book of Ukraine are developed and monitoring of populations in the wild is carried out.

Threatened species of medicinal plants listed on Red Data Book of Ukraine

Red Data Book of Ukraine is the main state document that generalizes the current state of rare and endangered species under which development of scientific and practical measures for their protection, restoration and sustainable use is done. The harvesting of wild medicinal plant in Ukraine governed by national legislation and the collection of species listed in the Red Book of Ukraine and species under regional protection is prohibited.

There are 63 threatened species of medicinal plants in Ukraine, which are no listed in the IUCN Red List, but have national protection status in Ukraine and included in Red Book (2009). There are mainly relict species on the boundary of the spread or local endemics, its populations are highly fragmented and small with a few numbers of individuals. The factors, limiting their vitality are both natural (mainly weak adaptive properties) and various anthropogenic. The main threats to this species are habitats alteration (incl. dehumidification), fragmentation and loss, as well as forestry, pasture and other agricultural activities. Moreover, the illegal collecting of plants for ornamental or/and medicinal purposes is also one of the main threats for threatened species as *Astragalus dasyanthus*, *Campanula carpatica*, *Lycopodium annotinum*, *Crocus speciosus*, *Crocus banaticus*, *Daphne cneorum*, *Delphinium elatum*, *Gladiolus imbricatus*, *Pulsatilla pratensis*, *Salix myrtilloides*, *Tamarix gracilis*, *Tulipa schrenkii* and all species from family Orchidaceae, which are protected at the state level in Ukraine.

For many medicinal plant currently highly threatened by direct or indirect human activities, which lead to transformation and loss of habitat. It has been identified as the main causes of decline in medicinal plants populations. Any further significant impact of limiting factors listed above on populations of assessed plants lead to increase their stress levels and habitat loss therefore threatens their survival, according to the assessment.

The threats for 193 species, listed on European Red List, are not sufficiently important or unknown in order to assign of any protection category in Ukraine. The current trend of it population may be estimated mainly as a stable or increasing; for example: *Daucus carota*, *Equisetum arvense*, *Heracleum mantegazzianum*, *Lepidium ruderale*, *Papaver rhoeas*, *Persicaria maculosa*, *Rumex alpinus*, *Sambucus ebulus*, *Sambucus nigra*, *Urtica dioica*, *Verbascum densiflorum*, *Verbascum thapsus* and other. Many of them have large resources, which may be harvested from wild.

Among of analyzed threatened medicinal plants, more than half (281 sp., 52%) were assessed as having a decreasing population trend in Ukraine, whilst for Europe it is equivalent near to 20%. The population trend near of 44% Ukrainian species (234 sp.) considered as stable and it customary for 56% assessed species in Europe. The state of only 3.7% medicinal species of Ukraine may be assessed as increasing.

Conclusions

Most of medicinal plants, which are listed in the IUCN Red List of Threatened Species (version 3.1), have limited distribution and resources in Ukraine. Their populations are decreased, fragmented and depleted as a result of irreversible changes of its habitat. The population trend near 44% accessed species considered as stable. Many of them are widespread species, their populations are not very sensitive to changes of the ecological balance of their habitats, and therefore, threats to their loss have not been identified at present. The state only of 3.7% of medicinal plants populations, accessed as threatened on Global and/or European level, was estimated as increasing in Ukraine. Their resources are large and harvested, or, perhaps, will be use in future from wild.

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MAXENT MODELLING OF THE POTENTIAL DISTRIBUTION OF *GANODERMA LUCIDUM* IN NORTH-EASTERN REGION OF ROMANIA

Ovidiu COPOȚ[1*], Cătălin TĂNASE[2]

Abstract: *Ganoderma lucidum* is one of the most valued mushrooms in the World, because of its medicinal properties. In the context of North-Eastern Region's development, any forest product could have a valuable contribution. Therefore, it is important to understand the mushroom's ecology and generate a map of its optimal distribution. For this, we used one of the most performant species distribution models available – Maxent, field occurrences and climatic-topographic-biotic variables. After multi-collinearity testing and step-wise Maxent modelling, we came to an end with a 0.8 final model based on two predictors. Thus, in the region, the optimal habitat distribution is found in oak, beech, riparian or mixed forests bellow approximately 800 m altitude. The species can be found in almost all forests across lowland, colline and submontane regions according to tree host presence. The approach could be promising for other fungal species for the sustainable development of the region.

Keywords: *Ganoderma lucidum*, Maxent, Romania, species distribution modelling, suitable habitat.

Introduction

Ganoderma lucidum is one of the most known medicinal fungus in the World, being used for its immunomodulatory, anti-inflammatory, antiviral, antioxidative, antiaging and antitumor properties [BARBIERI & al. 2017].

Ganoderma lucidum (Photo 1) belongs to the cosmopolitan genus *Ganoderma* [RYVARDEN, 1991], Ganodermataceae family of Polyporales order, within Agaricomycetes class of Basidiomycota phylum [Index Fungorum 2016, http://www.indexfungorum.org/]. The sporocarp is perennial, having the cap and the stem inseparable. The kidney-shaped cap can reach up to 20 cm in diameter. Its colour is red to reddish brown when mature, but has bright yellow and white zones toward the margin. It has whitish zone of pores which becomes dingy brownish in age. The stem grows side to cap, rarely in a central position and can reach 12 cm in length and 3 cm thick. The spore print is brown. The spores have 8-11 x 6-8.5 µm. Setae and cystidia are absent and the hyphal system is dimitic [SĂLĂGEANU & SĂLĂGEANU, 1985; TĂNASE & MITITIUC, 2001; TĂNASE & al. 2009]. *G. lucidum* is found all over the World, growing on multiples hosts [BARBIERI & al. 2017]. In Europe, it grows on living trees or stumps of oaks or chestnuts, rarely on coniferous trees [BERNICCHIA, 2005; GERHARDT, 1999; EYSSARTIER & ROUX, 2013; SĂLĂGEANU & SĂLĂGEANU, 1985].

[1] Alexandru Ioan Cuza University from Iași, "Anastasie Fătu" Botanical Garden, 7-9 Dumbrava Roșie, 700487, Iași – Romania.
[2] Alexandru Ioan Cuza University from Iași, Faculty of Biology, Department of Biology, 20A Carol I, 700505, Iași – Romania.
[*] Corresponding author. E-mail: copot_ovidiu2008@yahoo.com

Listed as the most appreciated superior tonics in Chinese traditional medicine, *G. lucidum* was used also for its benefit effects on preventing aging, tonifing the heart and improving memory [NAHATA, 2013].

Ganoderma lucidum is rich in antioxidant components that are absorbed quickly after ingestion, it has been observed that the total antioxidant activity in the blood plasma is increasing in human subjects [HAPUARACHCHI & al. 2016]. Therefore, the production of oxygen free radicals decreased, helping anti-aging process. Also, linghzi's polysacharides are known for their antibacterial, antifungal and antiviral activities. As some researchers found, Gram-positive and Gram-negative bacteria development are inhibited by some compounds from *Ganoderma lucidum* [HAPUARACHCHI & al. 2016; KAMBLE & al. 2010; VAZIRIAN & al. 2014]. Bacteria like *Escherichia coli*, *Helicobacter pylori*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium*, *Mycobacterium tuberculosis* and fungi like *Aspergillus flavus*, *Candida albicans* – common pathogens in humans, were found to react negatively to *G. lucidum* extracts [HAPUARACHCHI & al. 2016; NWANNEKA & al. 2011]. Also, plant pathogens like *Botrytis cinerea*, *Alternaria alternata*, *Aspergillus niger*, *Fusarium oxysporum* were inhibited by *Ganoderma lucidum* extracts [BAIG & al. 2015; HAPUARACHCHI & al. 2016].

Photo 1. *Ganoderma lucidum* on: (**a**) oak roots (Coverca Forest, Neamț County, 2016) and (**b**) oak stump (Heltiu Forest, Bacău County, 2016).

Because of its great medicinal value, there is much interest for this mushroom – the annual global turnover exceeds 2.5 billion dollars [LI & al. 2013], the vast majority being cultivated. But as TAKSHAK & al. (2017) pointed, *G. lucidum* collected from the wild can prove also as a good diet supplement, due to its high contents of nutrients. Therefore, it is essential to understand the ecology and to delineate the optimal habitat in order to use this mushroom as a valuable wild forest product. The importance of wild fungi can be therefore defined through their development perspective [BOA, 2004], which can also be applied to this species. It is also imperative for paying also attention to protect it from excessive collecting, as other wild medicinal mushroom populations declined in some parts of the World [YUAN & al. 2015].

For this, we analyzed the species-environment relationship through species distribution modelling or SDM. This tool uses species observations and environmental properties to generate predictions across different types of environments [ELITH & LEATHWICK, 2009].

One of the best prediction accuracies in spatial distribution analyses is given by Maxent or maximum entropy model, developed by PHILLIPS & al. (2006). It is a self-contained Java application which uses occurrence records and environmental variables for the study area [PHILLIPS & al. 2017]. Therefore it belong to non-parametric statistical group of methods, being different from other methods such as regression methods or profile methods. The method used by Maxent is about finding the distribution which has the maximum entropy [PHILLIPS & al. 2017]. It was chosen in this study because it outperforms other distribution models at small spatial record numbers [VAN GILS & al. 2012].

Materials and methods

The study area is North-Eastern Region in Romania. We chose this surface area in order to provide an insight of the one of the World's most valuable wild product in terms of medicinal applications [MONEY & al. 2016] in one of the poorest development regions in European Union [MAXIM, 2014].

We collected the data for the species occurrences in field surveys deployed in 2015-2017. Their distribution in region's space covers all counties and all broadleaved dominant or codominant types of forests (oak, beech, riparian and mixed forests). There were registered 18 occurrences of the species.

For the environmental layers, we used nineteen bioclimatic variables and altitude data downloaded from WorldClim database [HIJMANS & al. 2005] at finest resolution. For the topographic layers we used altitude and derived topographic variables (aspect - in cardinal directions classes - and slope) calculated in Quantum GIS Development Software 2.18. As the plant species has great influences on lignicolous fungi, and fungal species in general [KUTSEGI & al. 2015; SHI & al. 2013], we added a vegetation layer figuring the most common types of forests across the region. For this, we used the ICAS Forest Types Map (1997).

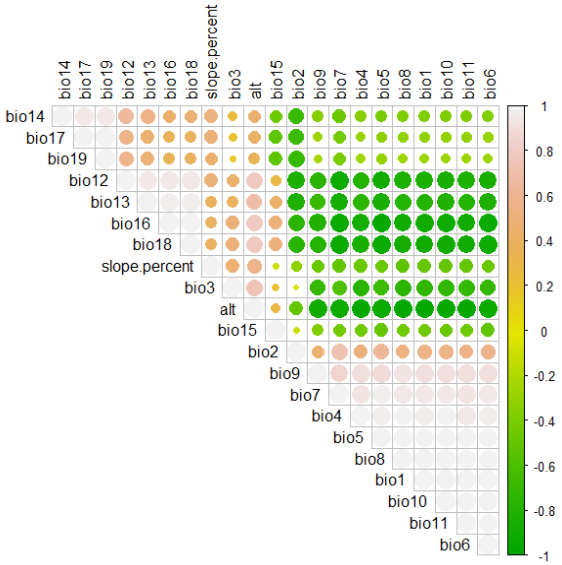

Fig. 1. Correlogram result of multi-collinearity test showing that elevation, bio15, bio19 and slope are not correlated at a 0.7 threshold.

Because multi-collinearity is a serious statistical problem in species distribution modelling and that of the large data set of environmental layers, from which some are highly correlated [CRUZ-CÁRDENAS & al. 2014], we tested the predictors using pair-wise correlation with the Spearman's rho coefficient at 0.7 threshold [MAGHIAR & STOICA, 2016].

After applying the multi-collinearity test, 4 predictors resulted as uncorrelated (Fig. 1): elevation, BIO15 (precipitation seasonality) and BIO19 (precipitation of the coldest quarter), slope (%). Those, together with qualitative variables (aspect and vegetation type) were used as predictors in Maxent modelling.

For modelling we used a machine-learning method for presence-only point data analysis called Maxent. The model estimates the probability distribution for a species in a geographic space using the maximum entropy algorithm based on species occurrences and environmental layers, known as constraints [PHILLIPS & al. 2017]. Having a high prediction accuracy [PHILLIPS & DUDÍK, 2008], it has been used for different purposes throughout researches, from conservation planning and exploring expanding distributions of invasive species to diversity predictions and endemism patterns [ELITH & al. 2010].

Using random selection 10,000 background points were generated according to geographic coordinates. The full dataset was k-fold partitioned into 75% training and 25% testing dataset.

In order to estimate the performance of the model, we used Area Under the Receiving Operator Curve (AUC). It takes values from 0 (lowest performance with random prediction) to 1 (best performance with perfectly fit prediction), [QIN & al. 2017]. A number of 10 runs were set for model building. In order to improve the model, we removed the least contributing predictors (shown by the Jackknife test) until AUC reached the minimum value of 0.8 [VAN GILS & al. 2012]. From those models, the model with highest AUC value was considered as best performer [YANG & al. 2013; YUAN & al. 2015].

For variable importance for each of the step-wise model, we used the Jackknife procedure. This test calculates the highest gain when the variable is used in isolation and the most decreasing gain when the variable is omitted. Furthermore, we removed the variables that contributed less than 5% to model accuracy gain [MOREHOUSE & TOBLER, 2013].

Fig. 2. The Jackknife test of final Maxent model for evaluating the relative importance of environmental variables for *Ganoderma lucidum* in North-Eastern Romania (''veg'' is vegetation type, ''alt'' is elevation)

In the final distribution map, we regrouped the values in four classes of habitat suitability: a. optimum habitat (>0.6); b. suitable habitat (0.4–0.6); c. least suitable habitat (0.2-0.4); d. unsuitable habitat (<0.2), as proposed by QIN & al. (2017). We also obtained a species optimal distribution, as a binary map delineated by 0.6 value [QIN & al. 2017].

Finally, we used the forest vegetation layer to filter out areas without forests as these do not support a suitable environment for wild populations of *G. lucidum*.

All the statistical analyses and Maxent modelling were done in the program R [R Development Core Team, 2009]. For Maxent modelling we used *dismo* package within R software.

Results and discussion

The final Maxent model provided a good accuracy, with a testing AUC value of 0.807 (Tab. 1). There is little difference from the first model (0.766) because the variables that contributed to the first and were omitted from the last had little contribution (12.2%). An interesting insight offers the aspect (in classes) which maintains a good contribution over the first 3 models but backwards under 1% in the first over 0.8 AUC model. Even if the final model have a lesser testing AUC than the 4th model, the only variables that contributes over 5% to model accuracy are altitude and vegetation type. From the first model, the percent contribution of elevation, respectively vegetation type are increasing with approximately same amount ~12%, respectively 15%, which is the approximately amount of the other non-semnificative predictors.

Tab. 1. Model's performance indicator AUC and predictor contribution in step-wise Jackknife test, run for the highest testing AUC model

model	training AUC	testing AUC	vegetation contribution (%)	elevation contribution (%)	other variables contribution (cumulated, %)	only > 5% variables contribution
1st (6 variables)	0.848	0.766	61.2	26.6	12.2	aspect 9.6%
2nd (5 variables)	0.848	0.766	61.2	26.5	12.3	aspect 9.7%
3rd (4 variables)	0.845	0.763	63.3	26.4	10.3	aspect 9.9%
4th (3 variables)	0.819	0.857	70.3	29.2	0.5 (aspect only)	-
final (2 variables)	0.836	0.807	70.6	29.4	0 (no other variable)	-

Vegetation type and elevation were part of the final model. The most contributive predictor, both as isolate and as omitted is vegetation type, confirming the strong relations existing between trees and the fungal species.

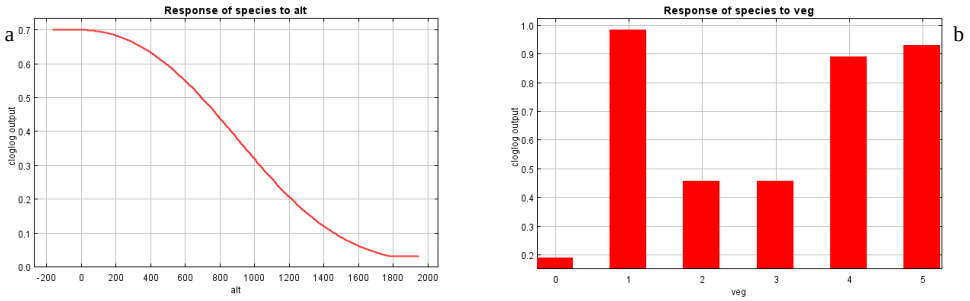

Fig. 3. Maxent taxa response curves shape for elevation (**a**) and for vegetation type (**b**); Y axis: Probability of presence; X axis: (**a**) Altitude (m) and (**b**) Vegetation type: "0" is no forest vegetation, "1" is other forests, "2" is mixed forests, "3" is coniferous forests, "4" is oak forests, "5" is beech forests.

The response curves of presence probability to predictors (Fig. 2) shows a typical response and a specific one. As the elevation grows, the presence of *Ganoderma lucidum* is decreasing in probability, thus showing a classic example of elevation-dependent taxa (Fig. 4). The curve shows a small decreasing trend at the beginning because of the association of oak and lowland riparian forests with low elevations (under 500 m). Then, the curve presents an accelerated downward trend because of the forest shift from oak to beech and mixed forests. At the end of the response curve, the trend decelerated because of the low/no-presence probability of *G. lucidum* in coniferous forests.

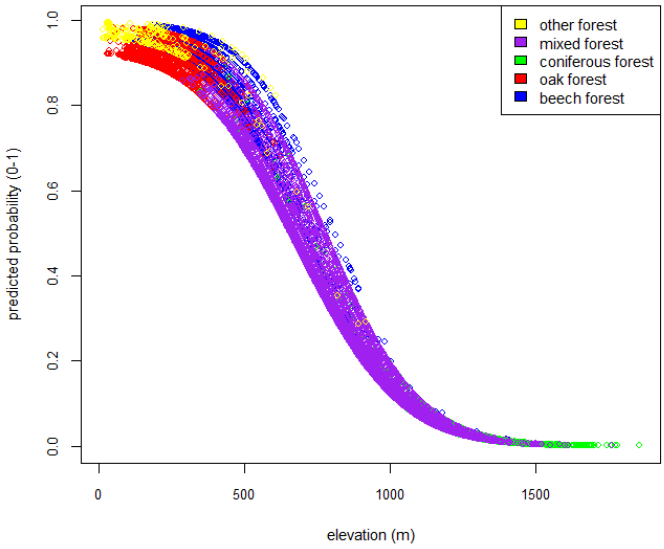

Fig. 4. The relation between *G. lucidum* predicted probability and elevation (m). The points are colored according to their forest vegetation type (5 forest classes) association.

Because of the correlation with important ecological variables, like mean annual temperature (MAT) and mean annual precipitations (MAP), this geographic distribution is

also correlated with those predictors. As the temperature grows and precipitations decreases, the chance to find *G. lucidum* in the respective location also grows. The environmental space defined by MAT and MAP (Fig. 5) shows that the most suitable conditions for finding *G. lucidum* (over 60% presence probability) are places within the lowest third of MAP difference and within the highest third of MAT difference.

MAT and MAP have been found as predictors for white rot decomposers diversity at global scale [TEDERSOO & al. 2014]. The affinity of *G. lucidum* for warmer areas is confirmed by Ellenberg indicator values for MAT: 6 which corresponds to an intermediate class between fairly warm and warmth conditions, from submontane to colline and lowland sites [SIMMEL & al. 2017].

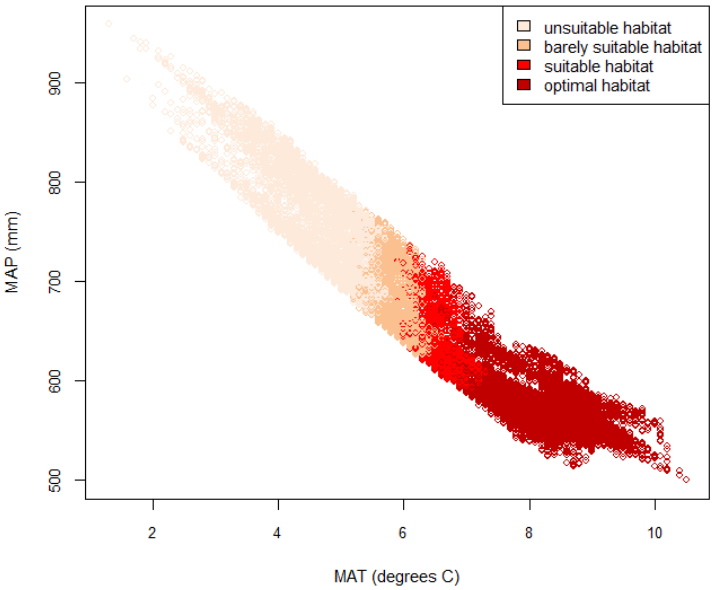

Fig. 5. A two-dimensional perspective of *Ganoderma lucidum* environmental space, defined by mean annual temperature (MAT) and mean annual precipitations (MAP). Red areas represent optimal habitat of the species according to Maxent prediction probabilities thresholded at 0.6.

This can be related to the spatial distribution of the most important predictor: forest vegetation type (Fig. 2).

The optimal suitability according to forest vegetation surfaces is divided among forest types (Fig. 6). The highest probability of presence (in terms of associated pixels) is in "other forests" class, dominated by riparian forests (100%). It is followed by oak dominated forests (99.2%) and beech dominated forests (94.2%). The least relative surfaces with optimal habitat belongs to coniferous forests (under 2%). Mixed forests takes approximately 15% of the optimal habitat, because of the elevation limitation.

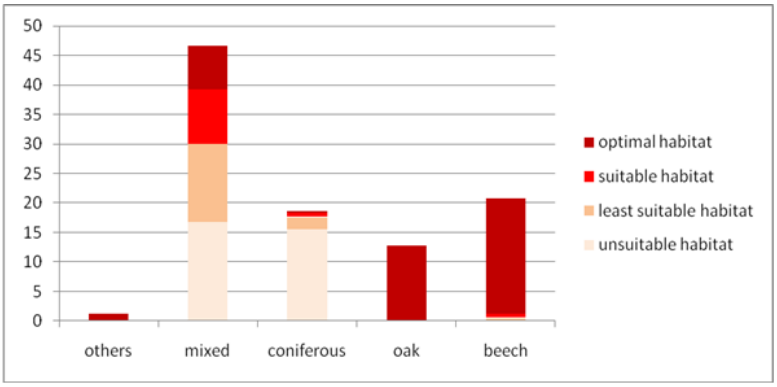

Fig. 6. Forested area (% to total) suitable for *Ganoderma lucidum* in North-Eastern Romania. Beech and oak forests represents the vast majority of optimal habitat.

The final model shows a widespread potential distribution across North-Eastern Region of Romania (Fig. 7). From an economical perspective, rural communities from all counties can access this resource.

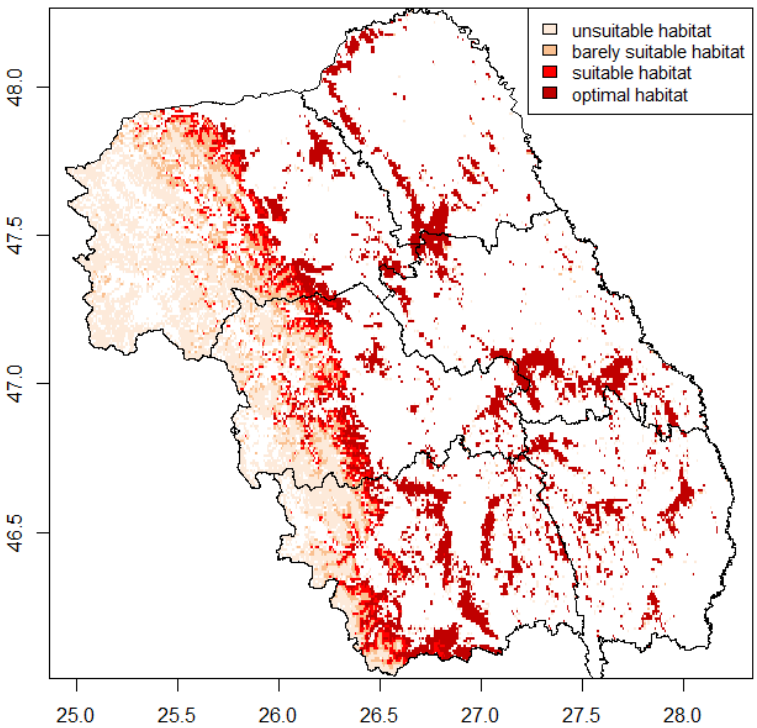

Fig. 7. *Ganoderma lucidum* habitat suitability map according to Maxent presence probability prediction.

As the mushroom's presence is highly predicted by the forest type, especially oak and beech ones, it is enough for the local people to engage in gathering this resource. A more complicated situation is in the Carpathians, where the suitability of the habitat is departing among different levels of elevation, in the presence of broadleaved forests.

The second purpose of this paper was not only to predict areas with *G. lucidum* for financial collecting but also for its protection. Over-collecting could affect wild *G. lucidum* populations. Therefore it is essential to find robust conservation measures that can be applied for effective conservation management.

Conclusions

The potential distribution in geographical space of *Ganoderma lucidum* was accurately predicted through Maxent modelling, having a 0.806 testing AUC. By applying multiple predictor filtering, overfitting was removed and non-important predictors were not included in the final model. The species distribution was influenced by the variation of elevation, as in other classical environmental-species relationship. But the most important predictor was the forest vegetation type, *G. lucidum* having the optimal habitat in oak and beech forests. Those forests have a good covering across North-Eastern Romania, therefore the mushroom can be efficiently exploited through sustainable development.

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DISPERSAL OF *RAFFLESIA PATMA* BLUME ENDOPHYTE IN GRAFTED HOST PLANT (*TETRASTIGMA LEUCOSTAPHYLUM* (DENNST.) ALSTON)

Adhityo WICAKSONO[1], Jaime A. TEIXEIRA DA SILVA[2], Sofi MURSIDAWATI[3]

Abstract: This study provides a hypothetical discussion about the growth of *Rafflesia patma* Blume (syn. *R. horsfieldii* R. Br. (1821); Rafflesiaceae), an endophytic parasite, within a grafted host, a woody vine (*Tetrastigma leucostaphylum* (Dennst.) Alston). Based on the observation of a 10-year old (2007 to 2017) *R. patma – T. leucostaphylum* graft, we hypothesize that *R. patma* moves away from its host rather than remaining in an anchored position from which it flowers, although flower knobs that emerge later may vary in range from the grafting point, i.e., flower knobs may develop close to or far away from the grafting point. Our provisional macroscopic observations point towards a gradual creeping motion of the endophyte towards new host tissues, flowering opportunistically when sufficient nutrients have been found. Much has yet to be discovered about the growth and flowering behavior of *R. patma* and about the dynamics of the *R. patma – T. leucostaphylum* interaction.

Keywords: endophyte, parasitic plant, Rafflesiaceae, *Tetrastigma*, vine.

Introduction

Rafflesiaceae, which are the most minimalistic plants on the planet, having no leaves or roots, grow as an endophytic parasite inside a host, of the genus *Tetrastigma*. There are 12 recorded *Tetrastigma* species (or synonymous taxa) that are capable of hosting *Rafflesia*: *T. leucostaphylum* (Dennst.) Alston. (syn. *T. lanceolarium* (Roxb.) Planch.), *T. loheri* Gagnep., *T. papillosum* (Blume) Planch., *T. trifoliatum* Merr., *T. scortechinii* (King) Gagnep., *T. scariosum* (Miq.) Planch. (syn. *T. pisicarpum* (Miq.) Planch.), *T. coriaceum* (DC.) Gagnep. (syn. *T. tuberculatum* Latiff), *T. diepenhorstii* Miq., *T. quadrangulum* Gagnep. & Craib, *T. scortechinii* (King) Gagnep., *T. curtisii* (Ridl.) Suesseng., and *T. glabratum* (Blume) Planch. [NAIS, 2001; CHEN & al. 2011; MURSIDAWATI & IRAWATI, 2017]. As *Rafflesia* grow within the host woody vine, they are invisible until they reach the reproductive stage when flower bud or knob emerged through the surface layer of the host [WICAKSONO & al. 2016].

There are currently two hypotheses about how the endophyte is able to grow inside the host vine of *Tetrastigma* (Fig. 1). NIKOLOV & al. (2014) claimed that the endophyte grows radially towards the center of the stem in the xylem area. Personal communication with Nikolov in 2017 revealed that since it is very difficult to observe the transverse cross-sectional morphology of the endophyte by microtomy preparation (i.e., for microscopic observation) compared to radial cross-sections, it is difficult to determine whether *Rafflesia*

[1] Laboratory of Plant Breeding and Genetics, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Agro, Bulaksumur, Yogyakarta 55281 – Indonesia. E-mail: adhityo.wicaksono@gmail.com

[2] P.O. Box 7, Miki-cho post office, Ikenobe 3011-2, Kagawa-ken, 761-0799 – Japan. E-mail: jaimetex@yahoo.com

[3] Center for Plant Conservation, Bogor Botanical Garden – Indonesian Institute of Sciences (LIPI), Jl. Ir. Juanda no. 13, Bogor 16003 – Indonesia. E-mail: sofi_mdawati@gmail.com

grows within a vine. Nikolov hypothesized that *Rafflesia* grows in one certain place and does not traverse the graft. BARKMAN & al. (2017) revealed that for the *Rafflesia* endophyte, the host body serves as an "island" where it grows and creeps inside the host body rather than remaining anchored in the exact same place.

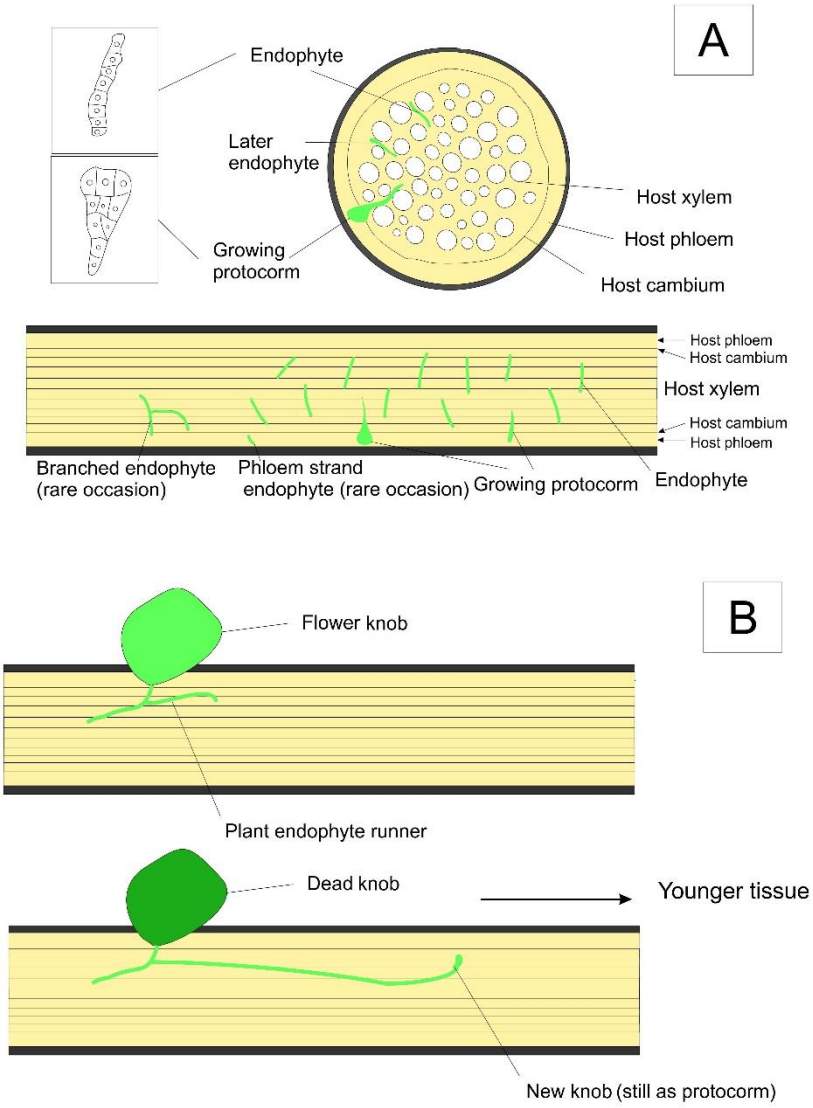

Fig. 1. Two hypotheses that currently describe the growth and distribution of *Rafflesia* endophytes: Anchored endophyte (A) (figure was inspired by NIKOLOV & al. (2014) and depicts a hypothetical growth form following personal discussion with Nikolov); a creeping endophyte as suggested by observations (this study) of a 10-year-old *Rafflesia patma* Blume (syn. *R. horsfieldii* R. Br. (1821)) – *Tetrastigma leucostaphylum* (Dennst.) Alston graft (B).

In this study, *Rafflesia patma* Blume (syn. *R. horsfieldii* R. Br. (1821)) was transplanted by veneer grafting [MURSIDAWATI & al. 2015] in 2007 using a scion from Pangandaran, West Java, Indonesia, and a rootstock from an uninfected host in Bogor Botanical Garden. *R. patma* first bloomed in 2012. The host was identified as *T. leucostaphylum*.

Since 2007, the original grafting spot has disappeared and has become covered by the convoluted stem of *T. leucostaphylum* vines. We made an approximated grafting site and pinned it as the center point of the distance measurement (Fig. 2). The distance of the knobs from the center point, diameter, and age of the knobs were measured (Tab. 1).

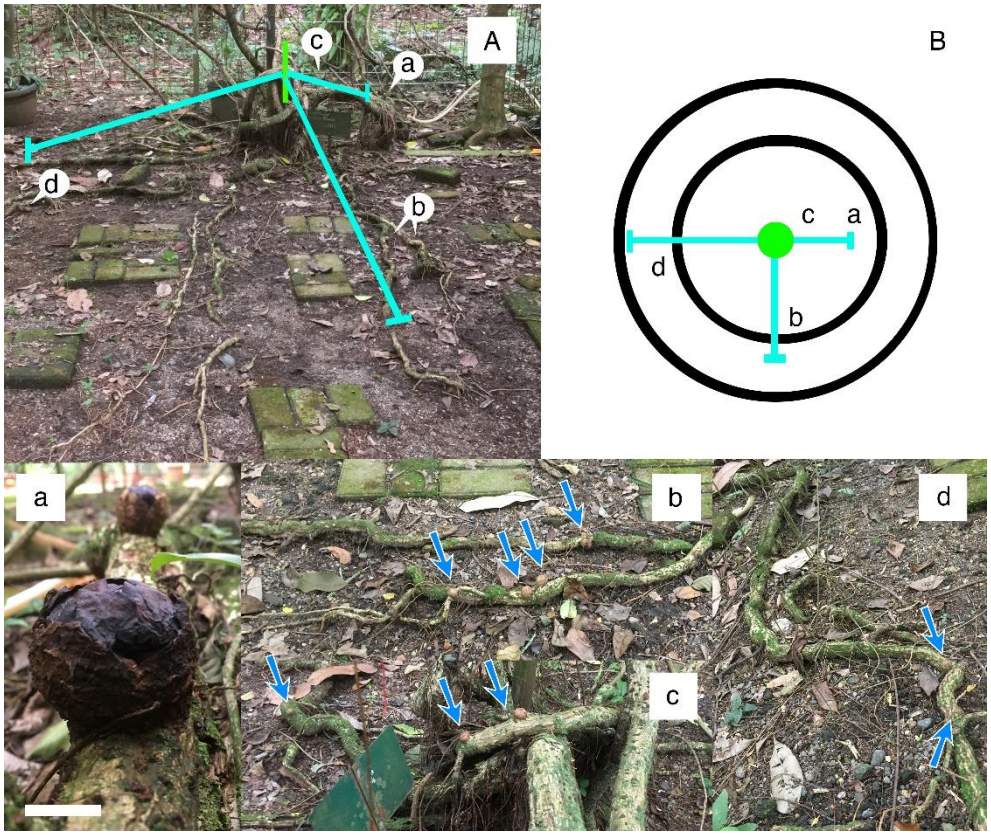

Fig. 2. Measurements of the host vine, *Tetrastigma leucostaphylum*, in Bogor Botanical Garden, Indonesia (A). Top view showing the center point (green) and distance (cyan) covered by the endophytic parasite, *Rafflesia patma* Blume (B). Spots in A have been enlarged in a-d for easier visualization. Flower primordial knobs (enlarged image in a) of *R. patma* are shown by blue arrows. Scale bar (in a): 2 cm.

Tab. 1. *Rafflesia patma* Blume (syn. *R. horsfieldii* R. Br. (1821)) flower knob distribution on/along the host vine (*Tetrastigma leucostaphylum* (Dennst.) Alston).

Year	Distance from grafting area (cm)	Flower/knob diameter (cm)	Knob age (months)	Notes
2017	26.5	2	3	
	27.5	1.8	3	
	62	2	3	
	142	3.5	5	
	163	1.7	3	
	167	4.5	6	
	177	2.5	3	
	182	1.7	2	
	188	3	3	
	191	2	3	
	203	1.2	3	
2015	3	44*	>12	Female flower
2014	58	38*	>12	Female flower
	53	44*	>12	Male flower
	95	34*	>12	Female flower, 6 perigones
2013	31	34*	>12	Female flower
2012	58	37*	>12	Female flower
	34	38*	>12	Female flower

Note: Numbers denoted by an asterisk (*) indicate a fully-grown flower rather than a knob (i.e., flower bud)

We observed that *R. patma* within a grafted host (*T. leucostaphylum*) grew away from its old original point in the transferred (i.e., infected) root into a healthy rootstock and stem of *T. leucostaphylum*, leaving behind the old and decayed host tissue. The growth of *R. patma* was shown to be distributed in several directions within *T. leucostaphylum* vines from its original point (Tab. 1, Fig. 2). This movement, which suggests active growth rather than passive sedentary placement and subsequent flowering, might indicate that *Rafflesia*, as an endophyte, in fact creeps inside its host, possibly to obtain more nutrients. HEIDE-JØRGENSEN (2008) stated, about *R. kerrii* Meijer, that flowers often appear on the younger part of *Tetrastigma*, suggesting that the endophyte grows away from older part before the tissue dies. Variation of the distance of flower knob formation in Table 1 also shows that new flowers do not always sprout far away from the grafting point, but might also sprout close to it. This either shows that the flower might grow from an already existing endophyte or from the reversed direction of endophyte growth (Fig. 3). All of these hypotheses related to the growth of the endophyte, as well as flower knob formation, will require detailed miscroscopic and even genetic studies to prove if the same endophyte is responsible for the formation of knobs close to and distant to the grafting point.

This paper provides distribution data for *R. patma* knobs growing on grafted *T. leucostaphylum* vines, suggests that the endophyte moves away in an active process from the point of origin, unlike current model that suggests sedentary growth, including flowering.

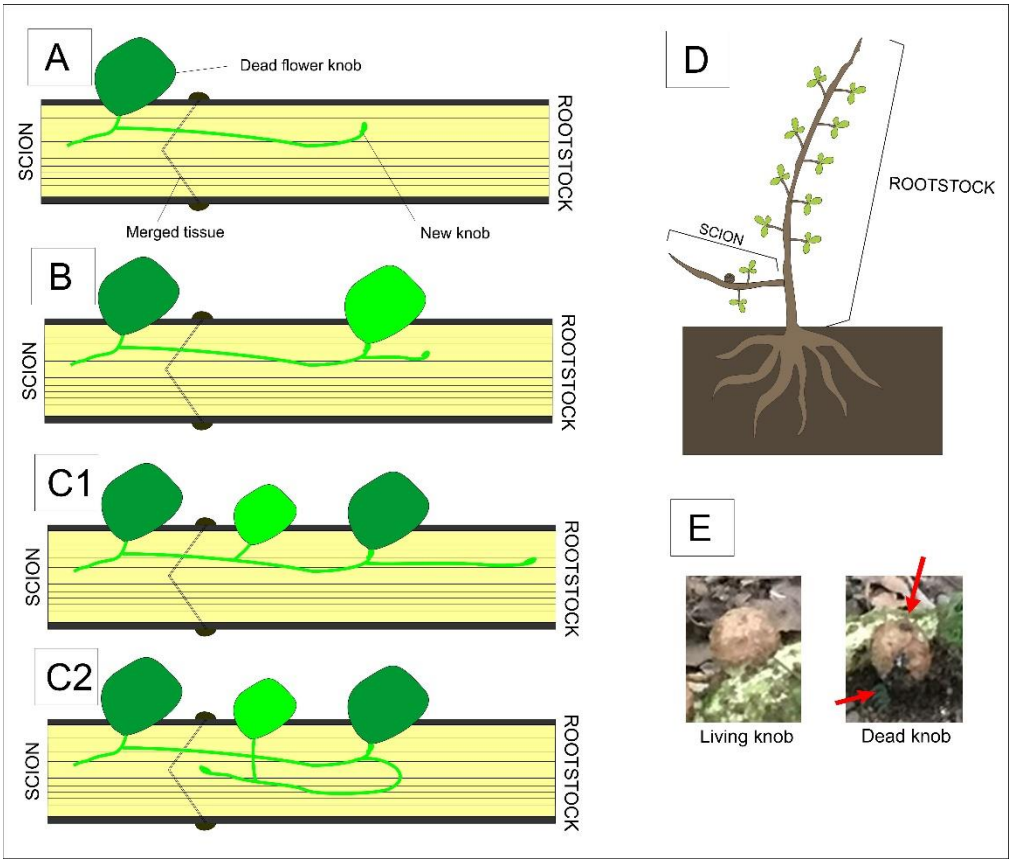

Fig. 3. Hypothetical growth and development of the *Rafflesia patma* Blume flower knob on its grafted host (*Tetrastigma leucostaphylum* (Dennst.) Alston) showing that the endophyte (green) grows towards the rootstock tissue (A) and develops a new flower knob (B). At a later stage, at some moment in time a new flower knob grows closer to the grafting site rather than growing away. Two possible scenarios are depicted: A new flower knob emerges from a previously established endophyte (C1), or the growing point of an endophyte turns back and forms a new flower knob (C2). The picture (D) shows a simplified illustration of a scion and rootstock in the grafted host plant, and (E) shows a living or newly emerged flower knob (left) and an aborted or dead knob (right) with red arrows pointing to the blotched and blackened dead tissues.

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PROFESSOR PhD MIHAI MITITIUC AT HIS 80TH ANNIVERSARY

Mihai MITITIUC was born on March 16th 1937 in Sinăuții de Jos, Bucovina (today Mihăileni, Botoșani County) as son of Rosalia and Ștefan Mititiuc. He attended the local school from Mihăileni and in 1955 he graduated the No. 1 Boys Middle School (today "Grigore Ghica Vodă" High school).

Immediately after the high school graduation he was enrolled as substitute teacher at Hărnava School and one year later he was accepted at the Faculty of Natural Sciences-Geography, "Alexandru Ioan Cuza" University of Iași. As student of this institution he proved seriousness and tenacity, dedication and rigor, qualities through which he was remarked throughout all 4 years of faculty and which recommended him as valedictorian. In 1960 he passes the *State Exam* and obtains the title of academic diplomat. In the same year he becomes Assistant at the Faculty of Natural Sciences-Geography within the Botany Department, for *Plant Morphology and Anatomy* and *Phytopathology* courses. In 1964 he becomes a PhD student at the Faculty of Biology, University of Bucharest, under the coordination of Professor PhD Olga Săvulescu and in 1968 he obtained his *PhD in Biology* with the thesis *Contributions to the knowledge of micromycetes and macromycetes from Ponoare and Frumoasa-Suceava natural reserves*.

He followed an academic career within the Faculty of Biology, "Alexandru Ioan Cuza" University of Iași as lecturer (1969-1990), associate professor (1990-1992) and professor (1992-2007) for several courses, such as: *Biogeography*, *Phytopatogens biology*, *Plant systematic*, *Phytopathology*, *Mycology* and *Plant protection*. Starting from 1991 he became a PhD coordinator in the field of *Botany*, training more than 20 PhD students.

He publishes over the years, as single author or co-author more than 300 scientific articles in Romanian and foreign journals, more than 23 volumes and monographs in the field of *Phytopathology* and *Mycology* and participates at numerous symposia and scientific sessions with valuable oral communications and conferences. His most recent book published in 2015 is entitled *Ciupercile genului Phyllosticta* and represents a thorough analysis of the fungal species belonging *Phyllosticta* genus regarding their morphology, pathogenesis and economical importance.

Throughout his career, **Mihai MITITIUC** discovers 35 new for science species of fungi, more than 430 new species for Romania and over 900 fungal species mentioned on new hosts.

He decisively contributed to the development of the *Herbarium* from the Faculty of Biology, "Alexandru Ioan Cuza" University of Iași, by handing in 2731 herbarium sheets that include micromycetes, macromycetes and vascular plants.

His passion for Botany and Mycology, but also his organizational and managerial skills were put to good use during his time as director of "Anastasie Fătu" Botanical Garden of Iași (1990-2007), period in which 3 new greenhouse compartments were built, the heating system was renewed and several functional spaces were rearranged.

He was also a member in numerous committees and boards, work groups and scientific societies: president of the Romanian Mycological Society (1995-2007), president of the Botanical Gardens Association from Romania (2004-2008), member in the editorial board of *Analele Științifice ale Universității „Alexandru Ioan Cuza" din Iași, seria Biologie Vegetală* and editor of *Buletinul Grădinii Botanice Iași*.

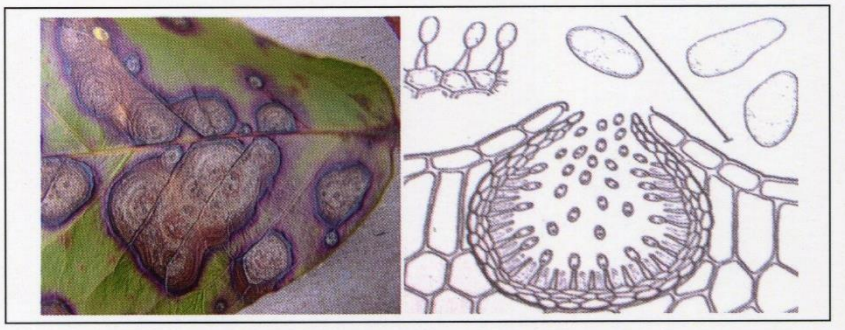
With this special occasion, on behalf of "Anastasie Fătu" Botanical Garden staff we wish **Professor PhD. Mihai MITITIUC** many healthy years, joy and fulfillments, ability to work and all the best!

HAPPY ANNIVERSARY!

Ana COJOCARIU, Cristiana Virginia PETRE, Cătălin TĂNASE

MITITIUC MIHAI

CIUPERCILE GENULUI
PHYLLOSTICTA



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DR. GAVRIL AUREL NEGREAN AT HIS 85TH ANNIVERSARY

On 30th of August 2017 we celebrated the 85th anniversary of Dr. Gavril Aurel Negrean, a remarkable personality of Romanian botany, with a great scientific career. It was an honor for the entire community of botanists but not only to highlight his diverse and very prolific activity.

The great botanist was born in 30th of August 1932 in Dindeşti village from Satu-Mare County. After he graduates the primary school in the natal village, follows the high-school courses in Oradea and Carei. In 1964 graduates the Faculty of Biology in Bucharest University. For the next five years works in "Louis Pasteur" Institute investigating some avian viruses. Recommended by the great botanist Constantin Zahariadi he works since 1970 in Institute of Biology of the Romanian Academy, first as biologist and then as scientific researcher. Since 2000 he collaborates with "Dimitrie Brandza" Botanical Garden of Bucharest University.

In these many years he significantly contributed to the knowledge of Romanian flora and mycobiota, identifying new taxa for the country, enriching the Mycological Herbarium of the Institute of Biology, thus making it the greatest collection of this type in Central Europe and working in numerous national and international projects etc. He dedicated his career to the study of Romanian flora and mycobiota, with an accentuated focus on endemic, threatened, vulnerable and rare species. For many of the rare as well as for some ambiguous species he clarified their taxonomy and completed their distribution. He found new taxa for Romania and studied almost all protected areas in the country, proposing the foundation of over 20 new ones. Moreover, Dr. Gavril Aurel Negrean begun and for the most part succeeded in an ambitious activity: to gather and publish the whole Romanian botanic bibliography.

Dr. Gavril Aurel Negrean is the author of numerous scientific articles and books in which he systematized the results of his long and prolific work. He published many articles in taxonomy, flora, mycobiota, phytosociology, sozology, plant ecology, nature conservation and biography of great personalities in biology. One of the most important is the *Red Book of Vascular Plants in Romania* (2009), published in collaboration with Dr. Gheorghe Dihoru. This red book presents for the first time, in a micro-monographic mode, the most threatened

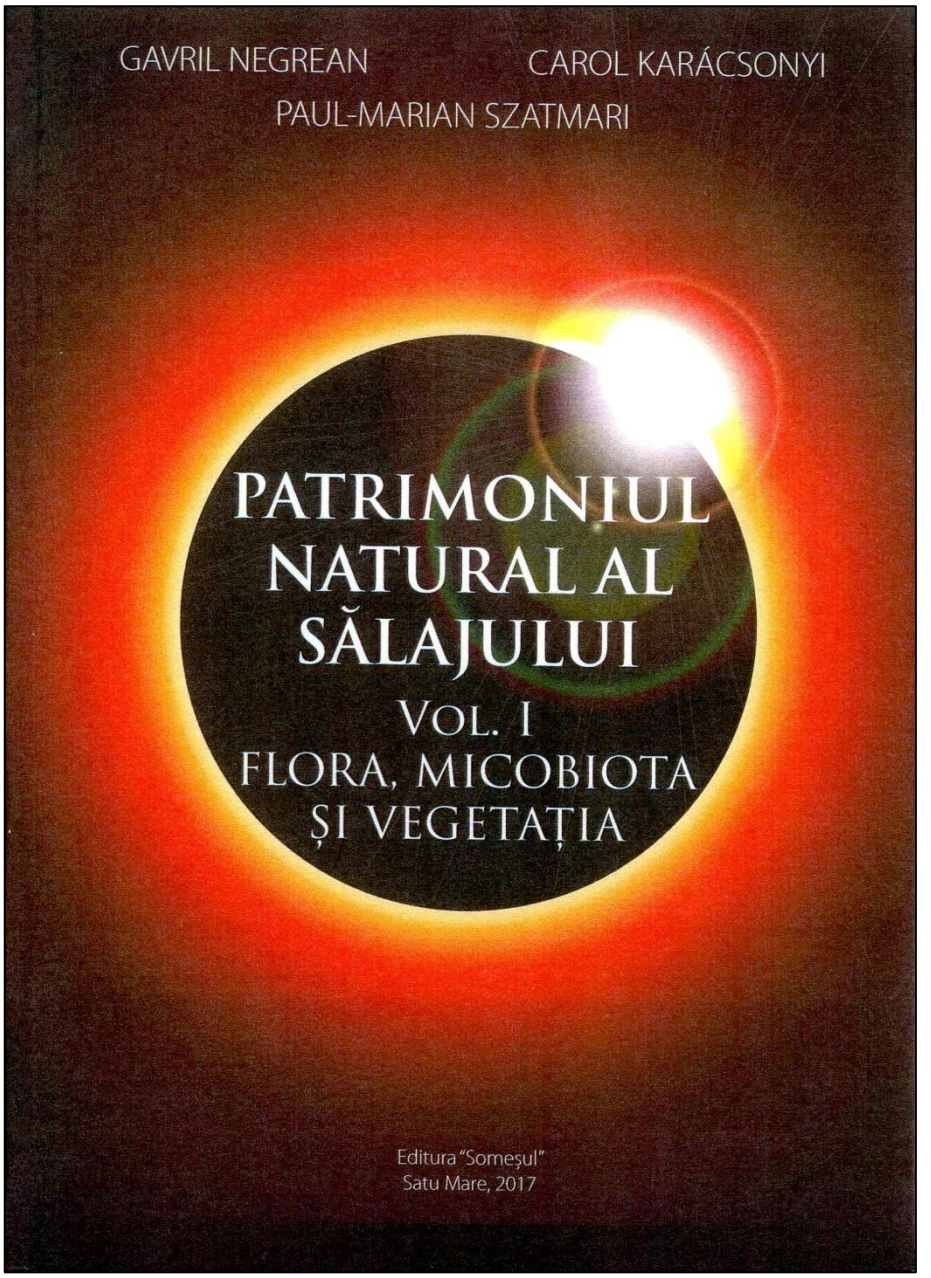
and vulnerable plant species in our country with very comprehensive information about their taxonomy, chorology, biology, areal, habitat, restrictive factors and conservation measures. This book was distinguished with the Romanian Academy Prize (2011). The results of five years of researches of plant and fungi species and also of plant communities from the north – western region of Romania were published in first volume the great monography "*The natural patrimony of Salaj*" (a collaboration with Carol Karácsonyi și Paul-Marian Szatmari) including 601 species of fungi, 139 bryophytes, 2050 cormophytes and 166 plant communities.

His scientific competence is widely recognized and consequently, he is a valuable collaborator in *Atlas Florae Europaeae* and scientific reviewer for *Acta Horti Botanici Bucurestiensis*, *Biológia* (Szombathely) and *Kanitzia* (Bratislava) journals. Also is member in Nature Monuments Committee within Romanian Academy, Micological Society of Romania, Biological Sciences Society from Romania, Association of Botanical Gardens in Romania etc. It is not surprising that for his overall activity and contribution to Romanian botany Dr. Gavril Aurel Negrean was awarded with the *Linnaeus Medal* granted by Planta Europa (2007).

His passion for plants, fungi and nature, his strictness and critic spirit determined the respect and admiration of his friends, colleagues and younger botanists. Therefore, on the behalf of all colleagues in "Anastasie Fătu" Botanical Garden from Iași, we wish Dr. Gavril Aurel Negrean great health, energy to continue his extraordinary research work and all the best for many years to come!

Happy Anniversary!

Constantin MARDARI, Cătălin TĂNASE


GAVRIL NEGREAN
CAROL KARÁCSONYI
PAUL-MARIAN SZATMARI
PATRIMONIUL NATURAL AL SĂLAJULUI
VOL. I
FLORA, MICOBIOTA ȘI VEGETAȚIA
Editura "Someșul"
Satu Mare, 2017

THE 70TH ANNIVERSARY OF THE BESSARABIAN MYCOLOGIST ŞTEFAN MANIC

Ştefan Manic was born on March, 22, 1947, in Stolniceni, district Hînceşti, a village located at the edge of the forest. He inherited, from his parents, the love for nature, which later made him become a student at the Faculty of Biology and Chemistry of Tiraspol State Pedagogical University (1965).

Since the early years as a student, he had a particular interest in research and was invited to participate in expeditions to collect paleobotanical material, organized by the assistants of the Department of Botany, the now late academician Andrei Negru and dr. hab. Ana Ştefârţă. Together, they gathered a rich collection of fossil plants, which served as a basis for further research on Sarmatian flora from around the village Bursuc (district Camenca). The results were presented in two monographs of great value. After graduating from university, he worked for a year as a biology and chemistry teacher at school, in his native village, then, for another year, he served in the army.

In 1972, he started working in the Flora and Geobotany Laboratory of the Botanical Garden of the Academy of Sciences of Moldova (ASM). Between 1972 and 1976, together with his colleagues Andrei Negru and Ana Ştefârţă, Mr. Manic undertook numerous expeditions to collect palaeobotanical material, at that time he specialized in researching fossil wood. In 1976, Ştefan Manic published his first scientific paper entitled: "The Remains of *Glyptostrobus* Endl. (Taxodiaceae) in the Pontic Sediments from the South of Ukraine". In the same year, the Flora and Geobotany Laboratory was reorganized, and Mr. Manic was transferred in the study group that researched lower plants and the theme of his doctoral thesis was approved – "Pileated Fungi in Moldova" – a new direction in the study of fungi.

At the Botanical Institute of St. Petersburg, one of the best equipped and most prestigious scientific centres, in the first two years of research, from 1977 to 1978, he inventoried about 150 species of macromycetes, included in the paper "Mushroom Composition of Agarics in the Central Part of Moldova". With this paper, he participated in the contest for young scientists and won the 2nd prize, awarded by the Presidium of the Academy of Sciences of Moldova.

Under the supervision of the famous mycologist, Boris Vasilcov, Ştefan Manic completed the doctoral theses and defended it in 1982, becoming PhD in biology. In the doctoral thesis, he presented about 300 new species of macromycetes, found on the territory of Moldova. As a result of the field research, he has gathered an impressive herbarium of macromycetes, which lists more than 2500 specimens, which today is kept in the Botanical Garden.

Along with the scientific work in the Flora and Geobotany Laboratory, Ştefan Manic actively participated in the construction of the Botanical Garden and, together with his

colleagues from the laboratory, took part in the creation of the exhibition sector "Flora of Moldova", based on the principles of typological classification of forests from Moldova.

In 1990, by contest, he became director of "Codrii" Scientific Reserve, where he worked until 2014. From the very beginning of his activity as a manager, Stefan Manic faced acute organizational and scientific problems, as well as problems with the staff. However, having a rich experience, gained at the Botanical Garden, Mr. Manic solved these problems – supported the scientific training of four PhD students and a *dr. habilitat* in biological sciences, who successfully defended their theses.

As director of "Codrii" Scientific Reserve, Mr. Manic, in 1991-1995, with the help of the collaborators of the Botanical Garden of ASM, initiated and conducted a floristic research on (secondary) inventory, determining the precise floristic composition of plant communities in the reserve. Besides, an inventory of flora and fauna in the strictly protected area was carried out for the first time. In order to study the dynamics of development of the vegetation of the main types of forest of the reserve, a network of stationary terrains was organized.

It is noteworthy that every 5 years since 1996, were organized in the Codrii reserve four symposiums entitled *"Codrii" Scientific Reserve: Achievements, Problems and Prospects*. They were followed by the publication of collected articles, totalling over 400 scientific papers.

The director of the reserve always paid a lot of attention to the Museum of Nature, which was modernized and completed with new exhibits from the reserve, and in 2005 launched a new concept of its organization and obtained the status of National Museum. He founded a collection of botanized plants found in the reserve, permanently completed with new exsiccate.

As an outstanding manager, Mr. Manic, at a time when computerization was progressing at a rapid pace, realized how important the knowledge of the staff in the field of information technology was. Thus, he equipped the offices of the institution with 10 computers. The botany and zoology workshops were re-equipped with everything necessary to run meetings, scientific conferences and practical work with pupils and students. He also made possible to open, in the reserve, a canteen and a hotel for visitors. As a result of these organizational activities, "Codrii" Scientific Reserve collaborates with other institutions from the Republic of Moldova and, annually, tens of students do their practical work and prepare their theses.

By the will of fate, at the end of 2013, Mr. Ștefan Manic returned to the Botanical Garden (I) of the ASM, to the Laboratory of Spontaneous Flora and Herbarium, where his scientific career had started.

The theme, addressed by the author in the past 5 years, focuses on the taxonomic diversity of macromycetes, and has been researched for more than three decades (1976-2016), in all its aspects, in order to develop a sustainable and flexible strategy for its effective and rational use and a more effective conservation of the gene pool of the biosphere.

He managed to carry out a generalization of the research on macromycetes and, in the summer of 2015, defended his habilitation thesis in biology, on the topic "Macromycetes in the Ecosystems of the Republic of Moldova". As a result, the taxonomic composition of macromycetes in the Republic of Moldova has been determined; it includes 836 taxa belonging to 227 genera, 81 families, 26 orders, 8 classes, 3 phyla and 2 kingdoms: Fungi and Protozoa. The biological and ecological characteristics of the identified species have been established.

The conducted study reflects the current state of the mycobiota of the Republic of Moldova and makes it possible to develop recommendations for the optimization of biodiversity conservation in this territory. For the 3rd edition of the Red Book, 15 species of macromycetes, of different categories of rarity, have been proposed and accepted by the National Committee for the Red Book. It is still necessary that the competent authorities ("Moldsilva" Agency and the Ministry of Environment) adopt measures to protect the 15, more or less endangered, species of macromycetes, as well as the edible species, by limiting the quantities allowed to be collected.

The results, obtained by the author, greatly enrich the knowledge about the biological diversity of the territory of the Republic of Moldova and represent a significant contribution to the assessment and the identification of fungal taxa, besides they are a starting point for future research.

In the future, the results of the research from the above-mentioned publications will supplement *Flora of Bessarabia* with a separate volume about the world of fungi.

Mr. Manic is the author of more than 60 scientific papers, including 9 monographs. He is the President of the Society of Mycologists and Botanists from the Republic of Moldova. For his prodigious work on the research and the conservation of biodiversity, Mr. Manic was awarded: 2nd Prize, for young scientists, of the Academy of Sciences of Moldova; Jubilee Medal "The 60th Anniversary of the Academy of Sciences of Moldova"; Honorary Diploma of the Academy of Sciences of Moldova; the Medal "Civic Merit"; Honorary Title "Emeritus Man of Science"; "Dimitrie Cantemir" Medal of the ASM; Prizes of the ASM "Ilie Untilă" and "Boris Melnic".

On the occasion of the anniversary, we wish you, Mr. Manic, good health and new scientific achievements!

Valentina CANTEMIR, Cătălin TĂNASE

JOURNAL OF PLANT DEVELOPMENT
GUIDE TO AUTHORS

***Types of contributions*:** Original research papers, as well as short communications. Review articles will be published following invitation or by the suggestion of authors. "Journal of Plant Development" also publishes book reviews, as well as conference reports.

Submission of a paper implies that it has not been published previously (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.

Authors are requested to submit their original paper and figures in digital format, to the Editor-in-Chief. The corresponding author should be indicated with an asterisk.

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The papers will be published only in a foreign language, structured as follows: title (the title would be also in the romanian language, if it is possible for the authors), authors, affiliation of the authors (including e-mails), abstract, keywords, introduction, material and method, results & discussions, conclusions, acknowledgements, references.

Titles would be written with bold, capital letters, 12 points, centered.

Names of the authors will be written with Times New Roman, 10 points, centered, capitals for surname (family name) and no capitals for first name (except initial letter). The names will not be abbreviated; 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.

Abstract: A concise and factual abstract is required (about 100-150 words). The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. 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.

Units: The SI system should be used for all scientific and laboratory data. In certain instances, it might be necessary to quote other units. These should be added in parentheses. Temperatures should be given in degrees Celsius.
The main text would be written at a single space, on A4 format page, Times New Roman, of 10 points.

The scientific names of taxa would be italicized.

Tables should be numbered consecutively in accordance with their appearance in the text and given suitable captions. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

Illustrations: photographs, charts and diagrams are all to be referred to as "Figure(s)", should be numbered consecutively in accordance with their appearance in the text. The mentions at the drawings, figures, pictures and tables will be placed inside the round brackets – for instance (Fig. 2); (Tab. 2); all illustrations should be clearly marked with the figure number and the author's name.

Obs.: all the schemes, drawings, etc. would be accompanied by a scale; the pictures must be very clear, being accompanied by the explanations. The diagrams should be made in Excel; pictures, ink drawings must be saved in JPG, JPEG, or BMP format, having a good resolution.

Other than the cover page, every page of the manuscript, including the title page, references, tables etc. should be numbered; however, no reference should be made in the text to page numbers.

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For **scientific papers**: the name of the author (s) would be given in capital letters. The Christian name(s) would be abbreviated. Before the last but one and the last author you must insert the sign "&". In the reference list you must mention all the authors of a certain paper.
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Title: it should be fully written. The title of a book is written in italics. Between the year and the title we recommend to be inserted a dot sign. Next to it is the town and the publishing house of it (for books) or the periodical for papers. For periodicals, the abbreviations would be according to the international standards (BRIDSON & SMITH, 1991 or BROWN & STRATTON (eds), 1963-1965). Each periodical name is to be written in italics. A certain volume must be given in bolds. After it is placed the number of the issue, inserted between the round brackets; next to it would be inserted the page numbers of the paper.

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Examples of papers quotation:

References for papers in periodicals:

CIOCÂRLAN V. 2008. *Lathyrus linifolius* (Reichard) Bässler in the Romanian flora. *J. Plant Develop.* **15**: 3-6.

MEHREGAN I. & KADEREIT J. W. 2008. Taxonomic revision of *Cousinia* sect. *Cynaroideae* (*Asteraceae*, *Cardueae*). *Willdenowia*. **38**(2): 293-362.

References for books:

BOŞCAIU N. 1971. *Flora şi Vegetaţia Munţilor Ţarcu, Godeanu şi Cernei*. Bucureşti: Edit. Acad. Române, 494 pp.

HILLIER J. & COOMBES A. 2004. *The Hillier Manual of Trees & Shrubs*. Newton Abbot, Devon, England: David & Charles, 512 pp.

Serials:

JALAS J., SUOMINEN J., LAMPINEN R. & KURTTO A. (eds). 1999. *Atlas Florae Europaeae*. *Distribution of vascular plants in Europe*. Vol. **12**. *Resedaceae to Platanaceae*. Helsinki: Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo. Maps 2928-3270, 250 pp., ill (maps).

TUTIN T. G., BURGES N. A., CHATER A. O., EDMONDSON J. R., HEYWOOD V. H., MOORE D. M., VALENTINE D. H., WALTERS S. M. & WEBB D. A. (eds, assist. by J. R. AKEROYD & M. E. NEWTON; appendices ed. by R. R. MILL). 1996. *Flora Europaea*. 2nd ed., 1993, reprinted 1996. Vol. **1**. *Psilotaceae to Platanaceae*. Cambridge: Cambridge University Press, xlvi, 581 pp., illus.

Chapters in books:

†TUTIN T. G. 1996. *Helleborus* L. pp. 249-251. In: † TUTIN T. G. & al. (eds). *Flora Europaea*. 2nd ed., 1993, reprinted 1996. Vol. **1**. *Psilotaceae to Platanaceae*. Cambridge: Cambridge University Press, xlvi, 581 pp., illus.

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