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MORPHOLOGICAL FEATURES CONCERNING EPIDERMAL APPENDAGES ON SOME SPECIES OF THE *SOLANUM* GENUS

Camelia IFRIM[1*], Iuliana GAȚU[1]

Abstract: The importance of studying the features of the prickles and hairs within the *Solanum* genus is unanimously acknowledged. In the present work 12 taxa have been examined, thus revealing the presence, density and micro morphological features of the epidermal appendages. The observed features of the eglandular hairs underline their diversity, the analysis focusing on aspects concerning the stellate type. The morphological observations can be of use in the taxonomy of the genus, as it is apparent from the key for identification elaborated based on the highlighted features.

Keywords: eglandular trichome, glandular trichomes, key for identification, prickles, *Solanum* morphology

Introduction

The *Solanum* genus, containing 1400 species, is considered the 10th most numerous one in the angiospermae group. It is particularly known through its representatives with culinary uses: *S. melongena*, *S. tuberosum*, or the less spread tropical species *S. aethiopicum*, *S. aviculare*, *S. betaceum*, *S. pimpinellifolium*, *S. quitoense*. The ornamental uses of the species within this genus are less known, mainly in the East-European areas; the species specified in the horticultural studies are *S. jasminoides*, *S. rantonnetii*, *S. wendlandii* [RICKARD, 2011], whereas in the online articles the ornamental features of the *S. atropurpureum* and *S. pyracanthos* species are very appreciated.

In the study Flora of Romania [CIOCÂRLAN, 2000] 9 species of the *Solanum* genus are mentioned, 5 of which are adventitious. In 2011, 2 other species were classified as adventitious. If apart from the species cultivated for alimentary purposes the others are considered weeds, some even quarantine Flora of Romania [CIOCÂRLAN, 2000], *S. citrullifolium* is mentioned as an ornamental plant, garden-grown, where it becomes subspontaneous [SÎRBU (coord.) & OPREA, 2011]. Although the first mentions of this nature date from 1941, the species does not appear in the ornamental plants registry nor is it mentioned in any relevant work/study.

The study of the *Solanum* genus is currently the focus of many specialists [ADEDEJI & al. 2007; BENITEZ DE ROJAS & FERRAROTTO, 2009; BUKENYA & CARASCO, 1995], and as of 2006 the *Solanum* Trichome Project has been initiated. The project is a collaborative functional genomics project funded by a national Science Foundation grant to Michigan State University, The University of Michigan and the University of Arizona. Part of this project proposes the morphological characterization of the *Solanum* genus trichomes, as their taxonomic [KNAPP, 2001], biochemical, ecological importance has often been acknowledged.

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Classical literature [ANELI, 1975; LINSBAUER, 1930; NAPP-ZINN, 1973, 1974; NETOLITZKY, 1932; UPHOFF & HUMMEL, 1962] does not provide significant information on the mentioned morphological aspect; also, in the Romanian literature there are only few articles [NIȚĂ & al. 1990], and only the contemporary ones are focused on highlighting the characteristics of eglandular hairs [MĂRGINEANU & al. 2014].

The epidermal appendages are among the plant surface features that are crucial for their effective adaptation to stress. The emergent protect the plants from enemies (offensive/defensive organisms). As there aren't many studies concerning the hairs present on the flowers, there are still uncertainties regarding the structure, ultrastructure, the secreted substances the functions these serve. Thus, depending on the area where they are found, they can have a protective role (against undesired insects/animals or against their visit during an inappropriate period of the flower's development) or they can attract the latter.

Materials and methods

The material (Tab. 1) used for this study consists of 12 taxa from the *Solanum* genus, grown in the Botanical Garden of Iasi. The floriferous shoot were collected the period of anthesis and were analyzed using the Optika binocular and the Optika optic microscopes.

Tab. 1. Analyzed species, their taxonomy and uses

	Infrageneric classification		**Species**	**Origin**	**Uses**
	Subgenus	Section			
1.	**Leptostemonum**	Oligacanthes	*S. aethiopicum* L., Gilo group	tropical Africa	Alimentary
2.		Acanthophora	*S. atropurpureum* Schank	tropical South America	
3.		Croatianum	*S. pyracanthos* Lam.	Madagascar	
4.		Melongena	*S. rostratum* Dunal	Mexic, W and Central N America	
5.			*S. sisymbriifolium* Lam.	Central and South America	Ornamental, medicinal
6.			*S. citrullifolium* A. Braun	SUA (Texas, New Mexico), Mexic	
7.			*S. linnaeanum* Hepper & Jaeger	Caep Provinces	
8.		Oligacanthes	*S. coccineum* Jacq.	South Africa	Medicinal
9.		Micracantha	*S. lanceifolium* Jacq.	tropical South America	
10.	**Solanum** sensu stricto	Lycopersicon	*S. pimpinellifolium* L.	Ecuador, Peru	Alimentary
11.		Archaesolanum	*S. aviculare* G. Forst.	New Zealand	Medicinal, alimentary, tinctorial
12.	**Potatoe**	Basarthrum	*S. caripense* Dunal	South America (Costa Rica, Venezuela, Columbia, Ecuador, Peru)	

Results and discussions

The representatives of the *Solanum* genus are known to have epidermal appendages such as hairs and prickles [BENITEZ DE ROJAS & FERRAROTTO, 2009]. Their morphological diversity and their distribution on different organs is quite variated, as it ensues from the descriptions of the studied species (S. – floriferous shoot, L. – leaves, P. – petiole, Lm. – lamina, Fl. – flowers, Ped. – peduncle, K. – calyx, C. – corolla, O. – Ovary, Fr. – fruit).

***Solanum aethiopicum* (Gilo group)** (Plate II, Fig. 5, 9): S. stellate stiped hairs, with 7-9 rays including a central one, approximately 3 times smaller the others, multicellular eglandular hairs. L. stellate white-ish hairs, numerous on the abaxial, present on the adaxial mainly on the veins, scarcely appearing between veins; among these hairs there are also found uniseriate hairs, stellated stipitate hairs whith 5-9 rays, of which one is centrally situated. Fl.: K. similar stellate hairs, very numerous, situated on the abaxial. C. stellate hairs situated on the abaxial and on the mid-vein of the adaxial (only). O. Stellate hairs.

S. atropurpureum (Plate I, Fig. 1.a): S. lilac-colored prickles - flattened, retrorse - growing in size towards the upper half. L. prickles only present on the veins; eglandular multicellular and glandular hairs appear on both sides; scarcely found stellate hairs. Fl.: Ped. small, very rare prickles. K. sepals with a single big prickle, lilac-colored, centrally situated; the base shows stellate hairs.

S. aviculare (Plate I, Fig. 7): S. short unicellular eglandular hairs present only on the veins. K. eglandular hairs on the edge of the sepals.

S. caripense: S.: eglandular multicellular falcate hairs, sparsely short stipitate glandular hairs, with 1-cellular head. L.: P. eglandular multicellular falcate hairs; Lm. adaxial eglandular multicellular falcate hairs, abaxial tomentose. Fl.: K. sparsely eglandular hairs and rarely glandular hairs with uni-cellular head. C. eglandular hairs only on mid-vein.

S. citrullifolium (Plate I, Fig. 1.e; Plate II, Fig. 12): S. erect white prickles less than 1cm in length, showing glandular hairs at the base much smaller than the ones present (directly) on the organs, rare; bicellular eglandular hairs, very numerous; glandular hairs with unicellular heads; uni-/bicellular stalk, appears in different sizes. L. very rare prickles, glandular and eglandular hairs similar to those present on the shoot; stellate hairs with 4 rays on the veins; similarly disposed on the abaxial, but more frequent. Fl.: K. greenish white prickles at a 30º angle; glandular hairs; very scarcely found eglandular hairs. C. very rare eglandular hairs and very small glandular hairs on the adaxial veins; on the abaxial veins there are found uni-/multicellular eglandular hairs, glandular hairs with unicellular head, stellate hairs with 2 rays and, very rarely, hairs with 5 rays, one of which is centrally situated. Fr. Whitish prickles at an angle of less than 45º; glandular hairs; rare eglandular hairs.

S. coccineum (Plate I, Fig. 2; Plate II, Fig. 6, 7): S. deltoid prickles of up to 4mm in lentgh, rare stiped stellate hairs aswell – very numerous, densely looking, with 7-8 rays. L.: P. subulate prickles on the mid-vein. Lm. dense yellowish stellate hairs. Ped. and K. rare prickles and dense stellate hairs. C. stellate hairs situated on the mid-vein of the adaxial and on the center of the petals on the abaxial – densely looking, growing more yellowish towards the mid-vein. O. subsessile stellate hairs.

S. lanceifolium (Plate I, Fig. 6): S. pointed eglandular hairs of 4-5 cells (sharp tip) L: P. whitish eglandular hairs, multicellular, present (scarcely) on the edges but mostly in between them. Fl.: K. eglandular hairs, multicellular (3-4 cells) on the abaxial. C. very numerous eglandular hairs on the abaxial, less on the adaxial. O. erect eglandular hairs. Fr. Ped. pointed multicellular eglandular hairs (2-3 cells), more scarcely present than on the fructiferous stem.

S. linnaeanum (Plate I, Fig. 1.d): yellowish deltoid prickles (more intensely colored towards the tip), stellate hairs freely disposed. L. prickels on the veins on both sides, stellated hairs on the lamina, more numerous on the abaxial. The stellate hairs have 9 rays, the central one longer than the rest. The prickles show stellate hairs at the base. Fl.: Ped. prickles, K. densely prickled. O. stellate hairs.

S. pimpinellifolium: S. uniseriate multicellular eglandular hairs (1-2-3 cells), hooked prickles and glandular hairs, with a tetracellular gland. L.: P. hairs similar to those on the stem; Lm. very numerous eglandular hairs on the abaxial veins, freely disposed on the rest of the area, glandular hairs appear among them; eglandular hairs uniformly disposed on the whole abaxial area, very scarce glandular hairs. Fl. K. hairs similar to those on the petiole, the eglandular hairs are predominant on the outside, while the glandular ones are predominant on the inside. C. eglandular hairs on the abaxial, and rarely glandular hairs and glabrous veins. F.: Ped. and K. glandular and eglandular hairs approximately as frequently disposed; C. very rare eglandular hairs.

S. pyracanthos (Plate I, Fig. 1.b, 3, 5; Plate II, Fig. 8): S. orange prickles of different sizes, dense stellate hairs. L. orange prickles on the veins, whitish stellate hairs on the lamina, very frequent and orange on the veins, very dense stellate hairs on the abaxial, less frequent on the mid-vein. Fl.: K. very dense orange stellate hairs on the abaxial. C. stellate hairs appear on the mid-vein of the adaxial, while on the adaxial they cover a "band" of 70% of the petal.

S. rostratum (Plate I, Fig. 1.f; 9; Plate II, Fig. 2, 4): S. rigid subulate prickles, appear whitish or yellowish; numerous stellate hairs. L.: P. prickles, stellate hairs. Lm. prickles on the veins, stellate hairs, less frequent on the mid-vein. Fl.: K. stellate hairs. C. rare prickles on the veins, numerous stellate hairs on the abaxial. Fr. yellowish prickles, numerous stellate hairs with 5-7 rays, uniseriate multicellular hairs.

S. sisymbriifolium (Plate I, Fig. 1.c, 4, 8; Plate II, Fig. 1, 3, 10, 11): S. light-yellow subulate prickles of up to 1cm in length, glandular hairs with a uniseriate stalk and unicellular head, stellate glandular hairs whose central ray ends with a unicellular gland, infrequent bristles. L. big prickles on the veins, scattered stellate hairs. Fl.: Ped. rare reddish-brown prickles, very numerous stellate hairs. K. pointed eglandular hairs on the abaxial (towards the tip), rare stellate hairs with a very long central ray (3-5 times longer). C. rare stellate hairs on the mid-third of the abaxial (like a band in length), glandular hairs with unicellular head.

The observed prickles of the 12 taxa are of subulate or deltoid type (*S. coccineum*), presenting a sharp tip. The prickles are retrorse only on the *S. atropurpureum*, whereas the on the other species who have them, the prickles are disposed vertically or oriented towards the upper half at an acute angle (especially on the fruits where they appear to be curved). In most cases, the prickles show glandular or eglandular hairs at the base, similar to those found on the respective organ, usually of smaller sizes. The color of the prickles differs;

white-yellowish for the most part, orange for *S. pyracanthos*, lilac for *S. atropurpureum* – for this species the color disappears once the plant is cut, whereas for the others the color persists.

The presence of the prickles within the *Solanum* genus is a classification criterion, but the morphological information is scarce, the best represented are the spiny species [KNAPP, 2013], and SYMON (1983) argues that the distribution and density of the prickles are more variated than those of the hairs. The roles of the prickles are also variated, especially depending on their location on the plant's surface. Thus, the prickles on the fruit are used for spreading the seeds (zoochory), while the prickles on the stem and leaves are mostly used for protection. SYMON's study (1986) on the Australian species concludes that the development of the prickles is a response to the navigation of marsupials between plants, especially of those from the group otherwise known as "wallabies".

The observed hairs of the taxa taken in study are both glandular and eglandular. The glandular hairs are particularly important due to their ability to synthesize, preserve and secrete specialized metabolites. The largely accepted classification of hairs is that of Lucwill [cf. KANG & al. 2009], who defines 4 morphological groups. The specimens taken in study have eglandular hairs with a unicellular (the most part) or multicellular stalk (*S. citrullifolium, S. pyracanthos*); the gland can be unicellular (*S. sisymbriifolium*), tetracellular (*S. pimpinellifolium*) or octocellular (*S. sisymbriifolium*). Thus, they correspond to Lucwill's type VIII. A divergent type is that of *S. sisymbriifolium* where the stellate hairs have 5-6 rays and a central one that ends with a unicellular gland.

The eglandular hairs of the analyzed species are uniseriate or pluriserial. The uniseriate hairs are unicellular, thick (*S. aviculare*) or thin, straight (*S. sisymbriifolium*) or hooked (*S. pimpinellifolium*). Multicellular hairs (3-5 cells) are found on *S. rostratum*, while unicellular bristles are found on *S. sisymbriifolium*. Stellate hairs usually have a unicellular stalk, but it can be multicellular as well with a robust aspect (*S. rostratum*). The number of rays may vary anywhere from 2 to 9. Usually one of the rays is radially disposed and can be short (1/5 of the other rays' length – *S. aethiopicum*, *Gilo* group) or very long (5 times longer than the others – *S. pyracathos, S. rostratum*). The morphologic diversity of the eglandular hairs is mentioned by METCALFE & CHALKE (1957); the works of Luckwill and Channarayappa (often used as reference) contain no mention of the stellate type [GLAS & al. 2012]. Seithe thought of the hairs type as being the only feature truly important for the major classification of the *Solanum* genus, and he used the possession of the stellate trichomes for defining its main subdivisions [KNAPP, 2001]. ROE (1971) is the one who performs a more detailed analysis and defines a much clearer terminology. The material analyzed here contains 5 of the types Roe describes: simple, uniseriate, multicellular: porrect-stellate sessile with a short median ray; porrect-stellate sessile with long median ray; multiserial porrect-stellate stipe with a short median ray; two-rayed stellate with raised base. A few examples of the terminology SEITHE & ANDERSON used in a previous work (1982) (which was not used by other authors) are worth noting: finger hair, bayonet hair (for multicellular uniseriate eglandular hairs), square gland, storied gland (for glandular hairs with tetra-/octocellular head) etc.

The color of the eglandular hairs varies, the majority being whitish, but there are some different cases too. One of these is *S. pyracanthos* where only the median ray of the

stellate hairs or the hairs around the prickles are orange, or *S. citrullifolium* where the hairs on the petals are of lilac color (like the petals).

Micromorphological features such as the size, density and aspect of the trichomes can be used for the correct identification of some invasive species (for a correct selection of herbicides and biocontrol agents) [ZHU & al. 2012] or of some medicinal species [ALVES & al. 2007; MAITI & al. 2002], for the study of pests [MEDEIROS & BOLIGON, 2007], for the study of herbivore diets [WIKEEM & PITT, 1983], or for the clarification of certain taxonomic aspects.

By using the features of the previously analyzed epidermal appendages, a key of identification for the 12 taxa can be developed:

1. Glabrous plants .....*S. aviculare*
1'. Plants with epidermal appendages 2
2. Prickle-less plants 3
2'. Prickled plants 6
3. Plants with stellate hairs 4
3'. Plants without stellate hairs 5
4. Plants with whitish stellate hairs .....*S. aethiopicum* (Gilo group)
4'. Plants with rare eglandular hairs .....*S. lanceifolium*
5. Plants with eglandular hairs which give a tomentose aspectand glandular hairs .....
.....*S. pimpinellifolium*
5'. Plants with massive eglandular hairsspread on the leavesand very rare glandular hairs ..
..... *S. caripense*
6. Plants with lilac-colored prickles .....*S. atropurpureum*
6'. Plants with prickles of another color 7
7. Plants with prickles of up to 1cm in length 8
7'. Plants with prickles of over 1 cm in length 9
8. Plants without uniseriate hairs, with prickles less than 4mm long .....*S. coccineum*
8'. Plants with uniseriate hairs, with prickles over 4mm long .....*S. sisymbriifolium*
9. Plants with yellow-orange prickles .....*S. pyracanthos*
9'. Plants with prickles of other color 10
10. Plants with glandular hairs and sets .....*S. citrullifolium*
10'. Plants with none of the above 11
11. Plants with very dense stellate hairs, which form a felt-looking layer .....*S. rostratum*
11'. Plants with very rare stellate hairs .....*S. linnaefolium*

Conclusions

The *Solanum* genus shows a great variety of epidermal appendages corresponding to each species and which are not holistically described in any relevant study. The micromorphological particularities of the studied species are generally congruent with the information found in scientific studies, but there have also been observed some structural aspects which bring new contributions, at least theoretical ones, to the general effort of studying the representatives of this highly numerous genus.

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http://www.trichome.msu.edu/about/overview_continued.html

EXPLANATION OF THE PLATES

PLATE I

Fig. 1. Caulinar prickles: a) *S. atropurpureum*, b) *S. pyracanthos*, c) *S. sisymbriifolium*, d) *S. linnaeanum*, e) *S. citrullifolium*, f) *S. rostratum* (x 2)
Fig. 2. Prickle and stelate hairs on off-shoot of *S. coccineum* (x 5)
Fig. 3. Prickle and stelate hairs on *S. pyracanthos* (x 25)
Fig. 4. Glandular and eglandular hairs on prickle of *S. sisymbriifolium* (x 25)
Fig. 5. Stelate hairs on adaxial calice and corolla of *S. pyracanthos* (x 10)
Fig. 6. Eglandular hairs on ovary of *S. lanceifolium* (x 25)
Fig. 7. Eglandular hair on leaf of *S. aviculare* (x 100)
Fig. 8. Bristle on leaf of *S. sisymbriifolium* (x 100)
Fig. 9. Eglandular hair on leaf of *S. rostratum* (x 100)

PLATE II

Fig. 1. Stelate hairs with 5 rays on leaf of *S. sisymbriifolium* (x 700)
Fig. 2. Stelate hairs with multiseriate stalk on leaf of *S. rostratum* (x 700)
Fig. 3. Stelate hairs with two rays on leaf of *S. sisymbriifolium* (x 700)
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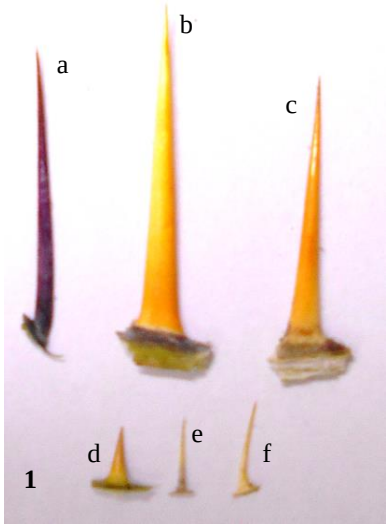
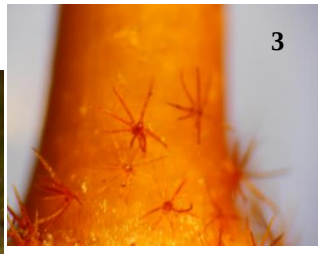
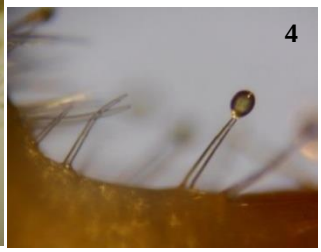
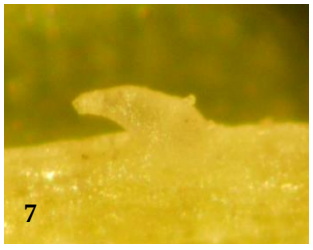
Fig. 12. Glandular and eglandular hairs on abaxial petal of *S. citrullifolium* (x 700)

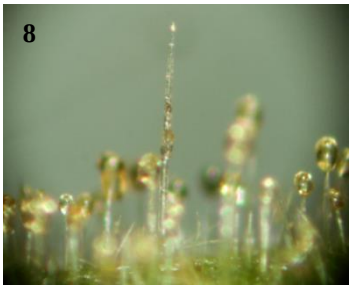
PLATE I











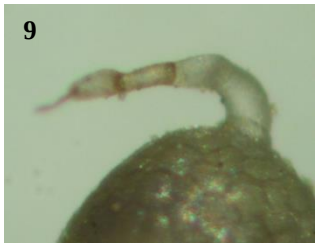
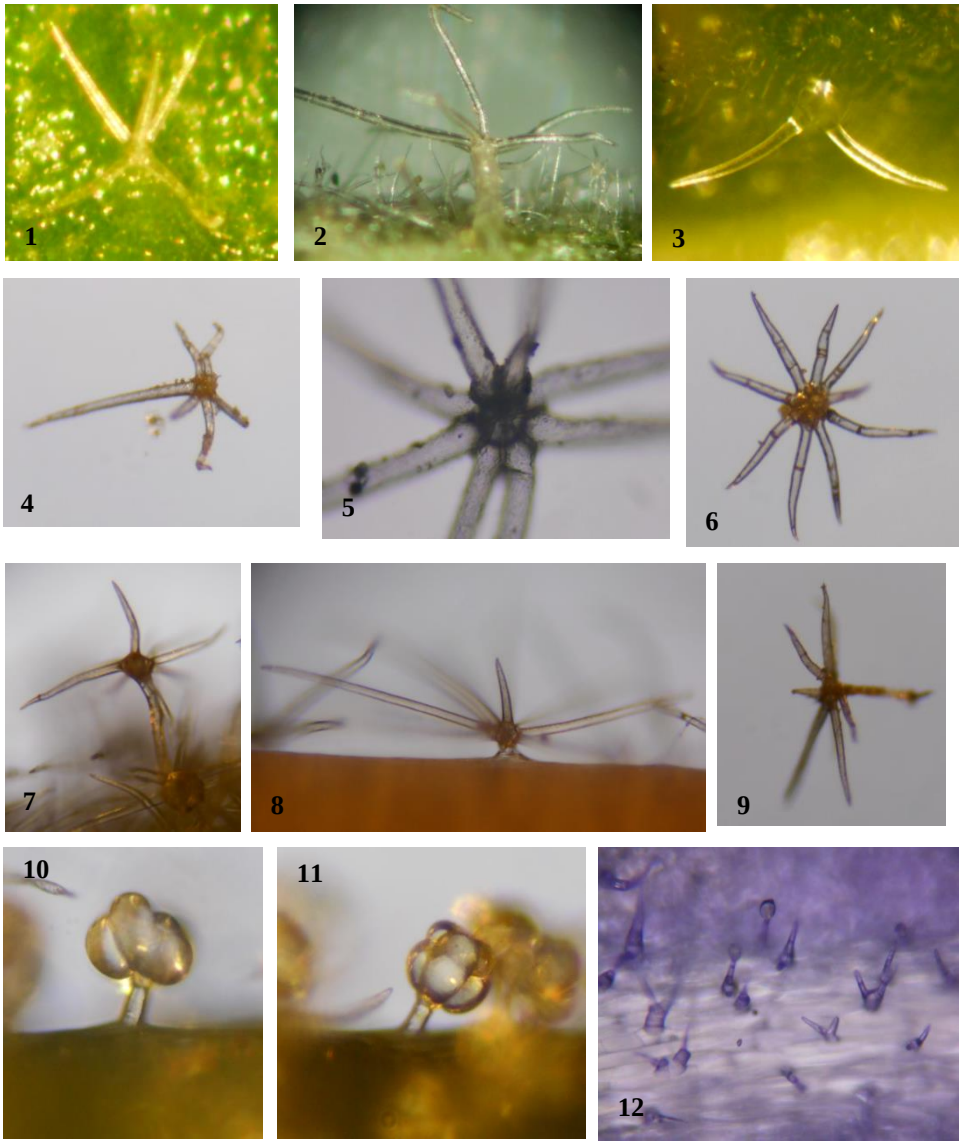


PLATE II



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ORGANOGENESIS OF *CYMBIDIUM* ORCHID USING ELICITORS

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Abstract: Elicitors are substances that induce protective responses in plants. In this study, methyl jasmonate (Me-JA) and lysozyme elicitation on PLBs culture of *Cymbidium insigne in vitro* was investigated. Elicitation by 0.1 mg/l Me-JA enhanced maximum PLB, shoot and root formation. The effects of lysozyme under white fluorescent tube, results indicated that every concentrations of lysozyme induced PLB, shoot and root formation and 0.1 mg/l lysozyme enhanced maximum formation of PLB, shoot and root compare with control. Lysozyme is known to play a vital role in medical industry and the present study firstly used lysozyme, as a plant growth regulator in *Cymbidium* tissue culture.

Keywords: lysozyme, methyl jasmonate, protocorm-like body, plant growth regulator, *in vitro*

Introduction

Elicitors are substances that induce protective responses in plants. In the beginning of their research era, they were alternatively called inducers, but because of the broad interpretation of this term, the term elicitors are now commonly accepted. It is well established that, upon the challenge by biotic or abiotic elicitors, plants respond with an array of defenses including the accumulation of secondary metabolites [DORNENBURG, 2004]. The method of elicitor-induced resistance to diseases in plants is characterized by a number of essential advantages: ecological safety, because the method is based on induction of the native immune potential of the host plant rather than on suppression of phytopathogens, a systemic and prolonged protective effect, involvement of multiple defense systems in induced resistance, which makes adaptation of pathogens to protected plants nearly impossible induction of nonspecific resistance to the number of fungi, bacteria, viruses, nematodes, etc. Plant cell culture has recently received a lot of attention as an effective technology for the production of valuable secondary metabolites. Plant cell cultures produce higher quantities of secondary metabolites, often with different profiles compared to their parent plants. A key factor to secondary metabolite production in plant cultures, however, is by elicitation among which treatment with jasmonate or methyl jasmoic is widely used [YUN-SOO & al. 2004; SEE & al. 2011; KOO & HOWE, 2009] and has been applied in orchid tissue cultures [SHIMASAKI & al. 2003; TEIXEIRA DA SILVA, 2012, 2012a]. However, whether it can be used in *Cymbidium kanran* Makino, hybride *Cymbidium* (Twilight Moon 'Day Light'), such as *Cymbidium insigne* has not been reported. *Cymbidiums* are among the most important orchids in horticulture. They are versatile plants, marketed as cut-flowers, buttonholes and as pot plants, producing many

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large, showy, long-lasting flowers [DU PUY & CRIBB, 2007]. Most *Cymbidium* spp. are commercially produced using tissue culture methods. The objective of this study is to investigate the effect of two elicitor; methyl jasmonate (Me-JA) and lysozyme on organogenesis of *Cymbidium insigne in vitro*. Me-JA (methyl jasmonates) is particularly interesting because of the myriad of plant responses associated with its synthesis and presence. Me-JA is emitted by wounded plants [MEYER & al. 2003] and therefore, may represent a means of communication between damaged plants. Lysozymes, also known as muramidase or N-acetylmuramide glycanhydrolase, are glycoside hydrolases. These are enzymes (EC 3.2.1.17) that damage bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. Lysozyme is abundant in a number of secretions, such as tears, saliva, human milk, and mucus. It is also present in cytoplasmic granules of the polymorphonuclear neutrophils (PMNs). Large amounts of lysozyme can be found in egg white. Lysozyme is widely used in medical industry. This is the first report demonstrating that lysozyme used as a plant growth regulator which increases PLBs, shoot and root formation of *C. insigne*.

Materials and methods

Plant material and explants source

Protocorm-like bodies (PLB) of *Cymbidium insigne* were proliferated in the modified Murashige and Skoog [SHIMASAKI & UEMOTO, 1990] medium by transferred new medium every two months. After excision of PLBs (ca. 3 mm in diameter) into pieces, they were used for explants.

Preparation of elicitors

Two elicitor including lysozyme, filter-sterilized (Wako Pure Chemical Industries, Ltd., Japan) with the concentration of 0 (control), 0.1, 1, 10 and 100 mg/l and also methyle jasmonate (purchased from Sigma) with the concentration of 0 (control), 0.1, 1, 10 and 100 mg/l were mixed with sterilized water used as aqueous solution (50 mg).

Culture methods

Experiment 1. Pick up single PLB with forceps and dipping into different concentrations of lysozyme aqueous solution for half an hour (30 minutes). After half an hour, PLBs were cultured on modified MS medium for 8 weeks (up to root formation).

Experiment 2. Pick up single PLB with forceps and dipping into different concentrations of lysozyme aqueous solution for one hour (60 minutes). After one hour, PLBs were cultured on modified MS medium for 8 weeks (up to root formation).

Experiment 3. Pick up single PLB with forceps and dipping into different concentrations of Me-JA aqueous solution for half an hour (30 minutes). After half an hour, PLBs were cultured on modified MS medium for 6 weeks (up to root formation).

Experiment 4. Pick up single PLB with forceps and dipping into different concentrations of Me-JA aqueous solution for one hour (60 minutes). After one hour, PLBs were cultured on modified MS medium for 6 weeks (up to root formation).

Modified MS medium supplemented with 412.5 mg/l ammonium nitrate, 950 mg/l potassium nitrate, 20 g/L sucrose and 2 g/L phytagel (Sigma KK, Japan) was adjusted to pH 5.5-5.8 before autoclaving. Jars (250 ml UM culture bottle; As One, Japan) with plastic

caps containing 30 ml of medium were used as culture vessels. Five explants were cultured in each culture vessel and three culture vessels were used for each treatment. All cultures were maintained at 25 ºC with a 16 h photoperiod and irradiance of 54 µmol m^{-2} s^{-1} under white fluorescent tube.

Statistical analysis

Experimental data were collected by counting the number of PLBs, shoot and root; percentage of PLBs, shoot and root; the fresh weight of PLBs were measured. The data were analyzed to a one-way analysis variance (ANOVA) and differences between means were tested using Tukey's honestly significant different test ($P \leq 0.05$).

Results

Experiment 1. In vitro growth of *C. insigne*, PLBs were dipping 30 minutes into lysozyme aqueous solution

The results indicated that PLBs of *C. insigne* were significantly increased fresh weight of PLBs, number of PLBs, shoot and root of lysozyme with modified MS media compare with control. Dipping 30 minutes at lysozyme aqueous solution, 0.1 mg/l lysozyme significantly increased the number of PLBs, shoot and root (Tab. 1). The highest average number of PLBs (7.1 PLBs/explant), the highest average number of shoot (1.1 shoots/explant) and the highest average number of root (0.3 roots/explant) were recorded at 0.1 mg/l lysozyme with modified MS media. The maximum PLB formation rate 100% and the maximum shoot formation rate 53% were observed at 0.1 mg/l lysozyme (Fig. 1A); whereas control had less formation of PLBs (67%) and shoot (20%) after 8 weeks of culture. The maximum root formation rate 33% was observed at 0.1 mg/l or 100 mg/l (Fig. 1B) lysozyme with modified MS media, comparatively control treatment had no root formation. The maximum fresh weight of PLBs (94.4 mg) was observed at 1 mg/l lysozyme with modified MS media.

Tab. 1. In vitro growth of *C. insigne*, PLBs were dipping 30 minutes into different concentrations of lysozyme aqueous solution and cultured on modified MS medium under white fluorescent tube for 8 weeks

Lysozyme (mg/l)	PLB			Shoot		Root	
	No. /explant	Rate (%)	FW (mg)	No. /explant	Rate (%)	No. /explant	Rate (%)
Control	2.6 ± 0.7b	67	41.3 ± 3.7c	0.2 ± 0.2b	20	0	0
0.1	7.1 ± 0.9a	100	86.8 ± 11.4ab	1.1 ± 0.5a	53	0.3 ± 0.2a	33
1	3.6 ± 0.7b	80	94.4 ± 16.8a	0.3 ± 0.3ab	27	0.1 ± 0.2b	13
10	3.3 ± 0.7b	87	57.3 ± 6.9bc	0.3 ± 0.2b	27	0.1 ± 0.2b	13
100	4.1 ± 1.5ab	80	45.2 ± 6.4c	0.7 ± 0.4ab	47	0.3 ± 0.2ab	33

*Value represents means±SE followed by the different letters show significant differences by Tukey HSD test ($P \leq 0.05$)

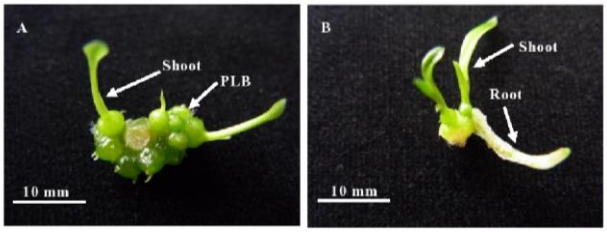

Fig. 1. In vitro growth of *C. insigne*, PLBs were dipping 30 minutes into different concentrations of lysozyme aqueous solution and cultured on modified MS medium under white fluorescent tube for 8 weeks. A: 0.1 mg/l lysozyme; B: 100 mg/l lysozyme.

Experiment 2. In vitro growth of *C. insigne*, PLBs were dipping 60 minutes into lysozyme aqueous solution

Dipping 60 minutes at lysozyme aqueous solution, 1 mg/l lysozyme significantly increased the number of PLBs and fresh weight of PLBs (Tab. 2). The highest PLB formation rate (80%), the highest average number of PLBs (4.9 PLBs/explant), the highest fresh weight of PLBs (79.6 mg) were observed at 1 mg/l lysozyme with modified MS media (Fig. 2A). The highest average number of shoot (0.5 shoots/explant) and the highest shoot formation rate 33% were recorded at 0.1 mg/l or 100 mg/l (Fig. 2B.) lysozyme with modified MS media. The highest root formation rate 33% was recorded which PLBs were dipping 60 minutes at 100 mg/l lysozyme aqueous solution. Comparatively, lowest number of PLBs and lowest fresh weight was observed at control. There was no shoot and root formation observed at control (which PLBs were dipping into water).

Tab. 2. In vitro growth of *C. insigne*, PLBs were dipping 60 minutes into different concentrations of lysozyme aqueous solution and cultured on modified MS medium under white fluorescent tube for 8 weeks

Lysozyme (mg/l)	PLB			Shoot		Root	
	No. /explant	Rate (%)	FW (mg)	No. /explant	Rate (%)	No. /explant	Rate (%)
Control	1.3 ± 0.7b	47	35.3 ± 4.7b	0	0	0	0
0.1	2.3 ± 1.2ab	47	45.9 ± 8.3b	0	0	0	0
1	4.9 ± 1.3a	80	79.6 ± 14.5a	0.5 ± 0.3a	33	0.1 ± 0.2a	13
10	1.7 ± 0.6b	60	45.7 ± 7.1b	0.3 ± 0.4a	20	0.1 ± 0.2a	13
100	2.1 ± 0.8b	53	45.2 ± 6.4b	0.5 ± 0.4a	33	0.3 ± 0.3a	33

*Value represents means±SE followed by the different letters show significant differences by Tukey HSD test (P≤0.05)

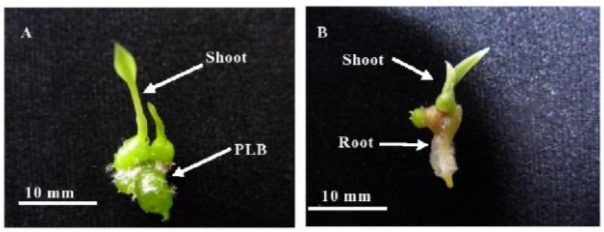

Fig. 2. In vitro growth of *C. insigne*, PLBs were dipping 60 minutes into different concentrations of lysozyme aqueous solution and cultured on modified MS medium under white fluorescent tube for 8 weeks. A: 1 mg/l lysozyme; B: 100 mg/l lysozyme.

Experiment 3. In vitro growth of *C. insigne*, PLBs were dipping 30 minutes into Me-JA aqueous solution

The results indicated that PLBs of *C. insigne* were significantly increased the fresh weight and number of PLBs at Me-JA treatment. Dipping into 30 minutes at Me-JA aqueous solution, 0.1 mg/l Me-JA increased the formation of PLB, shoot and root (Tab. 3). The highest average number of PLBs (4.4 PLBs/explant), the highest average number of shoot (0.4 shoots/explant) and the highest average number of root (0.4 roots/explant) were recorded at 0.1 mg/l Me-JA with modified MS media (Fig. 3). The highest PLBs formation rate 93%, the highest shoot and root formation rate 33% were observed at 0.1 mg/l Me-JA with modified MS media; whereas less formation of PLBs (27%) was observed high concentration of Me-JA (100 mg/l) aqueous solution. 100 mg/l Me-JA aqueous solution had no shoot and root formation observed after 6 weeks of culture. The maximum fresh weight of PLBs (68.3 mg) was observed at 10 mg/l Me-JA with modified MS media.

Tab. 3. In vitro growth of *C. insigne*, PLBs were dipping 30 minutes into different concentrations of Me-JA aqueous solution and cultured on modified MS medium under white fluorescent tube for 6 weeks

Me-JA (mg/l)	PLB			Shoot		Root	
	No. /explant	Rate (%)	FW (mg)	No. /explant	Rate (%)	No. /explant	Rate (%)
Control	2.1 ± 0.7abc	67	44.6 ± 8.2abc	0.3 ± 0.2a	27	0.2 ± 0.2a	20
0.1	4.4 ± 1.0a	93	67.8 ± 10.4ab	0.4 ± 0.3a	33	0.4 ± 0.3a	33
1	2.3 ± 0.8abc	73	38.7 ± 5.9abc	0.2 ± 0.2a	20	0	0
10	4.2 ± 1.0ab	73	68.3 ± 8.6a	0.7 ± 0.4a	27	0	0
100	0.9 ± 0.9c	27	20.9 ± 5.4c	0	0	0	0

*Value represents means±SE followed by the different letters show significant differences by Tukey HSD test (P≤0.05)

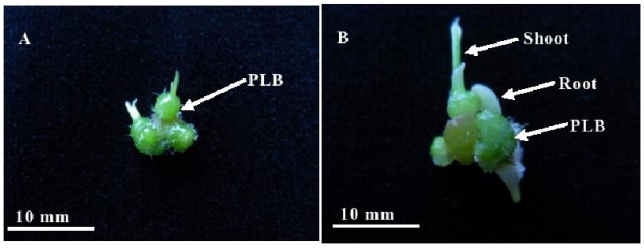

Fig. 3. In vitro growth of *C. insigne*, PLBs were dipping 30 minutes into different concentrations of Me-JA aqueous solution and cultured on modified MS medium under white fluorescent tube for 6 weeks. A: Control; B: 0.1 mg/l Me-JA.

Experiment 4. In vitro growth of *C. insigne*, PLBs were dipping 60 minutes into Me-JA aqueous solution

The results indicated that PLBs of *C. insigne* were significantly increased the fresh weight and number of PLBs at Me-JA treatment. Dipping into 60 minutes at Me-JA aqueous solution, 0.1 mg/l Me-JA with modified MS media increased the formation rate of PLB and shoot (Tab. 4). The highest average number of PLBs (3.6 PLBs/explant), the highest average number of shoot (0.7 shoots/explant) and the maximum fresh weight (58.5 mg) were observed at 0.1 mg/l Me-JA with modified MS media (Fig. 4A). The highest PLB

formation rate 87% and the highest shoot formation rate 40% were recorded at 0.1 mg/l Me-JA with modified MS media; whereas less formation of PLB (33%) was observed high concentration of Me-JA (100 mg/l) aqueous solution. Root formation rate 20% was observed only at 1 mg/l Me-JA aqueous solution after 6 weeks of culture (Fig. 4B).

Tab. 4. In vitro growth of *C. insigne*, PLBs were dipping 60 minutes into different concentrations of Me-JA aqueous solution and cultured on modified MS medium under white fluorescent tube for 6 weeks

Me-JA (mg/l)	PLB			Shoot		Root	
	No. /explant	Rate (%)	FW (mg)	No. /explant	Rate (%)	No. /explant	Rate (%)
Control	3.5 ± 0.9ab	73	51.2 ± 6.8ab	0	0	0	0
0.1	3.6 ± 0.7a	87	58.5 ± 6.8bcd	0.7 ± 0.4a	40	0	0
1	2.1 ± 0.7abc	73	54.4 ± 8.7ac	0.3 ± 0.3a	27	0.2 ± 0.2	20
10	2.3 ± 0.8abc	67	51.1 ± 8.5abd	0.3 ± 0.3a	27	0	0
100	0.4 ± 0.3c	33	20.3 ± 3.5c	0	0	0	0

*Value represents means±SE followed by the different letters show significant differences by Tukey HSD test (P≤0.05)

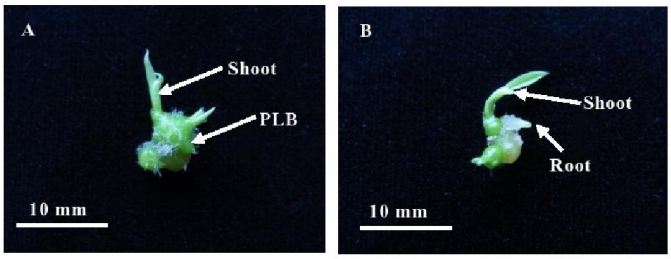

Fig. 4. In vitro growth of *C. insigne*, PLBs were dipping 60 minutes into different concentrations of Me-JA aqueous solution and cultured on modified MS medium under white fluorescent tube for 6 weeks. A: 0.1 mg/l Me-JA; B: 1 mg/l Me-JA.

Discussion

Plant organogenesis *in vitro* is a more controllable and reliable process. *In vitro* propagation of orchids as an option for rapid propagation of commercially valuable cultivars progressed well during the last decades. This is the first report demonstrating lysozyme, work as a plant growth regulator for increasing new PLB, shoot and root formation in *Cymbidium* tissue culture.

Lysozyme is an enzyme found in egg white, tears, and other secretions. It is responsible for breaking down the polysaccharide walls of many kinds of bacteria and thus it provides some protection against infection. The activity of enzymes is strongly affected by changes in pH and temperature. Each enzyme works best at a certain pH and temperature, its activity decreasing at values above and below that point. This is not surprising considering the importance of tertiary structure (i.e. shape) in enzyme function and non-covalent forces, e.g., ionic interactions and hydrogen bonds, in determining that shape. Since lysozyme is a natural form of protection from gram-positive pathogens like *Bacillus* and *Streptococcus* a deficiency due to infant formula feeding can lead to increased

incidence of disease. The effects of lysozyme under white fluorescent tube, results indicated that every concentrations of lysozyme induced best formation of PLB, shoot and root with modified MS media. Comparatively the PLBs which were dipping 30 minutes into lysozyme aqueous solution were best growth rate observed. Lysozyme is known to play a vital role in medical industry and the present study confirmed that lysozyme functions as a plant growth regulator in *Cymbidium insigne*. Lysozyme can stimulate PLBs proliferation of *Cymbidium in vitro*. Relatively low concentrations (0.1 mg/l) of lysozyme in culture media enhanced the maximum formation of PLB, shoot and root of *Cymbidium* spp. But the mechanism how it works in orchid tissue culture is unknown.

The response of methyl jasmonate (Me-JA) or jasmonic acid (JA) in the culture media as an elicitor enhanced the anthocyanin production of *Tulipa gesneriana* L. and *Vaccinium pahalae* Skottsb. [SANIEWSKI & al. 1998; FANG & al. 1999]. Methyl jasmonates (Me-JA) has successfully used as an elicitor in other plant species for enhancing the production of secondary metabolites in the cell cultures [AOYAGI & al. 2001; KIM & al. 2004; THANH & al. 2005]. Elicitation has been shown to be the most efficient strategy that direct to the enhancement in anthocyanin production in plant cell cultures [ZHANG & FURUSAKI, 1999). In *Cymbidium* tissue culture, Me-JA at 1 μM stimulated protocorm-like body (PLB) formation (from shoots) and shoot formation in epiphytic *Cymbidium eburneum* and in terrestrial *Cymbidum kanran* Makino [SHIMASAKI & al. 2003] while it stimulated, when applied at 1 mg/l, PLB formation from half-moon PLBs and PLB TCLs in a hybrid *Cymbidium* [TEIXEIRA DA SILVA, 2012]. According to this study result suggested that PLBs of *C. insigne* dipping 30 minutes at 0.1 mg/l Me-JA aqueous solution and cultured (after 30 minutes) on modified MS media, induced maximum formation of PLBs, shoot and root. When PLBs were dipping 60 minutes at different concentrations of Me-JA aqueous solution, 0.1 mg/l Me-JA induced maximum formation of PLB and shoot but root formation observed which PLBs were dipping at 1 mg/l Me-JA aqueous solution. Low concentrations of Me-JA (0.1 mg/l) induced best formation (PLBs, shoot and root) and 100 mg/l Me-JA aqueous solution had no shoot and root formation observed within culture period.

Conclusions

As biological control becomes more prevalent, useful, and important in horticultural crop production, targeted use of jasmonate-induced defenses may provide valuable augmentation of integrated pest management strategies. As reported by MIZUKAMI & al. (1993), jasmonic acid and its derivatives were involved in a part of the signal transduction pathway that induced particular enzymes catalyzing biochemical reactions for the synthesis of secondary metabolites and lysozyme is considered a “natural” antibiotic [GLYNN, 1968]. It is an important factor of innate immunity and a unique enzybiotic in that exerts not only antibacterial activity but also antiviral, anti-inflammatory, anticancer and immunomodulatory activities [SAVA, 1996; HELAL & al. 2012]. The results from this study indicate that Me-JA and lysozyme elicitation strategy was safe and useful to improve the PLBs culture of *Cymbidium insigne in vitro*.

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MICROPROPAGATION OF ADULT TREE OF *PTEROCARPUS MARSUPIUM* ROXB. USING NODAL EXPLANTS

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Abstract: Attempts were made for *in vitro* propagation of *Pterocarpus marsupium* Roxb., belonging to family Fabaceae, an economically important multipurpose tree. The tree is scared with noval antidiabetic properties. The tree shows poor seed germination capacity (30%) due to hard seed coat and conventional vegetative regeneration methods are a complete failure. Therefore, the propagation of this tree by tissue culture techniques is an urgent need and well justified. Nodal segments containing axillary bud from 10 years old tree of *P. marsupium* were evaluated for axillary shoot proliferation on Murashige and Skoog's (MS) basal medium fortified with BAP (6–benzylaminopurine) and kinetin (Kn) singly or in combinations with auxins at different concentrations. The best shoot proliferation was obtained with 13.95 µM Kn + additives (568 µM Ascorbic acid, 260 µM Citric acid, 605 µM Ammonium sulphate and 217 µM Adenine sulphate) in MS medium where 64.44% of the axillary buds responded with development of (2.51±0.10) shoots. Multiplication of *in vitro* shoots were achieved on MS Medium supplemented with Kn (9.30 µM) + NAA (0.54 µM) and additives. Half strength MS medium supplemented with 4.92 µM IBA induced *in vitro* rooting of *in vitro* shoots. *In vitro* regenerated plantlets with well developed roots were successfully hardened in a greenhouse.

Keywords: acclimatization, Fabaceae, *in vitro*, recalcitrant, tissue culture

Introduction

Pterocarpus marsupium Roxb. is a deciduous tree, commonly called as Indian Kino tree or malabar kino, belonging to the family Fabaceae. It is a medium to large sized tree reaching the height upto 15-20 meter with dark brown to grey bark having swallow cracks. The bark excudes a red gummy substance called 'Gum Kino' when injured. Leaves are compound and imparipinate. Flowers are yellow in terminal panicles. Fruit is circular winged pod. Seed is convex and bony. Tree flowers and fruits in the month of March to June. *P. marsupium* is distributed in deciduous forest throughout the India [VARGHESE, 1996]. It is a multipurpose leguminous tree. Heart wood is astringent, bitter, acrid, cooling, anti-inflammatory, depurative, haemostatic, revulsive and anthelmintic. The paste of seed and wood is useful in diabetic anaemia [TRIVEDI, 2006].The paste of heartwood is useful in body pain and diabeties. Wood of this tree is useful in making the waterglasses of diabetic [REDDY & al. 2008]. Due to overexploitation of the tree for its various useful applications coupled with low germinability, *Pterocarpus marsupium* has been included in the list of depleted plant species [CHOUDHARI & SARKAR, 2002].

Tissue culture method has been proved to be a promising technique for conservation and rapid multiplication of several forest rare woody species. However,

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member of Fabaceae have been found difficult to culture *in vitro* due to their recalcitrant nature [JHA & al. 2004]. *In vitro* regeneration protocols have been reported in *P. marsupium* using different explant sources including cotyledonary nodes, nodes and hypocotyl [CHAND & SINGH, 2004; TIWARI & al. 2004; ANIS & al. 2005; HUSAIN & al. 2007, 2008, 2010]. But till date, no report is available for *in vitro* regeneration of *Pterocarpus marsupium* from nodal segments. The present work is taken with aim to establish aseptic cultures of mature tree of *Pterocarpus marsupium* from nodal segments and to develop micropropagation protocol.

Materials and methods

Source of plant material and explant preparation

The plant material was collected from AFRI nursery, Jodhpur. Nodal segments containing axillary bud were collected and pretreated with Tween-80 for 5 min. and rinsed with distilled water. After that they were treated with 0.1% bavistin (w/v) solution and 0.05% streptomycin solution for 7 min. and thoroughly rinsed with distilled water. Later the explants were surface sterilized with 0.1% $HgCl_2$ for 7 min. and rinsed with autoclaved distilled water for three–four times.

Basal nutrient medium for shoot proliferation and multiplication

The surface – sterilized explants were inoculated on MS medium with various concentrations of BAP (2.22, 4.44, 8.86, 13.32 and 17.76 µM) or Kn (2.32, 4.65, 9.30, 13.95 and 18.50 µM) for shoot proliferation. MS medium with sucrose (3%) were used throughout the experiment. Additives (568 µM Ascorbic acid, 260 µM Citric acid, 605 µM ammonium sulphate and 217 µM Adenine sulphate) were also used in the MS medium. The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCL and the medium was solidified with 0.8% agar. The medium was then sterilized by autoclaving for 20 min. at 121 ºC. The percentage of explants responding to shoot proliferation, the number of shoots per explants and length of the shoots was recorded and evaluated after 4 weeks. One shoot or 2 shoots together were subcultured on MS medium supplemented with different concentrations of cytokinins (BAP and Kn) alone or in combinations with auxins for *in vitro* shoot multiplication.

Culture conditions

The cultures were maintained at 26 ± 2 °C under 16 h light photoperiod with light intensity of 1600 lux, obtained by cool white fluorescent tubes of 40 watts [Philips, India].

In vitro root induction of micropropagated shoots

Healthy shoots with 2-3cm length were used for *in vitro* root induction. Various concentrations of auxins IBA (0.49, 1.23, 2.46, 4.92, 7.32 µM) and NAA (0.27, 0.54, 1.34, 2.69, 5.37 µM) were studied for *in vitro* rooting.

Hardening and acclimatization

In vitro rooted plantlets were removed from culture vessels and washed with distilled water to remove adhered traces of nutrient agar. Plants were carefully transferred to bottles containing autoclaved soilrite moistened with ½ MS medium without organics. After rooted plantlets established in soilrite containing capped bottles, caps were gradually loosened and finally removed within 2 weeks. After 3-4 weeks, the *in vitro* rooted plantlets

were transferred into polybags containing mixture of farmyard manure, soil and sand (1:1:1).

Experimental design, data collection and statistical analysis

MS medium without hormone was treated as control in all experiments. All experiments were repeated three times. Observations were recorded after 4 weeks of interval. The results are expressed as mean ± SE of three experiments. The data was analyzed statistically using SPSS version 17 and significant difference between means were assessed by Duncan's multiple range test (DMRT) at $P = 0.05$.

Results

Axillary shoot proliferation

Mature nodal explants of *P. marsupium* (Fig. 2A) were inoculated on MS medium supplemented with different concentrations of cytokinins, BAP and Kn for axillary shoot proliferation. In 4 weeks axillary bud break was obtained (Fig. 2B). The percentage bud break response, average number of axillary proliferated formed per explants and average shoot length all varied considerably with the type and concentrations of growth regulator used in MS medium (Tab. 1).

Tab. 1. Effect of various cytokinins (BAP or Kn) in MS medium on *in vitro* axillary shoot proliferation of *Pterocarpus marsupium*

BAP(µM)	Kn (µM)	Percentage shoot proliferation	Mean shoot number	Mean shoot length (cm)
0.0	-	0.00	0.00	0.00
2.22	-	26.66	1.16 ± 0.11^{a}	0.71 ± 0.02^{a}
4.44	-	31.10	1.57 ± 0.20^{b}	0.79 ± 0.06^{a}
8.86	-	46.66	1.90 ± 0.19^{b}	1.10 ± 0.03^{b}
13.32	-	37.77	1.29 ± 0.14^{a}	1.08 ± 0.01^{b}
17.76	-	31.10	1.07 ± 0.71^{a}	0.77 ± 0.03^{a}
-	2.32	35.55	1.12 ± 0.08^{a}	0.90 ± 0.03^{a}
-	4.65	44.44	1.52 ± 0.11^{b}	1.03 ± 0.03^{b}
-	9.30	51.10	2.13 ± 0.14^{c}	1.19 ± 0.02^{c}
-	13.95	64.44	2.51 ± 0.10^{d}	1.47 ± 0.02^{d}
-	18.59	51.10	1.91 ± 0.13^{c}	1.26 ± 0.016^{c}

Values are in mean ± SE. Means followed by the same letter within columns are not significantly different (P =0.05) using Duncan's multiple range test.

Cytokinins played a significant role in inducing shoot proliferation from axillary bud. Of the two cytokinins used, Kn was found to be more effective than BAP for shoot proliferation from axillary bud. At lower concentration of Kn (2.32-9.30 µM), the bud break response was less. Increased concentration of Kn (13.95 µM), increased the response percentage and number of proliferated shoots. Whereas higher concentration of Kn beyond 13.95 µM resulted in decreased percentage bud break response. MS medium supplemented with 13.95 µM Kn, with additives (568 µM Ascorbic acid, 260 µM Citric acid, 605 µM Ammonium sulphate and 217 µM Adenine sulphate) was found to be optimal for maximum bud break response of 64.44% with 2.51 ± 0.10 numbers of axillary shoots.

Nodal segments containing axillary buds were cultured on MS medium supplemented with different concentration of BAP. Bud break response of 46.66% was obtained at 8.86 µM concentration of BAP supplemented MS medium. Response percentage as well as number of axillary shoot proliferated per explant was 1.16 ± 0.11 at 2.22 µM, which increased to 1.90 ± 0.19 shoots per explants at 13.32 µM. Further increase in BAP concentration beyond 13.32 µM resulted in decreased bud break response.

Nutrient medium plays a vital role in propagation through tissue culture. Three basal media (MS, WPM, and B_5) were tested to assess the effect on axillary bud proliferation. Results exhibited that MS medium was best for bud break response (64.44%) as compared to WPM (48.88%) and B_5 (39.99%). The number of shoots proliferated (2.51) and their length (1.47) found best on MS medium supplemented with 13.95 µM Kn (Fig. 1).

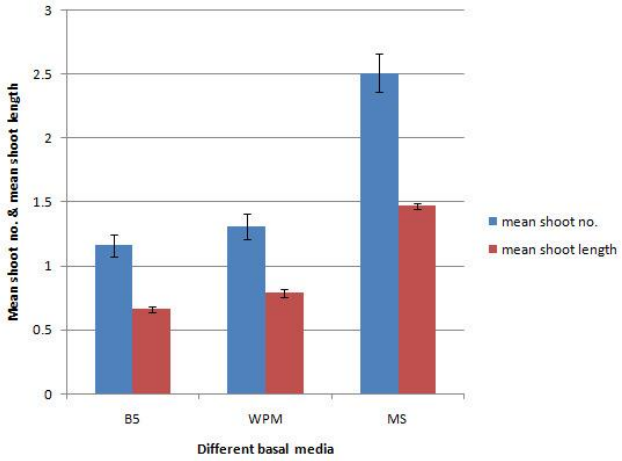

Fig 1. Effect of different basal media on shoot proliferation from nodal explants of *Pterocarpus marsupium*

In vitro shoot multiplication

The proliferated *in vitro* axillary shoots were excised from mother explants and subcultured on MS medium supplemented with cytokinins for establishment of cultures and multiplication of *in vitro* shoots. Response of *in vitro* shoot multiplication varied with cytokinin type and its concentration used in the medium (Tab. 2) BAP at 4.44 µM concentration found to be optimal, which gave an average of 4.82 *in vitro* shoots. Increased BAP concentration (13.32 µM) resulted decreased multiplication potential and only 4.17 *in vitro* shoots were obtained. Length of regenerated shoots also decreased with increase of BAP concentration. Regenerated shoots had an average length of 1.58 cm on MS medium supplemented with 4.44 µM BAP, which is decline to 1.31 cm at 13.32 µM BAP level.

On kinetin supplemented MS medium, 9.30 µM Kn gave optimal response for *in vitro* shoot multiplication 4.92 shoots per explants were developed (Fig. 2C). There was a decrease in number of shoots decreased at concentrations lower and higher than 9.30 µM Kn. At 2.32 µM Kn, average 4.62 shoots per explants were developed whereas 4.16 shoots per explants developed at 13.95 µM Kn. Average length of regenerated shoots on 2.32 µM

Kn supplemented medium was 1.32 cm which increased to 1.70 cm at 9.30 µM Kn and then declined to 1.31 cm at 13.95 µM Kn.

Tab. 2. Effect of various types of cytokinins (BAP, Kn) in MS medium on *in vitro* shoot multiplication of *Pterocarpus marsupium*

BAP(µM)	Kn (µM)	Mean shoot number	Mean shoot length (cm)
2.22	-	4.27 ± 0.09^{a}	1.32 ± 0.02^{a}
4.44	-	4.82 ± 0.11^{b}	1.58 ± 0.02^{c}
8.86	-	4.62 ± 0.12^{ab}	1.50 ± 0.02^{b}
13.32	-	4.17 ± 0.07^{a}	1.31 ± 0.01^{a}
-	2.32	4.62 ± 0.12^{ab}	1.32 ± 0.02^{a}
-	4.65	4.34 ± 0.11^{a}	1.54 ± 0.02^{c}
-	9.30	4.92 ± 0.12^{b}	1.70 ± 0.03^{b}
-	13.95	4.16 ± 0.06^{a}	1.31 ± 0.01^{a}

Values are in mean ± SE. Means followed by the same letter within columns are not significantly different (P =0.05) using Duncan's multiple range test.

Cytokinin alone did not influence *in vitro* shoot multiplication in *P. marsupium*. Auxins with cytokinin enhanced *in vitro* shoot multiplication. Therefore, cytokinin-auxin interaction was also studied for *in vitro* shoot multiplication (Tab. 3). Kn (4.65, 9.30, 13.95 µM) was used in combination with NAA (0.27, 0.54, 1.34 µM). It was observed that Kn in combination with NAA increased *in vitro* shoot multiplication response. An average number of 4.16 shoots per explant was obtained at 13.95 µM Kn alone, while addition of NAA with Kn in medium increased *in vitro* shoot multiplication. The maximum 6.21 shoots was observed on 9.30 µM Kn + 0.54 µM NAA supplemented with MS medium.

Tab. 3. Effect of cytokinin-auxin interaction (Kn + NAA) in MS medium on *in vitro* shoot multiplication of *Pterocarpus marsupium*

Kn(µM)	NAA(µM)	Mean shoot number	Mean shoot length (cm)
4.65	0.27	4.27 ± 0.08^{ab}	1.41 ± 0.02^{abc}
	0.54	5.00 ± 0.10^{e}	1.59 ± 0.02^{d}
	1.34	4.66 ± 0.09^{cd}	1.47 ± 0.02^{c}
9.30	0.27	4.86 ± 0.11^{de}	1.59 ± 0.03^{d}
	0.54	6.21 ± 0.11^{f}	1.67 ± 0.01^{e}
	1.34	4.55 ± 0.12^{bc}	1.38 ± 0.02^{ab}
13.95	0.27	3.44 ± 0.09^{bc}	1.44 ± 0.02^{bc}
	0.54	4.27 ± 0.07^{ab}	1.39 ± 0.01^{ab}
	1.34	4.11 ± 0.05^{a}	1.35 ± 0.01^{a}

Values are in mean ± SE. Means followed by the same letter within columns are not significantly different (P =0.05) using Duncan's multiple range test.

***In vitro* rooting**

The *in vitro* produced shoots were capable of inducing roots when cultured on half strength MS medium containing auxins (Tab. 4). Two auxins were tried for *in vitro* rooting. IBA had pronounced effect on *in vitro* rooting than NAA. On medium supplemented with 4.92 µM IBA, 42% rooting was observed. Any increase and decrease of 4.92 µM IBA

levels reduced the rooting percentage. *In vitro* roots were also induced when the *in vitro* shoots were cultured in NAA supplemented half strength MS medium, where rooting response of 28.88% was obtained on 2.69 µM NAA (Fig. 2D).

Tab. 4. Effect of different concentration of IBA and NAA on *in vitro* rooting of *Pterocarpus marsupium*

IBA (µM)	NAA (µM)	Rooting %	Mean root number	Mean root length
0.00	-	0.00	0.00	0.00
0.49	-	0.00	0.00	0.00
1.23	-	28.88	1.38±0.01[a]	0.58±0.05[a]
2.46	-	35.00	1.57±0.02[a]	0.82±0.06[a]
4.92	-	42.22	2.14±0.17[b]	1.24±0.06[b]
7.32	-	31.10	1.82±0.12[a]	0.76±0.05[a]
	0.27	0.00	0.00	0.00
	0.54	19.99	1.00±0.00[a]	0.50±0.04[a]
	1.34	26.66	1.19±0.08[a]	0.62±0.03[a]
	2.69	28.88	1.29±0.14[a]	0.94±0.05[a]
	5.37	26.66	1.25±0.13[a]	0.71±0.02[a]

Values are in mean ± SE. Means followed by the same letter within columns are not significantly different (P =0.05) using Duncan's multiple range test.

Hardening and acclimatization

The *in vitro* raised plantlets were successfully acclimatized first under culture room conditions and then in the green house. The four weeks old plantlets were transferred in screw cap glass bottles containing 1/3 volume of autoclaved soilrite. These plantlets were nurtured with half strength MS medium (without organics) twice a week and were kept for four weeks in culture room. Then these bottles containing plantlets were transferred to mist chamber. Plants were then transferred to polybags containing sand: soil: FYM in 1:1:1 proportion and were kept in the mist chamber (Fig. 2 E&F). Under the mist chamber the plants started to harden and were shifted to shade house conditions for further acclimatization.

Fig 2. Micropropagation of *Pterocarpus marsupium* from nodal explants (A-F) **A**. Nodal segments of *P. marsupium.* **B**. Axillary shoot proliferation from nodal segment containing axillary bud of *P. marsupium* on MS medium supplemented with Kn (13.95 µM) + additives. **C**. *In vitro* shoot multiplication on MS medium supplemented with Kn (9.30 µM), NAA (0.54 µM) and additives. **D**. Induction of rooting from microshoots of *P. marsupium* on ½ MS medium supplemented with IBA (4.92 µM). **E & F**. Hardening of *in vitro* raised plantlets.

Disscusion

In the present investigation, micropropagation protocol of *Pterocarpus marsupium* from nodal explants derived from about 10 years old tree was established. During the present investigation, effect of two cytokinins, BAP and Kn were studied for axillary bud proliferation of *P. marsupium*. Axillary bud proliferation was more on Kn as compared to medium supplemented with BAP. This result is contrasting with reports on leguminous species *Dalbergia sissoo* Roxb. and *Macrotyloma uniflorum* [ARYA & al. 2013; BISHT & al. 2013] where BAP gave maximum shoot proliferation.

The effect of different basal media (MS, WPM, B_5) on axillary shoot proliferation was also tested. During present investigation it was observed that MS medium was better for axillary bud proliferation compared to other media (WPM and B_5).The findings are in agreement with earlier reports on many woody tree species including *Swartzia madegascariensis* and *Lagerstromia parviflora* [BERGER & SCHAFFER, 1995; TIWARI & al. 2002]. Whereas, WARAKAGODA & SUBSINGHE (2013) suggested that B5 medium was superior to MS for plant regeneration of *Pterocarpus santalinus*.

Shoot multiplication is the major criterion for successful micropropagation. *In vitro* shoot multiplication is affected by numerous factors, such as physiological status of plant material, culture medium and culture environment. Cytokinins are essential for *in vitro* shoot multiplication. In the present investigation two cytokinins, BAP and Kn were studied for *in vitro* shoot multiplication. The inclusion of cytokinin and auxin in the culture media stimulated the *in vitro* multiplication and growth of shoots in several plant species [GEORGE, 1993]. Interaction of cytokinin-auxin was investigated for *in vitro* shoot multiplication and different combinations of Kn and NAA were tried. Results illustrated maximum shoot numbers (6.22) on MS medium supplemented with 9.30 µM Kn + 0.54 µM NAA + additives. The results substantiate with earlier findings of several workers, where the addition of low level of auxin with cytokinin promoted shoots in *Acacia catechu*, *Eucalyptus grandis* and *Lagerstromia parviflora* [KAUR & al. 1998; CID & al. 1999; TIWARI & al. 2002]. Higher concentrations of auxins resulted in callus formation at the base of shoots, which is undesirable feature for *in vitro* shoot multiplication. Additives have also been reported to improve the multiplication rate and length of the shoots when added in combination with kinetin [KHAN & al. 2014]. The promotive role of additives in shoot multiplication has been reported in different woody species namely, *Tectona grandis*, *Bauhinia vahlli* and *Melia azedarach* [DEVI & al. 1994; DHAR & UPRETI, 1999; HUSAIN & ANIS, 2009].

As a woody perennial, *P. marsupium* is difficult to root. The ability of plant tissues to form roots depends on interaction of many endogenous and exogenous factors. A varied effect of auxins IBA, NAA was observed by incorporating them in MS medium at different concentrations. Our observation on root induction in *in vitro* shoots of *P. marsupium* reveals that IBA is more effective than any other auxin on root induction. Effect of IBA for *in vitro* rooting has also been reported in leguminous species like *Acacia auriculiformis* [RANGA RAO & PRASAD, 1991] and *Prosopis tamurago* [NANDWANI & RAMAWAT, 1992].

Conclusions

To conclude, the present communication describes an *in vitro* propagation protocol for *P. marsupium* using nodal explants. The protocol outlined above offers *in vitro* propagation and conservation of this economically important multipurpose tree and would facilitate its use for future tree improvement programme using genetic transformation technology.

Abbreviations: Kn – Kinetin; NAA – α Naphthelene acetic acid; IBA – Indole butyric acid; min. – Minutes; h – hour.

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MACROPROPAGATION OF PLANTAIN (*MUSA* SPP.) CULTIVARS PITA 3, FHIA 21, ORISHELE AND CORNE 1: EFFECT OF BENZYLAMINOPURINE (BAP) CONCENTRATION

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Abstract: In Africa, plantain is one of the most important starchy food and cash crops. Nonetheless, one of the major constraints for its production was the unavailability of healthy planting materials at planting time. This constraint could be lifted using the cloning of planting materials via the *in vitro* micropropagation or *in vivo* macropropagation techniques. Shelled corms from four cultivars, known as PITA 3, FHIA 21, ORISHELE and CORNE 1, were used. Three treatments differing in three hormonal concentrations, especially 20.0, 30.0 and 40.0 mg L^{-1} were tested. The control one was hormone free. Tested treatments were laid out in a split plot design. The decorticated banana corms were sprayed twofold at 2 weeks interval with BAP solution when placed in sterilized soil in high humidity plastic tunnel. It emerged from results, regarding BAP concentration effect, that BAP treatment with 40 mg L^{-1} significantly reduced the emergence time of shoots at 20 days as against 25.1, 28.3 and 28.5 for the 2 tested other treatments as well as control, respectively. Likewise, the concentrations 40.0 mg L^{-1} both recorded the largest number of sprouted buds per corm and number of shoots per corm. With respect to banana cultivar effect, PITA 3 showed the largest number of shoots per corm. Basing on such findings, it is concluded that MSD technique combined with BAP at 40.0 mg L^{-1} is a suitable technique for improving of the *in vivo* macropropagation of plantain. This concentration increased at least 50 % of sucker production compared to control.

Keywords: Plantain, *in vivo* macropropagation, MSD, Benzlyamimopurine (BAP)

Introduction

Plantain is a staple food for many people in Africa. Nearly 30 million tons of plantain is yearly produced in Africa, mostly by small holders and consumed locally [FAO, 2010]. The demand for this local product is very high in rural and urban markets. Plantain is ranked among the most preferred foodstuffs, highly valued and contributes in feeding more than 250 million people in countries of West and Central Africa [TOMEKPE & al.

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2011]. It is also a major source of income for many people and actors in the supply chain in the rural and urban sectors [JACOBSEN & al. 2004].

In spite of its great socioeconomic importance, the cultivation of the crop has however never satisfied the domestic demands. Low production is due to pest and disease constraints such as, among others, nematodes [FOGAIN, 2000]. Likewise, banana weevils, and foliar diseases such as black leaf streak caused by (*Mycosphaerella fijiensis*) are part of these constraints. Such a situation is aggravated by poor agronomic practices.

Plantain as parthenocarpic and seedless is traditionally propagated by planting corms and suckers. Suckers are traditionally used by farmers as planting materials coming from their own plantations. Theses suckers are most of time affected with pests (e.g., nematodes and weevils) and diseases (e.g. viruses such as banana bunchy top, banana streak). The suckering ability of plantain is very low with an average of about 3 suckers per year per stool depending on agro-climatic conditions and cropping practices [JOAB, 2004]. The lack and poor quality of planting materials are threatening plantain production and limit the expansion of plantations [DZOMEKU & al. 2014]. The quantity and quality of the planting material are major factors for successful crop production [TENKOUANO & al. 2006]. This could be achieved through clonal planting materials obtained via the *in vitro* micropropagation or *in vivo* macropropagation techniques. *In vivo* macropropagation is an alternative technique for mass production of banana planting materials under *in vivo* conditions [KWA, 2003]. Compared to the *in vitro* one, this technique is relatively simple, less expensive and provides in a short period pest-free and genetically identical plantlets [KINDIMBA & MSOGOYA, 2014].

The *multiplication sur souches décortiquées* (MSD) is one of methods of banana for *in vivo* macropropagation technique. This method exploits the entire potential of the corms to produce large quantities of healthy planting materials within a short period from secondary buds [KWA, 2003; NJUKWE & al. 2005; MSOGOYA & MWAKISITU, 2014]. Nevertheless, information about the response of plantain to MSD method in combination with BAP at different concentrations is hardly known. Benzylaminopurine is an adenine-based cytokinin popularly used for *in vitro* induction of axillary and adventitious shoots in banana [KALIMUTHA & al. 2007; BHOSALE & al. 2011; DEVENDRAKUMAR & al. 2013] and rarely used for *in vivo* macropropagation [KINDIMBA & MSOGOYA, 2014]. The testing of this technique in combination with BAP at different concentrations might allow the identifying of a concentration which may trigger strong production of suckers from corms as a function of cultivars. The objective of this study was to evaluate the effect of BAP concentration on *in vivo* proliferation technique (MSD) of 4 cultivars tested.

Materials and methods

Plant material, culture preparation and conditions

The study was carried out in 2012 and repeat in 2013 during 6 months at the Centre National de Recherche Agronomique (CNRA, Côte d'Ivoire), Azaguie Station, at 05°18'N and 04°09'W, 20 m above sea level. Four cultivars were used in this study. The CORNE 1 (False Horn) and ORISHELE (False Horn) are triploid plantains (*Musa* spp. AAB group) and are the most important varieties popularly grown in Côte d'Ivoire. The

FHIA 21 and PITA 3 are plantain-like hybrids belonging to the genome group AAAB with a ploidy level of 4x. FHIA 21 and PITA 3 hybrids are respectively crossed between AAB Plantain cv. AVP-67 x SH-3142 and AAB Plantain cv. Obino l'Ewaï x Calcutta 4. Ten corms of plant mother of each cultivar of 8 months were carefully removed from field grown banana plant. Corns weighing between 7 and 8 kg were used for the experiment. The corms were cleaned, pared to remove roots. The leaf sheathes of the corms were carefully stripped away one by one, to expose axillary buds nodes at the basis of each leaf (Fig. 1). The apical meristem of each corm was destroyed by decortication to overcome the apical dominance. The materials generated were planted 5cm deep in sterilized soil in high humidity plastic tunnel with temperature of 25–30 °C.

Experimental design

Two factors, namely cultivar and hormonal (BAP) concentration, each with 4 variants, were used. In all, 8 treatments, obtained from combination of the variants of 2 aforesaid factors, were repeated threefold and laid out in a split plot design. A replication consisted of 10 corms of each cultivar. Banana cultivars were the main plot factor while BAP concentrations (0.0, 20.0, 30.0 and 40.0 mg L^{-1}) were the sub-plot factor. The decorticated banana corms were pulverized by 10 ml of each BAP concentration twofold at 1 week interval. Irrigation was regularly carried out to maintain moist environment.

Data collection and statistical analysis

Seven variables were measured. These were : i) the number of days to first shoot or sucker emergence, ii) the number of sprouted buds per corm, iii) the number of shoots per corm, iv) the shoot height, v) the shoot collar girth, vi) the number of roots per shoot and vii) number of leaves per shoot. The collected data were analysed using STATISCA 6.0 software. The Bartlett's test for equality of variance of sub-populations analysed as well as that of Shapiro-Wilks for normality of the distributions of measured variables were used. These tests were performed prior to analysis of variance (ANOVA). Means were separated according to Student-Newman-Keuls' test at 0.05 probability.

Results and discussion

Under field conditions, suckers production by plantains is very low. Indeed, in spite of the presence of several axillary buds, they produced only about 10 suckers during the crop cycle due to apical dominance. Even if, the apical dominance is removed at flowering, often it is only few of the primary buds that develop into daughter suckers. However, with this MSD technique, several of axillary buds could be activated to sprout as healthy seedlings for planting. The same result could be obtained with the PIBS (Plants Issus de Bourgeons Secondaires) technique [KWA, 2003; DZOMEKU & al. 2014]. The results of our study revealed a significant effect of BAP concentration on axillary buds activation. The number of axillary buds activated significantly ($P \leq 0.05$) increased as BAP concentration increased from 0.0 to 40.0 mg L^{-1} (Tab. 1). BAP at 40.0 mg L^{-1} resulted in the largest number of sprouted buds of 12.4 buds per corm followed by BAP at 30.0, 20.0 and 0.0 mg L^{-1} with 9.9, 8.5, and 7.0 sprouted buds per corm, respectively.

Results also indicated that BAP concentration had a significant ($P \leq 0.05$) influence on the number of days from corm sowing to first shoot emergence and number of shoot per corm (Tab. 1). Banana corms treated with BAP at 40.0 mg L^{-1} produced the first shoot earlier at 20.0 days followed by corms treated with BAP at 30.0, 20.0 and 0.0 mg L^{-1} with 25.1, 28.5 and 28.3 days, respectively (Tab. 1). This result quite agrees with the study of KINDIMBA & MSOGOYA (2014) who shows the effect of BAP on first shoot emergence. In another study, MSOGOYA & MWAKISITU (2014) reported the action of another cytokinin (Thidiazuron, TDZ) on the first shoot emergence and the number of shoot per corm. Cytokinins such as benzyl amino purine (BAP) and Kinetin are generally known to reduce the apical meristem dominance and induce both axillary and adventitious shoots formation from meristematic explants in banana [DEVENDRAKUMAR & al. 2013]. The effectiveness of BAP over other cytokinins in inducing multiplication of shoot tip cultures has been reported in different cultivars of banana *in vitro* micropropagation and *in vivo* macropropagation [BUAH & al. 2010; AZAM & al. 2010; JAFARI & al. 2011; BHOSALE & al. 2011; KINDIMBA & MSOGOYA, 2014]. Sometime, the BAP is combined with additives like bio-fertilizers such as *Bacillus subtilis* to induce more sprouting of axillary buds in banana [SAJITH & al. 2014].

With respect to BAP concentration effect, in relation to the number of shoots per corm, the number of shoots significantly ($P \leq 0.05$) increased with BAP concentration from 0.0 to 40.0 mg L^{-1} (Tab. 1). Four statistically different groups of means were evidenced. First, represented by BAP concentration of 0.0 mg L^{-1} was characterised by very low number of shoots per corm (165.0 shoots). Second, illustrated by BAP concentration of 20.0 mg L^{-1} differed from the first by low number of shoots per corm (181.2 shoots). Third, consisting of BAP concentration of 30.0 mg L^{-1} was marked by fairly high number of shoots per corm (204.8 shoots). Fourth, comprising BAP concentration of 40.0 mg L^{-1} stood out from the first 3 groups by the highest number of shoots per corm (280.8 shoots).

Number of leaves per sucker, sucker height and sucker collar girth significantly ($P \leq 0.05$) increased as BAP concentration increased from 0.0 to 40.0 mg L^{-1} (Tab. 2). Moreover, number of roots per sucker did not influenced by BAP concentration. Corms treated with BAP at 40.0 mg L^{-1} produced suckers with the largest number of leaves of 4.4 per sucker followed by corms treated with BAP at 30.0, 20.0 and 0.0 mg L^{-1} with 4.0, 3.4 and 2.9 leaves per sucker, respectively. Conversely, corms treated with BAP at 40.0 mg L^{-1} had largest collar diameter and tallest sucker followed by corms treated with BAP at 30.0, 20.0 and 0.0 mg L^{-1}. Similar result was obtained by KINDIMBA & MSOGOYA (2014) with the positive action of BAP at 1.5 and 3.0 mg L^{-1} on banana growth parameters and shoots production. Contrary to KINDIMBA & MSOGOYA (2014) work, positive responses of banana were obtained in our study with high BAP concentration (40.0 mg L^{-1}) where corms from mother plant were used compared to corms from suckers used by KINDIMBA & MSOGOYA (2014). In a similar experiment, MANZUR MACIAS (2001) increased suckers proliferation by injecting 4.0 ml of BAP at 40.0 mg L^{-1} in the cavity left by the removal of the apical meristem of the corms. Under *in situ* conditions where BAP at 40.0 mg L^{-1} treated sucker produced an average of 4 suckers at both G1s and G2s stages and the same technique applied to G3s produced an average of 13 plantlets, which are very similar to those obtained *in vitro*.

Banana cultivar had a significant ($P \leq 0.05$) effect on the number of sprouted buds per corm, number of days to first shoot emergence and number of shoots per corm (Tab. 3).

Cultivar PITA 3 (plantain-like hybrid) produced the first shoot earlier at 26.3 days and the largest number of sprouted buds with 8.0 buds per corm against 5.8, 6.3 and 7.8 buds sprouted per corm in ORISHELE, CORNE 1 and FHIA 21, respectively. PITA 3 also produced the largest number of shoots of 198.2 per corm compared with banana cv. CORNE 1, ORISHELE and FHIA 21 with 137.3, 147.8 and 178.6 shoots per corm, respectively. However, banana cultivar had no significant ($P \leq 0.05$) effect on sucker height, collar girth, number of leaves and roots per sucker. The results of the investigation revealed an influence of the banana variety to MSD technique on shoot proliferation. Indeed, varieties behavior is not the same *in vivo* macropropagation [DZOMEKU & al. 2014, MSOGOYA & MWAKISITU, 2014]. PITA 3 and FHIA 21 belonging to the same subgroup of plantain (French's group) produced the largest number of sucker compared to CORNE 1 and ORISHELE belonging to False Horn group. This result concurs with those of KWA (2003), who found that the average number of suckers was significantly higher for French clair (French) and French sombre (French) with approximately 18 and 17 suckers, respectively than Bâtard (False Horn) and Mbouroukou N° 1 (False Horn) with approximately 14 and 16 suckers, respectively. The best performance of FHIA 21 and PITA 3 seems to be linked to their ploidy level. FHIA 21 and PITA 3 are hybrids belonging to the genome group AAAB with a ploidy level of 4x, whereas, CORNE 1 and ORISHELE are triploid plantains belonging to the genome group AAB with a ploidy level of 3x. The superiority of tetraploid hybrids would be partly due to gene dosage effects at polyploid level [ORTIZ, 1995; TOMEKPE & al. 1995].

The interaction of banana cultivars and BAP concentrations had a significant ($P \leq 0.05$) effect on number of sprouted buds per corm (Fig. 2) and number of shoots per corm (Fig. 3). Banana cv. PITA 3 produced the largest number of sprouted buds of 13.3, 10.9, 9.5 and 8.0 buds per corm with BAP at 40.0, 30.0, 20.0 and 0.0 mg L^{-1} respectively, followed by FHIA 21, CORNE 1 and ORISHELE (Fig. 2). Shoot production by PITA 3 was the highest among the cultivars whatever BAP concentrations. Indeed, PITA 3 produced the largest number of shoot of 344.1, 240.5, 213.1 and 198.1 shoots per corm with BAP at 40.0, 30.0, 20.0 and 0.0 mg L^{-1} respectively followed by FHIA 21, ORISHELE and CORNE 1 (Fig. 3). The interaction of banana cultivars and BAP concentrations had no significant ($P \leq 0.05$) effect on sucker height, sucker collar girth and number of leaves per sucker.

Conclusion

We postulated that there may be a BAP concentration able to increase effectiveness of the *in vivo* macropropagation of plantain. After the testing, only the BAP concentration equal to 40.0 mg L^{-1} was the most effective. The *multiplication sur souches décortiquées* (MSD) technique is an effective method that could generate large quantities of healthy planting materials from any type of corms. The technique was more efficient when combined with BAP at 40.0 mg L^{-1}. This dose increased at least 50 % of shoots production. The response of banana to BAP depends on cultivars where cv. PITA 3 provides the highest *in vivo* multiplication rate with BAP at 40.0 mg L^{-1}. Further studies are required to test the responses of other plantain cultivars to *in vivo* macropropagation in combination with different BAP concentrations or other cytokinin growth regulators.

Tab. 1: Effect of BAP concentrations on *in vivo* multiplication of plantain cultivars

BAP concentration (mg L^{-1})	Number of sprouted buds per corm	Number of days to first shoot emergence	Number of shoots per corm
0.0	7.0^{a}	28.5^{a}	165.0^{a}
20.0	8.5^{b}	28.3^{a}	181.2^{ab}
30.0	9.9^{c}	25.1^{b}	204.8^{b}
40.0	12.4^{d}	20.0^{c}	280.8^{c}
CV (%)	15.1	12.3	17.4

Means followed by the same letters within the column are not significant different at 5% level after Student Newman-Keuls' test.

Tab. 2: Effect of BAP concentrations on growth parameters of plantain suckers

BAP concentration (mg L^{-1})	Number of leaves per suckers	Sucker height (cm)	Sucker collar girth (cm)	Number of roots per sucker
0.0	2.9^{a}	10.2^{a}	0.9^{a}	8.8^{a}
20.0	3.4^{b}	11.1^{b}	1.1^{b}	9.1^{a}
30.0	4.0^{c}	12.5^{c}	1.3^{c}	9.2^{a}
40.0	4.4^{d}	14.2^{d}	1.9^{d}	9.3^{a}
CV (%)	16.2	12.7	20.1	9.7

Means followed by the same letters within the column are not significant different at 5% level after Student Newman-Keuls' test.

Tab. 3: Response of plantain cultivar to MSD technique on *in vivo* shoot proliferation

Cultivar	Number of sprouted buds per corm	Number of days to first shoot emergence	Number of shoots per corm
CORNE 1(False Horn)	6.3^{a}	28.3^{a}	137.3^{a}
ORISHELE (False Horn)	5.8^{a}	28.1^{a}	147.8^{a}
PITA 3 (French)	8.0^{b}	26.3^{b}	198.2^{b}
FHIA 21 (French)	7.8^{b}	28.0^{a}	176.6^{b}
CV (%)	18.3	10.4	15.8

Means followed by the same letters within the column are not significant different at 5% level after Student Newman-Keuls' test.

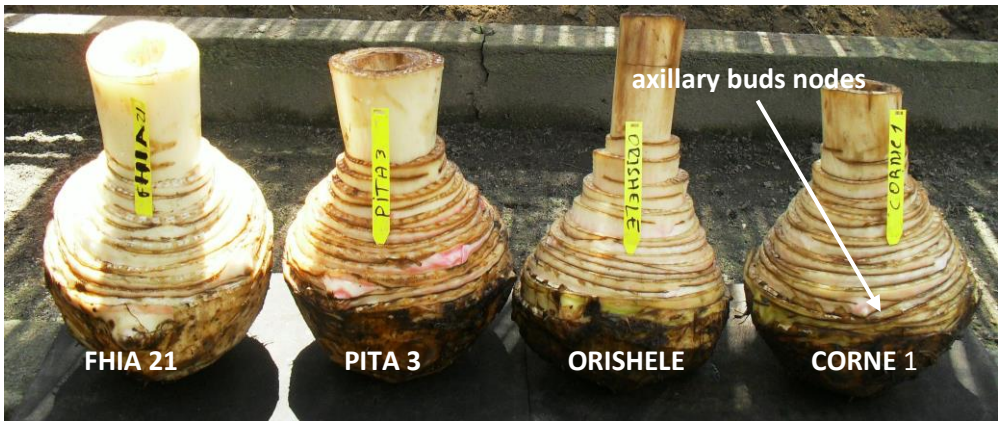

Fig. 1. Deshealthed and decorticated corms of plantain cultivars FHIA 21, PITA 3, ORISHELE and CORNE 1

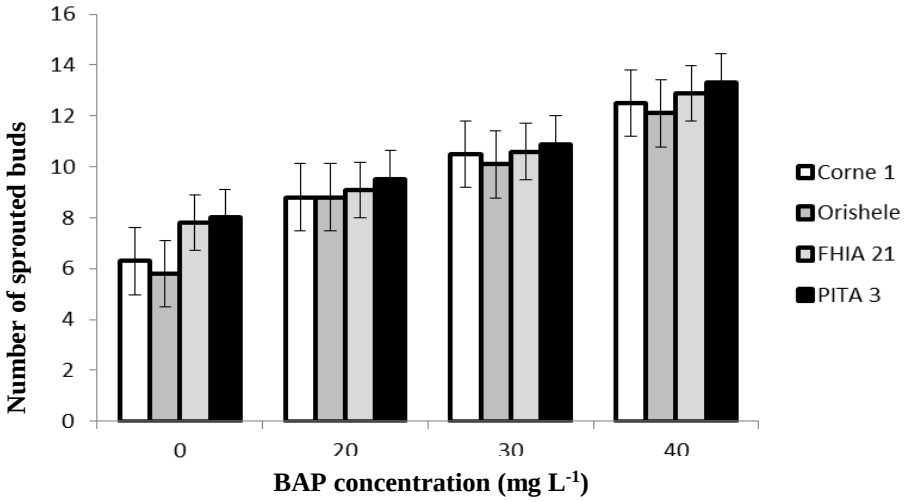

Fig. 2. The effect of different BAP concentrations on number of sprouted buds per corm of plantain cultivars CORNE 1, ORISHELE, FHIA 21 and PITA 3. Bar indicates the standard error of mean

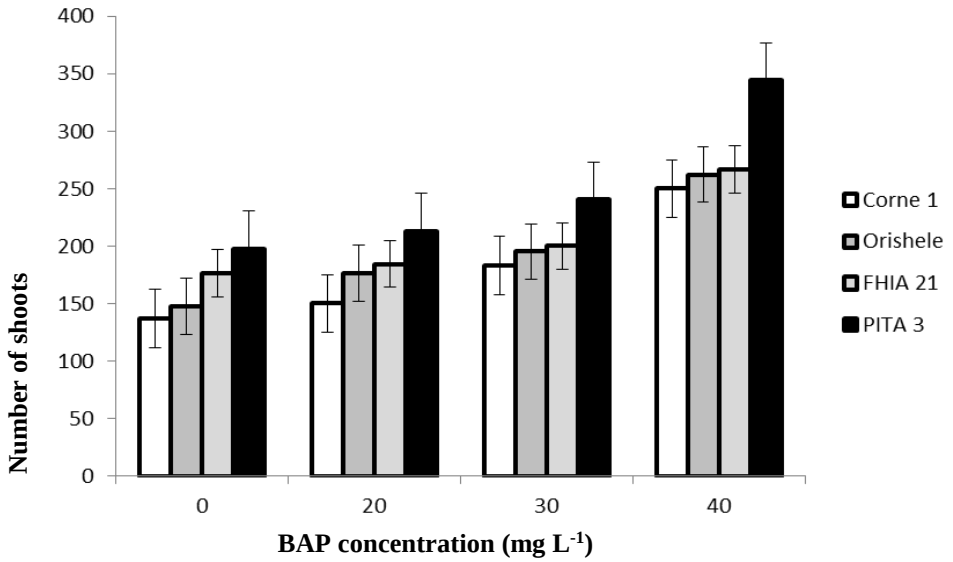

Fig. 3. The effect of different BAP concentrations on number of shoots per corm of plantain cultivars CORNE 1, ORISHELE, FHIA 21 and PITA 3. Bar indicates the standard error of mean

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ALTERATIONS TO PLBS AND PLANTLETS OF HYBRID *CYMBIDIUM* (ORCHIDACEAE) IN RESPONSE TO PLANT GROWTH REGULATORS

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Abstract: A previous study examined, in detail, the morphological response of hybrid *Cymbidium* Twilight Moon 'Day Light' protocorm-like bodies (PLBs) to 26 plant growth regulators (PGRs). In this study, flow cytometric analyses of the PLBs derived from several of these PGR treatments revealed changes in the ploidy of PLBs while the ploidy of plant leaves remained constant. The SPAD value of leaves of plants derived from PGR treatments changed significantly. The choice of PGR must be accompanied by careful scrutiny of the possible resulting changes to morphology and physiological parameters.

Keywords: flow cytometry; plant growth regulator; PLB; SPAD; Teixeira *Cymbidium* (TC) medium; thin cell layer

Introduction

Cymbidium (Orchidaceae) is a *de facto* model orchid genus in terms of *in vitro* development [HOSSAIN & al. 2013; TEIXEIRA DA SILVA, 2013a, 2013b]. The protocorm-like body (PLB) is an effective propagule for *in vitro* clonal micropropagation and is a *de facto* somatic embryo [TEIXEIRA DA SILVA & TANAKA, 2006). A previous study examined the effect of 26 plant growth regulators (PGRs) on the resulting production of new PLBs, including the development of new PLBs, or *neo*-PLBs, their fresh weight, with darkness generally having a more negative effect than light [TEIXEIRA DA SILVA, 2014a]. Using that study's experimental protocol, PLBs derived from treatment of 26 PGRs. In addition, the SPAD value of the leaves of plantlets derived from these treatments was measured, with the objective of understanding whether treatment with PGRs has any downstream effects on plant growth and development.

Materials and methods

Basal protocols, reagents, plant material

All basic protocols for the establishment of *in vitro* cultures, PLB induction and proliferation, and *neo*-PLB induction followed, broadly, [TEIXEIRA DA SILVA & al. 2005, 2006a, 2006b] for hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan). Teixeira *Cymbidium* (TC) medium 1 [TEIXEIRA DA SILVA, 2012a] was the basal medium used in this study, supplemented with 0.1 mg/l NAA, 0.1 mg/l Kin, 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA) after adjusting pH to 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. As indicated

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in TEIXEIRA DA SILVA (2014a), all chemicals and reagents, including PGRs, were of the highest analytical grade available and were purchased from either Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), unless specified otherwise. PLBs were kept on 40 ml medium in 100-ml Erlenmeyer flasks (10 PLBs/flask), double-capped with aluminium foil, and cultures were placed at 25 °C, under a 16-h photoperiod with a light intensity of 45 µmol/m^2/s provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan).

Plant growth regulator-derived treatments

Three groups of PGRs from a previous study [TEIXEIRA DA SILVA, 2014a], or from other studies referenced within that study, that induced some form of a growth response *in vitro*, in terms of PLBs, were employed (see abbreviations list and Tab. 1 footer for full definition) (all at 1 mg/l): 6 cytokinins (Ads; BA; 2iP; Kin; TDZ; *m*T), 6 auxins (2,4-D; IBA; NAA; picloram; TRIA; PG) and 6 other growth substances (ABA; dicamba; GA_3; JA; MeJA; SA). The control was PGR-free TC medium (i.e., excluding NAA and Kin).

Growth parameters assessed

Neo-PLBs that were 60 days old, prior to the development of shoot tips [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013], were used. Ploidy was determined in 6-day-old PLBs, as described next, and based on TEIXEIRA DA SILVA & TANAKA (2006) and TEIXEIRA DA SILVA & DOBRÁNSZKI (2014). Shoots that formed from PLBs were rooted individually on PGR-free Hyponex medium solidified with 7 g/l agar, as described in TEIXEIRA DA SILVA & al. (2006a, 2006b) and TEIXEIRA DA SILVA & TANAKA (2006). Well-rooted plantlets (i.e., shoots that had developed a robust root system) and had developed at least 6 fully developed leaves, were used to assess SPAD readings (chlorophyll content), based on TEIXEIRA DA SILVA & al. (2007) and TEIXEIRA DA SILVA & DOBRÁNSZKI (2014), in the third leaf (counting downward from the apex), with a chlorophyll meter (SPAD-502, Minolta, Japan).

Flow cytometry

PLBs or the leaves of plantlets (0.5 cm^2 of fresh material) were chopped in a few drops of nucleic acid extraction buffer (Partec Cystain UV Precise P, Germany) to isolate nuclei, then left to digest on ice for 5 min. Based on the protocol suggested by Mishiba and Mii (2000), the nuclear suspension was then filtered through a 30 µm mesh size nylon filter (CellTrics®) and five volumes of Partec Buffer A (2 µg/ml 4,6-diamidino-2-phenylindole (DAPI), 2 mM $MgCl_2$, 10 mM Tris, 50 mM sodium citrate, 1% PVP K-30, 0.1% Triton X-100, pH 7.5). After leaving this mixture at room temperature for 5 min, nuclear fluorescence was measured using a Partec® Ploidy Analyser with diploid barley (*Hordeum vulgare* L.) cv. 'Ryufu' serving as the internal control. Each biological sample (PLBs, leaves) was repeated in triplicate, and relative fluorescence intensity of the nuclei was analysed (coefficient of variation between samples < 3%; minimum of 5000 nuclei for each sample).

Statistical analyses

Experiments were organized according to a randomized complete block design with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate. Data was subjected to analysis of variance with mean separation by Tukey's multiple range

test using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leq 0.05$.

Results and discussion

Visually, PLBs formed under control conditions (Fig. 1A) or after exposure to BA (Fig. 1B) do not reveal visible differences. However, flow cytometry of *neo*-PLBs indicates that TDZ, mT, 2,4-D, IBA, picloram, TRIA, PG, ABA, GA_3, and MeJa induced some level of endopolyploidy (Tab. 1; Fig. 2), which is not unusual for *Cymbidium* [FUKAI & al. 2002; TEIXEIRA DA SILVA, 2014b] and other orchids [TEIXEIRA DA SILVA & al. 2014], even under control growth conditions, and is usually associated with the external layer of cells in the PLB [TEIXEIRA DA SILVA & TANAKA, 2006]. However, the leaf tissue of plantlets derived from PLBs, once placed on ideal regeneration medium, displays no polyploidy ([TEIXEIRA DA SILVA & TANAKA, 2006]; this study, data not shown), suggesting that endopolyploidy may be an evolutionarily disadvantageous genetic system that is naturally selected against. The chlorophyll content, and thus photosynthetic ability of plantlets, that were derived from different PGR treatments (2iP, TDZ, 2,4-D, picloram, ABA, dicamba, JA, and MeJA) showed a significant decrease (relative to the control) in SPAD value. Although *neo*-PLBs may appear morphologically similar after exposure to a PGR treatment, this study indicates the importance of testing the resulting *neo*-PLBs cytologically, and also the physiological performance of plants.

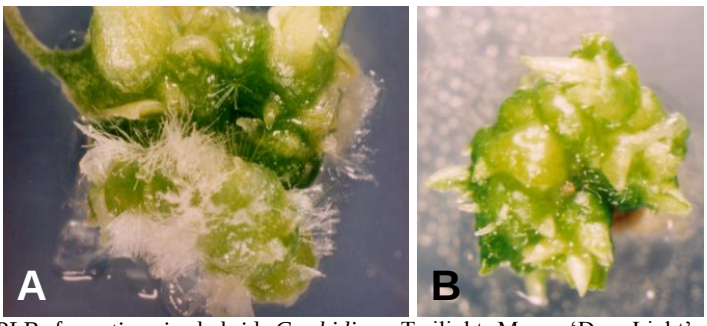

Fig. 1. *Neo*-PLB formation in hybrid *Cymbidium* Twilight Moon 'Day Light' under control conditions on TC medium [TEIXEIRA DA SILVA, 2012a] (A). The visual appearance of *neo*-PLBs in response to BA is similar, although productivity differs [TEIXEIRA DA SILVA, 2014a] (B)

Acknowledgement and conflicts of interest

The author thanks Prof. Michio Tanaka for research support and Prof. Shin Taketa for providing diploid barley used in the flow cytometric analyses. The author declares no conflicts of interest (financial or other).

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, N^6–[Δ2-isopentenyl] adenine (syn. 6(γ,γ-dimethylallylamino)purine); ABA, (±)-*cis*,*trans*-abscisic acid; Ads, adenine hemisulphate; BA, 6-benzyladenine (syn. BAP, 6-benzylaminopurine; Teixeira da Silva 2012b); dicamba, 3,6-dichloro-2-methoxybenzoic acid (syn. 3,6-dichloro-*o*-anisic acid); GA_3, gibberellic acid; IBA, indole-3-butyric acid; Kin, kinetin; MeJa, methyl jasmonate; NAA, α-naphthaleneacetic acid; PG, phloroglucinol; PGR, plant growth regulator; picloram, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; PLB, protocorm-like body; SA, salicylic acid; TC medium, Teixeira *Cymbidium* medium (TEIXEIRA DA SILVA, 2012a); TDZ, *N*-phenyl-N'-1,2,3-thiadiazol-5-yl-urea or thidiazuron; TRIA, 1-triacontanol (syn. melissyl alcohol or myricyl alcohol).

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Tab. 1. Flow cytometric analysis of *Cymbidium* Twilight Moon 'Day Light' PLBs derived from different PGRs (1 mg/l) after 60 days in TC medium. The SPAD value was calculated from the leaves (third fully developed leaf from the apex) of 6-month old plantlets derived from PGR treatments.

Treatment	PGRs	SPAD[1]	FC[2]
Control	None	41.1 a	92:8:0:0
Cytokinins	AdS	0	96:4:0:0
	BA	43.2 a	74:23:3:0
	2iP	33.7 b	71:27:t:0
	Kin	41.6 a	91:6:4:0
	TDZ	22.8 cd	64:31:2:t
	*m*T	35.8 ab	76:16:8:0
Auxins	2,4-D	25.7 cd	72:22:6:t
	IBA	0	90:8:2:0
	NAA	0	94:6:0:0
	Picloram	31.8 b	73:18:6:3
	TRIA	36.2 ab	72:24:4:0
	PG	0	80:16:3:1
Others	ABA[3]	19.7 d	71:28:1:0
	Dicamba[4]	26.9 c	91:9:0:0
	GA_3[5]	0	80:13:5:2
	JA[3]	30.8 b	88:12:0:0
	MeJa[3]	29.2 b	66:28:3:t
	SA[3]	42.1 a	92:6:t:0

Means followed by different letters within a column across all PGRs indicate significant differences at $P<0.05$ according to Tukey's multiple range test. SPAD = measurement of chlorophyll content on the third youngest leaf; all FC values represent 2C:4C:8C:16C relative ratios derived from three independent biological replicates; t = trace (≤2%).

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, N^6–[Δ2-isopentenyl] adenine (syn. 6(γ,γ-dimethylallylamino)purine); ABA, (±)-*cis*,*trans*-abscisic acid; Ads, adenine hemisulphate; BA, 6-benzyladenine (syn. BAP, 6-benzylaminopurine; see TEIXEIRA DA SILVA 2012b); dicamba, 3,6-dichloro-2-methoxybenzoic acid (syn. 3,6-dichloro-*o*-anisic acid); GA_3, gibberellic acid; IBA, indole-3-butyric acid; Kin, kinetin; MeJa, methyl jasmonate; NAA, α-naphthaleneacetic acid; PG, phloroglucinol; PGR, plant growth regulator; picloram, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; PLB, protocorm-like body; SA, salicylic acid; TC medium, Teixeira *Cymbidium* medium [TEIXEIRA DA SILVA, 2012a]; TDZ, *N*-phenyl-N'-1,2,3-thiadiazol-5-yl-urea or thidiazuron; TRIA, 1-triacontanol (syn. melissyl alcohol or myricyl alcohol)

[1] SPAD values of zero indicate that no plantlets could be derived from that treatment, thus SPAD could not be measured in leaves (these values were not included in the analyses.

[2] The 2C:4C value of leaves of plantlets derived from any treatment was not different (94:2 on average) and thus values are not presented.

[3] Also referred to as stress hormones.

[4] Also considered by some to be an auxin.

[5] Also referred to as a gibberellin.

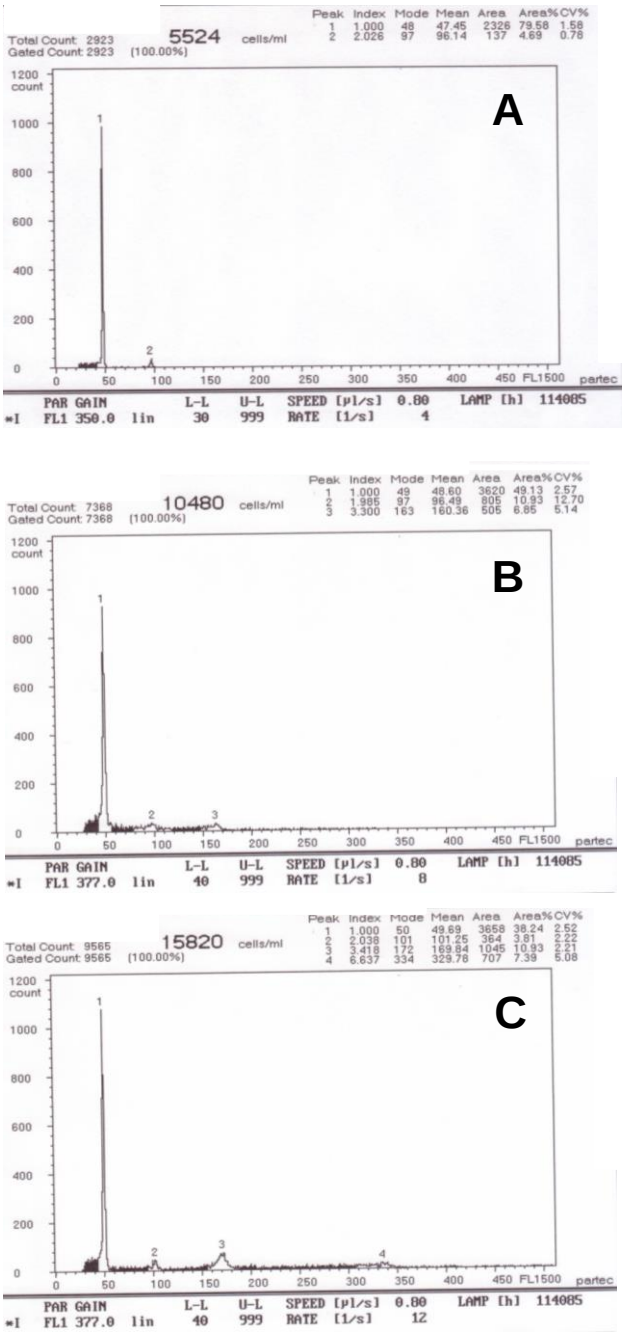

Fig. 2. Control versus endopolyploid *neo*-PLBs in hybrid *Cymbidium* Twilight Moon 'Day Light'. Control *neo*-PLBs on TC medium [TEIXEIRA DA SILVA, 2012a] (A). Endopolyploidy up to 8C in *neo*-PLBs in response to BA (B). Endopolyploidy up to 8C in *neo*-PLBs in response to picloram (C).

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22(2015): *47 – 58*

EVALUATION OF CALLUS BROWNING AND DEVELOP A STRATEGICALLY CALLUS CULTURING OF *BOERHAAVIA DIFFUSA* L.

Gulshan CHAUDHARY[1*], Prem Kumar DANTU[1]

Abstract: Callus browning is a major problem in *Boerhaavia diffusa*. This phenomenon was investigated in present study by evaluating major reason for callus browning, develop a strategy for the survivals of callus and study the accumulation of secondary metabolites. Torpedo shaped embryos were cultured on semisolid MS basal medium supplemented with n various combinations of hormones, with and without adjuvants. After a particular time callus used for cytological, fresh viz dry weight studies and later used for the secondary metabolite study by HPTLC method. Cytological studies of the callus were performed to understand the reason for low survival of the callus. Over a culture period of 30 days revealed that the callus was made up of three types of cells: small isodiametric cells, elongated cells and elongated enucleated cells. The isodiametric cells were meristematic and predominant during the initial days of the culture and subsequently their number decreased and elongated nucleated and enucleated cells increased. Towards the latter part of the culture period the enucleated cells were predominant. The increase in elongated cells coincided with increased browning of the callus and peroxidase activity. The HPTLC of extracted callus with different precursors confirmed the presence of some flavonoids likes kaempferol, quercetin, myrecetin. A strategic subculturing method was developed where in the small cells were isolated and subcultured every three weeks and the life of callus could thus be prolonged to almost 30-36 weeks. Based on these studies conclude that the life of callus could be prolonged to almost 30-36 weeks by strategic subculturing method. This study is important because as plant has various medicinal properties so its secondary metabolites can be collected by *in vitro* callus production at particular time period.

Key words: *Boerhaavia diffusa*, callus browning, peroxidase, strategic subculture

Introduction

Boerhaavia diffusa is an important medicinal plant, known as punnarnava in Sanskrit and is used in a wide number of Ayurvedic preperations. The plant harbours a large number of secondary metabolites such as geranylacetone, limonene, indoleresorcinol monoacetate, vanilin, eugenol and kaempferol 3-O-robinobioside in leaves and quercetin 3-O-robinobioside, caffeoyltartaric acid, eupalitin 3-O-galactosyl (1-2)-glucoside, and isomenthone in roots [PEREIRA & al. 2009]. It has been in use to alleviate a large number of ailments such as liver disorders, dyspepsia, jaundice, enlargement of spleen, and abdominal pain [KIRTIKAR & BASU, 1956; RAWAT & al. 1997; MALIK, 1980].

Plant cell cultures are proving to be effective alternative for producing *in vitro* secondary metabolites [ROBERTS & KOLEWE, 2010; LEE & al. 2010]. In this regard a study was undertaken to establish callus cultures of *Boerhaavia diffusa* for *in vitro* secondary metabolite production. However, a major problem was the sustenance of callus

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for long periods due to browning and early senescence of callus. This interfering phenomenon is known to be tissue browning due to the production of phenolic compounds. Now days it is consider being serious problem because tissue browning event involves many toxic compounds through the phenolization process eventually resulting in the necrosis of cells [BANERJEE & al. 1996; MURATA & al. 2001; WU & LIN, 2002; CHEN & al. 2012].

Browning of callus is a major problem for growth and long term maintenance of callus and in many cases inhibits adventitious shoot formation [LEE & WHITAKER, 1995]. This problem has been encountered in several plants such as peach [LEE & al. 1990], sugarcane [CHEN & al. 1990], *Taxus cuspidata* [FETT-NETO & al. 1992, 1993], oak [TOTH & al. 1994], *Zea mays* [DOWD & NORTON, 1995], *Pinus sylvestris* [LAUKKANEN & al. 2000], *Taxus chiensis* [CHOI & al. 2000], guava, date palm [DAAYF & al. 2003], *Taxus media* [BAEBLER & al. 2005], cotton [OZYIGIT & al. 2007], *Cicer arietinum* [NAZ & al. 2008], *Jatropha curcas* [HE & al. 2009], *Nigella glandulifera* [ZHOU & al. 2010], *Taxus brevifolia* [KHOSROUSHAHI & al. 2011] and *Senna occidentalis* [ISAH & MUJIB, 2013), *Taxus chinensis* [NAN & al. 2015].

The main reason for tissue browning of *in vitro* tissues has been correlated with excessive accumulation of phenolics [LEE & al. 1990; DOWD & NORTON, 1995; LAUKKANEN & al. 2000; DUBRAVINA & al. 2005; ISAH & MUJIB, 2013]. Plant phenolics are chemically active because of the presence of a hydroxyl functional group [SREENIVASULA & al. 1989]. Phenolic compounds though cause browning in exposed areas but play many important functions in higher plants. They may combine with proteins either reversibly by hydrogen bonding or irreversibly by oxidation. Phenolics modulate plant development by regulating indole acetic acid catabolism [ARNALDOS & al. 2001]. They are effective in plant growth regulation, cell differentiation and organogenesis [OZYIGIT & al. 2007]. Phenols on oxidation form compounds called quinones that polymerize to impart the characteristic brown colour and are inhibitory to plant cellular growth [MAYER & HAREL, 1979]. Phenol oxidation may be catalyzed by polyphenol oxidases or peroxidases [VAUGHN & DUKE, 1984; KE & SALTVEIT, 1988] which act synergistically. Polyphenol oxidase promotes peroxidase activity by generating H_2O_2 through the oxidation of phenolic compounds [RICHARD-FORGET & GAUILLARD, 1997].

Studies in Scots Pine have shown that browning is primarily because of peroxidase activity [LAUKKANEN & al. 2000], though, in *Jatropha* polyphenol oxidase has been reported to play a more important role [HE & al. 2009]. Peroxidase has been also reported to be associated with degradation of chlorophyll and peroxidation of lipid in senescing plant tissues [CAMPA, 1991]. In *Pinus virginiana* callus browning, an increased polyphenol activity was associated with a concomitant decrease in the antioxidant enzymes ascorbate peroxidase, glutathione reductase and superoxide dismutase [TANG & NEWTON, 2004].

Several methods have been adopted to alleviate browning of *in vitro* callus or shoot cultures. Chief among these have been the use of antioxidants as ascorbic acid – *Taxus* sp. [FETT-NETO & al. 1992]; *Magnolia officinalis* [FU & al. 2009] or cysteine – *Taxus* sp.[FETT-NETO & al. 1992]; growth adjuvants as casein hydrolysate – *Taxus brevifolia* [GIBSON & al. 1993]; activated charcoal – *Taxus* sp. [FETT-NETO & al. 1992]; *Magnolia officinalis* [FU & al. 2009]; polyvinyl pyrolidine – *Rollinia mucosa* [FIGUEIREDO & al. 2001]; *Magnolia officinalis* [FU & al. 2009]. Other strategies to reduce *in vitro* browning

have been to lower the salt strength of MS medium to half or one-fourth as in *Nigella* [ZHOU & al. 2010] and *Actinidia arguta* [HAN & al. 2010], or by replacing high salt strength medium MS with that of moderate strength medium of Schenk and Hildebrandt in *Taxus* [FETT-NETO & al. 1993].

To alleviate the problem of callus browning in *B. diffusa*, the present study growth dynamics of the callus with respect to variation in the cell types, their growth rates, changes in fresh and dry weights of callus was correlated with variation in peroxidase activity of the callus. Based on these results a strategy for subculture of callus was developed which prolonged the life of callus *in vitro* to several subcultures. Secondly, as plant cell cultures are useful for producing *in vitro* secondary metabolites so in this regard a study was undertaken to establish callus cultures of *B. diffusa* for *in vitro* secondary metabolite production and to identify some of important secondary metabolites from *in vitro* production of cell culture by HPTLC.

Material and methods

Plant material: young immature fruits were collected from the Botanical garden of the Institute, Dayalbagh Agra.

Sterilization of fruits: fruits were collected, washed under running tap water for about 30 min followed by a quick rinse in ethanol then surface sterilized with 0.1 % mercuric chloride for 8 min under aseptic conditions.

Embryo isolation: the sterilized young immature fruits were dissected under aseptic condition and embryos were cultured on appropriate medium.

Preparation of medium: for all studies MS basal medium [MURASHIGE & SKOOG, 1962] was used supplemented with various growth regulators such as 2,4-D, BAP or NAA in different combinations with various growth adjuvants such as silver nitrate, casein hydrolysate or activated charcoal. To increase secondary metabolite contents some precursors such as phenyl aniline, t-cinnamic acid were also added.

Callus establishment and maintenance: young immature embryos were cultured on MS basal medium supplemented with 2,4-D alone at 0, 0.5, 1, 1.5, 2, 2.5 and 3 mg/l. The resulting callus was subculture to their respective hormone combination media. Callus was subcultured every 20 days. Besides, the callus was also transferred to MS basal medium supplemented with NAA (0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg/l) + BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l). The NAA+BAP medium was also supplemented with various adjuvants: silver nitrate (5, 10 and 15 mg/l), casein hydrolysate (25, 50 and 100 mg/l), activated charcoal (5, 10, 20 mg/l) or proline (10, 25 and 50 mg/l).

Cell type composition of the callus: the cell type and composition of callus was studied in the 3% sucrose solution. Types of cells and their numerical variation were counted using a haemocytometer. Dimensions such as length and breadth of the cells were measured using the software package NIS Elements-D version 3.1 (Nikon, Japan).

Spatial distribution of cells in callus: spatial distribution of different types of cells in the callus was determined by cutting thin free-hand vertical sections of the callus. The sections were stained with acetocarmine and the distribution of different types of cells from portion proximal to medium to the portion distal from medium was studied in Nikon Stereozoom Microscope (SMZ 800) attached with Nikon digital camera.

Temporo-numerical variation in different type of cells in the callus: callus cultured on MS + 1 mg/l 2,4-D + 0.5 mg/l BAP was used for this study. Small isodiametric cells were carefully isolated and inoculated on fresh medium. Subsequently, differentiation into various cell types and their numerical variation over a period of four weeks was recorded at 5-day intervals for a culture period of 30 days.

Fresh and dry weight measurements: fresh and dry weights of callus from MS + 1 mg/l 2,4-D + 0.5 mg/l BAP + 25, 50, 100 mg/l casein hydrosylate, 25, 50, 100 mg/l phenylanaline and t-cinnamic acid were recorded every fifth day starting from day 0 to day 25. To get dry weight callus was dried in an oven maintained at 60 °C for 48 h or till the weight became constant.

Peroxidase assay: peroxidase activity of callus from day 0 to day 30 at five day intervals was assayed by guaiacol method[38].

Preparation of callus extract: callus obtained from immature embryos was extracted for quantifying their secondary metabolite content [CHAUDHARY & al. 2012].

Preliminary tests for secondary metabolites: Shinoda test conducted for flavonoid and Dragendorff test for alkaloids [SVENDSEN & VERPOORTE, 1983].

Preparation of standard solution and HPTLC conditions: a 100 ppm stock solution of standards kaempferol, quercetin, mercetin and leuteonine (Sigma-Aldrich, St. Louis) were prepared and scanned the samples according to HPTLC standard conditions (CAMAG, France) [CHAUDHARY & al. 2012].

Results

Callus study: MS basal medium without 2,4-D the embryos neither grew nor survived while lower concentrations of 2,4-D (0.5, 1 and 1.5 mg/l) supported good callus formation than the higher concentrations. Best callus initiation and growth from the torpedo embryos was obtained in MS + 0.5 mg/l 2,4-D. The callus was creamish, compact and friable (data not shown). The callus from initiation phase could not survive beyond 15 days when transferred to a medium containing either 2,4-D or BAP alone at all concentrations combinations. Presence of both 2,4-D and BAP and their concentration was critical for survival of the callus in subsequent subcultures. Concentration of 2,4-D with BAP at 1 and 0.5 mg/l supported the best callus growth (Fig. 1). Till the third subculture, the callus was healthy, creamish and friable. In subsequent cultures the callus slowly turned watery, brown and by fifth and sixth subculture (150-180 days) the entire callus became brown and died. MS medium supplemented with various growth adjuvants such as casein hydrolysate, activated charcoal, ascorbic acid, silver nitrate and proline did not improve callus growth but callus turned brown and died within 2-3 weeks of transfer (data not shown). Reducing MS salt strength to half also did not help reduce browning of the callus.

Fresh and dry weight changes in callus: a typical sigmoidal growth curve was observed for the weight changes. The lag phase lasted for the first five days, thereafter, fresh weight showed a consistent increase till day 25 beyond which weight became stationary. Dry weight increase followed almost a similar pattern except that dry matter accumulation was sluggish till day 20. Thereafter, in the next five days dry matter accumulation increased exponentially, and then became stationary in the final period of 25-30 days (Fig. 2A).

Types of cells in the callus: callus from the medium MS + 1 mg/l 2,4-D + 0.5 mg/l BAP at the end of 30 day growth period was composed of three types of cells: (i) small, isodiametric cells with centrally placed nucleus and intensely staining cytoplasm (Fig. 3A), (ii) elongated cells with sparsely stained cytoplasm and nucleus drifted to one side (Fig. 3 B & C) and, (iii) elongated cells with sparse cytoplasm without traceable nucleus. The distribution of these three types of cells in callus at the end of 30 days was shown in Figure 3 D. In a total of 3400 cells 72% were nucleated and only 28% of the total cells were enucleated (the enucleated cells were elongated). Of the nucleated cells 46% were small, isodiametric with a centrally placed nucleus and intensely staining cytoplasm. The remaining 54% of cells are elongated; their cytoplasm stained light and had a peripheral nucleus. The only cells that appeared to be meristematic were the small isodiametric cells as these were present in clumps. The elongated cells and the enucleated cells were mostly present as separate individual cells. The area of small cells doubled during the 30 day period while the area of elongated cells increased by slightly more than 10% (Fig. 2 B). A perusal of Fig. 2D indicates that balance sheet of small cells was mostly negative throughout culture period except on day 15 when a slight increment of about 50 cells was recorded.

Peroxidase activity: the callus turned brown with passage of time and at the end of subculture period entire callus became brown and died. Peroxidase activity at different intervals during callus growth and has been overlapped on the results of numerical variation in the cell types (Fig. 2C). The long as the small cells were in sufficient numbers (day 10) and actively dividing the peroxidase activity was almost absent, however, as the number of small cells start to decline and the number of elongated and enucleated cells increased the peroxidase activity increases and on day 20 the enzyme activity peaks, thereafter, by day 25 and 30 its activity declines. On day 20, corresponding to the peak activity of peroxidase, only a few small cells were present and the cumulative numbers of elongated and enucleated cells were sufficiently large. Thereafter, there was a rapid decline in the number of small cells and an increase in number of elongated and enucleated cells. Peroxidase activity though was minimum on last two intervals (day 25 and 30) of culture but it never became zero. This activity of peroxidase paralleled increased browning of callus and also increased dry weight. As based on the results obtained it could be argued that so long as a reasonable population of small meristematic cells was present the callus continued to proliferate.

Fresh and dry weight: The callus attains a maximum fresh and dry weight between the second and the third week of culture. Incidentally, it was during this period that the population of small cells stabilizes and peroxidase activity also peaks. Thus, a strategy for subculturing such a callus should aim at that period of callus growth which has the maximum number of senescing cells and optimal gain in fresh and dry weight. Such a stage in callus cultures of *B. diffusa* as revealed by the present study was in the second to third week of culture. Therefore, callus at the end of third week was subcultured using the portion proximal to the medium as it had the maximum number of small meristematic cells. The steps involved in the strategy for subculture of callus were: (a) subculture of callus to fresh medium was done after every three weeks instead of the earlier four weeks, (b) only the portion of the callus rich in small, isodiametric cells was transferred to fresh medium, and (c) the callus was cleaned of all the elongated, brown and dead cells. Following this procedure carefully the life of callus could be increased to almost 30 to 36 weeks.

The dried callus were fractionated and analysed on HPTLC against these flavonoids standards. It was estimated that the callus of *B. diffusa* contained 0.36 µg/µl of flavonoids as quercetin at maximum (0.95 µg/µl) followed by kaempferol (1.5 µg/µl) and myrcetin was the least (0.95 µg/µl) For details refer previous paper [CHAUDHARY & DANTU, 2011].

Discussion

The present study revealed that it was possible to establish healthy growing callus from young embryos of *B. diffusa*. However, the callus could not be maintained beyond five subcultures as it becomes brown and eventually dies. Cytological studies revealed that the well established callus was made up of three types of cells: small, isodiametric meristematic cells with central nucleus, elongated cells with peripheral nucleus and enucleated elongated cells. The study also revealed that the small cells are continuously differentiated into nucleated and enucleated elongated cells. Microscopic examination showed depositions of optically dense substances on the cell wall of the elongated cells (Fig. 3). Presumably these morphological changes and possible biochemical changes (as revealed by change in colour of the cells and depositions on cell walls) could be resulting in browning and early apoptosis of the callus cells. Further, it was also noted that increase in peroxidase activity in the callus cultures was associated with increase in browning of the callus and elongated cells and concomitant decrease in small cells. Accumulation of secondary metabolites in the callus cells has been implicated in browning and early death due to apoptosis [SOLOMON & al. 1999; KNIGHT & al. 2001; QIAO & al. 2003].

Morphological and biochemical changes in *Taxus chinensis* var. *meirei* cells occurred mainly in the non-dividing cell clusters indicating that the cells died by apoptosis. These authors found a close relationship between cell apoptosis and Taxol formation. Taxol concentration increased with increased number of apoptotic cells and reached a maximum after 23 days of culture which corresponded to a maximal ratio of apoptotic to total cells to about 13%. That the apoptotic cells mainly occurred in the cell clusters of brown colour was also observed by EXPOSITO (2009). Permanent loss of cell viability was observed in callus cells of *Taxus cuspidata* P991 that were producing high levels of Taxol upon elicitation with methyl jasmonate [KIM & al. 2005]. *B. diffusa* plants are known to be rich in flavonoids and other secondary metabolites [PEREIRA & al. 2009]. It could be possible that callus cultures of *B. diffusa* are actively producing some secondary metabolites [PEREIRA & al. 2009; MURTI & al. 2010; GOYAL & al. 2010]. The elongated nucleated cells probably undergo differentiation to produce some secondary metabolites and excessive accumulation of which lead to browning and eventual death of these cells.

Callus browning has often been associated with an increase in accumulation of phenolics such as lignin [LAUKKANEN & al. 2000]. This increase in phenolics has been linked to increase in polypenol oxidase (POP) and peroxidase (POD) activity in *Malus sylvestris*, *Heva brasiliensis*, *Panax ginseng*, *Camellia sinensis* [BERGER & al. 1985; HOUSTI & al. 1992; KORMUTAK & VOOKOVA, 2001; BONFILL & al. 2003; AOSHIMA & TAKEMOTO, 2006]. Present study also revealed an increase in peroxidase activity as callus browning intensified. In callus cultures of *B. diffusa*, present study, decrease in actively dividing small cells and concomitant increase in non-meristimatic elongated cells caused untimely senescence and increase in population of brown cells. Decrease in regenerability, poor growth and eventual death because of callus browning has

been observed in several species [BERGER & al. 1985; DOWD & NORTON, 1995; LAUKKANEN & al. 2000; KAWAOKA & al. 2003; HE & al. 2009; HAN & al. 2010]. *In vitro* browning of callus has been overcome in many species by changing the composition of the basal medium, or by reducing the salt strength, or by adding adjuvants such as casein hydrolysate, silver nitrate, proline or activated charcoal. Interestingly, in the present study, the callus of *B. diffusa* could not be prevented from browning either by changing composition of basal medium or by changing growth regulators or by adding any of the adjuvants mentioned.

The decline in peroxidase activity towards the end of culture period in callus cultures of *B. diffusa* could be attributed to decrease in small meristematic cells that were continuously differentiating into nucleated and enucleated cells. These elongated cells are continuously becoming brown and dying. The differentiation of the small meristematic cells into non-dividing elongated cells (nucleated and enucleated) and the consequent decrease in the number of small cells by the 15th day of culture resulted in senescence of the callus. This was reflected by browning of callus and an increase in the peroxidase activity. A subculturing strategy was developed where in the small meristimatic cells proximal to the medium were carefully separated and transferred to fresh medium and subsequent subculture was done every three weeks. This procedure enhanced the life of *B. diffusa* callus cultures to almost 30-36 weeks.

The HPTLC result of *B. diffusa* callus indicates the presence of flavonoids such as kaempferol, quercetin and myrecetin. The earlier reports also shows the presence of flavonoids and other compounds in the *in vitro* cultures of *Hypericum* [KARTNIG & al. 1996; BERNARDI & al. 2007; SHILPASHRE & RAI, 2009], *Mormodica charantia* [AGARWAL & KAMAL, 2004], and *B. diffusa* [CHRISTIAN & al. 2006]. The present study is only a preliminary work towards identification of *in vitro* biosynthesis of flavonoids.

Conflict of interest

The authors have not declared any conflict of interest.

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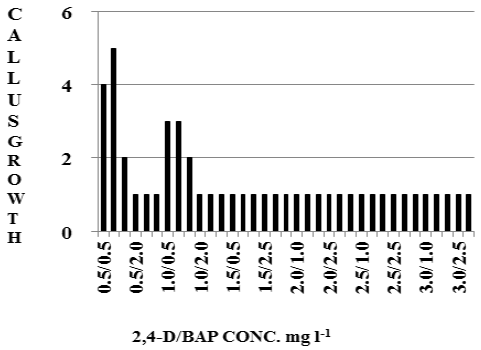

Fig 1. Effect of 2,4-D and BAP concentrations on growth of *B. diffusa* callus. On the horizontal axis concentration above the bar indicate 2,4-D and that below indicate BAP. The callus growth has been measured on a scale of 1 to 5 taking into consideration size of callus at the end of growing period, morphological quality of callus and subculturable or not.

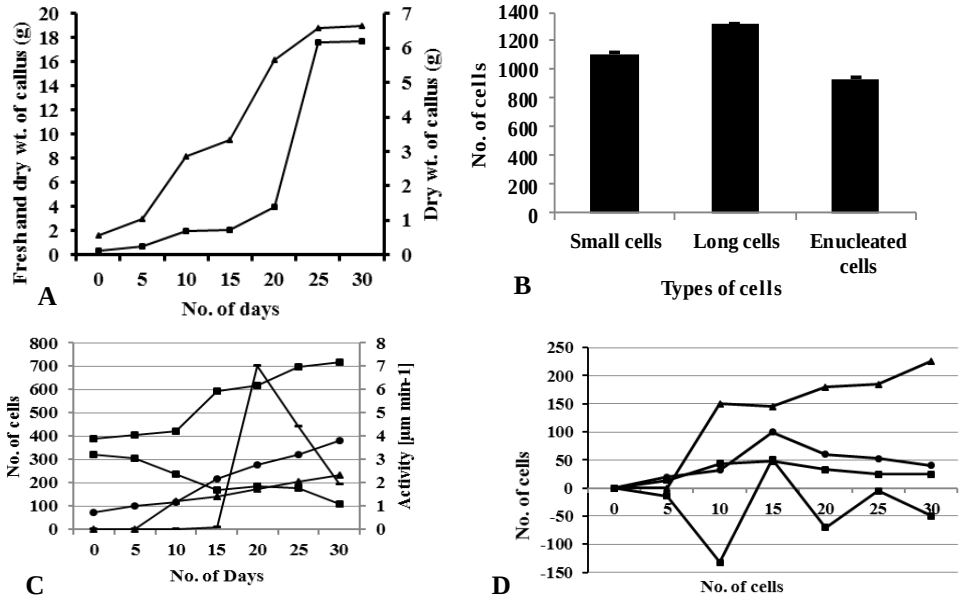

Fig 2 A. *B. diffusa* callus growth, elucidated by fresh weight (▲—▲) and dry weight (■ — ■) changes over a period of 30 days; **Fig 2 B.** Numbers of different types of cells at the end of a growth period of 30 days in the callus cultures of *B. diffusa*; **Fig 2 C.** Variation in small (■-■), long nucleated (●-●), long but enucleated (▲-▲), total Cells (**x-x**) and peroxidase (— - —) over a period of 30 days in the callus cultures of *B. diffusa*. This graph clearly represents that as the numbers of small nucleated cells turns to enucleated cells there is simultaneously increases of peroxidise activity. The small isodiametric cells were used to initiate callus; **Fig 2 D.** Is a balance sheet, small cells (■-■), was mostly negative throughout culture period except on day 15 when a slight increment of cells. long and nucleated cells (●-●), long but enucleated cells (▲-▲), and total cells (**x-x**) at five day intervals in callus cultures of *B. diffusa*. Total culture period 30 days.

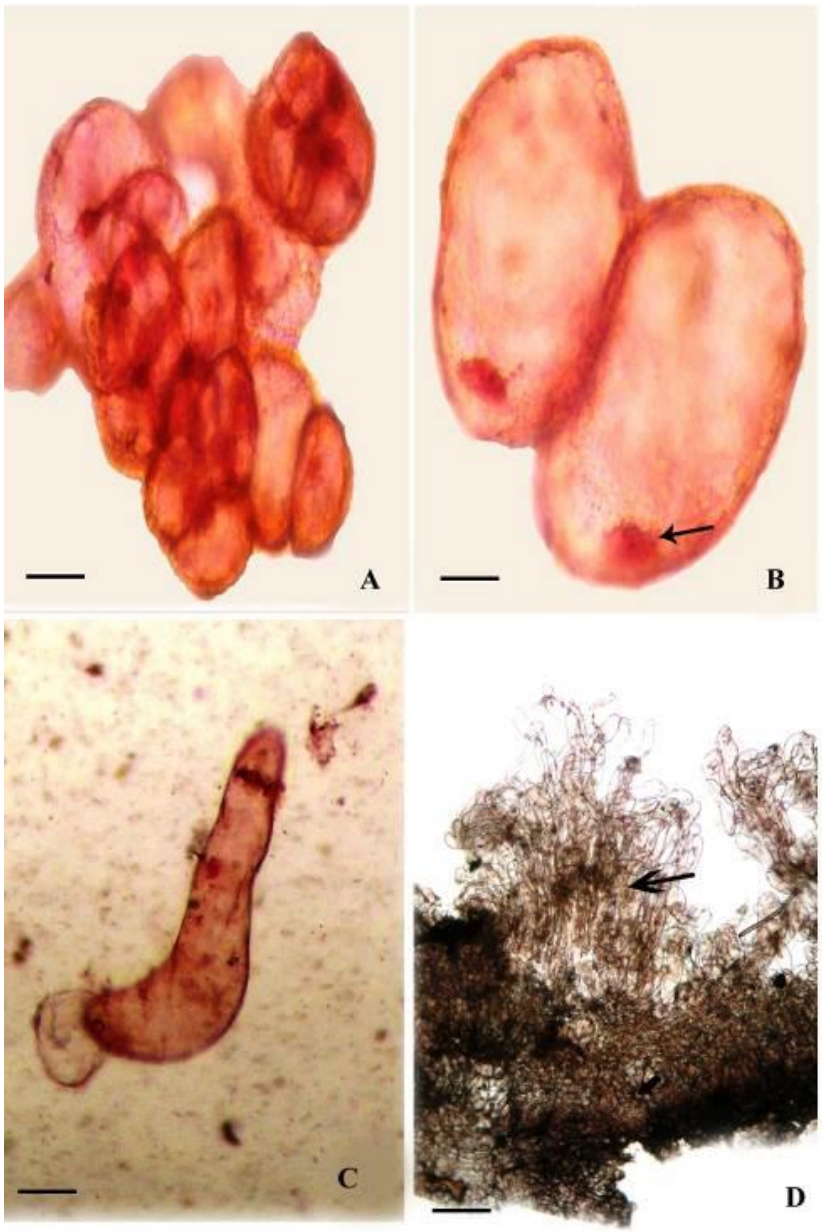

Fig 3. A-F: Types of cells in the callus of *B. diffusa*, A. small isodiametric cells with centrally placed nucleus, B. elongated cells with peripheral nucleus, C. enucleated cells, in which nucleus did not stained with acetocarmine, D. vertical sections of 15-day old callus. Note the small isodiametric cells (small, thick arrow), and the elongated cells (long, thin arrow); Cw = cell wall; dCyt = dense cytoplasm; dp = depositions; N = nucleus. (Bar: Fig A 13.5 µm; Fig B 13 µm; Fig C 40 µm; Fig D 41 µm; Fig E 19 µm; Fig F15.83 µm)

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COMPOSITION OF HERB AND SEED OIL AND ANTIMICROBIAL ACTIVITY OF THE ESSENTIAL OIL OF TWO VARIETIES OF *OCIMUM BASILICUM* HARVESTED AT SHORT TIME INTERVALS

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Abstract: An experiment was conducted to study the changes in the chemical composition of the essential oil of two varieties of *Ocimum basilicum* over a period of six months at short harvest intervals for two crop seasons.

In variety Vikarsudha, GC/MS analysis revealed the presence of eighteen essential oil constituents. Linalool (23.5-40.1% and 22.8-33.7%) and methyl chavicol (25.4-51.9% and 40.0-52.7%) were the major constituents in main and ratoon crops.

Similarly, in variety Kuhmohak GC/MS analysis revealed the presence of linalool (19.2-25.4 % and 16.1-31.3%) and methyl chavicol (34.7-53.4% and 39.4-59.2%) in large quantities in main and ratoon crops, respectively. β myrcene, limonene, 1,8 cineole, ocimene, camphor, terpinen-4-ol, bornyl acetate, eugenol, methyl eugenol, β elemene, β caryophyllene, α humulene, γ Cadinene and cadinol were present in small quantities.

Results pertaining to the zone of inhibition in the antimicrobial activity of essential oil indicated that *Chromobacterium violaceum* is more sensitive compared to *Staphylococcus aureus.* Among the fungal strains *Aspergillus niger* was found to be more sensitive.

GC-MS analysis of the fixed oils obtained from the seeds in the ratoon crop revealed the presence of unsaturated and saturated fatty acids. The unsaturated fatty acids averaged 89% consisting of α-linolenic (49.3%-52.4%), linoleic (23.4%-26.0%), and oleic (10.3%-12.3%) acids. The most abundant saturated fatty acids were palmitic and stearic acids.

Key words: *Ocimum*, Lamiaceae, Eugenol, β caryophyllene, methyl eugenol, relative humidity

Introduction

The genus *Ocimum* belongs to the family *Lamiaceae* consists of many species of herbs and shrubs and these are collectively called basils [SIMON & al. 1992]. The number of species reported in the genus varies from 50-60 [HEGNAUER, 1966; SUCHORSKA & OSINSKA, 1992] to 150.

The most important species of *Ocimum* genus is *O. basilicum* L., this species, usually named common basil or sweet basil, is considered economically useful because of their basic natural characteristics as essential oil producers [LAWRENCE, 1993]. Sweet

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basil is a tender herbaceous annual plant, which originates from tropical and warm areas, such as India, Africa and southern Asia. It is naturalized almost all over the world [HALVA, 1987; SORENSEN & HENRIKSEN, 1992]. The herb on steam distillation yields a bright yellow, volatile oil possessing a pleasant odour. The essential oils are composed of aroma compounds such as eugenol, methyl eugenol, citral, linalool, geraniol and thymol which are required as raw materials for the pharmaceutical, comestics and food industries [GUPTA, 1994; BIZZO & al. 2009]. Basil essential oils are known to possess antimicrobial [BERRINGTON, 2012], and insecticidal activities. Due to its pleasant aroma and antimicrobial activity, basil essential oil is a major aroma chemical with applications in various industries such as the food, pharmaceutical, cosmetic, and aromatherapy.

The variations in the essential oil compositions in *O. basilicum* cultivated in different geographical localities led to the classification of basil into chemotypes on the basis of the prevalent chemical components [LAWRENCE, 1992] or components having composition greater than 20 percent [GRAYER & al. 1996]. When grown for essential oil production, basil is harvested in full bloom, because the content and the composition of the oil are optimal at that stage [BERRINGTON & LALL, 2012; NYKÄNEN, 1989]. Depending on the climate, basil can be harvested one to three times during the cropping season. The essential oils with the finest aroma are obtained from European basil that contains linalool and methylchavicol as the main components. Reunion basil is characterized by high levels of methylchavicol, whereas the tropical chemotype of basil is known to have methyl cinnamate as the main component. Another basil chemotype that is high in eugenol is grown in North Africa, Russia, Eastern Europe, and India.

There is a great variation of essential oil composition (and aroma) among basil cultivars currently on the international market. There is a significant interest in basil as a new high-value essential oil crop in many countries. The objective of this study is to study essential oil content, composition, and bioactivity of two varieties of sweet basil (*O. basilicum*) harvested at short intervals of time in two crop seasons (main crop and ratoon crop). Stage of harvesting plays a dominant role in obtaining good quality oil. Chemical profiles of the essential oils harvested at short intervals of time in *Ocimum* sp are limited. Furthermore, this study also aims at analyzing the fatty acid composition of seed oils and antimicrobial activity of the essential extracted at different harvests.

The information provides scientific background for successful cultivation and production of quality essential oil. Considering these aspects the content and quality of essential oil obtained from two varieties of *Ocimum basilicum* harvested at short intervals of time were studied for two crop seasons.

Materials and methods

Experimental site and design of the experiment

The present study was undertaken to study the seasonal variation in the essential oil composition of two varieties of *Ocimum basilicum* (Vikarsudha and Kushmohak) during a six month period from October, 2012 to March, 2013 at the research farm of Central Institute of Medicinal and Aromatic Plants (CIMAP), Research Centre, Boduppal, Hyderabad, Telangana, India. The experimental site is located at an altitude of 542 m

above sea level with a geographical bearing of 78°8′ E longitude and 17°32′ N latitude. The mean annual rainfall of this region is generally 750 mm.

The soil is a red sandy loam (alficusto chrept) with pH 8.27 (1.25 soils to solution ratio), EC – 1.21 ds/m, organic C – 0.58%, available N (215.40 kg/ha), available P (10.30 kg/ha), and exchangeable K (103.08 kg/ha).

The experimental field was ploughed, harrowed and levelled with tractor drawn implements before starting the nursery. Seeds of CSIR-CIMAP varieties Vikarsudha and Kushmohak were sown in nursery and healthy well grown seedlings (25 days old) were transplanted in rows following a row spacing of 60 cm between rows and 45 cm between plants in 4.8 m x 6.0 m plots. The crop was planted during first week of October, 2012. A fertilizer dose of 60:40:40 Kg/ha of N:P:K was applied to the crop. Uniform doses of P and K were applied during ploughing. Nitrogen was applied in four splits. The crop was managed as per standard practices under irrigated conditions in 40 plots cultivated uniformly under similar conditions. The plots were kept weed free.

Between first week of October and middle of December 2012, the crop was harvested five times in different plots. The days of harvesting constituted the treatments and the treatments were replicated five times in a randomized complete block design. This is designated as the main crop. The field plots were again harvested commonly up to a height of 20 cm above ground level in the third week of December 2012. During the seventy days period from 15th December 2012 to the end of February 2013 the crop was harvested seven times at 10 days interval up to the end of February 2013. The dates of harvesting constituted the treatments and replicated five times. This was designated as ratoon crop.

Basilicum varieties (Kushmohak and Vikarsudha) were harvested five times at 15 days interval at 15,30,45,60 and 75 days in the main crop and seven times at ten days interval (10, 20, 30, 40, 50, 60 and 70 days) during the ratoon crop. Observations were recorded at each harvest on the morphological characters, essential oil content, and composition.

Observation on morphometric traits

The herb was harvested from ten randomly selected plants in each treatment plot replication wise and data were recorded for morphometric traits viz., number of leaves/plant, leaf area, and leaf weight/plant. The fresh weight of plant was also taken for calculating the essential oil yield/plant.

Essential oil extraction/distillation

The aerial parts of ocimum were collected from ten random plants in each plot. For the extraction of essential oils, freshly collected herbage was subjected to hydro-distillation using a Clevenger-type apparatus for 4.0 h. The essential oils obtained were dried over anhydrous sodium sulphate and stored at 4 °C until the GC analysis was carried out. The oil content and quality were observed five times in the first phase and seven times in the second phase replication wise.

Gas Chromatography analysis of essential oil

The essential oils were analyzed on a Varian CP-3800 model gas chromatograph with Galaxy software system equipped with flame ionization detector (FID) and an electronic integrator. Separation of the compounds was achieved employing a Varian CP-Sil 5CB capillary column (ID: 50 m X 0.25 mm; film thickness 0.25 μm). Nitrogen was

used as the carrier gas at a constant flow rate of 0.4 ml/min. The column temperature was programmed from 120 °C (held for 2 min.) to 240 °C (held for 5 min.) at a rate of 8 °C/min. The injector and detector temperature were set at 250 °C and 300 °C respectively. Samples of 0.2 µL were injected with a 20:100:20 split ratio. Retention indices were generated with a standard solution of n-alkanes (C_6-C_{19}). The composition was reported as a relative percentage of the total peak area without FID response factor correction.

Gas Chromatography/Mass Spectrometry (GC/MS) analysis of essential oil

GC-MS analysis was carried out on a SHIMADZU GCMS-QP2010 PLUS using a Zebron ZB5MS capillary column (ID: 30 m X 0.32 mm; film thickness 0.25 µm). The column initially held at 90 °C for 4.5 min, then heated to 150 °C at a rate of 7 °C /min and to 170 °C at a rate of 10 °C /min, held for 8 min. Injector and detector temperatures were kept at 250 °C. Helium was used as carrier gas at 86.1 KPa (12.48 Psi). Mass detection was performed by an electron ionization mode with ionization energy of 70 eV and ion source temperature of 250 °C.

Chemical compounds identification

The identification of the essential oil constituents was based on comparison of their retention indices relative to homologous series of n-alkanes (C_6-C_{19}; Poly Science; Niles, USA) and published data. Chemical constituents were further confirmed by correlating the GC data with GC-MS data and compared to the NIST mass spectral library.

Evaluation of antibacterial activity

Staphylococcus aureus and *Chromobacterium violaceum were* used in the present study were collected from Microbial Type Culture Collection and Gene Bank (M.T.C.C) Institute of Microbial Technology Sector, Chandigarh, India. The antibacterial activity of these ocimum oils against *Staphylococcus aureus* (MTCC No: 9542) and *Chromobacterium violaceum* (MTCC No: 8071) was determined using the disk diffusion method.

The cultures were cultivated on Nutrient Agar (HiMedia) at 25 °C for 48 hours. The medium and petridishes were autoclaved at a pressure of 15 lb/inc^2 for 20 min. The medium was poured into sterile petridishes under aseptic conditions in laminar flow chamber. When the medium in the plate solidify, 0.5 ml of 24 h cultured of test organism was inoculated and uniformly spread over the agar surface with a sterile L-shaped rod. Controls were maintained with DMSO and pencillin G (for gram-positive) and streptomycin (for gram-negative). The treated materials and the controls were kept in an incubator at 37 °C for 24 h. Ocimum essential oils were applied on filter paper (2.0 and 4.0 µL/disk) disks of 6 mm in diameter separately. 10 mg/mL of pencillin G (for gram-positive) and streptomycin (for gram-negative) were used. These disks were placed on the surface of seeded agar plates at equal distance. Inhibition zones were measured and diameter was calculated in mm. Two replicates were maintained for each treatment.

Evaluation of antifungal activity

Aspergillus niger (MTCC No: 9687) and *Candida albicans* (MTCC No: 7253) were used for Antifungal assay and collected form Institute of Microbial Technology Sector, Chandigarh, India. The method followed for antifungal bioassay is similar to that followed for antibacterial assay where in the medium is Potato Dextrose Agar (HiMedia) and the control is Fluconazole. Control was maintained with DMSO with Fluconazole. The treated and the control were kept in an incubator at room temperature for 48 h. Incubation

zones were measured and diameter was calculated in mm. Two replicates were maintained for each treatment.

Seed oils extraction

Seeds of CIMAP developed two *Ocimum* varieties were cleaned and powdered. Each powdered material was exhaustively extracted for fixed oil by solvent hexane in a soxhlet apparatus. All the extracts were concentrated at reduced pressures to obtain crude residues.

Preparation of fatty acid methyl esters

All the samples were treated with 2% H_2SO_4 in methanol (10 ml) at reflux temperature (65 °C) for 4 hr for the conversion of lipid component to its fatty acid methyl esters. The reaction mixture was extracted with ethyl acetate (3 x 15 ml) and the washed with water. The organic layer was dried over anhydrous sodium sulphate and concentrated to get the fatty acid methyl ester (FAME) mixture.

GC/GC-MS Analysis for the Determination of Fatty acid composition: FAME mixture was dissolved in minimum amount of chloroform and analyzed by GC and G-MS for fatty acid composition.

GC analysis fatty acids

Agilant 6890 N Series gas chromatograph equipped with a FID detector. The GC was performed using DB-225 capillary column (30 m x 0.25 mm x 0.25 µm) and the oven temperature: 160 °C (2min)-5 °C.min-230 °C (20 min); N2 flows: 1.0 mL/min and the split ration – 50:1.

GC-MS analysis fatty acids

Agilant 6890 N series gas chromatograph connected to Agilant 5973 mass spectrometer (Palo Alto, USA). The GC-MS detection was performed at 70 eV (m/z 50-550; source at 230 °C and quadruple at 150 °C) in the EI mode attached with DB-225 ms capillary column (30 m x 0.25 mm x 0.25 µm). The oven temperature: 2 min at 160 °C (2min)-5 °C/min °C (20 min): He flow: 1.0 mL/min and the split ration 50:1.

Statistical analysis

Analysis of variance was performed to determine the effect of different times of harvest on morphological traits, essential yield and quality parameters using statistical software IRRISTAT [IRRI, Manila, Philippines]. Means were compared using least significant differences [LSDs] at 5% probability levels.

Results

Leaf number, leaf weight /plant and leaf area

In Vikarsudha leaf weight (58.4-368.4 g/plant) and leaf number (163-553/ plant) increased up to 60 days harvest in the main crop (Tab. 1). In the ratoon crop leaf weight and leaf number /plant increased up to 60 days harvest and later decreased, whereas leaf area increased up to 75 days harvest.

In the variety Kushmohak, leaf weight (55-350 g/plant) and leaf number (32-314/plant) increased up to 60 DH where as leaf area showed significant improvement up to 70 DH (14-32 cm^2) in the main crop (Tab. 1). Similar results were noticed in the ratoon crop also. Results of the present investigations confirm morphological and

developmental variability of basil with respect to leaf characteristics which are inconformity with those reported earlier [SIMON & al. 1992; GRAYER & al. 1996].

Oil content and oil yield/plant

In Vikarsudha, oil content decreased from 0.6 to 0.3 with advancement in the harvest date (DH) from 15 to 75 days in the main crop (Tab. 1) where as oil yield/plant increased from 1.1 to 4.4 g/plant up to 60 DH and then decreased to 2.8 g/plant at 75 DH during the same period in the main crop.

In the ratoon crop oil content increased up to 40 DH and later showed a study decline where as oil yield showed a study increase from 0.3 to 4.17 g/plant at 60 DH and later decreased.

In the variety Kushmohak, oil content exhibited an increase decrease pattern in both the seasons and varied around 0.45 on an average (Tab. 1). Oil yield/plant showed a significant improvement up to 60 DH and decreased later in both the seasons.

Differences in the essential oil content in different varieties of the same sps. and variable composition of the essential oil with time and changes in weather parameters in the same variety were reported by many workers. The oil content of sweet basil varieties reported in this study was similar to several literature reports [SUCHORSKA & OSIŃSKA, 2001; MAROTTI & al. 1996; WETZEIL & al. 2002; SUCHORSKA & OSINSKA, 1992].

Essential oil composition at different days of harvest

Vikarsudha: In *Ocimum basilicum* variety Vikarsudha, the essential oil distilled at different harvest dates was subjected to GC/GCMs analysis and nineteen chemical constituents in the oil (> 94% of the total) were identified. The constituents identified are (Tab. 2) camphene, β myrcene, limonene, 1-8-cineole (2.7 to 5.2), ocimene, linalool (23.5 to 40.1 %), camphor, terpinen-4-ol, methyl chavicol (25.4 to 51.9 %), neral, geranial, bornyl acetate, eugenol, methyl cinnamate, methyl eugenol, β caryophyllene, α humulene, δ cadinene, and cadinol in the main crop. Similar constituents were observed in the ratoon crop also (Tab. 2). In the ratoon crop the major constituents in the essential oil were linalool (22.8-33.7%) and methyl chavicol (39.0-52.7%).

Linalool exhibited an increase-decrease pattern (28.0-40.1-23.5%) in the main crop and showed a decrease-increase pattern in the ratoon crop (33.7-22.8-30.6%). Methyl chavicol exhibited an erratic pattern in the main crop and an increase-decrease pattern in the ratoon crop (40.0-52.7-38.9%).

Kushmohak: In *Ocimum basilicum* variety Kushmohak, the essential oil distilled at different harvest dates was subjected to GC/GCMs analysis and 92% of the chemical constituents in the oil were identified. The constituents identified were (Tab. 3) camphene, β myrcene, limonene, 1,8 cineole (3.2-4.5%), ocimene, linalool (19.2 to 25.5 %), camphor, terpinen-4-ol, methyl chavicol (34.7-53.4%), bornyl acetate, eugenol (2.1-5.4%), methyl eugenol (1.9-10.1 %), β elemene, β caryophyllene, α humulene, γ Cadinene and cadinol (1.4 to 2.1%).

During the main crop season, linalool remained constant around 25% up to 45 DH and later it decreased. Other major constituent methyl chavicol showed a definite increase from 47.1 to 53.4 at 45 DH and later it decreased to 34.7% at 75 DH. Ocimene, β

caryophyllene, γ cadinene and cadinol showed decreasing pattern with time, whereas limonene, terpinen-4-ol, bornyl acetate (0.16-1.2%), eugenol (2.1-5.4%) and methyl eugenol (1.9-10.1%) showed increasing pattern with time (Tab. 3).

In the ratoon crop, though the content of linalool showed a variable pattern with time of harvest the overall trend was a decrease in linalool content of the oil with late harvesting up to 70 DH. Methyl chavicol increased from 52.7 to 59.2 at 30 DH and it decreased in the later harvests. β pinene (0.4-1.4%), limonene (0.1- to 0.3%) and 1,8 Cineole (2.2-5.1%) showed increasing pattern (Tab. 3).

The chemical composition of basil oil has been the subject of considerable studies. In basil cultivars from Australia, methyl chavicol, linalool, methyl cinnamate, a mixture of linalool/methyl cinnamate, and linalool/ methyl chacivol were reported as the main components [LACHOWICZ & al. 1997]. In the oils obtained from aerial parts of basil grown in Colombia and Bulgaria, linalool and methyl cinnamate were reported as major components of oils, respectively [VIŇA & MURILLO, 2003].

Antimicrobial activity

The essential oil of *O. basilicum* extracted from both the varieties exhibited strong antimicrobial activity against both bacterial strains whereas antifungal activity (Tab. 4) was noticed at higher dose of application only. Results pertaining to the zone of inhibition indicated (Tab. 4) that among bacterial strains tested, *Chromobacterium violaceum* is more sensitive organism compared to *Staphylococcus aureus.* Similarly among the fungal strains *Aspergillus niger* was found to be more sensitive than *Candida albicans*.

Among the pure compounds eugenol exhibited strong antibacterial activity compared to methyl chavicol, β caryophyllene and linalool (Tab. 5). Eugenol also exhibited strong antifungal activity compared to methyl chavicol.

Essential oil obtained from plants harvested at 10 and 20 days exhibited higher antibacterial activity compared to the oil extracted from old plants. The activity was more at higher dose (4 µl) compared to lower dose in case of all organisms tested in both the varieties.

The activity of the oil decreased with advancement in age of harvest in case of bacterial strains *Staphylococcus aureus* and *Chromobacterium violaceum.* Both the varieties of basil exhibited significant antibacterial activity compared to control pencilin G and streptomycin at the early stage harvested oils. Only in case of fungal strain *Aspergillus niger* higher activity was noticed with the essential oil obtained from late harvests.

Seed fixed oil

Composition of basil seed oils evaluated in this study are shown in Tab. 6. Oil content in the seeds varied from 21.6 % in Vikarsudha to 12.4 % in Kushmohak. The seed yield observed was 230 kg/ha in Vikarsudha and 250 kg/ha in Kushmohak.

Unsaturated fatty acids averaged 85.6-88.1%, including α-linolenic (49.3-52.4%), linoleic (23.6-26%), and oleic acids (10.3-12.3%). The most abundant saturated fatty acids were palmitic (8.0 % -9.2%) and stearic (3.6-3.8%).

Discussion

Essential oil content and oil yield/plant

Oil content: In Kushmohak, the oil content ranged from 0.4 to 0.5% during the five harvest dates in the main crop and, the oil content increased from 0.2 to 0.5% at 30 DH and remained the same up to 60 DH and later decreased to 0.30 at 70 DH in the ratoon crop.

In Viukarsudha the oil content decreased with age whereas in the ratoon crop oil content exhibited a variable pattern and ranged from 0.3 to 0.7% and cantered around 0.45 % in the ratoon crop.

Differences in the essential oil content in different varieties of the same sps. and variable composition of the essential oil with time and changes in weather parameters in the same variety were reported by many workers. The oil content of sweet basil varieties reported in this study was similar to several literature reports. The oil content in sweet basils from different sources (Germany, Romania, Hungary and Egypt) was reported to be varied from 0.1 to 0.55% [SUCHORSKA & OSIŃSKA, 2001]. The content of essential oil in herb of ten Italian basil cultivars ranged from 0.3 to 0.8% [MAROTTI & al. 1996]. In another study essential oil content varied from traces to 2.65% in 270 sweet basil accessions studied in Germany [WETZEIL & al. 2002]. Such variations in the essential oil content of basil across countries might be attributed to the varied agroclimatic conditions of the regions besides their genetic makeup [SUCHORSKA & OSINSKA, 1992; GALAMBOSI & SZEBENI, 2002; SEIDLER-ŁOŻYKOWSKA & KRÓL, 2008].

Oil yield/plant: In both the varieties, oil yield per plant showed an increasing tendency with age due to the increase in leaf weight and area and when there was a drop in leaf weight after 60 days there was a decrease in oil yield per plant which was a product of oil content and herb weight. Though the oil content was very high at early stages the oil yield was less due to low herb weight. The results obtained in this study are in agreement with most of previous works which reported that the full-flowering stage is characterized by the highest essential yield. Oliveria and his coworkers (2005) reported that the essential oil yield increased during plant development to reach a maximum during the flowering stage. In earlier studies highest essential content and oil yield at full flowering stage was reported in *Thymus vulgaris* [OZGUVEN & TANSI, 1998], *Artemisia pallens* Wall [MALLAVARAPU & al. 1999] peppermint [ROHLOFF & al. 2005], *Satureja rechingeri* [SEFIDKON & al. 2007], and in oregano [KIZIL & al. 2008]. The essential oils yields in another crop *Melissa officinalis* were reported to vary considerably from month-to-month and vary significantly with harvesting stages [KEIVAN SAEB & al. 2012].

Essential oil composition

Essential oil distilled at different harvest dates was subjected to GC/GCMs analysis and 92% of the chemical constituents in the oil were identified. In Vikarsudha variety, a total seventeen constituents were identified and linalool and methyl chavicol were found to be the major constituents in the essential oil.

Linalool exhibited an increase – decrease pattern (28.0-40.1-23.5%) in the main crop and showed a decrease – increase pattern in the ratoon crop (33.7-22.8-30.6%). Methyl chavicol exhibited an erratic pattern in the main crop and an increase – decrease pattern in the ratoon crop (40.0-52.7-38.9%).

In *Ocimum basilicum* variety Kuhmohak, linalool (19.2 to 25.5%) and, methyl chavicol (34.7-53.4%) were found to be the major constituents. During the main crop season, linalool remained constant around 25% up to 45 DH and later it decreased. Other major constituent methyl chavicol showed a definite increase from 47.1 to 53.4 at 45 DH and later it decreased to 34.7 % at 75 DH.

In the ratoon crop, though the content of linalool showed a variable pattern with time of harvest the overall trend was a decrease in linalool content of the oil with late harvesting up to 70 DH. Methyl chavicol increased up to 30 DH and it decreased in the later harvests.

In both the varieties methyl chavicol is present in large quantities compared to linalool and both exhibited variable patterns with date of harvest. Linalool exhibited a decreasing tendency with age whereas methyl chavicol exhibited an increase in the vegetative phase and with the onset of flowering it started declining.

The chemical composition of basil oil has been the subject of considerable studies. Methyl chavicol, linalool, methyl cinnamate, a mixture of linalool/methyl cinnamate, and linalool/methyl chacivol were reported as the main components in basil cultivars from Australia [LACHOWICZ & al. 1997], linalool and methyl cinnamate were reported as major components of oils from Colombia and Bulgaria respectively [VIŇA & MURILLO, 2003].

Due to the high content of linalool, and methyl chavicol the studied cultivars may become applied in food and perfume industries, food seasoning and flavouring, aromatheraphy, and medicinal application.

In the present study higer seasonal variation was not noticed in the essential oil composition, whereas high percentage of linalool (60.6%) [HUSSAIN & al. 2008] and estragole (52.6 and 58.26%) were reported in winter grown basil compared to summer [CHALCHAT & OZCAN, 2008]. Higher solar irradiance level increased the contents of linalool and eugenol in *Ocimum basilicum* [CHANGA & al. 2008]. The hydro-distilled essential oils content ranged from 0.5% to 0.8%, the maximum amounts were observed in winter while minimum in summer [HUSSAIN & al. 2008].

Anti microbial activity of essential oil

The major constituents in the essential oil linalool and methyl chavicol showed increasing g pattern up to 30-40 DH and later showed a decreasing pattern. The decrease in antimicrobial activity against the bacterial strains with a later harvest dates may be due to the decrease in the concentration of the major constituents. In the varity Kushmohak exhibited a increased activity agains fungal strains with essential oil obtained from later harvests, which might be due to increase in eugenol and 1-8 cineole which showed increasing pattern with late harvest dates.

Some earlier reports showed that the changes in chemical composition of an essential oil directly affected their biological activities [CELIKTAS & al. 2007; VAN VUUREN & al. 2007; SUPPAKUL & al. 2003]. Literature indicates that basil essential oils exhibited good to moderate antimicrobial activity against a wide range of microorganisms [SUPPAKUL & al. 2003; WANNISSORN & al. 2005]. Gram-positive strains of bacteria showed higher sensitivity to *O. basilicum* essential oils than those of their counterpart [BOZIN & al. 2006; LOPEZ & al. 2005].

Linalool in the essential oil was reported to be responsible for the antifungal [SOKOVIC & VAN GRIENSVEN, 2006] and antimicrobial activities of essential oils from *O. basilicum* [KOUTSOUDAKI & al. 2005; SARTORATOTTO & al. 2004]. Evaluation of antimicrobial activity of the essential oils and linalool, the most abundant component, against bacterial strains: *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pasteurella multocida* and pathogenic fungi *Aspergillus niger*, *Mucor mucedo*, *Fusarium solani*, *Botryodiplodia theobromae*, *Rhizopus solani* indicated that all the tested microorganisms were affected [HUSSAIN & al. 2008].

The essential oils from *O. basilicum* varieties showed broad activity against bacteria and pathogenic fungi. The production of essential oils and their utilization as potential natural food preservants could be of economical value. However, further investigation to establish how components interact to provide the antioxidant activity is needed.

Seed oils

Oil content in the seeds varied from 21.6% in Vikarsudha to 12.4% in Kushmoh and seed yield from 230 kg/ ha in Vikarsudha and 250 kg/ha in Kushmohak.

Unsaturated fatty acids averaged 85.6-88.1%, including α-linolenic (49.3-52.4%), linoleic (23.6-26%), and oleic acids (10.3-12.3%). The most abundant saturated fatty acids were palmitic (8.0-9.2%) and stearic (3.6-3.8%). Values from the literature range from 5.3% to 15.4% for oleic acid, 14.0% to 66.1% for linoleic acid, and 15.7% to 65.0% for linolenic acid [PATWARDHAN, 1940; HENRY & GRINDLEY, 1944].

Basils are multi harvest multi utility crops. The main crop can be used for extraction of essential oil and the second crop can be used for production of dry leaf and seed oil. The seed oil content is high and the seed yield /ha is in the range 230-250 kg/ha a good quality seed oil to the extent 34.5 to 37.5 kg/ha can be obtained in the ratoon crop. This provides more income to the cultivator as this system facilitates production of essential oil for aroma chemicals, seed oil for food industry and also dry herb for traditional medicine industry and herbal tea manufacturers.

Basil cultivation technology is a very well known process through modern plant breeding techniques seed and oil content yields could be increased. Seeds of basil do not readily dehisce and can be harvested using a combine. A high linolenic acid oil, such as that found in *O. basilicum* and *O. canum*, could be used in the paint, varnish and ink industries, and as a source of linolenic acid, while oils with lower linolenic acid contentcan be used by the food industry.

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Tab. 1. Morphological characters, oil content and essential oil yield /plant in of *Ocimum basilicum* varieties Vikarsudha and Kushmohak at different times of harvest during October 2012 to February, 2013

Harvest date	leaf wt,g	Leaf n o	leaf area, cm^2	Oil content	Oil yield, g/plant	leaf wt, g	Leaf no	leaf area, cm^2	Oil content	Oil yield, g/plant
				Vikarsudha				Kushmohak		
15	58.4	163.0	15.0	0.6	1.1	55.0	32.0	14.0	0.5	0.8
30	253.6	221.0	23.0	0.6	4.6	165.0	143.0	21.0	0.4	2.0
45	358.4	435.0	24.0	0.5	5.4	197.0	212.0	24.0	0.5	3.0
60	368.4	553.0	28.0	0.4	4.4	350.0	314.0	26.0	0.5	5.3
75	306.0	427.0	31.0	0.3	2.8	302.0	221.0	32.0	0.4	3.6
CD	13.8	10.75	2.47	0.12	0.04	5.8	7.5	1.91	0.04	0.07
CV	11.06	4.05	7.27	16.84	5.92	6.34	3.63	5.45	6.17	9.52
10	32.5	68.0	24.0	0.3	0.3	52.0	**25.0**	16.0	0.2	0.3
20	43.5	196.0	28.0	0.6	0.8	142.0	96.0	23.0	0.4	1.7
30	97.3	212.0	31.0	0.7	2.0	267.0	146.0	26.0	0.5	4.0
40	157.0	324.0	31.0	0.7	3.3	289.0	198.0	29.0	0.5	4.3
50	217.5	412.0	34.0	0.5	3.3	352.0	324.0	31.0	0.5	5.3
60	343.5	522.0	34.0	0.4	4.1	387.0	392.0	32.0	0.5	5.8
70	326.9	512.0	34.0	0.4	3.9	321.0	265.0	36.0	0.3	2.9
CD	12.89	0.04	0.04	0.09	0.04	12.18	0.04	0.04	0.05	0.09
CV	9.26	0.01	0.09	14.34	5.59	4.81	0.01	0.08	6.58	6.1

Tab 2. Chemical composition of the essential oil of *Ocimum basilicum* variety Vikarsudha at different times of harvest during October 2012 to February, 2013

Harvest date	Chemical constituents of essential oil, %																		
	Camphene	β myrcene	Limonene	1,8 Cineole	Ocimene	Linalool	Camphor	Terpinen-4-ol	Methyl chavicol	Neral	Geranial	Bornyl acetate	Eugenol	Methyl cinnamate	Methyl Eugenol	β Caryophyllene	α Humulene	γ Cadinene	Cadinol
15	0.2	0.9	0.2	5.2	0.3	28.1	0.9	0.2	45.0	0.8	0.9	0.1	1.9	0.8	5.1	1.5	0.3	0.5	1.5
30	0.1	0.6	0.1	4.7	0.2	35.5	0.8	0.4	36.4	1.0	1.1	0.4	1.1	2.3	2.9	1.8	0.4	0.6	1.6
45	0.1	0.6	0.1	4.5	0.1	40.1	0.8	0.8	32.7	0.8	0.9	0.1	2.8	0.8	5.1	1.6	0.2	0.5	1.3
60	0.1	0.7	0.2	4.3	0.2	26.6	1.1	1.0	51.9	0.3	0.2	0.2	2.7	0.4	1.9	1.3	0.2	0.4	1.2
75	0.3	0.5	0.1	2.7	0.1	23.5	1.5	1.0	25.4	1.4	0.4	2.6	4.2	1.6	6.0	1.1	0.3	0.5	0.9
CD	0.04	0.12	0.04	1.40	0.04	10.79	0.17	0.14	9.22	0.11	0.11	0.10	0.92	0.91	1.40	0.18	0.04	0.12	0.17
CV	15.33	11.59	17.55	22.41	16.01	24.12	11.38	13.98	16.82	9.10	10.73	32.68	24.92	35.42	22.87	8.42	10.38	15.11	9.34
10	0.2	0.8	0.2	4.2	0.2	33.7	0.7	1.0	40.0	1.3	1.4	0.3	1.7	1.7	1.9	1.2	0.2	0.4	1.0
20	0.2	0.8	0.1	5.1	0.1	29.5	0.8	0.5	48.4	0.4	0.4	0.2	2.1	1.1	4.1	0.9	0.2	0.3	0.9
30	0.2	1.0	0.2	5.9	0.1	22.9	0.6	0.7	52.3	0.4	0.4	0.3	2.6	0.8	4.1	1.3	0.2	0.5	1.1

40	0.2	0.8	0.2	4.8	0.2	22.8	0.6	0.7	52.7	0.6	0.7	0.3	1.5	1.8	3.7	1.4	0.2	0.6	1.5
50	0.2	1.2	0.2	5.0	0.2	25.8	1.2	0.6	47.9	0.5	0.6	0.5	2.9	0.5	2.3	1.3	0.2	0.6	1.8
60	0.2	0.9	0.2	4.8	0.2	26.0	0.8	1.1	47.6	0.6	0.5	0.6	1.6	1.7	4.7	1.2	0.2	0.6	1.4
70	0.3	1.3	0.3	5.2	0.2	30.6	0.7	0.8	39.0	0.4	0.3	0.5	3.2	0.5	6.0	2.0	0.3	0.9	1.9
CD	0.03	0.18	0.03	0.78	0.03	5.75	0.09	0.09	7.86	0.11	0.13	0.09	0.25	0.11	0.77	0.14	0.03	0.08	0.16
CV	11.73	12.34	11.33	10.61	13.58	13.73	7.74	7.66	11.90	12.70	15.04	15.07	7.69	6.43	13.73	7.02	11.43	10.35	7.86
RI Values	949	984	1030	1032	1034	1105	1130	1165	1186	1222	1240	1274	1340	1348	1376	1422	1456	1507	1650

Tab. 3. Chemical composition of the essential oil of *Ocimum basilicum* variety Kushmohak at different times of harvest during October 2012 to February, 2013

Harvest date	Chemical constituents of essential oil, %																		
	Camphene	β pinene	Limonene	1,8 Cineole	Ocimene	Linalool	Camphor	Terpinen-4-ol	Methyl chavicol	Bornyl acetate	Eugenol	Methyl Eugenol	βelemene	β Caryophyllene	α Humulene	γ Cadinene	Cadinol		
15	0.3	0.8	0.2	3.8	0.2	25.5	1.0	0.6	47.1	0.3	2.7	6.2	0.6	1.5	0.3	0.7	2.1		
30	0.3	0.6	0.2	3.5	0.2	25.4	0.8	0.5	47.7	0.2	2.8	4.5	0.9	2.5	0.4	0.7	1.8		
45	0.2	0.5	0.2	3.2	0.2	25.3	0.9	0.7	53.4	0.2	2.1	1.9	0.5	1.8	0.2	0.8	1.8		

	60	0.2	0.7	0.2	4.5	0.1	19.2	0.7	0.6	51.5	0.3	3.7	6.9	0.8	1.5	0.2	0.5	1.4
	75	0.1	0.9	0.2	3.6	0.2	20.8	1.1	1.0	34.7	1.2	5.4	10.1	0.8	1.0	0.2	0.5	1.4
CD		0.04	0.08	0.04	0.94	0.04	4.62	0.14	0.09	9.20	0.10	1.38	2.30	0.08	0.23	0.04	0.10	0.92
CV		13.09	7.91	16.31	17.25	16.91	13.66	10.26	9.61	13.49	16.65	28.44	26.59	8.05	9.47	10.51	11.04	37.13
	10	0.1	0.4	0.1	2.2	0.1	23.0	0.7	0.4	52.7	0.2	1.3	5.4	0.6	1.9	0.3	0.9	2.0
	20	0.2	0.9	0.2	4.9	0.2	16.1	0.8	0.7	52.9	0.2	3.2	7.4	0.6	1.3	0.3	0.6	1.6
	30	0.1	0.5	0.1	3.4	0.1	22.9	0.7	0.6	59.2	0.2	1.7	2.3	0.5	1.5	0.2	0.6	1.4
	40	0.2	1.2	0.2	5.8	0.2	24.9	0.8	0.8	51.2	0.3	2.2	3.2	0.5	1.2	0.2	0.5	1.2
	50	0.1	0.7	0.2	3.7	0.1	18.8	1.3	1.0	43.9	0.7	2.6	8.9	0.9	1.7	0.3	0.8	2.0
	60	0.2	1.2	0.2	5.2	0.1	21.9	1.0	0.6	45.0	0.7	2.3	8.3	0.8	1.2	0.2	0.6	1.7
	70	0.3	1.4	0.3	5.1	0.3	21.3	1.0	1.0	39.4	0.6	3.8	4.7	0.7	1.8	0.2	0.7	1.7
CD		0.03	0.15	0.05	0.78	0.04	7.78	0.13	0.11	11.65	0.12	0.78	1.94	0.12	0.21	0.03	0.15	0.37
CV		12.21	11.40	18.45	12.35	16.49	23.57	9.95	10.76	16.29	21.23	21.99	23.26	12.80	9.76	8.62	15.39	15.26
RI Values		949	984	1030	1032	1034	1105	1130	1165	1186	1274	1340	1376	1396	1422	1456	1501	1650

Tab. 4. Antimicrobial activity[§] of the essential oil of two varieties of *Ocimum basilicum* and one variety of *Ocimum tenuiflorum* at different harvest dates during December, 2012 to February, 2013

Variety	Harvest date	**Antibacterial activity**				**Antifungal activity**			
		Staphylococcus aureus		***Chromobacterium violaceum***		***Aspergillus niger***		***Candida albicans***	
		2 µl*	4 µl**	2 µl	4 µl	2 µl	4 µl	2 µl	4 µl
Ocimum basilicum variety Vikarsudha	10	14.5	16.5	22.5	40.0	0.0	0.0	5.5	12.5
	20	9.5	12.5	14.5	19.5	0.0	6.0	0.0	5.5
	30	7.5	13.0	12.0	22.0	0.0	7.0	0.0	5.5
	40	8.5	10.5	17.5	25.5	0.0	14.0	0.0	6.0
	50	6.5	11.5	17.5	25.5	0.0	5.5	0.0	6.0
	60	7.5	10.0	14.5	29.5	0.0	7.5	0.0	6.0
	70	11.0	13.0	12.0	19.0	0.0	16.5	0.0	7.0
Ocimum basilicum variety Kushmohak	10	11.0	12.5	21.0	30.0	0.0	7.0	5.5	12.5
	20	11.0	12.5	19.5	29.0	0.0	8.0	4.0	5.5
	30	7.5	11.5	16.0	21.5	0.0	7.0	5.5	11.5
	40	8.5	11.5	19.0	14.0	0.0	6.0	9.0	13.5
	50	8.5	10.0	20.5	24.5	0.0	8.0	7.5	11.0
	60	8.5	10.0	15.0	25.0	0.0	16.5	8.0	11.0
	70	9.0	11.0	14.5	30.5	0.0	16.0	9.0	12.0

Tab. 5. Antimicrobial activity§ of the essential oil components of *Ocimum* sps.

Components	Antibacterial activity				Antifungal activity			
	Staphylococcus aureus		***Chromobacterium violaceum***		***Aspergillus niger***		***Candida albicans***	
	2 µl	4 µl	2 µl	4 µl	2 µl	4 µl	2 µl	4 µl
Eugenol	23.0	25.0	24.0	28.5	20.0	30.0	20.0	35.0
Linalool	4.5	7.0	6.5	9.5				
Methyl Chavicol	18.0	30.0	27.0	34.0	14.0	18.0	9.0	9.0
Beta Caryophyllene	6.5	9.5	15.5	17.5				
Penicillin G	8.0	17.5	0.0	0.0	0.0	0.0	0.0	0.0
Streptomycin	0.0	0.0	12.5	15.0	0.0	0.0	0.0	0.0
Fluconazole	0.0	0.0	0.0	0.0	15.0	20.0	15.0	20.0

§: Inhibitory zone diameters in mm

Tab. 6. Fatty acid composition of fixed oil from seed

Variety	**Seed yield , kg/ha**	**Fatty acid composition of fixed oil from seed (%, w/w)**							
		16:0	**16:1**	**17:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:0**
Kushmohak	**230**	9.2	0.2	0.1	3.8	10.3	26.0	**49.3**	0.3
Vikarsudha	250	8.0	-	0.1	3.6	12.3	23.4	**52.4**	0.2

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VEGETATIVE IMPROVEMENT OF THREE NIGERIAN SESAME VARIETIES AFTER FNI TREATMENT

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Abstract: The effects of Fast Neutron Irradiation (FNI) from an Americium Beryllium source with a flux of 1.5 × 10^4 n cm^{-2} s^{-1} on select vegetative parameters of three Nigerian sesame (*Sesamum indicum* L.) varieties were investigated. Seeds of Kenana-4 , Ex-Sudan and E-8 were irradiated with 4, 8, 12 and 16 µSv doses of FNI before they were grown to maturity, alongside their respective controls (0 µSv dose of FNI). The vegetative parameters investigated included percentage survival, petiole length, number of leaves per plant, leaf surface area and plant height. There were significant differences ($p<0.05$) between the different doses of FNI for all three varieties. Correlations between irradiation doses and morphological parameters were generally highest in E-8, followed by Kenana-4, while Ex-Sudan showed the weakest correlations, suggesting that E-8 was the most sensitive to FNI. Thus, FNI can induce genetic variability in sesame and may be important for sesame breeders who seek to expand the genetic base of their breeding material.

Key words: sensitivity, FNI, morphological parameters, sesame, genetic improvement

Introduction

Sesame (*Sesamum indicum* L.; Pedaliaceae), an annual oilseed plant, has a rich history of cultivation in Asia [BISHT & al. 1998]. The most cultivated *Sesamum* species is *S. indicum* [ASHRI, 1998], an important edible oil crop in many regions of the world that may have originated from Africa, which is where the greatest diversity of the genus *Sesamum* and its family, Pedaliaceae, is present [FALUSI & al. 2001]. Mutation technology has been used to produce many cultivars with improved economic value and to advance the study of plant genetics and development [RANALLI, 2012]. Genetic variability for desired characters can be successfully induced through mutations, with high practical value in plant improvement programs [FAHAD & SALIM, 2009]. Mutation breeding employing fast neutron irradiation (FNI) has been used to develop new varieties of pepper [FALUSI & al. 2012].

Ionizing radiation has been routinely used to generate genetic variability in sesame for breeding and genetic studies and is a way to supplement existing germplasm with additional variation and to improve existing cultivars [BOUREIMA & al. 2009]. Since FNI-induced mutations in sesame could be useful as a new source of altered germplasm, our objective was to assess the impact of FNI on vegetative parameters of three sesame varieties grown in Nigeria. A previous study using the same experimental design and cultivars indicated that FNI could improve reproductive characteristics and oil-related properties [MUHAMMAD & al. 2013].

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Materials and methods

Following the protocol of [MUHAMMAD & al. 2013], seeds of three varieties of sesame (Ex-Sudan, Kenana-4 and E-8) 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 FNI and served as the control. The remaining four groups were irradiated with FNI for 30, 60, 90 and 120 min (resulting in 4, 8, 12 and 16 µSv, respectively) at the Centre for Energy and Research Training (CERT), Ahmadu Bello University, Zaria, Kaduna State, Nigeria. Pot experiments were conducted during the 2012 rainy season (May-August) at the Biological Garden, Federal University of Technology, Minna, Niger State, Nigeria. A randomized block design with 30 pots/block was used. The experiment was replicated four times, with a total of 120 pots. Ten seeds were planted per pot (i.e. 5/hole/pot). Three weeks after planting, each pot was thinned to two plants/pot and 8 pots/treatment combination were used. The physical and chemical properties of the soil were analysed at the Nigerian Institute for Science Laboratory Technology (NISLT), Ibadan, Nigeria (see Tab. 1, [MUHAMMAD & al. 2013]). No fertilizer was applied. When the crop began to flower, an insecticide (pyrethroid cypermethrin, 10-15 L/ha with controlled droplet application using spinning disc sprayers) was applied to prevent insect-borne diseases.

Plant height (cm) was measured at 2, 4 and 6 weeks after planting (WAP) and plant survival (%) was assessed 21 days after planting (DAP). Two morphological parameters were assessed at 6 WAP (petiole length (cm) and leaf surface area (LSA; cm^2)), two leaf samples randomly from every plant and 10 plants/treatment.

All data (except survival %) were subjected to analysis of variance (ANOVA). Significant differences between means were assessed by the least significant difference (LSD) test ($P \leq 0.05$). Pearson's correlation was used to assess the relationship between treatments and parameters.

Results and discussion

Significant differences ($P \leq 0.05$) were observed for all the vegetative parameters of sesame plants after exposure of seeds to different doses of FNI except for number of leaves/plant and LSA, which were statistically equal in Kenana-4 and E-8 (Tab. 1). Similar effects of FNI on morphological and yield traits were reported by FALUSI & al. (2012) in *Capsicum annuum* (peppers) and on reproductive (floral) and oil-related parameters by MUHAMMAD & al. (2013) in the same sesame cultivars. There were both positive and negative correlations between vegetative parameters and FNI level (Tab. 2). The strong positive correlation (0.953) observed for LSA (Tab. 2) in E-8 is an indication that an increase in FNI dose also increased this parameter. However, the negative correlations (Tab. 2) between morphological parameters and FNI doses are in agreement with MUHAMMAD & al. (2003) who studied the sensitivity of five *Oryza sativa* (Basmati rice) varieties to different doses of gamma-rays and observed that seedling emergence, panicle fertility and grain yield declined with increasing dose in all varieties. The positive correlations observed are, on the other hand, in agreement with FALUSI & al. (2012a, 2012b) on pepper in which yield parameters such as number of fruits/plant, number of seeds/fruit, length of fruit, width of fruit and fruit weight increased significantly as

irradiation exposure period increased. FALUSI & al. (2012a) observed that an increase in FNI irradiation dose in *Capsicum annuum* var. *accuminatum*, *C. annuum* var. *abbreviatum* and *C. annuum* var. *grossum* increased plant height, number of leaves/plant, fruits/plant, seeds/fruit, weight, length and width of fruits. The survival percentages of the three sesame varieties at different doses of FNI are shown in Tab. 3. In this study, an increase in FNI dose increased select vegetative traits. The differences observed among the varieties might be due to varietal responses to irradiation as reported by PATHIRANA & SUBASINGBE (1993), also in sesame in response to gamma radiation, and by MUHAMMAD & al. (2013) for the same sesame cultivars as this study. In contrast, the IAEA (1994) reported that sesame seeds are resistant to gamma irradiation. Moreover, seeds may be resistant to irradiation, as reported by PATHIRANA & SUBASINGBE (1993) and the IAEA (1994). In this study, vegetative parameters were most sensitive to 12 µSv. FNI has the potential to create genetic variability in sesame, and this is important for breeders seeking to expand the genetic base of their breeding material.

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Tab. 1. LSD of or select morphological parameters of three sesame varieties at different doses of FNI

	Plant height (cm)			At 6 weeks		
Treatment combinations	2 weeks	4 weeks	6 weeks	Length of petiole (cm)	Number of leaves/plant	Leaf surface area (cm^2)
Kenana-4						
0 µSv	6.67 ± 1.67 a	23.43 ± 5.29 bc	59.65 ± 14.61 a	3.72 ± 1.80 b	10.00 ± 1.16 a	36.64 ± 14.76 a
4 µSv	5.53 ± 1.29 b	20.83 ± 5.78 c	58.19 ± 16.03 a	3.80 ± 1.28 b	9.00 ± 1.25 a	33.04 ± 9.32 a
8 µSv	6.43 ± 1.71 a	21.24 ± 3.64 b	35.52 ± 19.76 b	3.53 ± 0.76 b	11.00 ± 1.90 a	35.52 ± 19.76 a
12 µSv	6.42 ± 1.28 a	25.79 ± 3.38 c	32.21 ± 10.32 b	5.08 ± 1.33 a	11.00 ± 2.18 a	32.21 ± 10.32 b
16 µSv	6.40 ± 1.84 a	20.75 ± 2.41 c	35.87 ± 13.71 b	2.11 ± 0.53 c	10.00 ± 0.95 a	35.87 ± 13.71 a
Ex-sudan						
0 µSv	7.15 ± 1.75 a	27.21 ± 4.69 a	69.90 ± 12.4 a	4.05 ± 1.48 c	11.00 ± 1.77 b	38.49 ± 17.25 b
4 µSv	7.33 ± 1.75 a	21.65 ± 2.87 d	67.54 ± 14.25 ab	4.53 ± 1.13 ab	11.00 ± 0.97 b	38.06 ± 6.86 b
8 µSv	6.97 ± 0.87 ab	26.31 ± 4.24 b	27.64 ± 8.77 c	3.88 ± 1.39 c	9.00 ± 0.82 b	27.64 ± 8.77 b
12 µSv	6.76 ± 1.52 ab	26.30 ± 3.73 bc	62.17 ± 21.51 b	5.80 ± 2.74 a	16.00 ± 7.65 a	62.17 ± 21.51 a
16 µSv	6.55 ± 1.12 c	24.49 ± 3.48 c	31.95 ± 7.68 c	4.18 ± 1.02 b	10.00 ± 0.57 b	31.95 ± 7.68 b
E-8						
0 µSv	6.51 ± 1.12 b	20.91 ± 1.93 c	49.19 ± 13.35 b	2.01 ± 0.55 c	10.00 ± 1.29 a	36.64 ± 6.787 a
4 µSv	6.89 ± 1.25 a	22.72 ± 3.10 bc	53.75 ± 12.34 a	3.05 ± 0.90 b	9.00 ± 0.99 a	33.04 ± 7.11 a
8 µSv	6.13 ± 1.36 b	21.27 ± 3.44 c	30.80 ± 6.49 c	2.34 ± 0.78 bc	11.00 ± 1.73 a	35.52 ± 6.49 a
12 µSv	5.76 ± 1.50 b	24.61 ± 3.83 a	36.20 ± 10.47 c	3.75 ± 1.34 ab	10.00 ± 1.16 a	32.21 ± 10.47 a
16 µSv	6.38 ± 0.84 b	24.04 ± 3.14 b	37.83 ± 15.34 c	4.26 ± 2.11 a	10.00 ± 1.42 a	35.87 ± 15.34 a

*Values are mean ± SD. Values followed by the same letter(s) within the same column, assessed separately for each cultivar, do not statistically differ at the 5% level according to LSD, analysed for the treatment combinations.

Tab. 2. Correlations between various morphological parameters of three sesame varieties for all doses of FNI

	Plant height (cm)			At 6 weeks			
Variety	2 weeks	4 weeks	6 weeks	PL (cm)	NOL/P	LSA (cm^2)	Survival (%)
Kenana-4	0.126	-0.029	-0.865	-0.994	-0.834	-0.874	-0.048
Ex-Sudan	-0.907	-0.056	-0.631	-0.125	-0.129	-0.245	-0.233
E-8	-0.52	0.788	-0.667	0.998	0.757	0.953	-0.850

*PL = petiole length, NOL/P = number of leaves/plant, LSA = leaf surface area

Tab. 3. Survival percentage three sesame varieties at different doses of FNI (n = 11 per dose)

	E-8	Ex-Sudan	Kenana-4
0 µSv	50.00 ± 22.52 a	50.91 ± 26.96 a	38.18 ± 17.27 a
4 µSv	46.36 ± 30.68 a	37.27 ± 21.00 ab	32.73 ± 19.23 a
8 µSv	40.00 ± 26.43 a	36.36 ± 19.23 a	41.82 ± 22.52 a
12 µSv	45.45 ± 20.53 a	21.82 ± 14.14 b	27.27 ± 18.52 a
16 µSv	34.55 ± 28.25 a	46.36 ± 23.75 a	41.82 ± 34.62 a

*Values are mean ± SD. Values followed by the same letter(s) within the same column do not statistically differ at the 5% level according to LSD, analysed for the treatment combinations.

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BASIDIOMYCETE-BASED METHOD FOR BIOCONTROL OF PHYTOPATHOGENIC NEMATODES

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Abstract: Phytopathogenic nematodes represent one of the most important groups of pathogens in crops. The use of chemical to control the nematodes attack in crops is decreasing every year due to the concern of the toxicity and side effects of such compounds. In the course for finding alternatives to the use of chemicals, biological control of nematodes is gaining much attention. Some saprotrophic fungi are able to feed on invertebrates, thus becoming efficient agents of control. In this study, three species of basidiomycetes were analyzed for their potential to be used as control agents of phytopathogenic nematodes. Through on *in vitro* investigation of these potential, one strain – *Gymnopilus junonius* was further selected for a pot test against *Meloidogyne incognita*, a very important phytopathogenic species of nematodes. The fungal treatment strongly decreased the *M. incognita* population on the tested pots, proving the potential of *G. junonius* strain to be used in biocontrol.

Keywords: biocontrol, phytopathogenic nematodes, *Meloidogyne incognita*, nematophagous fungi, *Gymnopilus junonius*

Introduction

The nematodes are primarily aquatic organisms, or organisms that develop in humid soils, in many types of habitats. Most of the nematodes have microscopic dimensions (0.3–3.0 mm). The soil is very rich in nematodes, representing approximatively 26% of all the invertebrates genera [WHARTON, 1986]. The root knot nematodes from the genus *Meloidogyne* is one of the most worldwide-spread group of plant parasites that affect most of the crops [PERRY & MOENS, 2009; BOROŞ & al. 2015], being obligate-parasites. The use of nematicidal compounds have significantly decrease the *Meloidogyne* populations sizes, but due to the strong toxicity and side-effects of these chemical, many of nematicidal compounds have been banned for commercializing [RAVICHANDRA, 2010]. For this reason, new types of treatment for controlling the phytopathogenic nematodes are required, and the biological ones are the most promising.

Lignicolous saprotrophic fungi degrade dead wood, but can also use other substrates as an alternative source of nutrients. In this respect, many species of lignicolous basidiomycetes capture and consume invertebrates for an increase uptake of nitrogen and phosphorous [DIX & WEBSTER, 1995].

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Although most of the studies on biocontrol of nematodes involved imperfect nematophagous specialized fungi [YANG & al. 2007], the effectiveness of these species is limited due to their reduced development in agricultural soils. In this respect, the basidiomycete species appear to be better adapted to agricultural environment, many of these species having the ability of colonizing nematodes [KUMAR & KAVIYARASAN, 2012; LI & al. 2007; TRUONG & al. 2007; TZEAN & LIOU, 1993]. The involved mechanisms are either biological, based on specialized structures used for actively capturing nematodes [LUO & al. 2004; KARASIŃSKI, 2013; TRUONG & al. 2007] or biochemical ones, with the secretion of nematicidal compounds [DONG & ZHANG, 2006; LIU & al. 2008].

When nutrients are available in large quantities, the carnivory of fungal species involved do not occur. However, in the agricultural soils, the characteristic substrata for lignicolous fungi are missing, but the mycelium develops in vegetative form, using different organic compounds as sources of nutrients. In a previous study concerning the potential of basidiomycetes in biocontrol of nematodes (*in press, corrected proof*) we have tested 68 strains of basidiomycetes for selection of strains with high potential of capturing / killing nematodes. Three saprotrophic species of fungi – *Gymnopilus junonius*, *Fomitopsis pinicola* and *Daedalea quercina* proved a strong efficiency in colonizing nematodes cadavers when the medium was low in nutrients.

In the present study, we have analyzed three species o lignicolous basidiomycetes for their potential as biological control agents. The most promising strains, *G. junonius* was selected for further investigations against phytopathogenic nematodes – *Meloidogyne incognita.* The obtained data suggest a biochemical mechanism involved, as *Gymnopilus junonius* produces gymnopillins, gymnoprenol and related products [KUSANO & al. 1986] with various biological effects, such as antibacterial [AL-FATIMI & al. 2013], antimycotic [VAHIDI & al. 2006] and cytotoxicity against different types of animal cells [KAYANO & al. 2004; TOMASI & al. 2004].

Our results proved a good potential of *G. junonius* strain for the production of nematicidal compounds, opening new perspectives in biocontrol of phytopathogenic nematodes.

Materials and methods

Fungal strains and nematodes

The tested fungal strains: *Daedalea quercina*, *Fomitopsis pinicola* and *Gymnopilus junonius*, previously isolated from fruit bodies [BALAEŞ & TĂNASE, 2012] belong to the Culture Collection of Fungal Research Laboratory (RECOSOL), Faculty of Biology, Alexandru Ioan Cuza University of Iasi. The nematode species – *Steinernema feltiae* was purchased as a commercial available product, Entonem® (Koppert B.V, The Netherlands). The product consists in a powder with juveniles at the same stage of development. A suspension of approximatively 10,000 individuals per milliliter was prepared in distilled sterile water and used for inoculation. The phytopathogenic nematodes – *Meloidogyne incognita*, was obtained from the collection of Regional Laboratory of Nematology – Brasov, Romania [BOROŞ & al. 2015]. The juvenile nematodes (second stage, J_2) were extracted from tomatoes roots of 12 weeks old and treated with 1% streptomycin sulfate for 3 minutes.

Media and cultures

All the media have been prepared using reagents of analytical grade. An organic medium was used for preparation the inoculum used in all experiment (L^{-1}): glucose 8 g, malt extract 20 g, yeast extract 2 g, peptone 2 g, agar 15 g. Except when stated, no other minerals have been added to the media composition. Sterilization of media was done using a 75 liters upright model autoclave (Raypas, Barcelona, Spain), at 120 ºC. The pH was adjusted at value 5.5, with hydrochloric acid 0.1 M or potassium hydroxide 0.1 M, using an electronic pH/ion-meter (model INOLAB, WTW, Weilheim, Germany).

Influence of carbon and nitrogen sources. For testing the influence of the carbon and nitrogen sources over the colonization of nematodes by the fungal mycelium, eight types / concentrations of carbon sources (C1-C8) and seven for nitrogen sources (N1-N7) have been used (Tab. 1) in solidified media (15 g agar L^{-1}).

Tab. 1. Composition of nutrient media used for screening of C and N sources

Media	C (g L^{-1})	N (g L^{-1})	Mycelium development (cm radius)		
			D. quercina	*F. pinicola*	*G. junonius*
C1	sucrose (2)	peptone (0.5)	1.7	1.8	2.2
C2	maltose (2)	peptone (0.5)	4.0	4.1	3.2
C3	sorbitol (2)	peptone (0.5)	1.6	3.0	1.6
C4	starch (2)	peptone (0.5)	2.4	4.5	3.1
C5	glucose (1)	peptone (0.5)	3.0	4.5	2.4
C6	glucose (3)	peptone (0.5)	4.5	4.1	4.1
C7	glucose (5)	peptone (0.5)	4.5*	4.2	4.5
C8	glucose (7)	peptone (0.5)	4.5*	4.2	4.5
N1	glucose (2)	peptone (0.5)	4.0	4.1	4.0
N2	glucose (2)	peptone (1)	4.1	4.2	4.1
N3	glucose (2)	peptone (2)	2.3	4.0	3.1
N4	glucose (2)	peptone (4)	2.2	3.1	2.4
N5	glucose (2)	ammonium sulphate (0.5)	4.1	3.7	4.0
N6	glucose (2)	urea (0.5)	3.5	4.0	2.4
N7	glucose (2)	sodium nitrate (0.5)	4.0	3.7	4.5

*the mycelium reached the edge of plate prior to analysis, and develop a very thick net

Influence of nutritive salts. The influence of salts, has been tested both on solid (15 g agar L^{-1}) and liquid media, containing 2 g of glucose and 0.5 g of peptone L^{-1} for solid media (S1-S3) and for first variant of liquid medium (S4) and respectively 4 g of glucose and 1 g of peptone L^{-1} for second variant of liquid media (S5). Five variants of media have been used (L^{-1}): **S1** 2 g KH_2PO_4 + 1 $MgSO_4 \cdot 7H_2O$; **S2** 1 g KH_2PO_4 + 0.5 $MgSO_4 \cdot 7H_2O$; **S3** 0.5 g KH_2PO_4 + 0.1 $MgSO_4 \cdot 7H_2O$; **S4** 1 g KH_2PO_4 + 0.5 $MgSO_4 \cdot 7H_2O$ + 0.1 $FeSO_4 \cdot 7H_20$ + 0.1 $NaNO_3$; **S5** 1 g KH_2PO_4 + 0.5 $MgSO_4 \cdot 7H_2O$ + 0.1 $FeSO_4 \cdot 7H_20$ + 0.1 $NaNO_3$. The test tubes with liquid media were shaken at 150 rpm.

Influence of metals. The influence of metals was tested only using *G. junonius*, on liquid media (L^{-1}: 2 g glucose, 0.5 g peptone, 1 g KH_2PO_4, 0.5 $MgSO_4 \cdot 7H_2O$) supplemented with sulfates of heavy metals: Cu^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+}, in concentration of 1 mM and 2 mM respectively. The cultivation was performed in tubes of 10 mL, with 2 mL working

volume, and the addition of nematodes have been done after 7 days of incubating fungi. The control consisted in fungi-free medium with nematodes.

Influence of inoculum quantity. To assess the optimum quantity of inoculum, for the colonization / nematicidal effect to occur, and for establishing economical variants of media for fungal cultivation, three types of media have been used (L^{-1}): A: 2 g glucose + 0.5 g peptone; B: 2 g sucrose + 0.5 malt extract; C: 10 g sucrose + 5 g malt extract. The sucrose and malt extract used, were cheap supplements acquired from commerce, as food sugar for human consumption and malt extract for beverages. Nematodes were added as a suspension of 0.1 mL, containing 1000 individuals. In order to keep the pH at an approximately constant value, a lactate buffer of 0.05 M, 5.5 pH was used. Quantities of inoculum from 0.1 to 1 mL were used.

Each sample / combination on Petri dishes were made in three replicate, while on test tubes five replicates were used for each. The plates / test tubes have been incubated in the dark at 25 ºC, for 7 days before suspensions with nematodes being added.

The inoculation of Petri dishes have been achieved using agar plugs of 11 mm diameter, taken from actively growing colonies (2 weeks old cultures, grown on the inoculum medium) and placed in the center of each Petri dishes. 0.1 mL of nematodes suspension (1000 nematodes) has been pulverized after 7 days of incubation, on the surface of each Petri dishes, then being analyzed for 15 days further. Controls, consisting of Petri dishes inoculated with nematodes but with no fungi, were used for each type of media. From the three fungal species tested, *G. junonius* was selected as the most efficient species, and used further in experiments. For test tubes, inoculation was performed using mycelium grown on liquid medium (the inoculum medium described above, without agar) for two weeks and homogenized thereafter using a Heidolph Homogenizer, at 9000 rpm.

Analytical methods and measurements

The effect of fungal mycelium over the viability of nematodes was assessed after 7 days of co-incubation (after nematodes addition to experimental variants). Visual observation made both under the stereomicroscope (stereomicroscope SZM2 Optika, at a magnification of 20-45x) and at microscope using glass slides (phase contrast microscope NIKON, at a magnification of 200-1000x) were made, and the viability of nematodes was tested by mechanically stimulating the bodies with a very thin needle. The degree of nematodes colonization by fungal mycelium was assessed visually, after staining. Pictures have been taken using a photo camera at all stages.

Pots experiment

A total number of 10 pots filled with a mixture of 6:3:1 clay-sand-peat, pH 7.2, sterilized through autoclaving at 120 °C, 20 minutes. Seeds of *Lycopersicum esculentum* "ClaussF1" variety were placed in the pots, and after 14 days from the germination, the substrate was inoculated with root-knot nematodes from *Meloidogyne incognita* species, 80 individuals (second stage juveniles, J_2) per pot. From these pots, five were co-inoculated with tested fungi (*G. junonius* cultivated on liquid medium for inoculum, as described above) and five remained un-inoculated and used as control. After 20 days of cultivation of nematodes-inoculated tomatoes, the nematodes extraction from soil was performed using the Cobb's method, through sieving and decantation and afterwards using the Baermann modified method [Southey, 1985]. The nematodes were collected in aqueous suspension

during no more than three days, numbered on counting dish, using a binocular stereomicroscope (Leica MZ95). The features of roots and the morphological aspect of juveniles were also analyzed.

Data interpretation and calculations

The rate of fungal colonization of nematodes bodies and viability of nematodes was evaluated under the microscope by randomly choosing seven squares of one cm^2 for each Petri dishes and counting the nematodes in designated area, or by taking 0.1 mL of liquid for samples in liquid media. For each Petri dishes, 200-250 nematodes were observed and analyzed. The data presented in current paper represents the mean value for all the replicates of each sample (after normalizing and eliminating from calculation the values with a high standard deviation). The finally obtained value for each set was considered the percentage of mortality / colonization.

Results and discussion

Many lignicolous basidiomycete species kill and colonize invertebrates when nutrients availability is very low. Among these basidiomycete species, some have particular adaptations for actively capturing insect larvae and worms, especially nematodes. Synthesis of compounds with nematicidal activity is another mechanisms involved in capturing nematodes for using their cadavers as sources of nutrients. As it can be observed in the Fig. 1 (A-E) and Fig. 2 (A-E), the nematodes cadavers are being gradually colonized by fungal hyphae until the total degradation of cadavers occur.

Influence of carbon and nitrogen sources. In order to optimize the process and to develop an easy and cheap method for using these fungi against phytopathogenic nematodes, we have tested different sources of carbon and nitrogen (Tab. 1) in preparing the media. In the same time, the quantity of glucose and peptone (the two nutrients widely used in our previous study that gave good rates of both mycelium development and nematicidal effect) ranged from 1 to 7 g L^{-1} for glucose, and from 0.5 to 4 g of peptone L^{-1}.

The mycelium development varied widely. Among the sugars, maltose had the strongest stimulating effect for the three fungal species. Increased concentration of glucose lead to a very fast development of mycelium. The preferences for the nitrogen sources was different from a species to another. While peptone and sodium nitrate stimulated the mycelium development for all the fungi, urea gave positive results only for *F. pinicola* and ammonium sulfate stimulated the development for *D. quercina* and *G. junonius*. Increasing concentrations of peptone from 0.5 to 1 g L^{-1} stimulated the mycelium development and extension of it on the medium surface, but increasing the concentration above this value lead to a very slow extension of mycelium, forming a thick and dense net around the inoculation point.

These observations are very important for elaborating a biocontrol strategy for field trials, as the concentration and type of nitrogen sources are affecting positively or negatively the process. Uptaking inorganic compounds of nitrogen, such as ammonia or nitrate, is an advantage as many of the used fertilizers in agriculture contain such compounds.

The used nutrients have had a different effect over the nematicidal activity and fungal colonization of nematodes. Overall, *G. junonius* presented the strongest nematicidal

effect and the highest colonization rate (Tab. 2). All the carbon sources used in quantity of 2 g L^{-1} were satisfying the fungal requirements. Although the results were different for the three fungi, on the maltose media, strong nematicidal activity was observed for all the fungi. *G. junonius* killed all the nematodes also on the medium with starch. Regarding the nitrogen sources, both ammonium sulfate and sodium nitrate gave good results concerning the nematicidal activity of fungi. For *G. junonius* was effective as well.

Concerning the colonization rate, the results were comparable, the same nutrients stimulating the process. However, the increasing concentration of peptone strongly decreased the colonization rate. As the fungi meets their nutritional requirements in the medium, no longer colonize nematodes.

Tab. 2. The effect of various C and N sources on colonisation and nematodes mortality (after 15 days of co-incubation with fungi)

Media	*D. quercina*		*F. pinicola*		*G. junonius*		Control
	colonization %	nematodes mortality%	colonization %	nematodes mortality%	colonization %	nematodes mortality%	nematodes mortality%
C1	0	68	0	55	11	92	17
C2	14	59	13	71	26	100	16
C3	0	19	12	71	0	89	17
C4	15	38	0	28	31	100	15
C5	8	12	11	49	14	93	16
C6	26	59	27	56	61	100	15
C7	31	61	36	62	56	100	17
C8	30	60	34	61	31	100	16
N1	17	49	0	49	78	100	17
N2	15	47	12	42	49	88	16
N3	11	40	10	36	37	67	16
N4	0	36	0	22	0	25	14
N5	16	62	54	62	17	100	17
N6	8	25	0	21	0	79	18
N7	15	64	16	63	16	100	17

Influence of nutritive salts. Ions such as Mg^{2+}, K^{+} and PO_4^{3-} are important for both plant and fungal development. Although less important, SO_4^{2-} is also required in sufficient concentration for fungi to grow. In agricultural practices, magnesium, potassium and phosphorus are usually added as fertilizers if the soil do not contain such minerals in adequate concentrations. In this study, we have tested the influence of these minerals, added as salts in nutritive media. For liquid media, other two salts – iron sulfate and sodium nitrate have been added in small concentrations.

It can be observed from the results that, except the case of *F. pinicola*, increasing concentration of minerals led to significantly decreasing the colonization rate (Tab. 3), while the nematicidal activity remained constant. This phenomenon suggests that fungi are colonizing nematodes cadavers not only for sugars and nitrogen compounds, but also for essential minerals.

Tab. 3. Mortality of nematodes on solid (S1-S3) and liquid (S4-S5) media supplemented with nutritive salts (after 15 days of co-incubation with fungi)

Media	*D. quercina*		*F. pinicola*		*G. junonius*		Control
	colonization%	nematodes mortality%	colonization%	nematodes mortality%	colonization%	nematodes mortality%	nematodes mortality%
S1	25	58	12	62	43	100	11
S2	51	59	0	37	54	100	10
S3	53	59	0	37	55	100	10
S4	5	14	8	39	39	100	8
S5	6	20	10	50	33	90	7

For liquid media (with previously mentioned minerals in concentration equal to the richest solid medium) *D. quercina* and *F. pinicola* presented the highest activity on the medium with higher concentration of glucose and peptone, while *G. junonius* did the opposite. As already stated, for the fungal metabolisms to be switched in the direction of using invertebrates as sources of nutrients, it is very important that in the substratum these fungi develop, there are enough nutrients for mycelium to grow, but in concentration small enough to represent a limitative factor.

Influence of heavy metals ions. In agriculture, there are frequently used pesticides that contain ions of heavy metals, particular fungicides. The cupric fungicides were historically used. Considering that for biocontrol strategy fungi will be applied as a liquid inoculum to soil, it is very important that these fungi resist to the concentration of heavy metals as high as in the agricultural soils. In this respect, the strain that presented the highest activity, *G. junonius*, was further tested for its resistance to heavy metals. As it can be observed in the Tab. 4, addition of copper and zinc manifested o strong inhibitory effect over the mycelium development and nematicidal activity as well. Iron and manganese ions had a stimulating effect and gave positive results, especially on the media with higher concentration of these ions (2 mM). Other researchers [CAMPOS & al. 2009] have proved that *G. junonius* is a metal accumulator, meaning that it resists to high levels of metals. The results suggest that when applying fungi as biocontrol agents, the use of chemical compounds containing copper or zinc should be limited.

Tab. 4. Mortality of nematodes on media supplemented with heavy metals (after 15 days of co-incubation with fungi)

Metals	plates inoculated with *Gymnopilus junonius*		Control
	fungal development	nematodes mortality%	nematodes mortality%
1mM Cu^{2+}	weak	20	16
1mM Mn^{2+}	strong	55	9
1mM Fe^{2+}	strong	85	18
1mM Zn^{2+}	weak	12	7
2mM Cu^{2+}	no development	25	25
2mM Mn^{2+}	strong	95	17
2mM Fe^{2+}	strong	100	30
2mM Zn^{2+}	weak	13	11

Pots experiment. In order to test the nematicidal activity of fungi in laboratory simulated agricultural condition, plants of tomatoes infected with phytopathogenic nematodes were treated with liquid inoculum of *G. junonius.* We have used five pots for control and five for fungi-inoculated plants, with 80 individual of nematodes in each pot (Tab. 5). After incubation time, the average number of individuals were recovered on pots inoculated with fungi was approximatively three time lower that the number recovered from control pots, showing a significant nematicidal activity of *G. junonius*'s mycelium. The treated (with fungi) and un-treated plants did not look different (there was any significant evidence of the nematodes attack), due to the small incubation period (20 days). For severe symptoms to occur in attacked plant an entire generation of nematodes (60 days) should develop. The nematodes obtained from control pots, through extraction, at the end of the experiment were in the pre-adult stage (J3 and J4), meaning their life cycle was normal, and not affected by the culture condition, but individuals on treated pots were affected by the presence of fungi. The strong decrease of nematodes' number on the treated pots is an evidence of *G. junonius* efficiency in controlling nematodes.

The possible involved mechanisms might be the production of nematicidal compounds, as different authors [KAYANO & al. 2004; KUSANO & al. 1986] observed the production of gymnopilins, gymnoprenols and other related compounds, with different physiological action on animals, such as cytotoxicity against human tumor cell lines [KIM & al. 2012] or mobilization of Ca^{2+} from nervous cells [MIYAZAKI & al. 2012]. In the same time, other species from family Strophariaceae, such as *Stropharia rugosoannulata*, are nematophagous [LUO & al. 2006] and there is a possibility of these mechanisms to occur in the case of *G. junonius* as well.

Tab. 5. The effect of *G. junonius* inoculum addition on the tomatoes inoculated with *Meloidogyne incognita* (after 15 days of fungal inoculum addition)

Type of experiment	Pot	Initial number of juveniles (J2)	Final number of juveniles (J2)	Examination of nematodes	Symptoms on tomatoes
with *J. junonius*	1	80	16	The cuticle thinned, abnormal positions, faint stylet, granulations on digestive tube	Small zoocecidia in formation
	2	80	18		
	3	80	29		
	4	80	21		
	5	80	19		
	average	80	20.6		
without fungi	1	80	68	Strong movements for advancing, prominent stylet, retractile, the hyaline part of the tail prominent	Small zoocecidia in formation
	2	80	71		
	3	80	55		
	4	80	63		
	5	80	59		
	average	80	63.2		

Tab. 6. The mortality of nematodes in dependence of *G. junonius* inoculums quantity (after 1 day of co-incubation)

Inoculum quantity (mL)*	Type of medium		
	medium A	medium B	medium C
	nematodes mortality%		
0.1	53	22	30
0.2	75	27	48
0.4	92	39	51
0.6	100	52	100
0.8	100	100	100
1.0	100	100	100
control	20	18	17

*out of a total mixture of 2 mL (inoculum, fresh medium and 100 µL nematodes suspension)

Influence of inoculum's quantity. After one day of incubation, a quantity of inoculum as high as 0.6 mL/2 mL (30%) was enough for killing all the nematodes. Considering the short period for incubation, the involved mechanism should be of biochemical type, as no colonization occur. Concerning the media used in this experiment, the standard medium was the most effective, but also the cheap variants gave positive results, especially on the C medium, rich in nutrients, where mycelium developed very strong. There are necessary additional studies for testing the chemical compounds with nematicidal activity, purifying and researching for understanding the conditions in which these compounds present the nematicidal activity.

Conclusions

The tested fungi are colonizing nematodes when nutrients are available in low quantity. This property gives the possibility of using fungi in biological control strategies.

G. junonius presented the highest nematicidal activity and was effective in simulated conditions of plants attacked by nematodes.

The chemical compounds used as fertilizers in agriculture have a positive effect over the process if they are not used in very large quantities.

The tolerance to the high concentrations of iron and manganese ions of *G. junonius* is an advantage and recommends the use of this species in biocontrol of nematodes. However, the concomitant usage of fungicides or compounds containing copper or zinc should be avoided.

The proved biochemical mechanism involved in the process is very important and can lead to isolation and purification of the active compound for using it in biocontrol. In this respect, additional studies are required for completely understanding the mechanisms.

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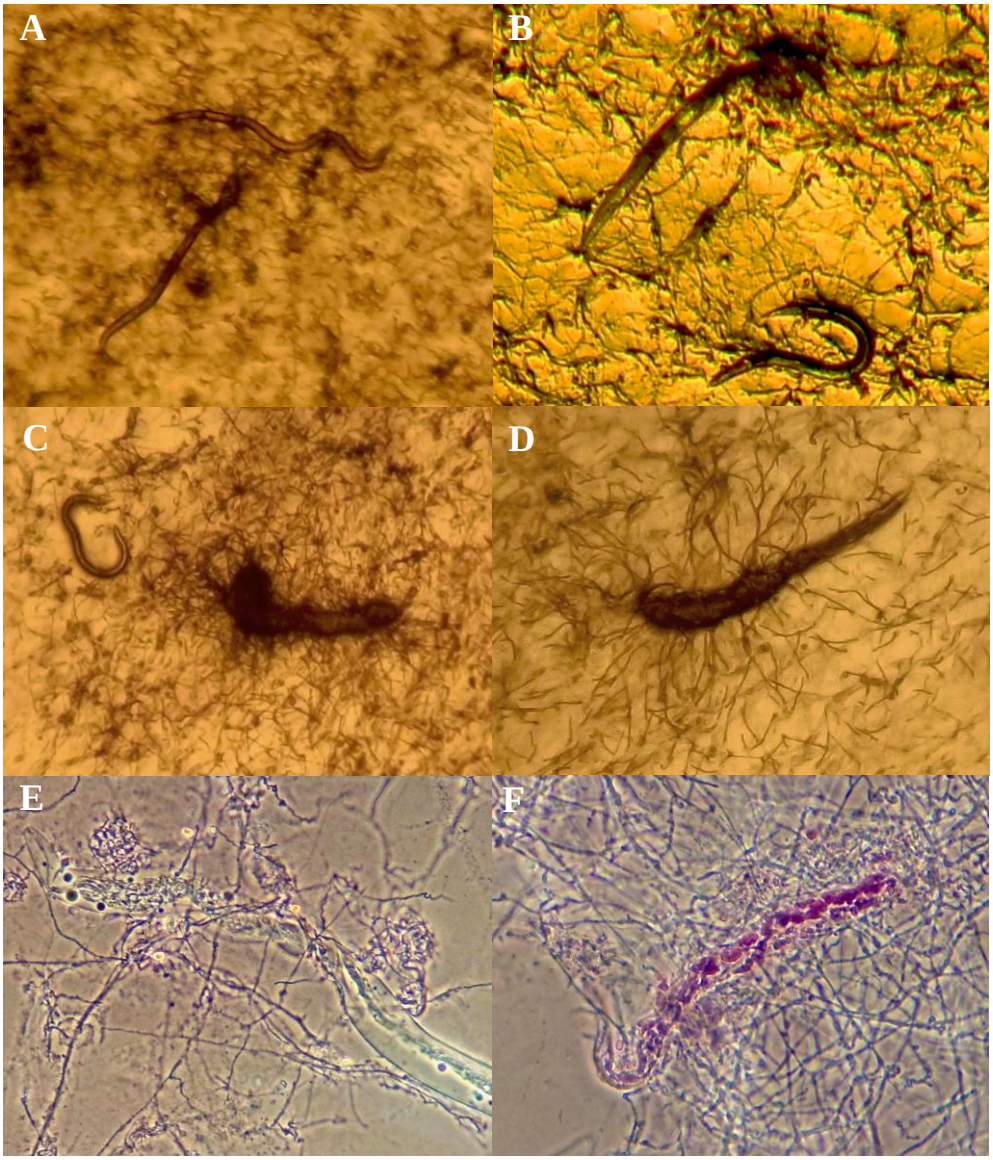

Fig. 1. Colonisation of nematode's bodies: A and B – initial stages of infection, stereomicroscope image, 25x; C and D – the end of colonisation and degradation, stereomicroscope image, 25x; E and F – different stages of colonisation, phase contrast microscope image, after Acid Fucsine staining, Microscope 400x.

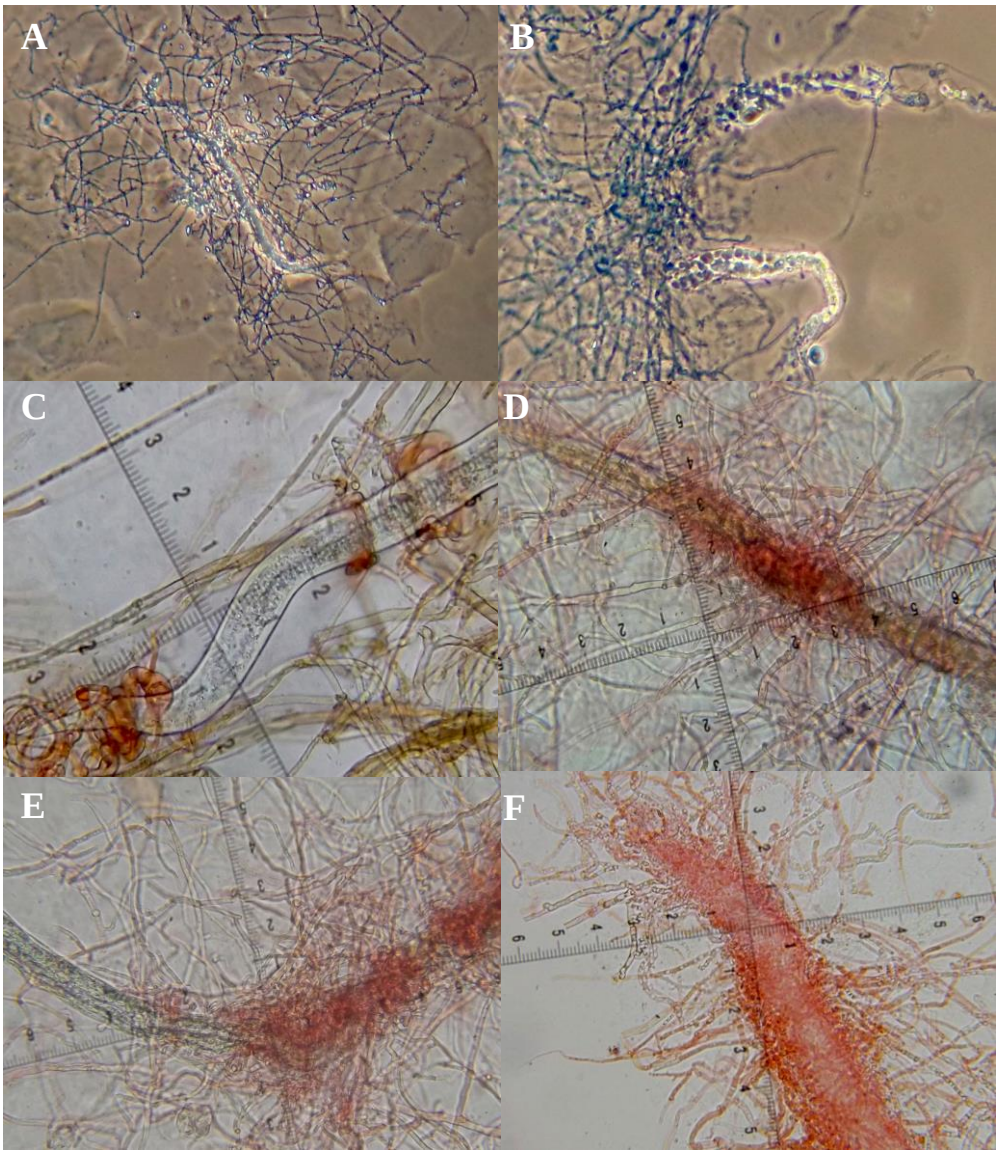

Fig. 2. Fungal degradation of nematode's bodies: A and B – phase contrast microscope image, after Methyl Blue staining, 400x; C – F – upright normal microscope image, Congo red staining, Microscope 400x.

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PLANT COMMUNITIES WITH *ARNICA MONTANA* IN NATURAL HABITATS FROM THE CENTRAL REGION OF ROMANIAN EASTERN CARPATHIANS

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Abstract: *Arnica montana* is a species of European Union interest, whose harvest from the wild and exploitation should be made under certain management measures. In Romania it is a vulnerable species due to excessive collection. It is a species with European areal occuring in pastures, meadows, forest glades, shrubs communities of mountain to the subalpine regions and, isolated, up to the alpine belt. Most of the plant communities with *Arnica montana* are semi-natural, with a floristic composition in which there are numerous rare or threatened species also supporting the need of their conservation. Our study was focused on a numerical classification (hierarchical, using Flexible ß algorithm and Bray-Curtis dissimilarity) based on 48 plots, of the plant communities with *Arnica montana* from the central region of Romanian Eastern Carpathians and on the investigation of the effect of some environmental variables (Ellenberg indicator values, altitude, heat load index) on their floristic composition (100 m^2 scale). Vegetation – environment relationship was assessed via detrended correspondence analysis and canonical correspondence analysis with Monte Carlo test. Six plant communities with *Arnica montana* were identified (communities of *Festuca rubra* with *Agrostis capillaris, Festuca nigrescens, Vaccinium myrtillus, Nardus stricta, Vaccinium gaultherioides* and *Juniperus sibirica*) with a floristic composition mainly shaped by altitude, temperature and soil nitrogen content. Details related to location and sites characteristics, diagnostic species, floristic composition, presence of other rare or threatened species and *Arnica montana* abundance were presented for all these plant communities.

Key words: vegetation, *Arnica montana*, floristic composition, ecology, habitats

Introduction

Arnica montana, a species well-known for its medicinal properties, has an European (montane) areal: it is native in numerous countries (e.g. Italy, Germany, Austria etc.), or large-scale cultivated as in Estonia [EURO+MED PLANTBASE, 2006-]. In some other countries (as Hungary) it is considered extinct [EURO+MED PLANTBASE, 2006-]. In Romania, due to excessive collection it is considered a vulnerable species and is included in the Red List of Plants from Romania [OLTEAN et al. 1994]. Also the species was inserted in the Habitats Directive of the European Union (1992), in Appendix 5, in which there are listed the animal and plant species of community interest whose taking in the wild and exploitation may be subject to management measures. In Romania, *Arnica montana*

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frequently occurs in secondary communities developed after the cutting of forest vegetation or in mesophytic grasslands, preferring more acid and poor in nutrients soils [ELLENBERG, 1992]. It is a species which grows in pastures, meadows, forest glades, shrubs communities from mountain to the subalpine regions and, isolated, up to the alpine areas. It is considered as diagnostic species for *Calluno-Ulicetea* Br.-Bl. et R. Tx. ex Klika et Hadać 1944 (*Nardo-Callunetea* Preising 1944), a vegetation class including grasslands and shrubs communities on acid substrata developed in montane and subalpine vegetation belts [MUCINA, 1997]. For Romanian vegetation, significant constancies were recorded in vegetal communities within *Potentillo-Nardion* Simon 1958 alliance [COLDEA & al. 2012; CHIFU & al. 2015].

In the present study, the main objective was to identify the main plant communities with *Arnica montana* in the central region of Romanian Eastern Carpathians using a vegetation dataset with 48 relevés and hierarchical clustering procedures. For each identified plant community we wanted to highlight the diagnostic and dominant species, the floristic and phytosociological composition, the cover of *Arnica montana* individuals, other rare and threatened species and the habitat type.

Material and methods

The study area (Fig. 1), situated in the central region of Romanian Eastern Carpathians, included different localities (Farcaşa, Borca, Broşteni, Barnar, Chiril, Ortoaia, Dorna Arini, Neagra Şarului, Gura Haitii) from several mountain ranges (Stânişoarei, Bistriţei, Rarău, Călimani Mountains and Dorna Depression) in two counties (Neamț and Suceava). This area's geology is varied and represented by crystalline rocks (Bistriţei Mountains), limestones (Rarău and Stânişoarei Mountains), volcanic rocks (Călimani Mountains) and sedimentary deposits (Dorna Depression) [MIHAILESCU, 1963]. The main soil types are the cambisols (corresponding to mixed beech forests), spodosols (corresponding to spruce forests) and lithosols (corresponding to subalpine and alpine grasslands) [BARBU & al. 1984]. The climate is temperate continental, with mean annual precipitations ranging between 600 (in lower areas) and 1100 mm (1200, in upper areas) and mean annul temperatures ranging between 0 and 4 ºC. From a phytogeographical perspective, the study area is included in the Euro-Siberian floristic region with Carpathian

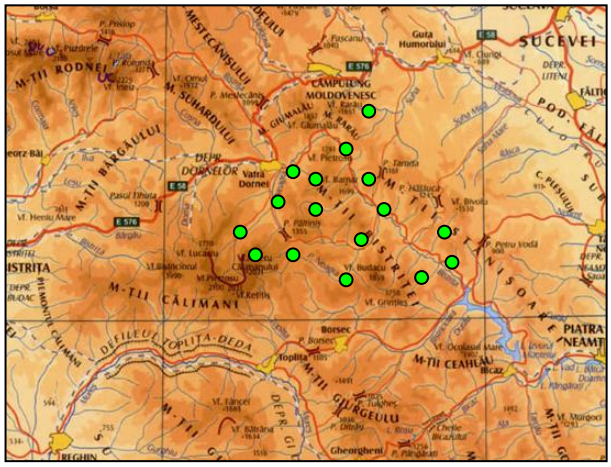

Fig. 1. Geographical localization of the study area. The green points represents the main localities where vegetation plots were sampled.

Province [CHIFU & al. 2006]. According to Habitats Directive of the European Union (1992), the studied territory is considered as a part of the alpine biogeographical region.

In order to identify the main plant communities with *Arnica montana* in the central region of Romanian Eastern Carpathians, 48 relevés (including 208 species) were used. The relevés were realized using the method of the Central European School for vegetation study adapted for Romanian vegetation [BORZA & BOȘCAIU, 1965]. Plant species cover was visually estimated using a 6 level scale: + (<5%); 1 (5–10%); 2 (10–25%); 3 (25–50%); 4 (50–75%); 5 (>75%). All relevés had 100 m^2 in size and were made at altitudes ranging between 700-2000 m in the summer periods of 2014-2015. Species present only in one relevé were removed (final dataset included 48 relevés x 156 species). Vegetation classification was performed by hierarchical agglomerative clustering (using Bray-Curtis dissimilarity and Flexible β (β = -0.25) algorithm). Optimum number of clusters was detected using the corrected Rand index and the silhouette index. For each cluster the diagnostic species were identified by the indicator value index [DUFRÊNE & LEGENDRE, 1997] and validated by a permutation test [DE CÁCERES & LEGENDRE, 2009] (the threshold value, subjectively chosen, for a species considered as diagnostic was 0.400, $p < 0.5$). The environmental factors with significant influence on the floristic composition were identified via detrended (square root transformation, detrending by segments and non-weighted average values of the Ellenberg indices -EIVs- for light (L), temperature (T), soil moisture (U), soil pH (R) and nutrients (N) [ELLENBERG & al. 1992], alongside altitude and heat load index) and canonical correspondence analysis (square root transformation, same indices as in DCA and Monte Carlo test). Differences among ecological characteristics the six associations were highlighted using the Kruskal-Wallis non-parametric ANOVA and the Mann-Whitney post-hoc test (Bonferroni corrected). Nomenclature of plants species follows CIOCÂRLAN (2000).

For each plant community were presented the localities, some characteristic of the stations, diagnostic and dominant species, floristic and phytosociological composition, cover of *Arnica montana* individuals, the rare and threatened species [OLTEAN & al. 1994; Habitats Directive, 1992; Bern Convention, 1979] and the habitat type according to GAFTA & al. (2008) and DONIȚĂ & al. (2005).

Results and discussion

The dendrogram resulted from hierarchical clustering was cut into 15 partitions with 2-14 clusters (Fig. 2). The optimal number of clusters was identified by the corrected Rand index which had the highest values when partitions with 6 and 7 clusters were compared. In addition, the Silhouette index showed a local maximum for the partition with 6 clusters, and consequently, the partition 6 clusters was considered for the association level (Fig. 2). Next, for each of the six clusters were identified the diagnostic species and, based on them, the clusters were related to plant associations described in the literature.

In the central region of Eastern Carpathians, plant communities with *Arnica montana* were represented by secondary grasslands developed on moderate and nutrient richer soils and also by dwarf boreal and subalpine shrubs communities developed on more acid and nutrient poor soils. In terms of life-forms, all communities were dominated by hemicryptophyte species. In their floristic composition, an altitudinal transition was highlighted, from communities in which the European and Eurasian elements were more

frequent to communities where the circumpolar (and circumpolar-arctic-alpine) were more frequent. The plant communities were assigned to the next syntaxonomical system:

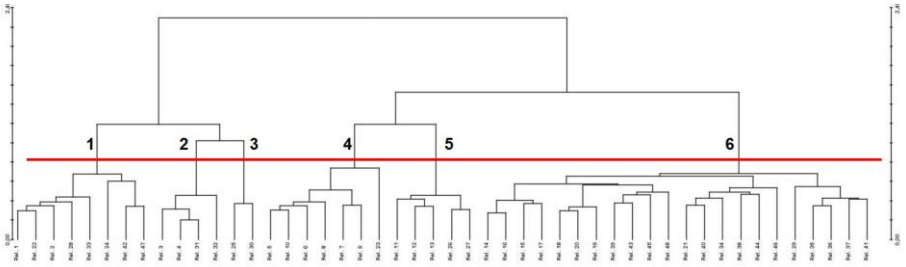

Fig. 2. Classification of plant communities with *Arnica montana* from the central region of Eastern Carpathians. The dendrogram resulted in hierarchical agglomerative clustering (Flexible β algorithm and Bray-Curtis dissimilarity) showing the reports of floristic similarity among plant communities. The red segment highlights the optimum number of clusters according to corrected *Rand* index and *Silhouette* index. 1 – *Campanulo abietinae-Vaccinietum*; 2 – *Campanulo abietinae-Juniperetum nanae*; 3 – *Cetrario-Vaccinietum gaultherioidis*; 4 – *Scorzonero roseae-Festucetum nigricantis*; 5 – *Violo declinatae-Nardetum*; 6 – *Festuco rubrae-Agrostietum capillaris*.

MOLINIO – ARRHENATHERETEA R. Tx. 1937
ARRHETANTHERETALIA R. Tx. 1937
Cynosurion R. Tx. 1947
1. *Festuco rubrae-Agrostietum capillaris* Horvat 1951

NARDO – CALLUNETEA Preising 1949
NARDETALIA Oberd. 1949
Potentillo ternatae – Nardion Simon 1958
2. *Scorzonero roseae-Festucetum nigricantis* (Puşcaru et al. 1956) Coldea 1987
3. *Violo declinatae-Nardetum* Simon 1966

LOISELEURIO – VACCINIETEA Eggler ex Schubert 1960
RHODODENDRO – VACCINIETALIA Br.-Bl. in Br.-Bl. et Jenny 1926
Juniperion nanae Br.-Bl. et al. 1949
4. *Campanulo abietinae-Juniperetum nanae* Simon 1966
Loiseleurio – Vaccinion Br.-Bl. in Br.-Bl. et Jenny 1926
5. *Cetrario-Vaccinietum gaultherioidis* Hadač 1956
Rhododendro – Vaccinion J. Br.-Bl. ex G. Br.-Bl. et J. Br.-Bl. 1931
6. *Campanulo abietinae-Vaccinietum* (Buia et al. 1962) Boşcaiu 1971

A) *Festuco rubrae – Agrostietum capillaris* Horvat 1951 (**6520** Mountain hay meadows. **R 3803** South-Eastern Carpathian meadows of *Agrostis capillaris* and *Festuca rubra*).

Location and sites characteristics: is the most frequent plant community with *Arnica montana* in the studied region, occurring on significant areas from Farcaşa, Borca, Broşteni, Chiril, Ortoaia, Dorna Arini, Şaru Dornei, Gura Haitii localities (Stânişoarei, Bistriţei, Rarău or Călimani Mountains and Dornelor Depression), at altitudes ranging from 693 to 1500 m.a.s.l (973±184 m.a.s.l.), on terrains with various slopes and aspects (from

plane terrains to 50 º slopes). In certain sites, the field management - excessive grazing facilitated its invasion by *Nardus stricta* and *Vaccinium myrtillus*.

Diagnostic species: *Centaurea phrygia* (0.853***), *Agrostis capillaris* (0.838***), *Festuca rubra* (0.831***), *Alchemilla xanthochlora* (0.603**), *Carum carvi* (0.603*), *Leucanthemum vulgare* (0.596**), *Campanula glomerata* (0.564*), *Holcus lanatus* (0.564*).

Floristic composition: the community includes secondary meadows edified by *Festuca rubra* and *Agrostis capillaris*, in various co-dominance reports. The floristic composition was diverse and the herbs layer had (almost in all sites) 100% cover. Besides diagnostic species, the most frequent (and sometimes with significant cover) were: *Cynosurus cristatus, Arrhenatherum elatius, Briza media, Stachys officinalis, Deschampsia caespitosa, Trifolium pratense, Anthoxanthum odoratum, Viola tricolor, Luzula luzuloides, Veratrum album* etc. From the phytosociological perspective, high constancy presented plants considered as diagnostic species for Cynosurion (*Cynosurus cristatus, Gentiana cruciata, Leontodon autumnalis* etc.), Arrhenatherion (*Centaurea phrygia, Stellaria graminea* etc.), and Deschampsion alliances (*Deschampsia caespitosa, Carex pallescens* etc.), for Arrhenatheretalia (*Thymus pulegioides, Dactylis glomerata* etc.) and Molinietalia orders (*Succisa pratensis, Lychnis flos-cuculi, Linum catharticum* etc.) and also for Molinio – Arrhenatheretea class (*Rhinanthus angustifolius, Lotus corniculatus, Polygala vulgaris, Trifolium repens, Prunella vulgaris, Lathyrus pratensis* etc.). Also, the floristic composition included diagnostic species for the meso-xeric grasslands of Festuco – Brometea class (*Pimpinella saxifraga, Anthyllis vulneraria* etc. and species infiltrated from the vegetation of Epilobietea angustifolii (*Digitalis grandiflora, Fragaria vesca* etc.), Vaccinio – Piceetea (*Campanula abietina, Luzula luzuloides, Vaccinium myrtillus* etc.) or Trifolio – Geranietea classes (*Trifolium ochroleucon, Astrantia major, Veronica chamaedrys, Agrimonia eupatoria* etc.). *Arnica montana* (Photo 1) had, in certain sites (as in Gura Haitii village), significant covers (up to 25% of plot area).

Rare/threatened/endemic species: *Arnica montana* (RL, HD), *Trollius europaeus* (RL), *Primula elatior* ssp. *leucophylla* (RL), *Phyteuma tetramerum* (RL), *Gymnadenia conopsea* (RL), *G. odoratissima* (RL), *Traunsteinera globosa* (RL), *Anacamptis pyramidalis* (RL), *Dactylorhiza maculata* (RL), *Dactylorhiza majalis* (RL).

Photo 1. *Arnica montana* in mountain hay meadows (*Festuco rubrae – Agrostietum capillaris*)

B) *Scorzonero roseae – Festucetum nigricantis* (Puşcaru et al. 1956) Coldea 1987, *Violo declinatae – Nardetum* Simon 1966 (**6230*** Species-rich *Nardus* grasslands, on siliceous substrates in mountain areas (and sub-mountain areas, in Continental Europe). **R 3608** South-Eastern Carpathian meadows of *Scorzonera rosea* şi *Festuca nigrescens*; **R 3609** South-Eastern Carpathian meadows of *Nardus stricta* and *Viola declinata*).

Location and sites characteristics: secondary communities with *Festuca nigrescens* and *Nardus stricta* (with maximum cover of 50%) were identified on smaller areas compared to the previous one, in Bistriței and Călimani Mountains, in an altitudinal range of 972 – 1700 m.a.s.l. (1464±264 m.a.s.l.), preponderantly on south-western and north-western slopes, with pronounced inclinations (35 º).

Diagnostic species: *Scorzonero roseae – Festucetum nigricantis*: *Festuca nigrescens* (0.953***), *Scorzonera rosea* (0.702*); *Violo declinatae – Nardetum*: *Luzula campestris* (0.827**), *Viola declinata* (0.825**), *Nardus stricta* (0.796***), *Prunella vulgaris* (0.587*).

Floristic composition: is species-rich and relative homogenous. The herbs layer presented 80 – 100% cover and included, besides diagnostic and dominant species, some other species with high frequency and cover: *Hieracium pilosella, Briza media, Euphrasia stricta, Rumex acetosella* etc. High constancies had diagnostic species Potentillo ternatae – Nardion alliance (*Campanula serrata, Hieracium lactucella, Gentiana acaulis, Carex pallescens, Hypochoeris uniflora, Hypericum maculatum* etc.), for Festucetalia spadiceae order (*Hieracium aurantiacum, Carlina acaulis, Antennaria dioica*) and Nardo – Callunetea class (*Antennaria dioica* etc.). Also, in the floristic composition there were species from the mountain grasslands of Molinio – Arrhenatheretea (*Agrostis capillaris, Thymus pulegioides, Cynosurus cristatus, Centaurea phrygia, Stellaria graminea* etc.) or from the alpine communities of Juncetea trifidi class (*Potentilla aurea, Phleum alpinum* etc.). ***Arnica montana*** was commonly identified in small groups but, in some locations, had significant cover, up to 10% plot area.

Rare/threatened/endemic species: *Arnica montana* (RL, DH), *Dianthus barbatus* subsp. *compactus* (RL), *Phyteuma tetramerum* (RL), *Scorzonera rosea* (RL), *Gymnadenia conopsea* (RL), *Campanula serrata* (DH), *Phyteuma orbiculare* (RL).

Photo 2, 3. *Arnica montana* in species-rich *Nardus* grasslands, on siliceous substrates in mountain areas (and sub-mountain areas, in Continental Europe). 2) *Scorzonero roseae – Festucetum nigricantis*; 3) *Violo declinatae – Nardetum*

C) *Campanulo abietinae – Vaccinietum myrtilli* (Buia et al. 1962) Boşcaiu 1971, *Campanulo abietinae – Juniperetum nanae* Simon 1966, *Cetrario – Vaccinietum gaultherioidis* Hadač 1956 (**4060** Alpine and Boreal heaths. **R 3111** South-Eastern Carpathian *Vaccinium myrtillus* heaths; **R 3108** South-Eastern Carpathian *Juniperus sibirica* heaths; **R 3109** South-Eastern Carpathian *Vaccinium gaultherioides* heaths).

Location and sites characteristics: these subalpine dwarf-shrubs communities occured on the slopes and around Căliman Izvor and Căliman Cerbuc peaks (Călimani Mountains) or Pietrosul Bistriței (Bistriței Mountains), from the upper limit of the forests (widespread communities with *Vaccinium myrtillus*) to highest mountaintops (communities with *Juniperus sibirica* and *Vaccinium gaultherioides* restricted to higher elevations). They were developed on terrains with various slopes and aspects, on moderate humid, acid and nutrient poor soils.

Diagnostic species: *Campanulo abietinae – Vaccinietum myrtilli*: *Vaccinium myrtillus* (0.850^{***}), *Vaccinium vitis-idaea* (0.660^{*}); *Campanulo abietinae – Juniperetum nanae*: *Juniperus sibirica* (0.948^{***}), *Gnaphalium supinum* (0.866^{**}), *Calamagrostis villosa* (0.757^{**}), *Hieracium alpinum* (0.750^{**}), *Homogyne alpina* (0.747^{**}), *Luzula luzuloides* (0.659^{*}), *Pulsatilla alba* (0.588^{*}); *Cetrario-Vaccinietum gaultherioidis*: *Vaccinium gaultherioides* (0.947^{***}), *Festuca supina* (0.728^{**}), *Deschampsia flexuosa* (0.678^{*}).

Floristic composition: the communities of *Vaccinium gaultherioides* or *Juniperus sibirica* (from the subalpine belt) and *Vaccinium myrtillus* (which descends in the montane belt) were characterized by an compact shrubs layer, with covers ranging between 70 – 90% and a species poor floristic composition. Besides dominant species it included also other shrubs species: *Rhododendron myrtifolium*, *Vaccinium vitis-idaea*, *Pinus mugo* etc. The herbs layer had decreased cover (5 – 20%) and included few species among the most frequent were: *Ligusticum mutellina, Antennaria dioica, Homogyne alpina, Festuca supina, Nardus stricta, Luzula sudetica, Veratrum album, Phleum alpinum, Hypericum richeri* ssp. *grisebachii* etc. In some communities from higher elevations a well-developed lichen layer was highlighted. High constancies had species considered as diagnostic for Rhododendro – Vaccinion alliance, Rhododendro – Vaccinietalia order (*Calamagrostis villosa*, *Ligusticum mutellina*, *Vaccinium gaultherioides*) and Loiseleurio–Vaccinietea class (*Primula minima*, *Cetraria islandica*, *Vaccinium myrtillus*, *Thamnolia vermicularis* etc.). In addition, in these plant communities were identified species from the alpine grasslands of Juncetea trifidi class (*Juncus trifidus*, *Hypochoeris uniflora*, *Pulsatilla alba*, *Potentilla ternata*, *Hieracium alpinum* etc.) and from the forests of Vaccinio – Piceetea class (*Lycopodium selago*, *Pinus mugo* etc.). *Arnica montana* was identified as isolated individuals or in small groups (2-3 individuals).

Rare/threatened/endemic species: *Arnica montana* (RL, HD), *Rhododendron myrtifolium* (RL), *Campanula abietina* (BC), *Lycopodium clavatum* (HD), *Leucorchis albida* (RL).

Photo 4, 5. *Arnica montana* in Alpine and Boreal heaths. 4) *Campanulo abietinae – Vaccinietum myrtilli*; 5) *Campanulo abietinae – Juniperetum nanae*

Tab. 1. Ecological characteristics of the six plant communities with *Arnica montana* in the central region of Eastern Carpathians. Means and standard deviations for EIVs for light (L), temperature (T), soil moisture (U), soil reaction (R), soil nutrients (N), altitude and heat load (Hload) are given. *P*-values are derived from Kruskal-Wallis test. Different letters (on columns) indicate significant differences among communities ($\alpha \leq 0.05$ according to Mann-Whitney post hoc test, n.s. – not significant).

Com. no.	EIV L	EIV T	EIV U	EIV R	EIV N	Alt	Hload
1.	7.03 ± 0.21^{a}	4.55 ± 0.43^{a}	4.93 ± 0.24^{a}	4.95 ± 0.68^{a}	3.25 ± 0.29^{a}	973.04 ± 183.45^{a}	0.06 ± 0.52^{a}
2.	7.20 ± 0.18^{a}	4.40 ± 0.38^{ab}	4.78 ± 0.19^{a}	4.58 ± 0.14^{ab}	3.06 ± 0.05^{ab}	1277.00 ± 130.96^{ab}	-0.05 ± 0.28^{a}
3.	7.10 ± 0.40^{a}	3.80 ± 0.27^{bc}	4.91 ± 0.13^{a}	3.98 ± 0.34^{bc}	2.80 ± 0.36^{ac}	1418.57 ± 169.05^{bc}	0.09 ± 0.19^{a}
4.	6.83 ± 0.36^{a}	3.35 ± 0.51^{bd}	4.78 ± 0.12^{a}	3.25 ± 0.57^{bd}	2.46 ± 0.38^{bcd}	1493.12 ± 341.55^{bd}	0.12 ± 0.30^{a}
5.	7.00 ± 0.14^{a}	2.95 ± 0.07^{acde}	4.95 ± 0.07^{a}	2.95 ± 0.21^{acde}	2.25 ± 0.07^{ade}	1797.25 ± 109.60^{acde}	0.50 ± 0.70^{a}
6.	6.97 ± 0.17^{a}	2.92 ± 0.09^{be}	5.15 ± 0.05^{a}	3.10 ± 0.24^{be}	2.50 ± 0.14^{bce}	1821.30 ± 25.28^{be}	0.17 ± 0.20^{a}
p	n.s.	< 0.001	n.s.	< 0.001	< 0.001	< 0.001	n.s.

The ecological characteristics of the six plant communities with *Arnica montana* are shown in Tab. 1 – averages and standard deviations of the factors expressing plant species preferences (ELLENBERG & al. 1992) for light (L), temperature (T), soil moisture (U) soil reaction (R), soil nutrient content (N), altitude and heat load (relating land slope and aspect). It was observed that among the investigated communities there was no significant difference in terms of plant species preferences for light, soil moisture and heat load (in all communities the most of the plant species in the floristic composition were heliophyte, mesophyte species and which can grow on any aspect and slope of the land). The temperature, soil nutrients and pH and altitude differentiated the communities from low altitudes apart from those of high altitudes. Thus, concomitant with the altitude increase, the floristic composition changed, from species adapted to boreal climate from the mountain vegetation belt to species preferring the colder conditions of subalpine and alpine belts. Also there was a transition from moderate acidophilous species growing on nutrients-rich soils at lower elevations, to the more acidophilous species on very nutrients-poor soils at higher altitudes.

In order to confirm the results on variation of the floristic composition, a detrended correspondence analysis was performed (Fig. 3), analysis in which the relevés were arranged along the first two axes depending on the gradients of floristic similarity. From this analysis resulted that the first two axes are the most important, they explained both the largest variation in species data and mostly from the relationships between species and the environment. At the same time, the length of the gradients of floristic similarity along the first axis (the most important according to its eigenvalue) was about 4 units of standard deviation, indicating a unimodal pattern of variation in the floristic composition. Therefore a canonical correspondence analysis with Monte Carlo test was applied, in order to observe the effect of each variable on the floristic composition.

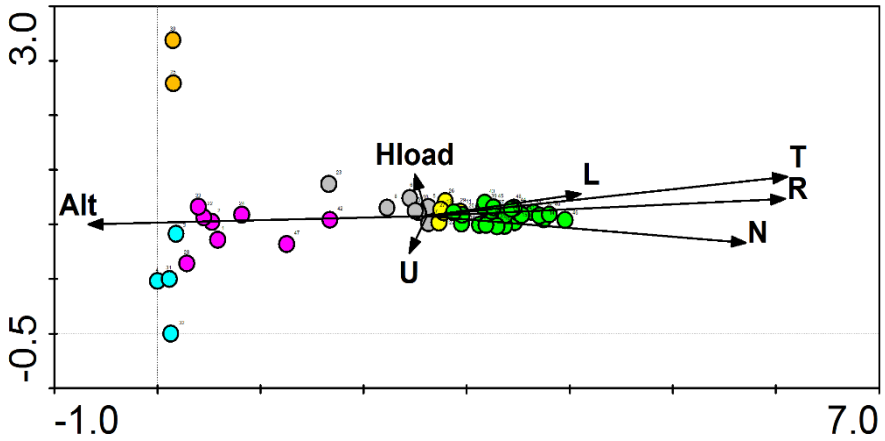

Fig. 3. DCA ordination of the 48 relevés. First two axes are presented, with EIVs for light (L), temperature (T), soil moisture (U), soil reaction (R) and soil nutrients (N), altitude (Alt) and heat load (Hload) passively projected on the ordinogram. The groups generated by agglomerative clustering were colored as follow: *Campanulo abietinae-Vaccinietum myrtilli* - grey, *Campanulo abietinae-Juniperetum nanae* - blue, *Cetrario-Vaccinietum gaultherioidis* - brown, *Scorzonero roseae-Festucetum nigricantis* - violet, *Violo declinatae-Nardetum* - yellow, *Festuco rubrae-Agrostietum capillaris* - green. Correlations with first two axes: L (0.384 and 0.056), T (0.900 and 0.106), U (-0.043 and 0.077), R (0.894 and 0.060), N (0.795 and -0.033), Alt (-0.844 and -0.040) Hload (-0.029 and 0.086).

Tab. 2. Effect of each variable on the floristic composition of plant communities with *Arnica montana* in the central region of Romanian Eastern Carpathians - CCA analysis and Monte Carlo test

Variabilă	EIV T	EIV N	Alt	EIV U	EIV L	EIV R	Hload
***F*-ratio**	8.42	1.98	1.78	1.43	1.24	1.20	1.18
p	0.002	0.002	0.012	0.086	0.12	0.18	0.28

The results of this analysis were presented in Tab. 2 where in the values of the F-ratio expresses the strength of the effect of each variable floristic composition. It could be noted, as in Tab. 1, that temperature, nutrients in the soil and altitude are the main

ecological factors which shape these communities floristic structure and, unlike previous analysis, soil reaction no longer has a significant effect on these communities.

Conclusion

Vegetal communities with *Arnica montana* in the central region on the Romanian Eastern Carpathians are represented by secondary mesophytic montane grasslands (*Festuco rubrae – Agrostietum capillaris*, *Scorzonero roseae – Festucetum nigricantis*, *Violo declinatae – Nardetum strictae*) and also boreal and subalpine dwarf shrubs communities (*Campanulo abietinae – Vaccinietum myrtilli*, *Campanulo abietinae – Juniperetum nanae*, *Cetrario – Vaccinietum gaultherioidis*). These communities corresponds to three habitat types: 6520 Mountain hay meadows, 6230* Species-rich *Nardus* grasslands, on siliceous substrates in mountain areas (and sub-mountain areas, in Continental Europe) and 4060 Alpine and Boreal heaths. The abundance of *Arnica montana* was higher in mown meadows at relative low altitudes. At higher elevations the species was represented by solitary individuals or in numerically small groups.

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THE USE-POTENTIAL OF *QUERCUS ALIENA* VAR. *ACUTESERRATA* FOR URBAN PLANTATIONS – BASED ON HABITAT STUDIES IN THE QINLING MOUNTAINS, CHINA

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Abstract: Traditionally, a limited number of species and genera dominate the tree stock in streets and urban sites, and recent surveys in European and North American cities show that few species/genera continue to dominate. Yet, over the past decades, a growing proportion of those commonly used species have shown increasing difficulties to cope with urban sites. This has led to considerable and persistent arguments for using a more varied range of trees, including stress-tolerant species, at urban paved sites. This study examined forest systems occurring between 1300-2200 m asl. in the Qinling Mountains, China, in order to evaluate the oriental white oaks (*Quercus aliena* var. *acuteserrata* Maximowicz ex Wenzig) growth and development in warm and dry forest habitats and hence evaluate its potential for urban paved sites in northern parts of central Europe and in adjoining milder parts of northern Europe. In total, 102 oriental white oak where found in the studied plots and here showed very promising development in habitats experiencing drier conditions than those in park environments in Copenhagen, and is therefore interesting for urban paved sites were the demands of a greater catalogue of tolerant trees are highly needed.

Key words: Urban tree, Drought tolerance, Oriental white oak, Urban forestry

Introduction

Traditionally, a limited number of species and genera dominate the tree stock in streets and urban sites, and recent surveys in European and North American cities show that few species/genera continue to dominate [RAUPP & al. 2006; SJÖMAN & al. 2012a; COWETT & BASSUK, 2014]. Yet, over the past decades, a growing proportion of those commonly used species have shown increasing difficulties to cope with urban sites. Impermeable surfacing affecting both storm water run off and the urban heat island effect have resulted in tree decline and the increase of disease in the urban tree habitat. This negative trend, combined with the challenges of climate change and the threat of further future disease and infestations of vermin [e.g. TELLO & al. 2005; RAUPP & al. 2006; TUBBY & WEBBER, 2010] have led to considerable and persistent argumentation for the necessity of a more varied use and stress tolerant selection of tree species for urban sites [PAULEIT, 2003; SJÖMAN & al. 2012a].

A number of selection programmes with focus on trees for urban sites are in progress in several countries [SÆBØ & al. 2005]. However, the majority of these concentrate on the genetic aspect of species in current use, with the aim to select suitable varieties and genotypes [SANTAMOUR, 1990; MILLER & MILLER, 1991; SAEBØ & al. 2005]. In the case of northern Europe the majority of species used in cities originate from

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the native dendroflora, representing cool and moist site conditions were limitations of drought and pest tolerance continue to frame the main complications, albeit the intentions from these selection programmes [SAEBØ & al. 2005]. To supplement these selection programmes, additional tree species still awaits discovery and testing [DUHME & PAULEIT, 2000].

In order to achieve knowledge of a greater diversity of species adapted to urban sites, new innovating methods have to be developed. As water stress is widely argued to be the main constraint for tree growth and health in the urban environment [e.g. CRAUL, 1999; SIEGHARDT & al. 2005], research on drought tolerance of trees has classically focused on physiological reactions in the water balance/water use like transpiration rates, sap flow measurement and the hydraulic architecture of the tree [e.g. KOZLOWSKI & al. 1991; SPERRY & al. 1998; BREDA & al. 2006; DAVID & al. 2007; WEST & al. 2007]. These investigations give valuable information at the tree level but they are limited in their practical "every day use" for urban tree planners, arborists etc. [ROLOFF & al. 2009]. Instead, dendroecological studies can contribute to evaluate different tree species reaction and tolerance of e.g. drought. According to ROLOFF & al. (2009) this kind of dendroecological descriptions are seldom or not at all available for most species, which clearly points out the importance of this type of research in the selection process for "new" tree species for urban sites.

In natural habitats, trees have been stress-tested and selected over evolutionary periods of time. Some species have developed an extensive plasticity and tolerance of a range of environmental conditions while others have specialised in certain habitat types [RABINOWITZ, 1981; GUREVITCH & al. 2002]. For instance, steep mountain slopes with thin soil layers represent distinct habitat types, where the environmental parameters that define the particular habitat and separate it from other habitats have shaped the evolution of plants and acted as a filter that screens out many potential colonizing species not suited to the particular habitats. Investigating habitats experiencing similar conditions as urban environments in nature and studying the ecological background of these species would be of special interest for future selection of trees for use in urban fabric [FLINT, 1985; WARE, 1994; SÆBØ & al. 2005; ROLOFF & al. 2009]. Starting this process now is urgent, as tree selection is a long-term process.

From the perspective of the northern parts of Central Europe and in the adjacent, mild parts of Northern Europe (in the following abbreviated to the "CNE-region") it is unlikely that the species poor native dendroflora can contribute to a larger variation of tree species with extended tolerance of the environmental stresses characterizing urban sites of the region [DUHME & PAULEIT, 2000]. In comparison, other regions with a comparable climate yet having a rich dendroflora may hold the potential to contribute new tree species and genera well adapted to the growing conditions in urban sites in the CNE-region [TAKHTAJAN, 1986; BRECKLE, 2002].

During the last decade extensive fieldwork have been carried out in the Qinling mountain range, China, in order to obtain an overall understanding of the species composition, structure and dynamics of the forest systems in the elevational zone where the climate is similar to the inner city environment across the CNE region [e.g. SJÖMAN & al. 2010]. This paper presents a study where the oriental white oak, *Quercus aliena* var. *acuteserrata* Maximowicz ex Wenzig, use-potential for urban sites in the CNE-region have been evaluated based on habitat studies in the Qinling Mountains. This study is initiated by

the Swedish University of Agricultural Sciences to examine selection of site-adapted species for urban sites. The research hypothesis in this selection programme is that identification of "new" tree species for urban use can be gained through studies of natural habitats with similar site conditions as urban paved environment – where the field study in Qinling is one of the case studies on order to test this hypothesis. With the long-term aim to contribute to the selection of "new" tree species and genera well adapted to the growing conditions in urban sites in the CNE region the field work in China specifically focused on:

- identification of habitats in the Qinling Mountains where the oriental white oak are exposed to seasonally dry and harsh conditions;
- characterisation of the oriental white oaks performance in these habitats;
- presentation and discussion of the use-potential of the oriental white oak for urban sites in northern Europe.

In order to evaluate the use potential of the oriental white oak for the CNE-region origin from the Qinling Mountains, China, the field data is compared to urban environments of Copenhagen. In the comparison, the Copenhagen case is divided into paved respectively park environment in order to evaluate the broadness of the use potential.

Method and materials

Case study area

China is considered the most species rich region of the world [KÖRNER & SPEHN, 2002; TANG & al. 2006]. The Qinling Mountain range in the central, temperate part of the country forms a botanic border between the southern and northern regions of China, and consequently, it hosts a species rich flora [YING & BOUFFORD, 1998]. Shaanxi province, where the Qinling mountain range is situated, harbours 1224 wooded species [KANG, 2009], which can be compared to a total of only 166 wooded plants in the Scandinavian countries [MOSSBERG & STENBERG, 2003]. The relatively northern location of the mountain range combined with its altitudinal levels, makes it possible to find steep, south facing rocky and craggy slopes. Here, plants are exposed to cold winters and warm summer months with periods of intense drought [TAKHTAJAN, 1986; BRECKLE, 2002] much comparable to the climate expected in urban paved sites of the CNE-region.

The oriental white oak grows in the Qinling Mountains in the altitude 1300-2200m asl, belonging to the deciduous broadleaved oak forest zone [LIU & ZHANG, 2003]. The oriental white oak is the main canopy species throughout the zone. In the lower part (< 1200 m asl) the oriental white oak is co-dominating with *Quercus variabilis*, and in higher parts of the zone together with *Quercus wutaishanica.* These oak species dominate particularly on slopes, independently of direction, whereas the moist river valleys are characterised by mixed broadleaved forests with a large number of other canopy species [SJÖMAN & al. 2010].

Site description

The research was conducted in the northern part of the Qinling Mountain range within three different areas – Taibai Forest Reserve (34° 05'10" N 107° 44'46" E), Red

Valley Forest Reserve (34° 05'08" N 107° 44'52" E), and Siboshan (33° 42'08, 30" N 106° 47'16, 69" E).

Based on climate data for the Qinling Mountains, the altitude-zone from 1000-2000 m above sea level (asl.) was identified as the altitude where mean annual temperature and precipitation match the climate of urban sites in the CNE region. The mean annual temperature in the altitude 1000-1500 is 9-12 °C with a yearly precipitation on 650-1000 mm while the mean annual temperature in the altitude 1500-2000 is 8-9 °C with a yearly precipitation on 800-1000 mm (Tab. 1) [LIU & ZHANG, 2003; TANG & FANG, 2006]. The present situation of urban paved sites in Copenhagen represent a mean annual temperature of 8-12 °C when urban heat island effect is included (+1-3 °C) (DMI 2015; US EPA 2015) additionally with a yearly precipitation of 525mm (DMI 2015).

Tab. 1. Mean monthly temperature (°C) and precipitation (mm) at the study site.

Month	*Precipitation distribution (%)*	*Precipitation distribution at 1000-1500m asl (mm)*	*The mean monthly temperature at 1000-1500m asl (C)*	*Precipitation distribution at 1500-2000m asl (mm)*	*The mean monthly temperature at 1500-2000m asl (C)*
January	3 %	25	2	27	**0**
February	6 %	49,5	3	54	**1,9**
March	10 %	82,5	8	90	**3,9**
April	12 %	99	11,5	108	**7,9**
May	22 %	181,5	13	198	**8,9**
June	17 %	140,5	21,5	153	**14,5**
July	15 %	124	22,5	135	**15,5**
August	8 %	66	19,5	72	**13,9**
September	3 %	25	14,5	27	**11,9**
October	1 %	8	11	9	**7,9**
November	1 %	8	5,5	9	**2,9**
December	2 %	16,5	- 2	18	**- 4,1**
		Total 825,5 mm		**Total 900 mm**	

Location of plots

The field investigation was conducted during March-October with the assistance of botanical experts from the Northwest Agriculture and Forestry University, Yangling during the first two months. The task was to obtain an overall understanding of the species composition, structure and dynamics of the forest systems in relation to altitude and variation within the site conditions [SJÖMAN & al. 2010]. Special attention was given to identify exact locations of steep, south facing slopes with shallow soils and rock outcrop in order to establish the range of tree species that would grow in these locations. Subsequently, 20 study plots were strategically placed on recognized S facing slopes where extent of mature tree population on exceedingly rocky and/or steep gradients was the main criterion (Fig. 1). Homogeneous site conditions including oriental white oak trees determined the exact location and size of each plot. Plot sizes were of 10x10 m or 20x20 m and were located between 1150 and 1720 m asl. (Tab. 2). Due to human interference to vegetation and species composition plots below 1150 m asl, were not selected for the survey.

Fig. 1. The study plots were located at steep south facing slopes with shallow soils and rock outcrop

Measurement of plot data

For each plot, slope direction and steepness were measured and rock outcrop and cover of the herbaceous field layer were estimated. The exposure of bedrocks was based on FAO´ (2006). Field layer cover was estimated with intervals of 10%.

With the aim to parallel natural habitats and urban conditions in the CNE-region, soil texture, humus content and pH value was of special interest and focus. Soil samples were collected in three different depths (0-20, 20-30, 30-50 cm) from 10 pits randomly distributed in each plot [KLUTE, 1986; FAO, 2006]. For each depth, the samples were mixed before analyses [FAO, 2006]. Soil texture was analysed using the soil grain analyzer method **[**EHRLICH & WEINBERG, 1970] (Tab. 2), and organic matter was analysed with the $K_2Cr_2O_4$ method (Tab. 2), and pH using the potentiometric determination method (soil/water = 1:2.5) [TAN, 2005] (Tab. 2).

All trees were measured for diameter at breast height (DBH), total height and age in order to determine growth and development. To establish age, all trees were subjected to drilling as close to the ground as possible [GRISSINO-MAYER, 2003]. Tree positions were surveyed to distinguish canopy from understorey.

Tab. 2. Compilation of plot data. Rock outcrops in the plots were classified as N (None 0%), V (Very Few 0%–2%), F (Few 2%–5%), C (Common 5%–15%), M (Many 15%–40%), or A (Abundant 40%–80%).

Plot nr.	Altitude (m asl)	Slope direction	Slope steepness - degree	Number of soil sample to 30-50cm	pH	Rock outcrops	Fieldlayer cover (%)	Plot size (m)	Organic matter (g/kg)	Clay content (%)	Silt content (%)
1.	1720	South	53	10	6.5	V	40	10x10	9.6	1.7	40.6
2.	1620	South/Southeast	58	5	6.5	V	30	10x10	16.1	2.7	56.4
3.	1640	South	36	10	7.9	N	10	10x10	21.9	1.6	45.9
4.	1630	South	47	10	7.8	F	10	10x10	41.6	2.3	47.4
5.	1635	South	45	10	8.0	F	30	10x10	18.2	2.4	47.3
6.	1610	Southwest	45	10	7.5	F	10	10x10	27.1	2.1	44.4
7.	1650	South/Southwest	40	10	6.9	N	40	10x10	55.1	2.1	54.9
8.	1660	Southeast	45	9	6.1	C	30	10x10	12.1	1.7	44.3
9.	1620	Southeast	57	5	8.1	A	20	10x10	49.5	2.3	45.7
10.	1610	South	45	9	6.8	F	50	20x20	26.4	2.2	42.8
11.	1490	South	64	7	6.7	F	20	10x10	17.4	3.0	63.0
12.	1400	Southwest	43	10	6.4	F	10	10x10	18.8	2.0	48.2
13.	1590	South	40	10	7.2	V	20	10x10	41.3	2.7	59.4
14.	1560	South/Southeast	43	10	7.6	N	20	10x10	23.0	2.5	52.7
15.	1400	South/Southwest	38	5	7.0	C	30	10x10	44.5	1.8	44.3

16.	1350	South/Southwest	44	6	6.5	C	40	10x10	22.6	3.0	60.2
17.	1390	Southeast	43	7	5.8	F	30	10x10	16.8	3.0	58.6
18.	1360	South	45	5	6.5	A	10	10x10	44.8	1.9	47.5
19.	1260	South	45	2	6.4	C	30	10x10	51.1	1.6	45.7
20.	1370	South	44	6	6.9	V	40	10x10	31.0	2.5	53.8
Mean					**7.1**		**24.0**		**29.5**	**2.3**	**50.2**

Calculation of potential water stress

The potential water stress in the study plots was calculated and compared with data for the inner-city environment of Copenhagen, Denmark (Tab. 3). For the calculation of potential evapotranspiration, the regression by THORNTHWAITE (1948) was used, where monthly potential evapotranspiration was based on the values of temperature, number of sunshine hours per day and cloudiness. Sunshine hours per day were estimated on a monthly basis by combining information about day length [MEEUS, 1991] and days with rainfall as indicator for cloudiness [LIU & ZHANG, 2003]. Cloudiness is 10% of the total day length except the rainiest month (May, June and July) where cloudiness is 50% [LIU & ZHANG, 2003]. Since data of water runoff was not available for the study plots, a similar area of topography and vegetation characteristics in the region of Yangping was applied as a criterion [LIN & al. 2007]. The annual precipitation rate in Yangping exceeds Qinling with 215mm, yet data was considered suitable as the distribution and intensity of rain closely correlated with the studied terrain.

Estimates of water runoff data for park respectively paved environments in Copenhagen was based on P90 (2004), concluding a 10% runoff from park environment and an expected 70% water runoff for paved sites.

Tab. 3. The accumulated water netto difference (mm) in the study sites additionally with park respectively paved environments in Copenhagen

Qinling Mountains	jan	feb	mars	april	maj	juni	juli	aug	sep	okt	nov	dec
1000-1500m asl	2.5	11.4	12.3	-0.7	26.3	-1.5	-39.3	-114.4	-168.4	-206.8	-221.2	-215.0
1500-2000m asl	2.7	8.6	17.6	11.6	49.7	48.1	36.7	-16.0	-69.3	-106.5	-121.0	-114.2
Copenhagen												
Park environment	25.9	49.9	63.1	66.1	41.1	34.3	13.0	-40.6	-63.0	-79.1	-63.2	-42.1
Paved environment	6.7	12.1	6.7	-22.1	-84.9	-152.3	-223.4	-310.6	-361.8	-392.9	-398.0	-395.5

Calculation of growth data

In order to evaluate any difference between oak trees growing in lower terrain (<1500m asl.) in a warmer and drier climate compare with oak trees in higher altitudes (>1500m asl.) a growth pattern where calculated by a regression in Minitab (Minitab 16 Statistical Software).

Results

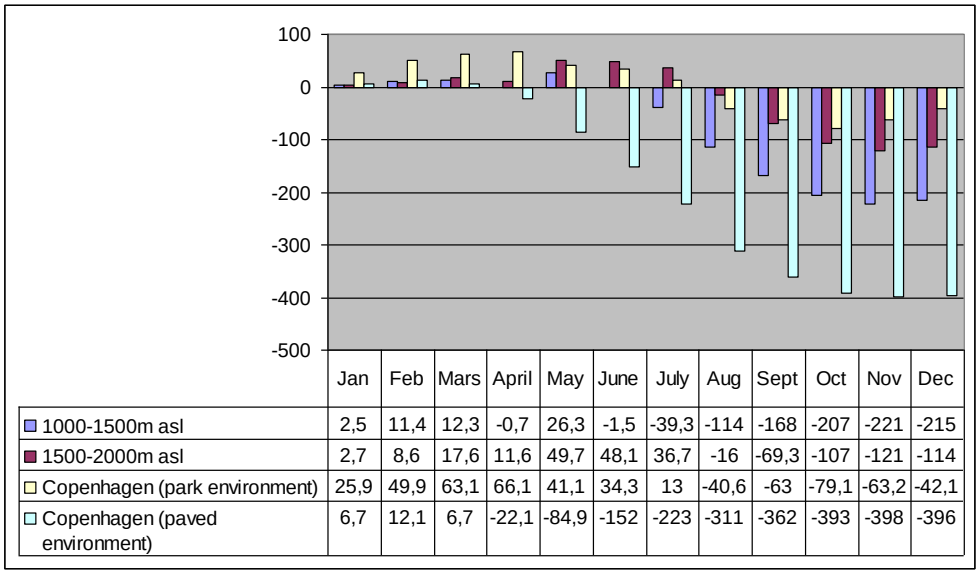
Site conditions

In all plots the soil depth was at least 50 cm, indicating tree root penetration into deeper grounds (Tab. 2). However, shallow bedrock and rock outcrops partly limit the soil depth for some of the plots (Tab. 2). The texture composition is comparable between all plots, with high to very high levels of silt (mean 50.2%) and low contents of clay (mean 2.3%) (Tab. 2). Also the organic matter content is low across the plots (mean 29.5 g/kg) (Tab. 2).

Cumulative water net difference

Due to higher precipitation and lower temperatures in higher altitudes (1500-2000 m asl) the water stress status is apparently smaller and occur later in the season compare to the sites in lower terrains (1000-1500 m asl) (Tab. 3). As Fig. 1 illustrates, current conditions in Qinling Mountains at 1000-1500 m asl, experience partial water stress in April and June and more severe water stress towards July and the remaining part of the growing season. In the altitude 1500-2000 m asl, a partial water stress occur first in August and thereafter in a less dramatically trend compare to the situation in lower terrains (Fig. 2).

In a compilation with Copenhagen, the study sites, regardless the altitude, experience warmer and drier site conditions compare to park environments in Copenhagen while they experience less water stress compare the situation in paved sites (Fig. 2).



	Jan	Feb	Mars	April	May	June	July	Aug	Sept	Oct	Nov	Dec
1000-1500m asl	2,5	11,4	12,3	-0,7	26,3	-1,5	-39,3	-114	-168	-207	-221	-215
1500-2000m asl	2,7	8,6	17,6	11,6	49,7	48,1	36,7	-16	-69,3	-107	-121	-114
Copenhagen (park environment)	25,9	49,9	63,1	66,1	41,1	34,3	13	-40,6	-63	-79,1	-63,2	-42,1
Copenhagen (paved environment)	6,7	12,1	6,7	-22,1	-84,9	-152	-223	-311	-362	-393	-398	-396

Fig. 2. The accumulated water netto difference (mm) in the two studied altitudes compare to park respectively paved sites in Copenhagen.

Species composition and performance

In total, 102 oriental white oak where found in the studied plots, 11 below 1500 m asl, and 91 above 1500 m asl.

Among the oak trees the majority have their vertical position in the canopy layer in the vegetation structure, regardless the altitude zone. Among the oak trees found in the plots below 1500 m asl., only one out of 11 where found in the understorey layer while 56 out of 91 oak trees in the plots above 1500 m asl where found in the canopy layer which indicating a high tolerance for warmer and thereby drier conditions existing in the canopy layer compared to underneath the tree crowns (Fig. 3).

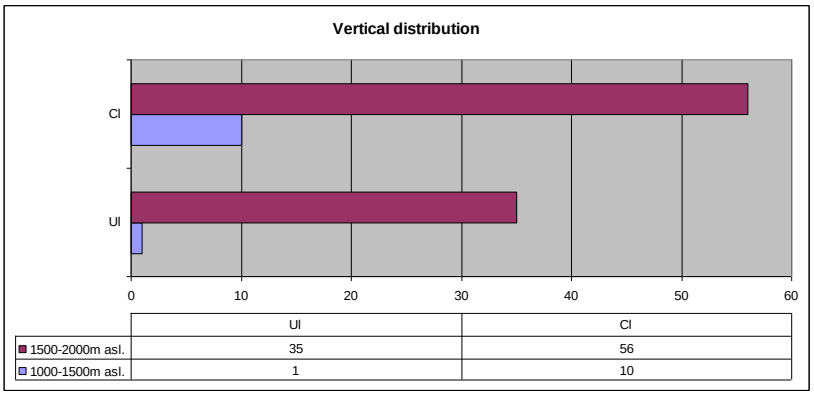


Fig. 3. The vertical distribution of the oriental white oak found in the studied plots separated between understoery layer (Ul) and canopy layer (Cl).

In a attempt to evaluate the growth pattern of the oriental white pine in the two studied altitude zones, growth tables have been completed, where height and diameter growth is match with the age (Fig. 4 and 5). Concerning height growth the oak trees in lower altitudes (<1500 m asl.) have a yearly mean growth rate of 0.28 m compared to 0.23 m tress in plots >1500 m asl. (Tab. 4). The calculations presented in Tab. 4 and 5 are based on rather few individuals (102 trees), especially in lower elevation (11 trees), but can still be used as an indicator of their growth rate in this climate and site conditions. Concerning the diameter growth the oak trees in lower altitudes have a slightly larger average growth compare to trees in higher terrains (Tab. 4). This above mentioned pattern is also illustrated in Fig. 4 and 5 where the trees in lower altitudes have a slighter stronger growth. However, concerning diameter growth illustrated in Fig. 5 show that the studied oak trees in higher altitudes show a stronger growth after 50 year.

Tab. 4. Yearly mean increment in height (m) and DBH (cm) of oriental white oak in the study sites divided between altitudes.

Plot area	Yearly Height Growth (m)	Yearly Diameter Growth (cm)	Number of trees	Size of an 15 year old tree	Size of an 50 year old tree
1000-1500 m asl	0.28	0.38	11	4.2/5.7	14/19
1500-2000 m asl	0.23	0.34	92	3.5/5.1	11.5/17

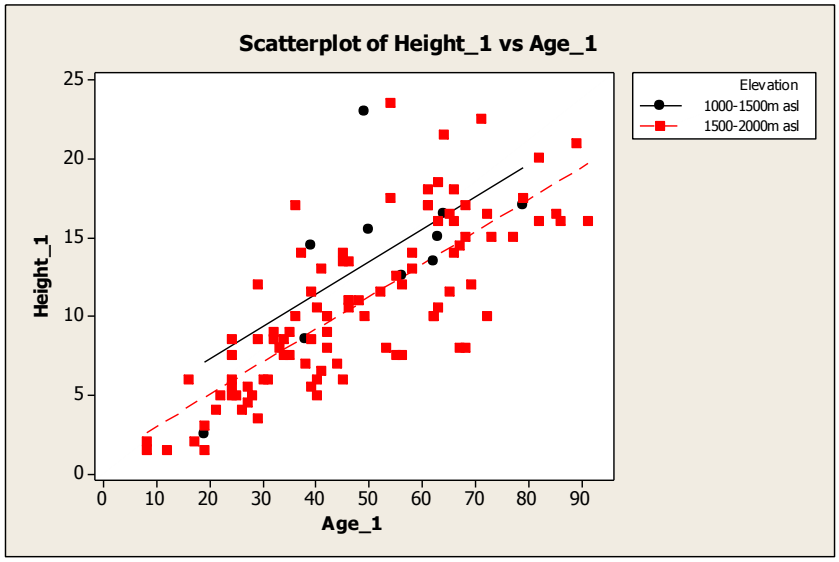


Fig. 4. Height increment (cm) of oriental white oak in two altitudes (1000-1500 m.a.s.l. and 1500-2000 m.a.s.l.) as a function of tree age (years).

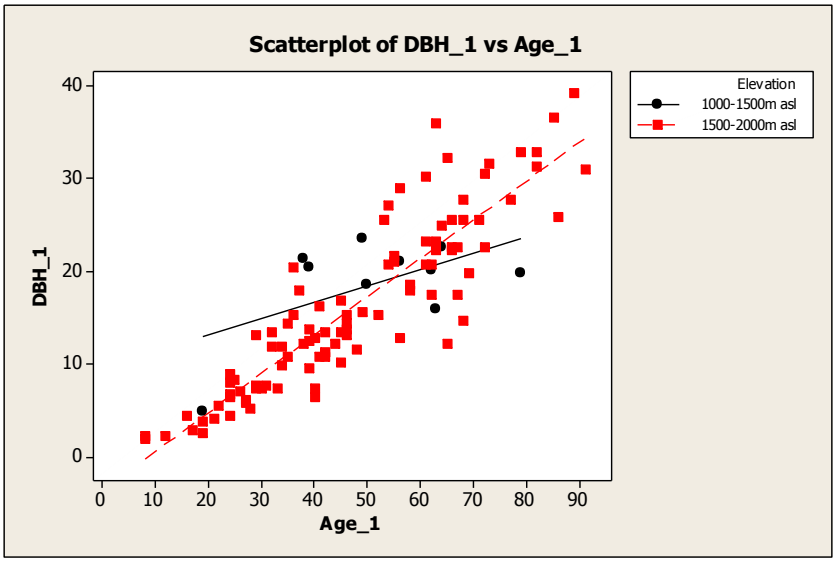


Fig. 5. DBH increment (cm) of oriental white oak in two altitudes (1000-1500 m.a.s.l. and 1500-2000 m.a.s.l.) as a function of tree age (years).

Discussion

As has been suggested by a number of authors, investigating the ecological background and performance of species growing in habitats that naturally experience drought during the growing season and winter temperatures similar to those of inner-city environments provides a sound and reliable selection method [FLINT, 1985; WARE, 1994; DUCATILLION & DUBOIS, 1997; BROADMEADOW & al. 2005; SÆBØ & al. 2005; ROLOFF & al. 2009; SJÖMAN & al. 2012b]. This study examined forest systems occurring between 1300-2200 m asl. in the Qinling Mountains, in order to evaluate the oriental white oaks (*Quercus aliena* var. *acuteserrata*) growth and development in warm and dry forest habitats and hence evaluate its potential for urban paved sites in the CNE-region. When comparing the study sites with urban paved environments in Copenhagen, Denmark, the trees in lower altitudes (<1500 m asl.) had a closer match with urban paved sites but had a later negative water netto difference and also a less extreme development during the season compare to paved environments in Copenhagen (Fig. 2). The trees in higher altitudes (>1500 m asl.) had an even less match with paved environments due to a cooler climate and hence a less dramatic evapotranspiration over the season. The conclusion from this is that in order to succeed growing oriental white oak in inner-city environments it is necessary to create larger planting pits or/and complement the plantations with storm water management which makes it possible to increase the soil water content compare to traditionally planting pits in paved environments [SIEGHARDT & al. 2005]. Furthermore, even the high levels of silt in the study plots indicate a rather good water holding capacity [BRADY & WEIL, 2002]. However, the high level of silt and the lack of vegetative field layer cover in many plots the surface can have a tendency to form a hard crust, which can cause extensive water runoff [BRADY & WEIL, 2002]. This water runoff in the plots can be of significant importance and to a rather large proportion due to rather steep slopes within the study sites which can in fact create much drier conditions in the studied sites that the data in his paper present [SJÖMAN & al. 2010]. Therefore it is possible to rank the oriental oak as a promising species for paved environment, especially the genotypes from lower altitudes since they have over evolution adapt to a warmer and dryer climate compare to trees in higher altitudes. Yet, further evaluation has to be done, including evaluation of the traits behind the genotypes tolerance towards drought and the capacity of these traits. For example, it is necessary to evaluate differences between avoiding respectively tolerating traits and how well these are and its combination such as turgor loss point and other leaf traits [e.g. SCHULZE & al. 2005; LAMBERTS & al. 2008]. Through this following evaluation more detailed information concerning their tolerance can be gained.

The majority of the oaks studied had their vertical position in the canopy layer in the vegetation structure, regardless the altitude zones studied, indicating that the species is rather shade intolerant, which is also presented in other literature [MENITSKY, 2005]. Noticeably, is that there were only one out of 11 trees that were found in the understory in the plots below 1500 m asl., while 35 out of 91 oak trees in higher altitudes (>1500 m asl.) were found in the understory. From a plant physiological perspective, shade and drought is a very hard combination of stresses for plants in order to capture resources for survival

and/or competitions [GRIME, 2001], which might make the number of trees in the understory few in lower altitudes compare with the number of trees in cooler and moister habitats in higher altitudes. Nevertheless, it is important to keep in mind that the number of oak trees found in lower altitudes is rather few which makes above conclusion weak and need further studies. From an urban forest perspective this might however be a useful reflection since the built up structure in urban environments be able to create dry and shaded sites where the oriental white oak might is a less appropriate plant material. Furthermore, when the age distribution between analyse oak trees (Fig. 4 & 5) it is obviously that the main age distribution is between 20-70 years, indicating a very limited occurrence of young individuals in the plots. The lack of young trees indicates a pioneer strategy, with high demands for sunlight and has therefore difficulties in establishing under an existing tree canopy, which is a trait among many broadleaved oak species [JOHNSON & al. 2009].

This first stage in the selection process with dendroecological habitat studies can screen out species showing slow and/or underdeveloped growth in habitats similar to urban inner-city environments. This allows the focus to be directed towards the species in these natural sites that develop rapidly into large trees. This first stage consequently identifies genotypes of the species that ought to be included in the following steps at an early phase of the procedure [SJÖMAN & al. 2012b]. In the Qinling Mountains of China the oriental white oak shows very promising development in habitats experiencing drier conditions than those in park environments in Copenhagen, and is therefore interesting for urban paved sites were the demands of a greater catalogue of tolerant trees are highly needed.

This study focused on trees that in their natural sites are exposed to warm and dry growth conditions, since water stress is argued to be the main constraint for tree growth and health in urban environments [e.g., CRAUL, 1999; HOFF, 2001; SIEGHARDT & al. 2005; NIELSEN & al. 2007; ROLOFF & al. 2009]. It is important to bear in mind that this process with dendroecological habitat studies in order to identify potential urban trees is just the first step in the selection process. Further research is necessary in order to evaluate the species tolerance towards warm and periodically dry growth conditions in another geographical area and towards other stressors, such as de-icing substrates or air pollution. Nevertheless, this approach constitutes a faster and more effective route, since subsequent selection work can focus on species with high potential for the purpose instead of testing species randomly. Dendroecological studies, as presented in this paper, contribute to an ecological understanding that provides for a much wider knowledge base in the selection process, thus helping to evaluate the reaction, tolerance, and performance of different tree species to different stressors. Furthermore, dendroecological studies provide valuable guidance regarding the use potential of species, which can be of importance in their subsequent evaluation in full-scale plantations in urban environments.

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IDENTIFICATION OF THE MAIN FUNCTIONAL GROUPS IN THE DRY GRASSLANDS OF *FESTUCETALIA VALESIACAE* FROM NORTH-EASTERN ROMANIA

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Abstract: Plant functional traits and plant functional groups are increasingly used to assess the effect of the change in land use on plant species or plant communities, in nature conservation projects, to detect patterns in the expansion some invasive species or to asses the processes of succession or competition in plant communities. In this study, the main objective was to identify the main plant functional groups (based on plant traits) which co-exist in different plant communities of the dry grasslands (*Festucetalia valesiacae*) from North-Eastern Romania using the RLQ analysis (considering the plant traits, environment characteristics and vegetation). As RLQ analysis mainly revealed a soil moisture-soil nutrients gradient along the first axis, a transition from species with traits specific to more dry conditions and less available nutrients to moister and higher nutrients availability sites was observed (from perennial species with short flowering range and traits oriented to resources retention in storage organs to annual species with long flowering range and traits oriented to resource acquisition). Plant functional groups were identified using species scores along the first two RLQ axes via k-means clustering which generated six groups displayed along the above mentioned gradients. The floristic composition of the identified functional groups suggested that, in the context of *Festucetalia valesiacae* vegetation type from North-Eastern Romania, plant communities developed in areas with lower values for soil moisture and nutrients could possibly be richer in autochore and barochore geophyte and hemicryptophyte species with short flowering range and mixed reproduction type. As soil moisture and nutrients increase, in the floristic composition could possibly occur more anthropochore and zoochore therophyte species with long flowering range, mainly reproducing by seeds and, also, some taller endozoochore shrubs species.

Key words: xeric grasslands, functional approach, RLQ, North-Eastern Romania

Introduction

Plant functional traits are morphological, physiological or life-history characteristics influencing the growth, reproduction and even the survival of species and highlights the strategies by which the plant respond to abiotic and biotic environment [NI, 2003; GUBSH & al. 2011; SEEBACHER & al. 2012]. Plant functional groups include non-phylogenetic related species with similar eco-physiological and life-history traits, which respond in a common way to environmental factors and present a similar effect on ecosystem functioning [LAVOREL & GARNIER, 2002; NI, 2003; MOONEN & BARBERI, 2008; FRANKS & al. 2009]. The study of functional groups in different plant communities can be used to assess the effect of the change in land use [ANSQUER & al. 2009; FRANKS & al. 2009] on plant species or plant communities, in nature conservation projects by identification of the best management measures applied to the target species

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[DROBNIK & al. 2011], or to detect patterns in the expansion some invasive species [FARNSWORTH, 2007]. Also, plant functional types can be used to asses the processes of succession or competition in plant communities because certain plant traits are important for the competitive ability of a certain species [DUCKWORTH & al. 2000].

The majority of the xeric grasslands within *Festuco-Brometea* vegetation class are semi-natural communities of herbaceous species, sometimes of an extraordinary diversity, and with a floristic composition including numerous rare or threatened species. As a number of studies concluded that some of the dry grasslands represents the most species rich plant communities at least for small spatial scales [DENGLER & al. 2012; TURTUREANU & al. 2014; WILSON & al. 2012], they present a great conservation value and most of the dry grasslands habitats, as 6240* Sub-pannonic steppe grasslands and 62C0* Ponto-Sarmatic steppes (in Romania) were included in the community interest category [GAFTA & MOUNTFORD, 2008]. One of the possible explanations of the coexistence of a great number of species in relative small areas could be represented by the differences in plant traits which make possible the complementary use of resources. Plant functional traits can be used in order to group the plant species according to their functions, to understand and to predict the assembly and stability of plant communities [GUBSCH & al. 2011]. In this context the plant functional groups could represent also ones of the main determinants of the species composition of the plant communities [MOONEN & BARBERI, 2008].

In the present study, the main objective was to identify the main plant functional groups (based on plant traits) that co-exist in different plant communities of the dry grasslands (*Festucetalia valesiacae*) from North-Eastern Romania using a vegetation dataset (including 45 releves) realized in 2014 and a RLQ analysis in which the plant traits, environment characteristics and vegetation are considered.

Material and methods

The study area (Fig. 1: 46°30’ – 47°40’ N and 26°40’ – 28°00’ E), situated in the Moldavian Plateau, is characterized by a fragmented relief and a mainly agricultural landscape, with mean altitudes of 150-250 m. The general climate is temperate continental, with mean annual temperatures of 9-10 °C and mean annual precipitations of 400-600 mm/m^2. The main soil types are the mollisols and chernozems. The region is situated at the intersection of two floristic regions: the Euro-Siberian region and the Irano-Turanian region, each presenting their particular floristic elements [CHIFU & al. 2006]. From another perspective almost all territory is included in the continental biogeographical region and only the southern, smaller, part is included in the steppic biogeographical region [HABITATS DIRECTIVE, 1992].

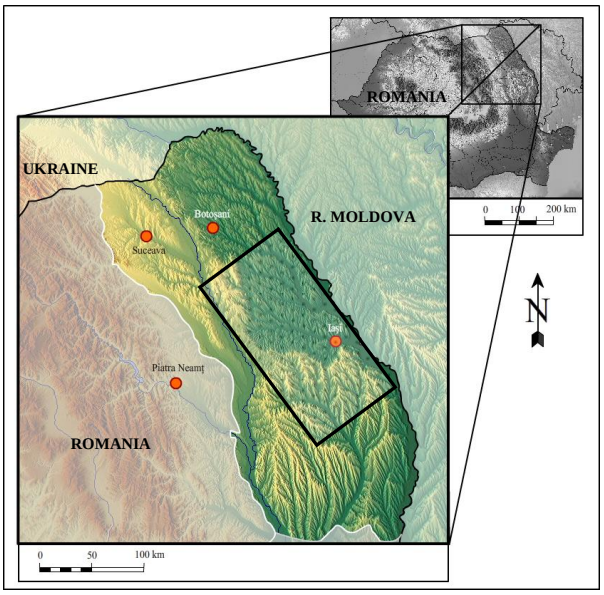

Fig. 1. Geographical location of the study area within Romanian territory

In order to identify the main functional groups in the dry grasslands from North-Eastern Romania, 45 relevés (including 273 species) were used. The relevés covered the main plant communities within *Festucetalia valesiacae* in the region (communities of *Stipa capillata*, *Stipa lessingiana*, *Festuca valesiaca*, *Dichanthium ischaemum*, *Chrysopogon gryllus* etc.), and were realized using the method elaborated by the Central European School for vegetation study adapted for Romanian vegetation [BORZA & BOȘCAIU, 1965]. Plant species cover was visually estimated using a 6 level scale: + (<5%); 1 (5–10%); 2 (10–25%); 3 (25–50%); 4 (50–75%); 5 (>75%). All relevés have 100 m^2 in size. Species present only in one relevé were removed (final dataset included 45 relevés x 208 species). Each plant species was characterized by the following seven traits (factors in the RLQ analysis):

a) grasses (gr), non leguminous forbs (fo) and legumes (le) – a priori classification of plant species in the above functional categories [WRIGHT & al. 2006]. In adition to this classification, some species as *Rosa gallica*, *Rosa canina*, *Prunus spinosa* were treated as shrubs (sh);
b) life form – six categories: therophyte (t), hemitherophyte (he), hemicryptophyte (h), geophyte (g), chamaephyte (c) and phanerophyte (ph) [SÂRBU & al. 2013];
c) reproduction type - two categories: reproduction by seeds (s) and mixed (m) - by seeds and vegetative [KOVÁCS, 1979];
d) dispersal mode – nine categories: anemochory (wind dispersal – an), endozoochory (seeds passed through digestive system of various animals – en), anthropochory (dispersal by humans – at), myrmecochory (ant dispersal – my), mixed mode of dispersal (anemochory / anthropochory – mi), epizoochory (dispersal by animals – ep), autochory (self dispersal – au), barochory (unassisted dispersal – ba) and hydrochory (water dispersal – hy) [KOVÁCS, 1979];
e) pollinating mode – three categories: wind (w), insects (i) and mixed (m) [KOVÁCS, 1979];
f) plant height [SÂRBU & al. 2013] – three categories: low (< 0.3 m), medium (0.3 – 0.7 cm - med) and high (> 0.7 m);
g) flowering range [SÂRBU & al. 2013]: six categories (from one to six months).

Next, a RLQ analysis [DOLÉDEC & al. 1996; DROBNIK & al. 2011] was conducted using three matrices previously constructed: a sites x species matrix (the L matrix), a sites x environment matrix (the R matrix), including the non weighted average values of the Ellenberg indicator values [ELLENBERG & al. 1992] for light (EIV L), temperature (EIV T), continentality (EIV C), soil moisture (EIV F), soil pH (EIV R), soil nutrients (EIV N), altitude and heat load (derived from aspect and slope [OLLSON & al., 2009]) and a species x traits matrix (the Q matrix). A correspondence analysis was firstly performed on the L matrix, on the arcsin-square root transformed values of the plant species cover percentages. After that, Hill-Smith ordinations were conducted on the R matrix (using the row species scores from the correspondence analysis as canonical factor) and on the Q matrix (using the column species scores from the correspondence analysis as canonical factor). The RLQ analysis link the vegetation data, species traits and environmental characteristics [BORCHARDT & al. 2013] finding two sets of coefficients (representing linear combination of traits and vegetation data and linear combination of environment variables and vegetation data) between which the covariance is maximized and equal to the square root of the corresponding eigenvalue [MINDEN & al. 2012]. Correspondence analysis, Hill-Smith ordinations and the RLQ analysis were made in the 'ade4' package [DRAY & al. 2015] from R software [R Development Core Team, 2004]. In order to identify the main functional groups, species scores from the RLQ analysis (only the first two axes) were further subjected to a k-means clustering procedure, trying partitions with 2 to 10 clusters. Optimal number of clusters was assessed using the average *silhouette* width and *Calinski-Harabasz* criteria. K-means clustering was conducted in the fpc package [HENNING, 2015] from the R software [R Development Core Team, 2004].

Results and discussion

Analysis of vegetation plots – environmental variables relationship – In the separate analysis of vegetation plots – environmental variables (Fig. 2), the first ordination axis (the most important one, presenting the highest eigenvalue) explained almost half of the total variance (47.14%) in species composition and was correlated with plant species preferences for temperature, soil moisture and soil nutrients. The second ordination axis explained only 15.40% of the variation and was correlated with the heat load and soil reaction. The first axis distinguished the dry sites, with plant species tolerating high temperatures growing on soils with less available nitrogen (left side) from the more moist sites with higher amounts of available nitrogen and species preferring lower temperatures. The second axis represented a gradient of heat load and soil reaction separating the vegetation plots from steep, south and south-western slopes and lower values of soil pH (upper part of the ordinogram) from the plots from north and north-eastern slopes, with higher values of soil ph (the lower part of the ordinogram).

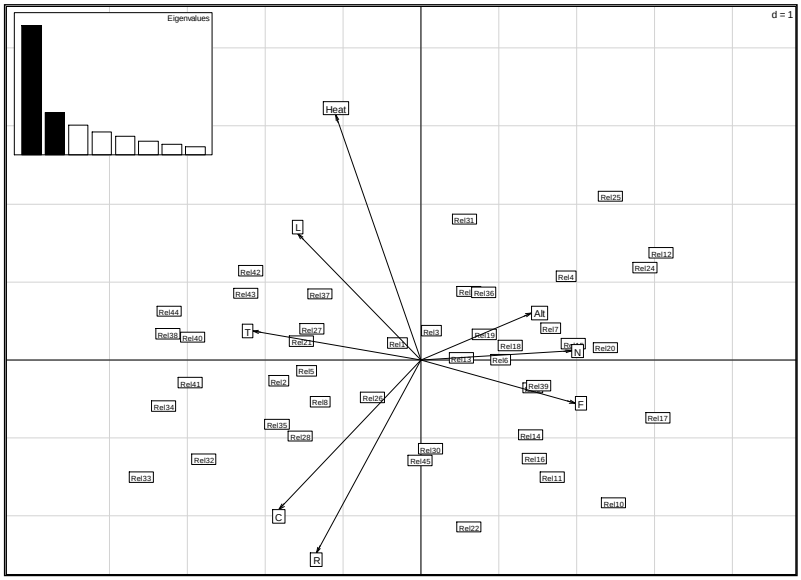

Fig. 2. Hill-Smith ordination of sites and environmental variables (R table: sites x environment) using the row weights from the site scores of the CA on L table as canonical factors.
Eigenvalues: Axis 1: 3.771, Axis 2: 1.233. Variance explained (%): Axis 1: 47.143, Axis 2: 15.406. Squared correlation coefficients: Axis 1: T (0.7577), F (0.6411), N (0.6099), C (0.5391), L (0.4065), Alt (0.3291), R (0.2933), Heat load (0.1943); Axis 2: Heat load (0.5263), R (0.3266), C (0.1965), L (0.1392), Alt (0.0191), F (0.0164), T (0.0072), N (0.0006).

The soil moisture-soil nutrients gradient along the first ordination axis separated the *Chrysopogon gryllus* phytocoenoses from those edified by *Stipa lessingiana*, *Dichanthium ischaemum* and *Festuca valesiaca* while the soil reaction and heat load gradients along the second axis differentiated the *Stipa capillata* community from *Festuca valesiaca community* (Fig. 2).

Hill - Smith ordination of the species traits – Relationship among species traits revealed by the Hill-Smith ordination of the traits table (Q), also emphasized the first axis as the most important one, accounting for 11.28% of total variance, compared to axis two which explained only 7.65%. As all of the plant traits were qualitative, the correlation ratios suggested that plant classification into grasses, forbs and legumes (0.83), pollination mode (0.81) and reproduction type (0.52) are the main traits correlated with the first axis while life form (0.61) and the dispersal mode (0.4) were correlated with the second axis. The first axis distinguished the grass species pollinated by wind and mixed reproduction type (left side of the ordinogram) from the forbs having a mixed pollinating mode and reproduction (mainly) by seeds (the right side of the ordinogram). The second axis opposed the therophyte and hemitherophyte species with long flowering range and mixed

(anemochory/anthropochory) dispersal mode to endozoochore geophyte and phanerophyte (shrubs) species presenting a short flowering range.

RLQ analysis (relationship species traits – environmental variables) – The first two axes of the RLQ analysis explained 70.01% and 12.87% of the total inertia of the plant traits – environmental variables relationship and for this reason we further took into consideration these two axes, as they accounted for 82.88% of the variance in the analysis. Compared to the separate analysis of the sites x environmental variables (R table) the RLQ analysis (three tables ordination) accounted for 95.04 for the first axis and 88.69% for the first two axes of the separate analysis. Also, compared to the separate analysis of the species x traits (Q table) and sites x species (L table) the RLQ analysis accounted for 39.14 (the first axis) and 54.73% for the first two axes of the separate analysis (Fig. 3.). The climatic variables (continentality and temperature) showed negative correlations with the first RLQ axis while soil properties (represented by moisture and nutrients content) were positively correlated with the same axis. The second axis was more strongly positively correlated to light and altitude (Fig. 3). Thus, a soil moisture-soil nutrients gradient along RLQ axis one showed a transition from more dry conditions, with less available nutrients to more moist and higher nutrients availability sites, and, also, a climate continentality-temperature gradient along the same axis showed a transition from the sites characterized by a more continental climate, exposed to higher temperatures to sites with species preferring lower values of temperatures and climate's continentality. In this way, the plant traits aggregated at the negative end of the first RLQ axis separated the geophyte and chamaephyte species with a short flowering range, able to self disperse (autochory) or unassisted dispersal (barochory) from the therophyte species, characterized by a long flowering range and anthropochory (e.g. separating *Chrysopogon gryllus*, *Iris aphylla*, *Muscari comosum*, *Muscari tenuiflorum*, *Elymus hispidus* from *Trifolium arvensis*, *Anagallis arvensis*, *Acinos arvensis*, *Arenaria serpyllifolia*, *Centaurium erythraea*, *Sideritis montana*) and, also, from the few shrub species with a medium flowering range and mixed dispersal mode (*Rosa gallica*, *Rosa canina*, *Prunus spinosa*). Another transition along the first axis is that from grasses to forbs and legumes (*Stipa capillata*, *Stipa lessingiana*, *Stipa tirsa*, *Ajuga laxmannii*, *Adonis vernalis*, *Allium rotundum*, *Crambe tataria* to *Cytisus austriacus*, *Medicago falcata*, *Trifolium pratense*, *Trifolium campestre*, *Trifolium repens*). This could be interpreted as a transition from perennial species with short flowering range and traits oriented to resources retention in storage organs to annual species with long flowering range and traits oriented to resource acquisition.

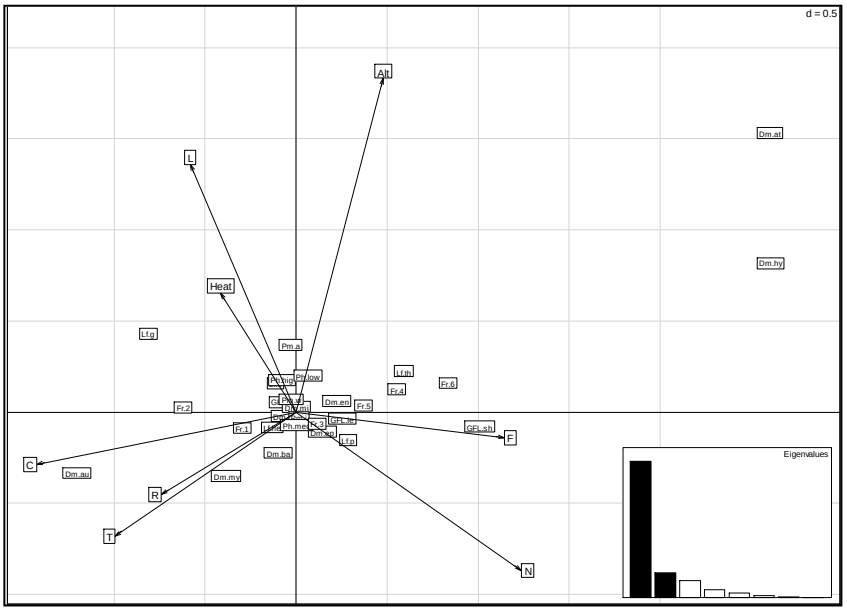

Fig. 3. The RLQ-analysis (relationship plant traits - environmental variables): Eigenvalues: Axis 1: 0.299, Axis 2: 0.055. Variance explained (%): Axis 1: 70.01, Axis 2: 12.87. Covariance: Axis 1: 0.546, Axis 2: 0.234. **Inertia & coinertia R:** Table R (separate ordination) - Axis 1: 3.584, Axis 1+2: 4.438; Table R (RLQ) - Axis 1: 3.771, Axis 1+2: 5.003; % RLQ - Axis 1: 95.04, Axis 1+2: 88.69. **Inertia & coinertia Q:** Table Q (separate ordination) - Axis 1: 1.192, Axis 1+2: 2.799; Table Q (RLQ) - Axis 1: 3.047, Axis 1+2: 5.114; % RLQ - Axis 1: 39.14, Axis 1+2: 54.73. **Correlation L:** Table L (separate ordination) - Axis 1: 0.264; RLQ - 0.409.

Plant functional groups

The k-means clustering algorithm generated six groups according to the position of species along the first RLQ axes (according to average *silhouette* width and *Calinski-Harabasz* criteria) based on their particular traits and environmental variables. The groups are mainly displayed along gradients of soil moisture and nutrients.

The first group comprised a mix of low and some taller species, predominantly grasses and few forbs (16 species, with no legume species in this cluster), most of them geophytes, pollinated by wind, having an short (only two months) flowering range with mixed (anemochory/anthropochory) dispersal mode and reproduction type (seeds/vegetative); *Arrhenatherum elatius*, *Chrysopogon gryllus*, *Asparagus officinalis*, *Dactylis glomerata*, *Elymus hispidus* etc. were some of the component species of the group.

The second group included preponderantly hemicryptophyte forbs and (a lower proportion of) grasses (but no legumes) with short flowering range (two months), medium to low height and mixed dispersal, pollination and reproduction types. Among the 36 species within this group there were: *Artemisia austriaca*, *Bromus squarrosus*, *Carthamus lanatus*, *Cerinthe minor*, *Phlomis tuberosa*, *Stipa tirsa*, *Teucrium chamaedrys* etc.

The third group contained predominantly low and medium height perrenial forbs and legumes, characterized by a mixed (anemochory/anthropochory) dispersal mode and a

three-four months flowering range, most of them pollinated by insects. Among the 75 species within this group there were: *Achillea setacea*, *Anthemis tinctoria*, *Astragalus onobrychis*, *Ferulago campestris*, *Hypericum perforatum*, *Medicago falcata*, *Silene otites*, *Trifolium montanum*, *Veronica spicata* etc.

Tab. 1. Characteristics of the five clusters (groups) resulted from k-means clustering of species scores in the RLQ analysis

	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**
No. of species	16	36	75	63	15	3
EIV L	7.80±1.20	8.20±1.14	7.59±1.05	7.76±1.16	7.60±1.80	7.50±0.55
EIV T	6.58±1.16	6.86±1.19	6.24±1.17	6.61±1.01	6.16±0.83	5.75±0.75
EIVC	5.07±0.70	6.02±1.16	5.55±1.21	5.90±1.04	5.07±0.75	5.25±0.50
EIV U	3.84±1.21	3.00±1.13	3.57±1.19	3.36±1.08	4.07±1.20	4.33±0.57
EIV R	5.75±1.48	6.68±1.71	7.00±1.82	6.80±1.38	6.00±1.30	7.50±0.50
EIV N	3.30±2.28	3.56±1.45	3.40±1.76	3.15±1.54	3.63±1.96	5.50±2.25
Functional category	gr (68.7%); fo(31.3%)	gr(33.3%); le (66.7%)	fo (65.5%); le (24.3%)	fo(98.5%)	fo (93.3%)	sh (100%)
Life form type	g (75%)	t (27.7%); h (52.7%)	h (65.5%)	h (79.4%)	t (86.6%)	ph (100%)
Reproduction type	m (87.5%)	s (41.6%); m (58.4%)	s (50%); m (50%)	s (73%); m (27%)	s (93.3%)	m (100%)
Dispersal mode	mi (75%)	an (25%); mi (63.9%)	mi (66.6%)	an (22.2%); mi (47.6%)	mi (73.3%)	en (100%)
Pollination mode	w (62.5%)	w (33.3%); m (44.4%)	i (57.7%); m (24.3%)	i (57%); m (43%)	i (33.3%); m (66.7%)	i (100%)
Plant height category	low (43.7%); high (56.3%)	low (25%); med (54.7%)	low (59%); med (21.8%)	med (93.6%)	low (80%)	med (25%) high (75%)
Flowering range	2 (62.5%); 3 (37.5%)	2 (88.8%)	3 (46.2%); 4 (34.7%)	2 (31.7%); 3 (68.3%)	4 (60%); 5 (20%)	2 (100%)

In the fourth group there were mostly hemicrytophyte forbs (63 species, but no grass species included) as *Adonis vernalis*, *Aster amellus*, *Campanula sibirica*, *Dianthus membranaceus*, *Echium maculatum*, *Falcaria vulgaris*, *Inula germanica*, *Oxytropis pilosa*, *Salvia nemorosa*, *Verbascum blattaria* etc. Most of the component species were pollinated by insects, of medium height and medium flowering range (three months). The predominant reproduction type was by seeds and the predominant dispersal mode was the mixed one (but a significant proportion was represented by anthropochore species).

Almost all species of the fifth group (15 species) were low height therophyte forbs (no grass species included), reproducing by seeds, with a long flowering range, mixed (anemochory/anthropochory) dispersal mode and mixed pollination mode (wind/insects). As representatives for this group there were: *Anagallis arvensis*, *Centaurium erythraea*, *Sideritis montana*, *Trifolium campestre*, *Viola arvensis* etc.

The last group comprised the shrubs species (as *Rosa gallica*, *Rosa canina*, *Prunus spinosa*) with short flowering range (two months), medium to increased height and endozoochory as dispersal mode, pollinated by insects and mixed reproduction type.

The floristic composition of the six groups generally suggest a shift from low to medium height geophyte species (not necessarily *Liliaceae*) with short flowering range, pollinated by wind adapted to grow in soils with water and nutrients deficit (short time of optimum conditions for plant development) to: a) low height therophyte with long

flowering range, reproduction by seeds and mixed dispersal and pollination modes (anthropic influence) and b) to increased height phanerophytes (shrubs) pollinated by insects and endozoochore dispersal mode developed on soils with higher availability of water and nutrients. From another perspective the increase of the mean height of species in grasslands with N availability was observed and highlighted in other studies [SCHELLBERG & PONTES, 2012].

As a final remark, in the context of this particular vegetation type (*Festucetalia valesiacae*) and this particular region (North-Eastern Romania), plant communities developed in areas characterized by higher climate continentality, higher values of temperatures and soil reaction and lower values for soil moisture and nutrients will possibly be richer in autochore and barochore geophyte and hemicryptophyte species (grasses and forbs) with short flowering range and mixed (seeds and vegetative) reproduction type. As soil moisture and soil nutrients increase, in the floristic composition could possibly occur more legume species and, in the richest in nutrients areas the floristic composition could possibly be infiltrated one by more anthropochore and zoochore therophyte species with long flowering range, mainly reproducing by seeds and, second, by some taller endozoochore shrubs species occur with short flowering range also reproducing by seeds.

Conclusion

Six main plant functional groups in which species with similar ecological requirements and life-history traits co-exist were identified in the dry grasslands (*Festucetalia valesiacae*) from North-Eastern Romania via RLQ analysis and k-means clustering. The groups are mainly displayed along gradients of soil moisture and nutrients. Their floristic composition generally suggest a shift from low to medium height geophyte species with short flowering range, pollinated by wind adapted to grow in soils with water and nutrients deficit to low height therophyte with long flowering range, reproduction by seeds and mixed dispersal and pollination modes and to increased height phanerophytes (shrubs) pollinated by insects and endozoochore dispersal mode developed on soils with higher availability of water and nutrients. The groups' species composition suggests transition from perennial species with short flowering range and traits oriented to resources retention in underground organs to annual species with long flowering range and traits oriented to resource acquisition.

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PLANT BIOPRINTING: NOVEL PERSPECTIVE FOR PLANT BIOTECHNOLOGY

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Abstract: Bioprinting is a technical innovation that has revolutionized tissue engineering. Using conventional printer cartridges filled with cells as well as a suitable scaffold, major advances have been made in the biomedical field, and it is now possible to print skin, bones, blood vessels, and even organs. Unlike animal systems, the application of bioprinting in simple plant tissue cells is still in a nascent phase and has yet to be studied. One major advantage of plants is that all living parts are reprogrammable in the form of totipotent cells. Plant bioprinting may improve scientists'understanding of plant shape and morphogenesis, and could serve for the mass production of desired tissues or plants, or even the production of plant-based biomaterial for industrial uses. This perspectives paper explores these possibilities using knowledge on what is known about bioprinting in other biosystems.

Keywords: biomaterial, bioprinting, plant biotechnology, micropropagation, tissue engineering

Introduction: historical framework and basic bioprinting concepts

The concept of bioprinting emerged in the early 2000s. The patent for bioprinting using a common inkjet printer was filed in the US in 2003 and granted in 2006 to Dr. Thomas Boland at Clemson University [DOYLE, 2014]. Since then, studies in the fields of engineering, material science, cell biology, and regenerative medicine have assessed the impact of bioprinting, with the greatest impact being on biomedical science.

The earliest bioprinter used protein and endothelial cells placed in an inkjet cartridge for 2D printing. To create protein, four cartridges consisting of biotin, streptavidin, biotinylated bovine serum albumin (BSA) or biotin-BSA, and only BSA were used to create a pattern in the shape of the word "Biotin" in Times New Roman font size 8. Using the same biopritnter, trypsinized bovine aortal endothelial cells and smooth muscle cells (i.e., cells that had dettached from each other after bonding proteins broke)were suspended in modified Eagle's [EAGLE, 1955] medium (MEM) and 10% fetal bovine serum with a cell concentration of 1×10^5 cells/ml. The cells were printed in a reconstituted basement membrane gel with 3 mg/ml collagen gel. After printing, the resulting single layer of cells was incubated at 37 °C in a CO_2 environment for 30 min to maintain pH before adding liquid medium. Cells were visualized after 72 hunder an epifluorescent microscope revealing that 75% of the mass of printed cells survived [WILSON JR. & BOLAND, 2003]. Separately, 3D printing technology allows the creation of a 3D biological shape by using cells and a scaffold of desirable shape. This technology covers the limitation of 2D printing that is useful only for a surface area instead of a 3D solid object. In the field of 3D

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bioprinting, the bovine aortal endothelial cells is used for 3D bioprinting [BOLAND & al. 2003] to form cell aggregates in layered thin gel alignments.

3D bioprinting in animal cells using an arranged aggregation principle (i.e., the organized alignment of cells, like pixels, in an orderly fashion, similar to printed letters) as a key protocol for tissue engineering and organogenesis. In biomedics, bioprinting can be used for skin grafting by applying skin tissues [LEE & al. 2013] thus playing a significant role in plastic surgery or wound healing. Organ transplants may be possible by 3D bioprinting that woud allow for the growth of a fully functional and fully developed organ [MIRONOV & al. 2011]. Other uses of 3D printing might be in the production of meat *in vitro* from stem cell tissue and lab-grown *in-vitro* meat may contain designated target nutrients and adjustable shape for aesthetic purposes [POST, 2012; MATTICK & ALLENBY, 2013].

Other than bioprinting involving animal cells and tissues, the application of bioprinting to other complex multicellular organisms, especially plants, has not yet been tested or studied. Unlike its direct function in biomedical sciences, plants might be considered less interesting as a bioprinting subject, also because tissue culture and micropropagation already provide a suitable and robust system for producing plant cells, tissues or organs in a sterile *in vitro* environment. Plant bioprinting may be difficult due to rigid plant cell walls, unlike animal cells that do not have a cell wall, although plant cells have a distinct advantage, totipotency, which allows a plant cell, under strict environmental conditions, to develop a tissue scaffold that serves as the precursor for an organ, and then the whole plant itself, organogenic steps that are under strict genetic control. A second possible problem might be the efficiency of cell and tissue regeneration once a scaffold has been printed. In principle, bioprinting would be required to shape plant cells into a scaffold, which would serve as a building block for engineering plant tissues for partial organogenesis to produce specific products rather than a whole plant. Where necessary, the printer cartridge could overlay different building blocks of different cellular origins onto media or substrates containing different inducers such as plant growth regulators (PGRs). This concept is explored in a bit more detail later.

This paper is the first ever proposal for the theoretic possibility of using 2D and 3D bioptinting in plants by relying on earlier successful cases of animal bioprinting and on a rich literature of basic concepts of plant cell, tissue and organ culture.

Plant bioprinting: basic requirements

1. Cells and tissues for *in vitro* culture

A plant cell or tissue can be made to survive, grow and develop artificially *in vitro* when placed on a suitable medium that contains macro- and micronutrients, carbohydrates, vitamins, and PGRs. The most commonly used basal medium is Murashige and Skoog (MS) [MURASHIGE & SKOOG, 1962]. To obtain plant cell aggregates that could form multiple cell clusters and eventually a tissue, cell suspension cultures in liquid media might serve as the optimal printing form when placed in the printer cartridge rather than the use of "dry" cells, which can die easily due to oxidation.

2. Growth scaffold as a template for shaping the product: importance of basal medium, medium additives and plant growth regulators

A scaffold is essential in organic tissue printing as a base to direct tissue growth. Gel material for plant tissue can be calcium alginate, agar, agarose, polyacrylamide, gelatin, or even synthetic material like polyurethane [NEUMANN & al. 2009]. Other cheaper soft material like starch from various source like sago, cassava, and corn can also be used

[HENDERSON & KINNERSLEY, 1998; NAIK & SARKAR, 2001; DABAI & MUHAMMAD, 2005; PUROHIT & al. 2011]. In plant bioprinting, a scaffold would be used as a mold to shape cells so that they will be aligned prior to differentiation using induction by PGRs.

The proposed printing process can take place in two ways: 2D printing or 3D printing. For 2D printing or monolayer printing, cells are simply directed to spread over a 2D area of nutrient gel scaffold before they are left to grow when placed under optimized conditions (Fig. 1). On the other hand for multilayer or 3D printing, a gel is added gradually to adjust the surface before placing another layer of cells or to enclose the surface. In some cases, a gel may also contain a specific concentration of PGRs to match the desirable level that would result in a product (tissue (e.g., parenchyma, sclerenchyma, etc.) or organ (leaf, stem, petiole, stigma, tuber, bulb, etc.). Thus, more than one gel cartridge may be possible, or necessary. 3D or multilayer printing is expected to be followed by directed organogenesis and differentiation in response to PGRs or other optimal conditions (Fig. 2), either optimized *a priori* using assays, or following the published literature. The difference between traditional plant tissue culture (PTC) and the use of a bioprinter will lie in the automation and the precision associated with it.

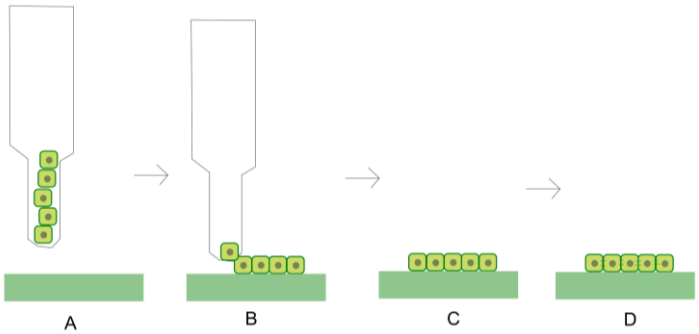


Fig. 1. Monolayer plant cell printing (i.e., 2D) using a simple inkjet printer. From the cartridge nozzle (A), cells are placed over a basal medium (B-C). Some time after, the cells interact and begin a process of differentiation or dedifferentiation (D). Even with a small surface area, bioprinting a layer of cells would take no more than a few seconds to achieve, similar to a regular printer that prints on paper.

When cells are printed, a bioprinter will align each cell to form a layer of equal size forming the desired scaffold. When a 2D printer is used, the printing result will only be a single thin layer of cells of variable sizes depending on the size and capacity of the bioprinter. On the other hand, a 3D printer will add more layers of cells joined by a gelling agent to a desirable height that is limited to a printer's maximum height capacity. A larger printer would thus be able to print alarger scaffold (3D) or wider base (2D). A printed cell or layer of cells (2D horizontal scaffold; Fig. 3A) or cellular mass (3D scaffold; Fig. 3B) may be placed on a basal medium carrying a gradient of PGRs or any other nutrient. The concept of a gradient is not usual in conventional PTC, and is made by using tilted agar medium (Fig. 3A) to make two different concentration gradients that would theoreticallyaffect the growth of the cell layer overlaying it by diffusion. In a 3D gradient (Fig. 3B), the precision of a computer that guides the printing process is important and a step that is impossible to achieve at present in conventional PTC. This gradient medium

serves as an attempt to try and direct the growth of cells that have been printed. The basal medium can either be printed, or is not printed, i.e., it is set *a priori*, e.g., in Petri dishes.

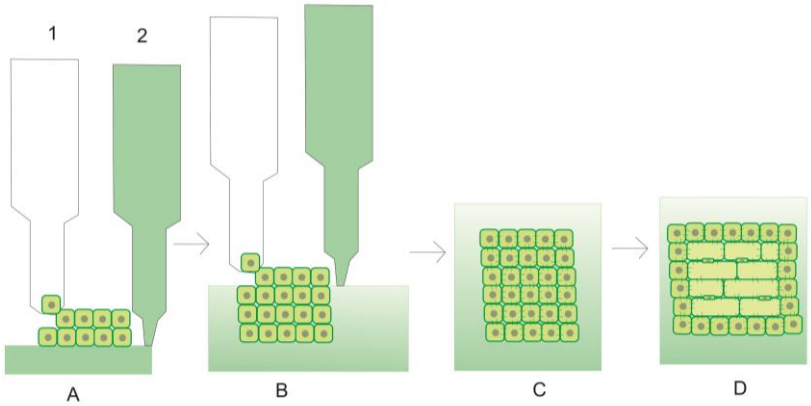
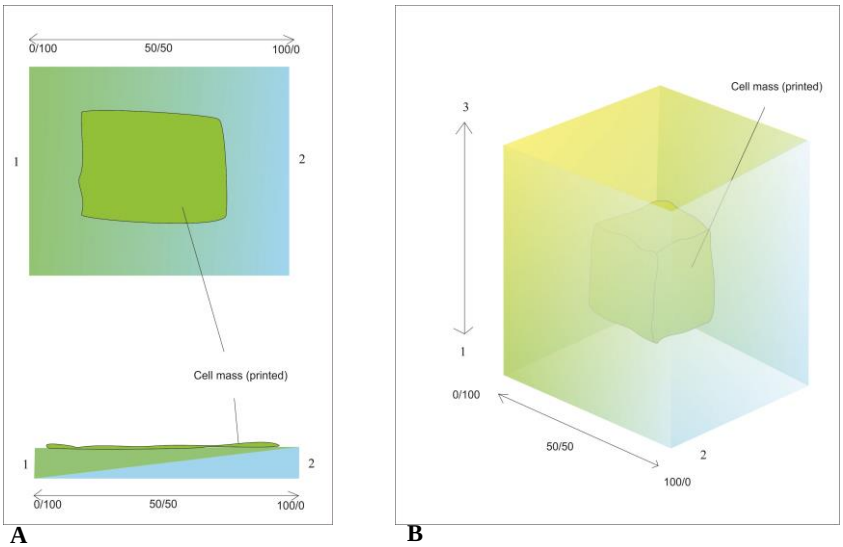


Fig. 2. Multilayer (3D) plant cell printing in which the speed and other specifications can be adjusted. Other than the cell-containing cartridge (1), a gel scaffold with plant growth regulators (PGRs) is placed in another cartridge (2). After one layer is printed (A), the gel is adjusted to add a layer of different cells or a different scaffold (B) to follow the surface of printed cells. Once printing is complete (i.e., resulting in a desired pattern, scaffold or basal structure (C), the interaction between cells, nutrients and PGRs in a basal medium induces the differentiation of cells to form an undifferentiated cellular mass(i.e., callus)or a differentiated mass(i.e., tissue or organ like a leaf or bulb) with a distinct epidermis (D).Depending on the complexity and on the number of different cell types and scaffolds used, which would require different cartridges to be inserted, printing could last from between minutes to a few hours.



A **B**

Fig. 3. Gradient in 2D printed cells (A) showing the ratio between PGR1 and 2.Gradient in 3D printed cells (B). Instead of only two PGRs (1 and 2), the third one (3) can also be added. The intention is to manipulate cellular differentiation for later stages of growth so that a plant product with a desirable shape can be created.

Plant bioprinting: potential applications and perspectives

Bioprinting plant material has foreseeable benefits for ornamental and agricultural purposes, and for biomaterial production (Fig. 4). The main concept in bioprinting using plant cells is to arrange cells into a suitable scaffold of a specified area (2D) or volume (3D)that will allow them to develop, in response to an ideal basal medium and additives, including PGRs, directly into a specific organ (i.e., direct organogenesis).By being able to bioprint a living structure of living cells with desirable shape, the most obvious manipulation that could be envisioned is in the improvement of aesthetic in ornamental plants such as bonsai or *in vitro* flowers (Fig. 4A).

It is also conceivable that plant tissue can be printed as a base for the production of plant-based biomaterial, e.g., lab-grown wood planks or wood blocks for construction (Fig. 4B), decreasing deforestation and creating bioprinted blocks of wood of rare and valuable wood such as sandalwood. Such wood blocks could be printed by tabletop-sized or larger printers.

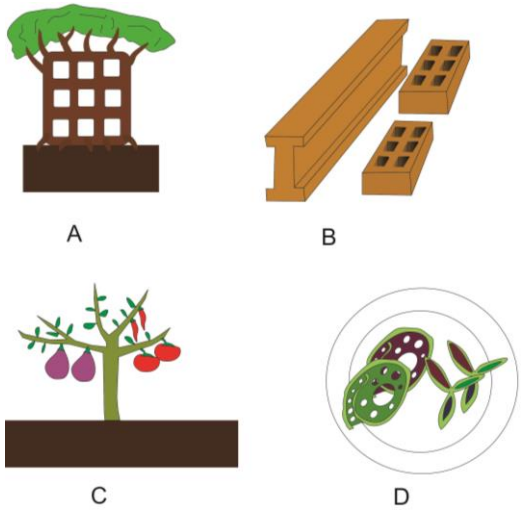


Fig. 4. Applications of plant bioprinting for ornamental plants (A), printed plant-based biomaterial (B), chimeric grafting for horticultural plants (C), and printed plant-based food (D).

A handheld bioprinter could be used for *in-vivo* bioprinting of *in vitro* tissues or onto plants growing under a sterile and controlled environment. For example, a small graft (as a single layer using a 2D printer, or a mass of cells or tissues using a 3D printer) could be printed onto the part of a plant that was damaged by an abiotic stress (e.g., cold- or heat-induced injury) or by a biotic stress (e.g., a fungus or pest) allowing for recovery of dead tissue or covering and strengthening scarred tissue (Fig. 5). As result, chimeric plants that yield multiple fruits can be created (Fig. 4C).

A fourth possibility is the use of plant bioprinting to produce designer plant-based food that combines aesthetics, nutraceuticals, and productional aspects, e.g., lab-grown vegetable products, a procedure equivalent to lab-grown meat. This 3D method would create a desirable and edible plant-based product that can be specific (e.g., only a leaf, root or fruit; Fig. 4D) or a whole printed plant. The procedure would also apply to transgenic material. Such a bioprinter would allow individuals to manufacture their own fruits or

vegetables at home although, relative to presently used forms of producing fresh produce in mass, the costs of producing a single item would likely be prohibitive. However, as for most technologies, costs tend to become lower over time. Initially, while the prototype is small, the concept would take the form of a table-top printer, using plant suspension cells in liquid medium within cartridges. As the system develops into a robotized format, the printer interface would allow the user to define the desired cell or tissue to be printed, with the desired shape, nutrients, or colours (Fig. 6). Such a concept would benefit tissue engineering science and PTC. In addition, there could be untold benefits of plant bioprinting for the production chain and mass production of rare or valuable plant material. The production in space of plant products rich in nutrients and with reduced volume through plant bioprinting would be suitable where space may be limited, where delivery of renewable resources may be difficult, or impossible, and thus where plant-based resources would be needed to be printed as needed.

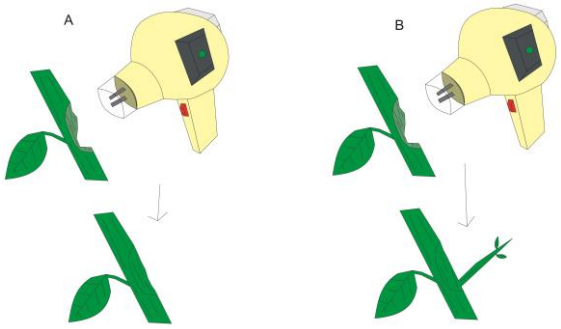


Fig. 5. Handheld plant bioprinter with two applications: (A) healing plant scars and damaged tissue; (B) grafting.

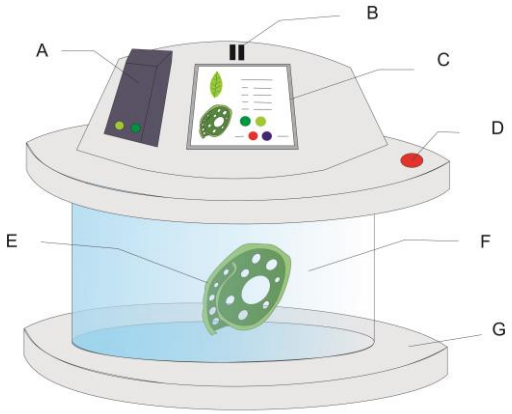


Fig. 6. Future concepts of plant bioprinters for plant-based food. The device consists of a cellular replaceable cartridge (A), USB drive slots (B), a touchscreen display panel (C), a power switch (D), the product to be printed (user interface selection) (E), a print chamber (F), and a device pad that can serve for axillary functions such as medium (scaffold) sterilization, temperature regulation, etc. (G).

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PROFESSOR CONSTANTIN TOMA AT HIS 80TH ANNIVERSARY

Marking this memorable anniversary is proven to be a very honorable mission but also a very difficult one.

As disciples of the Iaşi Botany's Magister we are grateful that we had the privilege to be influenced by the fascinating personality of the one who will always remain the **PROFESSOR**. His special mentor endowment marked the student years and the further professional development of many generations that were polished by the clear and precise voice that shined the botany's unknown, by the piercing and determined look, by the distinct attitude always based on the certainty of science. Respected, admired, maybe feared – **Professor Constantin Toma, PhD**. was always and everywhere an imposing figure: in front of the students, in the laboratory, on the filed or in the Academy's halls.

The respect for his forerunners, for their work and results was a desideratum that marked the professor's entire professional activity proof of that being his efforts to enforce and consolidate **Plant Morphology and Anatomy School of Iasi**. Far from seeking peace at this venerable age, he uses his knowledge, sharpness of mind and energy to support and coordinate the activity of his followers.

His prodigious activity as born researcher was often evoked with different occasions in which the various areas covered by the Professor were mentioned: histology and compared anatomy, experimental and teratological anatomy, ecological anatomy, blastology, *in vitro* cultures, cytogenetics.

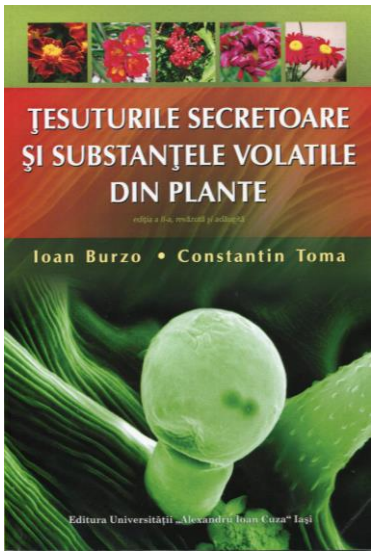
Probably the highest recognition of his scientific merits and not only came in 2012 with his election as full Member of the Romanian Academy. This prestigious distinction crowned the activity of a lifetime rewarded by several orders, medals, diplomas, "Doctor Honoris causa" titles, "Emanoil Teodorescu" prizes, Corresponding Member of the Romanian Academy.

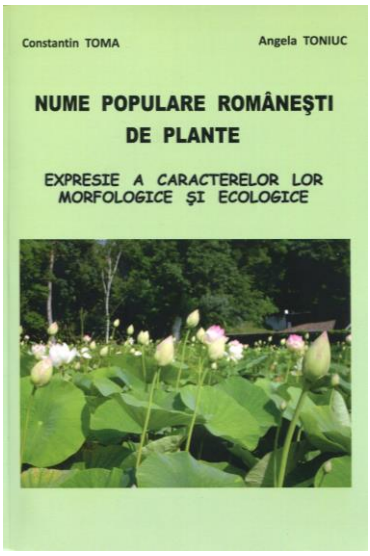
The tirelessly work of the distinct professor recently materialized in some bibliographical titles that prove once again the diversity of the approached scientific areas: ***Țesuturile secretoare şi substanțele volatile din plante / Secretory tissues and volatile substances in plants*** (published together with Professor Ioan Burzo in 2012, first edition and in 2013, second edition), ***Nume populare româneşti de plante – expresie a caracterelor lor morfologice şi ecologice / Common names of Romanian Plants – expression of their morphological and ecological characteristics*** (published together with botanist Angela Toniuc in 2014), ***Biologi de altădată şi de azi. Portrete. Amintiri. Viață ştiințifică / Biologists of yesterday and today. Portraits. Memories. Scientific life*** (published in 2015).

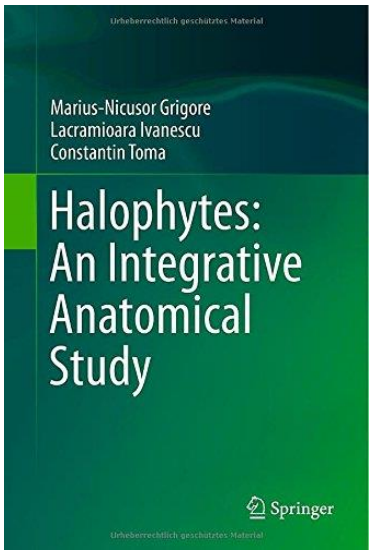
Halophytes: An Integrative Anatomical Study (published together with Marius-Nicuşor Grigore, PhD and Associate Professor Lăcrămioara Ivănescu in 2014) is a book of great value emphasized by its publication by the prestigious publishing house Springer.

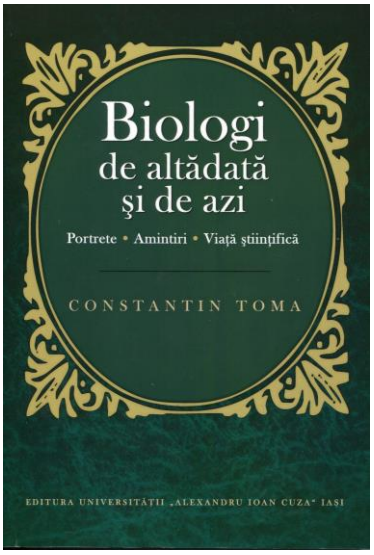
The richness and depth of a life dedicated to scientific truth are hard to be comprised in just a few words; the only thing remaining is to wish always having the living example of the one to whom everybody wishes **Happy Anniversary PROFESSOR!**

Camelia IFRIM, Lidia ADUMITRESEI
"Anastasie Fătu" Botanical Garden, "Alexandru Ioan Cuza" University of Iaşi



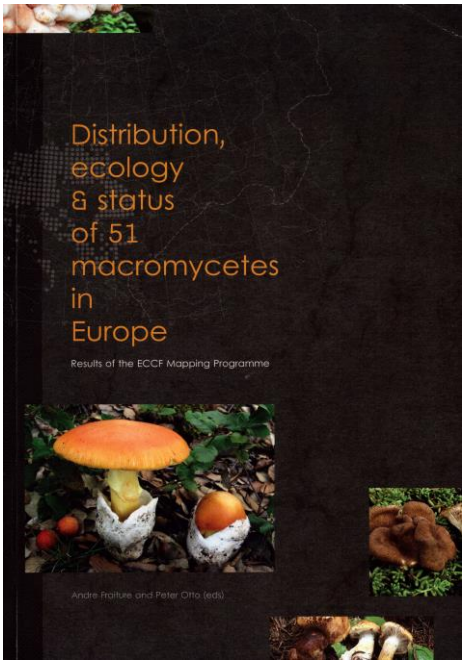






BOOK REVIEW

ANDRÉ FRAITURE, PETER OTTO (eds.), ***Distribution, ecology & status of 51 macromycetes in Europe***. *Results of the ECCF Mapping Programme.* Meise, Botanic Garden Meise, Belgium, 2015, 247 p., 72 figures, 67 photo, ISBN 9789082352559, 25 x 17 cm.



The authors (editors), André Fraiture and Peter Otto, are both mycologists with interest in different Mycology branches, such as systematics, ecology, distribution, chorology, phenology, mycocoenology. Their results and published works recommend them as reputable researchers. André Fraiture is researcher at the Botanic Garden Meise, Belgium, involved in many projects related to mycological subject and member of different mycological societies. Peter Otto is research assistant at Faculty of Life Sciences, Pharmacy and Psychology, University of Leipzig, Germany and also the curator of the Faculty's Herbarium.

The presnt volume presents the distribution, ecology and status of 51 macromycete species, assessed in a framework of the European Council for the Conservation of Fungi (ECCF), entity that has been created at the 9th Congress of European Mycologists. During the discussions at the first meeting of ECCF (Poland, 1988), has been stated the idea of a program for mapping of particular species of macromycetes considered threatened, species selected based on criteria that are being presented in this work.

Out of the total number of countries that have their territory completely or partially on the European continent, are missing 4 small states (Vatican, San Marino, Monaco and Andorra) and other 5 states (Belarus, Bosnia-Herzegovina, Albania, Moldova and Kazakhstan). For Russia and Turkey, only the European part of these countries were considered for this program.

In the first part of this volume, the authors presents the methods for collecting, processing the data, the main phyto-geographical regions in Europe and aspects related to the interpretation and variability of data correlated with the country. Also, discussions with examples are being made about the distribution models of some species, influenced by their ecology, distribution that can be wide, can follow the distribution of the characteristic host/substrate or can be limited by the geographical conditions to certain area. The updated maps are compared to maps older than 40 years (Lange, 1974) and the observed changes are being commented.

At the end of the first part, a list with the main collaborators and the coordinators responsible for collecting the data for each country. The work compiles data supplied by over 300 mycologists, collected by a coordinated for each of the 38 contributing country.

The second part of the volume contains distribution maps of the 51 species of macromycetes, specifying the data collected before 1970 or after that year, along with a brief description of the sporophor, commented aspects related to the distribution in world and in Europe, ecology (nutrition type, hosts and substrata, characteristic vegetation, type of soil, phenology) and the status (frequency, threats and conservation) and photographs for each species. For objective reasons based on the limited availability of data, the authors did not classified the 51 species according to IUCN criteria, the present volume being an intermediate phase for achieving a Red List of Europe.

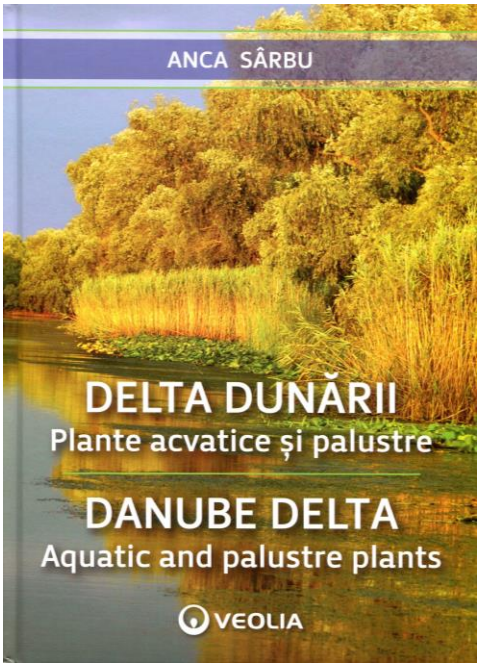
The volume is documented with a high number of published works, being accompanied at the end with a list of almost 600 titles.

We consider this volume as being very valuable through the complex that it offers and very useful to all of those who have interests in the field of mycology as well as for those who are activating in the field of environmental protection and biodiversity conservation, especially for the conservation of mycological diversity!

Cătălin TĂNASE, Tiberius BALAEŞ
"Anastasie Fătu" Botanical Garden, "Alexandru Ioan Cuza" University of Iaşi

BOOK REVIEW

ANCA SÂRBU, ***DELTA DUNĂRII. Plante acvatice şi palustre*** [***DANUBE DELTA. Aquatic and palustre plants***], 2015, CERES Publishing House, Bucharest, 304 pages, ISBN 978-973-40-1100-1.



The new book, DELTA DUNĂRII. Plante acvatice şi palustre / *Danube Delta. Aquatic and palustre plants*/, conducted by Professor dr. ANCA SÂRBU, from the Faculty of Biology, University of Bucharest, is an excellent scientific and cultural event, welcomed particularly in the botanic literature in Romania and elsewhere. Mrs. Anca Sârbu is a well-known romanian botanist, publishing many botanical papers, both in romanian journals and abroad.

This book was born out of love for nature and especially for the plant world, coupled with her passion for photography. Thus, resulted a very good book, in which the color images occupies the largest part.

By its content and form, this book addresses both specialists and the general public lover of nature, representing an invitation and a useful guide to understanding the fascinating worlds, both terrestrial and aquatic or palustre levels. This delta of Danube river is a biosphere reserve, a Ramsar site, and is included in Natura 2000 network of protected areas in European Union, as SCI's and SPA's. The Danube Delta has also been included in the UNESCO World Heritages, since 1990.

The first part of the book give brief, but enough information for: location of the Danube Delta in Romania, its origin, physical and geographical data, climate, flora and vegetation, natural habitats, as well as an aquatic and palustre plants classification. Two natural habitats, namely 3150 Natural eutrophic lakes with *Magnopotamion* or *Hydrocharition*-type vegetation, and 3260 Watercourses of plain to montane levels with the *Ranunculion fluitantis* and *Callitricho-Batrachion* vegetation, are fully described in this chapter, highlighting their main features and the characteristic plant species inside this great river delta of Europe. A good idea was to introduce people some of the most offensive alien plant species into this area, as *Elodea nuttallii*, *Paspalum distichum*, or *Eclipta prostrata*., together with their mechanisms of multiplying and spreading.

At the end of the first part is inserted a table with the taxa included in this book, along with their family and their preferences against the water, as a main environmental factor.

The second part of this book, representing over 80% of the pages, contain short descriptions of those 128 ferns and flower plants. Each species is accompanied by one or more color images, all original, with detailed characteristics, their natural habitats, other accompanying species etc. All the plant species are displayed in alphabetical order, to ease the search for those who are less familiar with botanical classifications.

There are described and photographically shown a number of 128 of aquatic and palustre plant species, found on river banks and wetlands; these vascular plants belonging to ferns and to the flowering plants, some of them being common, some rare, in the flora of Romania, belonging to ferns (5 species) and angiosperm plant families (the others 123 plant species).

Each plant species is accompanied by a brief description of morphology, useful to recognize more easily a plant on the field, one or more color, high resolution photographs, which enhances the charm of this guide into the water plant world.

The second part concludes with an index of the scientific and vernacular names of all the taxa included in this book, both in Romanian language and translated into English language, and each page where they are to be found inside the book.

There are inserted many full plates inside the book, with beautifull pictures from the Danube Delta, with yellow and white lily waters, willows, birds, channels, amazing images of sunsets or sunrises etc.

The bibliography includes a total of 30 references, selected and used strictly for the purposes intended.

The text of this book is written in two columns per each page, one in Romanian language, the other in English language.

Achieving this book is the fruit of many field trips, starting with spring until late fall, during which the author accumulated an impressive amount of data, from which she elected this series of images, that delight the readers, urges to excursions, induce love and respect for nature itself.

We congratulate the author for this instructive, delightful, and relaxing book, waiting for fulfiling other books of this kind.

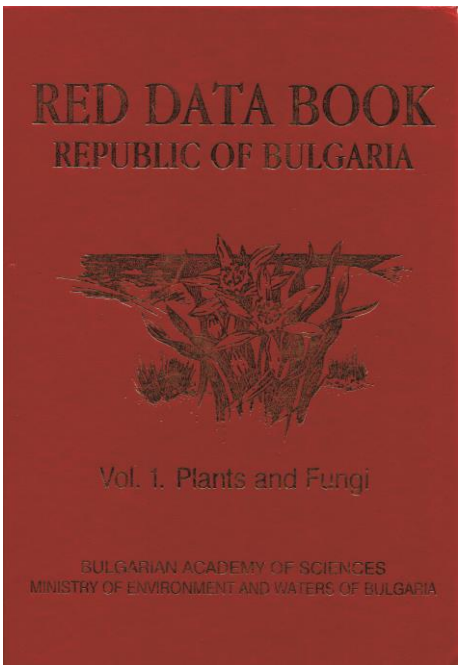
Ion SÂRBU, Adrian OPREA
"Anastasie Fătu" Botanical Garden, "Alexandru Ioan Cuza" University of Iaşi

BOOK REVIEW

DIMITAR PEEV et al. (Eds.), ***Red Data Book of the Republic of Bulgaria*. Volume I. *Plants and Fungi***, 2015, BAS & MoEW, Sofia, 881 p., 242 photos, 60 maps, and 104 references.

A very valuable book was published at Sofia, under the auspices of the Bulgarian Academy of Science and Ministry of Environment and Waters of Bulgaria, in 2015, namely *Red Data Book of the Republic of Bulgaria*. Volume I. *Plants and Fungi*, as an output of a national scientific team.

The book has been published in very good technical conditions, with some color, detailed photographs, and many color ink drawings, accompanied by references for every described plant species inside, on A4 size pages.



In the first part of the book, the authors present an introduction to the Red Data Book of the Republic of Bulgaria, with interesting historical data on the attempts of the Bulgarian specialists in preparation of the previous red books on flora and fauna of Bulgaria. In *Introduction* to volume 1 – *Plants and Fungi*, there are highlighted aspects on flora of Bulgaria, where, 20.5% of the vascular flora is threatened with extinction under various pressures, as trampling, grazing, infrastructure development and stone-pitting, alien plants etc.

Important thing is that, for the first time in Bulgarian literature on flora, this up-to-date book includes algae, bryophytes and fungi, besides the vascular plants. The authors make themselves a summary of this book. Thus, it includes "...all extinct, regionally

extinct, critically endangered and endangered species of plants and fungi...". More, ca. 20% from the vulnerable species have been included in the current red book.

The book is divided in four large chapters, namely: Algae (pages 29-36), Bryophytes (pages 37-141), Vascular Plants (pages 143-720), and Fungi (pages 721-874). Each of the four large sections is ended with a pretty comprehensive list of references. All the species are displayed by their category of threats, within each taxonomic group, as: Extinct (EX), regionally extinct (RE), critically endangered (CR), endangered (EN), the last one being those vulnerable (VU). Altogether, this book includes descriptions for 808 species.

For each species, the author(s) compiled the next data: latin name, followed by the authors(s); synonyms (in some cases); family or division (for algae) (both in latin and as vernacular names); illustrations; conservation status in Bulgaria; essential data on morphology and biology of each species; habitats and populations; distribution on Bulgarian territory, given in floristic regions and subregions, followed by a geographical map of Bulgaria, divided in grids of 10 x 10 km UTM squares (each taxa is located by red/black points on the grids); general distribution (area of distribution); threats; conservation measures taken; conservation measures needed; very specific references used for each taxa; the author(s) of each micromonography.

The last section of the book is an alphabetical index of latin names of the taxa included in, with the corresponding page number(s).

The first chapter dedicated to Algae list 6 species, as CR (5) and EN (1). All 6 species (*Chara kokeilii*, *Nemalion helminthoides*, *Padina pavonica*, *Thorea hispida*, *Tolypella intricata*, *Bryopsis hypnoides*) are very rare in Bulgaria, being seriously threatened by water pollution, habitats destruction, collecting etc.

The second chapter dedicated to Bryophytes (Liverworts, Mosses) list 102 species, as: CR (27) and EN (42), VU (33). Most of the species (e.g. *Fossombronia husnotii*, *Jungermannia caespiticia*, *Porella pinnata*, *Riccia crustata*, *Scapania apiculata* etc.) are very rare in Bulgaria, being seriously threatened by habitat disturbance, cutting down of forests, road constructions, rivers drying, trampling, and so on.

In the third chapter, dedicated to the vascular plants, is to be highlighted some of the rarest species, existing in Bulgaria, such as the next ones: *Ephedra fragilis* subsp. *campylopoda*, *Quercus thracica*, *Aethionema arabicum*, *Alchemilla bandericensis*, *A. mollis*, *Alopecurus thracicus*, *Alyssum orbelicum*, *Anchusa davidovii*, *Androsace obtusifolia*, *Anthemis argyrophylla*, *A. jordanovii*, *Arenaria rigida*, *Astragalus alopecurus*, *A. exscapus*, *Avena eriantha*, etc. (in a single grid), *Asplenium lepidum*, *Botrychium matricariifolium*, *Osmunda regalis*, *Pinus brutia*, *Aegilops comosa*, *A. dichasians*, *Alchemilla asteroantha*, *Aldrovanda vesiculosa*, *Arbutus andrachne*, *Artemisia involucrata*, *Asperula involucrata*, *A. suberosa*, *Centaurea jankae* (present also in Romania), and so on (in two grids), *Isoetes lacustris*, *Achillea kotschyi*, *Amygdalus* × *delipavlovii*, *Arbutus unedo*, *Artemisia chamaemelifolia* etc. (in three grids). In total, there are listed 8 ferns, 4 gymnosperms, and 539 angiosperms. Regarding the threat category, they are categorized as the next ones: EX (2), RE (12), CR (204), EN (295), VU (38).

In the fourth chapter, a list of fungi with macroscopically fruit bodies (macromycetes), both ascomycetes and basidiomycetes, is presented, with color drawings and distribution in Bulgaria. Every sheet is signed by renowned Bulgarian mycologists. There are presented 149 species of fungi, classified as CR (37 species), EN (104 species) and VU (8 species), making this list a large and comprehensive one. Some of the species

from this list are also listed in a recent Mapping Programme of European Council for the Conservation of Fungi, 51 species being studied concerning their distribution, ecology and status. In the present Red Book, one species critically endangered (*Tricholoma colossus*), 7 species endangered (*Amylocystis lapponicus*, *Battarrea phalloides*, *Boletus dupainii*, *Hericium erinaceus*, *Hydnellum suaveolens*, *Phylloporus pelletieri*, *Polyporus rhizophillus*, and *Suillus sibiricus*) and three vulnerable species (*Gomphus clavatus*, *Helvella atra*, *Sarcosphaera coronaria*) are also listed in the mentioned programme, as being threaten in different way.

Among the causes which threat all these species, the most common are: changes in land use, intensification of agriculture, trampling and grazing, drainage of the wetlands, habitat loss and destruction, water pollution, forest fires, afforestation, deforestations, climate aridisation, alien species, fires, tourism infrastructure, habitat fragmentation, low reproductive potential, and so forth.

As the authors already said, this new "Red Book" could contribute to the halting of the species/habitat loss in Bulgaria. Also, it could be a good guide for those future steps toward a better management in conservation actions and plans, in order to preserve the biodiversity in the region.

Not least, it is worth noting this fruitful collaboration among specialists in various plant groups and fungi.

We consider this book as an exceptional editorial issue and recommend it warmly to anyone interested in botany, not only in the study of nature conservation.

Adrian OPREA
"Anastasie Fătu" Botanical Garden, "Alexandru Ioan Cuza" University of Iaşi

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.

Manuscripts must be single-spaced, with wide margins. A font as Times New Roman, normal, is required.

The mirror of the page would be as follows: 13 x 20 cm (top 4.85 cm, bottom 4.85 cm, right 4 cm, left 4 cm).

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.

All publications cited in the text should be presented in a list of references following the text of the manuscript. In the text, references are made using the author (s) name of a certain paper (e.g.: other authors [GÉHU, 2006] mentioned that…). The full reference should be given in a numerical list in the end of the paper. References should be given inside the square brackets.

Obs.: if there are two authors only, there must be written down both names (ex. [BOX & MANTHEY, 2006]); if there are more authors, there would be written the first author followed by "& al." (ex. [AMORFINI & al. 2006]).

References
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.
The year of a paper publication is put after the author (s).

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.

For **books**, after the title, is placed the name of the town, the publishing house and the number of pages.

The chapter in books: author (s), year, title, pages, a dot sign, followed by "In": author (s) of the book, city, publishing house, number of pages.
Serial papers or chapters in serial papers: like the previous, but with the specification of the serial volume.

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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), ISBN 951-9108.
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. ISBN 0-521-41007-X (HB).

Chapters in books:

†TUTIN T. G. 1996. *Helleborus* L. Pp. 249-251. In: †T. G. TUTIN et al. (eds). *Flora Europaea*. 2nd ed., 1993, reprinted 1996. Vol. **1**. *Psilotaceae to Platanaceae*. Cambridge: Cambridge University Press, xlvi, 581 pp., illus. ISBN 0-521-41007-X (HB).

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Proofs will be sent to the corresponding author and should be returned within 48 hours of receipt. Corrections should be restricted to typesetting errors. All queries should be answered.

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JOURNAL OF PLANT DEVELOPMENT

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