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IN SITU AND *IN VITRO* COMPARATIVE STUDY ON THE BIOPRODUCTIVITY OF *ARNICAE FOLIUM ET CAULIS* FROM THE NORTHERN AREA OF THE ROMANIAN EASTERN CARPATHIANS

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Abstract: *Arnica montana* L. is an important plant bioresource, being traditionally used as medicinal plant, for which the scientific and economic interest remains at a high level. The aim of our study was to evaluate the bioproductivity for *Arnicae folium et caulis* in terms of biomass and content in biological active compounds originating from the wild populations and *in vitro* experimental cultures – under controlled environment. *In situ* biomass production registered variations, probably due to the different environmental features and also to the management strategies for each site. The development of *in vitro* cultures had as purpose the evaluation of the biomass production and production of plant material for the phytochemical screening. The *Arnicae folium et caulis* samples harvested from the wild populations have a higher content in phenolic acids than the *Arnicae flos* from the same area, but the total content in sesquiterpen-lactones was significantly lower. In contrast, the samples originating from the *in vitro* cultures had a low content in phenolic compounds and a high content in sesquiterpen-lactones, comparable with *Arnicae flos*.

Keywords: *A. montana*, biomass, phenolic acids, flavonoids, sescviterpen-lactones, HPTLC, HPLC

Introduction

In the current context of the development of basic research in the field of life sciences, with applicability in the improvement of the quality of the life, the plant species diversity, by the bio-resources offered by it, establishes a domain of priority study to identify and to isolate new biologically active compounds, including the evaluation of their activity.

Plant resources in the mountain areas are a valuable source of raw material, since pedo-climatic peculiarities give distinct biosynthetic potential with adaptive significance for the plants. This is reflected in the quality of plant material, in terms of the content of biologically active principles.

In this context, *Arnica montana* L. is one of the species of traditionally used medicinal plants for which the scientific and economic interest remains at a high level and is aimed at conservation and sustainable issues. *A. montana* is a perennial herbaceous species

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of the Asteraceae family with traditional medicinal use in Europe, arnica extract being used topically for its anti-inflammatory action.

The phytocomplex specific to *A. montana* includes sescviterpenic lactones, mainly helenalin, dihidrohelenalin and esters, phenolic acids and flavonoids, respectively. The phytochemical studies envisaged the characterization of the biological active compounds content for *A. montana* plant material harvested from wild populations and experimental cultures [AIELLO & al. 2012; GANZERA & al. 2008; NIKOLOVA & al. 2013; SCHMIDT & al. 1998] and the identification of the environmental factors that influence the content in biological active substances [ALBERT & al. 2009; SEEMAN & al. 2010; SPITALER & al. 2008].

Sescviterpen-lactones have an anti-inflammatory effect, through different mechanisms of action involved in inflammatory processes [LYSS & al. 1998]. Phenolic acids and flavonoids have antioxidant, cytoprotective and anti-inflammatory activity [ZHELEVA-DIMITROVA & BALABANOVA, 2012; WOERDENBAG & al. 1994].

In addition, for the plant itself, these biological active compounds present adaptive significance with defensive role for *A. montana* plants as a response to biotic and abiotic stress, characteristic for the mountain areas.

The species *A. montana* can be a therapeutically valuable bio-resource with multiple applications. Studies in the last decade have opened new perspectives on the applicability of arnica extract and helenaline in the treatment of some types of cancer and autoimmune diseases [HUANG & al. 2005; BERGES & al. 2009]. Also, *A. montana* is used in human and veterinary homeopathy, as well as in a wide range of products in the cosmetics industry.

The over-exploitation of the species *A. montana* and the environmental changes have led to problems of sustainability; the species has special conservation status in most areas of distribution in Europe, in Romania being considered rare or vulnerable [OLTEAN & al. 1994; NEGREAN, 2001].

Romania is one of the main European countries, providing plant material of *A. montana* on the global market. Although the interest in the species *A. montana* has remained high worldwide, the species is still poorly capitalized in Romania, for both economic and scientific purposes. Thus, there is a lack of comprehensive studies integrated into the current status of the species in natural populations, i.e., strategies for conservation and recovery by creating and promoting local products. Most studies in Romania concentrated on the Apuseni Mountains, while in the Romanian Eastern Carpathians the species is mentioned only in studies of flora [ZAMFIRESCU, 2006; MICHLER, 2007; MARDARI, 2008].

Evaluation of the quality and availability of native plant material gives added value to marketing strategies, as the scientific basis leads to the reduction of the vulnerability on a competitive international market.

The aim of our study was to evaluate the bioproductivity for *Arnicae folium et caulis* in terms of biomass and content in biological active compounds originating from the wild populations from the northern area of the Romanian Eastern Carpathians and *in vitro* experimental cultures – under controlled environment. The results of the *in situ* and *in vitro* studies may be used in cultivation strategies. Considering that the cultivation of the species involve high costs, and the flower heads production lowers after the 4th year, the exploitation of the underground parts of the plant may lead to the fulfilment of the

investment plan [PLJEVLJAKUSIC & al. 2012]. Thus, based on the phytochemical screening, *Arnicae folium et caulis* can be also promoted as raw material.

The assessments on *Arnicae flos* are the object of a parallel study [STEFANACHE & al. 2013], being well documented in literature since *Arnicae flos* is the official drug included in the European Pharmacopoeia. Currently, in human and veterinary homeopathy it is used only the *Arnicae folium et caulis*, respectively *Arnicae radix.*

Materials and methods

Plant material

Within the study 5 natural populations of *A. montana* (Ortoaia 1 – O1, Ortoaia 2 – O2, Arini 1 – A1, Arini 2 – A2 and Arini 3 – A3) from the northern area of the Romanian Eastern Carpathians, Neamt County, Dorna Arini commune, during the vegetation seasons 2012 and 2013. The plant material which was the object of this study consists of *Arnicae folium et caulis* samples collected from plants harvested from the 5 natural areals mentioned above (*in situ*), respectively from plant regenerated through the *in vitro* plant tissue culture, before and after the stage of acclimatization to the *ex vitro* environment (*in vitro* and *ex vitro*).

Assessment of the bioproductivity in terms of biomass

In situ studies

The plant material samples were harvested during the vegetation seasons 2012 and 2013, in the second half of June, in the stage of full flowering. The samples were dried at room temperature in the shadow, for about 1 week.

The assessment of the bioproductivity, in terms of biomass, was determined for an average sample of 25 plants. For this samples the fresh biomass, dry biomass and the drying ratio was determined. The drying ratio was calculated by dividing the fresh biomass to the dry biomass.

In vitro studies – development of the plant tissue culture

For the development of the plant tissue culture, the explants consisted of sterile plantlets, obtained from the achenes harvested from the natural populations Ortoaia 1 and Arini 3. The culture media used within this experiment are presented in the Tab. 1.

Culture initiation. The plantlets were placed on the MS basal media (M1) [MURASHIGE & SKOOG, 1962]. For the shoot inducing and growth variant of the MS supplement with the phytohormone BAP (6-benzylaminopurine) were used: 0.5 mg/l (M2) and 1.0 mg/L BAP (M3).

Subcultivation. The first subcultivation was performed after 8 weeks from the culture initiation, the following subcultivations being performed every 4–6 weeks depending on the multiplication and growth rates.

Rhisogenesis. The new formed shoots were isolated and transferred on MS culture medium, without phytohormones (M4 and M5).

Acclimatization. After the shoots developed a vigorous root system, they were transferred in pots with a mixture of soil and perlite (1:1), placed in an environment with high and constant humidity.

Tab. 1. Culture medium variants used for in the different stages of the *in vitro* plant tissue culture

Culture stage	Medium variant (code)	Components					
		mg/L				g/L	
		Macroelements	Microelements	Vitamins	BAP	Sucrose	Agar
Initiation	**M1**	MS	MS	MS	-	30	8
Multiplication	**M2**	MS	MS	MS	1.0	30	8
	M3	MS	MS	MS	0.5	30	8
Rhisogenesis	**M4**	MS	MS	MS	-	25	8
	M5	MS	MS	MS	-	25	7.5

Assessment of the bioproductivity in terms of content in biological active compounds

Sample preparation

Sesquiterpen-lactones. The extracts where prepared according to the European Pharmacopoeia, using santonin as internal standard. The percentage content of total sesquiterpene lactones, expressed as dihydrohelenalin tiglate, was calculated using the formula given in the European Pharmacopoeia.

Phenolic acids and flavonoids. Several extraction methods were tested, an optimum recovery of the compounds being obtain when implementing the protocol developed by ALBERT & al. (2009).

Phytochemical analysis

HPTLC analysis

The qualitative assessments for the flavonoids and phenolic acids were achieved by means of HPTLC. *Stationary phase* HPTLC 20x10 cm, silica gel 60 F_{254}, plates (Merck); *mobile phase:* anhydrous formic acid, water, ethylacetate **(**10:10:80, V/V/V); *development distance:* 7 cm; *derivatization:* NP solution (10 g/L, in ethylacetate) and PEG solution (Macrogol 400, 50 g/L, in dichloromethane); *vizualization:* 366 nm.

HPLC analysis

Sesquiterpene-lactones. The extracts were subjected to HPLC analysis (Nucleodur 100-5 C18 EC, 4 x 125 mm, 5 µm), flow 1 mL/min; injection volume 20 µL; 225 nm detection; mobile phase water + 1 mL/L phosphoric acid (A) and methanol (B); gradient 55-50-40-35-15-55% solvent A for 0-1.55-9.45-15.95-16.95-18.45-20.95 min (Fig. 1).

Phenolic acids and flavonoids. The extracts were subjected to HPLC analysis (Zorbax SBC18, 3 x 150 mm, 5 µm), flow 1 mL/min.; injection volume 10 µL; DAD detection; mobile phase acetonitrile (A) and sodium acetate buffer (2 mM), pH = 3.5 (B); gradient : 2-14-20-30-25% solvent A for 0-20-40-50-60 min, after which we switched back to the initial conditions for 10 min (Fig. 2).

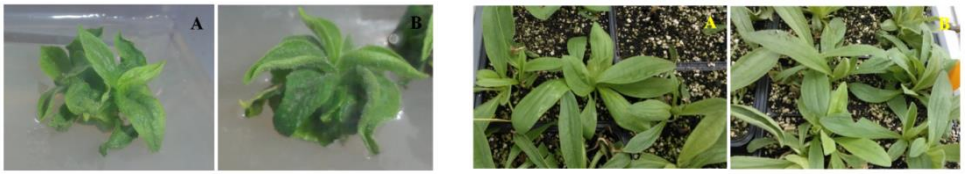

Fig. 1&2. Multiplication and growth, experimental variant O1 (**A**) and A3 (**B**), 2 weeks after subcultivation, respectively 20 days after the transfer in the *ex vitro* environment

Results and discussion

Assessment of the bioproductivity in terms of biomass

In situ studies

The *in situ* assessment of the bioproductivity in terms of biomass highlighted inter-populational variability for the same vegetation season, as well as the inter-populational variability registered during the 2 vegetations seasons (Fig. 1).

For the dry weight – *Arnicae folium et caulis*, the highest values were registered for Ortoaia 1 population in 2012, and for the Ortoaia 2 population in 2013. Although for the vegetation season 2012 Ortoaia 1 population registered the highest values, for the vegetation season 2013 it registered the lowest values compared with the other wild populations. From the field observations during 5 consecutive years we observed that Ortoaia 1 population has been established relatively recent after the deforestation of the area. In the first stage (first 2–3 years) the species had optimum developing conditions with low competition rate with other oligotrophic plant species and especially shrub species (*Vaccinium* sp.). Having a higher growth rate than the woody species, the *A. montana* population developed more quickly and spread in this area. In the second stage, when the woody species developed more and had a substantial covering percentage, the *A. montana* population declined due also to the high competition rate (for space and nutrients). This situation was not observed in the meadows that are properly managed by the owners (removing the woody plant species and mowing in order to obtain hay).

The inter-populational variability registered during the 2 vegetations seasons may also be explained through the meteorological peculiarities of each season.

The drying ratio for *Arnicae folium et caulis* had values of 4.02 to 5.69, varying both between the 5 populations in the same vegetation season and within the same population during the 2 vegetation season, fact that can be explained by the different water content in the plants and different relative air humidity at the time of harvest. The assessment of the drying ration is important in order to develop harvest strategies.

In vitro studies – development of the plant tissue culture

After the transfer of the plantlets on the shoot inducing medium, in the 22^{nd} – 29^{th} day new formed shoots were observed. After the first 2 subcultivations the multiplication rate was of 3 new shoots/explants. By reducing the BAP concentration from 1.0 mg/L (M2) to 0.5 mg/L (M3) both the multiplication and elongation were stimulated (Fig. 2). SMURMACZ-MAGDZIAK & SUGIER (2012) obtained similar results by reducing the BAP concentration. After 2–3 subcultivations on M3 we obtained an average multiplication rate of 7–8 new shoots per explant, with a maximum of 12–13 new shoots per explant.

After about 6 to 8 weeks, 95 % of the shoots developed a vigorous root system, being transferred for the acclimatization stage in pots in an environment with high and constant humidity. After 2 weeks the pots were transferred in a glasshouse, a survival rate of 90 % being obtained for both experimental variants (Fig. 3).

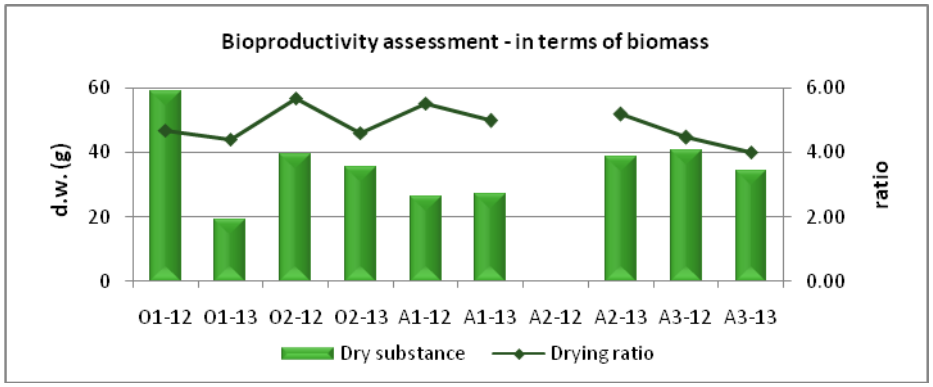

Fig. 3. Bioproductivity (in terms of biomass) assessment of the *Arnicae folium et caulis* harvested from the 5 studied wild populations during the vegetation seasons (2012 and 2013): dry substance (d.w. – dry weight) and drying ratio

Assessment of the bioproductivity in terms of content in biological active compounds

Qualitative assessments

The qualitative analysis of the phenolic acids and flavonoids contents was performed by means of High Performed Thin Layer Chromatography (HPTLC).

The phytochemical analysis of the *A. montana* samples harvested from the studied natural populations, highlighted the presence of the phenolic acids (isochlorogenic acids, Rf = 0.70 – 0.85; cynarine, Rf = 0.50 and chlorogenic acid, Rf = 0.63) and of flavonoids (luteolin-7-O-glicoside, Rf = 0.47; isoquercitrine, Rf = 0.44 and hyperoside, Rf = 0.40) (Fig. 4).

The analysis differentiated on the type of organs (leaves and stems) performed for the plant material harvested in 2013 had as purpose the assessment of the optimum raw material type depending on the final use of the plant material. The phenolic acids were present both in *Arnicae folium* and *Arnicae caulis*, while the flavonoids were present in all *Arnicae folium* samples and only in some *Arnicae caulis* samples. Thus, the HPTLC fingerprint of *Arnicae caulis* samples showed a narrower phytochemical spectrum than the *Arnicae folium*. In addition, the size and intensity of the spots corresponding to the flavonoids were lower in *Arnicae caulis* samples, which correlate with a lower content of this compounds (Fig. 5).

For the samples originating from the *in vitro* culture, harvested both before and after the stage of acclimatization to the *ex vitro* environment (*in vitro* and *ex vitro*), the HPTLC fingerprint highlighted only the presence of phenolic acids, the phytochemical spectrum being narrower when compared with the samples harvested *in situ* (Fig. 5).

Quantitative assessments

The quantitative phytochemical analysis, performed by means of High Performance Liquid Chromatography (HPLC), envisaged the total content in phenolic acids (expressed in caffeic acid equivalents), flavonoids (expressed in rutin equivalents) and sesquiterpen-lactones (expressed in dihydrohelenalin tiglate). All values are expressed as % dry weight.

In the *Arnicae folium et caulis* samples harvested from the natural populations the phenolic acids were found in total amounts of 1.01–3.02%, and the flavonoids in total amounts of 0.11–0.77%. For the samples originating from the *in vitro* culture, the total content in phenolic acids was of 0.05–0.23% for the samples harvested before the acclimatization stage (*in vitro*) and of 0.54–0.82% in the samples harvested after the acclimatization stage (*ex vitro*). Thus, it was observed, for the *in vitro* experimental variants, a significantly lower amount of phenolic acids, the flavonoids being under the detection limit, confirming the HPTLC analysis (Fig. 7).

The total content in sesquiterpen-lactones content, for the *in situ* samples varied from 0.08 to 0.33%, being under the lower limit of 0.4% stated by the European Pharmacopoeia for *Arnicae flos.* For the samples originating from the *in vitro* experimental variants the total content in sesquiterpen-lactones was of 1.15–1.29% for the samples harvested before the acclimatization stage (*in vitro*) and of 1.15–1.38% in the samples harvested after the acclimatization stage (*ex vitro*), being above the lower limit stated by the European Pharmacopoeia and comparable with the total content in the *Arnicae flos* samples harvested from the same area or presented in literature: 0.40–1.55% d.w. [SEEMAN & al. 2010; AIELLO & al. 2012].

SCHMIDT & al. (1998) obtained, for samples of leaves harvested from a convential culture, a total content in sesquiterpen-lactones of about 0.1%, while in the samples originating from the *in vitro culture* the total content was about 1.3%, with qualitative traits similar with the ones of the flower heads samples.

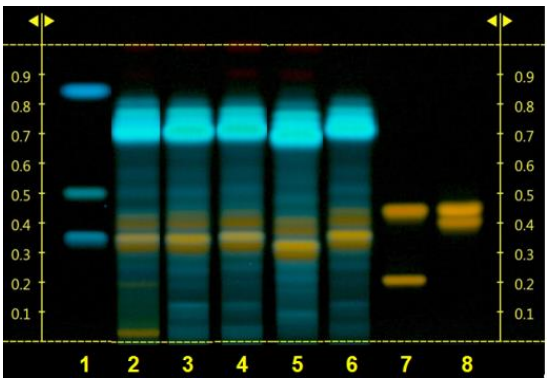

Fig. 4. HPTLC chromatogram for phenolic acids and flavonoids for samples harvested from the populations of Ortoaia and Arini (2012): 1. *Caffeic acid* + *Cynarine* + *Chlorogenic acid*; 2. O1FC – *Arnicae folium et caulis*, O1; 3. O2FC – *Arnicae folium et caulis*, O2; 4. A1FC – *Arnicae folium et caulis*, A1; 5. A2FC – *Arnicae folium et caulis*, A 2; 6. A3FC – *Arnicae folium et caulis*, A3; 7. *Isoquercitrine* + *Rutin*; 8. *Luteolin-7-O-Glucoside* + *Hyperoside*

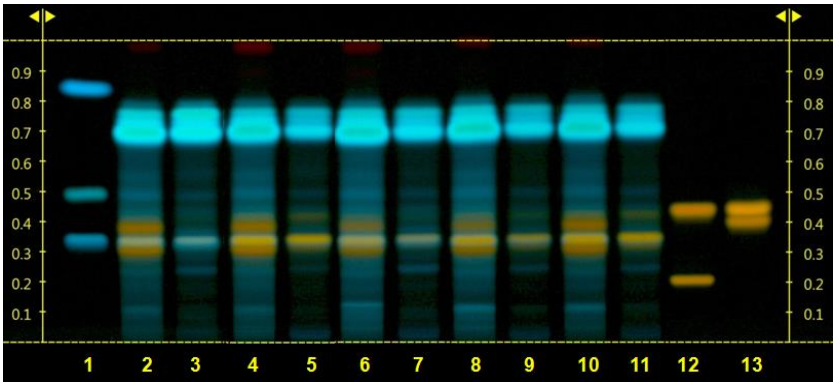

Fig. 5. HPTLC chromatogram, for phenolic acids and flavonoids for samples harvested from the populations of Ortoaia and Arini (2013): 1. *Caffeic acid* + *Cynarine* + *Chlorogenic acid*; 2. O1F – *Arnicae folium*, O1; 3. O1C – *Arnicae caulis*, O1; 4. O2F – *Arnicae folium*, O2; 5. O2C – *Arnicae caulis*, O2; 6. A1F – *Arnicae folium*, A1; 7. A1C – *Arnicae caulis*, A1; 8. A2F – *Arnicae folium*, pop. A2; 9. A2C – *Arnicae caulis*, A2; 10. A3F – *Arnicae folium*, A3; 11. A3C – *Arnicae folium*, A3; 12. *Isoquercitrine* + *Rutin*; 13. *Luteolin-7-O-Glucoside* + *Hyperoside*

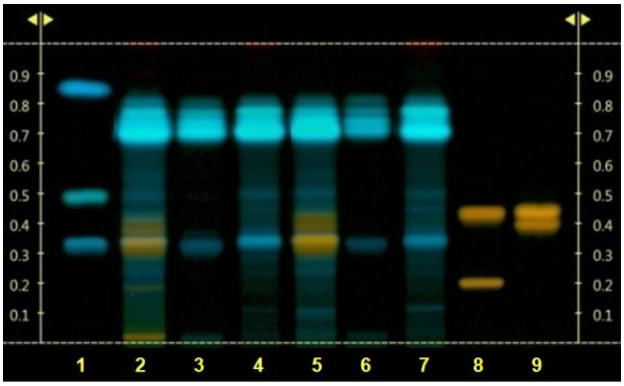

Fig. 6. HPTLC chromatogram for phenolic acids and flavonoids for samples harvested from *in situ* and from the *in vitro* plant tissue culture (experimental variants O1 and A3): 1. *Caffeic acid* + *Cynarine* + *Chlorogenic acid;* 2. O1FC12 – *Arnicae folium et caulis*, O1; 3. O1FCIv – *Arnicae folium et caulis*, *in vitro* plants, O1; 4. O1FCEv – *Arnicae folium et caulis*, *ex vitro* plants, O1; 5. A3FC12 – *Arnicae folium et caulis*, A3; 6. A3HIv – *Arnicae folium et caulis*, *in vitro* plants, A3; 7. A3FCEv – *Arnicae folium et caulis*, *ex vitro* plants, A3; 8. *Isoquercitrine* + *Rutin*; 9. *Luteolin-7-O-Glucoside* + *Hyperoside*

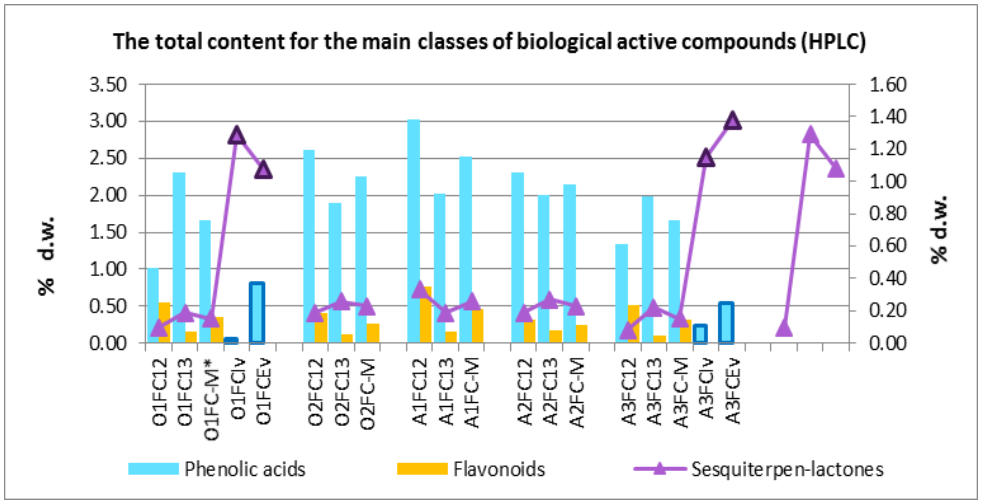

Fig. 7. The total content for the main classes of biological active compound: phenolic acids (exp. in caffeic ac. equiv), flavonoids (exp. in rutin equiv.) and sesquiterpen-lactones (exp. in dihydrohelenalin tiglate), in the samples harvested from the natural populations of Ortoaia and Arini in 2012 and 2013 (*in situ*) and from the *in vitro* experimental variants (*in vitro* and *ex vitro*); * mean value for 2012 and 2013.

Conclusions

The *in situ* biomass production and the content in biological active compounds is fluctuant due to the different environmental features and also to the management strategies for each site, parameters that can be partially controlled under cultivation conditions.

The *Arnicae folium et caulis* samples harvested from the wild populations have a higher content in phenolic acids than the *Arnicae flos* from the same area, but the total content in sesquiterpen-lactones was significantly lower. Thus, based on these results and on further studies regarding the qualitative featrures, *Arnicae folium et caulis* can be considered as a raw material for extractive fraction enriched in phenolic compounds.

In contrast, the samples originating from the *in vitro* cultures had a low content in phenolic compounds and a high content in sesquiterpen-lactones, comparable with *Arnicae flos* – above the lower limit stated in the European Pharmacopoeia. Thus, besides the applicability of the biotechnological methods in the production of planting material and in conservation strategies, these methods can be used in order to produce raw material in a controlled environment.

The origin of the plant material (*in situ* or *in vitro*) can be selected depending on the final use of the raw material.

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CALLUS INDUCTION FROM 15 CARNATION (*DIANTHUS CARYOPHYLLUS* L.) CULTIVARS

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Abstract: Plant growth regulators (PGRs) were used to induce callus in 15 carnation (*Dianthus caryophyllus* L.; Caryophyllaceae) cultivars: Orange Sherbert, Avalanche, Magenta, La France, Stripe Red, Marie, Concerto PVP, Snap, Lucky Pierot, Cinnamon Tea, White Love, Siberia, Magesta, Spark Bruno, and Honono no Estejo. Seeds were initially sown on autoclaved moistened filter paper and internodes of surface-sterilized seedlings were used as explants. Most callus was induced in the presence of 0.5 mg/L α-naphthaleneacetic acid used together with 1 mg/L 6-benzyladenine or 1 mg/L 2,4-dichlorophenoxyacetic acid on basal Murashige and Skoog medium. Callus is not a desirable method to clonally propagate important germplasm but can serve as one possible way of deriving periclinal mutants as a result of somaclonal variation.

Keywords: carnation, internode, plant growth regulator, thin cell layer

Introduction

The induction of friable and/or embryogenic callus from carnation (*Dianthus caryophyllus* L., family Caryophyllaceae), an economically important ornamental, has been well studied. α-Naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) [GUTIÉRREZ-MICELI & al. 2010] or BA and 2,4-dichlorophenoxyacetic acid (2,4-D) [KARAMI & al. 2007] were able to stimulate callus formation. KANWAR & KUMAR (2009) also found that a combination of 2,4-D + BA was most efficient for the induction of callus, primarily from leaf and internode explants. In all these studies, shoots were then induced to form from callus in the presence of other media and plant growth regulators (PGRs). A previous study [TEIXEIRA DA SILVA, 2014] found that shoots could be regenerated directly on MURASHIGE & SKOOG (MS; 1962) medium from the same 15 cultivars tested in this study using different explants (nodes, internodes, leaves, thin cell layers (TCLs)), although each explant showed a wide range of variation depending on the explant and PGR used. TCLs are important explants as they allow for a greater fine-scale control of plant organogenesis [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013]. In the TEIXEIRA DA SILVA (2014) study, it was found that internodes were most responsive *in vitro*, and were thus used as the explant for this study on callus induction.

The objective of this study was to induce organogenic or embryogenic callus from 15 as-yet unexplored carnation cultivars. Although callus is generally not a desired pathway for carnation or even for other ornamentals, since somaclonal variation can occur, it can

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also have positive benefits, including the discovery of new mutants with novel growth form, flower colour or other ornamentally attractive growth characteristics.

Materials and methods

The materials and methods related to seeding, explant sterilization and *in vitro* establishment strictly follow the protocols outlined by TEIXEIRA DA SILVA (2014). All PGRs were of the highest analytical grade available and were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals and reagents were purchased from either Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), the cheapest choice at the highest tissue-culture grade, unless specified otherwise.

Plant material and seeding conditions

As described in TEIXEIRA DA SILVA (2014), "seeds of 15 carnation cultivars (Orange Sherbert, Avalanche, Magenta, La France, Stripe Red, Marie, Concerto PVP, Snap, Lucky Pierot, Cinnamon Tea, White Love, Siberia, Magesta, Spark Bruno, and Honono no Estejo) were purchased from a Japanese online dealer. Seeds, some of which were very small, were soaked in between two layers of filter paper in 10-cm diameter glass Petri dishes overnight, then placed, 10-50 seeds/Petri dish, depending on the size of seeds, on top of two sheets of autoclaved, moistened filter paper (Advantec Toyo 2, 110 mm, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). Prior to autoclaving, filter paper was trimmed to 90 mm circles to fit glass Petri dishes. Approximately 4 mL of sterilized double-distilled water (SDDW) was applied per Petri dish, and SDDW was added, as needed, to maintain the filter paper moist at all times. The seeds of all cultivars germinated within one week, and seed viability or percentage seed germination were not measured as these were not important aspects of this study. The external surface of Petri dishes was sterilized by swabbing with 80% (v/v) ethanol and Petri dishes were placed under environmental conditions identical to those employed in the *in vitro* experiments outlined next".

Explant sterilization and *in vitro* establishment

A modified protocol of CASAS & al. (2010) was used for explant sterilization, as described in TEIXEIRA DA SILVA (2014): "nodal segments 1 cm long from 6 cm long seedlings growing in Petri dishes were cut with sterile feather blades, gently rinsed in SDDW, rinsed three times in 70% (v/v) ethanol, surface sterilized for 8 min in 2% (w/v) sodium hypochlorite containing 0.01% (v/v) Tween-20, then rinsed three times in SDDW. The ends of nodal segments were trimmed to obtain a section 6 mm long with the node and axillary buds in the middle. Nodes were plated on MS medium with 1.0 mg/L thidiazuron (TDZ), 0.1 mg/L NAA, 30 g/L sucrose, and 2 g/L Gellan gum (Gelzan®; CP Kelco Inc., J.M. Huber Corp.; GA, USA) for 10 d and then subcultured on the same freshly made medium every 10 days until shoots formed. Individual shoots (1-2 cm long) were then transferred to 500 mL glass bottles (AsONe, Osaka, Japan) holding MS medium with 1.0 mg/L BA, 0.1 mg/L NAA, 30 g/L sucrose and 2 g/L Gellan Gum (shoot induction medium, or SIM). Bottles were not ventilated and lids were closed off with air-permeable Parafilm M® (Bemis NA, Neenah, WI, USA). pH of all media was adjusted to 5.8 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Cultures were placed on 25 mL medium in

9-cm diameter Petri dishes, closed off with air-permeable Parafilm M®, at 25 °C, under a 16-h photoperiod with a light intensity of 45 µmol/m^2/s provided by plant growth fluorescent lamps (40 W; Homo Lux, Matsushita Electric Industrial Co., Japan)". These shoots served as stock material for the callus-induction experiments.

Testing plant growth regulators for callus induction

To induce callus, 1 cm long internodes from nodes 1-4 of 8 cm tall stock plantlets of all 15 cultivars were excised and placed on MS basal medium containing 0, 1, 2, 4 or 8 g/L 2,4-D, Kin or BA (found to be the most responsive PGRs in the wider carnation tissue culture literature), together with 0, 0.5 or 1.0 mg/L NAA. Following initial trials, it was ascertained that explants were most responsive to 2,4-D or BA in the presence of NAA. Thus, callus formation was only quantified for the 2,4-D/BA+NAA combination. All callus cultures were placed under growth conditions specified for *in vitro* culture establishment.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate (n = 30, total sample size per treatment). Data was subjected to analysis of variance (ANOVA) with mean separation by Duncan's multiple range test (DMRT) using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leq 0.05$.

Results and discussion

The direct formation of shoots from node and leaf explants in carnation is generally genotype-dependent, although the use of TDZ tends to produce shoots without an intermediate callus phase [NONTASWATSRI & al. 2002]. In contrast, callus is generally not a desired organogenic route in clonal propagation since callus-derived shoots can result in somaclonal variation and thus phenotypic differences. However, somaclonal variation can be an important result for ornamentals like carnation since alternative phenotypic characters may be derived, such as novel flower colours, or leaf forms, all with ornamental and thus economic potential. The carnation literature reveals a broad trend: TDZ and/or BA, alone or in combination with NAA, can be used to induce shoots without intermediate callus formation (e.g., NONTASWATSRI & al. 2002; CASAS & al. 2010; VARSHNEY & al. 2013). However, for the 15 cultivars tested in this study, the absence of TDZ or NAA, as well as the use of a high concentration of either, alone or combination, resulted in no or poor shoot formation [TEIXEIRA DA SILVA, 2014]. Thus, in this case, where a direct route for shoot formation was not possible, an alternative, indirect route, through friable and organogenic callus, was tested in this study.

Magenta was the most responsive cultivar to callus formation, followed by Snap, while Le France was least responsive to all combinations of NAA + BA/2,4-D (Fig. 1). In this study, the use of 0.5 mg/L NAA together with 1 mg/L BA or 1 mg/L 2,4-D on basal MS medium induced most callus across cultivars (Fig. 1), although the visual aspect of callus from most cultivars was similar (friable, green or white) after 45 days, with limited shoot formation (Fig. 2). Interestingly, *Dianthus ciliatus* ssp. *dalmaticus* and *D. giganteus*

ssp. *croaticus* formed both green organogenic callus in which shoot initials were embedded, as well as white embryogenic callus that contained embryo-like structures when seedling-derived nodal segments were placed in MS medium supplemented with 2,4-D, BA and NAA [RADOJEVIĆ & al. 2010].

Callus induction in carnation tissue cultures is widely reported in the literature but only a few representative studies are listed here. FREY & JANICK (1991) found that TDZ and NAA resulted in callus formation from 'Scania', 'Improved White Sire' and 'Sandra' petal explants but the same three cultivars formed embryogenic callus from internodes in the presence of 2,4-D [FREY & al. 1992]. SEO & al. (2007) found that the same combination of PGRs resulted in callus induction from root explants. KANWAR & KUMAR (2009) produced most callus in 'Indios' from a 2,4-D+BA combination from leaves or internodes, and shoots could then be regenerated from callus. A subsequent study by the same authors [KANWAR & al. 2010] indicated that the use of TDZ in combination with indole-3-acetic acid could result in the most shoots from leaf- and internode-derived callus in 'Tempo'. White and green friable callus, as was observed in this study, was also induced from root segments of 'Grenadin' in the presence of a wide range of concentrations (0-3.0 mg/L) of NAA and BA [YAACOB & al. 2013], and also from internode explants in the presence of 2,4-D for an unspecified cultivar [ARIF & al. 2014].

Even though cultures in this study were not ventilated, no visible hyperhydricity was observed. This was not the case in a study by MOHAMED (2011), who employed *D. caryophyllus* 'White Sim' nodal explants to induce shoots directly in the presence of BA on MS medium when culture flasks were aerated, although in that study, both callus formation and hyperhydricity were observed in non-aerated vessels, suggesting that aeration, or the lack of it, can alter the organogenic state of explants in the presence of the same PGRs [CASAS & al. 2010], possibly as a result of the action of ethylene, which can build up in culture flasks.

Conclusions

In this study, friable and organogenic callus could be induced from 15 carnation cultivars in the presence of six combinations of NAA, BA and 2,4-D. Most prolific callus in all 15 cultivars was in the presence of 0.5 mg/L NAA, 1.0 mg/L BA and 1.0 mg/L 2,4-D, as assessed by callus fresh weight. Although not the most desirable route for the clonal propagation of plants through shoot induction, the induction of shoots via an indirect callus route can be beneficial where direct shoot induction is not possible, as was observed in several of the same cultivars in separate experiments [TEIXEIRA DA SILVA, 2014]. This study provides an additional and expanded set of data to support callus induction in unexplored or difficult-to-propagate carnation cultivars or *Dianthus* species. VARSHNEY & al. (2013) used biochemical and molecular marker-based analyses to verify the genetic stability of tissue culture-derived shoots. YAACOB & al. (2013) employed cytological analyses to confirm the cytological stability of regenerants. Such methods of verification are recommended for all carnation tissue culture studies.

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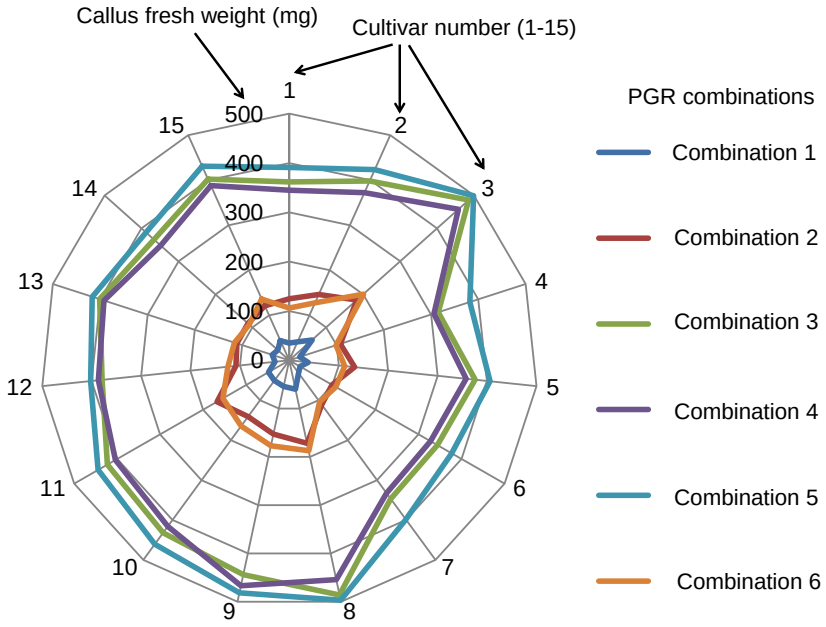

Fig. 1. The response (fresh weigh (mg) of callus) of 15 carnation (*Dianthus caryophyllus* L.) cultivar internodes to multiple combinations and concentrations (mg/L) of NAA, BA and 2,4-D on basal MS medium after 25 days. Cultivars: 1 = Orange Sherbert; 2 = Avalanche; 3 = Magenta; 4 = La France; 5 = Stripe Red; 6 = Marie; 7 = Concerto PVP; 8 = Snap; 9 = Lucky Pierot; 10 = Cinnamon Tea; 11 = White Love; 12 = Siberia; 13 = Magesta; 14 = Spark Bruno; 15 = Honono no Estejo. Plant growth regulator (PGR) combinations (in mg/L; NAA, BA, 2,4-D): combination 1 (0, 0, 0); combination 2 (0.5, 0.5, 0.5); combination 3 (0.5, 0, 1.0); combination 4 (0.5, 1.0, 0); combination 5 (0.5, 1.0, 1.0); combination 6 (1.0, 1.0, 1.0).

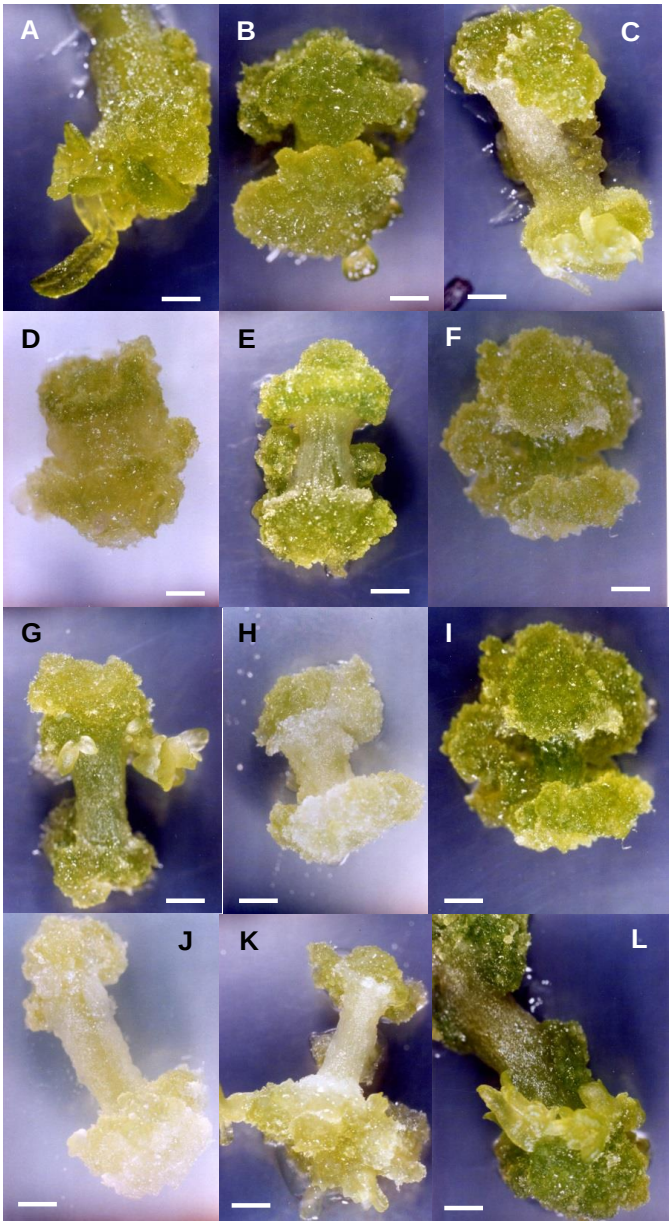

Fig. 2. The response of 12 carnation (*Dianthus caryophyllus* L.) cultivar internodes (Magenta, Snap and Spark Bruno not represented) to 0.5 mg/L NAA together with 1 mg/L BA or 1 mg/L 2,4-D on basal MS medium after 25 days. (A) Avalanche; (B) Cinnamon Tea; (C) Concerto PVP; (D) Honono no Estejo; (E) La France; (F) Lucky Pierot; (G) Magesta; (H) Marie; (I) Orange Sherbert; (J) Siberia; (K) Stripe Red; (L) White Love. Bars = 1 mm.

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IN VITRO PROPAGATION AND FIELD ESTABLISHMENT OF *HARDWICKIA BINATA* ROXB. AND ASSESSMENT POLYMORPHISM THROUGH MOLECULAR MARKERS

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Abstract: *Hardwickia binata* Roxb. is a leguminous tree of great economic importance. Yet a protocol for its *in vitro* propagation that is capable of taking the regenerated plants from lab to land does not exist. The plant is quite recalcitrant and has poor natural regeneration. Macropropagation techniques are also not standardized. Problem of leaching of phenolic compounds in culture condition, premature leaf fall during multiplication phase and callusing during rooting were sever problems encountered during the course of development of the protocol. These problems were addressed using various treatments and a working protocol for *in vitro* propagation of *H. binata* from axillary bud of the seedling nodal segments was perfected. The plantlets were hardened through a systematic two stage hardening procedure and were eventually transferred to experimental field. Here we report the development of an *in vitro* propagation protocol for *H. binata*, leading to successful establishment of plants thus obtained, in open field condition.

Keywords: micropropagation, shoot multiplication, rooting, acclimatization, RAPD

Introduction

Hardwickia binata Roxb. is a leguminous tree of great economic importance. It serves as a source of very heavy and durable timber, fiber, fuel and high quality fodder for the cattle. *H. binata*, is also an important component of several agroforestry systems of arid and semi-arid regions. However the tree suffers from various problems among which poor seed setting, low seed germination rate and lack of proper macropropagation methods are the most important ones. *In vitro* propagation of this marvelous nitrogen fixing tree species will help in production of quality planting material on a large scale throughout the year without depending on seed viability, germination and season. Woody plants, including leguminous trees appear to be recalcitrant to *in vitro* culture and plant regeneration [ZHAO & al. 1990]. Only a few reports of work on *in vitro* propagation of *H. binata* are available. ANURADHA & al. (2000) reported *in vitro* propagation using mesocotyls, shoot tips and axillary buds from 15 days old seedlings as source of explants. Direct somatic embryogenesis of *H. binata* with mean number of 18.3 somatic embryos per explant from semimature zygotic embryos was reported by CHAND & SINGH (2001). *In vitro* somatic embryogenesis was also reported by DAS & al. (1995) and DAS (2011). However, complete plants could not be obtained from somatic embryos.

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Here we report development of an *in vitro* propagation protocol of *H. binata* and analysis of genetic uniformity of tissue culture raised plants through RAPD (Random Amplified Polymorphic DNA) markers.

Materials and methods

Plant material and explants source: All explants of *H. binata* were collected from a healthy mother tree (Fig. 1) growing in and around AFRI (Arid Forest Research Institute), Jodhpur. For optimization of surface sterilization procedure for hard tissues (mature nodal segments and mature seeds) all explants were first washed in running tap water for 5-10 min to remove surface contaminants. They were then washed with detergent (Tween 80) for 7-10 minutes followed by rinsing with distilled water 4-5 times. The explants were immersed and agitated constantly in freshly prepared Streptomycin (0.5 g/l00 ml) and Bavistin solution (0.1 g/100 ml of distilled water) for 10-15 minutes followed by rinsing with distilled water. The explants were then treated with 5–20% NaOCl (Sodium hypochlorite) solution at various concentrations and different time duration.

Fig. 1. *Hardwickia binata* Roxb. **A.** Mother plant growing at AFRI Campus, Jodhpur (India); **B.** Close-up of a stem nodal segment with pinnate bifoliate leaved. The stem nodal segment was used as explants.

Leaching of phenolics and its control: The injury caused during the excision of explants, induces the cells to leach out phenolic compounds which are readily oxidised to produce quinones and cause discoloration. In case of *H. binata*, leaching occurs from seed coat. In order to control this problem cold treatment and antioxidant treatment was given for different time periods prior to inoculation. Seeds were treated with antioxidants – ascorbic acid (250 ppm), citric acid (100 ppm) for 10 minutes each. Cold water treatment was given for 40 minutes.

Bud break: For *in vitro* shoot induction nodal segments, apical buds, tender shoot from cut end of trunk of mature trees as well as stem nodal segments, apical buds and cotyledonary nodes from *in vitro* raised seedlings were inoculated on MS medium supplemented with 2.0 mg L^{-1} BAP (6-Benzylaminopurine). Mature nodal segments and apical buds from selected trees were inoculated on MS [MURASHIGE & SKOOG, 1962] medium supplemented with different cytokinins – BAP (0.5 - 5.0 mg L^{-1}), Kn (0.5 - 5.0 mg L^{-1}), 2ip (2-isopentenyl adenine) (0.5 - 5.0 mg L^{-1}) and TDZ (Thidiazuron) (0.1 - 2.0 mg L^{-1}), combination of BAP (0.5 - 5.0 mg L^{-1}) and NAA (1-Naphthylacetic acid) (0.5 - 5.0 mg L^{-1}) and combinations of BAP (1.0 - 4.0 mg L^{-1}) and Kn (Kinetin) (1.0 - 4.0 mg L^{-1}). To study the effect of different additives (glutamine and thiamine HCl), MS medium + 2.0 mg/l BAP was used along with glutamine (50.0 mg L^{-1}) and thiamine HCl (10.0 mg L^{-1}) alone and in combination. Seeds were germinated on liquid MS medium on filter paper bridges.

Shoot multiplication: The *in vitro* induced shoots obtained in above experiments were used for achieving further multiplication through axillary bud proliferation or through repeated subculturing. Effect of cytokinins used singly and in combination on multiplication from seedling derived nodal segments was seen. Among the additives tested, glutamine and thiamine HCl were used alone and in combinations.

Control of in vitro leaf fall: *In vitro* leaf fall was a persistent problem encountered during multiplication of micro-shoots of *H. binata*. In order to control the problem of leaf fall, silver nitrate (0.15 mg L^{-1}) and silver thiosulphate (0.15 mg L^{-1}) were used separately in MS medium along with 2.0 mg L^{-1} BAP, 50.0 mg L^{-1} glutamine and 10.0 mg L^{-1} thiamine HCl.

Rooting: Root induction in *H. binata* was studied on the basis of various parameters, which include plant growth regulators, strength of salts, concentration of sucrose, pulse treatment and nutrient media.

Acclimatization: This was a two stage process and carried out as follows:

i. *In vitro* hardening: Rooted plantlets were first of all transferred to jam bottles containing autoclaved soilrite and kept in culture room. They were irrigated weekly with ½ MS, gradually the caps were loosened and finally opened to expose them to decreasing humidity.
ii. *Ex vitro* hardening: *In vitro* hardened plantlets were shifted to polyhouse after 20 – 25 days. They were kept for around 1 month and then transferred to poly-bags containing soil: soilrite: manure (1:1:1) in polyhouse conditions. Hardened plants of *H. binata* were thereafter transferred to pots after two months. The field hardened plants were finally transferred to open-field condition.

The complete protocol for *in vitro* plant propagation for *Hardwickia binata* is pictorially represented (Fig. 2).

Evaluation of genetic fidelity of tissue culture raised plantlets: In order to test the polymorphism in tissue culture raised plants from seedling explants, DNA fingerprinting

using RAPD markers was carried out. Polymorphic RAPD markers were selected from the recommended marker list for leguminous tree species suggested by GOMEZ & al. (2011).

Data analysis: Each treatment consisted of 10 explants and all experiments were repeated thrice. The results were analyzed statistically using SPSS ver. 10 (SPSS Inc., Chicago, IL, USA). The results are expressed as the means ± SE of three experiments.

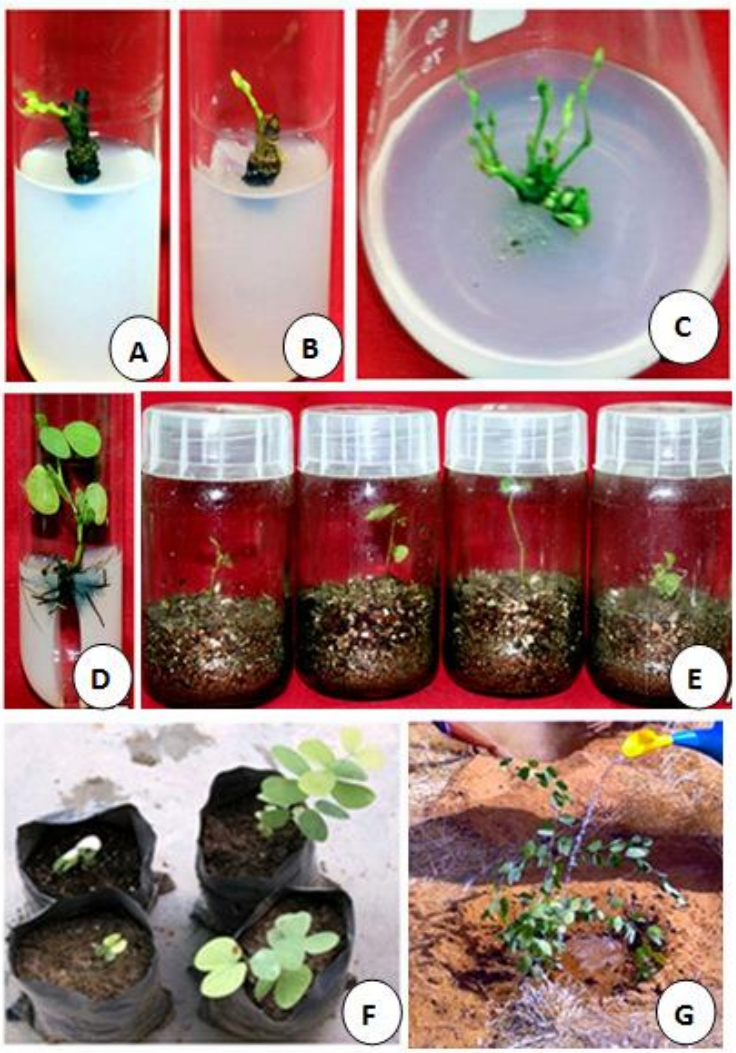

Fig. 2. *In vitro* propagation of *H. binata*. **A.** & **B.** Axillary bud-break; **C.** Shoot multiplication; **D.** *In vitro* rooting; **E.** *In vitro* hardening (acclimatization); **F.** Plantlets in poly – bags ready for field transfer; **G.** Tissue culture raised plant growing in field condition

Results and discussion

Surface sterilization of various explants of Hardwickia binata: For mature nodal segments, it was observed that 10% sodium hypochlorite was most effective at 9 minutes exposure showing only 1.67% contamination. For soft explants namely apical buds, immature seeds, young leaves, and mature leaves, it was observed that a treatment of 20% sodium hypochlorite for six minutes was most effective with only 3.33% contamination.

Effect of antioxidants on leaching of phenolics from seeds of H. binata: The percentage of leaching was observed to be 63% and 50% with the use of ascorbic acid and citric acid respectively. Cold water treatment was not found to have any positive role in reducing the extent of leaching. In the present work repeated subculturing was found most successful in reducing leaching.

In vitro shoot induction in H. binata: A maximum of 82% bud break was observed in case of stem nodal segments from *in vitro* raised seedlings, 69% in apical part from *in vitro* raised seedlings and 29% on cotyledonary nodes of seedlings. Thirteen percent bud break was observed in tender shoot from cut end of trunk (Tab. 1). A maximum of only 11% bud break was observed on MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l NAA. Maximum bud induction (11%) was observed on medium with both the additives (glutamine and thiamine HCl) in it.

Tab. 1. Screening of explants for bud break response on MS medium supplemented with BAP (2 mg L^{-1}), sucrose (3%) solidified with agar (0.8%) at pH 5.8. Bold figure represents best response

Explants	Bud Break	Bud Break percentage (%)
Source: Mature tree		
• Mature nodal segments	–	–
• Apical bud	–	–
• Tender shoots arising from cut end of trunk from mature tree	+	13.33
Source: *In vitro* raised seedlings		
• Nodal segments	+	**82.22**
• Apical bud	+	68.89
• Cotyledonary node	+	28.89

Treatment means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT). Values presented above are average of 10 explants, repeated thrice; observations were recorded after 4 weeks of inoculation.
– = no response, + = showed response.

Shoot multiplication: When *in vitro* raised nodal segments were transferred on MS media supplemented with BAP (2.0 mg L^{-1}), kinetin (2.0 mg L^{-1}) and combination of BAP (2.0 mg L^{-1}) and kinetin (2.0 mg L^{-1}), highest shoot multiplication was achieved on MS media supplemented with 2.0 mg L^{-1} BAP with 2.5 shoots per explant and mean shoot length of 3.1 cm (Tab. 2). When different media (MS, WP and B5) supplemented with BAP (2.0 mg L^{-1}), glutamine (50.0 mg L^{-1}) thiamine HCl (10 Mg L^{-1}) and silver nitrate (0.1 mg L^{-1}) were tested for shoot multiplication, MS medium proved to be the best providing mean number of 3.46 shoots per explant and mean shoot length of 3.86 cm followed by woody plant medium and B_5 medium.

Tab. 2. Effect of cytokinins alone and in combinations supplemented in MS medium having sucrose (3%) solidified with agar (0.8%) at a pH of 5.8 on multiplication from seedling derived nodal segments

Hormone concentration (mg L^{-1})		Number of shoots per explant (Mean ± SE)	Shoot Length in cm. (Mean ± SE)	Percentage of explants showing callusing (%)
BAP	Kn			
0.0	0.0	1.03 ± 0.03^{a}	0.72 ± 0.13^{a}	11.11
2.0	**0.0**	$\mathbf{2.50 \pm 0.33^{b}}$	$\mathbf{3.10 \pm 0.45^{c}}$	**22.22**
0.0	2.0	2.12 ± 0.19^{b}	1.89 ± 0.19^{b}	26.67
2.0	2.0	2.15 ± 0.16^{b}	2.29 ± 0.42^{bc}	28.89

Treatment means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT). Values presented above are average of 15 explants, repeated thrice; C = Control. Observations were recorded after 4 weeks of inoculation.

In vitro rooting of microshoots and acclimatization: Media supplemented with 4.0 mg L^{-1} IBA and 4.0 mg L^{-1} IAA resulted into 36% and 20% rooting respectively. Mean root number (2.22) and mean root length (1.30 cm) was observed to be highest on 4.0 mgL^{-1} IBA (Tab. 3). To achieve reduction of intermittent callus formation at root shoot junction, experiments were conducted using ½ MS media + 4.0 mg L^{-1} IBA supplemented with different percentages of sucrose (1.0, 2.0 and 3.0 %). Out of these 2% sucrose proved to be the best with 40% root induction. Percentage of callus formation was reduced by 50% (Tab. 4). Survival rate of 50% was observed during *ex vitro* hardening. Fully acclimatized plants were transferred to field in August, 2012 and out of six plants transferred to field conditions five are surviving and are in a healthy condition.

Tab. 3. Effect of auxins on rooting when supplemented in MS medium. Figures in bold indicate best performance for the particular auxin treatment

Hormone (mg L^{-1})			Percentage of explants showing rooting	No. of roots/shoot (Mean ± SE)	Shoot Length in cm. (Mean ± SE)	Percentage of explants showing callusing
IBA	IAA	NAA				
0.0 (C)	0.0 (C)	0.0 (C)	0.0	0.0	0.0	0.0
1.0	0.0	0.0	10.00	1.33 ± 0.88^{ab}	0.69 ± 0.35^{ab}	**53.33**
2.0	0.0	0.0	0.00	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	83.33
3.0	0.0	0.0	13.33	1.50 ± 0.50^{ab}	1.16 ± 0.24^{ab}	80.00
4.0	0.0	0.0	**36.67**	$\mathbf{2.22 \pm 0.40^{b}}$	$\mathbf{1.30 \pm 0.18^{b}}$	80.00
5.0	0.0	0.0	23.33	1.94 ± 0.24^{b}	1.18 ± 0.23^{ab}	83.33
0.0	1.0	0.0	0.00	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	**46.67**
0.0	2.0	0.0	6.67	1.00 ± 0.58^{a}	0.32 ± 0.16^{a}	63.33
0.0	3.0	0.0	10.00	0.83 ± 0.44^{a}	0.47 ± 0.26^{a}	76.67
0.0	4.0	0.0	**20.00**	0.67 ± 0.33^{a}	0.40 ± 0.23^{a}	76.67
0.0	5.0	0.0	16.67	$\mathbf{1.44 \pm 0.44^{a}}$	$\mathbf{0.50 \pm 0.16^{a}}$	83.33
0.0	0.0	1.0	3.33	0.33 ± 0.33^{a}	0.17 ± 0.16^{a}	**46.67**
0.0	0.0	2.0	6.67	1.00 ± 0.58^{ab}	0.70 ± 0.39^{ab}	60.00
0.0	0.0	3.0	13.33	1.22 ± 0.62^{ab}	0.66 ± 0.33^{ab}	80.00
0.0	0.0	4.0	**20.00**	2.06 ± 0.34^{b}	$\mathbf{1.24 \pm 0.06^{b}}$	80.00
0.0	0.0	5.0	23.33	$\mathbf{2.17 \pm 0.44^{b}}$	1.05 ± 0.09^{b}	90.00

Treatment means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT). Values presented above are average of 10 explants, repeated thrice; C = Control. Observations were recorded after 6 weeks of inoculation.

Tab. 4. Effect of different concentrations of sucrose on rooting with supplemented in ½ strength MS media having IBA (4 mg L^{-1}), and solidified with agar (0.8%) at a pH of 5.8. Figures in bold indicate best performance for the particular sucrose percentage.

Sucrose percentage (w/v)	Percentage of explants showing rooting (%)	No. of roots/shoot (Mean ± SE)	Root Length in cm. (Mean ± SE)	Percentage of explants showing callusing (%)
0.0 (C)	3.33	0.33 ± 0.33^{a}	0.33 ± 0.33^{a}	33.33
1.0	10.00	1.00 ± 0.58^{ab}	0.82 ± 0.42^{a}	**43.33**
2.0	**40.00**	2.11 ± 0.11^{b}	1.11 ± 0.02^{a}	50.00
3.0	33.33	**2.26 ± 0.14^{b}**	**1.16 ± 0.02^{a}**	83.33

Treatment means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT). Values presented above are average of 10 explants, repeated thrice; C = Control. Observations were recorded after 6 weeks of inoculation.

Silver nitrate proved to be better than silver thiosulphate in controlling leaf fall to 20% with 3.8 shoots per explant.

Selection of Molecular markers and analysis of polymorphism: RAPD analysis was performed based on earlier screening report by GOMEZ & al. (2011) for leguminous tree species *Acacia nilotica*, *Adenanthera pavonina*, *Prosopis juliflora*, *Pithecellobium dulce*, *Clitoria ternatea* and *Pongamia pinnata*, from which highly polymorphic primers were selected. These four highly polymorphic random and arbitrary 10-base primers [(5'AAGGCGGCAG (OPI-06), 5'TGCCCAGCCT (OPI-18), 5'TGGGCGTCAA (OPL-02) and 5'AGGTTGCAGG (OPL- 16)] were used for genetic fidelity test. The banding patterns from micropropagated plants through seedling material were slightly polymorphic (Fig. 3). The variations from the mother plant were due to the reason that *in vitro* seedlings were the source material for micropropagation.

Conclusions

Hardwickia binata is an important tree species that has nitrogen fixing capability, and hence also an important part of agro-forestry system. However due to its poor natural regeneration, and not-so-well developed propagation protocols, the present work was carried out leading to the development of a working protocol for *in vitro* mass propagation of the species (Fig. 4). Moreover, the DNA fingerprinting study using RAPD markers has also been carried out to establish the molecular polymorphism in the regenerated plants.

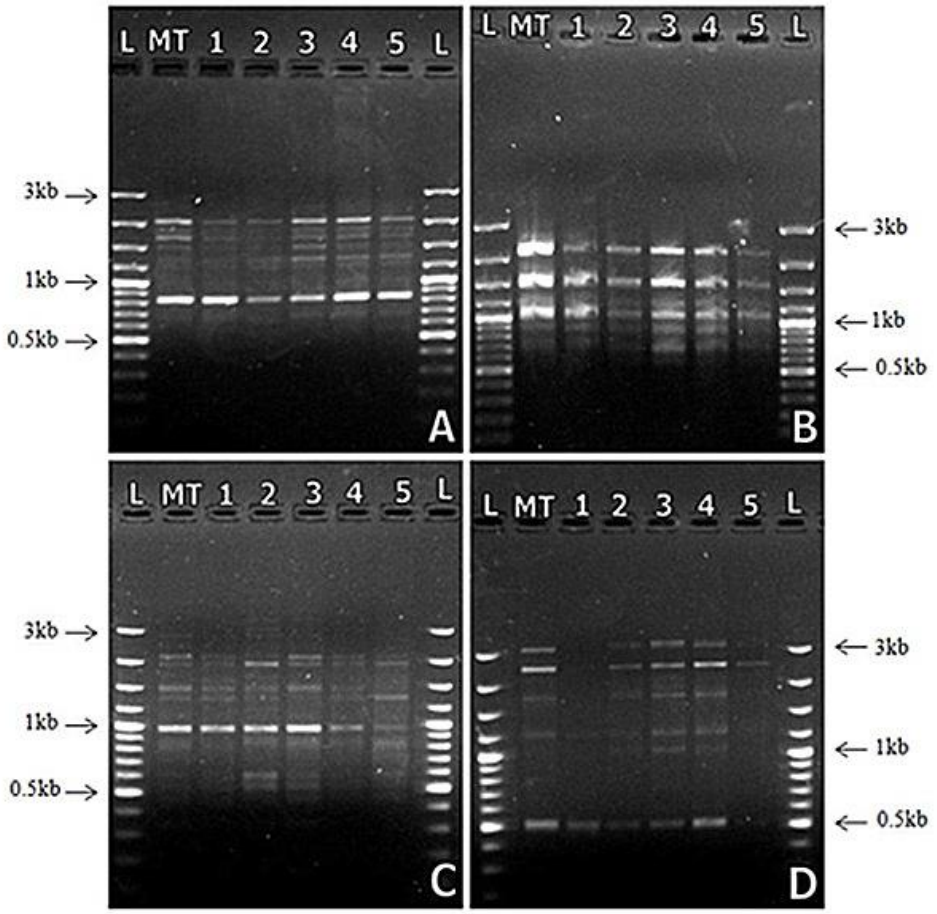

Fig. 3. DNA fingerprinting of mother tree (M) and tissue culture raised plants (1-5) of *H. binata*. **A.** RAPD analysis using primer OPL-16; **B.** Using primer OPI-06; **C.** Using primer OPL-02; **D.** Using primer OPI-18

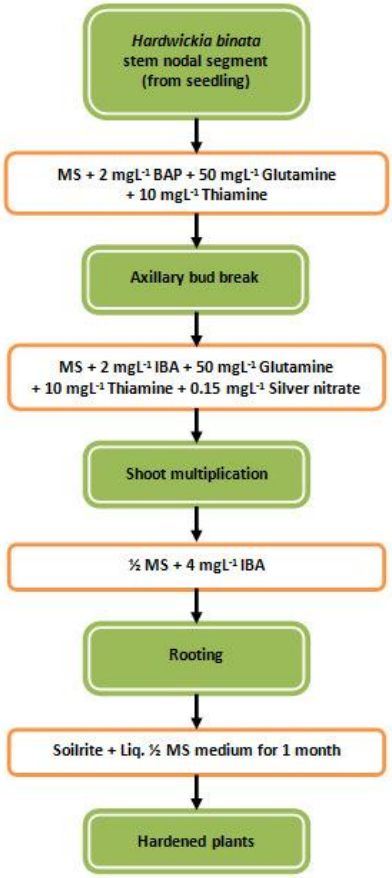

Fig. 4. Complete protocol for *in vitro* plant propagation of *H. binata*

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EMBRYOLOGICAL CHARACTERS TO STUDY THE *JUSTICIA-RUNGIA* COMPLEX (ACANTHACEAE)

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Abstract: Family Acanthaceae is characterized by very diverse plants whose taxonomic position is debated. Therefore, study of various macroscopic and microscopic characters had been used for studying the relatedness of the various taxa. Embryological characters are considered as one of the most stable characters. *Justicia–Rungia* Complex is one of the intriguing complexes present in family Acanthaceae. *Justicia* is basically a Linnaean Genus which included *Rungia* also. But later *Rungia* was separated from *Justicia* by Nees. Another offshoot of *Justicia* has been the formation of new genus *Rostellularia* which is also the creation of Nees. Due to this the *Rungia* is sometime confused as *Justicia* and some time it is considered as *Rostellularia.* In the present investigation embryological data is used to study the *Justicia–Rungia* Complex. The plants studied in present investigation are *Justicia procumbens*, *Rungia repens*, *Haplanthus verticillata* and *Blepharis repens*. The embryological study using UPGMA clearly indicates a very close similarity between *Justicia procumbens* and *Rungia repens*.

Keywords: Embryology, Acanthaceae, Justicia-Rungia Complex, UPGMA

Introduction

DANIEL (2009) considered the family Acanthaceae as a large pan-tropical family of about 229 genera and 3450 species. BREMEKAMP (1953) considered the family Acanthaceae to be highly heterogeneous and found that there is no single character which can be sufficient enough to classify. Therefore, study of various macroscopic and microscopic characters had been used for studying the relatedness of the various taxa. Embryological characters are considered as one of the most stable characters, since they are less prone to mutations. JOHANSEN (1945), CAVE (1953), KAPIL (1962), AREKAL (1963), JOHRI (1963, 1991), DAVIS (1966), BHANDARI (1971), PHILIPSON (1974), FALSER (1975), RAGHVAN (1997), BHOJWANI & BHATNAGAR (1999), IFRIM (2011) all of them effectively used reproductive and embryological characters for studying systematic placement of the taxon. DAHLGREN (1991) has also stressed the use of embryological characters as a next step towards the natural system of classification of the dicot plants. Family Acanthaceae have been reviewed by several authors from embryological point of view, in order to solve various taxonomic problems (MAURITZON, 1934; CRETE, 1951; MAHESHWARI & NEGI, 1955; JOHRI & SINGH, 1959; PHATAK & AMBEGAOKAR, 1963; MOHANRAM & WADHI, 1965; DAVIS, 1966; MAHESHWARI, 1963; LABHANE & DONGARWAR, 2011; LABHANE, 2011). *Justicia – Rungia* Complex is one of the interesting complexes present in family Acanthaceae, which needs to be studied from embryological stand point. In the present paper the

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reproductive and embryological characters are taken into consideration to study their relative affinities.

Materials and methods

The materials selected for the present investigation are *Blepharis repens* (Vahl) Roth., *Haplanthodes verticillata* (Roxb.) Majumdar, *Justicia procumbens* Linnaeus and *Rungia repens* (Linnaeus) Nees. All the taxa selected in the present investigation were collected mostly from the plants growing around Nagpur region, which is located in the central India, in the state of Maharashtra, with only exception, that *Haplanthodes verticillata* (Roxb.) Majumdar was collected from Sanjay Gandhi National Park (SGNP) Borivali, Mumbai. Nagpur falls within the tropical to sub-tropical region of central India, with temperature varying from 10-30 ºC during rainy and winter season to 40-45 ºC during summer. *Haplanthodes verticillata* was collected from SGNP, Mumbai belongs to North-western parts of Maharashtra, which shows more or less moderate climate throughout the year, with average temperate is 20-30 ºC. The plant material were identified with the help of standard flora namely viz., the Flora of Maharashtra [SINGH & al. 2001], Flora of Marathwada [NAIK, 1998], Flora of Nagpur [UGEMUGE, 1986] and Flora of British India [HOOKER, 1885]. The taxa under investigation were preserved in the form of herbarium specimen and deposited in the Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur with the accession numbers NML/201 – *Blepharis repens*, NML/202 – *Haplanthodes verticillata*, NML/203 – *Justicia procumbens* and NML/204 – *Rungia repens.*

The young buds and flowers in sufficient quantity were collected and fixed in formalin-acetic-alcohol and stored in 70% alcohol. The selected taxa were collected from Nagpur and Mumbai, during the months of August to March 2005-2010, when the plant bears buds, flowers and fruits. Customary methods of dehydration, infiltration and embedding were followed. Sections were cut 8-14 mm thick and stained with Heidenhain's iron-alum-haematoxylin. Light green was used as a counter stain. Twenty seven characters were recorded from the taxa whose reproductive and embryological studies are investigated (Fig. 2).

The cluster analysis was performed for the twenty seven reproductive and embryological characters by unweighted pair group method using arithmetic averages (UPGMA) [SNEATH & SOKAL, 1973]. The reproductive and embryological characters are used to study the *Justicia – Rungia* Complex, the closely related *Justicia procumbens* and *Rungia repens* are taken into consideration. *Blepharis repens* and *Haplanthodes verticillata* are included in the present investigation as out groups. The dendrogram was generated with the SAHN subroutine of NTSYS-PC to show similarity coefficient between the taxa [ROHLF, 1993].

Results and discussion

Twenty seven reproductive and embryological characters are selected carefully to estimate relative similarities (Tab. 1). The reproductive and embryological characters show close similarity between the two closely related *Justicia* and *Rungia* species (Fig. 2). The cluster analysis using un-weighted pair group arithmetic averages (UPGMA) shows the presence of three distinct clusters amongst the four taxa, when reproductive and embryological characters are taken into consideration (Fig. 1). The *Justicia–Rungia* cluster

shows a similarity coefficient of nearly 97%. The *Justicia–Rungia* cluster shows a similarity coefficient of 55% with the second cluster represented by *Haplanthodes*, which in turn shows a similarity coefficient of 22% with the *Blepharis* cluster. Thus the embryological characters assumes that the genera *Justicia* and *Rungia* are 97% similar to each other, whereas the most distantly placed taxa investigated is *Blepharis* which shows a similarity coefficient of just 22%.

BREMEKAMP (1938, 1944, 1953, 1955) gave a detailed account regarding his view on the subdivision of the family Acanthaceae and concluded in his paper titled 'The delimitation of the Acanthaceae' that the family is extremely heterogeneous and there is not a single morphological character by means of which it would be possible to delimit it from its allies. Thus the family Acanthaceae consists of very closely related taxa, whose placement is doubtful. Study of embryology, palynology, chemotaxonomy, anatomy etc had been used for studying the relatedness of the various taxa. *Justicia–Rungia* Complex is one of the exciting complexes present in family *Acanthaceae*. *Justicia* is a Linnean genus, based on *Justicia adhatoda* L. was split up by NEES (1832) into *Adhatoda, Justicia, Gendarussa* and *Rostellularia*. But this splitting was not widely followed. Later, BREMEKAMP (1944) resurrected these genera. GUNN & al. (1992) accepted *Adhatoda* Mill, *Justicia* L. and *Rostellularia* Reichb. This same course has been followed by NAIK (1998) in his flora of Marathwada with the submergence of *Rostellularia* in *Justicia*. Due to this the *Justicia* is sometime confused as *Rungia* and some time it is considered as *Rostellularia.* Hence, in order to understand the degree of similarity among the most closely related *Justicia procumbens* and *Rungia repens*, the reproductive and embryological characters are used to study the *Justicia–Rungia* Complex. Both *Justicia procumbens* and *Rungia repens* shows close similarity with respect to morphology. Another two taxa namely *Blepharis repens* and *Haplanthodes verticillata* are also included in the present investigation as out groups, since these taxa are morphologically quite distinct from the *Justicia–Rungia* Complex.

SCHNARF (1931) was the first to use embryology in solving taxonomic problems since embryological characters are considered as relatively stable and being less prone to adaptive stress. Embryological characters have acquired greater significance in plant taxonomy, especially when the external morphological characters are inconclusive and misleading as a result of convergence [KAPIL & BHATNAGAR, 1980]. MAHESHWARI (1950, 1963), and JOHN (1963) on the basis of their extensive work on embryology have provided list of families, genera, species, etc where embryology has either supported earlier classification or has proposed a new systematic position for the taxa concerned. Thus embryological evidences have been used in solving taxonomic problems at almost all levels and have helped to resolve the doubtful systematic position of several taxa. LABHANE & DONGARWAR (2011) based on embryological studies also confirmed the close relationship between the two species of *Justicia* and *Rungia*. However, previous attempts regarding the placement of taxonomic species lacks the statistics or quantitative element to descriptions. Statistical methods allow more rigorous comparisons between different forms and it has great significance in distinct grouping or separation of closely related species, which is used in the present study. The use of embryological characters and its analysis using the UPGMA for ascertaining the taxonomic alignment clearly indicates a very close relationship between the species of *Justicia procumbens* and *Rungia repens*.

Tab. 1. The reproductive / embryological characters selected for taxonomic alignment.

	Characters	*Blepharis*	*Haplanthodes*	*Justicia*	*Rungia*
1	Number of stamens	4	2	2	2
2	Anther cells *equal/unequal*	Unequal	Equal	Equal	Equal
3	Anther cell *spurred/not spurred*	Not spurred	Not spurred	Spurred	Spurred
4	Epidermal cells *small/large*	Small	Large	Small	Small
5	Stomium *pronounced/not pronounced*	Not pronounced	Not pronounced	More pronounced	More pronounced
6	Endothecium *1/2 layered*	1	2	1	1
7	Fibrous thickening *present/absent*	Absent	Present	Present	Present
8	Tapetal cells *2-3 / 2-4 nuclei*	2-4 nuclei	2-3 nuclei	2-3 nuclei	2-3 nuclei
9	Microspore tetrad *spherical/elongated*	Elongated	Spherical	Spherical	Spherical
10	Pollen grain *elongated/spherical/triangular*	Elongated	Triangular	Spherical	Spherical
11	Anther cells *parallel/superimposed*	Parallel	Parallel	Superimposed	Superimposed
12	Exine *uniform/not uniform*	Uniform	Uniform	Not Uniform	Not Uniform
13	Pollens *mono/dimorphic*	Monomorphic	Monomorphic	Dimorphic	Dimorphic
14	Number of ovules	2 Ovules	6-10 Ovules	4 Ovules	4 Ovules
15	Schizogenous Cavity *present/absent*	Absent	Present	Present	Present
16	Jaculator *long/short*	Long and lanceolate	Short and obtuse	Long and acute	Long and acute
17	Both micropylar and chalazal haustorium - *present/absent*	Absent	Present	Present	Present
18	Micropylar caecum *present/absent*	Absent	Absent	Present	Present
19	Micropylar haustorium at maturity- *present/absent*	Absent	Present	Absent	Absent
20	Chalazal haustorium at maturity- *present/absent*	Absent	Present	Absent	Absent
21	Secondary haustorium *present/absent*	Absent	Present	Present	Present
22	Endosperm *present/absent*	Absent	Present	Present	Present
23	Mature embryo *straight/curved*	Straight	Straight	Curved	Curved
24	Ornamentation on embryo *present/absent*	Present	Absent	Absent	Absent
25	Seed Coat *present/absent*	Absent	Present	Present	Present
26	Tubercles on seed *present/absent*	Absent	Present	Present	Present
27	Seed dispersal by *splitting/ degeneration*	Degeneration	Splitting	Splitting	Splitting

Conclusions

The present investigation shows that the two taxon, *Justicia procumbens* L. and *Rungia repens* (L.) Nees appears to be very similar with respect to the development of the embryological and reproductive characters. The genus *Rungia* is segregated from *Justicia* on the basis of minor morphological characters. However, the cluster analysis using UPGMA taking into consideration twenty seven embryological and reproductive characters showed 97% similarity coefficient between the two taxa and hence, the inclusion of species of *Rungia* under *Justicia* is justified. It seems that the two species might have got segregated very recently during the process of evolution leading to the formation of two distinct taxa, however the present study suggest that both the taxa *Justicia procumbens* and *Rungia repens* are reproductively very similar.

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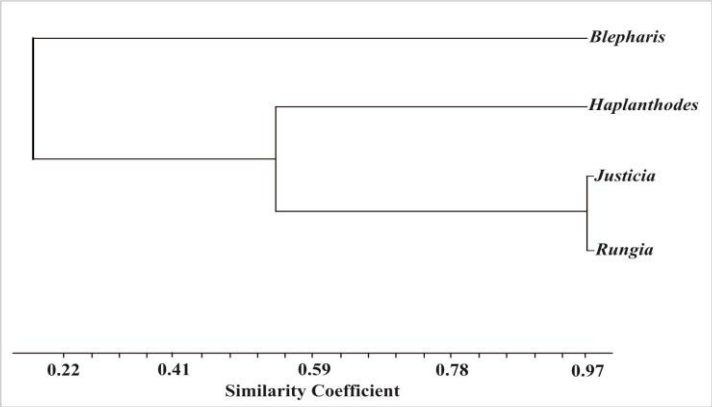

Fig. 1. Dendrogram based on embryological characters prepared by using unweighted pair group using arithmetic averages (UPGMA)

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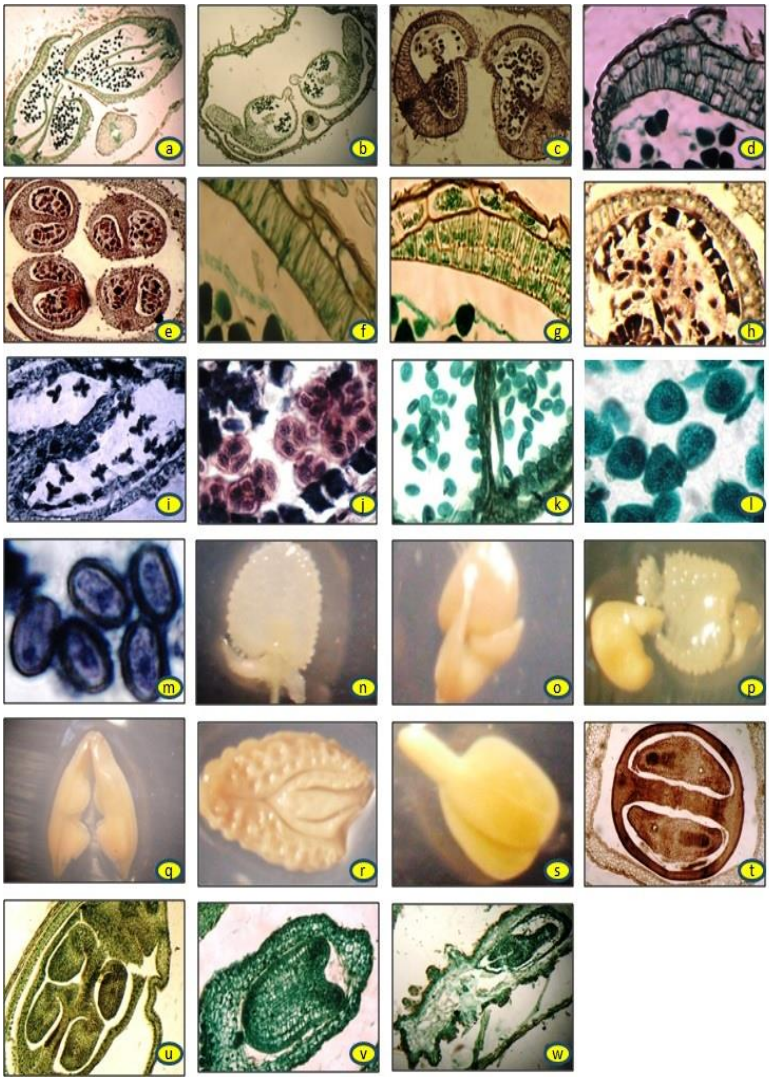

Fig. 2. a- Two anther with equal cells in *H. verticillata*; **b**- Spurred anther cell and prominent stomium in *J. procumbens*; **c**- Anther cells unequal and one layered endothecium in *B. repens*; **d**- Two layered endothecium *H. verticillata*; **e**- Four stamens without spurred anther cell in *B. repens*; **f**- Large epidermal cells in *H. verticillata*; **g**- Two layered fibrous endothecium in *H. verticillata*; **h**- Small epidermal cells without fibrous endothecium in *B. repens*; **i**- Elongated microspore tetrad in *B. repens*; **j**- Spherical microspore tetrad in *J. procumbens*; **k**- Pollen grains dimorphic and 2-3 nucleate tapetum in *J. procumbens*; **l**- Triangular pollen grains in *H. verticillata*; **m**- Monomorphic elongated pollen grains showing uniform thickening in *B. repens*; **n**- Long and acute jaculator in *J. procumbens*; **o**- Short and obtuse jaculator in *H. verticillata*; **p**- Mature embryo curved with seed coat as in *J. procumbens*; **q**- Four seeds in mature fruit in *J. procumbens*; **r**- Ornamentation (rumination) present with straight embryo in *B. repens*; **s**- No rumination with straight embryo in *H. verticillata*; **t**- Seed coat absent in *B. repens*; **u**- Four ovules in *J. procumbens*; **v**- Dicot embryo with endosperm in *H. verticillata*; **w**- Dicot embryo with endosperm and ornamentation on seed coat in *J. procumbens.*

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ANATOMY AND ONTOGENY OF *ASTRAGALUS REMOTIJUGUS* BOISS. & HOHEN. SEED

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Abstract: The aim of this study was to describe the anatomy and ontogeny of *Astragalus remotijugus* Boiss. & Hohen. seed using the usual techniques. The ovules are campilotropous, crassinucelate, and bitegmic. The nucellus cells disappear during an early stage of development. The following processes occur during integument development: anticlinal divisions and formation of palisade cells in the exotesta; predominantly periclinal divisions and cell expansion in the mesotesta; and endotesta differentiates in to an endothelium layer with thick and cubic in shape cells. The primary endosperm nucleus divides before the zygote nucleus, forming a nuclear endosperm. Endosperm cellularization begins when the embryo has developed the late globular stage. The embryological characters of *A. remotijugus* Boiss. & Hohen. are compared with other species of Fabaceae and those of other species of *Astragalus*. The remarked characteristic of the embryo was presence large suspensor with six columns of cells, and its large haustorial cells. Another difference in the development of *A. remotijugus* Boiss. & Hohen. seed was presence additional embryo at the globular stage that probably was developed from suspensor cells.

Keywords: embryogenesis, Fabaceae, polyembryony, seed coat, suspensor

Introduction

The Fabaceae family consists of approximately 650 genera and 18,000 species; it is one of the largest Angiosperm families [POHILL & al. 1981; JUDD & al. 1999]. *Astragalus* L., with about 3000 species worldwide, is the largest genus of flowering plants. The high variation of morphological characters has made infrageneric classification uncertain and problematic [SANDERSON & LISTON, 1995; SANDERSON & WOJCIECHOWSKI, 1996; WOJCIECHOWSKI & al. 1999; ZARRE, 2000; KAZEMPOUR-OSALOO & al. 2003, 2005]. It is important to emphasize that seed morphology usually shows little phenotypic plasticity. On the other hand, embryological characters, usually constant in the genera, function as a significant indicator of taxonomic affinity [RIAHI & al. 2003; RIAHI & ZARRE, 2009; FARHANGISABET & al. 2011]. According to these authors, there are few descriptive and ontogenetic studies on seed structure, which makes speculating about evaluative trends affecting seeds very difficult. Therefore, in the present study we investigate detailed embryology in *Astragalus remotijugus* Boiss. & Hohen. of *Astragalus* subgenera and *Caprini* section. The reason for selection this species is distribution at near of study place, because flower bud and fruits

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were collected at different stage. Also *A. remotijugus* Boiss. & Hohen. has big seed with relatively thin coat that is appropriate for section. The aim of this study was to describe the anatomy and ontogeny of *Astragalus remotijugus* Boiss. & Hohen. seed, emphasizing its testa, suspensor and endosperm structure.

Materials and methods

Flower buds, flowers and fruits were collected at different stage in May 2009 and 2010 from Alborze mountains north of Tehran province (Jajerood and Roodehen), Iran. Then fixed in formalin-glacial acetic acid and 70% ethanol (FAA_{70}, 5:5:90), stored in 70% ethanol, embedded in paraffin and sectioned at 6-8 µm with LEICA RM2255 rotary microtome. Staining was carried out using MICROM HMS70 and the periodic acid Schiff (PAS) and Meyer's Hematoxylin techniques. Sections were viewed with an OLYMPUS CX-31 light microscope.

Results

Astragalus remotijugus Boiss. & Hohen. ovules are campilotropous, crassinucelate, and bitegmic (Fig. 1A). The outer integument shows three to five layers of isodiametric cells; the inner integument has two to three layers. The micropyle is zigzag, with a larger number of cell layers in the exostome. The embryo sac cone- shaped just prior to fertilization in sagittal section. The embryo sac is 8- nucleate (7- celled) and of the Polygonum- type, which composed of 3 antipodal cells, 2 polar nuclei and a 3- celled egg apparatus. The egg apparatus consists of the egg cell and synergids (Fig. 1B). At the chalazal end of the embryo sac the nucellus consists of one or two cell layers as nucellus cap (Fig. 1C). The innermost of inner integument cell layer, the so called endothelium, is characterized by a thick layer of cuticle at the side adjacent to the embryo sac and large nucleated cells. These cells are cubic in shape (Fig. 1D).

Many alterations occur in the beginning of seed ontogeny, when the embryo is in proembryo stage (Fig. 1E). The first endosperm nucleus possesses a single large nucleolus. Few divisions of the primary endosperm nucleus occur before the first division of the zygote. Endosperm formation is free nuclear, that is, not followed by cytokinesis. In this manner the endosperm forms first at the micropylar chamber, then spreads towards the chalazal chamber as peripheral layer and remains free- nuclear for a while (Fig. 1E). The embryo development is the Onagrad type. Embryo differentiates in to a globular embryo proper and suspensor (Fig. 1F). Development of cellular endosperm begins during the late globular stage and showing great cytoplasmic density in two or three external peripheral layers (Fig. 2A). During the globular stage, the endosperm haustorium appears at the chalazal end. It is narrow and shows a tubular structure (Fig. 2A). The cells of the outer integument layer elongate and begin to differentiate to form the palisade layer (macrosclereid cells) characteristic for the testa of Fabaceae. The differentiation of the cells in this layer starts close to the hilum. The exotesta (palisade layer) is composed of a single cell layer, except for the region surrounding the hilum which is two layers thick (Fig. 2A). This layer is denser than the other layers, with cubic cells showing slight radial elongation. The mesotesta has two hypodermal layers which are composed of thin walled cells. Beneath this is endotesta with 3-4 parenchymatous cell layers with thick walls (Fig. 2A). The suspensor is large and is composed of six cell columns with several (Fig. 2B, E) inflated cells embedded in maternal tissue (Fig. 2B). After formation of cotyledon

primordia the embryo takes its heart- shaped form. By enlargement of cotyledons and embryo axis, the torpedo- shaped embryo grows in to the cellularized endosperm (Fig. 2C). In 10 % of ovule sections in globular embryo stage is an additional embryos that probably is originated from the suspensor cells (Fig. 2D, E). However, in 5% of ovules of *A. remotijugus* Boiss. & Hohen. there are more zygote to recognize, which represents most probably on abnormality. In such anomalous ovules three are 3 zygote, composed of polar nuclei, main egg and zygote cell that seem to be derived from one of the synergid (Fig. 2F).

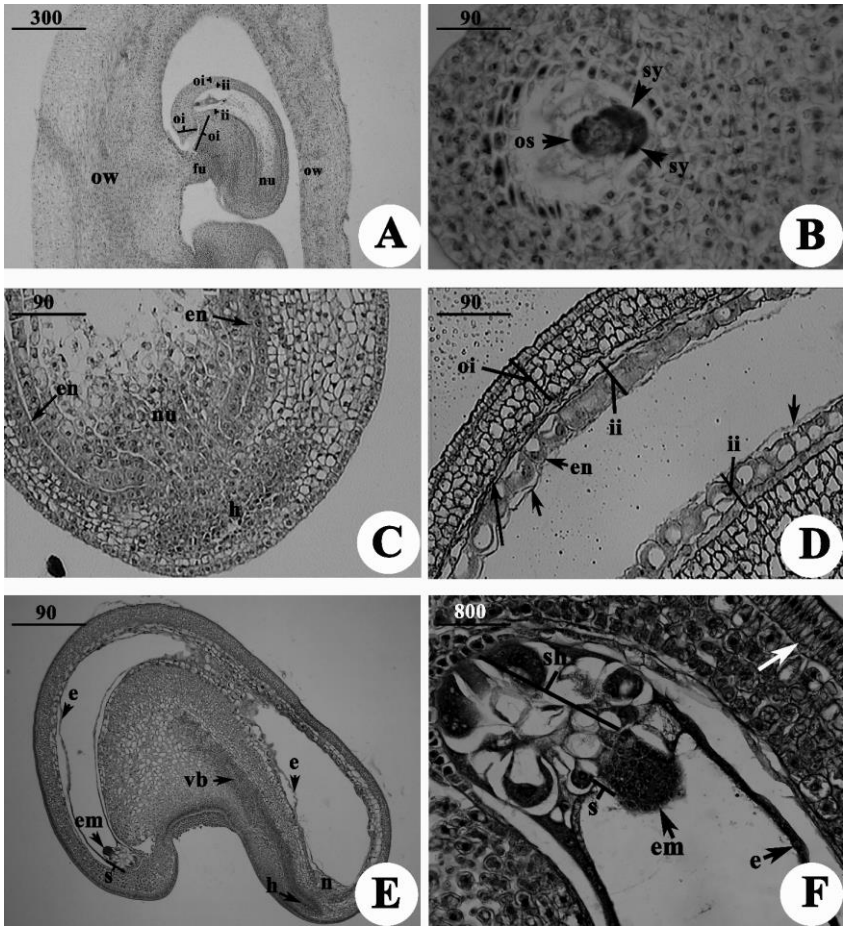

Fig. 1. A. Fertilized ovule in longitudinal section; the embryo sac (es) begins to be horse- shoe shaped; fu (funicle); ii (inner integument); oi (outer integument); nu (nucellus); ow (ovary). B. Transverse section of ovule before fertilization showing egg apparatus; os (egg); sy (synergid). C. Chalazal end of the embryo sac and nucellus cap; en (endothelium); h (hypostase); nu (nucellus). D. Detail of integument and endothelium, arrows indicate the cuticle layers. E. Section of the ovule showing the proembryo; coenocytic endosperm (e) forms a thin sheath around the embryo sac; e (endosperm); em (embryo); h (hypostase); nu (nucellus); s (suspensor); vb (vascular bundle). F. Detail of embryo with suspensor (s) and suspensor haustorium (sh); white arrow indicates the palisade layer; e (endosperm); em (embryo). Bars in µm.

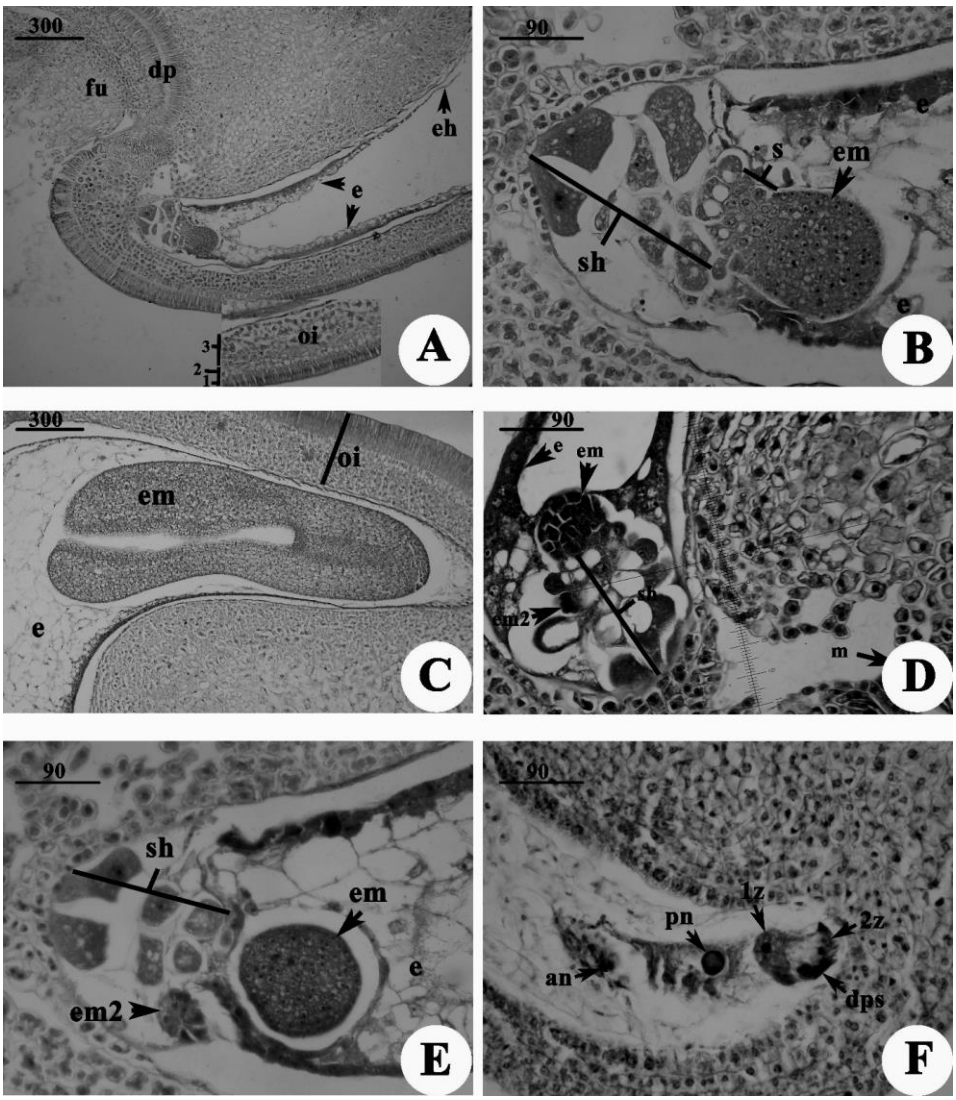

Fig. 2. A. General view of seed with detail the different parts of outer integument; e (endosperm); eh (haustorial endosperm); fu (funicle); dp (double palisade layer); oi (outer integument); vb (vascular bundle). B. Detail the embryo, suspensor (s) and suspensor haustorium (sh); glass arrow show peripheral endospermal cells are rich in cytoplasmic content; e (endosperm); em (embryo); suspensor (s); suspensor haustorium (sh). C. Cotyledon stage of the embryo (em); endospermal cells (e) are thin walled and begin to degerate; oi (outer integument). D. Early globular embryo (em) with additional embryo (em2) and coenocytic endosperm (e); suspensor haustorium (sh). E. Late globular embryo (em) with additional embryo (em2); e (endosperm); suspensor haustorium (sh). F. An abnormal young embryo sac including normal zygote (1z); fertilized synergid (2z); degenerative persistent synergid (dps); fertilized polar nuclei (pn) and antipodal cells (an). Bars in µm.

Discussion

Comparison with Fabaceae: Fabaceae seeds are typically testal, produced by bitegmic ovules, in which inner integument reduction frequently occurs [EAMES & MAC DANIEL, 1953; CORNER, 1951, 1976; LERSTEN, 1983; PRAKASH, 1987; AKHALKATSI & al. 1988; GVALADE & AKHALKATSI, 1996; OLIVEIRA & PAIVA, 2005]. In the Fabaceae endosperm varies from abundant to absent [CORNER, 1951, 1976; GUNN, 1981]. The studies by De CANDOLLE (1825) following which Fabaceae embryo structure have been considered of major important, resulted in the division of the Fabaceae in to two great subfamilies based on embryo axis curvature (Curvembriae and Rectembriae). The first subfamily includes the Faboideae and the second, the Caesalpinioideae and Mimosoideae. Although embryo axis curvature is presently not regarded as the best character for primary divisions in the family, it indicates better protection for the radical and may be one of a set of seed characters (especially hilar characters) used to separate the Faboideae from the other subfamilies [GUNN, 1981]. EAMES & MAC DANIELS (1953) generalized about the occurrence of complete absorption of the inner integument and nucellus during Fabaceae seed development. CORNER (1976) reported that because the tegument is crushed at maturity, it does not contribute to the seed- coat. Some species of the three Fabaceae subfamilies, in which the tegmen is absent in the mature structure, have been illustrated by CORNER (1951, 1976). BOESEWINKEL & BOUMAN (1984) also reported that the inner integument of Fabaceae is either crushed or reabsorbed. Several cases in the literature confirm this observation, e. g. *Indigofera enneaphylla* [DESHPANDE & UNTAWALE, 1971], *Indigofera parviflora* [MANNING & VAN STADEN, 1987], *Capaifera langsdorffi* [CRESTANA & BELTRATI, 1988], *Inga fagifolia* [OLIVEIRA & BELTRATI, 1993] and *Tipuana tipu* [MARTINS & OLIVEIRA, 2001]. Thus inner integument reduction is usual in Fabaceae, as is *Astragalus remotijugus* Boiss. & Hohen. seed, since has been demonstrated. Another distinguishing characteristic is embryo axis curvature in *A. remotijugus* Boiss. & Hohen.

Comparison with other *Astragalus* species: The embryo sac is Cone- shaped but finally differentiates in to the typical horse- shoe- shaped and this character is the same to other *Astragalus* species [AKHALKATSI & al. 1988; GVALADE & AKHALKATSI, 1996; RIAHI & al. 2003; RIAHI & ZARRE, 2009; FARHANGISABET & al. 2011]. The nucellus begins to degenerate when the embryo sac is formed. At the early globular embryo stage the nucellus is completely degenerated, although in some other *Astragalus* [RIAHI & al. 2003; RIAHI & ZARRE, 2005; FARHANGISABET & al. 2011] the disintegration of nucellus is completed at the formation of the proembryo.

The integuments development in *A. remotijugus* Boiss. & Hohen. was the same shown in other species of *Astragalus* [RIAHI & al. 2003; RIAHI & ZARRE, 2005; FARHANGISABET & al. 2011]. Differentiation of the integuments first occurs in the inner integument with the formation of the endothelium at an early stage of development. Then the inner integument degenerates at heart stage. The outer integument begins to take on the characteristic morphology of mature testa at proembryo stage, but reaches its maximal width and differentiation at the heart- shaped embryo stage. The main patterns in the development of endosperm in *A. remotijugus* Boiss. & Hohen. is similar with other cases recorded in *Astragalus*, i. e. *A. caucasicus* [AKHALKATSI & al. 1988], *A. denudatus* and *A. microcephalus* [GVALADZE & AKHALKATSI, 1996], *A. demavendicus* and *A.*

latifolius [RIAHI & al. 2003], *A. cemerinus* and *A. ruscifolius* [RIAHI & ZARRE, 2009], *A. eriocarpus*, *A. glaucacanthus*, *A. chrysostachys* and *A. compactus* [FARHANGISABET & al. 2011].

The shape of the embryo at different stages in *A. remotijugus* Boiss. & Hohen. is similar to other studied species of *Astragalus* [AKHALKATSI & al. 1988; GVALADZE & AKHALKATSI, 1996; RIAHI & al. 2003; RIAHI & ZARRE, 2009; FARHANGISABET & al. 2011], but considerable differences exist in the embryogenesis of this species that can be studied.

A typical characteristic of embryo is large suspensor which is composed of six columns of cells with several (10 to 15) inflated cells as haustorial cells. In comparing with other studies about *Astragalus* species, this type of suspensor is the largest type in studied *Astragalus* specieses [AKHALKATSI & al. 1988; GVALADZE & AKHALKATSI, 1996; RIAHI & al. 2003; RIAHI & ZARRE, 2009; FARHANGISABET & al. 2011].

An important character in this species is presence additional embryo at the globular stage. In this manner seems that additional embryo is developing from suspensor cells. The suspensor cells appear to be polyploidy, and the localization of the additional embryo indicates that the embryo arose from suspensor cells. According to articles published, this phenomenon is called polyembryony [BOTYGINA & VINOGRADOVA, 2007; CZAPIK, 1999]. In *Astragalus* genus this type of formation additional embryos has been described for the first time as well as some type of polyembryony. Another kind of polyembryony as perimitotic type of fertilization was reported in *A. caucasicud* [AKHALKATSI & al. 1988].

Conclusions

As a result of our work it becomes clear that such characteristics in the development of the ovule are most probably appropriate for separating taxa at species rank. More such studies on other species of *Astragalus*, can reveal the characteristics and differences useful for separating higher taxonomic ranks.

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ULTRASTRUCTURAL ASPECTS OF PROGRAMMED CELL DEATH IN THE EXOCARP OIL GLANDS OF MANDARIN (*CITRUS DELICIOSA* TEN.)

Artemios Michael BOSABALIDIS[1]

Abstract: In the exocarp of mandarin fruit (*Citrus deliciosa* Ten.), numerous globular/ovoid oil glands occur. In the centre of each gland, an essential oil-accumulating cavity is formed by a process of cell lysis. This process is induced by PCD which becomes ultrastructurally evident by the presence of a large number of fragmented ER-elements with a dark content. They appear only at the stage of PCD initiation and they disappear afterwards. ER-elements are scattered over the entire cytoplasmic area and do not locally aggregate or associate with other cell organelles and particularly the vacuoles. TEM observations favour the interpretation that ER involves in PCD of oil gland cells by releasing hydrolytic enzymes directly to the cytosol.

Keywords: *Citrus deliciosa* Ten., endoplasmic reticulum (ER), hydrolytic enzymes, oil cavities, programmed cell death (PCD)

Introduction

Programmed cell death (PCD) is a catabolic process which occurs during late development of specific tissues and results in their necrosis. It is genetically controlled and is associated with important physiological activities of the plant. PCD functionally deviates from deadly damages of tissues accidentally induced by biotic or abiotic agents. Plant tissues that undergo PCD are the following [BELL, 1996; RAVEN & al. 1999; EGOROVA & al. 2010; BOSABALIDIS, 2012]:

Xylem. Vessel elements of vascular bundles ultimately undergo disorganization of their protoplasts to constitute open tubular structures involved in water conduction.

Aerenchyma. Parenchyma cells in specific tissues disintegrate to create large intercellular spaces facilitating movement of gases during respiration, transpiration, and photosynthesis.

Sclerenchyma. Elongated cells with thick walls (fibers) undergo degeneration of their protoplasts contributing to the support of organs.

Cotyledons. During seed germination, cotyledon cells degrade after their content has been mobilized for growth of the seedling.

Pith. The central cells of some primary stems lyse resulting in the formation of an axial cavity which makes stems flexible.

Capsule. In the wall of the poppy capsule below the star-like stigma, groups of cells disintegrate to locally create pores through which seeds pass out and become dispersed.

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Cork. Dead cells with water-impermeable walls constitute the outer layer of the periderm in woody stems. They substitute the epidermis when herbaceous plants turn into woody plants.

Tapetum, embryo suspensor, nucellus. Their cells degrade after they have completed their nutritional mission.

Megasporogenesis. In the ovule of angiosperm flower, three out of four meiospores disintegrate to allow embryosac to develop.

Salt glands. Glands on leaves of halophytes differentiate, secrete NaCl, age, and ultimately die becoming replaced by other active glands. In this way the salt is continuously eliminated from the plant and does not accumulate in the tissues to create hyperosmotic phenomena.

Lysigenous oil glands. In the exocarp oil glands of citrus fruit, the central cells disorganize to create an internal cavity in which the secreted essential oil is accumulated.

In the present work, the manner of formation of the central cavity in the oil glands of the fruit peel of mandarin was studied as being associated with a process of programmed cell death.

Materials and methods

The present study was conducted at the mandarin orchard of the Agricultural School Farm of the Aristotle University, Thessaloniki. Small segments of ovaries (3-4 mm in diameter) randomly taken from ten open flowers of different plants of *Citrus deliciosa* Ten. (Rutaceae), were used. Ovary segments were initially fixed for 3h at room temperature with 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2). After washing in buffer, the specimens were post-fixed for 4h with 2% osmium tetroxide, similarly buffered. Samples were then dehydrated in an ethanol series (50-100%) and finally embedded in Spurr's resin. Semi-thin sections (1 μm thick) for light microscopy were obtained with a Reichert Om U_2 microtome (Reichert Optische Werke AG, Wien, Austria), stained with 1% toluidine blue in 1% borax solution, and observed in a Zeiss Axiostar Plus light microscope (Zeiss Microimaging GmbH, Göttingen, Germany). Ultrathin sections (80 nm thick) for electron microscopy were cut using a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a JEM 2000 FX II transmission electron microscope (Jeol Ltd, Tokyo, Japan).

Results

The oil glands in the exocarp of *Citrus deliciosa* exhibit, after conclusion of cell divisions, a globular/ovoid shape (Fig. 1A). The gland cells appear under the light microscope plasma-rich with small scattered vacuoles and they greatly differ from the surrounding parenchyma cells which contain large central vacuoles (Fig. 1A). Under the electron microscope, gland cells appear densely occupied by ribosomes and bear numerous mitochondria (Fig. 1B). The endoplasmic reticulum is represented by a few profiles of rough elements.

At late development of the gland, the cells of the central region enter PCD, i.e. they start to disintegrate and ultimately lyse creating a cavity (Fig. 2A, B). The cavity opens always prior to the stage of essential oil secretion to facilitate accumulation of the secreted oil. Opening of the cavity appears to initiate from a single cell in the centre of the gland which becomes disorganized (the degenerated cell organelles like plastids, dictyosomes,

mitochondria, etc. can be still discerned in Fig. 1D). Cell disorganization later extends to the surrounding gland cells, thus increasing the diameter of the cavity (Fig. 2A, B).

At early disorganization of the initial central cell of the gland, the endoplasmic reticulum (ER) greatly develops into many short elements having a dark content (Fig. 1C). Fragmented ER-elements have a normal thickness (66 nm) and do not aggregate at certain areas of the cytoplasm, but they are uniformly scattered all over the cytoplasm. Close associations of the ER-elements with other cell organelles or the vacuoles, were not observed. Vacuoles have a normal outline and do not form engulfments enclosing cytoplasmic portions.

At advanced disorganization of the gland central cell, the ground plasm becomes highly electron dense, the organelles deform, and the cell walls undergo an internal bending (Fig. 1D). Wall bending is probably due to the reduction of the osmotic pressure of the central degenerated cell, so that the surrounding turgid cells make protrusions into its lumen. Presence of dilated or deteriorated ER-elements was not observed. Finally, the central cell dies and lyses. These signs progressively extend to its bordering cells, ultimately leading to the formation of an open cavity (Fig. 2A, B). The gland central cavity is initially small and by PCD gradually increases in diameter (at the expense of the secretory cells) until it finally meets the peripheral sheath cells of the gland. After the gland cavity is fully-formed, secretory cells start secreting the essential oil which becomes released into it.

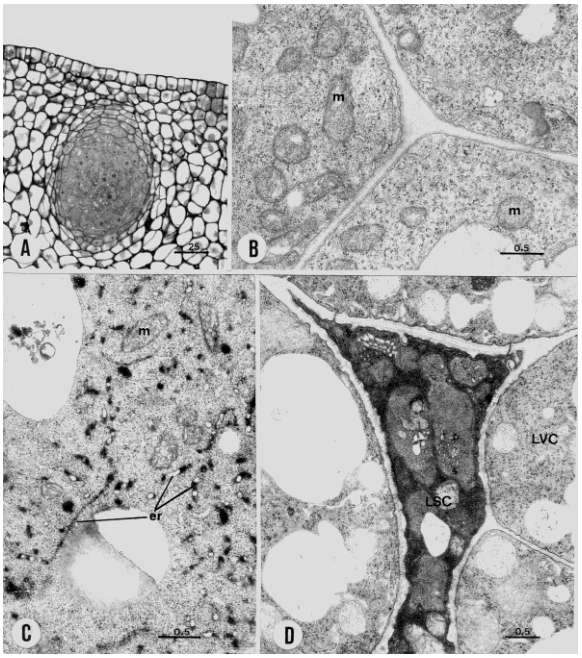

Fig. 1. *Citrus deliciosa.* A. Light-microscopical view of an exocarp oil gland after completion of cell divisions. B. Ultrastructural appearance of active gland cells. Mitochondria (m) are numerous and endoplasmic reticulum elements scanty. C. The central cell of an oil gland at early PCD. The ER is highly developed and consists of many short elements with a dark content (er). D. The central cell at advanced PCD. The ground plasm is electron dense and the organelles degenerated. LVC = living gland cell, LSC = lysed gland cell. Scale bars in μm.

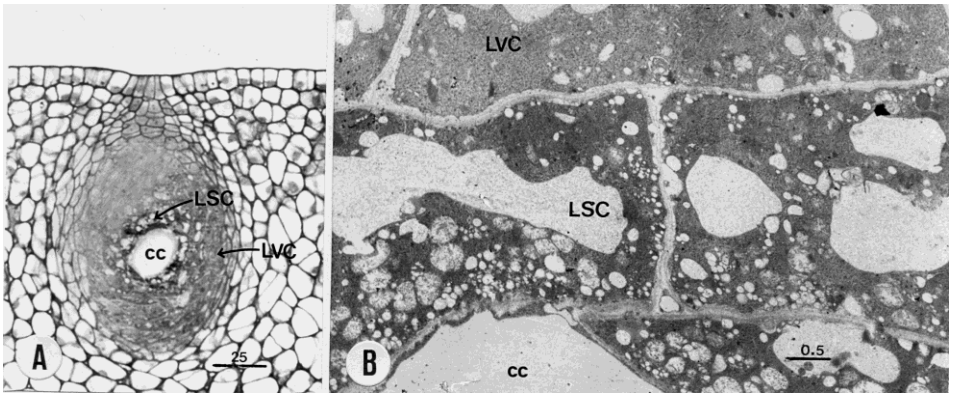

Fig. 2. *Citrus deliciosa.* A. Semi-thin section of an oil gland with an essential oil-accumulating cavity in the centre (cc). The gland cells facing the cavity (LSC) have undergone PCD, whereas those beyond the former (LVC) are still living. B. Ultrathin section illustrating at high magnification the difference in appearance between LSC cells and LVC cells, respectively. Scale bars in μm.

Discussions

Secretory oil cavities have been reported so far to initially open either lysigenously by disintegration of one or more cells [HEINRICH, 1969; BOSABALIDIS, 1982] or schizogenously by separation of two or more cells [BUVAT, 1989; TURNER & al. 1998]. Regardless whether the initial stage of cavity formation proceeds lysigenously or schizogenously, the important fact is that the whole cavity later develops (increases in volume) exclusively by disintegration of the secretory cells (lysigenously). In this process, PCD has a decisive participation. In the oil cavities of *C. deliciosa*, the central space appears to initially open by disintegration of a single cell. A prominent feature of advanced senescence of this cell is the high electron density of the ground plasm. The degenerated protoplasm remains closely attached to the cell wall and does not exhibit signs of plasmolysis [BOSABALIDIS, 2012]. At the stage just prior to senescence of the gland central cell (PCD initiation), the ER characteristically undergoes pronounced development. An analogous feature has been reported in the senescent cells of the abscission zone of apple flower and fruit [PANDITA & JINDAL, 2004]. The fact that ER greatly develops at the stage just prior to gland cell senescence (and not at any previous stage) and disappears after this stage, strongly indicates that this organelle has a crucial role in PCD initiation. A role of the ER as a PCD initiator has been also expressed by EICHMANN & SCHAEFER (2012).

Of interest is the observation that ER cisternae at early PCD of the gland central cell become fragmented into many short elements distributed all over the cytoplasmic area. ER fragmentation may be mediated by ER stress which in turn induces PCD [WOJTYLA & al. 2013]. ER stress is activated by misfolded or unfolded proteins that accumulate in the ER lumen [DENG & al. 2013]. Relevant to the above observation is that fragmented ER elements do not develop associations with other cell organelles and particularly with the vacuoles. The fusion of ER membranes with the tonoplast and the release into the vacuoles of lytic enzymes (autophagic vacuoles) has been well established [OUFATTOLE & al. 2005; YAMADA & al. 2005; MUENTZ, 2007]. The lack of association of the ER with the vacuoles in the senescing oil gland cell of mandarin, and also the lack of cytoplasm-

engulfing vacuoles, favour the interpretation that PCD in these cells is induced not by autophagic vacuoles, but by the autonomous operation of the ER (release of hydrolytic enzymes directly from ER to cytosol).

Presence of various hydrolytic enzymes in the ER has been reported in a number of studies. Thus, MOOR & WALKER (1981) identified in the cytosol of *Sedum* cells a high activity of acid phosphatase associated with cellular autolysis and cell death. They considered that the hydrolytic enzyme is biosynthesized in the ER (cytochemical localization) from where it becomes release into the cytosol leading finally to the necrosis of the cells. LAMPL & al. (2013) further reported that PCD in plants is promoted by the release into the cytosol of ER-compartmentalized proteases. Similarly, MULISCH & al. (2013) identified by immunogold labeling, ER-associated cysteine proteases in the tracheary elements and considered that they involve in PCD. Activity of the hydrolytic enzymes acetylesterase and nuclease was cytochemically localized also in the ER [DEJONG & al. 1967; FARAGE-BARHOME & al. 2011].

Conclusions

Conclusively, in the present study, anatomical and ultrastructural results indicated that ER might have a decisive role in PCD of mandarin oil glands as an independent organelle.

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A RUMINATE EMBRYO IN *BLEPHARIS REPENS* (VAHL.) ROTH. (ACANTHACEAE)

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Abstract: The study of morphology of embryo is very significant considering the fact that the embryo represents the important step in the determination of the viability of the seed. Ruminate endosperm has been reported in about 58 families of angiosperms. The rumination caused by the activity of the seed coat or by the endosperm itself is quite recurrent in angiosperm. Ruminate endosperm due to seed coat is reported from the family Acanthaceae in *Andrographis paniculata*. The rumination of endosperm is also considered as phylogenetically important. Rumination of endosperm is very common, however very little is known about rumination in embryo. The present papers reports the *de novo* development of ruminate embryo in *Blepharis repens*. The development of ruminate embryo is seen as an adaptation to ensure proper aeration and optimum germination for survival of the species.

Keywords: rumination, seed, ruminate embryo, endosperm

Introduction

Angiosperms are characterized by double fertilization, which initiates the development of two intimately interconnected multicellular structures, the embryo and the endosperm, which are derived from the zygote and the fertilized central cell, respectively [VIJAYRAGHVAN & PRABHAKAR, 1984]. The post fertilization product of ovule is seed, which harbor embryo and the endosperm [MAHESHWARI, 1950, 1963]. Embryo survives on the nourishment provided by the endosperm in case of albuminous or endospermic seed. However, in exalbuminous or nonendospermic seed, the nourishment to the developing embryo is provided by the surrounding tissues, since the endosperm whatsoever present is utilized during the early developmental stages of the embryo. Thus the role of endosperm and surrounding tissues for nourishment, leading to the normal growth and development of the embryo is very decisive for the survival of the species [RAGHAVAN, 1997]. The first developmental study on ruminate structure has been reported from family Araliaceae on taxon *Hedera helix* [HEGELMAIER, 1886]. The term ruminate is defined as an uneven endosperm surface that is often highly enlarged by ingrowths or in folding of surrounding tissues [PERIASAMY, 1962b]. The term ruminate endosperm is pertinent only when the endosperm is present, however many earlier reports were from exalbuminous taxa [PERIASAMY, 1962a; DAHLGREN, 1991]. Under the rubric of rumination those seeds are also included that at maturity showed a folded or ruminate structures on embryo [DAHLGREN, 1991]. Thus the term rumination is applicable to the embryo as well as the endosperm. WERKER (1997) considered the seed as ruminate if the surface of any part of

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the seed is irregular or uneven. JACOBS & al. (2008) considered three types of rumination one resulting from the shape and structure of the surrounding endocarp, while the last two types of ruminations are due to uneven growth of specific tissues of the seed, i.e., seed coat and endosperm, and which develops independently of the adjacent endocarp. The rumination or ornamentation in the seed is either by the activity of the seed coat or the endosperm of the seed itself. Ruminate nature of the seed has been reported in about 58 families of angiosperms [BAYER & APPEL, 1996]. However, *de novo* development of ruminate embryo is not reported in any angiosperms.

Materials and methods

The material selected for the present investigation *Blepharis repens* (Vahl) Roth. is collected mostly from the Nagpur district. Nagpur falls within the tropical to sub-tropical region of central India in the state of Maharashtra, India. Temperature of Nagpur during rainy and winter season varies from 10-30 ºC, while during summer it goes up to 40-45 ºC. The plant material was identified with the help of standard floras, viz., the Flora of Maharashtra [SINGH & al. 2001], Flora of Marathwada [NAIK, 1998], Flora of Nagpur [UGEMUGE, 1986] and Flora of British India [HOOKER, 1885]. The taxa under investigation were preserved in the form of herbarium specimen and deposited in the Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur with the accession numbers NML/201.

Young and mature fruits of *B. repens* (Vahl) Roth. were collected every year from 2006-2011, during the months of October to March from various locations, so as to nullify the localized effect on the morphology and development of the seed and the embryo. The fresh fruits, as well as fruits fixed in formalin-acetic-alcohol were collected and stored in 70% alcohol for carrying out further investigation. Under dissecting microscope, the fresh and preserved fruits were dissected to study the morphology of the seed and the embryo at maturity.

Results and discussion

The capsular fruit is more or less compressed (Fig. 1A) with a central partition wall, showing the presence of one seed in each chamber. The mature fruit of *B. repens* is green, before turning brown on drying and shows the presence of two seeds (Fig. 1B). The mature fruit is 0.8 cm long and 0.6 cm broad. The mature seed on dissection shows the presence of seed coat which encloses the embryo. The seed is 0.6 cm long and slightly triangular in shape. The seed coat is covered profusely with of long and hairy outgrowth, which becomes mucilaginous on hydration (Fig. 1C, D). Endosperm is completely absent at maturity, thus the seed is non-endospermic at maturity [LABHANE, 2011]. Thus, the formation of the ruminate structures due to endosperm seems to be distant reality. The endosperm was absent but still ruminate structures is seen on the mature embryo. The mature embryo is more or less triangular, dorsi-ventrally compressed showing the presence of two distinct cotyledons and radicle (Fig. 1E, F). The embryo is upright with radicle, cotyledon and plumule lying in one straight line. The mature embryo is 0.4-0.5 cm in length along the long axis of the embryo. The cotyledons show the presence of distinct ridges and furrows on its surface. The ornamentation on the surface of the cotyledon is more or less circular in outline and appears

as small craters or pits. Thus the mature embryo shows distinct ruminate structure (Fig. 1E, F). The ornamentation is also present on the cotyledons of the embryo, however ornamentation on stalk is less pronounced as compared to the cotyledons. The presence of ruminate structure is family Acanthaceae has also been reported in *Andrographis serpyllifolia* [MOHANRAM, 1960] and *Elytraria acaulis* [JOHRI & SINGH, 1959]. However in both the cases, the origin of ruminate structure is attributed to the seed coat or the irregular development of the endosperm. In the present investigation, the endosperm is completely absent in the mature seed and the seed coat is not having any kind of irregular outline or in growth inside the seed [LABHANE, 2011]. Thus the development of ruminate structure on the embryo in *B. repens* seems to be *de novo*.

The study of ruminate nature of embryo and endosperm is the pioneer work of PERIASAMY (1962a, 1962b, 1966, 1990), however the first developmental work on ruminate structures in angiosperms was done on *Hedera helix* [HEGELMAIER, 1886]. The number of families of angiosperms showing ruminate structure varies according to different authors. PERIASAMY (1962a, b, 1966) reported ruminate structure in 26 families, DAHLGREN (1991) 25 families, BHOJWANI & BHATNAGAR (1999) 32 families, whereas BAYER & APPEL (1996) reported in 58 families.

The numbers of ovules per ovary and the number of seeds per fruit vary considerably in different members of the family Acanthaceae [RENDLE, 1938]. LABHANE & DONGARWAR (2012) reported about 50% embryo abortion in *B. repens*, hence at maturity the fruits in most of the cases showed the presence of only one seed, since one of the seed is aborted. At early stages and till maturity the fruit shows the presence of two seeds (Fig. 1B), but when the mature fruits were dissected it showed the presence of one fully developed embryo and other embryo was found to be rudimentary and aborted. Embryo abortion has also been reported in some other members of family Acanthaceae such as *Justicia procumbens* L., *Rungia repens* (L.) Nees and *Haplanthodes verticillata* (Roxb.) Majumdar [LABHANE & DONGARWAR, 2012]. Embryo of *B. repens* seems to have evolved ruminate morphology, with distinct ornamentation all over the cotyledons to facilitate proper aeration and optimum germination, the same is also reported by GOEBEL (1933) and ARNDT (1967). As the fruit contains one or two seeds, the *B. repens* seems to have evolved ruminate embryo to ensure its own survival. The ingrowths seen in ruminate structure often contain phenolic substances or ethereal oils which led to the assumption that rumination might make the seeds less attractive to predators [MULLER, 1887; OSENBRUG, 1894; GOEBEL, 1933]. The various substances which provides chemical defense in ruminate structures have been detected in the ingrowths, but not in the endosperm [MULLER, 1887; MEYER, 1891].

The presence of ruminate structures in family Araliaceae showed graded variation in various taxa of that family, suggesting the importance of ruminate structures in angiosperms [HARMS, 1894]. GURKE (1890) was most probably the first to use the variation in the structure of ruminate endosperm for taxonomic segregation of the various species of the genus *Diospyros*. CHUANG & CONSTANCE (1992) considered the ruminate character to be very useful, while studying the seeds and systematics in *Hydrophyllaceae*. JACOBS & al. (2008) found the seed character as phylogenetically very important while classifying 31 species of *Viburnum* belonging to *Caprifoliaceae*.

The presence of ruminate structure in angiosperms both in the primitive as well as the advanced taxa suggest that ruminate character seems to have evolved several time

independently [NETOLITZKY, 1926]. PERIASAMY & SWAMY (1961) reported ruminate structure in family Annonaceae. PERIASAMY (1963, 1964, 1990) reported ruminate structure from related families such as *Rubiaceae* and *Apocynaceae*. The presence of rumination was considered as a primitive trait of angiosperms [TAMAMSHJAN, 1951]. The occurrence of ruminate endosperm might represents an ancestral feature but still present in both primitive and advanced taxa of the angiosperms [VIJAYARAGHAVAN & PRABHAKAR, 1984]. In fact, the occurrence of ruminate endosperm in several families of the *Magnolianae* and *Monocotyledoneae* further strengths the assumption that ruminate structure might have evolved several times in angiosperms, independently. It seems that the presence of ruminate morphology in angiosperms needs to be further exploited so as to assess its viability in phylogenetic consideration.

MOHANRAM (1960), JOHRI & SINGH (1959), PERIASAMY (1962b), DAHLGREN (1991), BAYER & APPEL (1996) reported ruminate morphology in some of the members of the family Acanthaceae, which is due to the uneven activity of endosperm or the seed coat; however *de novo* development of ruminate embryo as seen in *B. repens*, is not reported till date. Endosperm is absent at maturity (Fig. 1D), since it is completely utilized by the developing embryo at early stages of seed development [LABHANE, 2011]. Thus the seed is non-endospermic at maturity. Seeds being devoid of endosperm, the outgrowths present on the seed coat which forms a mucilaginous mass might function as the reserve food material for the developing embryo. The development of such embryo with outgrowths on the seed can be attributed to the ecological conditions in which the species is growing. At maturity even the hairy outgrowths are consumed by the voracious developing embryo, since the number of hairy outgrowths is very less in dried seeds as compared to seeds collected before drying [LABHANE, 2011]. The seed character in *Silene* L. has also been used for identification of various species [IFRIM, 2011]. The two species of *Blepharis* namely *B. repens* and *B. maderaspatensis* were found to phonologically and reproductively distinct [DABGAR & MALI, 2010]. The flower of *B. repens* is found to be having the covered by one bract and seven bracteoles, so the fruit in case of *B. repens* is found to be strongly attached to the mother plant, making it difficult for the seeds dispersal. Hence the seed in most of the cases germinates while the fruit is still attached to the mother plant under favorable conditions.

De novo development of ruminate embryo in *B. repens* is obvious, as the seed is exalbuminous and there is complete absence of uneven growth of specific tissues of the seed, i.e., seed coat and endosperm. The adaptation of the plant species for survival in order to have maximum aeration and germination of the seed is by the development of the rumination of the embryo.

Conclusions

The presence of ruminate embryo in *B. repens* seems to have evolved ruminate morphology, with distinct ornamentation all over the cotyledons to facilitate proper aeration and optimum germination. Thus the development of rumination in some plant species, such as *B. repens* appears to be a step towards its own conservation during the process of evolution, since it also shows the embryo abortion.

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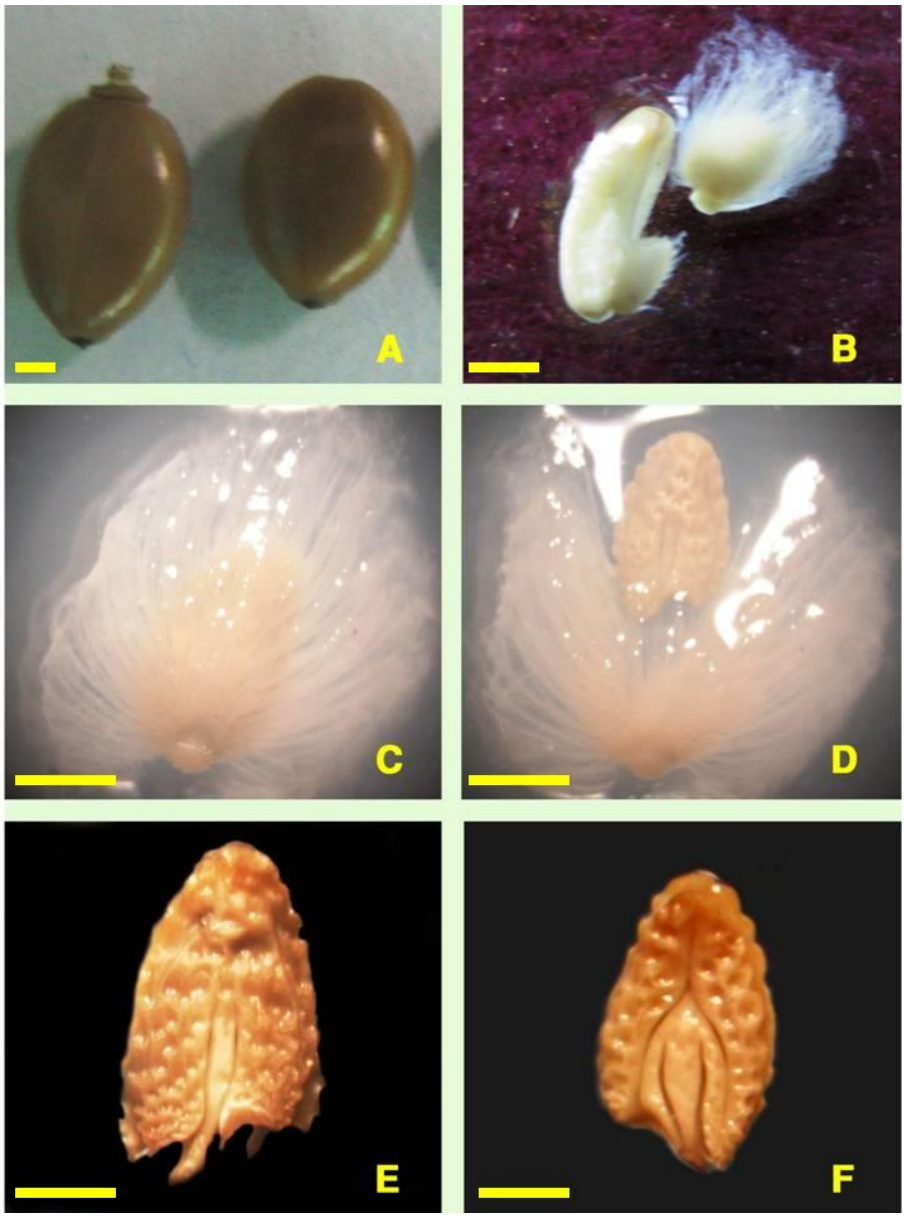

Fig. 1. A. Mature fruit; **B.** Seeds dissected out of the fruit; **C.** Seed at maturity; **D.** Seed dissected with mature embryo; **E.** Adaxial view of embryo; **F.** Abaxial view of embryo (Scale = 100 μm)

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NITRATES IMPROVED SEED GERMINATION PERFORMANCE IN *COMMIPHORA WIGHTII* (GUGGAL), A DATA DEFICIENT MEDICINAL PLANT FROM THE INDIAN ARID ZONE

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Abstract: The present article deals with seed germination behaviour of *Commiphora wightii* (Guggal) by using various concentrations (0, 5, 10, 15 & 20 mg L^{-1}) of different nitrate solutions such as NH_4NO_3, $Co(NO_3)_2$, $Ca(NO_3)_2$ and KNO_3 under nursery conditions. At present, plant is considered under data deficient category. The seeds were collected from four different sites of three districts in western Rajasthan, *viz*. Jodhpur, Jaisalmer and Barmer. Results revealed that seeds presoaked in $Ca(NO_3)_2$ and KNO_3 were found to be most suitable for germination under nursery conditions as compared to others. The best results for germination initiation, germination duration (days), germination percentage, peak value (PV), root and shoot lengths, collar diameter and biomass yield were also observed maximum in these two nitrate pretreatments.

Keywords: *Commiphora wightii*, nitrate solutions, germination initiation day, peak value (PV), data deficient, medicinal plant

Introduction

Commiphora wightii (Arnott) Bhandari (Family Burseraceae) locally known as Guggal, is a perennial branched shrub or small medium sized tree up to 1.5-3.0 m in height, with crooked and knotty branches ending in sharp spines (Fig. 1). The stem is covered with silvery white, papery bark that peels-off as flakes from the older parts of stem, whereas the younger branches are pubescent and glandular [ANONYMOUS, 2008]. The plant almost remains leafless except in rainy season. It is a very slow growing species on rocky substratum and grows in shallow, gravelly, unfertile soil, hilly terrains, open canopies and adaptable to high temperature (45 °C) in arid and semi-arid climates with an annual rainfall 225-500 mm [KUMAR & SHANKAR, 1982; KULHARI & al. 2012]. The plant produces fruits throughout the year, but maximum fruit production takes place from January to April. Fruits are ovoid, single, or 2-3 in a bunch, bright red when riped. Seeds are ovoid, bilobed, sometimes trilobed or rarely tetralobed. Immature seeds are reddish brown, while matured ones are yellowish- white and black [PRAKASH & KASERA, 2000; LAL & KASERA, 2010]. The plant is a source of oleo-gum resin, which exudes from bark, is an effective drug in several diseases in Indian Systems of Medicine.

Presently, over-exploitation, poor seed production, rare seed germination, slow growth rate, lack of cultivation, excessive and unscientific tapping method, over-grazing by domestic animals, mining activities and invasion of alien species, etc. are some of the major reasons to its destruction in natural habitats and make this plant an endangered species of

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Indian Thar desert [KASERA & PRAKASH, 2005; REDDY & al. 2012]. Recently, IUCN declared its status as under "Data Deficient Category ver. 2.3" [IUCN, 2012].

The regeneration of this plant takes place vegetatively either by stem cuttings or air-layering, but through seeds is extremely poor in nature [YADAVA & al. 1999; KASERA & PRAKASH, 2005]. A large number of chemical substances such as various nitrate solutions have been reported by many researchers for breaking dormancy in seeds, enhancing their permeability, inducing and hastening the germination and thereby acting as chemical regulator for seed germination. Chemical substances may behave as germination stimulator or inhibitor and their effects on inhibitions and germination may vary [SEN, 1977; SWAMI & al. 2011]. In view of this, in the present investigation an attempt has been made to study the morphological parameters of seeds as well as effect of different concentrations, *i.e*. 5, 10, 15 & 20 mg L^{-1} of various nitrate solutions such as NH_4NO_3, $Co(NO_3)_2$, $Ca(NO_3)_2$ and KNO_3 for enhancing seed germination potentialities as well as seedling parameters of *Commiphora wightii*, an important data deficient medicinal plant from the Indian Thar desert.

Fig. 1. *Commiphora wightii* – plant growing in natural habitat

Materials and methods

Mature ripened fruits of *C. wightii* were collected from four sites (Fig. 2) of western Rajasthan, *viz*. Machia Biological Park (MBP), Jodhpur (site-I; 11 km away from the University Campus in west direction); Beriganga, Jodhpur (site-II; 34 km in north-east); Kiradu, Barmer (site-III; 250 km in south-east) and Aakal Wood Fossils Park (AWFP), Jaisalmer (site-IV; 300 km in east direction from the University Campus, Jodhpur) during January-April (2011 & 2012). The pulp of air-dried fruits was removed gently through hand rubbing and seeds were washed in running tap water to remove any adhering pulp and other growth inhibitors (Fig. 3). Afterwards, the air-dried seeds were stored in plastic container with parad tablets to protect them from insets. Two types of seeds, *viz*. black and whitish-black have been observed from mature fruits (Fig. 4). The black seeds are viable, and a single seed produces more than one seedling due to its polyembryonic nature. However, whitish-black seeds were non-viable due to absence of embryo. Seed viability was tested by the tetrazolium method [PORTER & al. 1947]. Experiments were carried out at the University Campus during February-March 2011 & 2012 under nursery (*in-vivo*) conditions. The fresh black seeds were sown under *in-vivo* conditions in thermo cups, containing sand, clay and FYM (Farm Yard Manure) in 1: 1: 1 ratio at 0.5-1.0 cm depth.

Before sowing, seeds were presoaked for 24 h in different concentrations (5, 10, 15 & 20 mg L^{-1}) of nitrate solutions such as NH_4NO_3, $Ca(NO_3)_2$, $Co(NO_3)_2$ and KNO_3. For control experiments, seeds were presoaked in distilled water for same duration. Two seeds were sown in each thermo cup and total 45 replicates were maintained for each pretreatment. Irrigation was provided as and when required according to environmental conditions. To the germinated seeds, the seed counting process was begun with the day on which they were sown in soil mixture ratios to the end of last germination of seeds. A seed was considered to germinate when seedling was emerged out from soil surface. The germination initiation day, germination duration (days) and germination percentage values were recorded till last seed germination. While, seedling parameters such as root and shoot lengths, collar diameter and total dry weight were measured after completion of the three months of setting the experiments. The Peak Value (PV) was calculated by using the following formula:

$$PV = \frac{\text{Final germination percentage}}{\text{Number of days that took to reach the peak germination}}$$

Seed morphological parameters and the mean value of two years data pertaining to effect of different concentrations of nitrate solutions on seed germination are presented in the Tab. 1, 2 and 3. Data of three-months-old seedlings with various parameters were generated for analyses of variance using Randomized Block Design (RBD) in accordance with GOMEZ & GOMEZ (1984).

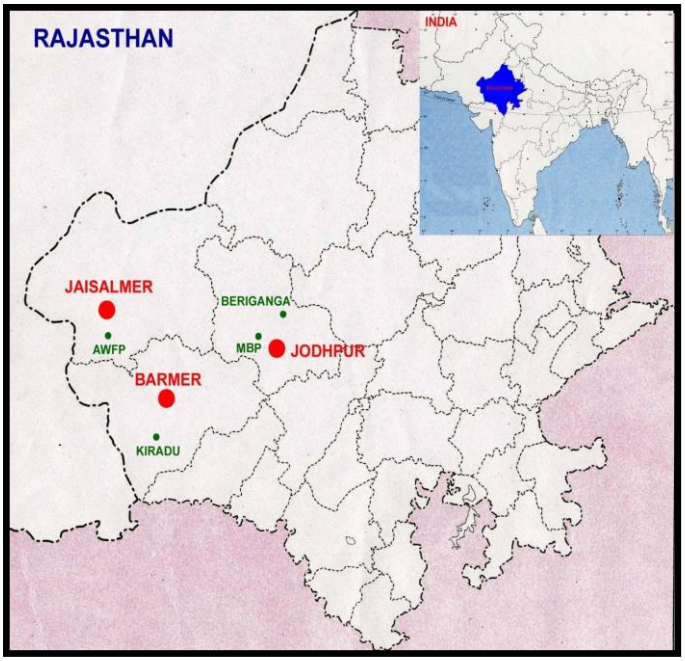

Fig. 2. Map of four selected sites of western Rajasthan for collection of *C. wightii* germplasm. MBP = Machia Biological Park; and AWFP = Aakal Wood Fossils Park

Results

The following experiments were conducted in the present investigations.

Weight, size, viability and ratio of black & whitish-black seeds

It is evident from Tab. 1 that there were significant variations in morphological parameters of seeds such as weight, size, viability and ratio of black and white seeds, collected from sites-I-IV. The seeds collected from site-III were heaviest in weight and largest in size, while those from site-I were lightest and smallest (Fig. 3). The highest viability and ratio of black and whitish-black seeds were observed in seeds collected from site-IV, while minimum from site-I.

Tab. 1. Morphological parameters of *C. wightii* seeds collected from sites-I-IV

Sites	Weight of 100 seeds (g)	Size (mm)			Viability (%)	Black and whitish-black seeds ratio
		Length	Breadth	Thickness		
I	2.557	5.1	4.5	3.2	53.33	0.563
II	2.790	5.5	4.7	3.4	61.66	0.693
III	4.426	6.8	5.8	4.1	68.33	0.617
IV	2.619	5.9	5.5	3.6	81.66	3.426
CD	0.240*	0.815*	0.452*	0.317*	18.763*	0.748*

*
= significant at $p<0.05$.

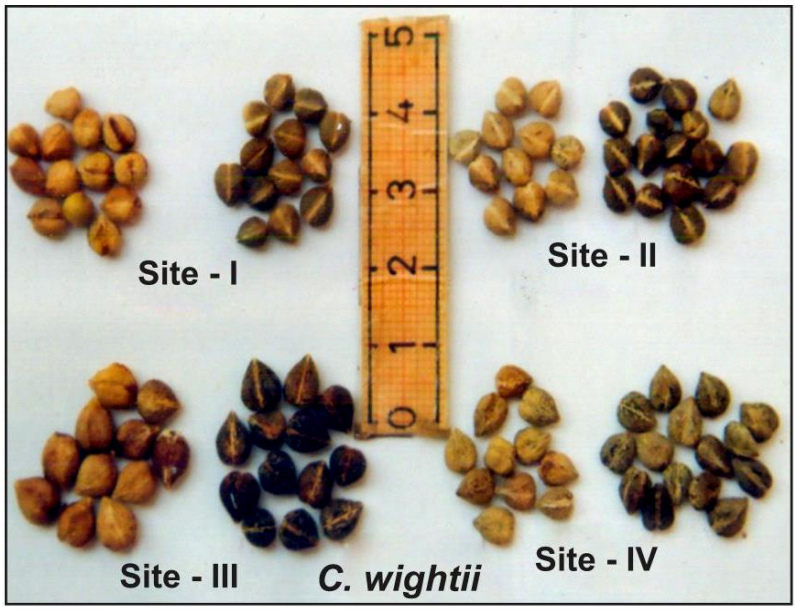

Fig. 3. Variation in colour and morphological parameters of *C. wightii* seeds collected from sites I-IV (adapted from LAL & KASERA, 2012)

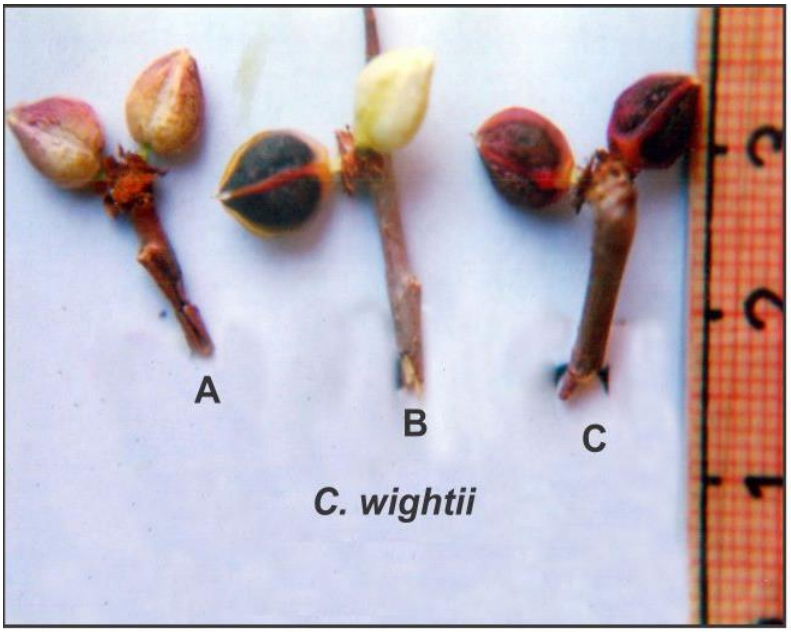

Fig. 4. Twig of *C. wightii* with immature reddish-brown (A), mature black and yellowish-white (B), and mature black (C) fruits, bearing black and whitish-black seeds

Tab. 2. Effect of different concentrations of nitrate solutions on seed germination behaviour in *C. wightii* under nursery conditions from sites-I-IV

Treatments	Concen-trations (mg L^{-1})	Germination initiation day				Germination duration (days)				Germination percentage				Peak value			
		Site-I	Site-II	Site-III	Site-IV	Site-I	Site-II	Site-III	Site-IV	Site-I	Site-II	Site-III	Site-IV	Site-I	Site-II	Site-III	Site-IV
Control	0	10.5	8.5	5.5	5.0	18.5	18.5	15.0	17.0	11.11	33.88	19.44	46.11	0.92	2.88	1.94	5.42
NH_4NO_3	5	-	9.0	6.5	5.2	-	19.0	15.5	16.0	-	28.33	18.33	43.73	-	2.36	1.67	4.79
	10	-	9.5	8.5	5.5	-	18.0	13.5	13.5	-	21.11	16.11	38.33	-	1.92	1.57	4.65
	15	-	10.0	-	5.5	-	16.0	-	12.5	-	19.33	-	38.11	-	1.12	-	4.49
	20	-	-	-	6.5	-	-	-	12.0	-	-	-	37.22	-	-	-	4.32
$Co(NO_3)_2$	5	8.0	8.5	6.0	5.5	17.0	16.5	14.5	13.5	11.66	39.44	21.66	46.66	1.11	4.15	2.10	5.62
	10	9.5	9.0	7.5	5.5	16.5	15.0	13.0	12.0	10.55	31.66	18.88	40.55	0.97	3.39	1.89	4.87
	15	9.5	9.3	-	5.7	13.0	14.5	-	12.0	8.89	28.33	-	38.22	0.56	2.72	-	3.60
	20	-	9.5	-	6.0	-	12.4	-	10.0	-	27.22	-	37.22	-	2.12	-	3.20
$Ca(NO_3)_2$	5	6.5	6.0	6.0	5.0	14.5	17.5	17.5	14.5	20.55	42.77	53.88	73.11	2.42	4.75	5.99	9.57
	10	8.0	5.5	6.5	4.0	14.5	16.0	16.5	13.5	14.44	37.72	35.00	59.44	1.52	3.92	3.68	8.17
	15	8.2	5.3	7.0	4.0	13.0	15.0	16.0	12.0	13.33	33.33	31.22	58.11	1.22	3.45	2.94	7.93
	20	8.5	5.0	7.5	4.0	11.5	14.0	14.5	11.5	12.22	32.77	29.44	57.22	1.10	3.15	2.55	6.35
KNO_3	5	6.0	5.0	6.5	4.0	16.0	20.0	17.0	13.5	23.73	51.66	40.55	70.55	2.92	5.11	4.20	10.08
	10	6.0	5.0	6.5	4.0	14.5	17.5	15.5	12.5	16.11	40.55	31.66	56.66	1.79	4.92	3.39	8.09
	15	6.2	5.3	6.3	4.3	13.5	15.0	15.0	12.0	14.11	38.66	29.11	51.33	1.33	4.27	3.02	7.44
	20	6.5	5.5	6.0	4.5	12.0	13.5	13.5	12.0	13.33	38.33	27.22	48.33	1.15	3.75	3.55	6.75
CD		1.036*	1.388*	1.198*	1.126*	1.507*	2.228*	2.061*	2.390*	6.897*	11.557*	7.727*	9.585*	0.170*	0.280*	0.231*	0.335*

* = significant at $p<0.05$; and - = no germination.

Tab. 3. Effect of different concentrations of nitrate solutions on various seedlings parameters of three months old seedlings of *C. wightii* under nursery conditions from sites-I-IV

Treatments	Concentrations (mg L^{-1})	Root length (cm)				Shoot length (cm)				Collar diameter (mm)				Total dry weight (g $plant^{-1}$)			
		Site-I	Site-II	Site-III	Site-IV	Site-I	Site-II	Site-III	Site-IV	Site-I	Site-II	Site-III	Site-IV	Site-I	Site-II	Site-III	Site-IV
Control	0	21.4	23.9	24.6	26.2	9.7	10.9	9.3	11.2	3.2	3.8	3.7	3.5	0.272	0.345	0.348	0.366
NH_4NO_3	5	-	21.9	22.5	23.9	-	11.7	9.9	10.8	-	3.7	3.5	3.9	-	0.294	0.301	0.310
	10	-	16.6	16.1	22.1	-	10.8	8.9	9.6	-	3.3	3.5	3.9	-	0.254	0.280	0.279
	15	-	15.6	-	22.1	-	10.1	-	9.6	-	3.2	-	3.8	-	0.248	-	0.250
	20	-	-	-	19.9	-	-	-	9.4	-	-	-	3.6	-	-	-	0.237
$Co(NO_3)_2$	5	22.5	27.6	23.4	25.5	9.7	10.9	10.3	10.7	3.6	3.7	3.7	3.7	0.268	0.365	0.348	0.402
	10	19.3	25.1	19.5	19.5	8.2	10.1	9.3	10.5	3.5	3.7	3.6	3.6	0.261	0.326	0.312	0.340
	15	18.7	20.3	-	18.9	8.0	9.9	-	9.9	3.1	3.5	-	3.6	0.213	0.314	-	0.315
	20	-	19.4	-	18.8	-	9.4	-	9.7	-	3.3	-	3.2	-	0.290	-	0.303
$Ca(NO_3)_2$	5	26.3	31.7	29.7	37.3	11.9	12.8	11.9	14.2	3.8	4.2	4.5	4.0	0.418	0.479	0.596	0.602
	10	22.4	29.6	27.6	33.8	10.4	12.7	11.0	13.8	3.6	4.2	4.3	3.8	0.366	0.420	0.515	0.535
	15	22.0	26.5	25.9	32.1	10.1	11.6	10.3	12.7	3.4	4.0	4.1	3.6	0.350	0.385	0.475	0.512
	20	21.8	24.7	25.8	30.2	9.9	11.5	10.8	11.5	3.3	3.9	3.9	3.5	0.326	0.389	0.460	0.457
KNO_3	5	28.8	34.1	33.6	39.4	10.7	12.5	10.9	13.3	4.0	4.8	4.9	4.3	0.456	0.482	0.532	0.547
	10	27.0	31.6	29.1	35.2	10.2	12.5	10.5	12.2	3.8	4.5	4.6	4.1	0.393	0.432	0.468	0.473
	15	25.3	27.5	28.1	33.5	9.8	11.4	9.9	11.5	3.6	4.3	4.4	3.8	0.356	0.421	0.435	0.454
	20	23.6	26.9	27.5	33.4	9.5	11.0	9.5	11.2	3.4	4.2	4.3	3.7	0.331	0.413	0.424	0.433
CD		4.854*	5.648*	5.848*	4.854*	1.235*	2.328*	0.696*	0.985*	0.089*	0.608*	0.525*	1.267^{ns}	0.048*	0.042*	0.011*	0.029*

* = significant at $p<0.05$; ns = non -significant; and - = no germination.

Germination initiation day and duration

The seeds pretreated with all the concentrations of KNO_3 germinate earlier as compared to others (Tab. 2). Results revealed that all concentrations of NH_4NO_3 totally inhibited germination from site-I, while 15 and 20 mg L^{-1} from site-III and only 20 mg L^{-1} from site-II. The higher concentrations of $Co(NO_3)_2$ also inhibited germination from sites-I & III. Further, increasing concentrations of all nitrates delay in germination from all sites. The early germination was observed on 4th day after seed sowing from site-IV in 10, 15 & 20 mg L^{-1} $Ca(NO_3)_2$ and in 5 & 10 mg L^{-1} KNO_3 and delayed up to 10.5th day from site-I in control. The values of germination duration (days) were decreased with increasing concentrations of nitrates from all sites. The maximum value (20 days) was found with 5 mg L^{-1} of KNO_3 from site-II, while minimum (10 days) in 20 mg L^{-1} of $Co(NO_3)_2$ from site-IV.

Germination percentage and Peak value

Data presented in Tab. 2 revealed that under controlled conditions seeds produced maximum seedlings (46.11%) from site-IV followed by site-II and minimum from site-I. Further, results revealed that maximum germination percentage values from sites-I and II were recorded in 5 mg L^{-1} of KNO_3. The same concentration of $Ca(NO_3)_2$ showed maximum germination from sites-III and IV. $Ca(NO_3)_2$ and KNO_3 showed positive results with higher concentrations only. It is evident from Tab. 2 that maximum peak values were reported in 5 mg L^{-1} of KNO_3 from sites-IV followed by site-III in 5 mg L^{-1} of $Ca(NO_3)_2$ and minimum from site-I in 15 mg L^{-1} of $Co(NO_3)_2$. The peak values were also decreased with increasing concentrations of nitrate solutions.

Root, shoot lengths and collar diameter

Results revealed from Tab. 3 that maximum root length was found from site-IV in 5 mg L^{-1} of KNO_3 and minimum from site-II in 15 mg L^{-1} of NH_4NO_3. The highest shoot length was observed in 5 mg L^{-1} of $Ca(NO_3)_2$ from site-IV and lowest in 15 mg L^{-1} of $Co(NO_3)_2$ from site-I. The maximum values of collar diameter were observed from site-III in 5 mg L^{-1} of KNO_3 and minimum from site-I in 15 mg L^{-1} of $Co(NO_3)_2$.

Biomass yield (g plant^{-1})

The sum of root and shoot dry weights were considered as biomass of plant. The maximum biomass yield was observed from site-IV followed by site-III in 5 mg L^{-1} $Ca(NO_3)_2$, whereas minimum in 15 mg L^{-1} of $Co(NO_3)_2$ from site-I (Tab. 3). The lower concentration (5 mg L^{-1}) of $Ca(NO_3)_2$ and KNO_3 were found to suitable for maximum biomass yield. Further, the biomass yield decreased significantly with increasing concentrations of nitrate pretreatments.

Data pertaining two years for various parameters of seed germination and seedling growth behaviour were significant at 95% level at all sites, except for collar diameter at site-IV, which were non-significant.

Discussion

Nitrogenous compound in various forms has been used to stimulate germination [CHOUDHARY & al. 1996; MC INTYRE & al. 1996]. They play a critical role in increasing the physiological efficiency [BHARGAVA & BANERJEE, 1994] and influence germination may be due to change in water relationship [NIKOLAEVA, 1977]. Ammonium nitrate was proved to inhibit or promote seed germination dependent on species type [SINGH & AMRITPHALE, 1992]. CHOUDHARY & KUMAR (2003)

reported that the higher concentration of NH_4NO_3 inhibited seed germination in *Plantago ovata*. HASSAN & al. (2011) also reported that higher concentrations of NH_4NO_3 delayed germination in *Striga hermonthica*. In the present studies, NH_4NO_3 showed very poor results as compared to control from all sites. The maximum germination (43.73%) was reported from site-IV in 5 mg L^{-1} and minimum (16.11%) from site-III in 10 mg L^{-1} solutions. Further, with increased concentrations, it completely inhibit seed germination from site-I. Our results were confirmative with the above findings.

Cobalt generally considered toxic to cells and it can cause various toxic effects on plant such as inhibition of seed germination, plant growth and yield reduction [SHAUKAT & al. 1999]. JAYAKUMAR & al. (2009) reported that cobalt at low concentrations has sobbed beneficial values in soybean germination. KHAN & KHAN (2010) observed that higher concentrations of cobalt (200 and 400 ppm) are detrimental to seed germination in chickpea. The results are in accordance with all these above-mentioned findings. Only, the lower concentration, *i.e*. 5 mg L^{-1} was found to be beneficial for improving germination as compared to control.

Calcium plays an essential role in protecting plants such as preserving the structural and functional integrity of cell membrane, stabilizing plant cell wall structure, regulating ion transport & selectivity and controlling ion-exchange behaviour as well as enzyme activities [RENGEL, 1992; HOWLADAR & RADY, 2012]. GEHLOT & KASERA (2011) observed cent percent germination in *Withania coagulans* when seeds were pretreated with 0.50% $Ca(NO_3)_2$ and also reported that higher concentrations retarded it. In the present studies, $Ca(NO_3)_2$ pretreatments showed the highest germination, *i.e*. 73.11% from site-IV in 5 mg L^{-1} and lowest 12.22% from site-I in 20 mg L^{-1} solutions. Our results are confirmative with above all these findings.

Use of KNO_3 has been an important seed treatment in seed-testing laboratories for many years without a good explanation for its action [HARTMANN & al. 1997]. The ion is bound to pyruvate kinase and other essential enzymes, regulating respiration and carbohydrate metabolism [SALISBURY & ROSS, 1991]. SINGH & al. 1998 reported 40% germination in 0.25% KNO_3 in *Commiphora wightii*. TIWARI & CHAUHAN (2007) reported that KNO_3 significantly enhanced seed germination (32-36%) in *Rhododendron niveum*. KARIMMOJENI & al. (2011) reported that 0.02 M concentration of KNO_3 increased germination up to 61.0% in *Lepidium latifolium*, while higher one retarded it. LAL & KASERA (2012) observed that seeds of *C. wightii* pretreated with 5 mg L^{-1} of KNO_3 were most favorable for optimizing germination and seedling development under *in-vivo* conditions. The results confirm all these findings presented above.

In the present investigation, the lower concentration (5 mg L^{-1}) of $Ca(NO_3)_2$ and KNO_3 performed best results of seedling parameters such as root & shoot lengths, collar diameter and total dry weight. KSHETRAPAL & SHARMA (1992) reported maximum root length in stem cuttings of *C. wightii* with 0.3% solution of KNO_3. GEHLOT & KASERA (2011) also reported maximum root and shoot lengths in *Withania coagulans* with 0.25 and 0.10% solutions of KNO_3 and $Ca(NO_3)_2$, respectively. This may be due to nitrate salts with an osmotic role on water uptake, which exerted a nutritional effect on protein synthesis as suggested by MC INTYRE & al. (1996).

Conclusions

Results obtained from the present studies revealed that morphological parameters of seeds such as weight and size does not affect the seedling emergence percentage, whereas the viability and ratio of black & whitish-black seeds affected them significantly. The seeds of *C. wightii* pretreated with 5 mg L^{-1} of $Ca(NO_3)_2$ and KNO_3 were found to be most suitable for optimizing germination and seedlings development under *in-vivo* conditions. The lower concentrations of nitrate solutions promote the germination as well other parameters of seedlings, while higher ones retarded them. Further, the lower concentration (5 mg L^{-1}) of $Ca(NO_3)_2$ showed best growth of shoot length and total dry weight, while the same concentration of KNO_3 performed with root length and collar diameter. Finally, it is concluded that KNO_3 and $Ca(NO_3)_2$ significantly influenced all parameters of seed germination at all sites. Hence, for large-scale cultivation and conservation of this plant, seeds collected from sites-II (Jodhpur), III (Barmer) and IV (Jaisalmer) should be pretreated with 5 mg L^{-1} of $Ca(NO_3)_2$ and KNO_3 before sowing under nursery conditions.

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EFFECTS OF CHLORIDE SALINITY ON NON-ENZYMATIC ANTIOXIDANT ACTIVITY, PROLINE AND MALONDIALDEHYDE CONTENT IN THREE FLUE-CURED CULTIVARS OF TOBACCO

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Abstract: High salinity causes ion poisoning and subsequent oxidative stress. During oxidative ion poisoning, non-enzymatic defense systems such as carotenoids, phenols and flavonoids are activated. In this study, the effects of various concentrations of chloride in the irrigation water (10, 20, 40 and 80 mg/l) on carotenoids, phenols, flavonoids and proline content in three varieties of tobacco plant (Virginia, Kentucky and Cooker) were investigated. Malondialdehyde content was also measured, as a lipid peroxidation index. The highest level of β-carotene was observed in Virginia at 10 mg/l chloride and the lowest quantity was seen in Kentucky at 20 mg/l chloride. Kentucky had the highest and lowest levels of flavonoids at 80 and 40 mg/l concentrations of chloride respectively. Highest phenol content was observed in the presence of 10 mg/l chloride in Virginia. Maximum and minimum MDA concentrations were observed respectively in 20 and 80 mg/l concentration of chloride in Cooker cultivar. Raising chloride concentrations in irrigation water caused a substantial decrease in proline content only in the Kentucky variety.

Keywords: Oxidative stress, β-carotene, chloride, flavonoids, tobacco, total phenol

Introduction

Chloride is generally abundant in the environment. It exists as a monovalent micronutrient in soil water. Chloride ion is extremely mobile and easily lost in soils, which have a leaching effect on the substance. Since chloride is present in different sources, such as irrigation water, soil, rain, fertilizers and air pollution, more attention is given to its poisoning effect rather than to its deficiency. On the other hand, chloride is one of the necessary components for plant growth. For instance, chloride is taken up very rapidly by tobacco plant [RHOADS, 1975]. Tobacco can accumulate chloride very rapidly in considerable amounts, equivalent to up to 100 g Cl per kg leaf dry weight [McCANTZ & WOLTZ, 1967]. While small quantities of chloride in nutrients has positive effects on tobacco yield and market value of the leaf, high concentrations in the soil cause abnormal growth and unfavorable properties in the cured leaf. Increasing quantities of chloride in cured leaves reduces the burning rate and causes undesirable effects, such as an increase in hygroscopy, dinginess or uneven colors and adverse odors. In recent years several researchers have focused on studying the role of chloride in creating such effects [PEELE & al. 1960, FLOWER, 1999].

High salt content has a decreasing effect on the osmotic potential of soil and causes water stress in plants. Interaction between salts and inorganic nutrients may result in

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an imbalance in feeding [McCUE & HANSON, 1992]. It decreases the regeneration of $NADP^+$ in Calvin cycle, which as a consequence reduces the photosynthetic electron transport chain dramatically and eventually generates super oxide radicals [SHUN-WU & KE-XUAN, 2004; LI & JIN, 2007]. In case of oxidative stress in plants, natural antioxidants, which prevent the adverse effects of oxidative stress, will increase. Some of the non-enzymatic antioxidants present in plants are polyphenols. These substances play key roles in the plant defense system and growth. In addition, these components contain the necessary information on the cell oxidation status and regulate gene expression related to optimizing defense via responses to biotic and abiotic stress [SHAO & al. 2008]. On the other hand, the amino acid proline is one of the most effective compounds for regulation of osmosis in plants under drought and salinity stress. Osmosis regulators are very soluble chemicals which increase resistance and tolerance of plants against stress by maintaining cell turgor and stability of the cytoplasmic enzymes. Despite the economic value of tobacco in the north of Iran, there has been no recent investigation on the effect of increasing soil chloride concentrations on tobacco growth responses. One of the reasons for the higher chloride concentrations in soil is that irrigation water at present contains higher levels of chloride than before, whereas in the past it was not considered a serious problem in tobacco growing areas. Recent measurements confirmed a rise in chloride concentration in soil and irrigation water in the north of Iran. This study was conducted with three main purposes:

1) To investigate the effects of different chloride concentrations on quantity and activity of some non-enzymatic antioxidants, such as total phenol and flavonoids.
2) To study the effects of different chloride concentrations on proline and MDA content in tobacco.
3) To determine the critical concentration of chloride in the three studied cultivars of tobacco and to establish an acceptable tolerance level for chloride in the water used for irrigating tobacco.

Materials and methods

***Sample preparation*:** Effects of four concentrations of chloride in irrigation water (10, 20, 40 and 80 mg/l) on proline content and some non-enzymatic antioxidants such as β-carotene, total phenol and flavonoids were investigated in three flue-cured cultivars of tobacco, namely Cooker 347, Virginia E1 and Kentucky 326, by spectrophotometry using factorial design (4×3) with 4 pot replications. The selected cultivars are the highest quality commercial cultivars in the north of Iran. Data were analyzed using standard analysis of variance (ANOVA), and means were separated using Duncan comparisons at $P \leq 0.05$.

***Plant culture and treatment application*:** An outdoor pot experiment was established during 2000 growing seasons at the Institute of Tobacco Research in Rasht in the northern part of Iran. Seedlings were prepared using the float system. In this study, plants in pots were grown according to normal practices used for tobacco production in Guilan. Plastic pots were filled with a mix consisting of 4 volume of soil and 1 volume of fine perlite. Defined basic fertilizer N-P-K was added to every pot upon soil analysis results before planting. Uniform seedlings (12-15 cm) were selected and transplanted to pots, one plant per pot. There are three vegetative growth phases in tobacco plants, including root development, fast growth phase and leaf ripening. Naturally, the fast growth phase is usually the most sensitive. Irrigation was therefore carried out twice a week with 2 liters of salt solution for each 6 liter pot. This amount of water did not provoke leaching and

chloride ions were added to water as $CaCl_2$. Electrical conductivity (EC) of the solutions was measured before irrigation. The analysis of all factors in this study was carried out using fresh and well developed leaves harvested from seedlings 60-65 days after transplanting.

***Extraction and assay of β-carotene*:** To estimate total β-carotene, leaf tissues (0.25 g) were first homogenized in a chilled (v/v) acetone-hexane mixture; the homogenates were centrifuged at 5000 rpm for 10 min at 4 ºC in the dark. The absorbance of the acetone-hexane extracts was measured at 663, 645, 505 and 453 nm. The amount of β-carotene was calculated according to NAGATA & YAMASHITA (1992).

***Non-enzymatic antioxidants extraction*:** For the preparation of tobacco extracts for non-enzymatic antioxidants, including phenols and flavonoids, the leaves of the three cultivars of tobacco were well ground separately in a mortar with liquid nitrogen. 0.25 g of the homogenized sample was weighed and transferred to falcon pips with label. 5 ml 80 % methanol was added to each falcon pip and stirred. Then the extract was centrifuged at 5000 rpm for 10 min at 0 ºC. The supernatants were collected and kept in a refrigerator at -20 ºC.

***Total flavonoid assay*:** The assessment of total flavonoid content in the obtained extracts was done using a colorimetric method [CHANG & al. 2006] with some modification. Sample absorbance was read at 510 nm. Different concentrations of catchin were used to draw the standard curve.

***Total phenol assay*: The method of SINGLETON & ROSSI** (1965) was used for total phenol assay using Folin-Ciocalteu solution. Total phenolic content (mg/g f.w.) was determined using the standard curve of gallic acid ($R^2 = 0.987$). The use of this method is mainly restricted due to the fact that it cannot distinguish between different types of phenols and only total phenol is measured.

***Proline measurement*:** Extraction of proline was carried out in accordance with BATES & al. (1973) and absorbance of solution color (included toluene and proline) was read at 520 nm. Pure proline (Merck) was employed as a standard reference and results were expressed as proline equivalents (ng of tobacco leaves on fresh weight basis).

***Extraction and measurement of malondialdehyde (MDA)*:** About 0.5 to 1.0 gr of tissue was homogenized in 5 ml of 5% (w/v) trichloroacetic acid and the homogenate was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was mixed with an equal volume of thiobarbitoric acid (0.5% in 20% (w/v) trichloroacetic acid), and the mixture was boiled for 25 minutes at 100 ºC followed by centrifugation for 5 minutes at 7,500 rpm to clarify the solution. Absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the A600. MDA content was calculated using an extinction coefficient of 155 M-1 cm^{-1}. Values of MDA content were taken from measurement of three independent samples and SEs of means were calculated [HEATH & PACKER, 1968].

Results

***β-Carotene assay*:** Changes of β-carotene content in mature leaves of tobacco cultivars Cooker 347, Virginia E1, and Kentucky 326 were investigated at different chloride concentrations (10, 20 40 and 80 mg/l). Studying mature leaves shows that the highest content (26.56 mg/g f.w.) of β-carotene is to be found in Virginia at 10 mg/l chloride and the lowest quantity (13.24 mg/g f.w.) in Kentucky at 20 mg/l chloride (Fig. 1).

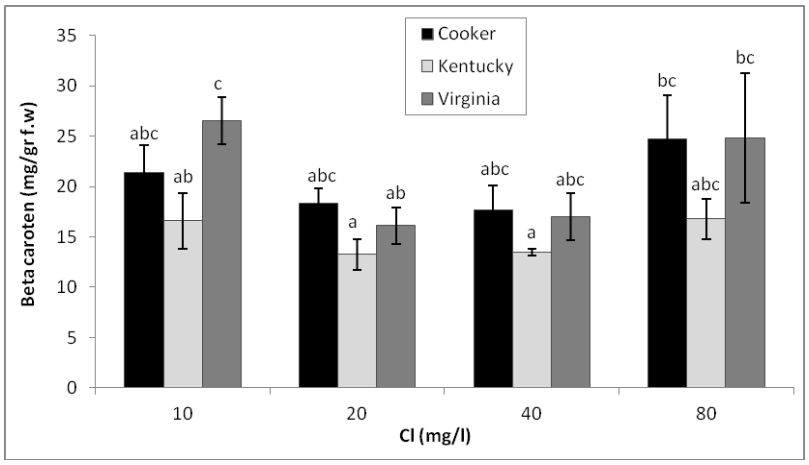

Fig. 1. β-carotene concentration in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively)

***Total flavonoid assay*:** Changes of the flavonoids were investigated in the tobacco cultivars under different concentrations of chloride. The total flavonoid content in samples varied from 0.16 to 0.45 mg/g f.w. with the lowest and the highest levels observed in 40 and 80 mg/l chloride concentration for Kentucky (Fig. 2).

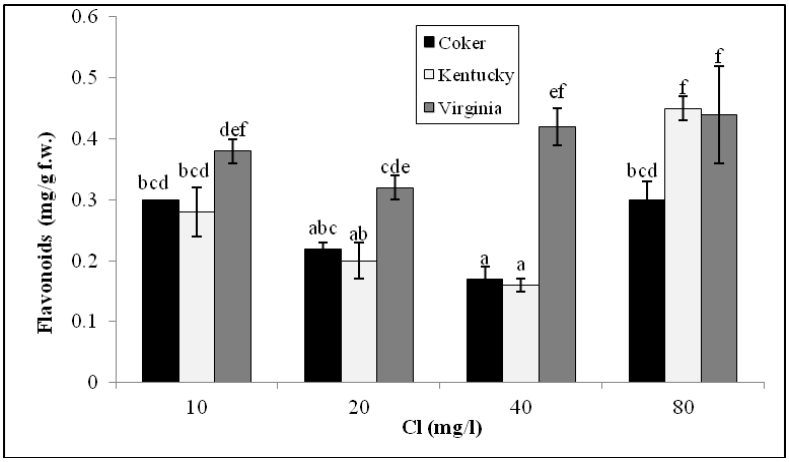

Fig. 2. Flavonoid content in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively). Different concentrations of catchin were used to draw the standard curve

***Total phenol assay*:** Results obtained show that total phenol varied from 56.64 mg/g f.w. in the Kentucky cultivar under 20 mg/l concentration of chloride to 79.44 mg/g in Virginia under 10 mg/l concentration of chloride (Fig. 3).

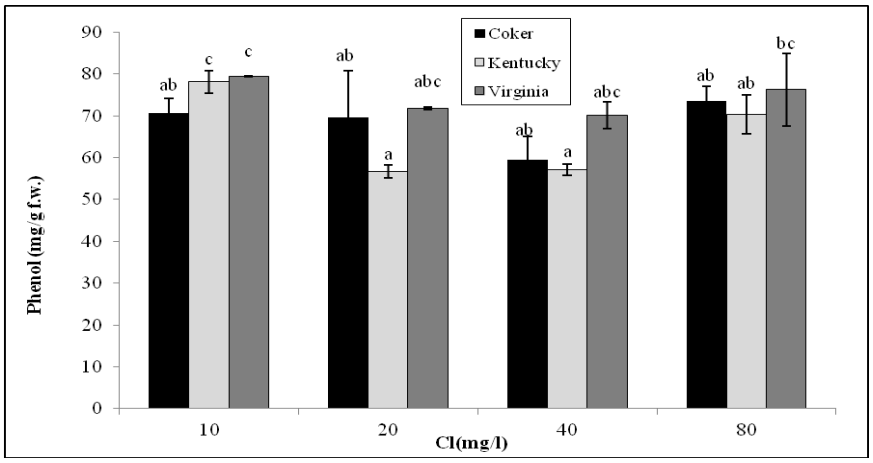

Fig. 3. Phenol concentration in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively). Total phenolic content was determined using the standard curve of gallic acid

***Measurement of Proline*:** The results showed that proline content in Kentucky cultivar varied from 437.8 to 2,169.4 mg/g f.w. The highest and the lowest proline content were observed at 10 mg/l and 20 mg/l chloride, respectively (Fig. 4).

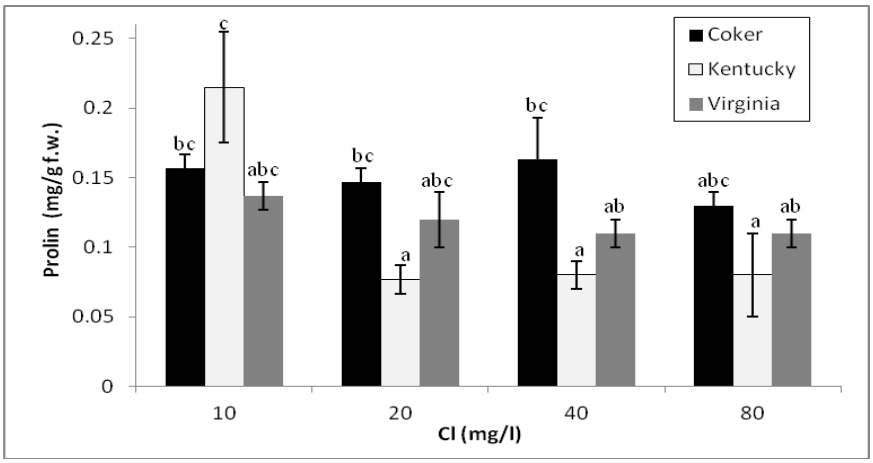

Fig. 4. Proline concentration (mg/g f.w.) in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively). Pure proline was employed to obtain the standard curve

***Lipid peroxidation*:** Lipid peroxidation is a process related to free radicals and its concentration is generally used as an index for oxidative stress and also as a marker for plant sensitivity to environmental stress. MDA assay in mature leaves of different cultivars showed different results. Maximum and minimum MDA concentrations were observed respectively in 20 and 80 mg/l concentration of chloride in Cooker cultivar (Fig. 5).

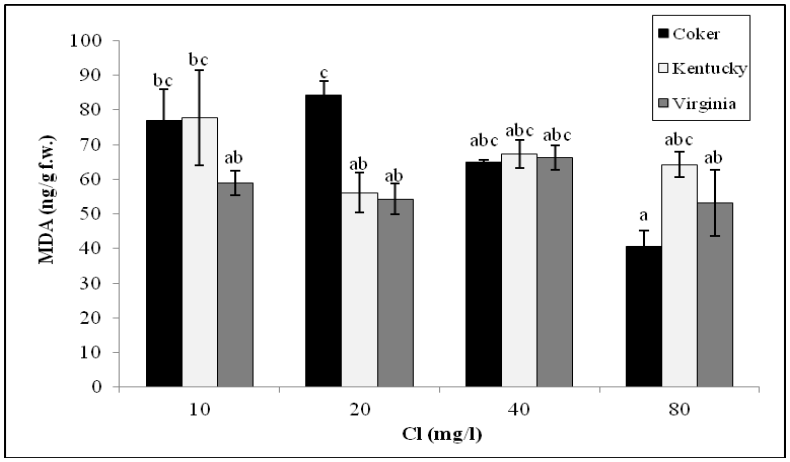

Fig. 5. MDA concentration in mature leaves of three cultivars of tobacco at different concentrations of chloride (error bars and letters above of the column are added upon standard error and two way analysis of variance respectively)

Discussion

As is shown in the results, non-enzymatic antioxidant responses to different concentrations of chloride in irrigation water are different, but the differences do not have the same statistical significance. Not only different concentrations, but also different cultivars have a significant impact on how the samples under treatment do react. In this study, MDA content was used primarily as a lipid peroxidation index and as a marker of oxidative stress.

***β-carotene*:** As seen in figure 1, with some exceptions, there are no meaningful differences between treatments and control; and this may be interpreted as absence of oxidative stress in the used range of chloride concentrations. Studies on other plants show a decrease in carotenoids content, especially β-carotene, in salt stress with chloride concentrations of up to 100 mM [STOEVA & KAYMAKANOVA, 2008; AYALA-ASTORGA & ALCARAZ-MELENDEZ, 2010]. In contrast, other studies seem to show an increase in other carotenoids during salt stress [RAO & al. 2007].

***Flavonoids*:** Figure 2 shows the differences between flavonoids content in the three cultivars. It emphasizes the results of interaction between two factors, namely chloride concentration and cultivar. Decreasing levels of flavonoids with chloride concentrations of 20 and 40 mg/l indicate that these concentrations are optimal and result in the least stress. Conversely, increasing chloride concentration to 80 mg/l results in higher levels of flavonoids and stress, except in the Kentucky variety, which exhibited more sensitivity and flavonoid levels increased from 40 mg/l chloride). The correlation between

the two antioxidant factors – β-carotene and flavonoids – in addition to changes in MDA, can lead us to an understanding of optimum chloride concentrations in the irrigation water.

***Total phenol*:** Investigation of the total phenols, as non-enzymatic antioxidants, shows that 10 mg/l concentration of chloride is a stressful concentration. Phenol content is maximal at 10 mg/l of chloride and with increasing chloride concentrations (20 and 40 mg/l) phenol values decrease; finally at 80 mg/l chloride, phenol content increase to its original level at 10 mg/l chloride concentration (Fig. 3). Interaction between concentrations and cultivars in the section indicates that the optimal response for the Kentucky cultivar is 80 mg/l chloride. This finding reinforces the results obtained from studies on flavonoids. Increasing phenol content during salt stress has been reported in several studies [MUTHUKUMARASAMY & al. 2000; AGASTIAN & al. 2002; NAVARRO & al. 2006].

***Proline*:** As mentioned in the introduction, proline has a significant role in salt and drought stress [HUA & al. 1997]. It is therefore desirable to measure proline in order to shed light on this question. Fig. 4 shows that in matured leaves we can see a significant decrease in proline content for Kentucky and a slight decrease in other cultivars together with increasing chloride concentrations. It is important to note that chloride ion probably causes more disorder in ion exchange in the rhizosphere than in osmosis. Given the situation we need to use a more multifaceted metabolism for a deeper explanation.

***Lipid peroxidation*:** Figure 5 shows that MDA content in mature leaves of Cooker cultivar increases with increasing chloride concentrations up to 20 mg/l, after which it decreases. Although variations are not meaningful in treatments which received chloride concentrations of less than 80 mg/l chloride, at 80 mg/l there was significantly reduced lipid peroxidation in the Cooker variety, as opposed to control. This means that concentrations below 80 mg/l chloride caused higher stress, and so led to higher concentrations of MDA. This result is not unexpected with regards to tobacco, as it easily absorbs chloride and needs it as a macroelement [DAVIS & NIELSEN, 1999]. It may therefore be concluded that chloride content of 10, 20 and 40 mg/l in irrigated water and soil could not supply sufficient chloride to provide the plants with the necessary minerals necessary for optimal growth. As a result, increasing chloride content to 80 mg/l created the conditions for a reduced lipid peroxidation. This situation was seen more or less in all three cultivars. Nonetheless, responses were not identical; in cultivars Virginia, Kentucky and Cooker lipid peroxidation decreases with the increasing chloride content, while the decrease is significant in the Cooker and not significant in the other two cultivars. The result obtained in our study show that high chloride concentrations in the soil result in increases in MDA levels; however this result needs to be verified in further studies. Investigations on other plant species show that stress due to increasing chloride content, especially where sodium ions are also present, results in lipid peroxidation, but does not significantly affect MDA content; these changes seem to be dependent on both chloride concentration and plant cultivar. So, in mean concentrations of chloride either no changes were reported [NOR'AINI & al. 1997] or even a decrease in lipid peroxidation occurred [KSOURI & al. 2007]. Conversely, in other species the same concentrations could induce an increase in lipid peroxidation meaningfully [KSOURI & al. 2007; ESFANDIARI & al. 2007].

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MERISTEM STRUCTURE, DEVELOPMENT OF CONES AND MICROSPOROGENESIS OF TEHRAN PINE (*PINUS ELDARICA* Medw.)

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Abstract: Tehran pine (*Pinus eldarica* Medw.) belongs to Pinaceae family with significant economic and ecological benefits. To gain further insight into anatomical-developmental structure of *P. eldarica*, both the vegetative and generative meristematic tissues and microsporogenesis were been studied during certain stages of development. To do this, meristematic tissues and male cones were initially fixed in FAA solution (37% formaldehyde, 96% ethanol and glacial acetic acid with a 2:7:1 ratio, respectively). They were then embedded in paraffin and sectioned using a rotary microtome. Prior to visualization and photography under a camera-equipped light microscope, they were stained with hematoxylin-eosin (Zeiss model). Our results revealed the vegetative meristem of *P. eldarica* to be in the Cryptomerya-Abies category. The results also indicated it is a protuberant (dome-like) type containing four regions. The meiosis occurs before the winter dormancy and continues through the winter. The pollen is shed at the four-cell stage of development.

Keywords: cone development, generative meristem, microsporogenesis, pollen grain, *Pinus eldarica*, Tehran pine

Introduction

Tehran pine (*Pinus eldarica* Medw.) belongs to Pinaceae family, it has significant economic and ecological benefits, for example its wood is used extensively as timber and as a source of pulp for papermaking and related industries. It is likely that this species is a variety of *P. halepensis* Mill., imported to Iran from Georgia, inbred and even naturally reproduced. *P. eldarica* has been given several different names such as, Tehran pine, garden pine, Iranian pine etc. There have been a great number of studies on the structure and resin components, as well as ecological studies on the quality of wood [DJAVANSHIR & REID, 1975; IRAVANI, 2013; KIAEI, 2011; MOTAHARI & al. 2013; PHILLIPS & al. 1986; SAFDARI & al. 2012]. However, to our knowledge, there is no information has yet been prepared on the anatomical-developmental of *P. eldarica*. Therefore, the aim of this study is to further investigate the vegetative and generative meristematic tissues and microsporogenesis of *P. eldarica* during certain stages of development.

Materials and methods

Plant material was collected from 30-year-old *Pinus eldarica* trees in Kharazmy University (Iran-Tehran). Meristematic tissues and male cones were collected during certain stages of their development (from June to March) and initially fixed in FAA solution (37% formaldehyde, 96% ethanol and glacial acetic acid in a 2:7:1 ratio,

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respectively), stored in 70% ethanol and embedded in paraffin. To study the meristematic tissues and microsporogenesis, median-longitudinal sections of 8-12 and 8-10µm thick, respectively, were cut and stained using hematoxylin-eosin. For each stage of development, several sections were analyzed using a camera-equipped light microscope (Zeiss model).

Results

Meristematic tissues

Four meristematic zones of the shoot apical meristem (SAM) of *P. eldarica* were observed: surface meristem (sm), central mother cells (cmc), rib meristem (rm) and peripheral meristem (pm) (Fig. 1). The unspecified axillary buds emerged at the sides of SAM on June 21st through September 1st. With shoot elongation during September to the end of October, the unspecified axillary buds grew separate from each other (Fig. 2).

Following expansion of the unspecified axillary buds, differentiation started and formed microsporophylls towards the end of October (Fig. 3). The axillary buds started differentiating from the lower to the upper regions (Fig. 4). This is the transition phase from the vegetative to the generative meristem. The different developmental stages of cones are shown in Fig. 5.

Microsporogenesis

Results showed that archesporial cells divided repeatedly and formed the archesporial tissue (Fig. 5a). The peripheral cells of the archesporium divided to form a parietal layer and sporogenous cells. The former divided and gave rise to a four- layer structure, with the most inner layer differentiating to tapetum, the two middle layers degenerated at the later stages of development, while the epidermal layer cells also known as the endothecium developed a fibrous wall with a few cells in a section not doing so, marking the dehiscence suture (Fig. 9b, c). The sporogenous cells divided and gave rise to pollen mother cells (microsporocytes), with callose walls around them (Fig. 5b, c). At this stage, the newly formed tapetal cells could not be distinguished from other wall layers (Fig. 9b, c), but during maturation, they acquired a compact cytoplasm and in a number of cases were bi-nucleate (Fig. 6a; 9e, d). On the other hand, each pollen mother cell divided meiotically and formed microspores in a tetrad. They were surrounded by a callosic coat of pollen mother cells. Cytokinesis was non-synchronous and we saw both dyads and tetrads in microsporophylls (Fig. 5c). At the early tetrad stage, the microspores contained a big, central nucleus (Fig. 5c), but gradually the ratio of cytoplasm to nucleus increased. The nuclei became marginal, and microspores started to separate from each other (Fig. 5d; 6a, b). Once released from tetrads, microspores formed regular shapes, while containing a marginal large nucleus (Fig. 6c, d). The microspore's unequal division resulted in the first small prothallial cell and a large central cell (Fig. 7a). The later divided unequally to form a second small prothallial and a large generative initial (Fig. 7b). Both prothallial cells were gradually pushed to one side forming two lens- shaped stacks (Fig. 7c, d; 8). Then, the generative initial cell divided unequally to form a large vegetative cell and a small generative cell (Fig. 7c). During these stages, tapetal cells degenerated while some microspores were still in contact with them (Fig. 6a-c and 9d, e). They later moved towards microspores for nutrition. Finally, tapetum was degenerated completely (Fig. 9f).

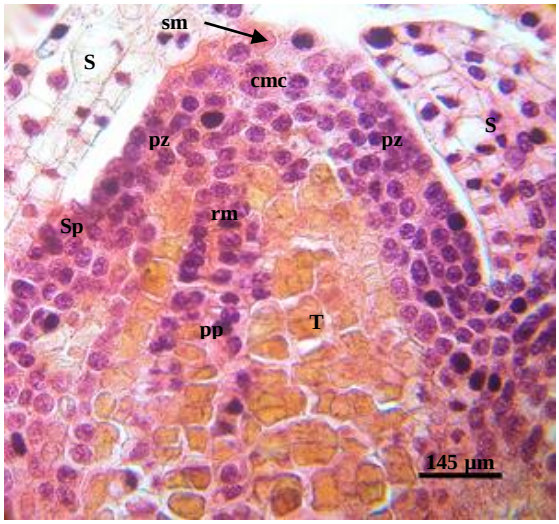

Fig. 1. SAM zonation. S – scale leaf; Sp – scale leaf primordia; sm – surface meristem; cmc – central mother cell; rm – rib meristem; pz – peripheral zone; pp – pith meristem; T – tannins.

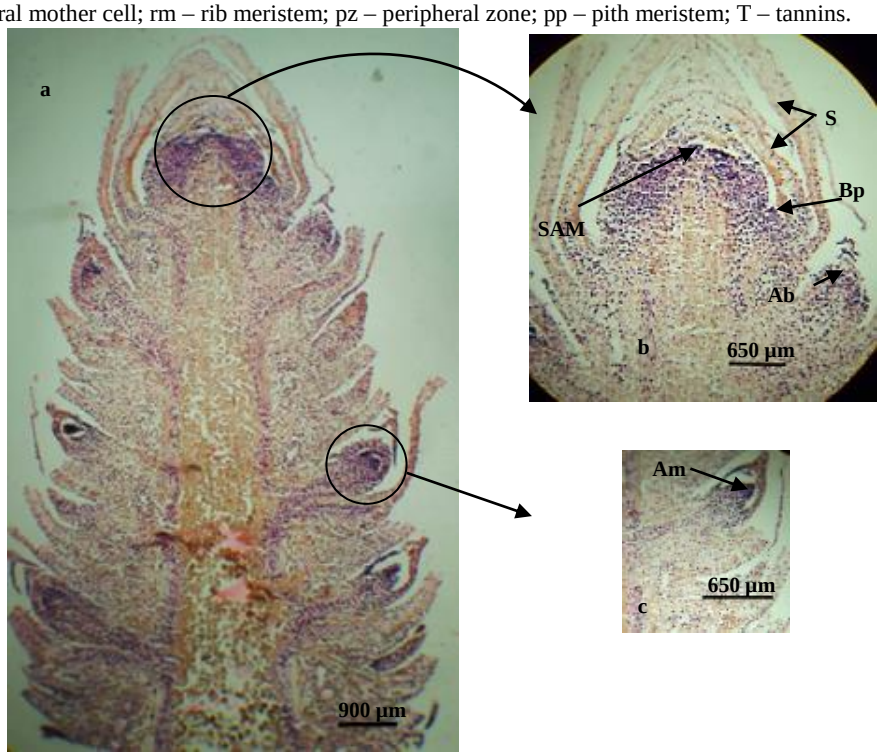

Fig. 2. (a) Apical and axillary meristems; (b) apical bud; (c) axillary bud. S – scale leaf; SAM – stem apical meristem; Bp – bud primordia; Ab – Axillary bud; Am – Axillary meristem.

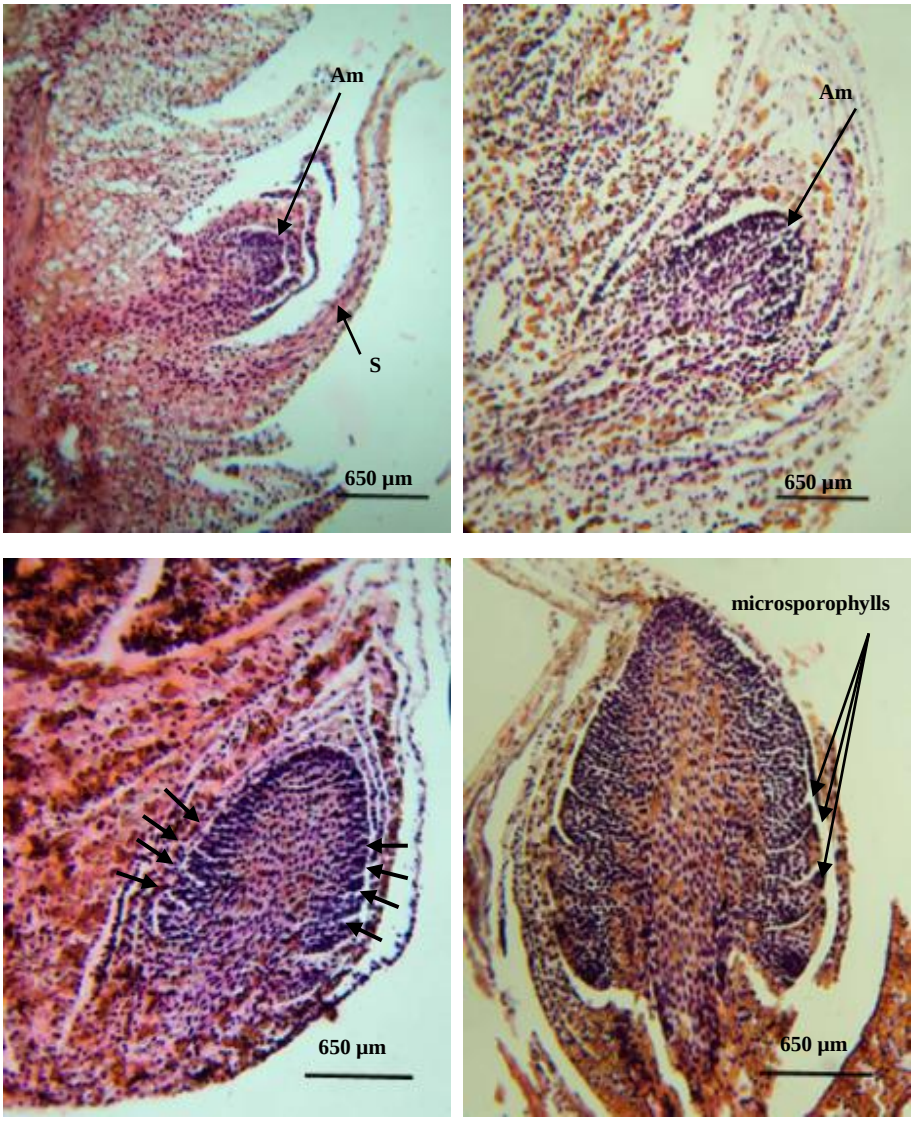

Fig. 3. Transition of the developmental stages of meristem, from the vegetative to the generative state. (a) indeterminate axillary bud; (b) increasing the size and volume of axillary meristem (arrow); (c) differentiation of axillary meristem and formation of microsporophylls (arrows); (d) a young male cone, notice to the disappearing of axillary meristem apex and completing differentiation. S – scale leaf; Am – axillary meristem.

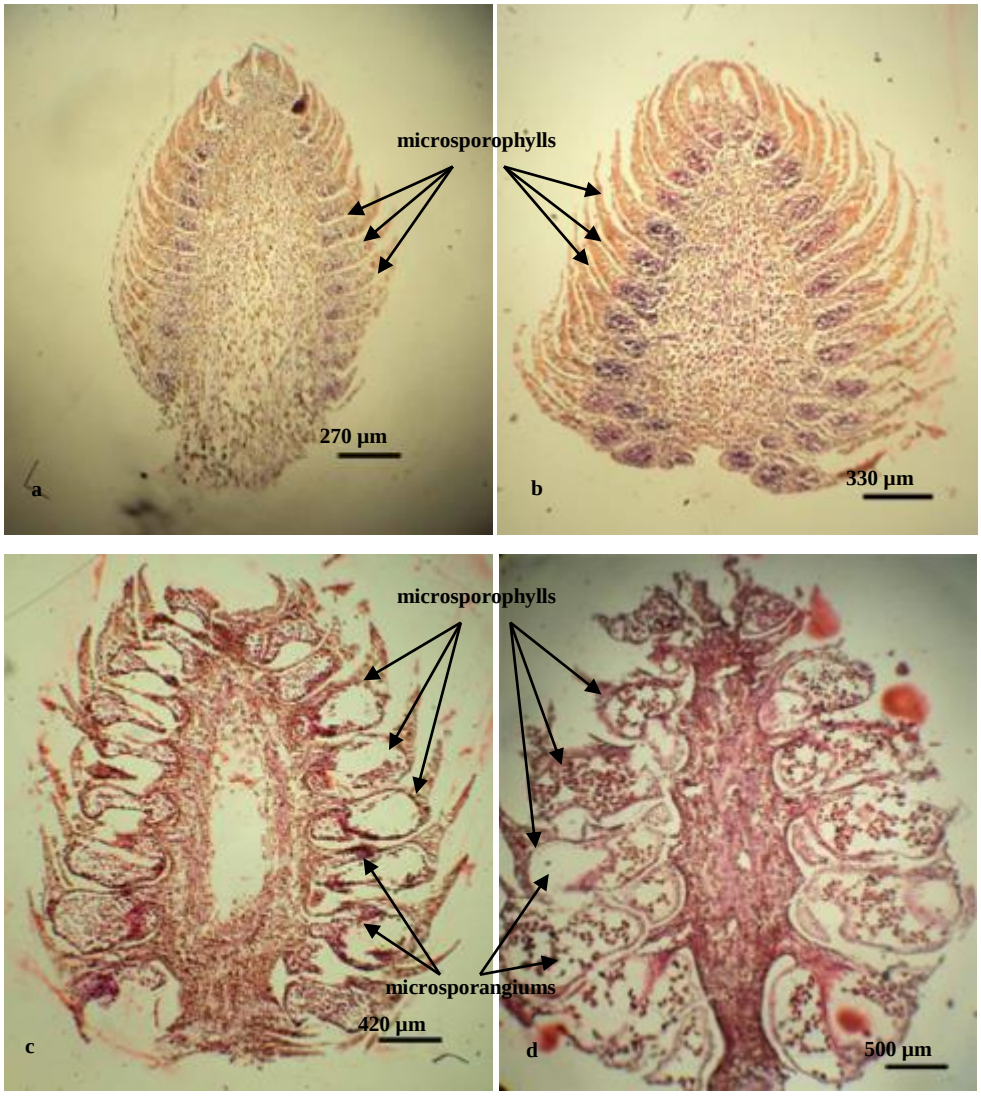

Fig. 4. Young to mature cone development stages. (a) Production of the microsporophylls of young male cone. At this stage, there are archespore tissues in upper microsporophylls and sporogenus tissues and pollen mother cells in lower microsporophylls; (b) in this size of cones, there were diads, tetrads and pollen mother cells in pollen sacs; (c) young and mature microspores are in the pollen sacs; (d) maturing pollens and also mature pollens are in the pollen sacs. Note the increasing size of cones and pollen sacs.

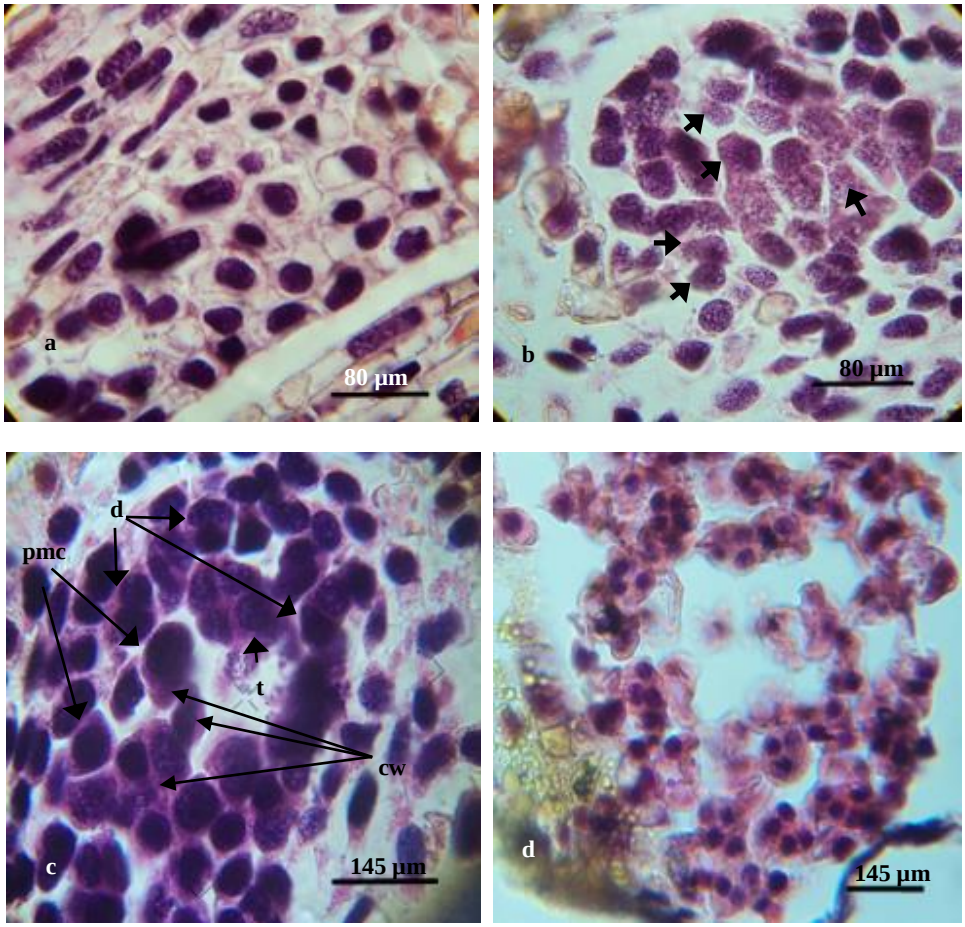

Fig. 5. Different stages of pollen development, from sporogenus tissue to tetrads. (a) archespore tissue; (b) sporogenus cells separation and the production of pollen mother cells are shown (arrows show the pmcs); (c) pmcs undergo meiose I and II; (d) at this stage pmcs have disappeared and tetrades are separating from each other. Increased cytoplasm/nucleus ratio is notable. d – diad; t – tetrad; pmc – pollen mother cell and cw – callosic wall.

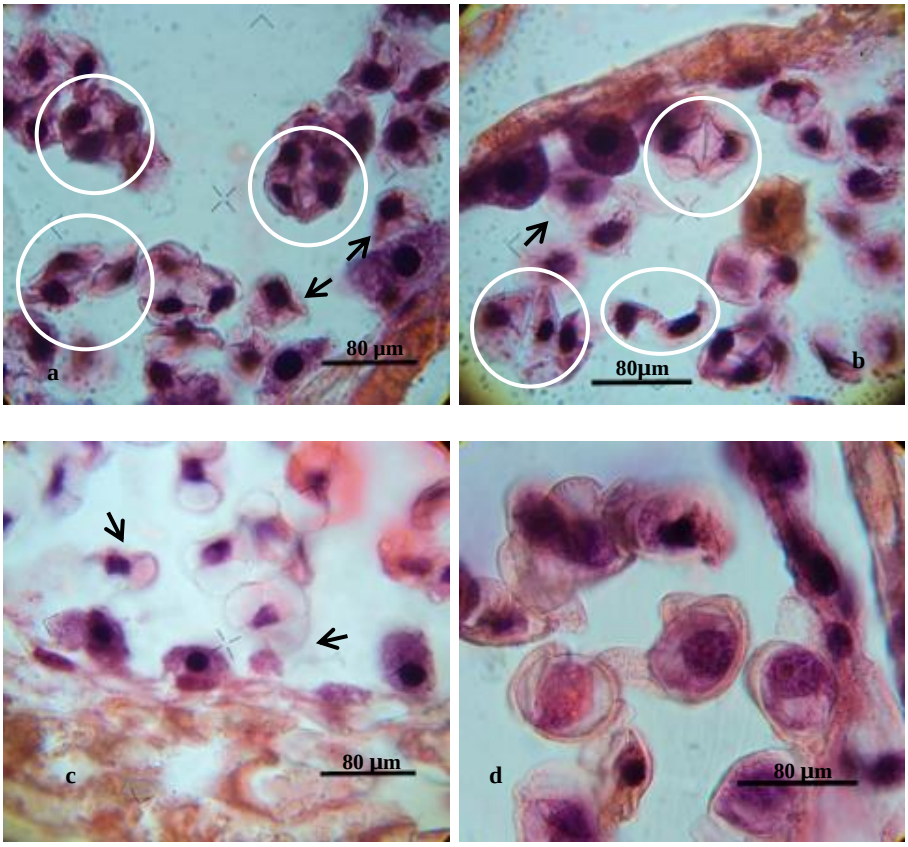

Fig. 6. Different stages of pollen development, from separating tetrads to mature microspores. (a-b) Microspores are separating from each other; (c-d) the size of microspores is gradually increasing. Interaction of diads and tetrads and tapetum cells is notable.

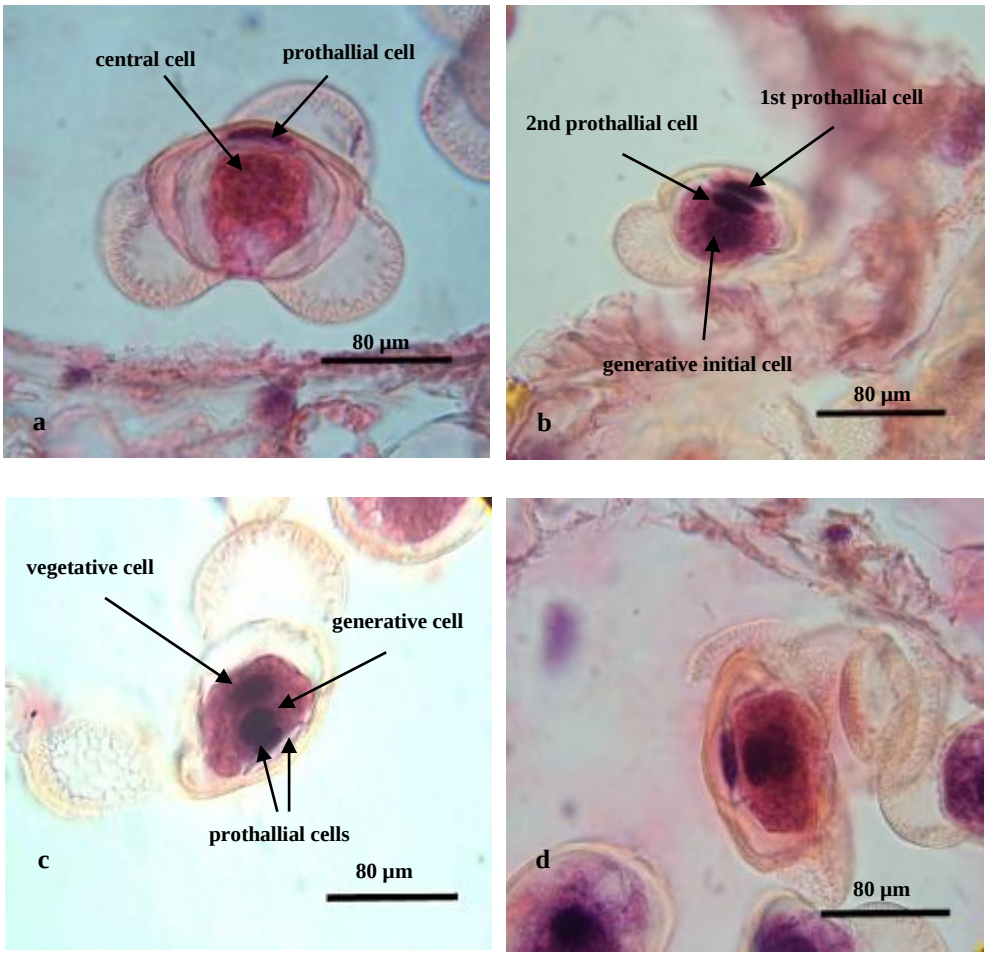

Fig. 7. Different stages of pollen development, from immature to mature pollens; (a) first mitosis and the formation of the first prothalian and central cell; (b) division of the central cell and formation of the second prothallial cells and also the initial generative cell; (c) generative initial cell is divided to generative and vegetative cells; (d) degeneration of the two prothallial cells is notable.

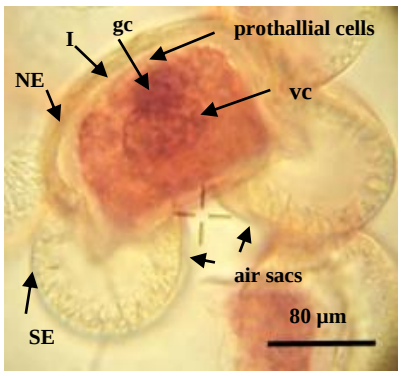

Fig. 8. Mature pollen. SE – sexine; NE – nexin; I – intine; gc – generative cell; vc – vegetative cell.

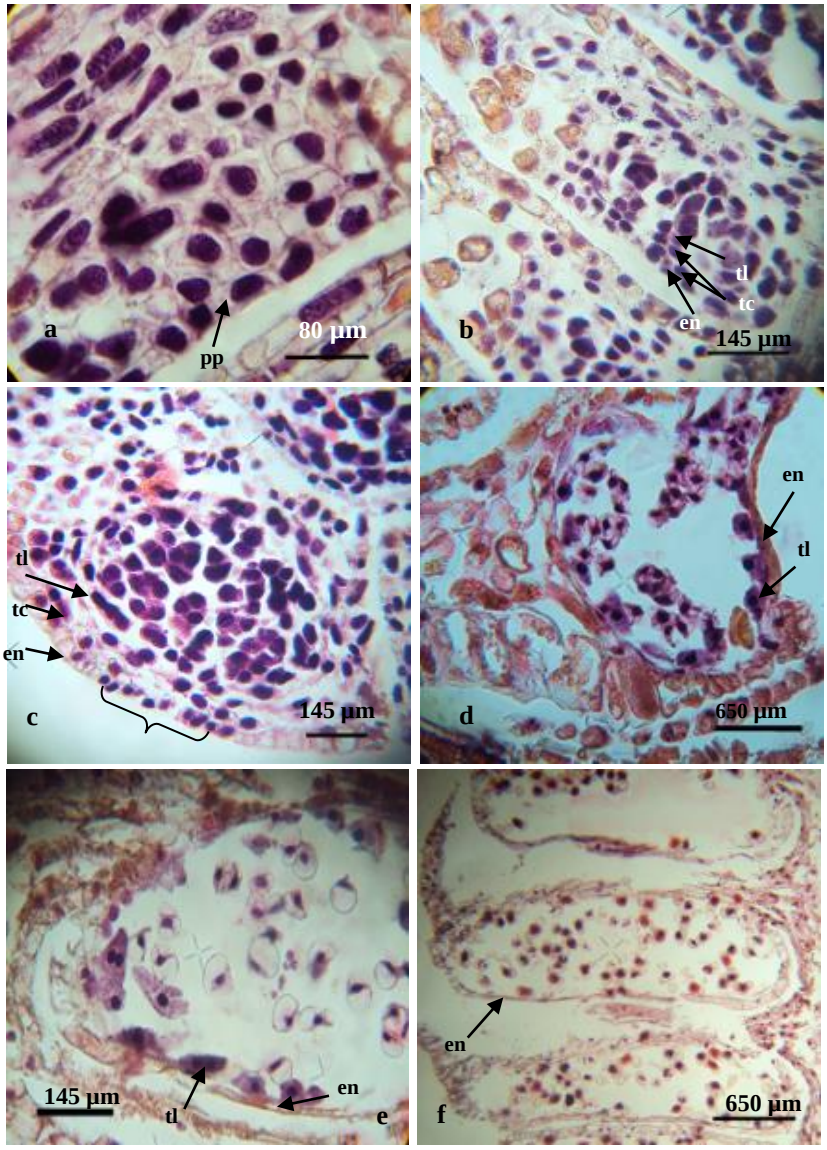

Fig. 9. Formation of different layers of pollen sac. (a) archespore tissue and the primary parietal layer; (b) the primary parietal layer divided periclinally to form the endothecium layer, two transition layers and tapetum layer; (c) transition layers gradually degenerated and the endothecium layer anticlinally divided to smaller cells (accolade shows the dehiscence location of pollen sac); (d) only tapetum cells with a large nucleus impact cytoplasm and endothecium layers have remained from the pollen sac layers. Some of the tapetum cells have degraded and have fallen off; (e) separation and degradation of the tapetum cells continue. pp, primary parietal layer; en, endothecium; tc, transition cells, tl, tapetum layer

Discussion

Results of this study showed that apical meristem of *Pinus eldarica* have four meristematic zones: (1) surface meristem, (2) central mother cell zone, (3) rib meristem and (4) peripheral zone. Observations of this study are in consent with JORDY (2004) on *P. pinaster* and MACDONALD & LITTLE (2006) on *P. sylvestris*. According to the theory of POPHAM (1951), there are three SAMs in the gymnosperms: (1) *Cycas* type, (2) *Ginkgo* type and (3) *Cryptomeria - Abies* type. Our results showed a *Cryptomeria - Abies* type of SAM in *P. eldarica*.

In the initial stages, the side axillary buds (Abs) of SAM are indeterminate, but according to their position on shoots, season and the age of the tree, they can be differentiated to (1) short shoot buds, that produce two needle leaves; (2) male cone buds and (3) female cone buds. According to our observations Abs in trees older than 30 - years old differentiate to male cones from the middle to the upper shoots or needles in the final days of summer. They differentiate to female cones (in the case of the nearest Abs to SAM) in mid- fall from the middle to lower shoots and only to needle leaves in mid- winter. The Abs greatly enlarge at first without any leaf primordial initiation. Later on, a large number of microsporophylls initiated using up the unspecified surface produced earlier from basal up to the Abs. This model has been reported for vegetative and generative meristems in some conifers [KWIATKOWSKA, 2004], our results show that the generative meristem of *P. eldarica* fits in this model.

Their prominent nuclei and compact cytoplasm are features of archesporial cells. In this developmental stage, young microsporophylls walls are single layered. When the sporogenous cells start to separate, the wall of microsporophylls consists of four layers and by the time the tetraspors start to separate from each other, the middle layers have been degenerated. Tapetal cells are large and may have two nuclei as shown by PANDEY & al. (1986).

The cytokines of the pollen mother cells are non- synchronous type. This process may occur before winter dormancy (some *Chamaecyparis* and *Juniperus* species in the Cupressaceae), or meiosis might begin before the winter, pause at a diffuse diplotene stage, then resume and form microspores after winter dormancy (*Larix, Pseudotsuga* and *Tsuga* in the Pinaceae and *Thuja* in Cupressaceae). Results of this study show that meiosis and pollen development of *P. eldarica* meiosis occurs before the winter dormancy and continues through the winter. Whereas in some conifers, all stages of meiosis and pollen development occur after winter dormancy [FERNANDO & al. 2005]. Our observations show that tetrad dissociation can be both of synchronous and non- synchronous type.

The tapetum cells at first are secretory with multiplication of the nuclei (2 nuclei). Later on they change to amoebic type (thus the nursery type). In *P. eldarica* before the pollen has shed, mature microspores go through three uneven division to form two lens shape prothallial cells, vegetative cell (tube cell) and generative cell (antheridial cell). Therefore, the pollen is shed at the four- cell stage of development. According to the FERNANDO & al. (2005), in the Pinaceae, pollen may be shed at the four- (like *Picea asperata*, LÜ & al. 2003) or five- cell (like *Pinus contorta*, OWENS & al. 1981) stage and pollen of *P. eldarica* is of the former type.

Conclusions

According to this anatomical-developmental study, the vegetative meristem of *P. eldarica* is in the *Cryptomerya-Abies* category. The meiosis occurs before the winter dormancy and continues through the winter and the pollen is shed at the four- cell stage of development.

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PRODUCTIVITY AND CYTOGENETIC STABILITY OF PROTOCORM-LIKE BODIES OF HYBRID *CYMBIDIUM* CRYOPRESERVED BY ENCAPSULATION-DEHYDRATION AND VITRIFICATION

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Abstract: Transformed and untransformed protocorm-like bodies (PLBs) of hybrid *Cymbidium* Twilight Moon 'Day Light' were cryopreserved using two methods (encapsulation-dehydration and vitrification) to form new PLBs (*neo*-PLBs) on Teixeira *Cymbidium* (TC) medium. The organogenic response was quantified and genetic stability was assessed by flow cytometry. Intact PLBs produced significantly more *neo*-PLBs and fresh weight of *neo*-PLBs than half-PLBs or PLB longitudinal thin cell layers (lTCLs), for two vitrification protocols and for an encapsulation-dehydration method. The addition of 2% sucrose significantly improved the number of *neo*-PLBs than half-PLBs or PLB lTCLs but any other concentration of sucrose, and all other osmotic agents (mannose, PEG-6000, DMSO) at any concentration resulted in significantly worse *neo*-PLB formation. However, the length of exposure to sucrose did not significantly affect the number or fresh weight of *neo*-PLBs. The desiccation (air current or silica gel) method applied negatively affected the number and fresh weight of *neo*-PLBs. The length of the cryopreservation period negatively impacted the number of *neo*-PLBs and fresh weight of transformed or untransformed *neo*-PLBs. Most severe negative effects were registered after one year of cryopreservation. Cryopreserved *neo*-PLBs showed high levels of endopolyploidy (8C-64C) relative to non-cryopreserved PLBs.

Keywords: alginate bead, orchids, PLB, thin cell layers, Teixeira *Cymbidium* (TC) medium, transgenic

Introduction

Orchids are an important group of flowering plants due to their ornamental value. Cryopreservation, or preservation in liquid nitrogen (LN; –196 °C), provides a useful way of preserving rare or endangered orchid tissue over a long period of time and can be used to create cryobanks [BENSON, 2008]. For example, many members of the orchid family (Orchidaceae) are included in the Convention on International Trade in Endangered Species (CITES) Appendix II (2014), making the search for suitable regeneration and preservation protocols all the more important. To date, several orchid explants (seed, meristems, tissue-cultured shoot primordia, immature seeds, somatic embryos or protocorm-like bodies (PLBs), callus) have been cryopreserved with different levels of success [WANG & PERL, 2006; HOSSAIN & al. 2013]. The number of studies reporting on the cryopreservation of orchid PLBs or protocorms remains limited, for example *Geodorum densiflorum* [DATTA & al. 1999], *Doritaenopsis* [TSUKAZAKI & al. 2000], *Dendrobium* Walter Oumae [LURSWIJIDJARUS & THAMMASIRI, 2004], *Dendrobium candidum* [YIN & HONG, 2009], *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011], *Cymbidium eburneum*

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[GOGOI & al. 2012], hybrid *Cymbidium* Twilight Moon 'Day Light' [TEIXEIRA DA SILVA, 2013c], and *Aerides odorata* [HONGTHONGKHAM & BUNNAG, 2014] while more work has been achieved with orchid seeds. The cryopreservation of *Dendrobium* germplasm was recently reviewed [TEIXEIRA DA SILVA & al. 2014]. SAIPRASAD & POLISETTY (2003) encapsulated *Dendrobium*, *Oncidium* and *Cattleya* PLBs at the leaf primordium stage, 13-15 days after culture. Half-PLB explants that had developed for two weeks prior to encapsulation resulted in higher survival of alginate beads [TEIXEIRA DA SILVA, 2012a]. NAGANANDA & al. (2011) encapsulated the PLBs of *Flickingeria nodosa* and achieved 95% conversion to plants after 3 months' storage at 4 °C. Cold storage, however, was not successful for *Cymbidium* hybrid PLBs [TEIXEIRA DA SILVA, 2012a].

In all cases, however, once the cryopreserved tissue emerges from cryostorage, it must have the ability to resume growth and regenerate. In that sense, tissue culture remains an essential complement to cryostorage, at least for orchids. This study employs one form of cryopreservation, encapsulation-dehydration [FABRE & DEREUDDRE, 1990] which generally involves three steps: 1) encapsulation in alginate beads, the synseeds (the former term will be used throughout this manuscript); 2) treatment with a high concentration of sucrose to reduce moisture content to 20-30%; 3) rapid freezing in LN. In another popular cryopreservation method, vitrification, explants are treated with a concentrated vitrification solution for variable periods of time (15 min to 2 h), followed by a direct plunge into LN, which results in both intra- and extra-cellular vitrification, which refers to the physical process of transition of an aqueous solution into an amorphous and glassy (non-crystalline) state [LAMBARDI & al. 2006]. The vitrification solution consists of a concentrated mixture of penetrating and non-penetrating cryoprotectant substances. The most commonly applied solution, named PVS2 (Plant Vitrification Solution n° 2), consists of 30% glycerol, 15% ethylene glycol, 15% DMSO (all v/v) and 0.4 M sucrose [SAKAI & al. 1990]. For example, PVS2 was used for the cryopreservation of *Vanda coerulea* [JITSOPAKUL & al. 2007]. Due to its popularity, vitrification was also tested in this study.

Generally, 4 °C has been found to be most suitable for storage of orchid alginate beads or synseed [SAIPRASAD & POLISETTY, 2003; IKHLAQ & al. 2010] although there is a wealth of literature on the choice of optimum temperature and light conditions (reviewed by SHARMA & al. 2013). Encapsulation-dehydration is likely to be the most suitable form of cryopreservation because it results in a high survival frequency. One reason is that the encapsulation of PLBs within calcium alginate beads protects them from direct damage when subjected to desiccation [KHODDAMZADEH & al. 2011] while avoiding toxic cryoprotectants through the use of more "natural" osmotica such as sucrose. The sucrose molarity in a bead (synseed) is further increased by desiccation which results in a glass transition during cooling in LN, preventing ice crystal formation (a cause of lethal damage to living cells) during exposure to ultra-low temperature [ENGELMANN, 2011].

A synthetic seed is an encapsulated clonal product that can eventually grow into a plant, either *in vitro* or *ex vitro*, through "conversion" or "germination", somewhat like a conventional zygotic seed [SHARMA & al. 2013]. Since PLBs are considered to be somatic embryos in orchids with a high degree of cytogenetic stability [TEIXEIRA DA SILVA & TANAKA, 2006], they have been used as explants in this study and, when encapsulated for cryopreservation, are termed alginate beads. TEIXEIRA DA SILVA (2012a) preserved hybrid *Cymbidium* PLBs as alginate beads in the short- and mid-term (1-12 months). In that study, in one treatment that involved cryopreservation, PLBs were

cryostored in LN in the dark for 1, 6 and 12 months without any pre-treatment, i.e., a crude or simple form of cryostorage. The result was understandably poor, and only 6% of cryostored PLBs formed *neo*-PLBs (i.e. new PLBs) after plating on optimized *Cymbidium* PLB regeneration medium, Teixeira *Cymbidium* (TC) medium [TEIXEIRA DA SILVA, 2012b]. Direct cryopreservation would not surprisingly result in cellular damage, in the form of ice crystal formation, due to the expansion of cells without a suitable osmotic treatment to prepare them for the cold shock treatment by LN. Based on a previously established protocol for the cryopreservation of a popular *Cymbidium* hybrid cultivar, Twilight Moon 'Day Light' [TEIXEIRA DA SILVA, 2013c], *neo*-PLB formation from PLB explants was further quantified in this study. Furthermore, the viability of explants post-cryopreservation and pre-re-plating was also assessed by flow cytometry.

Materials and methods

All protocols strictly follow TEIXEIRA DA SILVA (2012a, 2013c), almost *verbatim* in parts, except for flow cytometry.

Chemicals and reagents

All chemicals and reagents were of analytical grade and were purchased from either Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Tokushima, Japan) originally developed from shoot-tip culture on VACIN & WENT (VW, 1949) agar medium without PGRs, were induced and subcultured (PLB induction and proliferation medium) every 2 months on VW medium supplemented with 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin (Kin), 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA), following TEIXEIRA DA SILVA & al. (2005) and TEIXEIRA DA SILVA & TANAKA (2006). All media were adjusted to pH 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 ml medium in 100-ml Erlenmeyer flasks, double-capped with aluminium foil, at 25 °C, under a 16-h photoperiod with a photosynthetic photon flux density of 45 µmol/m^2/s provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally dissected as two pieces of PLB (3-4 mm in diameter) segments, 10/flask, were used as explants for PLB induction and proliferation. Culture conditions and media followed the recommendations previously established for medium formulation [TEIXEIRA DA SILVA & al. 2005], biotic [TEIXEIRA DA SILVA & al. 2006b] and abiotic factors [TEIXEIRA DA SILVA & al. 2006a] for PLB induction, formation and proliferation.

Explant preparation

Based on a protocol for the encapsulation of PLBs [TEIXEIRA DA SILVA, 2012a], three explant types were tested: intact PLBs, half-PLBs, and PLB longitudinal thin cell layers (lTCLs). lTCLs are useful explants in orchid regeneration due to the ability to control organogenesis at a fine scale relative to larger explants [TEIXEIRA DA SILVA & TANAKA 2006; TEIXEIRA DA SILVA, 2013a], although the risk is that they can also lose viability due to increased chances of damage during explant preparation. Intact PLBs were prepared by removing the terminal shoot apical meristem and white base of callus-like non-regenerative tissue, half-PLBs were prepared by cutting prepared intact PLBs along a

longitudinal axis while PLB lTCLs were prepared by 1-mm deep 1 mm^2 sections (5 or 6 per PLB) made from stock PLBs (see detailed preparation of three explant types in TEIXEIRA DA SILVA, 2013a).

Explant encapsulation and bead (synseed) preparation, and cryopreservation

Explant preparation and handling is described broadly in Fig. 1. Explants were encapsulated by immersing each explant with sterilized forceps in a 3.5% (w/v) sodium alginate solution made up in TC medium supplemented with 10% (v/v) coconut water [TEIXEIRA DA SILVA, 2012a] (Fig. 2A). Using wide-mouth glass pipettes (i.e., 5- or 10-ml pipettes with terminal end sawn off), this solution was fed into a complexing solution made of 100 mM $CaCl_2$ for 45 min, drop by drop, each drop containing a single explant. While half-PLBs and PLB lTCLs could pass through the neck of the 5-ml pipettes, 10-ml pipettes were required for intact PLBs. The complexing solution was stirred gently with a sterilized metal spoon once every 10 min to prevent the alginate beads from adhering to each other and fusing. After hardening, the alginate beads were rinsed gently for 10 min in sterile distilled water under sterile conditions on the clean bench to wash away any remaining $CaCl_2$ residue. Since the cryopreservation of raw (i.e., non-encapsulated) PLBs was poor in previous trials [TEIXEIRA DA SILVA, 2012a], in this study, encapsulated explants (intact PLBs, half-PLBs, PLB lTCLs) were removed from the alginate beads and transferred onto TC basal medium under the same conditions as in PLB initiation and proliferation (indicated above), following cryopreservation, to assess the ability to form *neo*-PLBs (Fig. 2B). Simultaneously, a set of explants was left in the alginate case and plated onto TC basal medium.

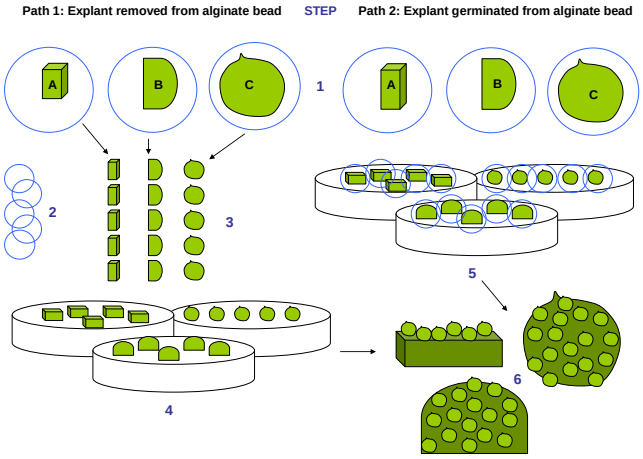

Fig. 1. General schematic of explant preparation and processing for different objectives of this experiment. For all studies, alginate beads (syn. synseeds) were created using 3.5% (w/v) sodium alginate-based TC medium [TEIXEIRA DA SILVA, 2012b] supplemented with 10% (v/v) coconut water [TEIXEIRA DA SILVA, 2012a] from three explants, in step 1: A = PLB lTCLs, B = half-PLBs, C = intact PLBs (see detailed preparation of all three explant types in TEIXEIRA DA SILVA, 2013a, 2013c). In path 1, any explant type was excised from the alginate bead, alginate bead sodium alginate cases were discarded (step 2) and explants were used in a "naked" form (step 3), plated separately on Petri dishes (step 4). In path 2, any explant type was retained within its alginate bead and alginate beads were separately plated on and germinated in Petri dishes (step 5). Finally, the development of *neo*-PLBs (i.e., new PLBs) from explants A, B and C was assessed from paths 1 and 2 (step 6). Explant sizes, shapes, distributions and densities are not proportional or to scale, and depending on the sampling time, can reveal completely different quantitative organogenic outcomes [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013].

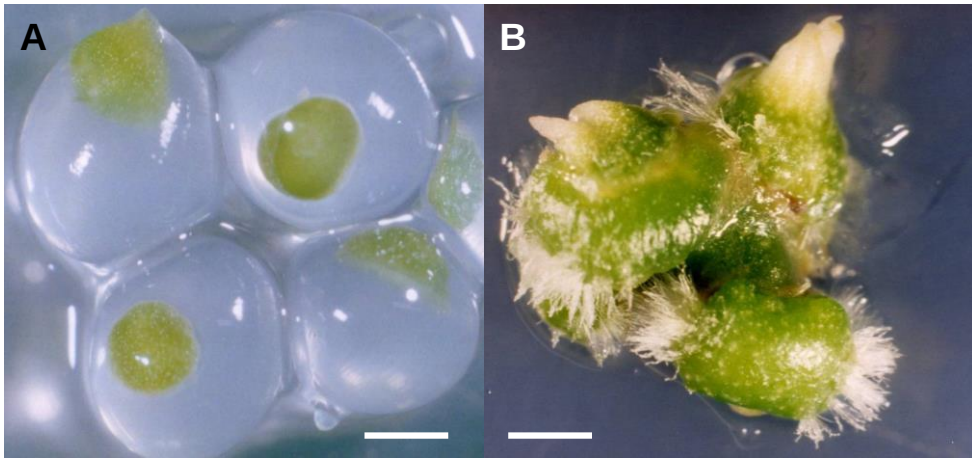

Fig. 2. (A) Encapsulated half-PLBs in sodium alginate synseeds. (B) Young (30 days old), actively dividing *neo*-PLBs from cryopreserved half-PLB growing on TC medium. Scale bars: A = 1 cm; B = 5 mm.

Preculture and priming of PLB-alginate beads

Two parameters were assessed: osmotic hydration (i.e., osmoticum loading) and desiccation.

1. Osmotic hydration

Intact PLBs were confirmed to be the best explant for encapsulation due to higher survival and percentage of explants forming PLBs (explants removed from the encapsulated alginate bead) or percentage re-growth (encapsulated explants) (see Tab. 1 of TEIXEIRA DA SILVA, 2013c). Encapsulated PLBs (25/dish) (i.e., alginate beads) were placed in 25-cm diameter, 1-cm deep Petri dishes (As-One, Osaka, Japan) and precultured in stationary liquid sucrose-free TC medium (50 ml/dish) (control), 1, 2, 3, or 4% (w/v or v/v) sucrose, mannose, polyethylene glycol (PEG-6000) or DMSO for 24 h. After it had been established that 2% sucrose was the best osmoticum, the same explants were plated under the same conditions for 0, 12, 24, 36 or 48 h. Precultured, encapsulated PLBs were plunged into LN (10 alginate beads/treatment, each within 5-cm wide stainless steel cages (maker unknown) or within 2-ml polypropylene tubes (2.0 ml Conical Screw Cap Microtube; Quality Scientific Plastics Inc., Kansas, USA), 5/tube ensuring that all vials and tubes were appropriately labeled). Pre-trials showed that there was no difference between the use of polypropylene tubes or stainless steel cages. After storage in LN for 1 h, the vials were rapidly re-warmed (3 min in a 35 °C water bath) and PLBs were retained in the alginate beads or removed from the alginate beads and replated on TC basal medium under the same conditions as PLB initiation and proliferation indicated above to assess survival and PLB-formation ability. Once it had been established that pre-culture of intact PLBs in 2% sucrose (Tab. 2 of TEIXEIRA DA SILVA, 2013c) for 24 h (Tab. 3 of TEIXEIRA DA SILVA, 2013c) was the best treatment and explant type combination, in terms of explant survival and ability to regenerate PLBs (i.e., germinate), intact PLBs pre-treated in 2% sucrose for 24 h were placed in LN for 1 h, 1 day, 1 week, 1 month or 1 year to assess the impact of cryopreservation period on the same survival and growth parameters.

2. Desiccation method and duration

After pre-culture of encapsulated intact PLBs under optimal conditions determined in TEIXEIRA DA SILVA (2013c) (liquid TC, 2% sucrose for 24 h), alginate beads were rapidly surface-dried by rolling them, in an air-flow cabinet, on two layers of sterile filter (Whatman No. 1) paper to remove any remaining liquid preculture TC medium and were subjected to dehydration by evaporation at room temperature. Two desiccation methods were tested at 25 °C: (1) dehydration under the air current of a laminar air flow cabinet; (2) dehydration in sealed (with a single strip of Parafilm® (Pechiney Plastic Packaging Co., Chicago, USA) Petri dishes (9 cm in diameter) containing 50 g of dry silica gel (Wako). Alginate beads from both dehydration methods were maintained in this state for 0 (control), 1, 6 or 12, 24 or 48 h. Processing of alginate beads (i.e., adding to LN for the same periods and post-cryostorage treatments) was identical to the preculture trials. PLB moisture content was determined as explained next.

Vitrification protocols

PLBs derived from growth on TC medium were prepared using the same protocol for the encapsulation of PLBs [TEIXEIRA DA SILVA, 2012a], except that PLBs were not encapsulated. Two protocols were followed. In protocol 1, unencapsulated PLBs were immersed in PVS2 [SAKAI & al. 1990, 2000] for 30, 60, 90 or 120 min. PLBs were then placed in 2-ml polypropylene tubes, 5/tube, and plunged directly into LN for 1 h, 1 day, 1 week, 1 month or 1 year. Protocol 2, originally established for *Rubus* [KOVALCHUK & al. 2010], was followed with modifications. Unencapsulated PLBs were submerged in loading solution (TC medium with 2 M glycerol and 0.4 M sucrose) for 20 min and then transferred to 2-ml cryovials on ice with 2 ml PVS2 for 80 min. Vials were then submerged in LN for 1 h, 1 day, 1 week, 1 month or 1 year, resulting in vitrified PLBs. All subsequent steps (rewarming and PLB regeneration) followed the alginate beads cryopreservation protocol described above. Controls were pretreated, exposed to PVS2 alone (protocol 1) or to loading solution plus PVS2 solution (protocol 2), then rinsed with liquid TC medium and plated on recovery medium solidified with agar. To assess the effectiveness for transgenic material, transgenic PLBs derived from particle bombardment containing the pWI-GUS plasmid vector [TEIXEIRA DA SILVA & TANAKA, 2009, 2011] were cryopreserved using vitrification Protocol 1 for the same period of time and compared.

Flow cytometry

This protocol was used previously for the same cultivar in TEIXEIRA DA SILVA & TANAKA (2006) and TEIXEIRA DA SILVA & al. (2005, 2006a, 2006b, 2007), although described with some modifications here, including the lack of an internal standard, which was barley in previous studies. Nuclei were isolated from about 0.5 cm^2 of control and treatment *neo*-PLBs by chopping in a few drops 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, pH 7.5; MISHIBA & MII, 2000). Nuclear fluorescence was measured using a Partec® Ploidy Analyser (Partec GmbH, Munich, Germany) after filtering the nuclear suspension through 30 µm mesh size nylon filter (CellTrics®, Partec GmbH) and adding five times of Buffer A for 1 min. Three samples were measured, and relative fluorescence intensity of the nuclei was analyzed when the coefficient of variation was < 4%. A minimum of 2500 nuclei were counted for any sample.

Morphological and photosynthetic parameters assessed

For all treatments, the number of *neo*-PLBs that formed from explants (intact PLBs, half-PLBs, PLB lTCLs) on TC medium was observed at 2 and 4 weeks, but scored at 4 weeks (30 days). The fresh weight (mg) of PLB explants + *neo*-PLBs that formed on them were also assessed at 30 days.

Statistical analyses

Experiments were organized according to a randomized complete block design with three blocks of 10 replicates per treatment (i.e., each medium and/or explant type). All experiments were repeated three times (n = 90, total sample size per treatment). Data was subjected to analysis of variance with mean separations by Duncan's multiple range test (DMRT) using SAS® ver. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were presented at the level of $P \leq 0.05$.

Results

Following the preparation of three different explant types (Fig. 1) and treatment with two cryopreservation protocols, this study has two main sets of novel findings: 1) the quantitative outcome of *neo*-PLBs that can be achieved through cryopreservation using two techniques, as outlined in TEIXEIRA DA SILVA (2013c); 2) the viability of *neo*-PLBs as assessed by flow cytometry.

Intact PLBs produced significantly more *neo*-PLBs than half-PLBs or PLB lTCLs, independent of the path (1 or 2) (Tab. 1). The same trend was observed for fresh weight of *neo*-PLBs. Similarly, within each cryopreservation method tested (vitrification protocol 1 or 2, or encapsulation-dehydration), intact PLBs produced significantly more *neo*-PLBs than half-PLBs or PLB lTCLs, independent of the path (1 or 2) (Tab. 1).

The addition of 2% sucrose significantly improved the number of *neo*-PLBs than half-PLBs or PLB lTCLs, independent of the path (1 or 2) (Tab. 2). All other osmotic agents (at any concentration) resulted in a significant decrease in the number of *neo*-PLBs and fresh weight of *neo*-PLBs than half-PLBs or PLB lTCLs, independent of the path (1 or 2) (Tab. 2). However, the length of exposure to sucrose did not significantly affect the number of *neo*-PLBs and fresh weight of *neo*-PLBs in path 1, except for 48 h exposure (Tab. 3). The trend was different, however, for path 2, in which there was a significantly negative effect on the number of *neo*-PLBs and fresh weight of *neo*-PLBs (Tab. 3).

In general, the method of desiccation (air current or silica gel) negatively affected the number of *neo*-PLBs and fresh weight of *neo*-PLBs in both paths (Tab. 4).

The length of cryopreservation had a negative impact on the number of *neo*-PLBs and fresh weight of *neo*-PLBs, with most severe negative effects being registered after 1 year cryopreservation period (Tab. 5). A similar trend was observed for transformed tissue, but the negative impact was even more pronounced (Tab. 5).

Cryopreserved *neo*-PLBs showed high levels of endopolyploidy relative to non-cryopreserved PLBs (Fig. 3), but shoots that regenerated from all *neo*-PLBs (cryopreserved, transformed, or not) showed no endopolyploidy (data not shown).

Tab. 1. Effect of explant type during encapsulation (i.e., alginate bead) of hybrid *Cymbidium* Twilight Moon 'Day Light' on number of *neo*-PLBs formed and fresh weight assessed 30 days after replating explants

		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
		Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control 1	Intact PLB	9.6 a	814 a	n/a	n/a
	Half-PLB	8.2 b	521 c	n/a	n/a
	PLB lTCL	2.2 e	186 e	n/a	n/a
Control 2	Intact PLB	7.9 b	763 b	6.4 a	718 a
	Half-PLB	5.0 d	486 cd	3.4 b	421 b
	PLB lTCL	0.4 f	156 ef	0.3 c	131 d
VP 1	Intact PLB	7.8 b	416 d	n/a	n/a
	Half-PLB	3.0 e	371 d	n/a	n/a
	PLB lTCL	0.2 f	91 f	n/a	n/a
VP 2	Intact PLB	6.5 c	456 cd	n/a	n/a
	Half-PLB	2.0 e	386 d	n/a	n/a
	PLB lTCL	0.1 f	108 f	n/a	n/a
E-D	Intact PLB	8.4 b	516 c	6.1 a	407 b
	Half-PLB	5.2 d	401 d	3.9 b	298 c
	PLB lTCL	0.4 f	126 ef	0 c	63 e

E-D, encapsulation-dehydration; lTCL, longitudinal thin cell layer; n/a, not applicable (because the vitrification protocols do not involve explant encapsulation as an alginate bead); PLB, protocorm-like body; VP = vitrification protocol

* see details of paths 1 and 2 in Fig. 1

[1,2] Controls: 1 = non-cryopreserved and unencapsulated state on TC medium [TEIXEIRA DA SILVA, 2012b]; 2 = non-cryopreserved but encapsulated state (i.e., alginate bead according to TEIXEIRA DA SILVA, 2012a) on TC medium.

Different letters within each column represent significant differences across treatments within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

Tab. 2. Effect of osmotic agent (24-h exposure) on efficiency of encapsulated PLB pre-treatment using intact hybrid *Cymbidium* Twilight Moon 'Day Light' PLBs[1] as assessed by number of *neo*-PLBs formed and fresh weight assessed 30 days after replating explants

Treatment	Concentration % (w/v)	Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
		Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	7.9 b	763 b	6.4 a	718 a
Sucrose	1	6.8 c	681 c	4.6 b	554 b
	2	8.8 a	874 a	6.1 a	699 a
	3	4.9 d	504 d	3.3 c	412 c
	4	2.2 f	238 f	1.7 ef	184 e
Mannose	1	3.4 e	381 e	2.6 cd	281 d
	2	3.0 e	314 ef	2.1 d	201 de
	3	1.1 g	109 g	0.9 e	106 f
	4	0 h	56 h***	0 f	58 g***
PEG-6000	1	0.3 h	71 gh	0.1 f	66 g
	2	0.8 f	99 g	0.2 f	68 g
	3	0.2 h	68 gh	0 f	61 g***
	4	0 h	57 h***	0 f	57 g***
DMSO	1	2.5 ef	271 ef	1.6 ef	173 e
	2	2.0 f	221 f	1.2 e	124 ef
	3	0.9 f	106 g	0.3 f	68 g
	4	0.2 h	63 h	0 f	54 g***

[1] Results from Table 1 indicated that intact PLBs were the best explant for cryopreservation

DMSO, dimethylsulfoxide; PEG, polyethylene glycol 6000; PLB, protocorm-like body

* see details of paths 1 and 2 in Fig. 1

** Control = non-cryopreserved but encapsulated state (i.e., alginate bead according to TEIXEIRA DA SILVA, 2012a) on TC medium [TEIXEIRA DA SILVA 2012b]

*** The weight is not 0, even though no new explants formed. The average weight of the basal explant is 54 mg (n = 10; data not shown). Different letters within each column represent significant differences across treatments within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

Tab. 3. Effect of length of exposure to 2% sucrose[1] (osmotic agent) on efficiency of encapsulated PLB pre-treatment using intact hybrid *Cymbidium* Twilight Moon 'Day Light' PLBs[2] as assessed by number of *neo*-PLBs formed and fresh weight assessed 30 days after replating explants

		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
	Duration (h)	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	7.9 a	763 a	6.4 a	718 a
	12	7.7 a	741 a	6.1 a	699 ab
	24[1]	7.8 a	756 a	5.6 ab	631 b
	36	7.6 a	749 a	5.2 b	608 b
	48	7.5 a	721 b	4.3 c	501 c

[1] Results from Table 2 indicated 2% sucrose to be the best osmotic treatment for cryopreservation

[2] Results from Table 1 indicated intact PLBs to be the best explant for cryopreservation

PLB, protocorm-like body

* see details of paths 1 and 2 in Fig. 1

** Control = non-cryopreserved state on TC medium [TEIXEIRA DA SILVA, 2012b]. Different letters within each column represent significant differences between treatments within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

Tab. 4. Impact of desiccation method on efficiency of encapsulated PLB pre-treatment using intact hybrid *Cymbidium* Twilight Moon 'Day Light' PLBs as assessed by number of neo-PLBs formed and fresh weight assessed 30 days after replating explants

		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
	Duration (h)	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	7.9 a	763 a	6.4 a	718 a
Air current	6	6.1 b	518 b	6.2 a	689 ab
	12	3.8 d	263 e	5.8 ab	651 b
	24	2.9 e	198 f	5.2 b	482 c
	36	1.1 f	104 g	3.8 cd	291 d
Silica gel	6	5.6 bc	472 c	4.1 c	701 a
	12	5.2 c	436 c	3.9 c	693 ab
	24	4.4 cd	318 d	3.7 cd	481 c
	36	2.9 e	206 f	3.3 d	388 d

PLB, protocorm-like body

* see details of paths 1 and 2 in Fig. 1.

** Control = non-cryopreserved state on TC medium [TEIXEIRA DA SILVA, 2012b]. Different letters within each column represent significant differences across treatments within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

Tab. 5. Effect of cryopreservation period on efficiency of encapsulated PLB pre-treatment using intact hybrid *Cymbidium* Twilight Moon 'Day Light' PLBs[1] following osmopriming in 2% sucrose[2] for 24 h[3] as assessed by number of *neo*-PLBs formed and fresh weight assessed 30 days after replating explants.

		Untransformed tissue			
		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
	Cryopreservation period	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	7.9 a	763 a	6.4 a	718 a
	1 week	7.6 a	731 ab	4.1 b	501 b
	1 month	7.4 a	716 b	3.0 c	298 c
	1 year	6.8 b	621 c	1.1 d	146 d
		Transformed tissue***			
		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
	Cryopreservation period	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	6.4 a	598 a	5.2 a	614 a
	1 week	6.1 a	571 ab	3.9 b	482 b
	1 month	6.0 a	556 b	2.6 c	238 c
	1 year	4.9 b	473 c	0.8 d	106 d

[1] Results from Table 1 indicated intact PLBs to be the best explant for cryopreservation

[2] Results from Table 2 indicated 2% sucrose to be the best osmotic treatment for cryopreservation

[3] Results from Table 3 indicated osmopriming with 2% sucrose for 24 h to be the best treatment for cryopreservation

PLB, protocorm-like body

* see details of paths 1 and 2 in Fig. 1.

** Control = non-cryopreserved state on TC medium [TEIXEIRA DA SILVA, 2012b]

*** Transformed PLBs containing pWI-GUS obtained following particle bombardment according to TEIXEIRA DA SILVA & TANAKA (2009, 2011) and transgenic tissue confirmed by GUS assay and growth on selective medium containing 100 mg/l kanamycin sulphate; Vitrification Protocol 1 was used after it was established that this was the superior protocol.

Different letters within each column represent significant differences between periods within each tissue (transformed vs untransformed) within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

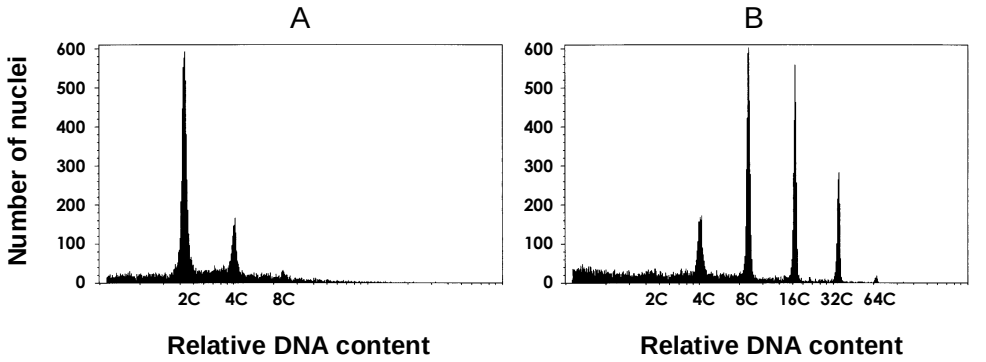

Fig. 3. Flow cytometric analyses of control and cryopreserved PLBs. (A) Young (30 days old), actively dividing *neo*-PLBs from control, non-cryopreserved half-PLB growing on TC medium. (B) Older (120 days old) *neo*-PLBs from which shoots and roots have formed derived from cryopreserved (pooled from encapsulation-dehydration and vitrification protocols) half-PLBs under optimized conditions indicated by this study and TEIXEIRA DA SILVA (2013a, 2013b).

Discussion

Cymbidium in the wider perspective of orchid cryopreservation

The cryopreservation of orchid tissue serves as an important biotechnological tool for the preservation of rare [e.g., GOGOI & al. 2012] or important tissue [e.g., TEIXEIRA DA SILVA, 2013c]. However, storage alone serves no purpose if the material is unable to regenerate into morphologically stable tissue after thawing. In an earlier study [TEIXEIRA DA SILVA, 2013c], an attempt was made to optimize cryopreservation parameters step by step for the same hybrid *Cymbidium* tissue. The cryopreservation of "raw" tissue, without appropriate osmotic protection can result, understandably, in near necrosis of tissue and extremely poor, if any, regeneration after thawing [TEIXEIRA DA SILVA, 2012a]. With this premise in hand, at first, encapsulation of hybrid *Cymbidium* PLBs and alginate bead production was optimized according to TEIXEIRA DA SILVA (2012a). Of note is the fact that TC medium was employed in the calcium alginate bead and also included CW. Most, if not all orchid studies on cryopreservation and alginate bead do not include CW [HOSSAIN & al. 2013]. Then, using this optimized alginate bead medium, factors such as explant source, choice and concentration of osmoticant, method of dehydration and vitrification protocol were all tested and optimized [TEIXEIRA DA SILVA, 2013c]. By priming PLB alginate beads by culture in liquid sucrose-free TC medium supplemented with 2% sucrose for 24 h positively impacted cryostorage [TEIXEIRA DA SILVA, 2013c and Tab. 2, 3, this paper]. This "basal" level of sucrose in the alginate bead may have provided an osmotic buffer against damage by cryostorage (personal hypothesis) although desiccation or air-drying following exposure to silica gel, which had different rates of moisture loss, negatively impacted *neo*-PLB survival (Fig. 2 in TEIXEIRA DA SILVA, 2013c) but did not negatively impact the number of *neo*-PLBs (Tab. 3, this study). This counters the claims by other scientists [e.g., KHODDAMZADEH & al. 2011] who stated that desiccation of *Phalaenopsis* PLB tissue improved explant survival. Although the constant presence of the alginate bead was a negative influence on *neo*-PLB formation (Tab. 1, 2 in TEIXEIRA DA

SILVA, 2013c and Tab. 3, this study), it appeared to afford some protection (or buffering effect) against the negative impacts of desiccation (Tab. 4 in TEIXEIRA DA SILVA 2013c and Tab. 4, this study). In that study, only two treatments were comparable to the control (i.e., regular *in vitro* culture of PLBs without the formation of alginate bead): preculture of half-PLBs left on TC medium for 2 weeks (Fig. 1B), and the addition of CW at 10% (v/v) into the sodium alginate solution (Tab. 1 in TEIXEIRA DA SILVA, 2013c). In the former, most likely the PLBs that were used had already survived any injury from explant preparation and had already initiated the development of *neo*-PLBs while in the latter, CW tends to include a large number of unknown substances, including PGRs, which may have stimulated the growth of PLBs even further, as found for other orchids [ZENG & al. 2012; TEIXEIRA DA SILVA, 2013b]. Even though the use of intact PLBs resulted in higher fresh weight of PLB-alginate bead + *neo*-PLBs, the actual number of PLBs/explant was low, so it was only given a mildly effective code (Tab. 1 in TEIXEIRA DA SILVA, 2012a). In a separate study on an epiphytic orchid, *Cymbidium eburneum*, encapsulation-vitrification was superior to vitrification in the cryopreservation of protocorms, with both techniques requiring the use of PVS2 [GOGOI & al. 2012]. In this study, vitrification (either protocol 1 or 2) was superior to encapsulation-dehydration (Tab. 1).

Cymbidium longifolium synthetic seeds or 'synseeds' were formed after the encapsulation of protocorms [SINGH, 1988]. *Cymbidium* PLB synseeds embedded in a fungicide, and cocooned in chitosan resulted in a 35% germination rate when sown directly on non-sterilized substrate [NHUT & al. 2005]. Protocorms 3-4 mm in diameter were suitable for optimal conversion frequency of encapsulated *Cymbidium giganteum* PLBs, but smaller PLBs could not withstand encapsulation or required a long time to emerge out of the capsule [CORRIE & TANDON, 1993]. High germination frequencies of encapsulated *C. giganteum* PLBs [SAIPRASAD & POLISETTY, 2003] or *C. longifolium* protocorms [CHETIA & al. 1998] occurred when they were stored at 4 °C.

The encapsulation-dehydration and vitrification protocols used in this study for hybrid *Cymbidium* Twilight Moon 'Day Light' were developed by TEIXEIRA DA SILVA (2013c). The following 13 main findings (some written *verbatim*) could be observed in that study: 1) the percentage of explants forming *neo*-PLBs was always significantly lower than the percentage of surviving explants, except for the control. 2) Encapsulation negatively impacted explant survival. 3) Intact PLBs had a significantly higher re-growth and percentage survival than half-PLBs or PLB lTCLs. 4) For all three types of explants, Vitrification Protocol 1 was significantly better than Vitrification Protocol 2. 5) Even though explant survival from encapsulation-dehydration tended to be higher than other protocols, the percentage of explants forming *neo*-PLBs and re-growth percentage was always significantly lower. 6) Any treatment involving the removal of the explant from alginate beads showed higher survival than when explants were left encapsulated. Pre-treatment of PLBs with sucrose and mannose significantly negatively affected PLB viability, but sucrose, especially 2%, had the most positive influential impact on growth parameters. 7) The percentage of explants forming *neo*-PLBs was always significantly lower than the percentage of surviving explants and encapsulation had a negative effect on explant survival. 8) Mannose, PEG-6000 and DMSO negatively impacted explant survival and alginate bead re-growth (relative to sucrose). 9) Hyperhydricity was not observed in any treatment. 10) Preculture of PLBs in stationary liquid sucrose-free TC medium supplemented with 2% sucrose for 24 h improved viability and survival while mannose had

a negative impact on both these parameters. 11) When alginate beads were desiccated on a laminar flow bench, there was a much greater loss of relative moisture content than when silica gel was used, and even though the regeneration of *neo*-PLBs was better in the latter, both methods were in most cases inferior to the control. 12) The survival of half-PLBs decreased significantly as the storage period increased for transformed or untransformed tissue, although the latter showed lower values, possibly because of explant damage caused by the biolistic treatment. Even so, considerable control and transgenic material could be recovered which, if replated onto TC medium, was able to regenerate. 13) PLBs from cryopreservation treatments or *neo*-PLBs that formed from encapsulated PLBs were morphologically normal, as assessed by light microscopy and SEM while shoots from *neo*-PLBs derived from cryopreservation and alginate bead-derived treatments were also normal. Except for point 10), where no significant differences were found between most treatments in paths 1 and 2 (Tab. 3), the trends are the same for number of *neo*-PLBs and *neo*-PLB fresh weight in this study.

Some hyperhydricity was observed in *neo*-PLBs (Tab. 1 in TEIXEIRA DA SILVA 2013c), but the frequency was low. RADY & HANAFY (2004) also observed hyperhydricity in regenerating *Gypsophila paniculata* (non-orchid) plantlets following alginate bead germination. In this study, using optimized conditions, hyperhydricity was not observed in *neo*-PLBs or in regenerated shoots (data not shown).

One of the possible reasons for the low level of germination (i.e., percentage of alginate beads forming *neo*-PLBs in Tab. 1 in TEIXEIRA DA SILVA, 2013c), in which germination is not the traditional formation of a shoot, but rather the *neo*-formation of PLBs or the formation of *neo*-PLBs, may be because half-PLBs were used rather that intact PLBs, suggesting that tissue injury may have negatively impacted the regeneration capacity. The reader is reminded that PLBs are in fact considered to be (i.e., synonymous with) somatic embryos in orchids [TEIXEIRA DA SILVA & TANAKA, 2006], and would thus represent the ideal form of a dipolar propagule for alginate beads, even though half-PLBs and PLB-derived TCLs can also be used.

Why use alginate beads?

Alginate beads (synonymous with synseeds; SHARMA & al. 2013) present a hypothetically excellent way to cryostore orchid material for weeks to months, even years, while maintaining the clonal stability of material. Alginate beads in orchid biotechnology have particular relevance considering that they produce tiny, non-endospermic seeds. CORRIE & TANDON (1993) encapsulated *Cymbidium giganteum* protocorms and healthy plantlets formed after synseeds were transferred either to nutrient medium or directly to sterile soil with a 100% conversion frequency *in vitro*. As described in the introduction, PLB encapsulation is well documented in several orchids.

The principle of desiccation (or air-drying) is to reduce the water content to a sufficiently low level and to induce an intrinsic tolerance to desiccation by triggering the genes responsible for desiccation tolerance. In hybrid *Cymbidium* (Fig. 2 in TEIXEIRA Da SILVA 2013c), and also for *Phalaenopsis* [KHODDAMZADEH & al. 2011], silica gel allowed a slower decrease in moisture content than laminar air flow, providing, most likely, an opportunity for PLBs to adapt to dehydration stress. If PLBs are not sufficiently dehydrated, injury after immersion in LN can occur due to intracellular ice formation but if over over-dehydrated, osmotic stress can cause damage [BIAN & al. 2002], this being the

most likely explanation for the extremely low survival (6%) of encapsulated *Cymbidium* PLBs following immersion in LN without a tissue-drying pre-treatment [TEIXEIRA DA SILVA, 2012a]. Incidentally, in *Dendrobium candidum* PLBs, dehydration tolerance was also induced by treatment with abscisic acid (ABA) for three days and optimal water content for cryopreservation was estimated to be 11-33% [BIAN & al. 2002]. Exogenous ABA treatment of the PLBs induced accumulation of soluble sugars, heat-stable proteins, and dehydrins [BIAN & al. 2002]. The synthesis of dehydrins was also enhanced by ABA treatment in *Spathoglottis plicata* protocorms [WANG & al. 2003]. Dehydrins are involved in the reactions of plants against drought, salinity, and dehydration and their accumulation during cell dehydration suggests their involvement in protective reaction, including in membrane stabilization, preventing denaturation and aggregation of macromolecules, detoxification of salt ion, and stabilization of transcription machinery [CLOSE, 1997; ALLAGULOVA & al. 2003; HANIN & al. 2011]. Thus it is effective for the acquisition of dehydration tolerance to accumulate dehydrins in PLBs. When the moisture content of encapsulated plant tissue lies between 20 and 40%, this ensures the highest survival after cooling in LN because this level is roughly the amount of un-freezable water in a plant cell, e.g., 38% in *Vanda coerulea* [JITSOPAKUL & al. 2007], or 30 or 43.5% in silica gel dehydration *versus* laminar-flow dehydration, respectively for *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011]. When membranes are excessively desiccated, they undergo structural changes and proteins denature [HOEKSTRA & al. 2001], this being a possible explanation why viability was much lower at 12 and 24 h dehydration than at 6 h (optimal) (Tab. 4 in TEIXEIRA DA SILVA, 2013c), while excessive moisture in tissues would result in the production of extracellular ice crystals [POPOV & al. 2006], also resulting in reduced viability, although this trend was not observed for *Cymbidium* tissue (Tab. 4 in TEIXEIRA DA SILVA, 2013c). Bucking this trend and logic, low levels of crystallizable water within poplar tissue ensured high levels of recovery percentages after cryostorage [LAMBARDI & al. 2000].

The alginate bead-cryopreservation interface: factors influencing successful regeneration

For seeds, synseeds or alginate beads to be successfully cryopreserved, seed moisture is an important factor because the presence of unbound water in seeds considerably reduces their germinability causing seeds to perish because of the formation of ice crystals in their cells during freezing in LN [ZHANG & al. 2001]. Most orchid cryo-researchers apply deep-freezing to seeds with a moisture content of less than 13% (i.e., vitrification-based protocols) while only scarce work has dealt with direct freezing of orchid seeds. In orchids, PRITCHARD (1984) (then later PRITCHARD & al. 1999) first cryopreserved terrestrial and epiphytic orchid seeds with a moisture level below 11%, and seed germinability did not change after cryoconservation for most species examined. In seeds of *Dendrobium candidum*, a high survival rate (about 95%) was also obtained when the desiccated seeds at 8-19% water content with silica gel were directly plunged into liquid nitrogen [WANG & al. 1998]. ISHIKAWA & al. (1997) successfully cryopreserved the zygotic embryos of *Bletilla striata* by vitrification. NA & KONDO (1996) described a cryopreservation protocol for preservation of tissue-cultured shoot primordia from shoot apices of cultured *Vanda pumila* protocorms following preculture in ABA and desiccation, which reduced relative humidity from 40-45% to 24%, with a 65.0 ± 7.5% survival rate and

no reported abnormalities in chromosome number or cell structure. NIKISHINA & al. (2001) found that cryopreservation of the mature seeds of eight orchid genera had no negative effect on the duration of germination, growth and development of protocorms or juvenile plants, although 24% water content for *Encyclia cochleata* was necessary. After cryopreservation by desiccation, protocorms derived from cryopreserved seeds of hybrid *Bratonia* developed faster than protocorms from non-treated seeds during the first 45 days [POPOV & al. 2004]. HIRANO & al. (2005a) applied three cryogenic procedures, namely (1) direct plunging into LN, (2) vitrification, and (3) vitrification with 3-day preculture on medium supplemented with 0.3 M sucrose to preserve immature seeds (2–4 months after pollination) of *Bletilla striata* with a 81-92% survival rate and development of seedlings into normal plantlets *in vitro*. In *Phaius tankervilleae*, when the seeds were dehydrated to 5% water content and preserved at a low temperature (4 °C), there was no decrease in viability or germinability after 3 months but after storage for 6 months, the seeds showed a drastic decrease (9%) in germinability, while cryopreservation of seeds by the vitrification method showed no deterioration after 12 months of storage [HIRANO & al. 2009].

Sucrose and mannose can serve as osmotic protectants, with sucrose accumulating within cells when exposed to sucrose in the surrounding medium, and while 0.75 M sucrose pretreatment resulted in maximum viability in *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011], and while 0.3 M sucrose resulted in a maximum of 30% survival in *Digitalis thapsi* after cryopreservation [MORAN & al. 1999], or 0.1 M sucrose for 7 days in *Doritaenopsis* [TSUKAZAKI & al. 2000], a high sucrose concentration was detrimental for hop [MARTINEZ & al. 1999], indicating that the response to and success of pretreatment is species-specific. High concentrations of sucrose or the use of other osmoticants (Tab. 2 in TEIXEIRA DA SILVA, 2013c), as well as the excessive exposure to an osmoticant (Tab. 3 in TEIXEIRA DA SILVA, 2013c) all had a negative impact on the survival of hybrid *Cymbidium*. Sucrose can reduce the freezable water through osmosis, protecting the cytoplasm by entering a vitrified state, although excessively high sucrose concentrations can lead to over dehydration of the cell, resulting in plasmolysis and membrane rupture [POPOV & al. 2006]. VERTUCCI & ROOS (1991) found that drying plant samples with silica gel enhanced the effect of their desiccation before immersion in LN. Since the -OH groups of sucrose are able to replace water and interact with membrane phospholipids, and subsequently stabilize cellular membranes and protect cells from damage during exposure to LN [TURNER & al. 2001], optimization of the preculture period is essential to minimize cell injuries during cryopreservation [TOUCHELL & al. 2002].

Cryopreservation offers other advantages relative to other available storage approaches, including the stability of phenotypic and genotypic characters, minimal storage space and maintenance requirements. Classical cryopreservation procedures include the following successive steps: pregrowth of samples, cryoprotection, slow cooling (0.5-2.0 °C/min) to a determined prefreezing temperature (usually around −40°C), rapid immersion of samples in LN, storage, rapid thawing and recovery which are generally complex since they require the use of sophisticated and expensive programmable freezers. Increasingly, improved and advanced cryopreservation procedures such as vitrification-based techniques are employed. To date, seven different vitrification-based techniques have been developed and applied: (i) encapsulation–dehydration, (ii) a procedure actually termed vitrification, (iii) encapsulation–vitrification, (iv) dehydration, (v) pregrowth, (vi) pregrowth–dehydration and (vii) droplet–vitrification [ENGELMANN, 2011]. Vitrification

involves the treatment of samples with cryoprotective substances, dehydration with highly concentrated vitrification solutions, rapid cooling and rewarming, removal of cryoprotectants and recovery. In total, three cryopreservation techniques have been applied to orchid species, including desiccation (air-drying), vitrification and encapsulation-dehydration [HIRANO & al. 2006; KHODDAMZADEH & al. 2011]. The desiccation technique involves direct dehydration of naked PLBs which are very sensitive to dehydration, as was observed by TEIXEIRA DA SILVA (2012a), while the vitrification technique uses high concentrations of chemicals which can be toxic, possibly explaining the differences in vitrification protocols used for *Cymbidium* (Tab. 1 in TEIXEIRA DA SILVA, 2013c). Encapsulation-dehydration may be the most suitable method as it results in a high survival frequency after cryogenic storage. The encapsulation of explants in alginate beads for cryopreservation has some benefits compared to the use of non-encapsulated samples. The alginate beads provide enhanced protection of dried materials from mechanical and oxidative stress during storage and ease of handling of small samples during pre- and post-cryopreservation [MANEERATTANARUNGROJ & al. 2007]. A limited number of studies have reported on the cryopreservation of orchid PLBs, including *Geodorum densiflorum* [DATTA & al. 1999], *Doritaenopsis* [TSUKAZAKI & al. 2000], *Dendrobium* Walter Oumae [LURSWIJIDJARUS & THAMMASIRI, 2004], *Dendrobium candidum* [YIN & HONG, 2009], *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011], and *Aerides odorata* [HONGTHONGKHAM & BUNNAG, 2014]. Only one other study in the *Cymbidium* genus, *C. eburneum*, exists [GOGOI & al. 2012]. Seeds of different orchids have also been successfully cryopreserved (reviewed in HOSSAIN & al. 2013).

The aim of vitrification is to increase the solute concentration inside the cells to avoid ice crystal formation [MATSUMOTO & al. 1994; HARDING, 2004; SAKAI & ENGELMANN, 2007]. This is progressively done by first exposing the explants to a constant source of carbon, which helps to stabilize the cell and to exchange inner water with the cryogenic liquids [UCHENDU & REED, 2008; VARGHESE & al. 2009]. In *Doritis pulcherrima*, when the seeds were osmotically dehydrated by PVS2 for 50 min, about 62% of the seeds thus treated survived after plunging into LN [THAMMASIRI, 2000]. 82-86% of *Bletilla striata* seeds were successfully cryopreserved by vitrification following the TTC test [HIRANO & al. 2005a], as were the immature seeds of *Ponerorchis graminifolia* var. *suzukiana* with 85-88% survival but 48-52% germination level [HIRANO & al. 2005b].

Survival, which, depending on the situation, could be synonymous with re-growth, has been variable in different orchid species following cryopreservation: 13.3% in *Dendrobium* Walter Oumae [LURSWIJIDJARUS & THAMMASIRI, 2004], 30% in *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011], 37% in *Dendrobium virgineum* [MANEERATTANARUNGROJ & al. 2007], or 40% in *Vanda coerulea* [JITSOPAKUL & al. 2007]. In this study, a wide range of PLB re-growth (assessed by explant survival and regeneration ability, i.e., *neo*-PLB formation) was possible, but this depended on the choice of explant and treatment, the highest value reached for any parameter being 21% (Tab. 1-5 in TEIXEIRA DA SILVA, 2013c). Such variability would depend on the physiological status of the explants, including its oxidation state [VERLEYSEN & al. 2004], or even on the cryopreservation protocol employed [REED & al. 2001]. Even though lTCLs yield much lower number of *neo*-PLBs/explant than intact or half-PLBs [TEIXEIRA DA SILVA, 2013a], once the Plant Growth Factor is employed [TEIXEIRA DA SILVA &

DOBRÁNSZKI, 2011], then the true number of *neo*-PLBs that can be derived from a PLB lTCL is in fact considerably higher than conventional PLB explants while the timing of the assessment of PLB development can also impact the conclusions drawn [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013]. Consequently, in this study, even though PLB lTCLs performed relatively poorly – relative to intact or half-PLBs – in terms of cryopreservation ability, the fact that lTCLs occupy a significantly smaller surface area and volume, and the fact that – depending on the cultivar – more explants can be derived from a single PLB than the number of explants from intact or half-PLBs [TEIXEIRA DA SILVA & TANAKA 2006], indicates that lTCLs should not be ignored as a potentially viable explant for future *Cymbidium* and orchid cryopreservation studies.

Cryopreservation of vegetative tissue involves several stages, specifically the establishment of *in vitro* cultures, conditioning of these tissues, addition of an appropriate cryoprotectant, exposure of cultures to ultra-low temperature, re-warming and regeneration of plant cells and tissues. Each stage plays an important role in determining the survival of tissue upon re-warming. Since somatic embryos and PLBs in orchid biotechnology are synonymous [TEIXEIRA DA SILVA & TANAKA, 2006], and since it is possible to generate alginate beads from PLBs [TEIXEIRA DA SILVA, 2012a], the modest success achieved in this study on the cryopreservation of PLBs, or related tissue, bodes well for the long-term preservation of *Cymbidium* germplasm, although conditions will almost inevitably need to be optimized for different cultivars and species.

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RESPONSE OF SELECTED LOCAL PLANTAIN CULTIVARS TO PIBS (PLANTS ISSUS DE BOURGEONS SECONDAIRES) TECHNIQUE

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Abstract: One major constraint to plantain production has been inadequate healthy planting materials at the time of planting. Several technologies for multiplying healthy planting materials exist but could not meet farmers' demand. A study was conducted to assess the performance of various landraces plantain to plants *issus de bourgeons secondaires* (PIBS) technique. Five cultivars of *Musa sapientum* (Apantu (False Horn), Asamienu (True Horn), Oniaba (intermediate French plantain) and FHIA-21 (tetraploid hybrid plantain) were tested to determine their response to the PIBS technique. Sword suckers of each cultivar with weight of between 0.2-0.5 kg were prepared and buried in fine sawdust in a humidity chamber built using transparent polyethylene sheets. Results at harvest showed that removal of rooted sprouts started three weeks after planting and every week thereafter for eight weeks. The intermediate French plantain cultivar (Oniaba) produced the least average number (about 20) of healthy planting. Apantu (False Horn) produced an average of about 75 healthy planting materials. The hybrid FHIA-21 on the other hand generated an average of about 85 healthy planting materials. Asamienu (True Horn) produced the highest healthy seedlings of about 90 healthy planting materials. The results revealed that the leaf scar carries a primary bud at the intersection of each leaf sheath and several eyes along the entire length of the leaf sheath which could not have developed into suckers. However, with this technique the eyes could be activated to sprout as healthy planting materials. The technique proved as an efficient method of multiplying healthy planting materials for plantain and could thus be recommended for adoption not only by peasant farmers but also to others who could become commercial seed producers. But there will be a need for certification guidelines for seed growing systems.

Keywords: *Musa*, plantain, macro-propagation, planting material, PIBS

Introduction

Plantains and bananas are classified according to genome group. Majority of cultivated plantains are triploid (2n = 3x = 33), that are derived from intra-specific crosses within *Musa acuminata* Colla (A genome) and inter-specific crosses between *M. acuminata* and *Musa balbisiana* Colla (B genome). The remainder is mostly diploid, while tetraploid clones are naturally rare. The tetraploid plantains are often as a result of breeding programmes by research. They are also classified as belonging to False Horn, True Horn and French plantain groups based on the morphology of the fruits. Local landraces of plantain a member of the AAB subgroup is among Africa's most important starchy food and cash crops [STOVER & SIMMONDS, 1987]. Nearly 30 million tons of plantain is produced yearly in Africa, mostly by small holders and consumed locally [FAO, 2010]. It is

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a crop suitable for the humid forest zones with high rainfall conditions. Despite the economic potential of plantain, farmers are confronted with high yield losses caused by pest and disease constraints such as nematodes [FOGAIN, 2000], banana weevils, and foliar diseases such as black leaf streak (*Mycosphaerella fijiensis*) and invasive weeds [ROBINSON, 1996] such as *Chromolaena odorata*, *Panicum maxima*, etc [HAUSER & MEKOA, 2009]. In the traditional low input systems, no pesticides are used and integrated control methods are still not user friendly for farmers.

Plantains are a perennial tropical and subtropical crop, which grow in a wide range of environments. However, the plantain production systems can be divided into three broad categories depending on the number of cultivars grown and the intensity of management. Plantains are starchy even when ripe compared to banana and are only eaten when cooked.

Though the average yield of local plantain in Ghana is 11.0 metric tons per hectare (mt/ha) [SRID-MOFA, 2011], the potential achievable yield of the landraces is 20.0 mt/ha. The yield gap of 9.0 mt/ha could be attributed to several factors. Nonetheless, the achievable yield potential of the crop could be attained if research efforts are geared toward using high-yielding landraces which are already tolerant to the adverse biotic and abiotic factors complemented with elite materials and other agronomic practices.

Plantain as parthenocarpic (produces fruit without fertilization) and seedless, it is propagated traditionally by planting corms and suckers (daughter plants that grow from the rhizomes at the base of the mother plants). Due to the unavailability of disease- and pest-free or clean planting materials, farmers in sub-Saharan Africa traditionally plant suckers derived from their own plantations, most of which are affected with pests and diseases. The morphology of the crop shows that each leaf scar carries a bud [SWENNEN & ORTIZ, 1997]. The quality of the planting material is one of the major factors for successful crop production [TENKOUANO & al. 2006]. In plantain production, farmers use planting material from old plantain fields, irrespectively of the health status of the mother plant. Often planting materials derived from these infected mother stocks results in perpetuation of diseases (e.g. viruses such as banana bunchy top, banana streak) and pests (e.g., nematodes and weevils) leading to low yields and poor quality fruits.

It is evident that farmers have no strong concept of infectious plant pests and diseases that are propagated by infested suckers. Poor sucker quality leads to high plant losses [HAUSER, 2000], and shortened plantation longevity [GOCKOWSKI, 1997] with occasional complete failure of the ratoon crop [HAUSER, 2007]. These could subsequently lead to over 50% yield losses.

The poor quality and inadequate planting materials is threatening plantain production. Unlike grains and legumes, plantain is vegetatively propagated. It is evident that quality planting material coupled with good agronomic practices could contribute to achieving the productive potential of plantain in Ghana. Bioversity International with their partners Latin America in their study reported that high quality planting material (genetic and phytosanitary) has been shown to contribute significant gains in productivity in smallholder systems in Latin America. Intra-varietal variability is well known to occur naturally in plantains, but only recently is this being considered as an opportunity for selecting improved planting materials [CÔTE & al. 2008]

Also, developing techniques for the rapid propagation of clean, healthy planting material through *in vitro*, which yield high performing and true-to-type plantlets through somatic embryogenesis, has been successful [CÔTE & al. 1993; VULSTEKE, 1998]. Simplified macro-propagation techniques and more traditional sanitation techniques of suckers have not been overlooked [AUBOIRON, 1997; KWA, 2002, 2003; TENKOUANO

& al. 2006; HAUSER & MESSIGA, 2010]. However, the high cost and low availability of planting material, especially healthy, good quality material with varietal traceability, is viewed as a major constraint and key obstacle for improved plantain productivity [NKENDAH & AKYEAMPONG, 2003]. While traditionally heavy emphasis has been placed on breeding activities, agronomic and pest management constraints have also received significant attention [SWENNEN & VUYLSTEKE, 1993; ORTIZ & VUYLSTEKE, 1998; BAIYERI & TENKOUANO, 2008].

In field production of plantain, numerous types of planting materials exist. They are classified into sword, maiden, peeper, and water suckers. The sword and maiden suckers are generally considered the most productive planting materials. Nonetheless, any type of sucker could be used for planting. Furthermore, corms of harvested plants could also be cut into small pieces and planted. This, however, would lengthen the crop cycle of the plant crop.

An important condition for the optimization of yield of any crop is the use of healthy planting material [DAS & BORA, 2000]. The planting material used in banana and plantain cultivation is mainly confined to its vegetatively propagated suckers because plantains and bananas are parthenocarpic and seeds are sterile.

Five methods are commonly employed to obtain planting material for the establishment of new planting material of plantain: (i) suckers extracted from plantain fields which are in production; (ii) suckers reproduced in field sucker multiplication plots; (iii) plants from micro-corms grown out in a nurseries; (iv) plants originating from secondary buds (PIBS), produced in a humidity chamber, seedbeds and grown in nurseries; and (v) tissue culture plants grown in two-phase nurseries [TEZENAS DU MONTCEL, 2005; FAO, 2010].

Tissue culture technique can produce large quantities of uniform disease-free healthy planting materials within a short time. The technique also requires small space. However, this is not accessible to farmers, as it requires sophisticated laboratory facilities. The micro-corm grown and sucker produced in field sucker multiplication plots on the other hand could be used by farmers but require space. These techniques could produce about four (4) suckers from a medium size sucker of about 0.2-0.5 kg. Some buds are also destroyed by these techniques. Planting materials produced from these techniques also require paring before planting. They also pass through a lag phase during transplanting compared to tissue culture-derived plants. The quality of planting materials produced from these processes is always of concern to the buyer and quality controllers.

The PIBS is the latest *in vivo* technology developed to optimize sucker production [KWA, 2002]. Like all other plants each plantain leaf bears an axillary (primary) bud at the point of overlapping of the leaf sheath. However, the architecture of the plant is such that several secondary buds occur along the entire length of the base of a leaf sheath [KWA, 2002]. Most of these buds remain dormant and never become suckers in the lifetime of the plant. These dormant buds could be activated to produce healthy planting materials within a short time. The entire potential of the corms and suckers could thus be exploited to produce large quantities of healthy planting materials within a short period. However, information on the amount of healthy planting materials that could be produced from an average sucker is scanty. Demand for plantain suckers in large quantities is currently very high. The objective of this study was to evaluate the response of the various cultivars to the new technique (PIBS).

Materials and methods

Plantain (*Musa* spp. AAB group) are triploid (2n = 33 chromosomes) starchy bananas, whose seedless parthenocarpic fruits are eaten cooked because they are unpalatable when raw. The Apantu, Asamienu and Oniaba used in this study are all triploid plantains. The FHIA- 21 belongs to the genome group AAAB with a ploidy level of 4x. The hybrid is cross between AAB Plantain cv. AVP-67 (French Plantain) x SH-3142.

Ten (10) sword suckers each of Apantu (False Horn), Asamienu (True Horn), Oniaba (Intermediate French) and FHIA-21 (Tetraploid hybrid) were removed, cleaned and pared. Suckers weighing between 0.2 kg and 0.5 kg were used for the experiment. The leaf sheaths were removed (de-sheathing) 2 mm above the collar till the apical meristem was exposed [KWA, 2002]. The materials generated (now called explants) were kept in a clean and cool environment until all the explants were ready. The apical dominance was destroyed with crosswise incision made to the collar of the first leaf from the base. The explants were planted 3cm deep in smooth redwood sawdust in a locally made humidity chamber. The experiment was set up in a Complete Block Design (CBD) and replicated three times and repeated three times. The column was watered regularly to maintain moist environment. Harvesting of sprouts began three weeks after planting (WAP) in sawdust. Harvesting of sprouts was done once a week from the third week to the eighth week after planting in the sawdust. The harvested sprouts were transplanted in polyethylene pots filled with sterile loamy soils and placed under 60% shade net. Data was collected on number of sprouts harvested, sources of sprouts, survival of sprouts in polyethylene bags, establishment six weeks after harvesting from sawdust. Data was analyzed using ANOVA.

Results and discussion

Sprouting was observed two weeks after planting in the sawdust. Harvesting of the proliferations started four weeks after planting and planted in polyethylene bags. The rooted plantlets and plantlets without roots were removed at weekly intervals for five weeks. Healthy rooted seedlings became ready for field planting after six weeks in the polybag.

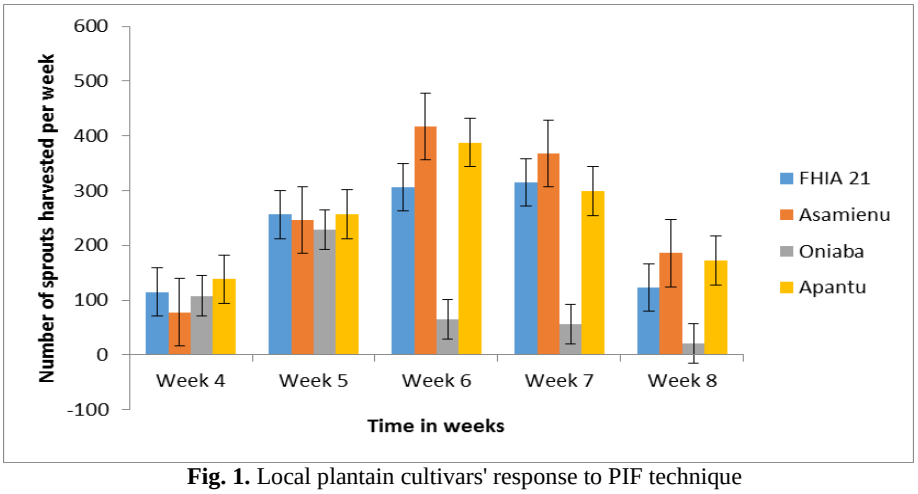

Fig. 1. Local plantain cultivars' response to PIF technique

The PIBS technique is an on-farm macro-propagation approach developed for mass propagation of healthy planting materials of plantains and bananas. The technique is applicable to other vegetatively propagated crops like cocoyams and pineapples. The technique is applicable all-year round and farmers can schedule to raise their planting materials to meet the planting season.

The results of the investigation revealed a significant effect of the chamber on healthy planting material production. Asamienu (True Horn) produced the largest number (92) of healthy suckers (Fig. 1). There was a significant increase in the number of sprouts harvested from week four to week six for Asamienu and Apantu and then declined after that. With regard to Oniaba, there was a sharp decline five weeks after planting in the chamber (Fig. 1). On the other hand, FHIA-21 produced constant numbers during the fourth and fifth weeks.

Asamienu produced the largest number of healthy seedlings with the highest harvested during the sixth week. However the number dropped drastically (Fig. 1). FHIA-21 (hybrid plantain) produced proliferations faster compared to all the other cultivars (Fig. 1).

Average production per sucker was at 86±7 for FHIA-21. Apantu produced consistent planting materials for the fifth and sixth weeks (Fig 1). Sucker production by Oniaba was the least (24±5) among the cultivars (Fig. 1). This result quite agrees with the study of SINGH & al. (2011) who estimates about 50 seedlings per sucker. In another study, [CTA & ISF, 2011] reported an average of 10 harvested sprouts per sucker using this technique.

The study discovered that each leaf scar on the corm, in addition to carrying a primary bud [SWENNEN & ORITZ, 1997], also has several latent secondary buds that will never have developed into daughter suckers.

It was evident that a high percentage (65%) of the sprouts was produced from the apical meristematic region (Plate 1 (a) and (b)).

Plate 1 (a and b). Sprouting meristems

Often it is only few of the primary buds that develop into daughter suckers when the apical dominance is removed at flowering. However, with this technique, several of these latent eyes could be activated to sprout as healthy seedlings for planting. The study further revealed that the ability of the secondary buds to sprout was also dependent on the removal of the leaf sheaths very close to the leaf collar (about 2 mm above the leaf collar). The ability of the eyes to sprout could be attributed to the high temperature (about 50 ^{o}C) generated within the growth chamber. The harvested sprouts when planted in the direct sun got scorched. The seedlings had to be acclimatized under 60% shade (Plate 2).

The study showed that the ability of the technology to exploit the full potential of the sucker planted in the sawdust was dependent on some key factors. Notable among them

include (i) ability to remove the leaf sheath at two millimeters above the leaf collar. Inability to remove the leaf sheath at the appropriate level results in a situation where the sheaths continue to grow hence prevents the sprouting of the buds. (ii) ability to destroy the apical dominance. Inability to break the apical dominance results in the apical tissue continue to grow. Also inability to bury the explants in the sawdust enough (3 cm) exposes the surface leading to surface dryness with not sprouts.

In a similar experiment, MANZUR MACIAS (2001) superimposed the technique on the suckers while still attached to the mother plant in the field and injected them with 4ml of benzylaminopurine (BAP). The results showed that second generation suckers were observed after three months.

Under field conditions, Asamienu (True Horn) could produce several buds, however, only few could develop into healthy planting materials. Similar behavior was exhibited under the PIBS nonetheless the warm condition within the humidity chamber forced the buds to develop into healthy planting materials.

Sucker production by Oniaba in the field was reflected in the PIBS. Under the field conditions, sucker production by Oniaba (intermediate French) is normally low. The results showed that the technology could exploit the entire potential of plantain to generate sufficient planting materials. In the field, False Horn plantain could produce about 39 leaves during its crop cycle; French plantain could produce over 50 leaves during the crop cycle. It presupposes that if each leaf produces one axillary bud, then the crop could generate several suckers, however, they produce only about 10 suckers during the crop cycle due to apical dominance. This technology could therefore break apical dominance hence activate all the dormant buds to become healthy planting material.

Plate 2. Acclimatization of plantain Seedlings from growth chamber

Conclusions

The technique is an effective on-farm method that could generate large quantities of healthy planting materials of plantain from any type of sucker. Latent eyes that would not have sprouted could be activated to generate healthy planting materials. The number of suckers produced using the technology and time period showed that the technique was efficient. The technology does not require any sophisticated equipment for its application but only skills; hence could be used by anyone for sufficient healthy planting material production.

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DISTRIBUTION OF THE ENDEMIC AND CRITICALLY ENDANGERED *DRABA SIMONKAIANA* JÁV. IN THE SOUTHERN CARPATHIANS

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Abstract: *Draba simonkaiana* Jáv. is a critically endangered, Southern Carpathian endemic species, distributed only in Parâng, Retezat and Cozia Mountains. As an European endemic, and restricted to a single European country, *D. simonkaiana* is treated as a "species of European concern".
Although of great international significance, the distribution of *Draba simonkaiana* is slightly known, without any georeferenced records on GBIF.
Based on field studies, analyses of herbarium material and literature data, the authors managed to record the occurrence of *D. simonkaiana* in Southern Carpathians and determined the threatened status according to criteria and categories of IUCN. Unfortunately, the species has not been found in locus classicus (Badea Hill) but the authors have discovered two new localities (in Parâng Mountains) of this important endemic species.

Keywords: *Draba simonkaiana* Jáv., endemic species, Southern Carpathians, Romania, threatened species, Red Book, chorology

Introduction

The species was discovered by Jávorka Sándor on 20th of July 1910 in the Parâng Mountains (Southern Carpathians), on the siliceous Badea Rocks (Piatra Crinului) (approximate 1,700 m altitude). On 23rd of November 1910 he published this species in "Botanikai Közlemények", a Hungarian botanical journal [JÁVORKA, 1910].

Based on a herbarium material (Hungarian Natural History Museum) (the plant collected by Hugó Lojka, a Hungarian botanist, on 26 of July 1880, in Retezat Mountains (Southern Carpathians), Râuşor valley and determined as *Draba carinthiaca*) Jávorka described one new form, namely *Draba simonkaiana* Jáv. f. *retyezátensis* Jáv. [JÁVORKA, 1918].

Draba simonkaiana Jáv. (Bot. Közl. IX (1910) 281, tab. III. A.) is a perennial, densely rosulate species. Stem up to 7 cm. Basal leaves 3-12 x 1-2 mm, oblanceolate, mostly entire, stellate-canescent and ciliate; cauline leaves 0-3, stem and pedicels with simple and branched hairs. Inflorescence 3- to 16- flowered. Petals 3-4 mm, white. Silicula 5-6 mm loosely covered with short simple and branched hairs. Stigma 0.7-1 mm, 2-lobed, filamentous [WALTER & AKEROID, 1996]. Flowering time: June. The habitat of the species is mostly siliceous rock crevices in the Southern Carpathians.

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Draba simonkaiana Jáv. differs from *Draba stellata* Jacq. principally in its shorter petals (3-4 mm), 2-lobed stigma (0.7-1 mm), and a chromosome number of 2n = 32.

Draba simonkaiana Jáv. is mentioned in volume I of Flora Europaea as microspecies under *Draba stellata* (of Alps). For this reason Beldie [BELDIE, 1977] appreciated it (illegitimately) as subspecies (*Draba stellata* Jacq. subsp. *simonkaiana* (Jáv.) Beldie).

In "The Plant List" version 1.1. (www.theplantlist.org) the accepted name of the species is *Draba simonkaiana* Jáv.

In "Euro+Med Plantbase" (www.emplantbase.org) also the accepted name of the species is *Draba simonkaiana* Jáv. In the same database, distribution of the species is not limited to Romania. Species is also cited in Armenia (without any bibliographical reference) [MARHOLD, 2011]. Consulted botanical literature concluded that the species it is a South Carpathians endemite present only in Romania.

Draba simonkaiana Jáv. is included in the list of endemic species of Romania by DIHORU & PARVU (1987) and by COLDEA [COLDEA & al. 2009].

In the complete list of pancarpathian and Southeastern Carpathians endemic species and their distribution per country along the Carpathians, *Draba simonkaiana* Jáv. is included by HURDU [HURDU & al. 2012; HURDU, 2012] in above mentioned list.

In the Carpathian List of Endangered Species [WITKOWSKI & al. 2003], *Draba simonkaiana* Jáv. is considered local endemic for Romanian Carpathians.

Also other authors such as: Borza [BORZA, 1931], Buia [BUIA, 1959], Ţopa [ŢOPA, 1960], Beldie [BELDIE, 1967], Buttler [BUTTLER, 1967], Pawłowski [PAWŁOWSKI, 1970], Heltmann [HELTMANN, 1985], Junk [JUNK, 1985], Dihoru & Pârvu [DIHORU & PÂRVU, 1987], Negrean & Oltean [NEGREAN & OLTEAN, 1989], Oprea [OPREA, 2005], Zachos & Habel [BÁLINT & al. 2011], Simon & Pócs [SIMON & PÓCS, 2012] consider that the species is endemic to Southern Carpathians.

In Romanian flora, *Draba simonkaiana* Jáv. is a rare species [OPREA, 2005]; [CIOCÂRLAN, 2009]; [SÂRBU & al. 2013], found in the Red list of superior plants of Romania as vulnerable/rare (VU/R) [OLTEAN & al. 1994], placed in the rare sozological category (R) [BOŞCAIU & al. 1994] and in vulnerable (VU) sozological category [DIHORU & DIHORU, 1994]. Species was also included in the Red book of vascular plants of Romania [DIHORU & NEGREAN, 2009], in the "Critically Endangered" (CR) category, but without indicating IUCN criteria. The threatened status of the species allocated by different authors is shown in Tab. 1.

Tab. 1. Threatened status of *Draba simonkaiana* Jáv. allocated by different authors

Sources	Threatened status
DIHORU & PÂRVU, 1987	VU
OLTEAN & al. 1992	V/R
DIHORU & DIHORU, 1994	VU
BOŞCAIU, 1994	R
WALTER & GILLETTE, 1998	VU
WITKOWSKI & al., 2003	VU
BUORD & LESOËUF, 2006	CR
DIHORU & NEGREAN, 2009	CR

Materials and methods

Our investigation was based on recent field studies, analysis of herbarium material, as well as literature data.

The identification of the taxa has been done using the following "floras" and papers: *Magyar Flóra* (*Flora Hungarica*) [JÁVORKA 1925]; Flora R. P. Române, vol. III [NYÁRÁDY, 1955]; *Flora Europaea*, vol. 1 [WALTER & AKEROYD, 1996]; *Flora ilustrată a României – Pteridophyta et Spermatophyta* [CIOCÂRLAN, 2009]; *Plante vasculare din România*: *determinator ilustrat de teren* [SÂRBU & al. 2013].

The distribution of *D. simonkaiana* in Romania, has been made based on data from the herbarium collections (Herbarium abbreviation follows Index Herbariorum, except Personal Herbarium) (BP, CL, B, EGR, GZU, BUCA, BUCA) [THIERS, 2012], Personal Herbarium "A. Bartók" (HAB) and also different bibliographical sources.

The collected materials were stored in the Eszterházy Károly College Eger – Department of Botany Herbarium (EGR) and in the Personal Herbarium A. Bartók (HAB).

To determine the conservation status we used IUCN category and criteria (IUCN Red List Categories and Criteria: Version 3.1. Second edition, 2012).

Results

During field trips over the last 134 years (since it was found for first time), *Draba simonkaiana* was collected from six localities, only in Southern Carpathians.

We hereby list all the known specimens, including those traced by us in herbarium collections (BP; CL; B; EGR; GZU; BUCA; HAB), arranged in the chronological order of gathering. It is worth mentioning that no voucher specimen was found in some herbaria (BVS; I; BUAG; SIB; CRAI).

1880 (BP): *Lojka H.*, Retezat Mountains, Râuşor valley
1910, 1918 (BP; CL; B; GZU): *Jávorka S.*, Parâng Mountains, Badea hill
1932 (BP): *Vajda L.*, Parâng Mountains, Badea hill
1935 (BP): *Kárpáti Z.*, Parâng Mountains, Badea hill
1956 (BP): *Pócs T.*, Parâng Mountains, Badea hill
1956 (BP): *Pócs T.*, *Simon T.*, Parâng Mountains, Groapa Mândrii valley
? (BUCA): *Negrean G.*, Cozia Mountains, Bulz peak
2013 (HAB): *Bartók A. & Bartók S. I.*, Parâng Mountains, Zănoaga Stânei lake
2013 (EGR): *Pócs T.*, *Vojtkó A.*, Parâng Mountains, E ridge of Mt. Cărbunele

Altogether 13 voucher specimens were deposited in (BP, B, CL, GZU, BUCA, EGR, HAB) from 6 different localities.

Herbarium data

Herbarium materials found by the authors is shown in Tab. 2.

Tab. 2. Herbarium sheets with *Draba simonkaiana* Jáv. in Southern Carpathians

Geographical region/ Mountains	Locality	Date	Collected by	Source	Observation
Transylvania/ Retezat	"In fissuris rupium umbrosarum vallis Kolcváriensis infra alpem Retyezát, com. Hunyad," "Transsylv."	26/VII/1880	H. Lojka (as *Draba carinthiaca*)	(BP)	*Draba simonkaiana* Jáv. f. *retyezátensis* Jáv. [JÁVORKA, 1918]
Transylvania/ Parâng	"Comit. Hunyad: montes Pareng infra Petrozsény: in rupibus Dealu Badea in altit. ca. 1,700 m, una cum Potentilla haynaldiana."	20/VII/1910	S. Jávorka	(BP72536)	Lectotypus (IX/1999, D. Kováts) [KOVÁTS, 1999]
Transylvania/ Parâng	"Comit. Hunyad: in fissuris rup. granit. montis Dl. Badea, alp. Pareng, alt. 1,700 m."	20/VII/1910	S. Jávorka	(BP)	Isolectotypus (IX/1999, D. Kováts) [KOVÁTS, 1999]
Transylvania/ Parâng	"Kom. Hunyad: in rupestr. Mtis. Dealu Badea alp. Pareng, 1,700-1,750 m. Granit."	20/VII/1910	S. Jávorka	(B0241589)	Note in herbarium label: "Original!"
Transylvania/ Parâng	"Comit. Hunyad, in fissuris rupium jugi "Virf Badea" alpium Pareng, in saxo unico in alt. ca. 1,750 m."	20/VII/1910	S. Jávorka	(GZU)	Isotypus [BUTTLER, 1967]
Transylvania/ Parâng	"Cott. Hunyad: in fissuris rupium mt. Badea in alpe Pareng ad opp. Petrozseny – locus classicus!"	20/VII/1910	S. Jávorka	(CL)	Duplicata
Transylvania/ Parâng	"Comit. Hunyad: in fissuris saxi granitici loco "Badea" dicto in alpe Pareng ad opp. Petrozsény."	12/VI/1918	S. Jávorka	(BP238189)	Isolectotypus (IX/1999, D. Kováts) [KOVÁTS, 1999]
Transylvania/ Parâng	"Comit. Hunyad: in fissuris rupium loci „Badea" dicti in alpe Pareng ad opp. Petrozseny – locus classicus."	12/VI/1918	S. Jávorka	(CL)	Duplicata

Geographical region/ Mountains	Locality	Date	Collected by	Source	Observation
Transylvania/ Parâng	"Parenghavas, Petrozsénynál, Hunyad m., Badea."	23/VI/1932	L. Vajda	(BP287704)	-
Transylvania/ Parâng	"Comit. Hunyad. In rupestribus graniticis montis Badea alpium Pareng supra opp. Petrozsény; alt. cca. 1,600 m."	17/VII/1935	Z. Kárpáti	(BP388318)	-
Transylvania/ Parâng	"In Carpat. Merid. In montibus Parîng. In rupibus schist. Badea, in decl. S montis Parîng. Alt. 1,860 m. (Locus classicus!)"	25/VII/1956	T. Pócs	(BP202151)	[PÓCS, 1961]
Transylvania/ Parâng	"In Carpat. Merid. In montibus Parîng. In rupibus schist. in decl. SE supra vallem glacialem Groapa Mândrii, in alt. 2,060 m s.m."	1/VIII/1956	T. Pócs, T. Simon	(BP202150)	[PÓCS, 1961]
Oltenia/ Cozia	"Versantul de nord al Muntelui Bulzu."	?	G. Negrean	(BUCA)	[NEGREAN & OLTEAN, 1989] [DIHORU & NEGREAN, 2009]
Transylvania/ Parâng	"In fissuris rupium prope lacum Zănoaga Stânei. Solo granitico-schistoso."	17/VI/2013	A. Bartók, S. I. Bartók	(HAB)	-
Transylvania/ Parâng	"On calciphyllite cliff of the E ridge of Mt. Cărbunele, at 2,020-2040 m alt."	8/IX/2013	T. Pócs, A. Vojtkó	(EGR)	-

The chorological map of *D. simonkaiana* Jáv. is shown in Fig. 1.

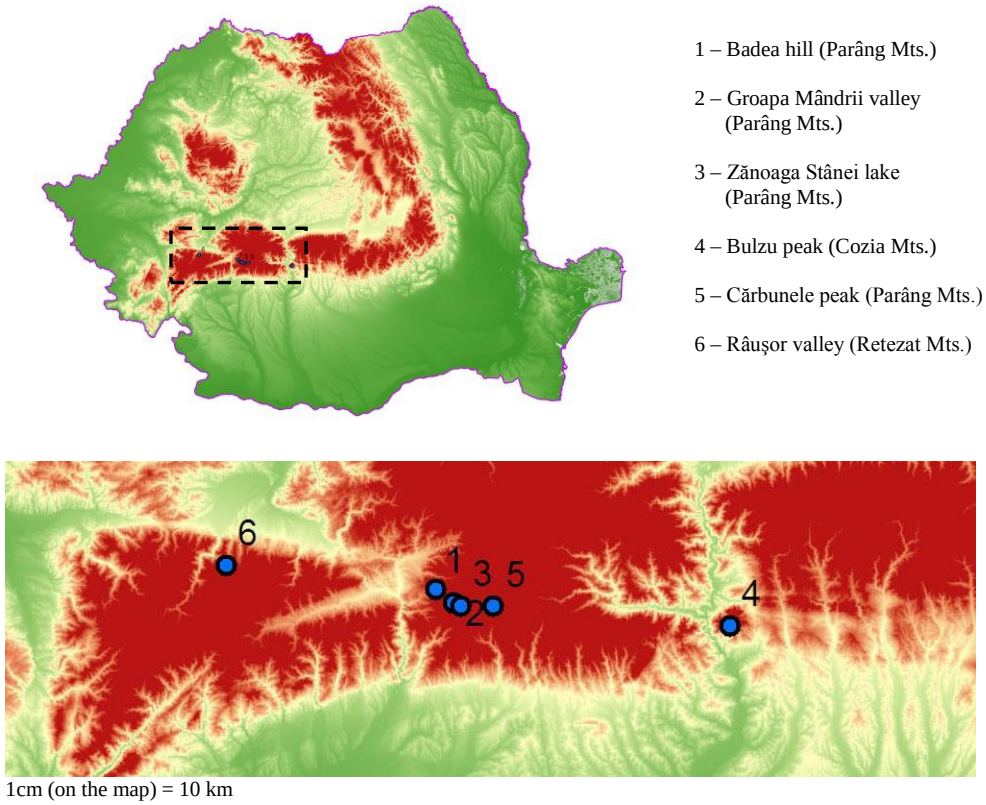

Fig. 1. The chorological map of *Draba simonkaiana* Jáv. in the Southern Carpathians

Field observation

1. During the floristical investigation performed in Parâng Mountains on 17 June 2013, we discovered one small population (10 individuals) of *Draba simonkaiana* Jáv. (Fig. 2), on a rocky cliff with granitic substrate.

Locality: Romania, Southern Carpathians, Parâng Mountains, Zănoaga Stânei lake, 2,076 m a.s.l., exp. SE, incl. 90°, rock cliffs, area 3 m^2, in a phytocoenosis of ass. *Sileno lerchenfeldianae – Potentilletum haynaldianae* Horvát, Pawł. et Walas 1937, leg. A. Bartók & S.I. Bartók; date: 17/VI/2013 with the following floristic composition: *Thymus praecox* subsp. *polytrichus* 1, *Juncus trifidus* 1, *Silene lerchenfeldiana* 1, *Draba simonkaiana* +, *Saxifraga pedemontana* subsp. *cymosa* +, *Potentilla haynaldiana* +, *Symphyandra wanneri* +, *Phyteuma confusum* +, *Campanula alpina* +, *Sedum alpestre* +.

2. During a trip to obtain *Pinus cembra* material for molecular investigation in the company of Maria Höhn and Endre Tóth, we discovered a small population (9 individuals forming small cushions) of *Draba simonkaiana* Jáv. (photo in Fig. 2) in a calciferous

Seslerio haynaldianae – Caricetum sempervirentis Puşcaru et al. 1956 community, accompanied by *Cerastium lanatum* 2, *Dianthus tenuifolius* 1, *Phyteuma nanum* 1, *Rhododenron myrtifolium* 1. *Saxifraga bryoides* +, *Saxifraga paniculata* 2.

Locality: Romania, Southern Carpathians, Parâng Mountains, E ridge of Cărbunele summit at 2,020-2,040 m a.s.l., on E exposed calciphyllite cliffs, incl. 90°, in an area of about 4 m^2, leg. T. Pócs & A Vojtkó; date 8/IX/2013.

In Romania only three very small populations of *Draba simonkaiana* were known which grow on a restricted area in Retezat, Parâng and Cozia Mountains (Southern Carpathians).

On the basis of chorological data and estimation of the geographical range (extent of occurrence) we can define *Draba simonkaiana* as IUCN CR B1ac (http://jr.iucnredlist.org/documents/redlist_cats_crit_en.pdf).

Conclusions

Based on field studies, analyses of herbarium material and literature data, the authors managed to record the occurrence of *D. simonkaiana* Jáv. in Southern Carpathians and determined the threatened status according to categories and criteria of IUCN: CRB1ac(iii).

Unfortunately, the species has not been found in its typically locality (Badea hill) but the authors have discovered two new localities (in Parâng Mountains) of this important endemic species: Zănoaga Stânei lake and Cărbunele Peak.

In the newly discovered locations the species is very rare.

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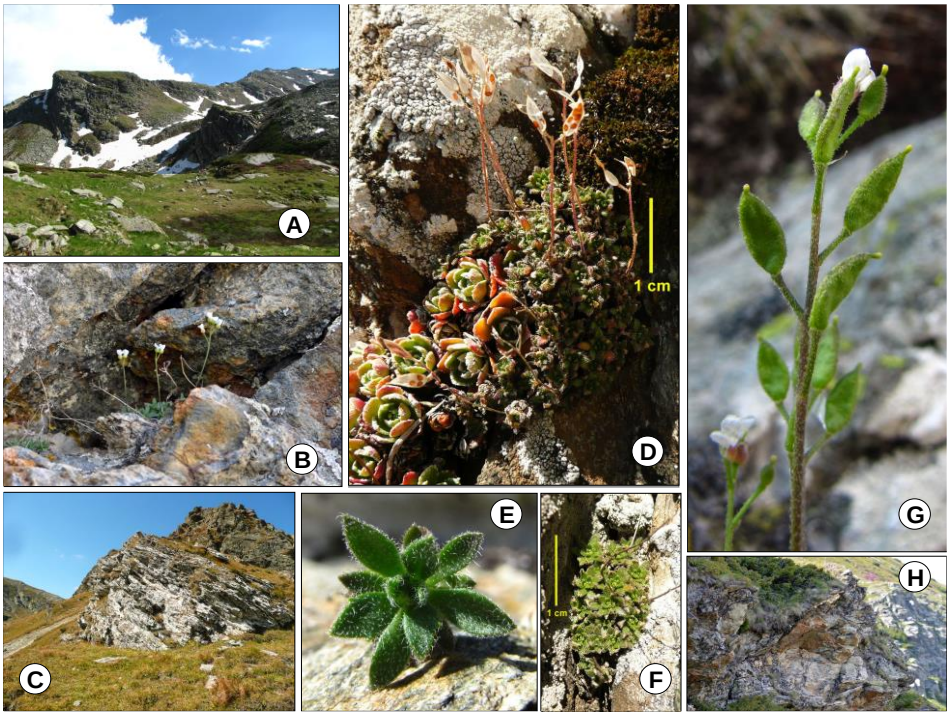

Fig. 2. A – Location lanscape near Zănoaga Stânei lake. **B** – Habitus of *Draba simonkaiana* on rocky cliff near Zănoaga Stânei lake. **C** – Natural habitat of *Draba simonkaiana* near Cărbunele peak. **D** – Habitus of *Draba simonkaiana* on rocky cliff near Cărbunele peak. **E, F** – Basal leaves of *Draba simonkaiana*. **G** – Silicules of *Draba simonkaiana.* **H** – Natural habitat of *Draba simonkaiana* near Zănoaga Stânei lake.

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DISTRIBUTION OF THREATENED SPECIES *TRIFOLIUM LUPINASTER* L., *HERACLEUM CARPATICUM* PORCIUS AND *RANUNCULUS THORA* L. IN ROMANIAN CARPATHIANS

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Abstract: Maramureşului Mountains Nature Park is widely known as one of the last wilderness areas in Europe and also represents a real oasis for naturalists eager to explore the flora and fauna of this special land not very researched.
During a botanical trip in the area of Farcău Peak (on 19 July 2014) the authors of this paper found three very rare species (all 3 threatened, included in Romanian Red Book of Vascular Plants): *Trifolium lupinaster* L., *Heracleum carpaticum* Porcius and *Ranunculus thora* L.
Based on field studies, analyses of herbarium material and literature data, the authors managed to record the occurrence of *Trifolium lupinaster*, *Heracleum carpaticum* and *Ranunculus thora* in the Romanian Carpathians and determined the threatened status of species according to criteria and categories of IUCN.

Keywords: chorology, Eastern Carpathians, endemic species, "Maramureşului Mountains" Nature Park, Red Book, threatened species

Introduction

"Maramureşului Mountains" Nature Park (incorporates Maramureşului Mountains) having a total surface of around 1.500 km^2, is the largest protected territory in the Romanian Carpathians and is located in the Maramureş county.

Maramureşului Mountains are made up of crystalline schist penetrated by eruptive and sedimentary rocks.

Within the natural park a rich flora including a significant number of rarities such as: *Lysimachia nemorum* L., *Ranunculus thora* L., *Silene rupestris* L., *Salix bicolor* Willd., *Trifolium lupinaster* L., *Woodsia alpina* (Bolton) Gray, *Sagina apetala* Ard., *Rhinanthus alectorolophus* (Scop.) Pollich and so on and a great number of dacic elements such as: *Gymnadenia carpatica* (Zapał.) Teppner & E. Klein, *Heracleum carpaticum* Porcius, *Pulmonaria filarszkyana* Jáv., *Poa rehmannii* (Asch. & Graebn.) K. Richt, *Cochlearia borzaeana* (Coman & Nyár.) Pobed., *Silene zawadzkii* Herb. and so on can be found.

There are four natural reserves in "Maramureşului Mountains" Nature Park: Sâlhoi-Zâmbroslavele Rocks (1 ha), Cornu Nedeii-Ciungii Bălăsinei (800 ha), Tomnatec-Sehleanu Narcissus Meadow (100 ha) and Farcău Peak-Vinderel Lake-Mihăilecu Peak (150 ha).

Three of the rare and threatened species in the "Maramureşului Mountains" Nature Park that in recent decades have not been seen by botanists are: *Trifolium lupinaster* L., *Heracleum carpaticum* Porcius and *Ranunculus thora* L.

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During a botanical trip on 19 July 2014 the authors of this paper found all three above mentioned species.

***Trifolium lupinaster* L.**

Trifolium L. sect. *Lupinaster* (Adans.) Ser. emend Lojac. contains 2 romanian species: *Trifolium lupinaster* L. and *Trifolium alpinum* L. (the presence of *Trifolium alpinum* in the Romanian Flora must be confirmed).

The species of this section can be distinguished by their exclusively perennial habit, their usually large flowers with essentially free standards and wing claws united for only a short distance to the staminal tube, the blades only lightly adhering to those of the keel.

The peduncles are usually long and often scapiform in the alpine species. The involucral bracts may be prominent or reduced but are not united into a continuous ring to form an involucre. The stigmas are exserted slightly beyond the cluster of anthers. All the species of the section appear to be outcrossers, often setting seed when hand triggered. The alpine members of sect. *Lupinaster* occupy old mountain ranges, the Rockies, the Alps, and the Caucasus. The disjunct distribution of these apparently related alpine species suggests that the group is an ancient one [GILLETTE, 1965].

In 1960 Iljin and Truchaleva published a paper on *Trifolium lupinaster* L. s.l. and concluded (on the basis of chromosome numbers) that *Trifolium lupinaster* comprises three species: *Trifolium lupinaster* with 32 or 40 chromosomes (covering the largest part of the area), *Trifolium ciswolgense* Sprygin with 16 chromosomes (spread in the Middle and Southern Urals, in the Volga Hills, and in trans-Uralian Western Siberia) and *Trifolium litwinowii* Iljin with 32 choromosomes which is identical to *Trifolium lupinaster* subsp. *angustifolium* [ILJIN & TRUCHALEVA, 1960].

In Flora of USSR *Trifolium lupinaster* subsp. *angustifolium* is accepted. The subspecies differs from the species by the following characters: leaflets narrowly lanceolate, the ratio of breadth to length one eight to one tenth, the plant as a whole more slender and lighter in colour. Also, *Trifolium lupinaster* var. *albiflorum* is accepted in Flora of USSR. The variety differs from the species by the following: flowers yellowish brown, somewhat smaller; leaflets slightly narrower than those of typical Siberian plants and it is distributed with the typical red-flowered form in the SW part of the distribution area of the species [BOBROV, 1945].

The taxonomical status of some species of genus *Trifolium* (section *Lupinaster*), according to different bibliographical sources is shown in Tab. 1.

Tab. 1. Taxonomical status of some species of genus *Trifolium*, section *Lupinaster*

Taxon name	**Taxonomic status**			
	Flora Europaea	The Plant List	Euro Med Plant Base	ILDIS
(1)*Trifolium lupinaster* L.	ACCEPTED	ACCEPTED	ACCEPTED	ACCEPTED
(2)*Trifolium lupinaster* L. subsp. *angustifolium* (Litv.) Bobrov	SYNONIM with (5)	ACCEPTED	ACCEPTED	ACCEPTED
(3)*Trifolium lupinaster* L. var. *albiflorum* Ser.	SYNONIM with (4)	SYNONIM with (1)	SYNONIM with (1)	SYNONIM with (1)
(4)*Trifolium ciswolgense* Sprygin ex Iljin & Trukh.	PROVI-SIONAL	SYNONIM with (1)	SYNONIM with (1)	SYNONIM with (1)
(5)*Trifolium litwinowii* Iljin	PROVI-SIONAL	SYNONIM with (1)	SYNONIM with (1)	SYNONIM with (1)
(6)*Trifolium romanicum* D. Brandza	-	SYNONIM with (1)	SYNONIM with (1)	SYNONIM with (1)

According to Ellison, genus *Trifolium* sect. *Lupinaster* (Fabricius) Ser. includes 3 species: *Trifolium eximium*, *Trifolium gordejevii* and *Trifolium lupinaster* with native distribution in East Europe-Siberia [ELLISON & al. 2006].

In the whole Carpathians *Trifolium lupinaster* L. was first discovered in Romanian Carpathians (Eastern Carpathians, Nemira Mts.) in subalpine forests, at the border between Transylvania and Moldova, at the altitude 1500 m, near to Băile Slănic town [BRÂNDZĂ, 1903].

Brândză described it as an independent species named *Trifolium romanicum* D. Brândză and he assumed that the plants he had collected differed from *Trifolium lupinaster* L. from other parts of its area and by tuberculously thick roots. The habitats of Carpathians plants are different from Poland or Ukraine habitats, where there are mostly of a semi-steppe to steppe character.

Degen specified [KLEIN, 1904] that the new species of Brândză does not differ from *Trifolium lupinaster* subsp. *angustifolium*.

Hendrych mentioned that the separation of the plants collected by Brândză and therefore also the other Carpathians plants from *Trifolium lupinaster* was unfounded [HENDRYCH, 1963].

Holub considered that the Carpathians plants remained in isolated enclaves from the rest of area (steppes), so he described the species as independent. Moreover, in 1984 he made a new nomenclatural combination and published a new species *Lupinaster romanicus* (D. Brandza) Holub [HOLUB, 1984].

However in "The Plant List" (www.theplantlist.org) taxonomical status of *Lupinaster romanicus* is "Unresolved", which shows that the species is not yet resolved taxonomically.

In 1948 Csűrös discovered the second localities of the plant in Carpathians (in Căliman Mts.), not far from where it was found by Brândză [CSŰRÖS, 1951; CSŰRÖS, 1956].

More than 30 years after, in 1980, Bârlea found the third localities in Carpathians of the species (Maramureşului Mts., Farcău Peak) on a rocky slope (on basalt soil) [BÂRLEA, 1984].

Recent Romanian botanical works [CIOCÂRLAN, 2009; OPREA, 2005; SÂRBU & al. 2013] mention that in Romania only *Trifolium lupinaster* subsp. *angustifolium* is present.

In most European countries *Trifolium lupinaster* is a threatened species. Threatened status of the species in different European countries is shown in Tab. 2.

Tab. 2. Threatened status of the *Trifolium lupinaster* L. in different European countries

Bibliographical source / Country	Poland	Slovakia	Romania	Ukraine
[JACKOWIAK & al. 2007]	EN	-	-	-
[TURIS & al. 2014]	-	CR	-	-
[DIHORU & NEGREAN, 2009]	-	-	CR	-
[KRICSFALUSY & BUDNIKOV, 2007]	-	-	-	EN

In romanian flora, *Trifolium lupinaster* L. is a rare species [OPREA, 2005; CIOCÂRLAN, 2009; SÂRBU & al. 2013], found in the Red list of vascular plants of Romania as vulnerable/rare (V/R) [OLTEAN & al. 1994], placed in the rare sozological

category (R) [BOȘCAIU & al. 1994] and in endangered (E) sozological category [DIHORU & DIHORU, 1994]. Species was also included in the Red book of superior plants of Romania [DIHORU & NEGREAN, 2009], in the "Critically Endangered" (CR) category, but without indicating IUCN criteria.

The threatened status of the species allocated by different authors is shown in Tab. 3.

Tab. 3. Threatened status of *Trifolium lupinaster* L. allocated by different authors

Bibliographical source	Threatened status
[OLTEAN & al. 1992]	V/R
[DIHORU & DIHORU, 1994]	E
[BOȘCAIU, 1994]	R
[WITKOWSKI, 2003]	EN
[DIHORU & NEGREAN, 2009]	CR

Another two species found by the authors of this paper in "Maramureşului Mountains" Nature Park – *Heracleum carpaticum* Porcius and *Ranunculus thora* L.– are also rare and threatened species in Romanian Flora. Moreover the distribution of *Heracleum carpaticum* is limited to the Eastern Carpathians (dacic elements).

The threatened status of the species allocated by different authors in Romania is shown in Tab. 4.

Tab. 4. Threatened status of *Heracleum carpaticum* Porcius and *Ranunculus thora* L. allocated by different authors

Bibliographical source / Species	***Heracleum carpaticum* Porcius**	***Ranunculus thora* L.**
[OLTEAN & al. 1992]	V/R	R
[DIHORU & DIHORU, 1994]	V	R(V)
[WITKOWSKI, 2003]	VU	VU
[DIHORU & NEGREAN, 2009]	CR	VU

Material and methods

Our investigation was based on recent field studies, analysis of herbarium material as well as literature data.

The identification of the taxa has been done using the following "floras" and papers: *Flora Europaea*, vol. 2; *Flora ilustrată a României – Pteridophyta et Spermatophyta* [CIOCÂRLAN, 2009]; *Plante vasculare din România*: *determinator ilustrat de teren* [SÂRBU & al. 2013].

The distribution of *Trifolium lupinaster* L., *Heracleum carpaticum* Porcius and *Ranunculus thora* L. in Romania, has been made based on data from the herbarium collections (BP, CL, SIB, BVS, P, I) [THIERS, 2012] and Personal Herbarium "A. Bartók" (HAB) and also different bibliographical sources. Herbarium abbreviation follows Index Herbariorum (http://sweetgum.nybg.org/ih/), except Personal Herbarium.

The collected materials were stored in the Personal Herbarium A. Bartók (HAB).

Results and discussion

Trifolium lupinaster L.

Literature data

Bibliographical data about *Trifolium lupinaster* L. (continental floristic element) from romanian botanical literature is presented in Tab. 5.

Tab. 5. Distribution in Romanian Carpathians of *Trifolium lupinaster* according to different bibliographical sources

Bibliographical source	Nemira Mts.	Căliman Mts.	Maramureşului Mts.
[BRÂNDZĂ, 1903]	+	-	-
[CSŰRÖS, 1951]	-	+	-
[CSŰRÖS, 1956]	-	+	-
[MITITELU & BARABAŞ, 1993]	+	-	-
[BÂRLEA, 1984]	-	-	+
[OROIAN, 1998]	-	+	-
[OPREA, 2005]	+	+	+
[CIOCÂRLAN, 2009]	+	+	+
[SÂRBU & al. 2013]	+	+	-

Herbarium data

During field trips over the last 110 years (since it was found for the first time) *Trifolium lupinaster* was collected from three localities, only in Eastern Carpathians.

We hereby list all the known specimens, including those traced by us in gathering.

? (CL): *Brândza*, Nemira Mountains
1948 (CL; BUCA): *Csűrös*, Câliman Mountains
1980 (BUCA): *Bârlea*, Maramureşului Mountains
2014 (HAB): *Bartók*, *Brener & Covâză*, Maramureşului Mountains

Altogether 6 voucher specimens were deposited in (CL, BUCA, HAB) from 3 different localities.

Herbarium materials found by the authors is shown in Tab. 6.

Tab. 6. Herbarium data of *Trifolium lupinaster* collected in Romanian Carpathians

Original label data	Collection date	Collected by	Source	Observations
Maramureş, Eastern Carpathians, Maramureşului Mountains				
"Mt. Farcău, 1,850 m, versant pietros, stânci de bazalt."	8/VIII/1980	L. Bârlea	(BUCA 144538)	(DIHORU & NEGREAN, 2009)
"In herbosis et saxosis sub cacumine Farcău. Solo basaltico. Alt. 1,791 m."	19/VII/2014	A. Bartók B. M. Brener G. Covâză	(HAB)	-
Transylvania, Eastern Carpathians, Călimani Mountains				
"Alpes „Călimani" – in saxosis sub mtis Izvor ad Fața Gardului, alt. 1,700 m."	2/VIII/1948	I. Csűrös	(CL 659790)	*Trifolium lupinaster* L. f. *albiflorus* Ser.

"Munții Căliman - lângă izvorul "La Fața Gardului", 1,700 m."	2/VIII/1948	I. Csűrös	(BUCA 10233)	*Trifolium lupinaster* L. f. *albiflorus* Ser. (DIHORU & NEGREAN, 2009)
"Alpes „Călimani"- in saxosis sub mtis Izvor ad Fața Gardului, alt. 1,700 m."	29/VII/1949	I. Csűrös	(CL 219619)	*Trifolium lupinaster* L. f. *albiflorus* Ser.
Moldova, Eastern Carpathians, Nemira Mountains				
"Creşce in pădurile subalpine printre tufele de Vaccinium la altitudinea de 1,500 metri, pe Muntele Nemira, lângă linia de frontieră in apropiere de Slănic in Districtul Bacău. Înfloresce în Iunie."	?	D. Brândza	(CL 49167)	*Trifolium romanicum* Brandza As HOLOTYPUS electus, but herbarium material missing (original herbarium material (BUC) burned). G. Negrean (14/IX/2003)

The chorological map of *Trifolium lupinaster* L. is shown in Fig. 1.

Herbarium data analysis shows that *Trifolium lupinaster* is extremely rare in Romania and in Maramureşului Mountains it has not been seen for more than 30 years.

On the basis of chorological data and estimation of the geographical range (extent of occurrence) we can define *Trifolium lupinaster* as IUCN: CR B1a.

Field observations

During the floristical investigation performed in Maramureşului Mountains on 19 July 2014 we found one population of *Trifolium lupinaster* L. (about 15-20 individuals) on a grassy place with basaltic substrate.

Locality: Romania, Eastern Carpathians, Maramureşului Mountains, Farcău Peak, 1791 m a.s.l., exp. S-SE, incl. 60°, grassy place, area 10 m^2, leg. A. Bartók, B. M. Brener, G. Covâză; date: 19/VII/2014.

Together with *Trifolium lupinaster* L. we also found the following species: *Juncus trifidus* L., *Vaccinium vitis-idaea* L., *Vaccinium uliginosum* L., *Dianthus carthusianorum* L., *Asplenium viride* Huds., *Scorzonera purpurea* L. subsp. *rosea* (Waldst. & Kit.) Nyman, *Sempervivum montanum* L., *Juniperus sibirica* Burgsd., *Phyteuma orbiculare* L., *Pulsatilla alba* Rchb., *Hieracium villosum* Jacq., *Campanula alpina* Jacq., *Ligusticum mutellina* (L.) Crantz, *Festuca supina* Schur, *Scorzoneroides crocea* (Haenke) Holub (syn. *Leontodon croceus* Haenke).

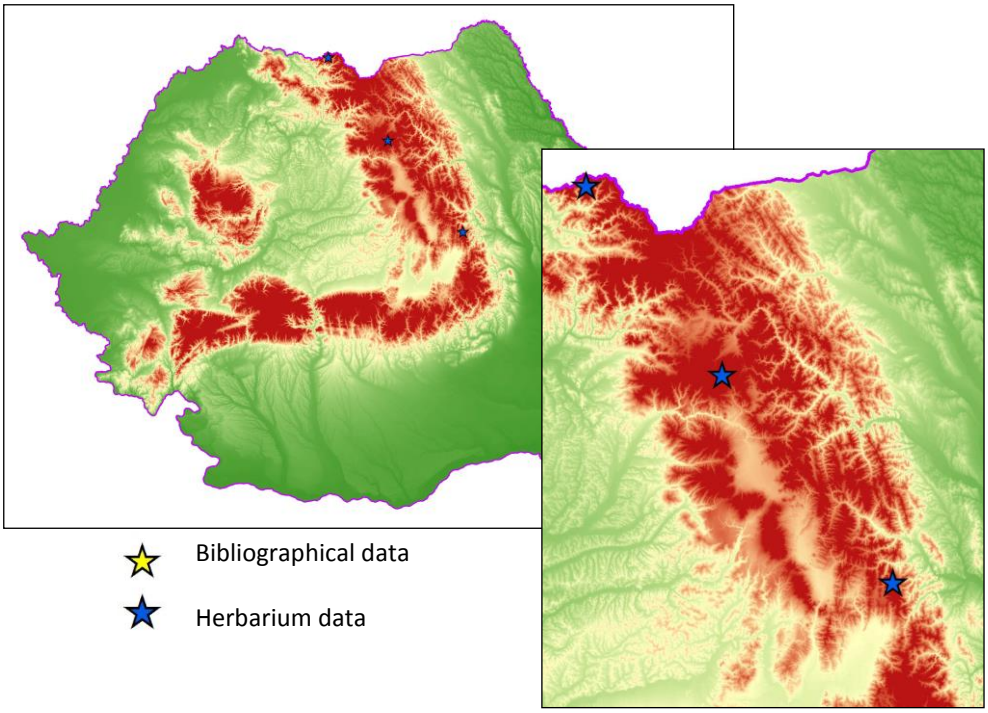

Fig. 1. Distribution map of *Trifolium lupinaster* L. in Romanian Carpathians

Heracleum carpaticum Porcius

Literature data

Bibliographical data about *Heracleum carpaticum* Porcius (dacian floristic element, Eastern and Southern Carpathians endemite, distributed only in Romania and Ukraine) from Romanian botanical literature is presented in Tab. 7.

Tab. 7. Distribution in Romanian Carpathians of *Heracleum carpaticum* according to different bibliographical sources

Bibliographical source	Maramureşului Mts.	Rodnei Mts.	Rarău Mts.	Bistriţei Mts.	Ceahlău Mts.	Nemira Mts.	Ciucaş Mts.	Bucegi Mts.	Făgăraş Mts.
[BAUMGARTEN,1816]	-	+	-	-	-	-	-	+	-
[FUSS, 1866]	-	+	-	-	-	-	-	+	-
[SCHUR, 1866]	-	+	-	-	-	-	-	+	-
[SIMONKAI, 1886]	-	+	-	-	-	-	-	+	-
[HAYEK, 1916]	+	+	-	-	-	-	-	-	-
[JÁVORKA, 1925]	+	+	-	-	-	-	-	+	-
[BELDIE, 1967]	+	+	-	-	-	-	-	+	-

[BELDIE, 1967]	-	-	-	-	-	-	-	+	-
[LUNGU, 1969]	-	-	-	+	-	-	-	-	-
[PUŞCARU-SOROCEANU & al. 1977]	-	-	-	-	-	-	-	-	+
[CIUCĂ, 1984]	-	-	-	-	-	-	+	-	-
[NEGREAN & OLTEAN, 1989]	+	+	+	-	-	-	-	+	-
[OPREA, 2005]	+	+	+	-	-	-	-	+	-
[DIHORU & NEGREAN, 2009]	+	+	+	-	-	-	+	+	+
[CIOCÂRLAN, 2009]	+	+	+	-	-	-	-	+	-
[SZABÓ, 2012]	-	-	-	-	+	+	-	-	-
[SÂRBU & al. 2013]	+	+	+	-	-	-	-	+	-

On the mentioned literature data *Heracleum carpaticum* Porcius can be found in 9 Mountain Massifs (Maramureşului, Rodnei, Rarău, Bistriței, Ceahlău, Nemira, Ciucaş, Bucegi, Făgăraş). It should be noted this is the first mention of *Heracleum carpaticum* Porcius in Ceahlău, and Nemira Mountains in a single bibliographical source [SZABÓ, 2012].

Herbarium data

Heracleum carpaticum was collected from ten localities, in Eastern Carpathians (Maramureşului, Rodnei and Rarău Mts.).

We hereby list all the known specimens, including those traced by us in gathering.

? (P): *Baumgarten*, Transylvania
1857 (CL): *Czetz*, Maramureşului Mts.
1860 (CL): *Czetz*, Rodnei Mts.
?, 1880 (CL): *Porcius*, Rodnei Mts.
1918, 1941(SIB): *Nyárády*, Rodnei Mts.
1936 (CL): *Borza & Pteancu*, Rodnei Mts.
1946 (CL): *Csűrös*, Rodnei Mts.
1950 (BVS): *Morariu*, Rarău Mts.
1987 (BUCM): *Negrean*, Rodnei Mts.
2001 (CL): *Puşcaş*, Rodnei Mts.
2013 (HAB): *Bartók*, Rodnei Mts.
2014 (HAB): *Bartók, Brener* & *Covâză*, Maramureşului Mountains

Altogether 18 voucher specimens were deposited in (CL, SIB, P, BVS, HAB) from 3 different localities.

Herbarium materials found by the authors is shown in Tab. 8.

Tab. 8. Herbarium data of *Heracleum carpaticum* collected in Romanian Carpathians

Original label data	Collection date	Collected by	Source	Observations
Transylvania				
"Transylvania"	?	J. C. Baumgarten	(P 2675039)	*Heracleum alpinum* L. Det. R.K. Brummit, 1965
Transylvania, Eastern Carpathians, Rodnei Mountains				
"Az Ünőkő éjszaki oldalán"	VIII/1860	A. Czetz	(CL 28730)	*Heracleum alpinum* L.
"In pascuis alpinis prope oppidum Rodna, in Transsilvania boreali-orientalis".	?	F. Porcius	(CL 210286) (CL 210287) (CL 210288) (CL 210289) (CL 210290)	-
"... auf Alpen in Grosse Corongis in bei Radna."	VII/1880	F. Porcius	(CL 28729)	*Heracleum alpinum* L. ß *carpaticum*
"Comit. Beszterce-Naszód. Montes Rodnenses. In declivibus graminosis et saxosis montis Nagy-Korongyis, alt. 1,600-1,990. Solo calc".	17/VIII/1918	E. I. Nyárády	(SIB 155944)	-
"Transilvania. Distr. Năsăud. Mte. Corongiş ad Valea Vinului. Calc. et schist. Alt. ad 1,400 m" .	28-29/ VII/1936	A. Borza P. Pteancu	(CL 503517)	-
"Transsilvania. Radnai-havasok. Lála csúcs- Vârfu Roşu déli, fűves oldalán, 1,600 m".	16/VIII/1941	E. I. Nyárády	(SIB 155946)	-
"Piatra Unică" ("Ünőkő")-Carpaţii Orientali	2/VIII/1946	I. Csűrös	(CL 599015)	Rev. G. Weimarck
"Muntele Negoiasa, alt. 1,940 m."	24/VIII/1987	G. Negrean	(BUCM 104934)	(DIHORU & NEGREAN, 2009)
"Căldarea Iezerul (Munţii Rodnei). Grohotiş înierbat. Alt. cca. 1,500 m."	28/VII/2001	M. Puşcaş	(CL 659100)	-
"Alpibus Rodnensibus. In pascuis alpinis montis Pietrosul Mare, supra lacum Iezer. Alt. 2,100 m s.m."	21/VII/2013	A. Bartók	(HAB)	-
Maramureş, Eastern Carpathians, Maramureşului Mountains				
"Máramarosi havasokban a Popp Iván tetejéről."	8/VIII/1857	A. Czetz	(CL 28731)	*Heracleum alpinum* L.
"Montibus Maramarosiensis. In herbosis et saxosis sub cacumine Farcău. Solo calcareo. Alt. 1,896 m s.m."	19/VII/2014	A. Bartók B. M. Brener G. Covâză	(HAB)	-
Moldova, Eastern Carpathians, Rarău Mountains				
"Rarău, prin pădure."	10/IX/1950	I. Morariu	(BV S022263)	-

The chorological map of *Heracleum carpaticum* Porcius is shown in Fig. 2.

On the basis of chorological data and estimation of the geographical range (extent of occurrence) we can define *Heracleum carpaticum* as IUCN: CR B1a, B1c(iii).

Field observations

During the floristical investigation performed in Maramureşului Mountains on 19 July 2014 we found one small population of *Heracleum carpaticum* Porcius (3 individuals) on a grassy place with limestone substrate.

Locality: Romania, Eastern Carpathians, Maramureşului Mountains, Farcău Peak, 1900 m a.s.l., exp. S-SE, incl. 30°, grassy place, area 2 m^2, leg. A. Bartók, B. M. Brener, G. Covâză; date: 19/VII/2014.

Together with *Heracleum carpaticum* Porcius we also found the following species: *Juncus trifidus* L., *Hieracium villosum* Jacq., *Ranunculus platanifolius* L., *Gentiana acaulis* L., *Anemone narcissiflora* L., *Hypericum alpigenum* Kit., *Solidago virgaurea* L., *Carduus kerneri* Simonk. subsp. *kerneri*, *Scorzoneroides crocea* (Haenke) Holub, *Campanula abietina* Griseb. & Schenk, *Laserpitium krapfii* Crantz.

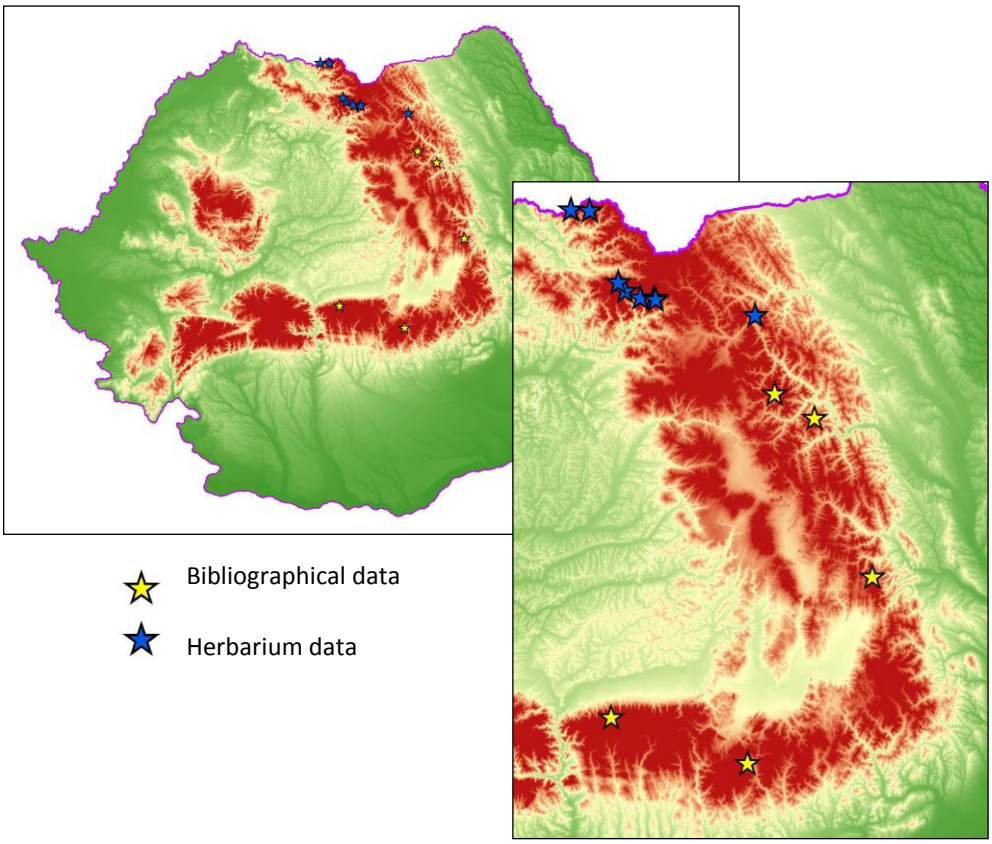

Fig. 2. Distribution map of *Heracleum carpaticum* Porcius in Romanian Carpathians

***Ranunculus thora* L.**

Literature data

Bibliographical data about *Ranunculus thora* L. (European endemite) from Romanian botanical literature is presented in Tab. 9.

Tab. 9. Distribution in Romanian Carpathians of *Ranunculus thora* according to different bibliographical sources

Bibliographical source	Maramureşului Mts.	Rodnei Mts.	Ceahlău Mts.	Ciucaş Mts.	Piatra Mare Mts.	Bucegi Mts.	Piatra Craiului Mts.	Iezer-Păpuşa Mts.	Făgăraş Mts.	Cindrel Mts.	Lotrului Mts.	Godeanu Mts.
[BAUMGARTEN,1816]	-	-	-	-	+	+	+	-	-	-	-	-
[FUSS, 1866]	-	+	-	-	-	-	-	-	-	+	-	-
[SCHUR, 1866]	-	+	-	-	-	+	+	-	-	-	-	-
[SIMONKAI, 1886]	-	+	-	+	-	+	+	-	-	+	-	-
[BRÂNDZĂ, 1879]	-	-	+	-	-	-	-	-	-	-	-	-
[GRECESCU, 1898]	-	-	-	-	-	-	+	-	+	-	-	+
[GRECESCU, 1909]	-	-	-	-	-	+	-	-	+	-	-	-
[GRECESCU, 1911]	-	-	-	-	-	+	-	-	-	-	-	-
[COMAN, 1939]	+	-	-	-	-	-	-	-	-	-	-	-
[BOŞCAIU, 1971]	-	-	-	-	-	-	-	-	-	-	-	+
[MIHĂILESCU, 2001]	-	-	-	-	-	-	+	-	-	-	-	-
[OPREA, 2005]	+	+	-	-	+	+	+	-	+	+	+	+
[DIHORU & NEGREAN, 2009]	+	+	+	+	+	+	+	+	+	+	+	+
[DRĂGULESCU, 2009]	-	-	-	-	-	-	-	-	+	+	+	-
[SZABÓ, 2012]	-	-	+	-	-	-	-	-	-	-	-	-

On the mentioned literature data *Ranunculus thora* L. can be found in 12 Mountain Massifs (Maramureşului, Rodnei, Ceahlău, Ciucaş, Piatra Mare, Bucegi, Piatra Craiului, Iezer-Păpuşa, Făgăraş, Cindrel, Lotrului, Godeanu).

Herbarium data

We hereby list all the known specimens, including those traced by us in gathering.

1853,1854 (CL): *Czetz*, Rodnei Mts.
1855 (CL): *Wolff*, Rodnei Mts.
1874,1883 (BVS, CL): *Porcius*, Rodnei Mts.
1883 (BP): *Simonkai*, Bucegi Mts.
1883 (BP): *Simonkai*, Piatra Craiului Mts.
1885, 1887, 1896, 1908 (BP, SIB, BVS): *Römer*, Piatra Mare Mts.
1886 (BP): *Barth*, Piatra Mare Mts.
1883 (BP): *Simonkai*, Bucegi Mts.
1891(BUC): *Grecescu*, Iezer-Păpuşa Mts.
1895 (BUC), *Grecescu*, Godeanu Mts.
1913 (BP): *Jávorka*, Maramureşului Mts.

1913 (BP): *Wagner*, Rodnei Mts.
1918, 1942 (SIB): *Nyárády*, Rodnei Mts.
1925 (CL): *Borza*, Rodnei Mts.
1939, ?, 1948 (SIB, CL, BUCF, I): *Coman*, Rodnei Mts.
1939, 1942 (BP): *Boros*, Rodnei Mts.
1941, 1942 (BP): *Andreánszky*, Rodnei Mts.
1941, 1942 (BP): *Kárpáti*, Rodnei Mts.
1941 (BP): *Soó, Nyárády & Felföldy*, Rodnei Mts.
1983 (CL): *Täuber & Groza*, Rodnei Mts.
1987 (BUCM): *Negrean*, Rodnei Mts.
2001 (CL): *Puşcaş*, Rodnei Mts.
2013 (HAB): *Bartók*, Rodnei Mts.
2014 (HAB): *Bartók, Brener & Covâză*, Maramureşului Mountains

Altogether 48 voucher specimens were deposited in (BP, CL, SIB, BVS, HAB, I, BUCF, BUCM, BUC). Herbarium materials found by the authors are shown in Tab. 10.

Tab. 10. Herbarium data of *Ranunculus thora* collected in Romanian Carpathians

Original label data	Collection date	Collected by	Source	Observations
Maramureş, Eastern Carpathians, Maramureşului Mountains				
"Com. Máramaros. In rupium fissuris montis Tomnatek."	5/VII/1913	S. Jávorka	(BP 64637)	as *Ranunculus carpaticus* Rev. A. Bartók 12/XII/2013
"Com. Máramaros: In rupium fissuris inter Farko et Mihailek supra pagum Havasmező."	7/VII/1913	S. Jávorka	(BP 64638) (BP 64639)	as *Ranunculus carpaticus* Rev. A. Bartók 12/XII/2013
"In saxosis inter cacuminis Farcău et Mihailecu. Solo calcareo. Alt. 1,728 m s.m."	19/VII/2014	A. Bartók B.M. Brener G. Covâză	(HAB)	-
Transylvania, Maramureş, Eastern Carpathians, Rodnei Mountains				
"Korongyis kupján éjszakra."	30/VI/1853 15/VI/1856	A. Czetz	(CL 40544)	-
"Alp. Radnensis. Korongyis la puarta."	VII/1855	G. Wolff	(CL 40545)	-
"Von der Alpe Corongişu bei Radna."	1874	F. Porcius	(BVS 037572) (BVS 037573)	-
"In gebirge Saca auf der gegen osten fallenden."	26/VI/1883	F. Porcius	(CL 40546)	-
"Rodnaer Alpen, Korongişului; Kalk., 1,990 m"	21/VIII/1895	F. Pax	(BP)	-
"Prope Rodnaborberek. Korongyis."	VIII/1913	J. Wagner	(BP)	-
"Comit. Beszterce-Naszód. Alpes Rodnenses. In saxosis montis Nagykorongyis supra balneas Radnaborberek. Alt. 1,800-1,900 m."	9/VII/1918 17/VIII/1918	E. I. Nyárády	(SIB 146838)	-

"Comit. Beszterce-Naszód. Alpes Rodnenses. In declivibus saxosis at graminosis montis Dosu Grasdiului supra pagum Major. Alt. 1,750 m".	10/VIII/1918	E. I. Nyárády	(SIB 146841)	-
"Transsilvaniae, distr. Bistrița-Năsăud. In saxosis calc. Corongiș. Alt. 1,994 m".	7/VIII/1925	A. Borza	(CL 504181)	-
"Transsilvania. Distr. Maramureș. In declivibus calcareis „Picioru Moșului montium Pietrosu Mare. Alt. cca. 1,888 m s.m".	17/VI/1939	A. Coman	(SIB 146844) (CL 197031) (CL 614422) (I 32465) (I 48548) (BVS 037571)	(FRE1940)
"Reg. Maramureș. Borșa. Fața Meselor. Alt. 1,700 m".	29/VI/1939	A. Coman	(CL 614388)	as *Ranunculus hybridus* Rev. ? 5/III/1985
"Muntii Rodnei: Iezer"	?	A. Coman	(BUCF 15703)	(DIHORU & NEGREAN, 2009)
"Com. Máramaros. In rupestribus arenosis montis prope Kőrösmező, 1,600-2,000 m".	30/VII/1939	A. Boros	(BP)	as *Ranunculus* ? Rev. A. Kurtto 1988
"Alpes Radnenses. In glareosis calcareis montis Kis-Korongyis, alt. cca. 1,600-1,700 m s.m".	22/VI/1941	G. Andreánszky	(BP 223582)	-
"Besztrece-Naszód. In alpe Korongyis supra pag. Radnaborberek. In rupibus. Alt. cca. 1,800 m."	22/VI/1941	Z. Kárpáti	(BP)	-
"Montes Radnenses. In rupibus calcareis ad „Portam" inter Montes Száka et Kiskorongyis. Alt. cca. 1,700 m s.m".	19/VII/1941	R. Soó E. I. Nyárády A. Nyárády L. Felföldy	(CL)	-
"Montes Radnenses. Transsilvaniae. In declivibus septentr. calcareis Vf. Repede. Alt. 1,900 m s.m".	8/VII/1942	A. Nyárády E. I. Nyárády	(CL 202218)	-
"Transilvania. Alpes Rodnenses. In graminosis montis Száka supra Radnaborberek. Alt. cca. 1,700 m s.m. Solo calc".	9/VII/1942	G. Andreánszky	(BP)	-
"Montes Radnenses, Transsilvaniae, Piatra Rea. In declivibus calcareis. Alt. 1,690 m s.m".	9/VII/1942	A. Nyárády E. I. Nyárády	(CL 202220)	-
"Montes Radnenses. Transsilvaniae. Nagy-Korongyis. In rupibus calcareis. Alt. 1,900 m s.m".	10/VII/1942	A. Nyárády E. I. Nyárády	(CL 202219)	-

"Com. Beszterce-Naszód. In alpe Korongyis supra pagum Radnaborberek, in rupibus calcareis. Alt. cca. 1,700 m"	18/VIII/1942	Z. Kárpáti	(BP)	-
"Com. Beszterce-Naszód. In rupestribus jugi sept. montis Korongyis prope pagum Radnaborberek. Alt. 1,800-1,994 m."	21/VIII/1942	A. Boros	(BP)	as *Ranunculus* ? Rev. A. Kurtto 1988
"Com. Beszterce-Naszód. Alpes Rodnenses. In rupestribus calcar. montis Száka pr. Radnaborberek. Alt. cca. 1,700 m s.m."	21/VIII/1942	A. Boros	(BP)	as *Ranunculus* ? Rev. A. Kurtto 1988
"Com. Máramaros. Mt. Nagy-Pietrosz. In rupibus calcareis infra lacum Mosolygó-tó supra pagum Borsa."	26/VIII/1942	Z. Kárpáti	(BP)	-
"Borşa, Aria Zimbrului, exp. N; 1,037 m."	29/VIII/1948	A. Coman	(BP 424061)	-
"Transsilvania, distr. Bistriţa-Năsăud, Mt. Rodnei."	VII/1983	F. Täuber Gh. Groza	(CL 649041)	-
"Turnul Roşu"	23/VIII/1987	G. Negrean	(BUCM 104877)	(DIHORU & NEGREAN, 2009)
"Căldarea Iezerul, Munţii Rodnei, stânci de calcar. 1,800 m."	5/VII/2001	M. Puşcaş	(CL 659102)	-
"Alpibus Rodnensibus. In declivibus calcareis "Piciorul Mosului", montium Pietrosul Mare."	15/VI/2013	A. Bartók	(HAB)	-
Transylvania, Eastern Carpathians, Piatra Mare Mountains				
"In alpibus: Piatra Mare."	VI/1885	J. Römer	(BP 64636)	Com. J. Barth
"In alpibus: Piatra Mare."	17/VII/1886	J. Barth	(BP)	-
"Hohenstein"	16/VI/1887	J. Römer	(SIB 38335)	-
"Hohenstein (Peatra Mare) 1,840 m."	19/VII/1896	J. Römer	(SIB 38336)	-
"Piatra Mare."	7/VI/1908	J. Römer	(BVS 037574)	-
Transylvania, Southern Carpathians, Bucegi Mountains				
"In alpe Bucsecs, locis saxosis alpinis."	30/VIII/1883	L. Simonkai	(BP 64621)	-
Transylvania, Southern Carpathians, Piatra Craiului Mountains				
"In graminosis alpis „Királykő" supra Zernyest, 2,000 m s.m."	27/VIII/1883	L. Simonkai	(BP 64622)	-
Muntenia, Southern Carpathians, Iezer-Păpuşa Mountains				
"Păpuşa, regiunea alpină, stânci cu muşchi."	12/VIII/1891	D. Grecescu	(BUC 322161)	(DIHORU & NEGREAN, 2009)
Transylvania, Southern Carpathians, Godeanu Mountains				
"Muntele Godeanu, micaşisturi, pe lângă zăpadă, la 2,200 m."	18/VII/1895	D. Grecescu	(BUC 322160)	(DIHORU & NEGREAN, 2009)

Checking herbarium materials for species *Ranunculus thora* two herbarium sheets were discovered (BP 64638 and BP 64639) with plants collected from the Farcău Peak, but it was recorded in the database of herbarium as *Ranunculus carpaticus*. Herbarium sheet

was reviewed by A. Bartók. The species was found by the authors of this paper in the mentioned area after 100 years after the collection of first herbarium material.

The chorological map of *Ranunculus thora* L. is shown in Fig. 3.

On the basis of chorological data and estimation of the geographical range (extent of occurrence) we can define *Ranunculus thora* as IUCN: VU C2a, B1(i).

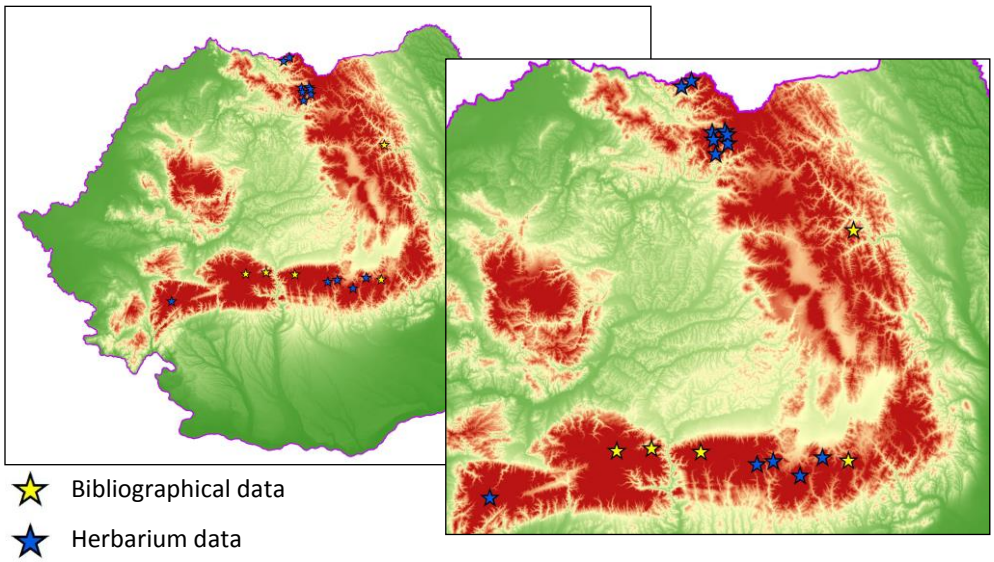

Fig. 3. Distribution map of *Ranunculus thora* L. in Romanian Carpathians

Field observations

During the floristical investigation performed in Maramureşului Mountains on 19 July 2014 we found one population of *Ranunculus thora* L. on exposed calcophyllite cliffs. Locality: Romania, Eastern Carpathians, Maramureşului Mountains, Farcău Peak, 1728 m a.s.l., exp. S-SE, incl. 90°, basalt (with dolomite inclusions) cliffs, area 25 m^2, leg. A. Bartók, B. M. Brener, G. Covâză; date: 19/VII/2014.

Together with *Ranunculus thora* L. we also found the following species: *Juncus trifidus* L., *Hieracium villosum* Jacq., *Saxifraga paniculata* L., *Asplenium viride* Huds., *Dianthus carthusianorum* L., *Aster alpinus* L., *Saxifraga adscendens* L., *Silene nutans* L. subsp. *dubia* (Herbich) Zapał., *Leontopodium alpinum* Cass., *Campanula alpina* Jacq., *Sempervivum montanum* L., *Hypochoeris uniflora* Vill., *Rhodiola rosea* L.

Conclusions

The authors of this paper during a botanical trip found in Farcău Peak (Maramureşului Mountains) area three very rare species (all 3 threatened, included in Romanian Red Book of superior plants): *Trifolium lupinaster* L., *Heracleum carpaticum* Porcius and *Ranunculus thora* L.

Herbarium data analysis shows that *Trifolium lupinaster* is extremely rare in Romania and in Maramureşului Mountains has not been seen for more than 30 years.

Authors rediscovered *Trifolium lupinaster* L. after 30 years from the collection this very rare and critically endangered species in Farcău Peak area.

Heracleum carpaticum is also rare in Romanian Flora and even though it was cited by several botanists in the Maramureş Mountains, herbarium data analysis indicates that the species was not collected from the Farcău Peak.

When checking herbarium materials for species *Ranunculus thora* L. two herbarium sheets were discovered (BP 64638 and BP 64639) with plants collected from the Farcău Peak, but the plant recorded in the database of herbarium as *Ranunculus carpaticus*. The species was refound by the authors of this paper in the mentioned area 100 years after the collection of the first herbarium material.

Based on field studies, analysis of herbarium material and literature data, the authors managed to record the occurrence of *Trifolium lupinaster* L., *Heracleum carpaticum* Porcius, and *Ranunculus thora* L. in Romanian Carpathians and determined the threatened status according to categories and criteria of IUCN.

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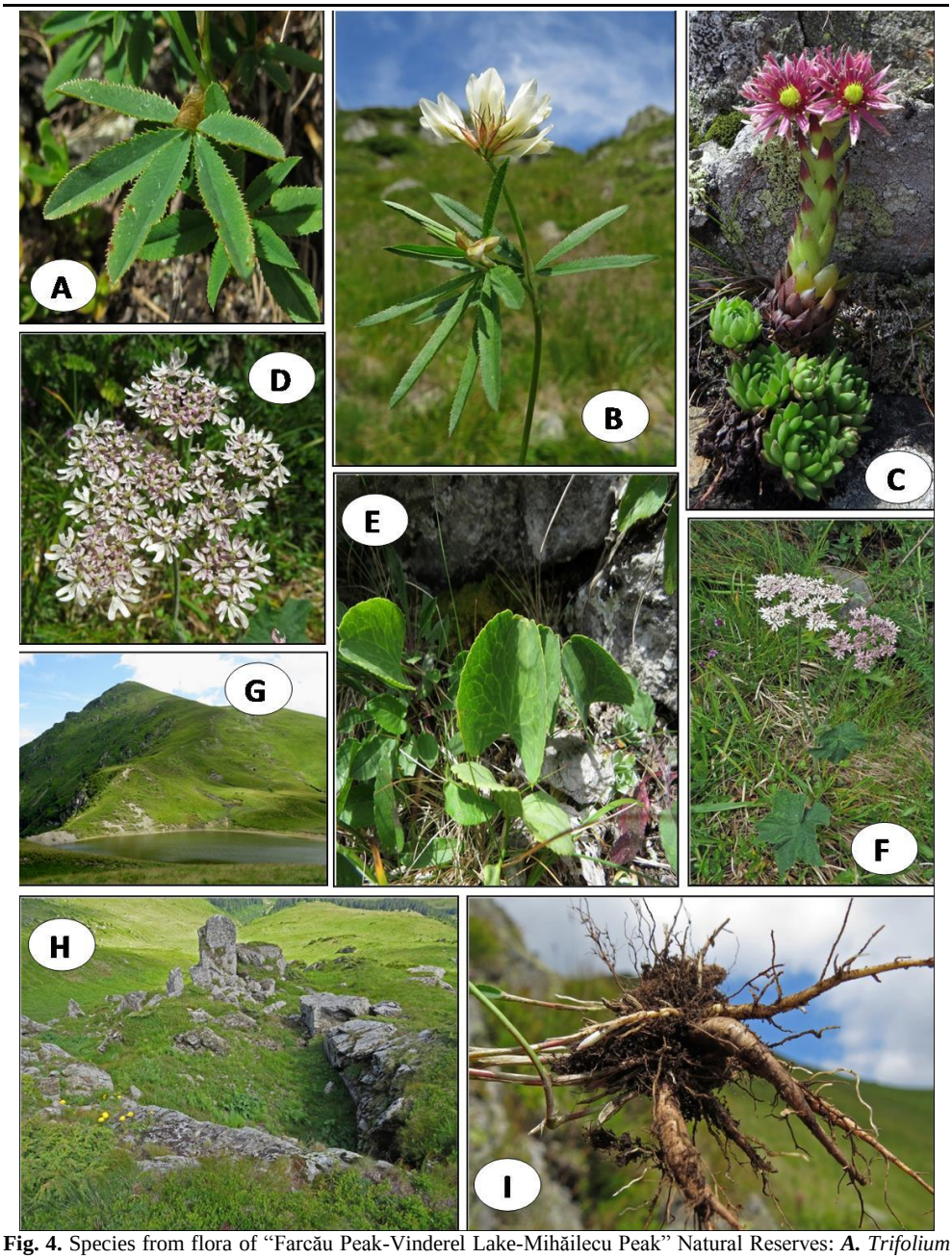

Fig. 4. Species from flora of "Farcău Peak-Vinderel Lake-Mihăilecu Peak" Natural Reserves: ***A.*** *Trifolium lupinaster* L. – leaf; **B.** *Trifolium lupinaster* L. – habitus; **C.** *Sempervivum montanum* L. – habitus; **D.** *Heracleum carpaticum* Porcius – inflorescence; **E.** *Ranunculus thora* L. – habitus; **F.** *Heracleum carpaticum* Porcius – habitus; **G.** Landscape – Vinderel Lake and Farcău Peak; **H.** Habitat of *Heracleum carpaticum* Porcius, near Farcău Peak; **I.** *Trifolium lupinaster* L. – root

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DIVERSITY AND ECOLOGICAL DETERMINANTS OF DEAD WOOD FUNGI IN TREE NATURAL RESERVES OF BROAD LEAVED FORESTS FROM SUCEAVA COUNTY

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Abstract: Dead wood fungi have a major importance for forests biodiversity as they produce wood degradation in forest habitats. In this paper are presented some aspects related to the diversity of dead wood fungi in tree deciduous forest types from tree natural reserves (Crujana, Dragomirna and Zamostea) from Suceava County and the effect of some ecological factors (host tree, diameter and decomposition degree of the dead wood and some microclimatic characteristics of sites) on their occurrence and diversity. Investigations carried out in 2013 resulted in the identification of 44 lignicolous fungi species. Analysis of similarities between lingnicolous fungi species from the investigated natural reserves (by hierarchical clustering) shows a separation of three fungi groups, depending on the host-trees species. The effect of the tree host species was highlighted also by detrended correspondence analysis, which, in addition presented the existence of an altitudinal gradient and a weaker effect of site conditions (slope and aspect) and microclimatic variables (solar radiation) on dead wood fungi occurrence. The effect of diameter and decomposition degree of fallen trunks and branches on dead wood fungi species was investigated using the redundancy analysis showing that wood debris with large surfaces are more easily colonized by the fungi species developing large sporocarps compared to small branches with low diameters colonized only by few or a single fungus species.

Keywords: forest, lignicolous fungi, wood debris, Detrended Correspondence Analysis (DCA), Redundancy Analysis (RDA)

Introduction

Dead wood fungi have a main role in the maintaining of ecosystems health, nutrient cycles, and, consequently, a major importance for forests biodiversity. Lignicolous fungi are, together with insects, the main agents of wood degradation in forest habitats [MÜLLER & al. 2007]. They are the main decomposing agents of the dead vegetal material as wood or litter. Dead wood quantities are lower in forests where the intensive silvicultural interventions are made by humans than in old growth forests and natural reserves where human interventions are minimized [KIRBY & al. 1998]. Host-tree species, wood debris size, microclimate conditions, decomposition degree and initial position of dead wood from tree are the variable keys influencing the fungi species composition [JACOB & MORTEN 2004; KÜFFER & SENN-IRLET, 2005]. The main task of this study was to investigate the dead wood fungi diversity in tree deciduous forest types from tree natural reserves from Suceava County and the effect of some ecological determinants (host tree, diameter and decomposition degree of the dead wood and some microclimatic characteristics of sites) on their diversity.

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The natural reserve from Dragomirna is represented by a natural arboretum of *Fagus sylvatica* (130 years old) with the main habitat type, 91V0, Dacian beech forests (*Symphyto-Fagion*). In Crujana forest (*Quercetum*) the natural arboretum is constituted of a mix of deciduous species, with oak species (*Quercus robur*) as dominant tree species, similar to the oak forests from Central Europe; the main habitat type is 9160, Sub-Atlantic and medio-European oak or oak-hornbeam forests of *Carpinion betuli* [SÂRBU & al. 2007]. Zamostea Forest is a natural reserve represented by mixed communities of *Quercus robur*, *Fraxinus* sp., *Salix* sp. and *Populus* sp. It represents an area of riverine forest with more lignicolous species compared to the above mentioned natural reserves (Tab. 1) [BLEAHU & al. 2006]. The main habitat type in this site is 91F0-Riparian mixed forests of *Quercus robur*, *Ulmus laevis* and *Ulmus minor*, *Fraxinus excelsior* or *Fraxinus angustifolia* along the great rivers (*Ulmenion minoris*) [GAFTA & MOUNTFORD, 2008].

Tab. 1. Important geographical features of the three natural reserves of broad leaved forests

Crt. No.	Name	Area (ha)	Altitude (m)	Main forest types	Geographical coordinates
1.	Crujana Reserve (Quercetum)	39.4	370-393	*Quercus robur* dominant	47^0 45’ N 26^0 11’ E
2.	Dragomirna Reserve (Beech-trees)	134.8	380-450	*Fagus sylvatica* dominant	47^0 45’ N 26^0 12’ E
3.	Zamostea Reserve	107.6	290	*Quercus robur*, *Fraxinus excelsior*, *Populus* sp., *Salix* sp.	47^0 52’ N 26^0 15’ E

Materials and methods

Diversity of lignicolous fungi was analyzed in three natural reserves of broad leaved forests from Suceava County: Crujana, Dragomirna and Zamostea (Fig. 1). Samples were collected in 63 randomly chosen points (21 in each natural reserve) on square shaped areas of 100 m^2 each. Investigations were made from April to October 2013. For each sample, geographical coordinates and altitude were recorded using a geographic positioning device (GPS II Plus Garmin Ltd.). All remnants of dead wood which had at least one sporocarp have been registered. For all dead wood debris we noticed: diameter, length, decomposition degree and host tree species. Sporocarps of unidentified species were investigated through laboratory specific methods based on micro-morphological and macro-morphological characters according to identification keys and reference guides [BERNICCHIA, 2005; BREITENBACH & KRÄNZLIN, 1986; JÜLICH & STALPERS, 1980; JÜLICH, 1989; SĂLĂGEANU & SĂLĂGEANU, 1985; TĂNASE & al. 2009]. Estimation of wood’s decomposition degree was semi-quantitatively and subjectively done using a knife [RENDVALL, 1995]. Analysis of similarities among lignicolous fungi species was realized using the Sorensen index (presence-absence data) in a hierarchical clustering procedure using the UPGMA algorithm. Detrended Correspondence Analysis (DCA) has been realized in order to distinguish the main gradients in lignicolous species composition and to characterize them from an ecological perspective. Detrending by segments and non-weighted average values of altitudes, heat load and potential annual incidence radiation [McCUNE & KEON, 2002] for each plot were used (as passive

projected variables). Redundancy analysis was used in order to observe the influence of the diameter and decomposition degree of the dead wood on fungi species. The hierarchical agglomerative clustering has been realized using the GINKGO software [DE CÁCERES & al. 2003]. DCA and RDA have been realized in CANOCO 4.5 [TER BRAAK & ŠMILAUER, 2002].

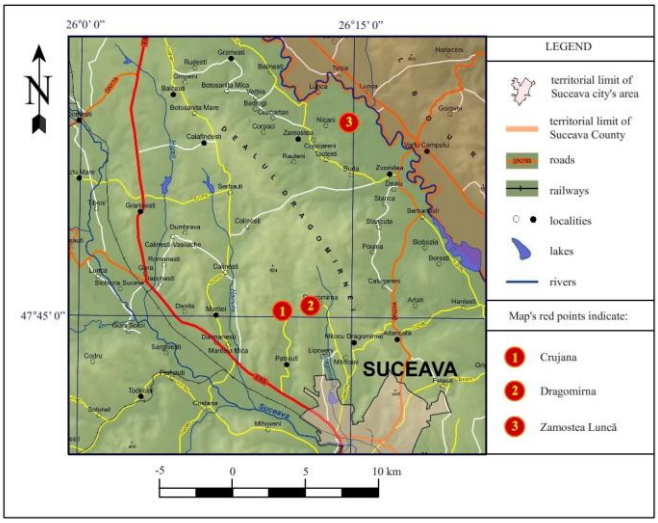

Fig. 1. Geographical positions of investigated Forests Reservations

Results and discussion

Investigations have been realized in the mentioned areas where we collect 486 samples belonging to 44 lignicolous fungi species (Tab. 2) with an average of 11 species per plot. *Trametes* and *Xylaria* were the most species rich genera, each with three species. Depending on their nutrition mode the fungi species belong to three categories: lignicolous saprophyte (28 species), lignicolous sapro-parasite (12 species) and parasite (4 species). The amount of dead wood, one of the important features of the maintaining of a high diversity of lignicolous macrofungi, varied among the sites (plots). The more dead wood was present, the greater diversity of species was registered, because of higher colonization probability.

Tab. 2. The species and main characteristics of lignicolous fungi recorded in the research plots

Species	Occurrence	Host-tree
1. Armillaria mellea	IX XI	Deciduous
2. Armillaria ostoyae	IX XI	Deciduous
3. Auricularia auricula-judae	I XII	Elder, Beech-tree, Acacia
4. Bjerkandera adusta	I XII	Deciduous, Beech-tree
5. Bulgaria inquinans	IX XI	Deciduous
6. Chondrostereum purpureum	I XII	Deciduous, Beech-tree, Birch-tree, Poplar
7. Daedalea quercina	I XII	Oak

8. Daedaleopsis confragosa	VII X	Deciduous
9. Daldinia concentrica	V X	Deciduous, Birch-tree, Beech-tree, Oak
10. Exidia glandulosa	I XII	Oak
11. Fistulina hepatica	VIII X	Deciduous, Oak
12. Fomes fomentarius	I XII	Beech-tree, Birch-tree
13. Fuscoporia ferruginosa	I XII	Beech-tree
14. Ganoderma applanatum	I XII	Deciduous
15. Ganoderma lucidum	I XII	Oak
16. Hyphodontia sambuci	I XII	Deciduous
17. Hypholoma fasciculare	V XI	Deciduous
18. Hypholoma lateritium	VIII XI	Deciduous
19. Hypoxylon fragiforme	I XII	Deciduous, Beech-tree
20. Laetiporus sulphureus	I XII	Deciduous, Willow
21. Lenzites betulina	IV XII	Deciduous, Beech-tree, Oak
22. Meripilus giganteus	VII X	Beech-tree, Oak
23. Merulius tremellosus	IX XII	Beech-tree, Poplar
24. Peniophora quercina	I XII	Oak
25. Phellinus igniarius	I XII	Deciduous, Willow
26. Phlebia radiata	IX XII	Deciduous
27. Pleurotus ostreatus	X XII	Beech-tree, Poplar, Willow
28. Plicaturopsis crispa	IX XII	Beech-tree
29. Pluteus salicinus	VIII X	Willow, Alder, Beech-tree
30. Polyporus arcularius	I XII	Deciduous
31. Pycnoporus cinnabarinus	I XII	Deciduous, Beech-tree, Cherry
32. Sarcoschypha coccinea	II IV	Deciduous, Hornbeam
33. Schizophyllum commune	I XII	Deciduous
34. Stereum hirsutum	I XII	Deciduous
35. Trametes pubescens	VII X	Deciduous
36. Trametes hirsuta	I XII	Beech-tree, Oak
37. Trametes versicolor	I XII	Deciduous
38. Tremella foliacea	I XII	Birch-tree, Beech-tree
39. Tremella mesenterica	I XII	Beech-tree, Oak, Ash
40. Vuilleminia comedens	I XII	Beech-tree
41. Xylaria hypoxylon	I XII	Deciduous
42. Xylaria longipes	I XII	Maple
43. Xylaria polymorpha	I XII	Beech-tree, Oak
44. Xylobolus frustulatus	I XII	Deciduous, Oak

Analysis of similarities between lingnicolous fungi species from the investigated natural reserves shows a separation of three fungi groups as follows (Fig. 2): a group with *Quercus* (in Crujana forest), another group with *Fagus* (in Dragomirna forest) and another group with *Quercus*, *Populus* and *Salix* (in Zamostea forest). This suggests that the installation of lignicolous fungi species depends on the host-trees. Thus, in the *Quercus* stands were identified species as *Daedalea quercina*, *Exidia glandulosa*, *Ganoderma lucidum*, *Peniophora quercina* which differentiate them from the *Fagus* stands where more frequent are *Bjerkandera adusta*, *Fomes fomentarius*, *Fuscoporia ferruginosa*, *Plicaturopsis crispa* or *Vuilleminia comedens*. However, there are also common species for these two forest types: *Meripilus giganteus*, *Trametes hirsuta*, *Tremella foliacea* etc. More similar to the almost pure oak stands from Crujana reserve is the mixed forest with oak, ash and willow or poplar from Zamostea natural reserve. As in this riverine forest the trees layer is more diversified, also the dead wood fungi diversity is more increased compared to

the pure oak forest or pure beech forests. The difference could be produced by *Populus*, *Salix* or *Fraxinus* individuals preferring places with higher humidity and presenting a different and more species rich fungi community. This fact is highlighted by the means of a simple linear regression (Fig. 3) showing that diversity (species richness) of dead wood fungi species increases with diversity (species richness) of woody plant species from investigated areas.

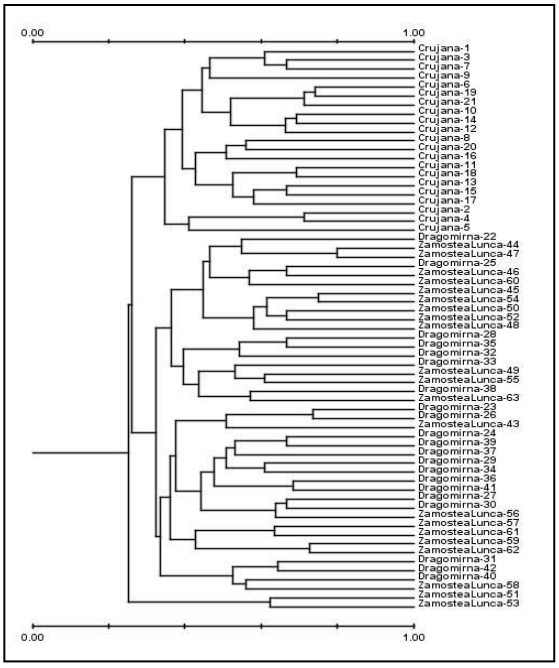

Fig. 2. Dendrogram generated by the hierarchical clustering presenting lignicolous fungi similarities among sites

Through Detrended Correspondence Analysis (DCA) (Fig. 4) three groups were also separated: the first group located at the right, in the lower region of the ordinogram includes the dead wood fungi from the *Fagus sylvatica* forest (Dragomirna), the second one also from the right part but in the upper part of the ordinogram include the dead wood fungi from the mixed forest of *Quercus robur* and other more hygrophilous trees species (Zamostea) and the third one includes dead wood fungi from the *Quercus robur* forest (Crujana). The first DCA axis is weakly negative correlated with heat load and PADI and explains only 5.9% of species-environment relation. The second axis is more strongly correlated with altitude and explains 37.6% of species-environment relation, indicating that the second axis is the most important one and the existence of a altitudinal gradient, from relatively low altitudes forests to higher altitude forests, suggesting that altitude represent the main factor with significant influence on the dead wood fungi composition in the investigated vegetal communities. Only secondarily, the dead wood fungi composition is influenced by the heat and incidence solar radiation indicating that variation in dead wood species among stands might be related to variation in local microclimate conditions. As

between investigated stands there are no significant differences regarding heat load (slopes and aspects) and solar radiation, these ecological factors have not a strong significant effect on dead wood fungi species occurrence. DCA could be also interpreted in terms of dead wood fungi affinity for host-trees. Thus, left group include species preferring oak wood (*Quercus robur*-dominant tree in Crujana forest): *Daedalea quercina*, *Ganoderma lucidum*, *Exidia glandulosa* and *Peniophora quercina*. The second group includes species showing a high affinity for beech wood (*Fagus sylvatica*-dominant tree in Dragomirna forest): *Pleurotus ostreatus*, *Fomes fomentarius*, *Plicaturopsis crispa* and *Vuilleminia comedens*. The third group includes species besides oak, also willow and poplar wood: *Pluteus salicinus*, *Phellinus igniarius* and *Laetiporus sulphureus*.

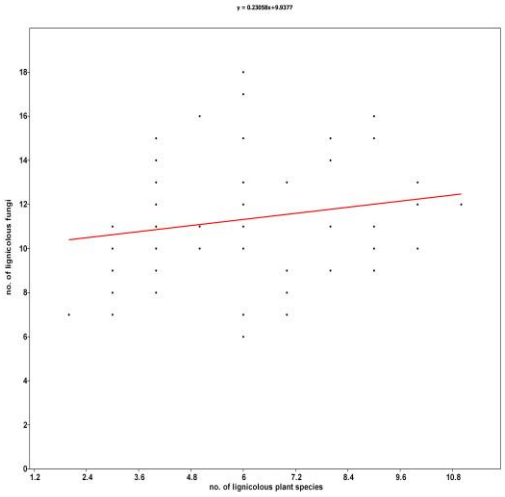

Fig. 3. Linear regression expressing the variation of fungi species richness as a function of lignicolous species richness

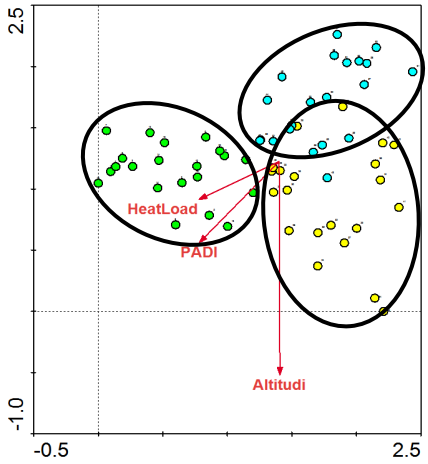

Fig. 4. DCA ordination diagram of the 63 samples using heat load index, potential anual direct radiation index and altitude as passive variables first two axes presented. Eigenvalues: 1st axis: 0.350, 2nd axis: 0.201, total inertia: 2.750.

Redundancy Analysis (RDA) (Fig. 5) suggests that diameter and decomposition degree of fallen trunks and branches have a significant importance for lignicolous fungi species. Thus, wood debris with large surfaces are more easily colonized by the fungi species (interspecific competition is avoided) developing large sporocarps (*Fomes fomentarius*, *Laetiporus sulphureus*, *Ganoderma applanatum*, etc.), as compared to small branches with low diameters, which allow only few or a single fungus species to colonize during a particular time (*Picnoporus cynnabarinus*, *Exidia glandulosa*, *Plicaturopsis crispa*, etc.). From another perspective, the monocentric species (which have only one starting point in growth) typically requires more substrate relative to their size than polycentric species. Thus, polycentric species have a physiological and competition advantage on monocentric species. Therefore, presence of these species can be determined not only by the diameter and volume of the substrate itself, but also by the great ability to

grow on such a substrate. Besides diameter, the decomposition degree is a determinant factor for the fungus species which can be observed at a certain stage of wood decomposition. Thus, for the incipient stages of wood decomposition *Bjerkandera adusta*, *Chondrostereum purpureum* can be observed; for increased decomposition degrees *Hypoxylon fragiforme* and *Peniophora quercina* are more frequent in the investigated forests.

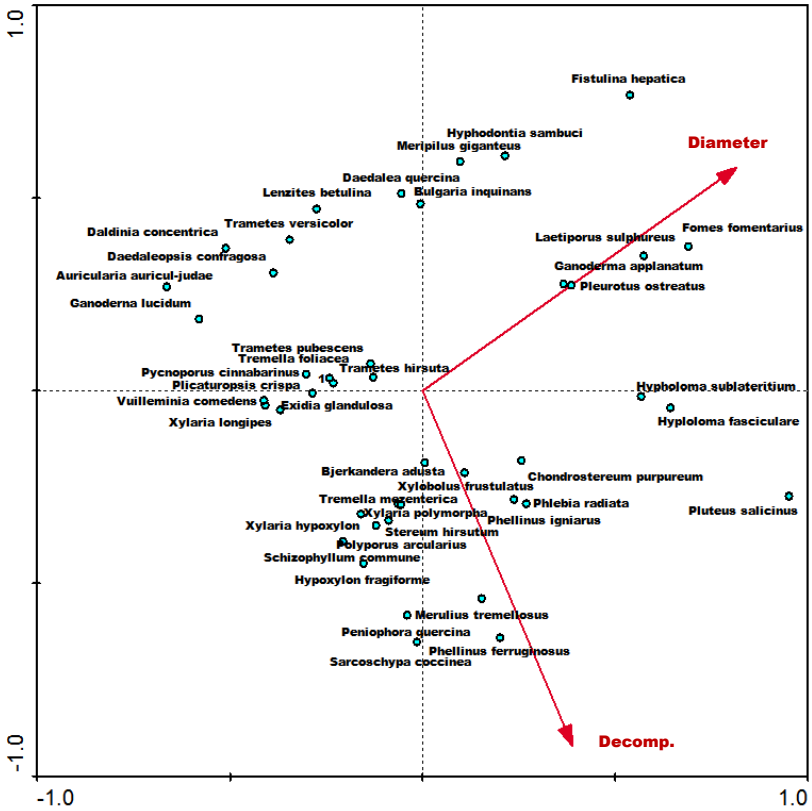

Fig. 5. RDA biplot of axes 1 and 2 with fungal species (44 species) constrained by two explanatory variables (diameter and decomposition degree)

Conclusions

Investigations carried out in tree deciduous forest types from tree natural reserves (Crujana, Dragomirna and Zamostea) from Suceava had as result the identification of 44 lignicolous fungi species. The diversity of lignicolous plant species (linear relationship between their diversity and dead wood fungi species diversity) and the host tree species have significant influence on the dead wood fungi species composition in the investigated areas. Also, the decomposition degree and dimensions of wood debris make clear distinctions among the fungal species which colonize the dead wood of different sizes and different decomposition stages.

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OPINION PAPER: SHOULD THE TERM PROTOCORM-LIKE BODY BE USED EXCLUSIVELY FOR ORCHIDS?

Jaime A. TEIXEIRA DA SILVA[1]

The term protocorm-like body (PLB) is traditionally used to describe an organ that develops in orchid *in vitro* culture that resembles, in morphology (structure) and function, an enlarged seed-derived zygotic embryo, the protocorm [ARDITTI, 1979]. The term protocorm was coined by Melchior Treub for seedlings of club mosses while studying the sporophyte developmental stages of cormophytes [TREUB, 1890]. Noel Bernard then directly applied Treub's concept of protocorm (for structures originating from seed) to the Orchidaceae [BERNARD, 1909]. A protocorm is "the tuber-like swollen part of an orchid seed, which appears during the early stage of germination" [XIONG, 2009] or, according to the online Merriam-Webster dictionary, "a tuber-shaped body with rhizoids that is produced by the young seedlings of various orchids and some other plants having associated mycorrhizal fungi". MOREL (1960) may very well have been the first orchidologist to describe a PLB as "an uncertain term that means a structure formed *in vitro* that looks similar to a protocorm" [YAM & ARDITTI, 2009]. However, a PLB is a *de facto* somatic embryo – an embryo that is derived from a somatic cell – in orchids [TEIXEIRA DA SILVA & TANAKA, 2006; LEE & al. 2013, and references therein]. This suggests that a PLB is an organ specific to the Orchidaceae. Indeed, the first public call for the strict use of the term PLB in orchids dates back to *Phalaenopsis* research [ISHII & al. 1998] and is a term that has been used exclusively for orchids in tissue culture in thousands of scientific papers. The plant literature thus suggests that the terms protocorm and PLB could be applied primarily to orchids, but could also be used more widely.

However, the term "PLB" has been used to describe, for at least 11 non-orchidaceous plants, round, globular structures that resemble PLBs in other crops, primarily ornamental plants: *Anthurium andraeanum* (Araceae) [GANTAIT & al. 2012, based on YU & al. 2009], Brodiaea (*Dichelostemma congestum*, *Triteleia laxa*, *T. ixioides*, *T. hyacintina*; ILAN & al. 1995), *Colocasia esculenta* (Araceae) [ABO EL-NIL & ZETTLER, 1976; NYMAN & al. 1983; NYMAN & ARDITTI, 1988; NYMAN & al. 1989; SABAPATHY & NAIR, 1992], *Heliconia psittacorum* (Heliconiaceae) [NATHAN & al. 1993; GOH & al. 1995; KUMAR & al. 1996], *Hippeastrum hybridum* (Amaryllidaceae) [HUANG & al. 1990], *Lilium longiflorum* (Liliaceae) [NHUT & al. 2001, 2002], *Musa* [VENKATACHALAM & al. 2006], *Philodendron micans* (Araceae) [XIONG, 2009], *Pinellia ternata* [WANG & al. 2009; LIU & al. 2010a, 2010b], *Rosa* [TIAN & al. 2008; LIU & al. 2014], and *Syngonium podophyllum* (Araceae) [CUI & al. 2008].

In fact, closer analysis of the literature reveals the following (when searching for the terms protocorm-like body or PLB): 4130 hits on Google Scholar (mostly orchids); 213

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hits on Elsevier's Sciencedirect.com (7 non-orchids); 180 hits on Springer Science + Business Medium's SpringerLink (9 non-orchids); 13 hits on Taylor and Francis, Wiley-Blackwell and deGruyter Online platforms (1 non-orchid). Thus, it is estimated that from the total main-stream literature, that approximately 95% of the plant science literature uses the term PLB for orchids.

The CUI & al. (2008) paper shows light-microscopic images of round structures which, using histological sectioning, appear to be somatic embryos. How then did the authors extrapolate to conclude that such structures were PLBs? The authors appear to rely on the literature to support their claims. For example, "Protocorm-like bodies are composed of many meristematic centers that are able to differentiate into shoots and roots [DA SILVA & al. 2000]." However, examination of the original DA SILVA & al. (2000) paper reveals that the authors did not use the term PLB at all, instead, the authors were describing the development of plantlets from pro-embryoids following anther culture. The CUI & al. (2008) paper thus incorrectly describes the literature and inadvertently introduces a significant error that affects the subsequent use of the term PLB in non-orchid genera. Is then the use of the term "PLB" for plants that are not orchids a misnomer (i.e., "a term or meaning that suggests a meaning that is known to be wrong" (Wikipedia 2014)? NATHAN & al. (1993) first used the term to describe PLBs in *Heliconia psittacorum* what were clearly somatic embryos, but no botanical explanation as to why the term PLB was used, was provided. The term PLB is then promulgated by the same authors in subsequent papers [GOH & al. 1995; KUMAR & al. 1996]. GANTAIT & al. (2012), despite accurately describing the exclusive use thus far in the plant science literature for the Orchidaceae, then suddenly employ it for *Anthurium*, the only rationale being that term had been used previously by YU & al. (2009). No other botanically-based rationale is provided. In fact, an examination of the YU & al. (2009) study reveals that the rationale for the use of the term PLB is incorrect, flawed and botanically unfounded: "Induction of protocorm-like bodies (PLBs) is a popular method to *Phalaenopsis* [ROY & al. 2007]. To our knowledge, there is no report of regeneration from protocorm-like bodies (PLBs) formation of anthuriums." Reference to the ROY & al. (2007) study is in fact also incorrect since that study is on *Dendrobium*, and not *Phalaenopsis*, calling into question thus the scientific and botanical accuracy of the NATHAN & al. (1993), CUI & al. (2008), YU & al. (2009) and GANTAIT & al. (2012) studies, specifically the use of the term PLB.

HUANG & al. (1990) decided to refer to the production of bulblets, a well-established term for the small bulblets that form from single or twin scales in the Liliaceae and Amaryllidaceae, PLBs, without any logical reason or explanation. It is unclear if any literature pertaining to bulblet formation in *Hippeastrum* adopted this term, but further scrutiny of the *Hippeastrum* literature is merited. In the Liliaceae, specifically *Lilium longiflorum*, NHUT & al. (2001) first introduce the term PLB to describe what is interchangeably referred to as pseudo-bulblets or somatic embryos, but then, without any clear explanation, adopted as PLBs. The potentially incorrect term is then carried forward to an ensuing study [NHUT & al. 2002]. A similar error to these studies was made by ILAN & al. (1995) in half a dozen members of the Brodiaea, in which cormlets were referred to as PLBs, without any botanical explanation or rational to substitute an already well-established term, or histological proof. NYMAN & ARDITTI (1988) observed several structures all forming simultaneously from the same explants, but refer to the round ones as PLBs. However, in their study, the nomenclature is inconsistent, sometimes referred to as

protocorm-like bodies, and sometimes as protocorm-like structures, weakening thus the rationale for the use of the term PLB for taro, a tuberous crop.

XIONG (2009) stated in the abstract "Histological analysis suggested that the globular structures were protocorm-like bodies (PLBs), a novel pathway for plant regeneration." Xiong then uses the term PLB for *Philodendron*, basing his defense of the use of this term and his rationale on the fact that "PLBs have been identified in a wide range of other plant genera." However, this characterization of the literature is incorrect and thus misleading. In fact, what Xiong observed was simply the use of the term PLB in the literature, but not necessarily the correct assignment of the term. However, the developmental evidence provided by Xiong in Fig. 3-1 A and B of the thesis seems to indicate that these round structures are simply undeveloped shoot initials while Fig. 3-2 suggests that indeed these structures may be somatic embryos or somatic-embryo-like structures. If so, then why did Xiong simply not refer to them as somatic embryos rather than PLBs?

TIAN & al. (2008) first induced callus and new rhizoids from *Rosa* spp. (*R. canina* L., *R. multiflora* var. *cathayensis* Rehd. & Wils., and *R. multiflora* f. *carnea* Thory.) rhizoids, then, after transfer to a "PLB-formation medium", which contained thidiazuron, then induced what they claimed were PLBs. However, examination of the structures they termed PLBs reveals structures that were anything except what is typically observed in orchids, thus begging the question: why did the authors select this term rather than creating a new term? The authors offer absolutely no botanical explanation for their choice of term, and the structures they observe appear to be either undeveloped shoot initials, or hyperhydric shoots, albeit with a roundish structure. Ironically, the biggest clue that in fact these are not PLBs, which develop only from epidermal layers, comes precisely from the evidence which the authors claim proves that these are PLBs: histological sections. Their Fig. 3d indicates a PLB forming in the center of parenchymatous cells (described in their figure legend as a meristematic center). Thus, this is clearly not a PLB, but some other structure. The same error exists in a subsequent paper by the same group that further tries to fortify the use of the term PLB for *R. canina* [LIU & al. 2014]. In that paper, despite an impressive display of histological analyses, essentially the authors observe "green ellipsoidal bodies", or "deep-green globular bodies" at the tips of rhizoids, i.e., inconsistent nomenclature, but then chose to use the term PLB to describe these structures, without indicating why botanically they should be named as such. Their histological analysis shows a mélange of shoots initials and "PLBs", which histologically are indistinct, once again reinforcing the question: why were these green round structures termed PLBs?

LIU & al. (2010a) identify the formation of round, green structures on *Pinellia ternata* leaf, petiole and tuber explants in the presence ofα- naphthalene acetic acid (NAA) and 6-benzyladenine (BA), but no histological proof is provided. The structure, claimed to be a PLB, is then used as the basis of the explants in a subsequent study [LIU & al. 2010b]. The use of the term PLB is extremely problematic since the same authors identified the exact same structures as micro-tubers in the same plant, using the same explants and the same plant growth regulators, BA and NAA [WANG & al. 2009]. In that study, the botanical name of the plant was also incorrectly spelt.

Thus, the initial use of the term PLB for non-orchids is, in my opinion, flawed, and unexplained, at least in botanical terms. Moreover, absolutely no rationale has been provided by these 11 non-Orchidaceous studies and very rarely (only 2-3 recent studies) has

cytological evidence been provided. Consequently, by association, all references that lend support to these potentially flawed studies would themselves be flawed, by association.

The ability of scientists to apparently easily introduce, even following "peer review", neologisms easily into the scientific literature without a sound or accurate scientific basis is one of the unintended (and unfortunate) consequences of the lack of defined standards regarding some terminology used in plant tissue culture and plant development, and also indicates one of the weaknesses of traditional peer review [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2015], even in established plant science journals. The real risk that the plant science community faces is that any round, somatic embryo-like or PLB-like structure that visually resembles a PLB will be termed a PLB in any plant species when in fact those plants do not form a protocorm from their seed. Thus, the botanically restricted term will lose its importance and impact, and exclusivity, for the orchids.

For example, it is difficult to imagine PLB formation in tobacco, potato, or *Arabidopsis thaliana*. However, by creating an exceptional precedent, these 11 studies now provide a spring-board for the unbased and expanded use of this term to non-Orchidaceous plants, which I feel is problematic. Undoubtedly, the "originality" factor would certainly score the authors of such papers a publication since reviewers who would be reviewing such papers would feel that a "new botanical structure" had been discovered in that plant when in fact no such botanical basis exists. However, botanically-speaking, would the use of this term for non-orchidaceous plants be accurate, or correct?

This paper then calls on the standardized use of the term protocorm-like body, or PLB, to describe somatic embryogenesis in orchids (all genera) and that structures that resemble PLBs in any other plant family be referred to more accurately as PLB-like bodies (i.e., protocorm-like body-like bodies). No doubt that this will be a topic of discussion until more irrefutable evidence, and solid logic, is provided that champions for the exclusive use of the term PLBs in the Orchidaceae. Moreover, provided that different levels of quality control exist in editor boards of plant science journals published by different publishers, and in a non-standard interpretation of the developmental aspect of orchids by so-called "peer reviewers", it will be difficult to ensure a literature-wide control of the use of suitable terminology. Studies like those by TIAN & al. (2008) and LIU & al. (2014) lend credence to the broader use of PLBs to non-Orchidaceous crops, while other studies that simply use the term PLB to describe a round, green structure without detailed histological or developmental analyses may very well be diluting, or even corrupting, the botanical literature.

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"I understand and agree with your point. To me, PLBs are exclusive to orchids, but technically, others might have an argument for expanding the use of the term. Just playing devil's advocate…" Wagner A. Vendrame

"Your question is a matter of opinion more than definition. If you want to be strict you are right. I would opt for a broader use." Joseph Arditti (Professor of Biology Emeritus, University of California – Irvine, USA)

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IN MEMORIAM

ASPECTS FROM THE BOTANICAL OPERA OF VALERIU ZANOSCHI (1934–1996)

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Abstract: This article contains a brief overview of the main aspects of the botanical opera of the Professor Valeriu Zanoschi, as well as a list of scientific papers and books published by him during the 38 years of activity in the service of the Romanian botany.

We marked this year the 80th anniversary of the birth and 18 years after the death of Professor Valeriu Zanoschi, a leading figure of his generation of Romanian botanists, an enthusiast of his work, to which he dedicated with all the power of his being.

All those who knew him personally, certainly remember his passion for the study of botany, his exigency with respect to itself, students or collaborators, his critical spirit, his sober and right character, communicative, even jovial sometimes, his admiration for the well done work, as well as his attachment to all those he appreciated.

His scientific activity carried out over a period of 38 years. Firstly (1958-1960) he acted as a researcher at the *Biological and Geographical Research Station "Stejarul" Pângărați*, later on (1960-1996) as a teacher at the discipline of Botany, in the *Agronomic Institute of Iaşi* (currently the *University of Agricultural Sciences and Veterinary Medicine*). His work is very impressive, both by the diversity of research areas (e.g. algology, mycology, bryology, ecology, plant physiology, history of botany, plant cytology and morpho-anatomy, medicinal herbs, toxicology, environmental protection, plant taxonomy, vascular flora and vegetation, segetal and ruderal weeds) and the valuable results published in numerous articles, books, treatises, monographs (see the list of scientific papers and books published, at the end of this paper).

One of the areas in which he obtained the most impressive results was that of **algology**. The algological studies have been conducted mainly in Eastern Romania (on Bistrița River and its tributaries; the meadow of Bahnei from Dersca and Lozna, Botoşani county; Bahna Mare-Bălăneşti, Neamț county; the Roşu Lake; the Ceahlău Mountain etc.), but also in other regions of the country (Bâlea Lake, the eutrophic marshes from the Bilborului Basin, Oltenia etc.). As a result, he discovered and published, in collaboration, a new species to science, namely *Cymbella bistritzae* Oltean & Zanoschi 1963, to which is added the identification of other 29 new taxa for the algoflora of Romania. Through this results, professor Zanoschi imposed himself among the Romanian algologists, as a senior specialist, which is why he was tasked with the monographic description of the subclass *Pennatibacillariophycidae* (from the phylum *Bacillariophyta*, class *Bacillariophyceae*), and

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the phylum *Dinophyta* (classification, characteristics of orders and main families and genera), in the 4th volume of the *Treaty of algology* (1981), edited under the direction of Şt. Peterfi and Al. Ionescu, a contribution for which he was awarded by the prize of the Romanian Academy. In addition, by studying over 1000 articles on algae in Romania, which were published until 1987, he prepared a *Conspectus of the algae of Romania*, a work of about 400 pages, unfortunately still in manuscript.

In **mycology**, he published important aspects on the systematic of wine "microflora", that were included in some treaties of oenology, as a base for classification of microorganisms involved in the production and storage of wine. In addition, a series of studies on fungi, in Ceahlău or Humosu scientific reserve, resulted in the discovery and publication (in collaboration) of 5 species of micromycetes new for Romania (i.e. *Metasphaeria isariphora* (Desm.) Johans., *Metasphaeria thalictri* Sacc., *Septoria bupleuri* Desm., *Septoria cardui* Tassi, *Leptostromella juncina* (Fr.) Sacc.), as well as other dozens of fungal species, new for the investigated areas.

Remarkable results he also published, alone or in collaboration, in the fields of **the vascular flora and vegetation** of Romania. For instance, his floristic research on vascular plants led to the publication of 5 new species for the Romanian flora (i.e. *Ambrosia trifida* L., *Capsella rubella* (L.) Reuter, *Genista sessilifolia* DC. – as *G. trifoliata* Janka, *Phleum subulatum* (Savi) Asch. & Graebn., *Pulsatilla slavica* (G. Reuss) Zamels, the latter, however, not confirmed subsequently); other 6 taxa were newly identified in Moldova and Dobrogea (i.e. *Ambrosia artemisiifolia* L., *Bupleurum apiculatum* Friv., *Lappula heteracantha* (Ledeb.) Borbás, *Lathyrus sphaericus* Retz., *Lithospermum glandulosum* Velen., *Veronica anagalloides* Guss.); over 64 rare taxa were also identified in the flora of the same two geographical provinces. As for the vegetation of Romania, the studies were carried out in the lower basin of the Jijia River, on the Siret floodplain, on the sandy Plain of Tecuci, on the valley of the Şomuz River, in the surroundings of Mărăşeşti town, various localities of the Botoşani county, but especially in the territory of the Ceahlău Massif. This research led to the description of two syntaxa, new to science (i.e. *Dryadetum octopetalae* Csűrös et al. 1956 *salicetosum retusae* Zanoschi 1972; *Brassicetum nigrae* Zanoschi, Turenschi, Viţălariu 1977), as well as the publication of a large number of other syntaxa, new or rare for the eastern part of Romania.

One of the leading contributions of this botanist to the study of the flora and vegetation of Romania is his PhD thesis entitled "*Flora şi vegetaţia masivului Ceahlău*" ("*Flora and vegetation of the Ceahlău Massif*") (University "Babeş-Bolyai" of Cluj, 1971, scientific coordinator: Professor E. Ghişa). The field research for the preparation of this thesis reached out over a period of 12 years (1958-1970). Although the flora of this emblematic mountain massive of the Romanian Carpathians has been studied with great interest by many illustrious naturalists, since the first half of the nineteenth century, beginning with I. Edel and J. Szabo (1835), continuing with V. Janka, D. Brândză, D. Grecescu, F. Pax, I. Römer, C. Petrescu and many others, however, the diligent botanist from Iaşi discovered a number of 341 new taxonomic units in the flora of this massive, of which: 83 species of algae, 80 species of fungi, 5 species of mosses, 105 species of vascular plants and 68 intraspecific taxa of algae, fungi and vascular plants. As for the vegetation of Ceahlău, his contribution was more substantial, identifying and describing, for the first time in this massive, a number of 39 plant associations, compared to only 5 associations (with a phytosociological table) previously reported in the literature. By all this, the monography "*Flora and vegetation of the Ceahlău Massif*" remains a landmark worth following, although difficult to achieve, of this kind of research, even on the national level. The results of the floristic research in Ceahlău were used (posthumous) in two synthesis books

published under the direction of PhD Al. Manoliu: "*Plante inferioare din Masivul Ceahlău*: *alge, ciuperci, muşchi*" ("*The inferior plants of Ceahlău Massif*: *algae, fungi, mosses*") (1998) and "*Flora Masivului Ceahlău*" ("*The flora of the Ceahlău Massif*") (2002). Much of the phytosociological research remained included, unfortunately, only in his PhD thesis, which is difficult to be consulted (due to the reduced number of copies held by few libraries and, therefore, little known).

The studies on **vascular weeds of crops**, carried out by V. Zanoschi, are not less important. Some studies of this type (1974-1989) were completed in the form of research reports and maps, carried out on a unitary methodology, developed at the national level, results which, unfortunately, still are waiting to be used by publishing some syntheses on the segetal flora of Romania. Other studies were published as articles, brochures and books, of which one especially stands out, namely "*Buruienile din culturile agricole şi bolile lor*" ("*The weeds in crops and their diseases*") (1996), a book published in collaboration, only a few days before the disappearance of the professor. This book is of a high utility for developing and implementing strategies for biological control of weeds in crops.

Also, the Professor Zanoschi had a significant contribution to the study of **toxic and medicinal plants**, by publishing (in collaboration) several contributions to the knowledge of these plants, from various regions of the country (the Ceahlău Massif, the upper basin of the Jijia River, the basin of the Crasna River etc.). The book "*Plante toxice din România*" ("*Toxic Plants in Romania*"), published in collaboration with E. Turenschi, and M. Toma (1981), remains a unique synthesis in our botanical literature, very useful for specialists in pharmacognosy, toxicology, and for the general public, eager to know the plants that could endanger human health or the animals bred by man.

Being a teacher at the Agronomic Institute of Iasi, V. Zanoschi was also preoccupied in the study of cultivated plants. In this area, the monographic book "*Morfologia şi anatomia plantelor cultivate*" ("*The morphology and anatomy of cultivated plants*"), authors V. Zanoschi & C. Toma (1985), is worth mentioning. At the time of publication, this book, containing detailed morpho-anatomical descriptions of a number of 46 species of cultivated plants, filled a gap in the literature, not only nationally but also internationally.

One of the most important projects of V. Zanoschi was developing a "*spontaneous and cultivated woody flora of Romania*". Unfortunately, although he dedicated his entire working power of this project, a ruthless illness killed him soon, not gave him the respite to publish than the first volume of this work, further publication of the other 3 volumes remaining in the responsibility of his close collaborators (I. Sârbu and A. Toniuc). In this monography, there are described and drawn a number of 2491 known species of woody plants of the spontaneous and cultivated flora of our country, from 253 genera and 81 families.

Other scientific publications of V. Zanoschi have addressed various issues such as: the knowledge and protection of rare plants, plant reproduction, adaptation to the environment in the plant world, the dynamics of physico-chemical properties of water of the Bistrița River, ecology and plant physiology, interspecific relationships ("sympathies and antipathies in the world of plants"), the history of the Romanian botany etc. His contribution to the drafting and publication of many courses and practical guidances for students is added to all these.

These are, therefore, in short, some aspects of the botanic opera of V. Zanoschi. Those willing will be able to enjoy more with the wonderful fruits of a life entirely devoted to botany, by reading the articles and books on which this diligent and competent botanist has left to posterity (see the list below, in the alphabetical order of the first author).

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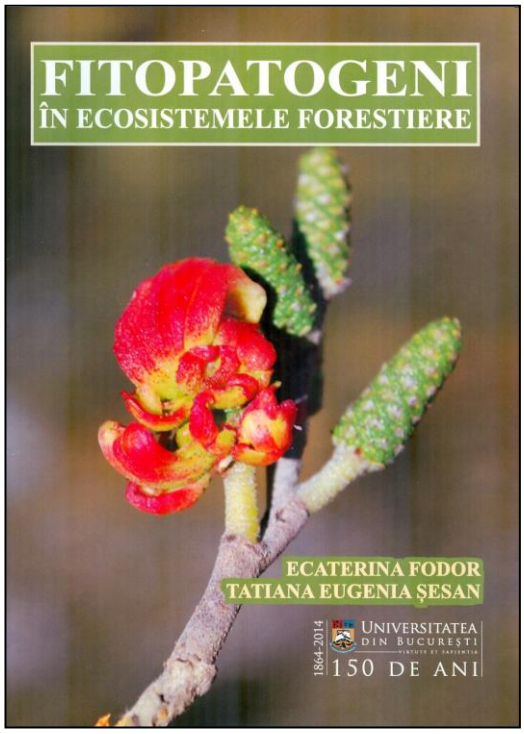
BOOK REVIEW

ECATERINA FODOR, TATIANA-EUGENIA ŞESAN, ***Fitopatogeni în ecosistemele forestiere*** [*Phytopathogens in forest ecosystems*], 2014, the University of Bucharest Publishing, 647 p., ISBN 978-606-16-0447-0.



Knowing the phytopathogens with forestry importance presents a major interest not only for the specialists activating in the field of the forestry sciences but also for those who love the nature, generally, and particularly for those interested in the plants health and protection. The study of the phytopathogens from the forest ecosystems requires combining vast knowledge from fields such as environment protection, biology and ecology, and thus, this study is bringing valuable additions to the romanian literature in the field.

The authors are teaching in the University, Ecaterina FODOR, associate Professor at the Faculty of Environment Protection, University of Oradea and Tatiana Eugenia ŞESAN is Professor at the Faculty of Biology, University of Bucharest, corresponding member of the Academy of Forestry and Agricultural Sciences from Romania. The vast experience gained over the years while activating in the University, teaching and researching, allowed them to combine the information selected form the scientific literature with numerous results of their own.

The volume *Phytopathogens in forest ecosystems* is structured in 2 parts: first part – ***The Study of the trees diseases agents*** – that is treating general aspects referring to the

agents producing diseases on the plants, which is presented throughout 6 chapters, aiming: the synthesis of the most important data from phytopathology, focalized especially on forest field; aspects that refer to the dynamic of the phytopathogens' populations; information on the preventing, controlling and fighting the phytopathogens agents from the forest ecosystems; features of nomenclature, classification and the main groups of phytopathogens (viruses, mycoplasma, bacteria, fungi) with representatives of the forest ecosystems; description of the most wide-spread diseases from the forest ecosystems in Romania, as their economic importance as well.

The second part – ***Applied mycology and phytopathology*** – is treating throughout 4 chapters the aspects that aim the main work methods for study in the field of mycology and forest phytopathology, techniques of microscopy for the analysis of the phytopathogens, as well as the main groups of pathogen agents from the forest ecosystems.

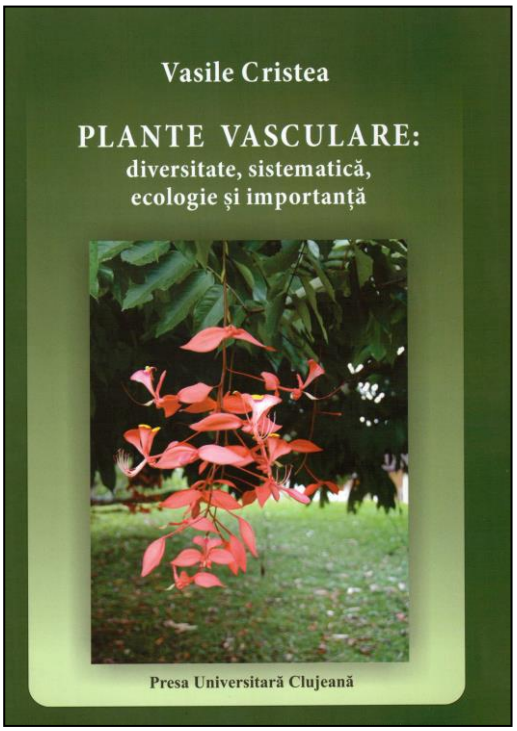
The volume has 647 pages and includes one extremely valuable Glossary, containing 1571 explained specialty terms, constituting a guide for both those less initiated and for the professionals in the field. The 306 images (pictures, drawings, diagrams – sometimes grouped as a plate) illustrate efficiently the information presented in this book, which is vast and well documented, referring to 1053 bibliographic titles, both romanian and foreign, and 50 web sites.

The book was presented in the XXth edition of the Scientific Communications Session *"Dimitrie Brândză"*, organized by the Botanical Garden of the University of Bucharest, on 8 November 2014, with the occasion of Mrs. Professor Tatiana Eugenia ŞESAN's celebration of her 70th anniversary. The volume is dedicated to the professors of the authors and to the University of Bucharest' Anniversary, at the celebration of 150 years from the foundation.

The work is very useful for the specialists in the field, students, master and PhD students who study biology, ecology, forestry and environment protection but also for those interested in other related fields.

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

Vasile CRISTEA, ***PLANTE VASCULARE: diversitate, sistematică, ecologie şi importanță*** [*VASCULAR PLANTS: diversity, systematic, ecology and importance*], 2014, Edit. Presa Universitară Clujeană, 575 p., ISBN 978-973-595-648-6.



Vascular plants: diversity, systematic, ecology and importance is a fascinating book exploring the botany science, aims of its study, the general features of the numerous groups of plant species, the diversity of vascular plants and their classification systems, their main evolutionary paths and also their ecology and practical importance. It is one of the most complex and actualized woks in domain of vegetal biology in Romania, in which, the author, a prominent researcher and professor of systematic botany, phytosociology and plant ecology, presents his opinion on the above mentioned aspects.

In the first chapter there is presented a short classification of the living organisms alongside the newest approaches focused on their classification: cladistics, phenetics and phylogeograpgy. Chapter II includes a general characterization of the Cormobionta subregnum and aspects related to the nomenclature, origin, evolution and classification of cormobiont species. In the third chapter the author describes the general characteristics of ferns (*sensu lato*), their evolution and classification alongside the importance in the subsequent evolution of the plant species and also their practical importance. The gymnosperms and angiosperms are presented in the next two chapters where there are highlighted the various hypotheses on their origin and evolution alongside explanation on

their classification in supra or infra-specific taxa. Very comprehensive knowledge is also provided on their practical importance.

The book is written in a clear and concise academic style, illustrated with numerous photographies and drawings and is based on a comprehensive literature (over 550 titles). Its high quality contents drew attention of numerous plant biologists and the book was presented within the Scientific Communications Session *"Biodiversity conservation in the context of sustainable development"*, organized by the Faculty of Biology of the "Alexandru Ioan Cuza" University of Iaşi, 23-25 October 2014, by the Senior Researcher PhD Ion Sârbu.

The contents and presentation form recommend the book *Vascular plants: diversity, systematic, ecology and importance* as a reliable information source for students (including MSc or PhD students), as it presents both theoretical and practical aspects and the description of the plants species includes original contributions concerning the biodiversity, chorology, ecology and specific habitats of occurrence. It is also very useful to all plant biology specialists, and in the same extent, too all the people fascinated by nature.

Constantin MARDARI, Cătălin TĂNASE
"Anastasie Fătu" Botanical Garden, "Alexandru Ioan Cuza" University of Iaşi

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The scientific names of taxa would be italicized.

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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:

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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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