VITROCULTURES OF *DROSERA ROTUNDIFOLIA* L. PRESERVED IN SUCEAVA GENE BANK

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ABSTRACT. We monitored the evolution of *Drosera rotundifolia* L. vitroplantules regenerated from propaguls, consisting of leafs rosettes, inoculated in fresh growth media that we produced. The phytoinoculs of *D. rotundifolia*, maintained in a "slow growth" conditions, either on Murashige – Skoog (1962) basic media, supplemented with manithol 30g/l or using daminozyde (B9) 30mg/l, kept their viability to subcultures, regenerating new plantules, and the colonies directly transferred to a septic media, in casolettes, being planted in an non sterile substrate made of flower soil and black peat managed to survive. Macroscopic investigations carried out on stereomicroscope as well as those on the scanning electron microscope favoured our encompassing of the regeneration phenomenon produced at the level of the superior epidermis of some leaf lamina of the microrosettes of new leafs, especially those situated at the extremities of the propaguls colony.

Keywords: *Drosera rotundifolia*, preserved vitroculture, propaguls

INTRODUCTION

Carnivorous plants, described since 1875 by the great Darwin as insectivorous plants, can be told to possess "bizarre" morphophysiological and metabolic adaptations, if we take into account some of their organs structured into "traps", i.e. elements meant to "capture", immobilise, digest, and eventually absorb, various chemical compounds – particularly amino acids – produced by the hydrolysis of proteins which compose the small living creatures (insects, molluscs, arachnids, etc.), captured by the leafs metamorphosed into "traps".

Carnivorous plants are preponderantly autotrophic, photosynthetic organisms. The lack of mineral nitrogen of the substrate on which they grow made them orient their metabolism towards "carnivory"; thus, they manage to complete their nitrogen necessities, an indispensable element for the synthesis of nitrogen organic compounds, particularly proteins, molecules which are necessary in order to "build" cellular biostructures.

As we know, "functions change the organ", and the propensity of the metabolism for carnivory in these plants exhibiting such mixotrophic nutrition determined the emergence of a metamorphosis which mainly takes place at a foliar level.

Generally, carnivorous plants grow on humid media.

From the over 535 species of carnivorous plants known to date, we focused on the *D. rotundifolia* (Sundew), a plant which populates the peat areas in our country, an ecological environment characterised by acid pH, low in nitrogen salts (Stănescu, 2008).

It is only through "carnivory" that this kind of plants can secure the integrity of their biological cycle, and by such adaptations not only they enhance their chance of survival, but also favour flower and fertile pollen formation, as well as that of seeds with a high germination capacity.

We should not overlook the fact that vitrocultures of *Drosera* are currently particularly interesting, not only for the use of leaf propaguls as vegetal models, in various investigations into plants' physiology, as initial material for subcultures – with demonstrative purposes (for practical approach with students, master and PhD candidates), for the discipline of vegetal biotechnology, but also for biomass production used in phytoterapy, as a source of secondary metabolic products, because the vegetative, supraterrestrial mass, is capitalized in phytopharmacy in the production of *Drosera herba* (Stănescu, 2008), as it contains a series of secondary metabolic products, with a naphtoquinonic structure such as: plumbagin, rossoliside, carboxy-naphtoquinone, various organic acids (malic, benzoic, citric, galic and ascorbic), flavones (quercetol), as well as some proteolytic enzymes.

According to Grigorescu et al. (1986), *Drosera* products help treating asthma and the fluid extract of *Drosera,* in homeopathic dosage, is used as an antitusive (especially in spastic cough); while the alcoholic (ethylic) extract of *Drosera* is used for the preparation of various pharmaceutical formulae and the extracts or tea of *Drosera* leafs are effective in the treatment of tuberculosis, arteriosclerosis, hepatic, gastric and intestinal diseases, as well as for the therapy of various ophthalmologic affections and ear-

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related problems, for syphilis, toothache, etc. (Pietropaolo and Pietropaolo, 1997).

All the above mentioned fully explain the interest shown by some vegetal biotechnology specialists in *Drosera* vitrocultures, as this technique gives the possibility of a constant provision of a fixed quantity of biomass, used as raw material in the extraction of secondary metabolic products, characteristic for this vegetal species and which can be used in the preparation of phytoproducts used in phytotherapy.

In the case of *Drosera,* the metamorphosis undergone by the leaflets consists in the development on the surface of the lamina of numerous tentacular, prehensile hairs, sensitive to chemical and physical stimuli. For instance, when they are touched by an insect, the tentacular hairs immobilise the "prey". Short after, the secretion of proteolytic enzymes begins, generated by the cells on this type of hairs, which eventually kills and favours the digestion of the captured organism, and, in the end, the absorption processes of the degradation compounds takes place. Gradually, the tentacles go back to their normal morphofunctional state, reassuming the vertical position, the lamina becoming flat again. An adult leaf may function as described above (at least in the case of *Drosera*) approximately 3 or 4 times.

As part of our preoccupations for vegetal biotechnology, we decided to extend our research not only in the direction of initiating *D. rotundifolia* vitrocultures (Cachiţă et al., 1991), but also of preserving this type of phytoinoculs in a "slow growth" rhythm, in acclimatised rooms, available at Suceava Gene Bank.

After initiating the *Drosera* vitrocultures, these were kept in the collection of multiple laboratories in our country, by periodically operating subcultures, at approximately 3-4 months intervals, their pricking out and subcultivation consisting in the separation of leafs colonies regenerated from an initial propagul (which consisted of a minirosette of leafs, with or without rhisogenesis as background), on basic agarised media, mainly Murashige – Skoog (1962), which were fresh and without growth regulators.

In 2003, we chose to perform a *Drosera* vitroculture at Suceava Gene Bank, under the form of "leaving collection". In order to do that, the *Drosera* propaguls were subcultivated on growth media adequate for the preservation – for a long period of time – of the vitality of the respective vitrocultures, without operating subcultures for 3 or 4 years. That is why we conceived special culture media (Table 1), using as a basis inorganic compound the Murashige – Skoog (1962) media.

Apart from the objective stated before, we also intended to study the behaviour of the *Drosera* phytoinoculs preserved at Suceava Gene Bank, in the conditions of their subcultivation on fresh media. Our previously performed investigations, of optical and electron microscopy, at the level of *Drosera* vitroplantules will make the object of further publications.

In this study, we will disclose some macroscopic and microscopic aspects noticed at the leafs of *D. rotundifolia* vitroplantules, resulted from vitrocultures which were preserved in an acclimatised chamber (fig. 1) for 4 or 3 years, without operating subcultures, as well as after operating a first postsubculture, or at the moment of acclimatisation, of some *Drosera* colonies, which, when the first experiment was dismantled, were transferred directly in the septic media of life, in plastic casolettes, in order to become acclimatised to the greenhouse environment conditions.

MATERIAL AND METHODS

In specialised literature (Cachiţă et al., 1991), the commonest preservation procedures – short - term – vegetal vitrocultures, consist in the preservation of the flasks containing phytoinoculs on shelves, in acclimatised rooms, adjusted from the point of view of the temperature either to a regular environmental temperature or to low, but positive temperatures, between 4 and 7^0 C, situation which characterized our experiment.

In order to stock the *Drosera* inoculs in Suceava Gene Bank, for the unaltered preservation both of their characteristic genome and their regenerative capacity (until cultures are used as head clones in propagation procedures), the propaguls were cultivated on special media that we produced, as follows: mineral medium MS ½; classic organic compounds, but with vitamins, sugar and the agar in modified concentrations, as stated in table 1.

In order to temporize the growth of *Drosera* vitrocultures, in the basic media we provided, we either added 30g/l *manithol* (subcultures performed in 2003, P - 21 variant), or substituted manithol with *daminozide* (Alar or B9, P - 22 variant), in a 30 mg/l concentration (subcultures performed in 2004). Finally, prior to autoclavation, the pH of the media was fixed to a 5.6 value. Vitrocultures were carried out in glass pots, 10cm high and having a 4cm diameter.

The sterilisation of the culture media was accomplished through the autoclavation of the flasks under a 1atm pressure for, 30 minutes.

Inoculations were performed under the vapour hood with laminar flux of sterile air, and the retention of the flasks – postinoculation – was performed by means of polyethylene foil, clamped with elastic rings.

The regime in the growth chamber corresponded to 14 hours of darkness, and a $6\text{-}7^0$ C temperature, alternating with 10 hours light, a $6\text{-}8^0$ C temperature and a 1000 – 1200lx lighting.

In vitrocultures where *Drosera* propaguls were inoculated in 2003 (P - 21 variant) the examination of the phytoinoculs was performed after 4 years and 3 months from their disposition in the stocking chamber, while for vitrocultures performed in 2004 (P - 22 variant), the final remarks were made 3 years and three months after the subcultivation of propaguls.

The most relevant image taken during this experiment are presented in figures 2 A-E and 3 A-E.

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Table 1

Variation of culture (growth) media, with inorganic compounds, structured according to the Murashige – Skoog (1962) basic recipe, and organic compounds elaborate by us for preservation in a "slow growth" regime of the *Drosera rotundifolia* L. vitrocultures

Organic compounds of varied nature (in mg/l)	Variations 2003 D 21	2004 D 22
Organic (mg/l)		
Myo-Inositol	100	100
Tiamine HCl	0.2	0.2
Pyridoxine HCl	0.2	0.2
Nicotinic acid	0.2	0.2
Glycine	0.2	0.2
Sugar	20	20
Agar-agar (g/l)	7	7
Growth Reg. (mg/l)		
K	0,01	0.01
BA	0.01	0.01
NAA	0.01	0.01
Daminozide (B_9)	-	**30**
Manithol (g/l)	**30**	-

Note: K – quinetine, BA – benziladenine, NAA –á naphtylacetic acid

Fig. 1 The aspect of a room in Suceava Gene Ban phytoinocul preservation

At the moment of dismantling the experiments, attachments of leafs were performed, especially those of foliar lamina, in order to examine them through scanning electron microscopy (Cachiţă et al., 2006), the most representative pictures being illustrated in figure 3 A and C.

A part of the *Drosera* vitrocultures, postpreserved for 3-4 years in the chamber destined for the collection of phytoinoculs, in a "slow growth" regime, were subcultivated on MS media (according to the original recipe) but devoid of growth regulators, *manithol* or *B 9*. In this case, sugar concentration in the media was increased to 30 mg/l, and the agar-agar in the media had a 7g/l concentration; the pH of the media was adjusted - prior to autoclavation - to a 5.6 value. The containers sterilizing, as in the previous experiment, was performed through autoclavation. In this experiment, culture containers consisted in 10cm high flasks, having a 2.5 cm basal diameter; obtained cultures were transferred on the shelves situated in a growth chamber where illumination corresponded to 16h light/8h dark (white fluorescent light was provided at 2500lx lighting); during all period of growth, temperature was constant, 24ºC.

The aspect of *Drosera* vitrocultures, performed as a result of the subcultivation of propagul clusters, created from the phytoinoculs preserved, each consisting of several minirosettes, one month after the operation of subcultures, may be noticed in figure 4 A and B.

A part of the *Drosera* colonies, presenting incipient roots, were retired from the P - 22 media and transplanted in plastic casolettes, on a mixed substrate made of flower soil and black peat. On a daily basis, the substrate was slightly watered using distilled water. Cultures were kept as such for several weeks (fig. 5 A and B).

RESULTS AND DISCUSSIONS

It was most rewarding that the *Drosera* vitroplantules kept in the preservation chamber of the MB – MS media phytoinoculs, with 30g/l manithol added (P 21 variant – experiments performed in 2003) survived in excellent conditions, until they were dismantled, 4 years and 3 months after their inoculation in the slow growth regime, without subcultivation needed, the environmental temperature being 4º-7ºC. As shown in figure 2 A and C, the *Drosera* rosette, generated by the initial propagul, was unusually dense, being made up of numerous minuscule leaflets, conglomerated in some sort of cluster, the lamina of the central leaflets being short petiolated, exhibiting only the curved superior parts of the lamina that will become the inferior part of the leafs; the leaflets at the edge of the central cluster presenting stretched leaf lamina. However, in the case of some rosettes, out of the cluster of propaguls small thick floriferous stems sprang up (fig. 2 B and C), presenting in their apical area an atypical inflorescence with hypertrophied rachis and flowers (fig. 2 C), abnormally shaped, different from the floriferous stem and miniature inflorescences, but normally formed, which developed in the vitrorosettes colony of the *Drosera* vitrocultivated for three years and three months, in the exact same conditions of light and temperature, but on MB – MS media, with 30mg/l *B9* added (fig. 2 D and E).

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Even though for the P - 21 variant (with *manithol*) the colonies of rosettes presented little roots, the survival capacity of *ex vitro* plantules was reduced, exhibiting necrosis after the first week of *ex vitro* culture, whereas the "bushes" subcultivated on fresh aseptic media (fig. 4A) managed to survive, and one month after the transfer they were green, lively and started to show off a part of their leaf lamina, without exhibiting evident processes of colony multiplication with new rosettes. It is interesting that, although *in vitro*, a small number of the peripheral leaflets, especially in *Drosera* vitrocultures grown on *manithol* media (P - 21 variant), presented neogenesis of microrosettes, an evident phenomenon at the level of the superior epidermis of their minuscule lamina (fig. 3 A and E). Eventually, the leaflets which formed new rosettes suffered necrosis. 3-4 days after the *ex vitro* plantation of the new microrosettes, although presenting a little root, they also suffered necrosis.

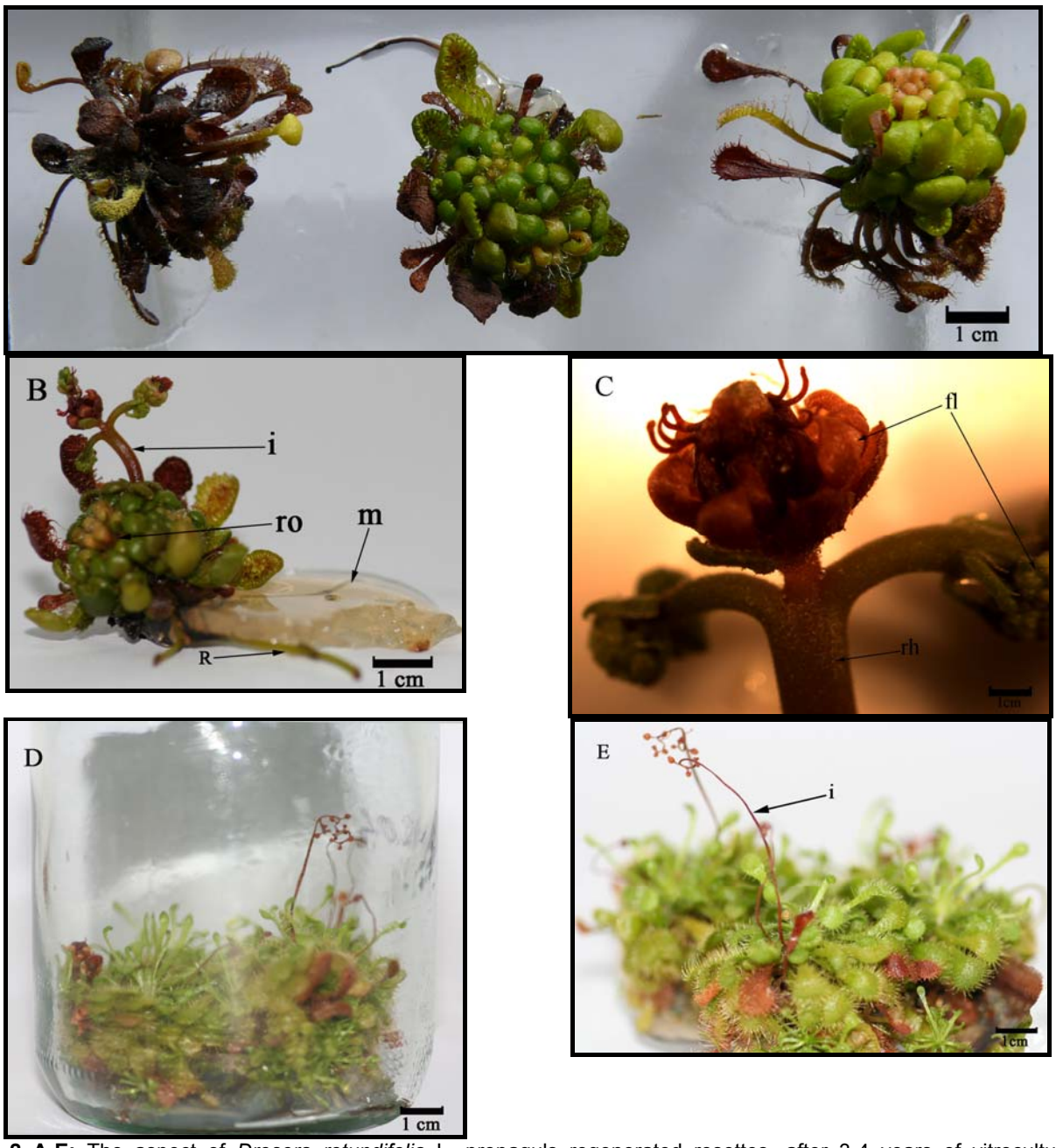

Fig. 2 A-E: The aspect of *Drosera rotundifolia* L. propaguls regenerated rosettes, after 3-4 years of vitrocultures preservation in Suceava Gene Bank, without subcultures operation. **A-C:** vitrocultures cultivated on Murashige-Skoog (1962) media with 30g/l *manithol* added; i – inflorescence; ro – minileaflets rosette; rh – rachis; **D-E:** rosette vitrocultures generated on Murashige – Skoog (1962) *daminozide* 30 mg/l (B 9) added. **D.** cultures *in vitro* culture container; **E.** *ex vitro* cultures, in the marked area one can distinguish a unique minirosette at the edge of the culture

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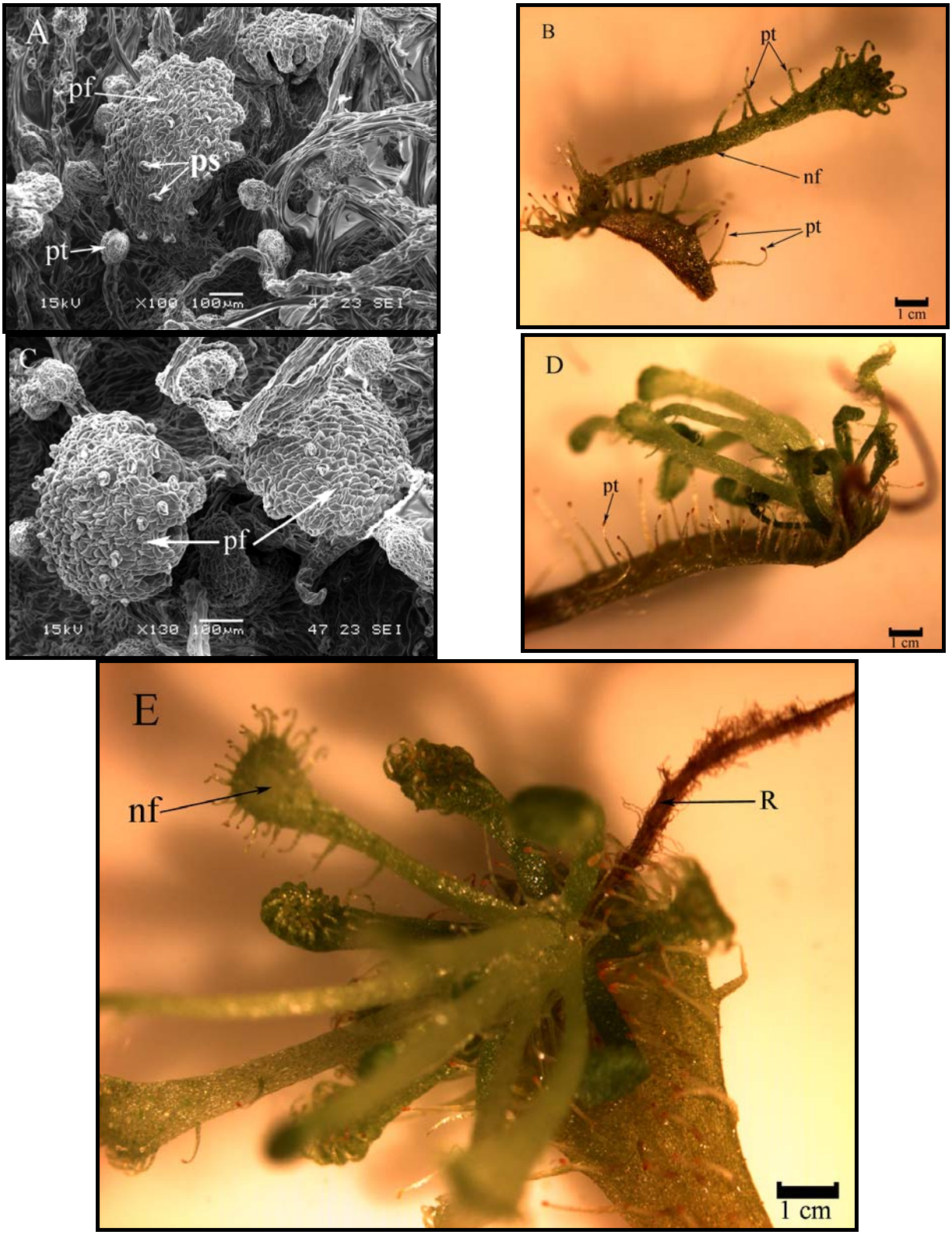

Fig. 3 A-E: Microscopic images, (A- C) of scanning electron microscopy, performed at the level of the superior epidermis of vitroleafs of *Drosera rotundifolia* L., (where: pf –primordials foil; ps –secretive sessile hairs; pt – tentacle secretive hairs); **B-E:** photographs on the regeneration of neomicrorosettes at the level of the lamina of a minileaflet (R – new little root; nf – neoleaflet; pt – tector hairs)

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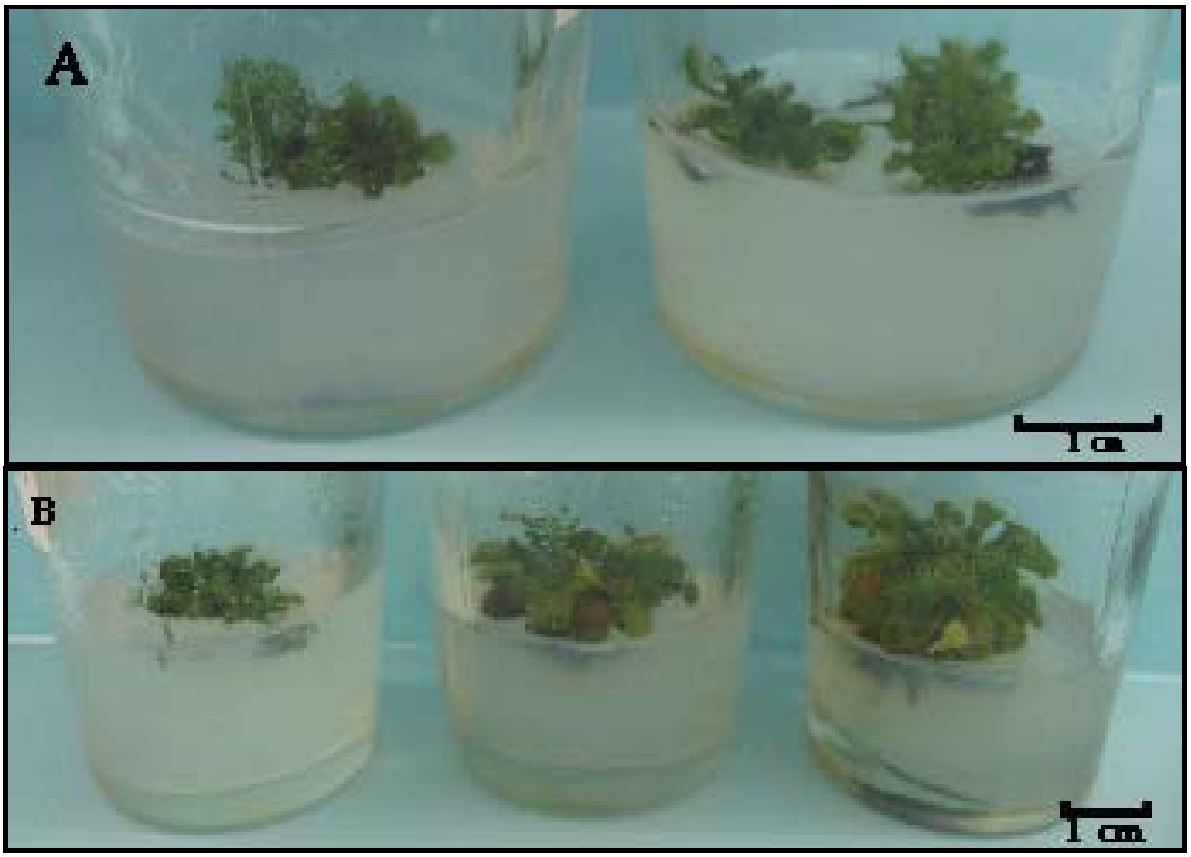

Fig. 4 The aspect of *Drosera rotundifolia* L. vitrocultures, performed after the picking out and subcultivation of the minirosette propaguls on fresh *Murashige – Skoog* (1962) media after their stocking in Suceava Gene Bank for four years and three months (A), on basic media with 30 g/l *manithol* added, or for three years and three months (B) on media with 30mg/l *daminozide* added instead of *manithol*

Fig. 5 The aspect of the rosettes of the *Drosera rotundifolia* L. plantules, two weeks after their plantation in unsterile substrate, transferred *ex vitro* after their preservation in Suceava Gene Bank for three years and three months, where: **A**. mixture of flower soil with black peat; **B.** detail of a macro- (a) and minirosette (b) regenerated from the upper epidermis of vitroleaflets (process which is illustrated in fig. 3 A-E)

Drosera vitrocultures, preserved in a "slow growth" regime for three years and three months, having as a substrate an MB – MS medium, with 30mg/l *B 9* added, grew very well, the propaguls colony newly formed at the level of the initiating inoculum for the subculture occupying the entire surface of the culture media (fig. 2 D and E). The leaflet rosettes were completely deployed and their leaf lamina exhibited dense tentacle hairs and well-developed roots. This type of rosettes colonies allowed their dismantling into fragments, which under *in vitro* subcultivation, on media frequently used for subculture (without *manithol, B 9* and growth regulators added), one month after their pricking out on fresh media, being transferred to a normal (optimum) regime of temperature, they presented a good evolution, they were green and particularly lively (fig. 4 B).

The sample of *Drosera ex vitro* plantules, transferred in a septic environment for their acclimatisation to normal conditions of life, had a good evolution and survived (fig. 5 A and B).

As a result of our research within these experiments, there are some particularly interesting aspects that we would like to emphasise. Macroscopic examinations carried out using a stereomicroscope and the scanning electron microscope (fig. 3 A and C) permitted our grasping of the regeneration phenomenon produced at the level of superior

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epidermis of some of the leaf lamina of microrosettes' neoleaflets, especially of those situated at the extremities of the propaguls colony (fig. 2 E). Such a process was also described by Kawiak et al. (2003) for another two species of *Drosera* vitrocultivated, respectively *D. binata* and *D. cuneifolia.* This regeneration process, occurred at the level of the leaf lamina, under a vitroculture regime, is probably present in nature as well, but we could not identify it.

Scanning electron microscope examinations revealed some incipient phases of the formation of the first (fig. 3 A) and second (fig. 3 C) leaflets, which generated a new minirosette. The leaflets we are referring to can be considered as leaf leaf primordia. In such a growth phase, leaflets do not exhibit a petiole, but their lower surface (which will become the inferior, abaxial or ventral surface of the lamina) is already endowed with *glandular sessile* hairs clearly noticeable on the surface of the epidermis. The existence of these hairs on the surface of the leaf primordials raises new questions concerning the potential role of this kind of hairs in the biology of the leaf, of the plants, respectively of the *Drosera* vitroplantules. As the regeneration process advances, the number of unformed *Drosera* microrosettes on the surface of the lamina grows, on the ventral side of the 'mother' leaf, at the level of the neorosettes a rhisogenesis process being initiated. Such a leaf functions as a leaf mini-seedling. Finally, the *Drosera* colonies become autonomous and can be used as propagul.

This phenomenon is extremely interesting and spectacular. The vitrocultures that we performed favoured the identification of a new side of vegetative reproduction for this species in the particular case of *D. rotundifolia*.

CONCLUSIONS

The phytoinoculs of *Drosera rotundifolia* L., maintained in a "slow growth" either on Murashige – Skoog (1962) basic media, supplemented with manithol 30g/l, or using daminozyde (B9) 30mg/l, kept their viability to subcultures, regenerating new plantules, and the colonies directly transferred to a septic media, in casolettes, being planted in an non sterile substrate made of flower soil and black peat managed to survive.

Macroscopic investigations carried out on stereomicroscope as well as those on the scanning electron microscope favoured our encompassing of the regeneration phenomenon produced at the level of the superior epidermis of some leaf lamina of the microrosettes of new leafs, especially those situated at the extremities of the propaguls colony.

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