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Pre-treatment of *Dryopteris cristata* (L.) A. Gray rhizome as a method of elimination of contaminants and explant browning micropropagation

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Abstract: Micropropagation of *Dryopteris cristata* (L.) A. Gray by rhizome explants requires a pre-treatment procedure as a «stage 0». Mycological examinations of fungi from fern rhizomes allowed to determine 24 species represented by 66 isolates and a group non-sporulating colonies. Most frequently fungi were represented by *Cylindrocarpon destructans* and *Mycelium radicis atrovirens*. The pre-treatment procedure reduces exogenous and endogenous contamination by fungi, actinomycetes and other bacteria in developing adventitious buds. Starvation of rhizomes during pre-treatment significantly reduces starch and phenolic contents and thereby prevents browning of the explants. The present data revealed that in decontaminated, actively growing buds there are less phenolics than in contaminated rhizomes.

Key words: *Dryopteris cristata*, pre-treatment procedure, contaminants eradication, micropropagation by rhizome explants, phenolic compounds, tissue browning

1. Introduction

Micropropagation of ferns from old rhizomes constitutes a difficult task. Their poor regenerative capacity is related to: (1) aging, peculiar growth patterns and lack of secondary growth (Kshirsagar & Mehta 1978; Materi & Cumming 1991); (2) inhibitory influence of released phenolic compounds, and (3) bacterial and fungal contaminations (Zenkteler & Kwaśna 1994). All these obstacles are encountered while establishing fern rhizome explants *in vitro*. Usually about 85-90% of explants are contaminated or 60-70% turn brown soon after disinfection and transferring onto the medium. A pre-treatment permits not only to eliminate microorganisms but also to reduce the amount of phenols and starch in explants and their regenerants (Bhat & Chandel 1991; Debergh & Read 1991). Our previous studies on micropropagation of explants from long-creeping, thin fern rhizomes showed that Nystatin (mycostatic) inhibits fungal growth in buds (Zenkteler 1995, 1997). The aim of this study was to evaluate the effectiveness of the pre-treatment of short-creeping, stout type of fern rhizome, as a method producing valuable stock material for micropropagation of Buckler-fern.

2. Materials and methods

Dryopteris cristata occurs in marshes, fens and wet heaths, mainly in the areas covered with birch and hummocks of *Sphagnum* bog moss, and in the habitats of the transition zone between reed swamp and old fen carr. Some populations of *D. cristata* are endangered in habitats subjected to antropopression (Kozlowski *et al.* 2002). The species reproduces mainly vegetatively by branching of rhizomes, forming several closely grouped crowns (Page 1997). The dark brown rhizomes are distinguished by erect axes and erect stipes of the fronds. The branching pattern is initiated phylogenously by buds developing on the outside surface of phyllopodia. Rhizomes of *D. cristata* were collected between October 2001 and May 2002 in a waterlogged ash-alder forest on the shore of Lake Strzeszynek, near Poznań.

2.1. Pre-treatment procedure

Rhizomes without roots and leaves were thoroughly washed in tap water, next shaked for 24 h in a Nystatin suspension (150 mg· l^{-1}) to which 0.25 ml Tween 20 had been added. (Nystatin/Mycostatin SIGMA, no 9767, 4.800 units = 1mg). Rhizomes were divided into 5-8 segments, 5-10 cm long, placed on moist filter paper in the glass jars in semisterile conditions and incubated at 18-20^{0}C in dark for 2-3 months. Afterwards, the segments were grown in continous light (20 µmol· m^{-2}· s^{-1}). When the adventitious buds protruded, half of these buds were used for detection of the presence of fungi, and the remainder was again shaken in Nystatin suspension (150 mg· l^{-1}) for 24 h, then surface disinfected for 5 min in 0.2% $HgCl_2$ (m/v solution acidified with three drops of 1n HCl), rinsed 3-5 times in sterile distilled water and asepticaly placed on the modified MFMM – Murashige Fern Multiplication medium (Miller & Murashige 1976).

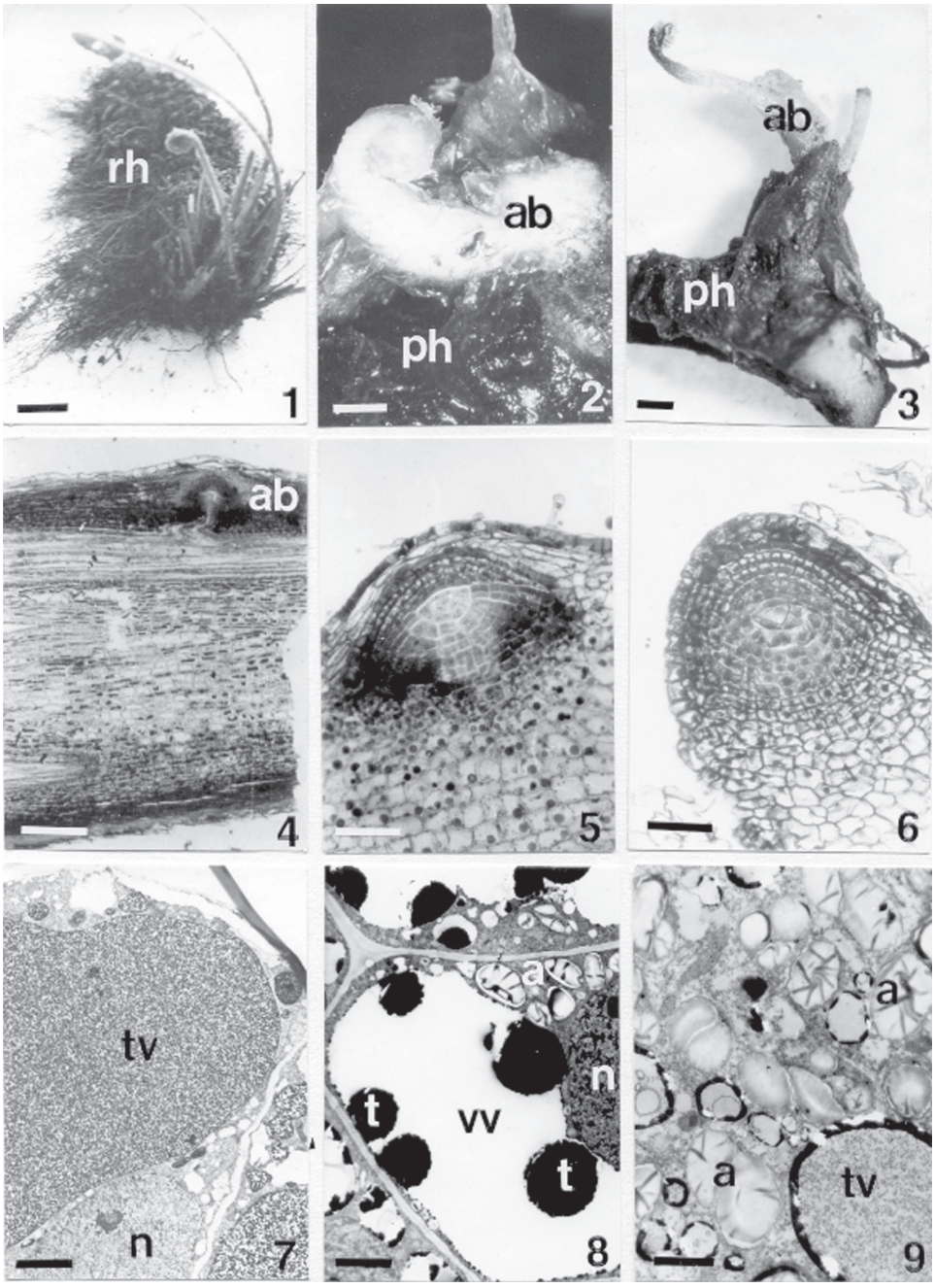

Fig. 1. Stage of rhizome *Dryopteris cristata* pre-treatment

Explanations: 1 – Rhizome (rh) before removing roots and stipes of leaves (Bar=5 cm); 2 – Crosier of first leaf of adventitious bud (ab) developed from the phyllopodium (ph); after 2 months of pre-treatment (Bar=1 mm); 3 – Adventitious bud (ab) in the stage of three leaves, (ph) – phyllopodium; 3 months of pre-treatment culture (Bar=1 mm); 4 – Longitudinal section of phyllopodium 1 month after initiation of pre-treatment (Bar=400 µm); 5-6 – Successive stages of a bud development (Bar=100 µm); 7 – Ultrathin section of a rhizome parenchyma cell (before pre-treatment) showing a large tannin vacuole (tv), nucleus (n) (Bar=2 µm); 8-9 – Ultrathin section of an adventitious bud (after pre-treatment) showing a vegetative vacuole (vv) with globular tannin deposites (t) and numerous amyloplasts (a) (Bar=2 µm and 4 µm)

2.2. Detection and identification of contaminants

Rhizome fragments before, and adventitious buds after pretreatments were surface disinfected with 70% ethanol (v/v solution) for 3 s and rinsed three times with sterile distilled water. Then they were dried on sterile filter paper and placed on potato dextrose agar (PDA) containing chlorotetracycline (50 mg· l^{-1}). Also explants with symptoms of microbial contamination from initiation stage of micropropagation were transferred from MFMM medium to PDA. After 7-10 days of incubation at room temperature, fungi were transferred to test tubes containing PDA, malt-agar or synthetic nutrient agar (SNA), and later identified by routine techniques (Domsch *et al*. 1980). All fungal sporulating isolates were classified to species level.

2.3. Electron microscopy analysis

Apical bud meristems and pieces of 1-3 mm in diameter of phyllopodia parenchyma were initially fixed in 2% glutaraldehyde in 0.05 M phosphate buffer at pH 6,8 for 3 h at room temperature and post-fixed overnight in 2% osmium tetroxide in a refrigerator. The tissues were dehydrated in a cold graded ethanol series and embedded in Spurr's epoxy resin (Spurr 1969). Ultrathin sections were cut on an ultramicrotome, post-stained with uranyl acetate and lead citrate, then analized and photographed under a transmission electron microscope (Jeol 100 C).

The resin slides were treated with 0.5% periodic acid for 15 min, washed in tap water for 10 min, placed in Schiff's reagent (PAS) reaction (Fischer 1968; Geier 1980) for 15 min and rinced in distilled water. Then slides were destained for 2 min in 2% potassium bisulphate solution and finally washed in tap water.

2.4. *In vitro* culture

After surface disinfection with 0.2% $HgCl_2$ (m/v solution) containing 2-3 drops of Tween 20 for 5 min, adventitious buds were aseptically excised and transferred into Erlenmeyer flasks, each containing 150 ml of the modified 1/2 MFMM medium (Miller and Murashige 1976). This medium consists of a half-strength solution of macro- and micronutrients, Fe EDTA and vitamins as formulated for ferns. Activated charcoal (250 mg· l^{-1}) as an absorbent of exudates and ascorbic acid (30 mg· l^{-1}) as an antioxidant were added to prevent tissue browning. Various modifications of MFMM medium (2.5; 5; 8 mg· l^{-1} Kin and 0.1; 0.2; 0.5 mg· l^{-1} NAA) were tested (Zenkteler unpubl. data), but results reported in this paper are based only on the most suitable formulation (8 mg· l^{-1} Kin and 0.5 mg· l^{-1} NAA)·

3. Results and discussion

3.1. Pre-treatment

In earlier studies, micropropagation from old rhizome explants of *D.cristata* proved to be unsuccessfull mainly because of tissue browning (Zenkteler & Urbaniak 1994). In the present study, a ‚stage 0' as a mean of rhizome introduction into semi-sterile culture was tested in order to obtain decontaminated and rejuvenated buds not subjected to browning on the medium.

Fragmentation of *Dryopteris cristata* rhizomes (Fig. 1.1) ensured rejuvenation of explants which became more capable to produce adventitious buds. After 2-3 months of culture in glass jars, each rhizome segment gave rise to 2-3 buds (Figs. 1.2-1.3) which had a high multiplication rate during the next months of *in vitro* culture. Buds development under conditions of pretreatment was analyzed on a series of anatomical sections (Figs 1.4-1.6).

The efficiency of rhizome pre-treatment and surface sterilization (in terms of contaminant eradication) was dependent on the type of fungicide and disinfectant applied. Previous studies revealed that Nystatin effectively controlled fungi colonizing the surface of rhizomes of other fern species (Zenkteler & Kwaśna 1994). Nystatin has a broad spectrum of mycostatic activity and does not reduce the growth rate of buds, their *in vitro* stabilization and callus proliferation. After 24 hours of rhizome exposure, the amount of microorganisms was greatly reduced, however some viable endobiotic bacteria and fungi still remained in cortex parenchyma not accessible to Nystatin penetration. However, the treatment of rhizomes with Nystatin, followed by 2-3 months of semi-sterile culture was sufficient for buds decontamination. The remainder of microorganisms inhabiting the surface of rhizomes was successfully eradicated by disinfection with 0.2% $Hg Cl_2$.

3.2. Detection and identification of contaminants

In this study, investigations were undertaken to ascertain the species composition of the microbial flora inhabiting *Dryopteris cristata* rhizomes. Results of identification of microorganisms inhabiting rhizomes and buds are shown in Table 1. Qualitative and quantitative

Table 1. Identification of the contaminants in *Dryopteris cristata* rhizomes (1*) – before pre-treatment, and in adventitious buds (2) – after pre-treatment

Contaminants	1*	2
Acremonium strictum W. Gams	2	-
Cylindrocarpon destructans (Zinss.) Scholten	7	5
Gliocladium roseum Bainer	1	-
Mortierella turficola Y. Ling.	1	-
Mycelium radicis atrovirens Melin	7	1
Nectria fuckeliana Booth	4	-
Penicillium roistrickii G. Smith	2	-
Phialophora cyclaminis J.F.H. Beyma	4	1
Rhizoctonia solanii Kuhn	3	2
Septonema chaetospira v. *pini* Bourchier	1	-
Sesquicillium candelabrum (Bon.) W. Gams	3	1
Trichoderma hamatum Rifai	1	-
Trichoderma harzianum Rifai	1	-
Trichoderma atroviride Pers. ex Fries	2	-
Verticillium albo-atrum Reinke & Berthold	2	-
Verticillium bulbillosum W.Gams & Malla	1	-
non-sporulating fungi	5	3
unidentified bacteria	14	11
Total isolates	61	24

differences in the composition of the fungal community in the rhizomes (16 species, 61 isolates) and buds (6 species, 24 isolates) were confirmed. (Non-sporulating fungi and unidentified bacteria were not taken into account). Rhizomes were inhabited by a much greater number of fungal species than buds. The most frequent species were *Cylindrocarpon destructans* and *Mycelium radicis atrovirens*.

Nearly all isolates belonged to Ascomycetes which are common saprophytic or pathogenic inhabitants of fern rhizosphere. Probably some of them form mycorrhizal associations with roots of *D. cristata*. This genus is regarded as strongly mycotrophic in spite of the fact that it is confined to wet habitats which are generally considered unfavourable for the development of mycorrhiza. There is little information in the available literature concerning fungi associated with *D. cristata* rhizomes. Endomycorrhizal associations in roots of *Dryopteris cristata* were found in Great Britain (Hepden 1960). Other autors described VAM (vesicular-abruscular mycorrhiza) in the roots of *Dryopteris* growing in wet Hawaiian forests (Gemma *et al*. 1992).

Not all identified species of fungi occurred at the two stages of *D. cristata* growth. In adventitious buds the frequency of microroganisms was significantly decreased (Table 1), which indicates a strong influence of pretreatment procedure on population of fungi. The buds developed well and did not turned brown during culture, which suggests that getting rid of contamination has also an influence on their phenol contents.

3.3. TEM and histological analysis

Ferns contain high levels of tannins (Michaux 1971) and hydrolysable or condensed phenolic compounds (Harborne 1980) which become oxidised very soon after explant isolation and transfer to the medium. For this reason, rhizome explants of *D. cristata* were not suitable for tissue culture or micropropagation. However, apical meristems of adventitious buds developed from rhizomes after the period of starvation revealed low contents of their tannin vacuoles (Fig. 1.7).

In parenchyma storage cells of the rhizomes some ultrastructural changes were present after the period of starvation (Figs. 1.8-1.9). Cells were much larger and

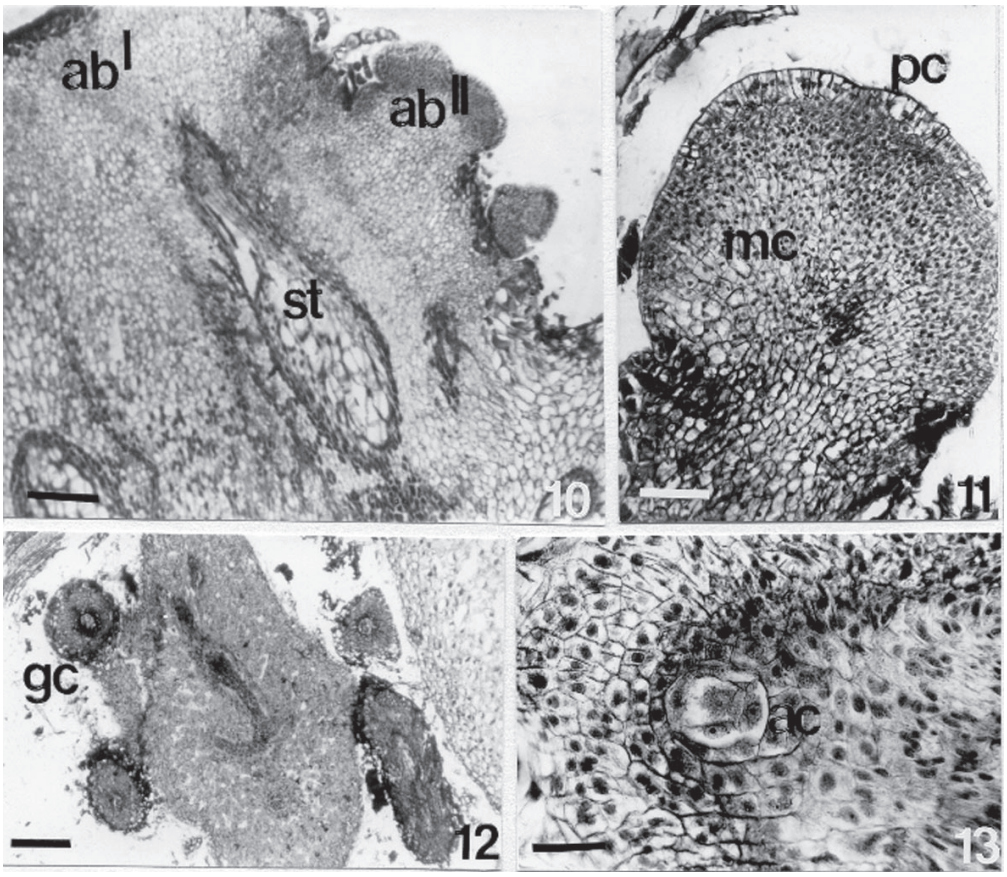

Fig. 2. Development of callus and buds of *Dryopteris cristata in vitro*

Explanations: 1 – Longitudinal section of an isolated primary adventitious bud (ab^{I}) after 5 weeks on the ½ MFMM medium. Note stele trace (st) and directly above it developed a secondary adventitious buds (ab^{II}) Bar=400 µm); 2 – Enlarged view of a bud (ab^{II}) from Fig. 2-1. Note a surface layer of prismatic cells (pc) surrounding the mass of small meristematic cells (mc) (Bar=50 µm); 3 – Callus regenerating from an adventitious bud on the modified ½ MFMM medium supplemented with 8 mg/l^{-1} of KIN and 0.5 mg/l^{-1} of NAA. Note a globular structure of calluses (gc) containing apical cell at the center (Bar=400 µm); 4 – Enlarged view of details from Fig 2-3. Callus with the area containing an apical cell (ac) (Bar=20 µm)

arranged more loosely than those from buds. Amyloplasts with the starch grains were missing, the cytoplasm contained few mitochondria, and tannin vacuoles coalesced into a few larger ones (tv). A strongly positive PAS reaction indicated that phenolic compounds, occurring as condensed tannins, were present at high concentrations in tannin vacuoles. According to Geier (1980) the tannins during fixation are converted into insoluble condensates that may give a PAS positive reaction for vacuolar contents.

In the cells of adventitious buds some structural signs of active metabolism could be distinguished at the time of their development induced by pre-treatment. Numerous amyloplasts (a) with large starch grains were located close to cell walls (Fig. 1.8). The cytoplasm contained abundant mitochondria, while in the nucleus (n) intranuclear crystals were present. The central vegetative vacuole (vv) was more electron-translucent than the small tannin deposites (t) with granular contents (Fig. 1.8). Probably, osmiophilic, tanniferous material deposited in vacuoles of buds and young leaves prevents the penetration of contaminants at the early stage of tissue colonisation by fungi.

3.4. *In vitro* culture

Isolation of apical meristems from adventitious buds for use as initial explants is an effective mean of establishing callus with a high morphogenetic potency. Buds of *Dryopteris cristata* were cultured in dark for the first three weeks. Then transferred to light (provided by warm-white fluorescent lampa at 20 mmol $m^{-2}s^{-1}$) and treated by frequent transfer (two weekly intervals) to a fresh, low salt modified MFMM medium supplemented with 8 mg· l^{-1} KIN and 0,5 mg· l^{-1} NAA and activated charcoal as an adsorbing agent. According to George & Sherrington (1984) addition of 100-250 mg· l^{-1} charcoal was sufficient to prevent the darkening of the medium caused by explant exudates. Also other autors found that there was a significant correlation between explant survival and their phenol exudation (Compton & Preece 1986; Preece & Compton 1991). Proliferating adventitious buds (Fig. 2.1) produced directly a secondary adventitious buds (Fig. 2.2) or indirectly a few small clumps of callus after some subcultures (Fig. 2.3). Those globular aggregates contain apical cells which generate new buds (Fig. 2.4). The addition of high concentrations of KIN significantly increased the rate of multiplication. After separation of the globular aggregates from primary explants they produced several clumps of leaves and short shoots. Culturing these clumps in the modified 1/2 MFMM medium without growth regulators enabled a development of the juvenile sporophytes during the next month of culture. The total number of newly formed shoots derived from one initial bud after six subcultures was 40-45. Afterwards, the sporophytes formed new roots within 2-3 weeks. The succesfully rooted plantlets were transferred to plastic cups containing autoclaved soil. Plantlets were acclimatized in laboratory conditions for about 4 weeks and finally transferred to the garden.

On the basis of the current study, efficiency of *D. cristata* micropropagation method was enhanced The literature reveals that there are different systems for mass propagation of ferns (especially for species representing a high decorative value) but this is the first report on propagation of *D. cristata in vitro*.

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