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Micropropagation of *Matteucia struthiopteris* (L.) Tod. through meristem proliferation from rhizomes

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Abstract: Micropropagation of *Matteucia struthiopteris* (L.) Tod. was undertaken to protect gene resources of the species in Poland. The main cause of low effectiveness of *in vitro* propagation has been the lethal browning of fern rhizome tissue after isolation. *In vivo* pretreatment of creeping rhizomes of ostrich fern induced their rejuvenation which was manifested by development of numerous buds. In the buds the amount of polyphenols and starch grains was decreased, as well as mitotic activity highly increased. The most effective in inducing buds break and increase of multiplication rate was Murashige Fern Multiplication Medium (MFMM) supplemented with KIN 5 $mg \cdot l^{-1}$ and NAA 0.5 $mg \cdot l^{-1}$. The regenerated microplantlets rooted best on half strength MFMM augmented with NAA 0.2 $mg \cdot l^{-1}$. In comparison with the low effectiveness of organogenesis from untreated rhizomes, their pretreatment *in vivo* proved to be better method to support the ostrich fern micropropagation.

Key words: fern rhizome pretreatment/rejuvenation, ostrich fern, tissue browning *in vitro*

1. Introduction

Matteucia struthiopteris (L.) Tod. occurs in some scattered localities in the lowland regions of Poland (in Lower Silesia near Nysa and Bobr river, in Borecka and Augustowska Primeval Forests). The species can be also found in submontane forests on the Carpathian and Sudetes Mts., in Roztocze Hills, in Holly Cross Mts., and more often in Bieszczady (Szafer 1966). In these localities *M. struthiopteris* was relatively widespread and dynamics of its populations appeared not to be declining (Zarzycki *et al.* 2002).

Riverside swampy carrs with *Alnus*, *Fraxinus* and *Ulmus*, on the more or less regularly flooded streambeds, constitute the optimum phytocoenosis for the species. There *M. struthiopteris* grows abundantly forming large patches confined to the lower flood subzone, very often submerged, on damp, fertile alluvial soils. The valuable function of this 'living fascine' is to prevent the erosion by water (Fabijanowski 1954). Unfortunately, the regulation of rivers and streams, as well as artificial drainage and irrigation operations which destroy river alluvial vegetation are the main threatening factors to ostrich fern (Fabijanowski 1954).

As the only representative of the genera, *M. struthiopteris* has been under legislative protection in Poland since 1946 (Szafer 1966). Despite all the protective measures, the resources of ostrich fern has been reduced in our country, due to transplantation from their natural habitats into gardens and cemeteries. The ornamental qualities of *M. struthiopteris*, its high tolerance to a different soil types and its luxuriance make it one of the most valuable garden ferns. Another serious hazard is that ostrich fern rhizomes are used as one of the components of the durable substrate for the culture of orchids. The above factors are responsible for the decline of their population in Poland. Although this species is mentioned in the Polish Red Data Book of Plants only in an appendix, it nevertheless occurs in the most endangered ecosystems (carrs, swamp woodlands) in Poland (Kaźmierczakowa & Zarzycki 2001). For those reason we have started micropropagation of this species to conserve their gene diversity.

2. Material and methods

2.1. Material

The plants of *Matteucia struthiopteris*, originating from natural habitat situated in southern Poland near the village of Skoczów, grow *ex situ* in the collection of rare and threatened species in the Botanical Garden of the University of Adam Mickiewicz in Poznań. One

randomly selected clone was excavated, cleaned, measured and analyzed in the field.

2.2. *In vivo* pretreatment culture of rhizomes

Excised long-creeping rhizomes were throughly washed with water and then soaked in suspension of Clotrimazol (1 tabl. in 500 ml of water) for 24h together with solution of KIN 2 mg·l^{-1}. Next, rhizomes were cut off into 10 cm sections and placed in conditions of carbohydrate starvation (into Petri dishes in darkness, at humidity of 80% and temp. of 20°C). After 3 weeks on the surface of rhizomes appeared 'dormant' buds which (according to Wardlaw 1943) developed from 'detached meristems'. Rhizomes together with 1-2 cm buds were disinfected in 0.1% mercuric chloride solution ($HgCl_2$ m/v) for 3 minutes, rinsed three times in sterile distilled water and then buds were excised from rhizomes and placed on the ½ Murashige Fern Multiplication Medium (MFMM).

2.3. *In vitro* establishment of explants

The initial source of the explants were: (*i*) creeping rhizomes removed from the erect mother rhizome; washed, disinfected in 0.1% mercuric chloride solution for 5 minutes, rinsed three times in sterile distilled water and then cut into 10 cm sections and placed on the ½ MFMM Modified Fern Multiplication Medium (Miller & Murashige 1976); (*ii*) dormant buds removed from creeping-rhizomes after their pretreatment culture.

The basal ½ MFMM medium was supplemented by monobasic sodium phosphate – 225; thiamine (HCl) – 0.6; nicotinic acid – 0.5; piridoxine (HCl) – 5.0; inositol – 100; kinetin (KIN) – 1.0, 2.0, 5.0; naphtalene acetic acid (NAA) – 0.1, 0.2, 0.5; active charcoal – 255 (in mg·l^{-1}); Difco Bacto agar 6 g·l^{-1} and sucrose 15 g·l^{-1}. The pH of all the media were adjusted to 5.8 before autoclaving at 121°C for 15 min. Four to five rhizome fragments (explant nr 1) or six buds (explant nr 2) were placed in 200 ml flasks containing 30 ml of medium and kept at 20±3°C under darkness for inhibition of phenolic compounds leakage and oxidation. After the first subculture (4 weeks) calluses were separated from initial explants and transferred into the same ½ MFMM medium and kept under continuous light (30 µmol m^{-2} s^{-1}). Multiplying tissues were sub-cultured every 21 days on fresh multiplication medium.

2.4. *In vitro* callus proliferation and multiplantlets development

In subsequent subcultures has callus developed which more or less rapidly produced a large number of spherical nodules. Continuously proliferating nodules were transferred to medium without growth regulators and vitamins to induce multiplication of plantlets. Every 4 weeks multiplantlets were excised and placed to the same ½ MFMM rooting medium with low concentration of NAA (0.2 mg·l^{-1}). For comparison the effects of micropropagation with and without *in vivo* pretreatment the statistical analysis was applied.

2.5. *Post vitro* establishment of plantlets

Rooted plantlets were removed from the culture vessel, washed gently under tap water and transferred to a potting mixture (peat, coarse sand, leaf mould – 2:1:1) into small pots. Plantlets were covered with polyethylene foil to maintain a high humidity (RWC 85-90%). Juvenile plants were shaded from direct sun radiation. After 2-4 weeks the humidity was reduced to common values. A formation of new roots was important to achieve a high percentage of juvenile plants survival and their transplantation to ground beds in partial shade.

2.6. Light and TEM microscopy

For anatomical observations rhizomes, apical meristems, callus and buds were fixed in FAA, dehydrated in ethanol-xylen series and embedded in paraffin. Serial transverse and tangential sections 10-12 um thick were then stained with safranin and fast green. For tannins and polyphenols detection rhizomes were fixed in Regaud mixture (bichromate potassium 3.27% water solution and formalin 30% according to Michaux-Ferriere 1975) and stained with Delafield hematoxylin. For TEM analysis longitudinally cut pieces of the creeping rhizome meristem of *M. struthiopteris* were prefixed in 2.5% glutaraldehyde, then postfixed in 1% OsO_4 (both in 0.2 M cacodylate buffer, pH 7.2), then dehydrated in graded series of ethanol, embedded in Spurr resine and polymerized at 65°C. Ultrathin sections were cut on ultramicrotome, stained with uranyl acetate and lead citrate and examined with a TEM.

3. Results

3.1. *Ex situ* reproductive biology

Matteucia struthiopteris rhizomes consists of few vertical mother rhizomes and many horizontal long-creeping rhizomatous shoots, which migrate at the distance 40-50 cm for a year and keep connection with mother ones for many years (Figs. 1A, B). Usually one new plant is produced each year at the end of every creeping rhizome. The aboveground vertical rhizome reaches 20-35 cm of height and contains axis with short internodes, numerous phyllopodia and adventitious roots (15-20 cm of length). Several long-term phyllopodia, relatively flat, covered on their both edges with many pelea, protect an axis of rhizome against water (Fig. 1C). Two or more short dormant roots 2-3 cm long (Fig. 1D) and one long-term dormant primordium of rhizome (Fig. 1D top row) develop at their bases. The buds

initiate new creeping rhizomatous shoots soon after the rhizome base becomes covered by soil deposited by flood. Those indicate the important role of dormant buds and roots in flooding-adaptative strategy and explain the reproductive behaviour of such facultative rheophytic species as to be *Matteucia*. The growth pattern is connected with flood-resistant features and influence on the architecture of rhizomatous matted-system tightly anchoring to stream-beds. The exact age of matted-system was difficult to estimate. However, the number of phyllopodia whorls of analyzed vertical rhizomes indicated a minimum age of about 9 years, and subsequently 7, 6, 5 and 3 years of younger ones (Fig. 1E).

M. struthiopteris is characterized by dimorphic fronds. Fertile ones are short, erect, brown and woody. They persist over the winter and shed their sporangia in February, sometimes over snow. Every year of the observations shows unopened sporangia contains 64 spores. Gametophyte growing in close contact which leads to intragametophytic selfing and to relatively short-lived gametophyte generation with limited capacities for vegetative reproduction.

3.2. *In vitro* callus proliferation and plantlets development

Without pretreatment culture callus developed only from explants containing apical meristem, those ones without meristem were not callusing (Fig. 2). Numerous contaminations and tissue browning were the most severe obstacle decreasing callus initiation.

The best subsequent callus proliferation was observed on ½ MFMM modified ferns medium supplemented with 5 mg·l^{-1} KIN and 0.5 mg·l^{-1} NAA (Table 1). On the medium callus developed rapidly producing a large number of sphaerical nodules constituting the stock cultures for further micropropagation (Figs. 3, 3a). Most of callus nodules have been maintained for over two years without changing their growth rate. These nodules were composed of loosely formed parenchymatic cells, a few vascular elements and three or more meristematic centers in the interior of nodules. Two subcultures, at 3-weeks intervals, on the medium without KIN were required to obtain the buds development from the meristematic centers of the callus. After the next subculture developed microplantlets without any decline

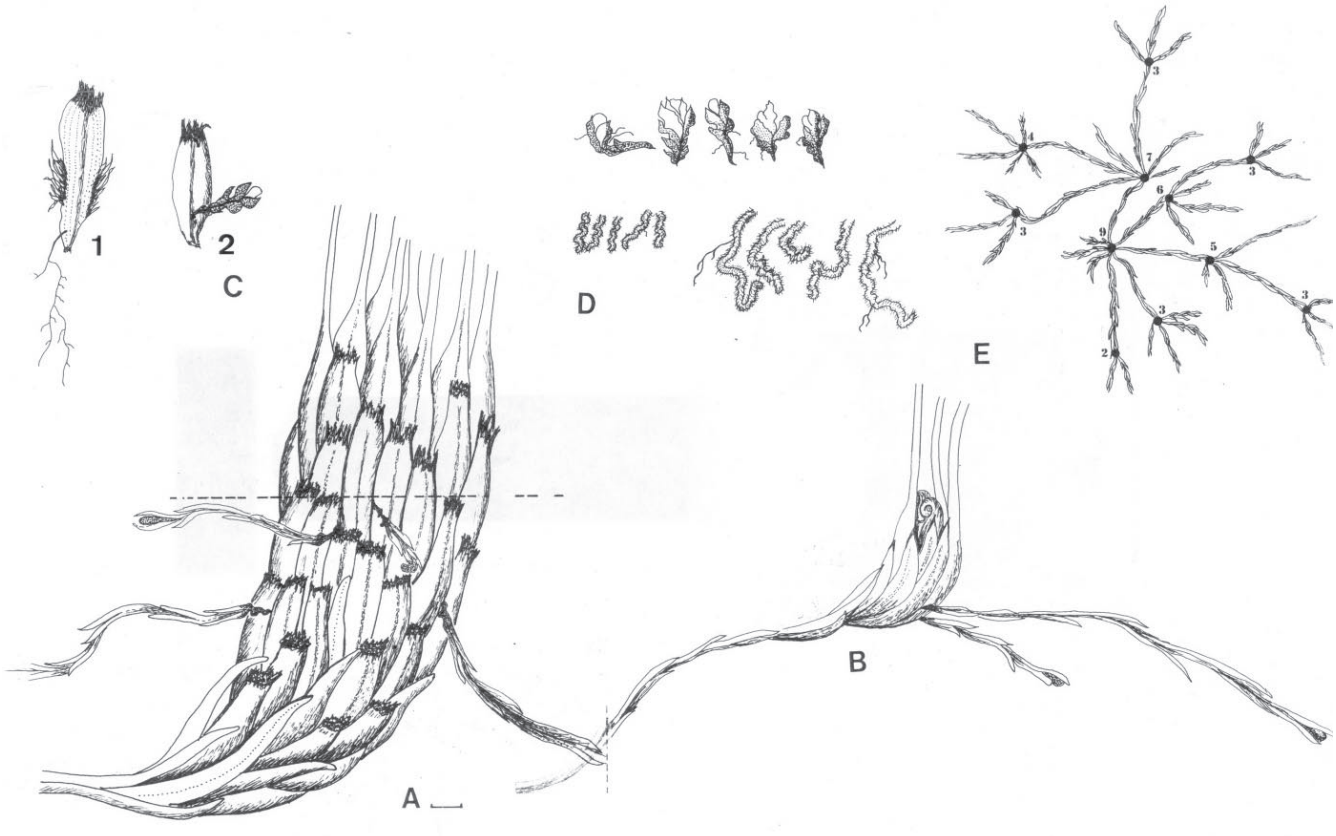
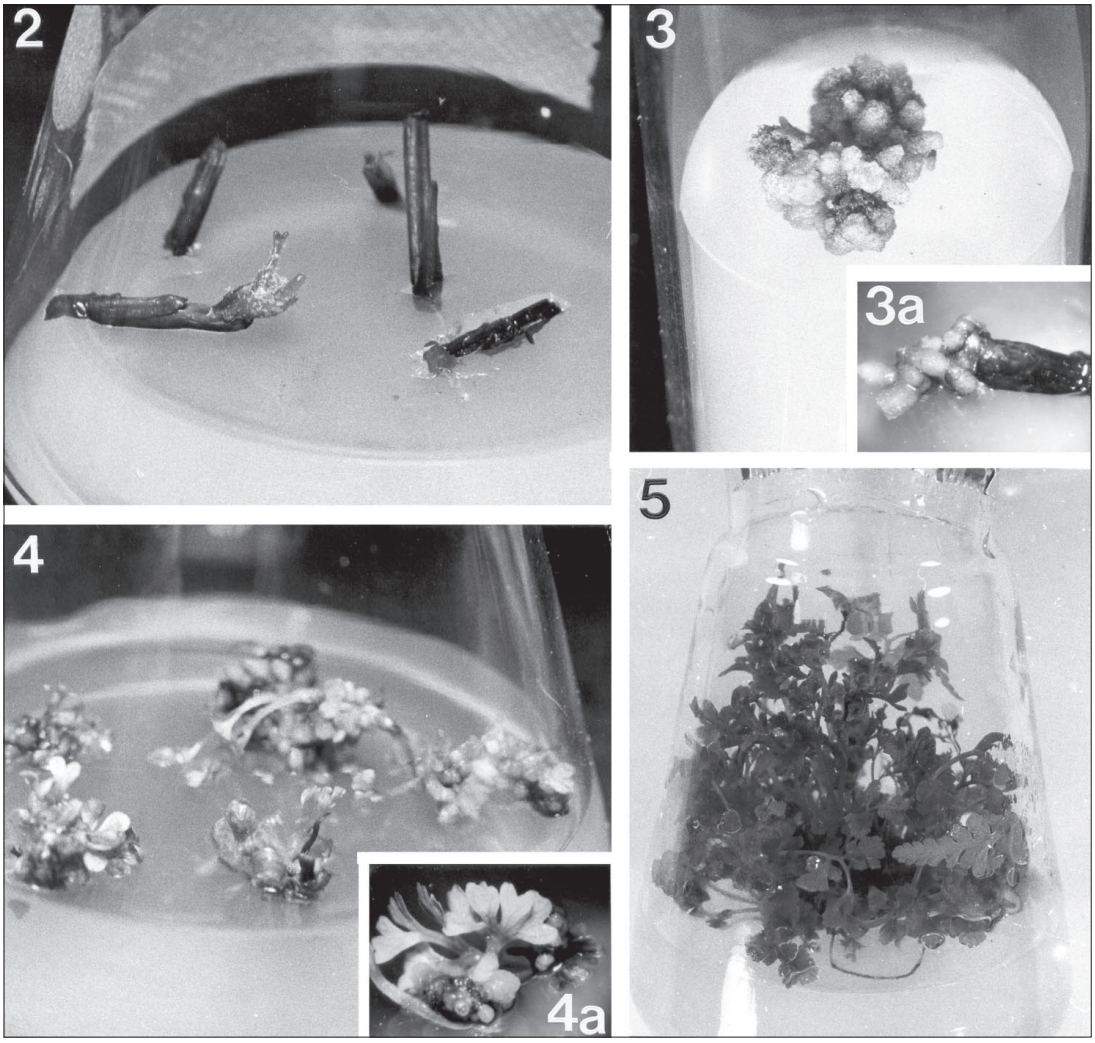


Fig. 1. *Matteuccia struthiopteris* rhizome habit in aspect of vegetative propagation
Explanations: A. Erect aboveground mother-rhizome with underground creeping rhizomes; B. Three years old long-creeping rhizome associated with current-year young rhizomes; C. Scale-like phyllopodia from outer (1) and lower (2) whorls bearing adaxialy regenerating buds; D. Dormant rhizome buds (top row) and dormant roots concealed under outer whorl of phyllopodia; E. Developmental sequences of rhizome matted-system of *M. struthiopteris* after nine subsequent years of growth of one clone

in multiplication rate (Figs. 4, 4a). The plantlets rooted best on MFMM medium augmented with 0.2 mg·l^{-1} NAA (Fig. 5). Plantlets established well in pots containing garden soil where they exhibited 90% survival.

Callus proliferated abundantly also from the buds developed after pretreatment culture. The dormant buds of *M. struthiopteris* (Fig. 6) developed from meristematic initials after 20 days of pretreatment (Figs. 9, 10). These initials were located directly beneath the epidermis of creeping rhizome (Fig. 7). For reason of their localization the initials are strongly affected by all kinds of surface disinfections which can completely destroy



Figs. 2-5. Callus proliferation, buds and plantlets regeneration from apical meristems of *Matteuccia struthiopteris* rhizomes
Explanations: 2 – Induction of callus from segments of rhizomes. Only explants with apical meristem began to proliferate callus on the ½ MFMM medium supplemented with KIN 5 mg·l^{-1} and NAA 0.5 mg·l^{-1}; 3 – Proliferation of large (0.5 – 0.8 mm) sphaerical nodules of callus after next two weeks on the same medium; 3a – A magnified view of the apical meristem which gave rise to callus masses; 4 – Development of plantlets from callus on a ½ MFMM medium without KIN after six weeks of culture; 4a – Detailed magnification; 5 – Complete sporophyte cultivated on ½ MFMM with NAA 0.2 mg·l^{-1}

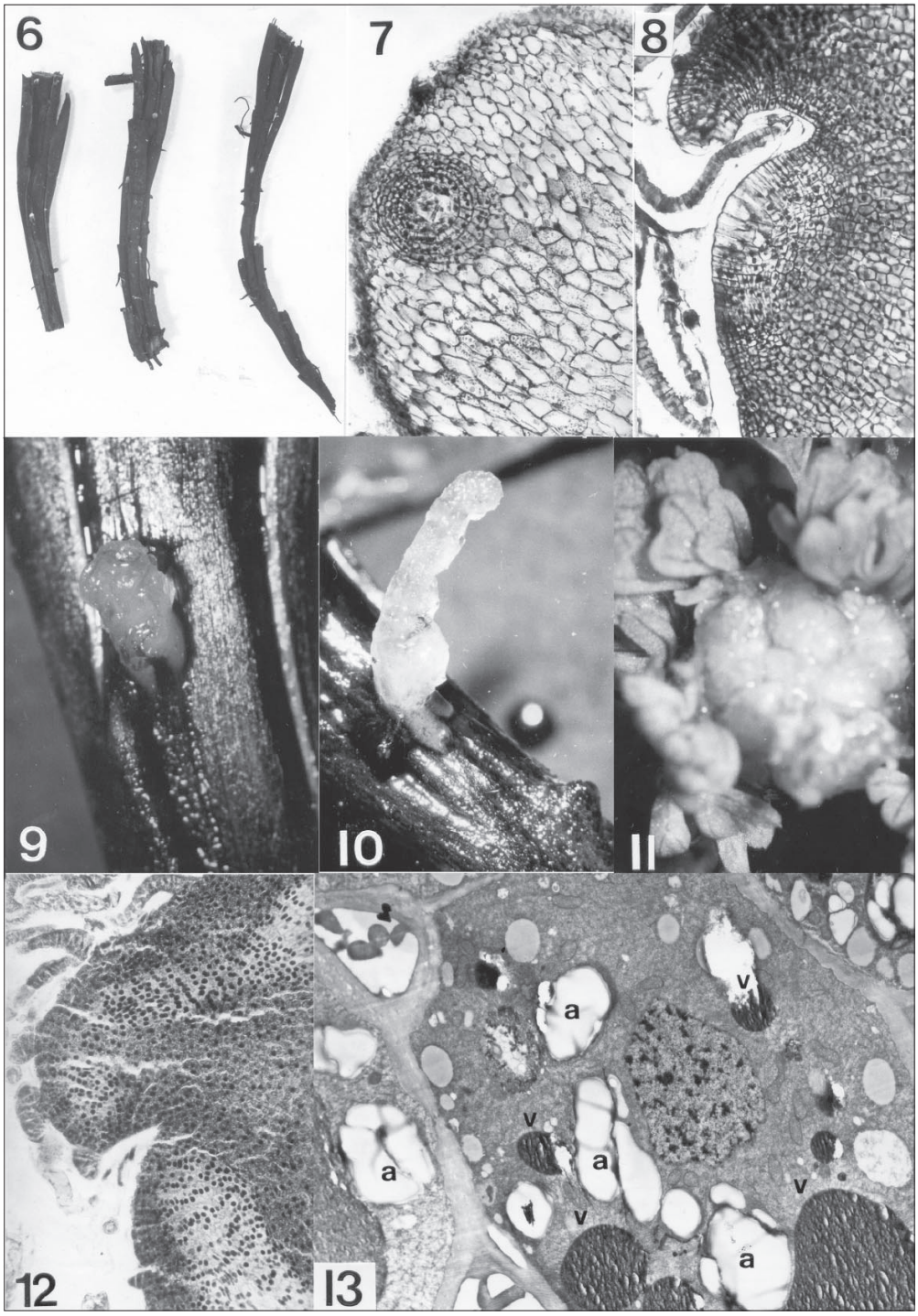
Table 1. Influence of pretreatment culture and growth regulators concentration on callus proliferation and plantlets regeneration in *Matteuccia struthiopteris* micropropagation

Plant growth regulators in mg·l^{-1}		Culture after pretreatment		Culture without pretreatment	
KIN	NAA	1	2	1	2
1.0	0.1	58.4b	72.2d	10.1a	75.4d
2.0	0.2	66.8c	84.0c	16.6a	64.8c
5.0	0.5	87.7e	87.7e	12.5a	80.7d

Explanations: Each value presents mean for 30 flasks per treatment: 1 – mean for explants proliferating callus; 2 – mean for plantlets regenerating from callus. Means followed by the same letter do not differ significantly according to Duncan's multiple test

them. This phenomenon we could observe also in this work.

Longitudinal sections of buds revealed the apical cell and its derivatives which formed a distinguishable layer of prismatic cells. The first leaf primordium arose from the leaf apical cell (Fig. 8). The extending procambial strands could be distinguished below the apical cell of shoot and leaf primordium shortly after transplantation to medium with high level of KIN. Intense cell divisions and proliferation altered a firm structure of apical meristem to more loose and soft (Figs. 11 and 12).



Figs. 6-13. Induction of 'detached meristems' to buds development during pretreatment culture
Explanations: 6 – Development of adventitious buds 20 days ofter rhizome fragmentation; 7 – Cross section through a 'deteched meristem' (initials of adventitive buds); 8 – Longitudinal section through an apical meristem of the bud which developed from the 'detached meristem'; 9-10 – Buds undergoing development after 20 and 30 days of pretreatment; 11 – Proliferation of callus and leaf primordia from isolated adventitious bud on the ½ MFMM medium supplemented with KIN 5 $mg \cdot l^{-1}$ and NAA 0.5 $mg \cdot l^{-1}$; 12 – Longitudinal section of bud showing profuse callusing; 13 – EM transverse section through meristematic cell with peripherally distributed tannin vacuoles (v) and several amyloplasts (a) with starch grains

On the basis of cytological EM studies it was found that cells of meristematic area of buds showed few amyloplasts, with small starch grains (Fig. 13). The most striking feature of fern meristematic cells was the double vacuole system, consisting of electron transparent non-tannin vacuoles and nontransparent tannin vacuoles. Circular or elliptical in cross section tannin vacuoles were filled with amorphous material of high electron density (Fig. 13).

Plantlets regeneration had an indirect character. Number of buds which initiated callus development increased with KIN concentration in the medium (Fig. 11). Addition of 5 $mg \cdot l^{-1}$ KIN and 0.5 $mg \cdot l^{-1}$ NAA was proved to be the most efficient for callus induction and their subsequent proliferation (Table 1). It was observed that in the absence of KIN in the MFMM medium, adventitious buds were formed from callus which contained meristematic centers. The buds developed to microplantlets which grew very well and multiplied at a high rate. Stimulation of rhizogenesis was observed under the influence of 0.2 $mg \cdot l^{-1}$ NAA. Rooted plantlets were successfully transferred to the pots containing sterile soil.

Such initial explants as the buds after pretreatment should compare well with rhizome explants. Those buds multiplied at a rate 10-fold higher producing nearly 50 clusters under this experimental conditions.

4. Discussion

Ferns like all plants growing on acidic, nutrient poor sites have elevated levels of phenolics substances in their tissues (Imperato 1991). Moreover, ferns rhizomes buried in the forest litter are exposed to influence of high amount of polyphenols, condensed tannins and flavonoids continuously released from decomposing leaf litter. At low pH conditions phenolic acids are taken up more easily as a result of the alternated root membrane permeability. Under nutrient deficit ferns are able to use phenolic substances as C-sources (Kuiters 1991).

The main difficulty with *in vivo* culture of fern rhizome explants is their lethal browning caused by the phenolic substances. Browning can occur as a result of unspecific reaction of the injured fern tissue, followed by exudation of phenolic compounds from damaged cells and their oxydation to more toxic quinnones. In vicinity of injury occurred transformation of starch grains (stored in abundance in fern rhizome parenchyma) to phenolic compounds during hydrolysis processes. Also, the polyphenols become more active after strong disinfection (Compton & Preece 1986), necessary for explants isolated from underground organs, often penetrated by soil-borne fungi (Prange 1985). However, disinfection with $HgCl_2$ probably caused subsequent death of 'detached meristems' (located close to the surface of the rhizome, Dykemann & Cumming 1985).

Pretreatment culture (a manipulation *in vivo* in semi-sterile conditions) has been an effective method for fern rhizomes rejuvenation in order to intensify the mitotic activity of tissues by numerous axillary buds development or adventitive buds regeneration (Compton & Preece 1986). In actively growing buds the concentrations of starch and phenolic compounds decrease. In contrary, progressive increase of regeneration possibility are noticed. This ontogenetic reversion was correlated with reduction of the size of apical cell and simplification of the structure of the solenostele to more simple protostele (Materi & Cumming 1991). The mitotic activity is more homogenous and higher in the young than in the adult phase. Adventitiously developed buds of *M. struthiopteris* have not only been considered as a means of vegetative multiplication of adult plant. Changes in morphology not always are adequate indicators of rejuvenation and can only show reinvigoration of rhizome materials (Pierik 1989). *M. struthiopteris* rhizome-buds after *in vivo* pretreatment exhibit easily distinguishable morphological and anatomical juvenile features; including small size of meristem which produce fine-featured leaves with high adventitious rooting ability (phyllorhiza). These changes in morphology indicate that rejuvenation occurs during *in vivo* pretreatment of rhizome. Recent studies show that the phase-related characteristic of buds developed from the 'detached meristems' of the *M. struthiopteris* can be obtained as a result of *in vivo* pretreatment and show morphological reversion to the juvenile state. The data presented in the paper indicated that rhizome pretreatment and adventitive buds development proved to have promotory effect on multiplication of *M. struthiopteris*. Both the rhizome apical and adventitive buds required a high concentrations of KIN for callus proliferation and plantlets development. Application of KIN combined with NAA caused subsequent callus proliferation and buds development also in other species of ferns (Kshirsagar & Metha 1978; Beck & Caponetti 1983). In *Pteris*, the *in vitro* culture of an adult meristem may produce under certain conditions a callus on which many apices are initiated, which give rise to new shoot meristems (Michaux-Ferriere 1975). Experimental callus plays a role of vegetative proliferation, including one of rejuvenation of the system (Wardlaw 1943).

To conclude, the successful micropropagation of *Matteucia* provides a system that is efficient in propagation of this valuable fern and could support their conservation, protecting the species from indiscriminate exploitation of the natural resources. The protocol described could be also useful for adapting micropropagation strategies to tissue culture storage in gene bank *in vitro*.

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