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## ANALYSIS OF ADVENTITIOUS BUD FORMATION IN *PINUS NIGRA* ARN. EMBRYO CULTURE

BRANKA KOLEVSKA-PLETIKAPIĆ and MARIJANA KRSNIK-RASOL

(Department of Botany, Faculty of Science, University of Zagreb)

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The development of adventitious buds in Black Pine (*Pinus nigra* Arn.) embryo culture was studied from its beginning to its end, i. e. from the 4<sup>th</sup> to the 21<sup>st</sup> day of culturing. The formation of meristemoids brought up in about 12, of primordial buds 16 and of leaf primordia in 19 days. The adventitious buds developed in 21 days and then started to grow.

During the culturing of Black Pine embryos the total activity of peroxidases was observed. From the 12<sup>th</sup> to the 19<sup>th</sup> day a large increase in peroxidase activity and a characteristic anodic separation pattern of two isoenzymes were stated.

### Introduction

Several papers describe the complete plant regeneration by induction of adventive buds in the culture of mature embryos in various species of gymnosperms. These species are: *Pinus palustris* (Sommer et al. 1975), *Pseudotsuga menziesii* (Cheng 1975), *Picea glauca* (Campbell and Durzan 1976), *Pinus radiata* (Reilly and Washer 1977), *Pinus contorta* (Arnold and Eriksson 1981a) and others. According to the results of these papers it is obvious that there are differences among the species in their morphogenetic potentials as well as in their demands for a specific composition of the nutrient medium.

The first author investigated the morphogenetic potentials of Black Pine (*Pinus nigra* Arn.), an economically important plant in our country. In this work a nutritive medium is defined, on which about 70% of cultured embryos formed adventitious buds thus obtaining regeneration of complete plantlets (Kolevska-Pletikapić 1981, Kolevska-Pletikapić et al. 1983).

In plant cells peroxidases are included in some basic metabolic processes. They play an important role in the catabolism of auxins and in the regulation of the content of free endogeneous auxins. Their overall distribution in the plant kingdom and the possibility to analyse them by simple methods have made them attractive for the studies of cell differentiation and growth. (Darimont et al. 1971, Lee 1972 a, b, Kevers et al. 1981 a, b, Gaspar et al. 1982).

In the present work in addition to histogenetic studies peroxidases have been observed as indicators of changes in metabolic processes occurring in cells during the differentiation of adventitious buds in the culture of the mature embryo of Black Pine.

## Materials and Methods

### *Tissue culture*

Mature embryos, isolated from seeds of wild population of Black Pine were transferred to the defined agar medium (Kolevska-Pletikapić et al. 1983). The characteristics of this medium are reduced salt concentration (to the half) in the MS-mineral medium and the use of auxins (NAA  $3 \cdot 10^{-8}$  mol and IBA  $3 \cdot 10^{-6}$  mol) in addition to BAP ( $10^{-6}$  mol). The cultures were grown at a temperature of 299 K under artificial light (fluorescent tubes IPR 40 W, 220 V and 4500 K) at an illuminating intensity of 1250 — 1500 lx and a daily photoperiod of 16 hours light and 8 hours darkness.

### *Peroxidases*

For the extraction of peroxidases, the buffer after Penel and Greppin (1972) with an addition of polyvinylpyrrolidone was used. The extract was centrifuged at 19000 g for 30 min and the supernatant was used as crude extract which was dialysed over night in a diluted extraction buffer (1 : 10). The total peroxidase activity was measured after Siegel and Galston (1967) and expressed as the change in the absorption of the light at 470 nm wavelength per min and per mg of protein. Proteins were quantitatively determined according to Bradford (1976). Isoperoxidases were separated after Davis (1964) and Ornstein (1964) the gels were stained by means of a reaction mixture after Siegel and Galstone (1967) ( $5 \cdot 10^{-6}$  mol guayacol and  $5 \cdot 10^{-6}$  mol  $H_2O_2$  in  $2 \cdot 10^{-1}$  mol of phosphate buffer, pH 5.8).

### *Histogenesis*

The explants, cultured from 1 to 29 days, used for histological analysis were fixed during the cultivation from the 1<sup>st</sup> to the 29<sup>th</sup> day. FAA (= formol-aceto-alcohol = formalin : glacial acetic acid : 70% ethanol — 5 : 5 : 90) was used as fixative. Dehydration was carried out by ethanol-butanol series procedure. The material was embedded in paraffin (Johansen 1940, Jensen 1962). Series of sections (thickness 10 — 15  $\mu$ m) were obtained by a Reichert rotary microtome. They were double stained by safranin and fast green and mounted in Canada balsam.

## Results and Discussion

*Histogenesis*

At the time when adventitious buds were induced and developed, the embryo culture was characterized by a specific sequence of events. During the first four days the embryos became considerably elongated. At the same time the cotyledons became green and elongated. From the 5<sup>th</sup> to the 19<sup>th</sup> day the hypocotyle tissue, the borders of the cotyledons and the zone around the apical meristem proliferated (Figs. 1 and 2). After the 20<sup>th</sup> day buds and leaflets became visible. The leaflets were intensely green and they developed on the border of the cotyledons or in the immediate vicinity of the shoot tips (Fig. 3). Their development continued till the transfer to the new medium (usually between the 28<sup>th</sup> and 36<sup>th</sup> day). A similar sequence of events has been described by Reilly and Washer (1975) for *Pinus radiata* as well as by Arnold and Eriksson (1981 b) for *Picea abies* and *Pinus sylvestris*.

On the basis of systematical analyses of series of histological preparations, obtained by sectioning of the explants (embryos) of various age, it could be stated that in Black Pine, like in *Pseudotsuga menziesii* (Cheah and Cheng 1978), the formation of adventive buds in vitro might be subdivided into four sequences of development: (1) the formation of the meristemoids, (2) the formation of primordial buds, (3) the development of buds and leaf primordia, (4) the formation of adventive buds.

*Meristemoids*

The survey of section series obtained from young cultures shows that the first cell divisions occur either in the hypodermal layer or in the epidermis, after the 4<sup>th</sup> day (Figs 4 and 5). These first cell divisions are more frequently observed on the border of cotyledons, but similar processes also occur in the tissue around the shoot apex. The cells, which begin to divide at first, have all characteristics of meristematic cells. After many succeeding divisions meristemoids arise. Their formation finishes on the 12<sup>th</sup> day (Fig. 6). The sequence of the events described is in many points similar to that in *Pseudotsuga menziesii* whose histogenesis of adventive buds in cotyledon culture in vitro has been described by Cheah and Cheng (1978). Meristemoids as developmental stages in the process of adventive buds formation have been described also in cotyledon culture of *Pinus radiata* (Yeung et al. 1981) and *Picea abies* (Jansson and Bornman 1981, Bornman 1983) as well as in the culture of needles of *Picea abies* (Arnold and Eriksson 1979).

*Primordial buds*

The survey of sections from cultures older than 12 days has shown that between the 12<sup>th</sup> and the 16<sup>th</sup> day a rapid proliferation of meristemoids occurs. The result of this proliferation are primordial buds of characteristic spherical shape, which are usually elevated over the surface of the plant organ on which they are formed (Fig. 7). The cells of primordial buds are always uniform and still have the characteristics of meristematic cells. This developmental stage has been mentioned in the description of adventitious buds in the cotyledon culture of *Pseudotsuga menziesii* (Cheah and Cheng 1978). Primordial buds have been described by Arnold and Eriksson (1978) in embryo culture

of *Picea abies*. While the development period of primordial buds in *Picea abies* equals that in Black Pine, in *Pseudotsuga menziesii* this developmental stage was prolonged even till the 21<sup>st</sup> day of culturing.

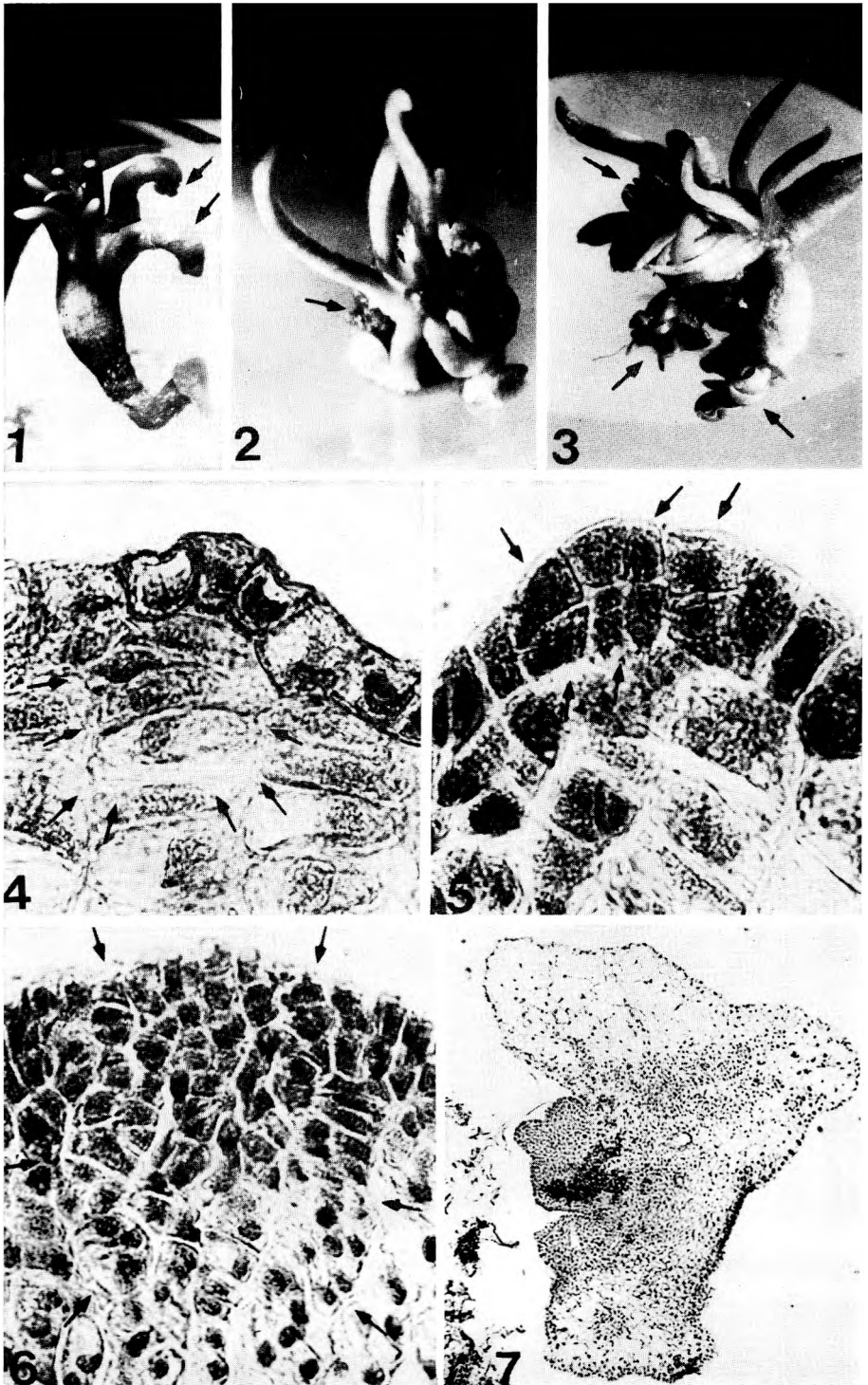
#### *Buds with primordial leaves*

After the end of primordial buds development, on the 16<sup>th</sup> day, a sudden proliferation of the tissue located on both sides of the primordial buds was observed. As a result of these processes on the 19<sup>th</sup> day of culturing primordial leaflets appeared. They soon reached the length of the buds and overgrew them rapidly (Figs. 8 and 9). For this developmental stage it is also characteristic that all cells of the primordial bud and primordial leaflets were uniform and still of meristematic appearance. Literature data concerning the in vitro formation of primordial leaflets are in accordance with those in Black Pine. There are some differences in leaflets appearance varying with the species and plant organ culture in which the adventitious buds have been induced (Yeung et al. 1981, Arnold and Eriksson 1978, 1979, 1981 b, Cheah and Cheng 1978).

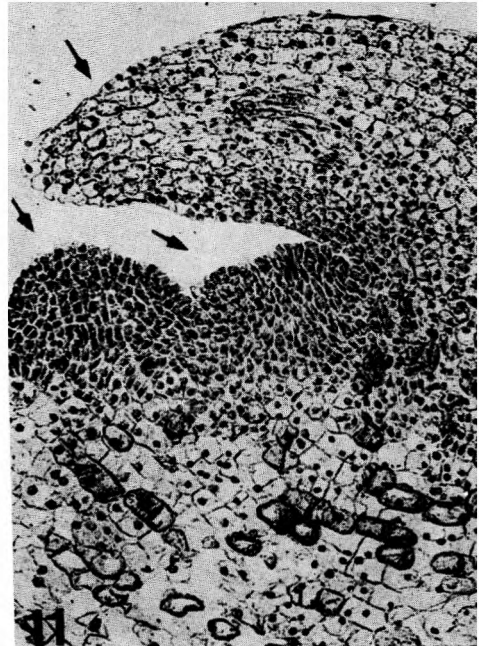
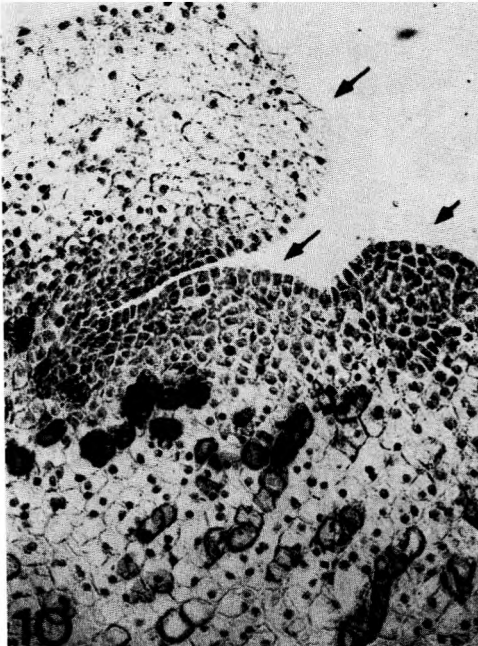
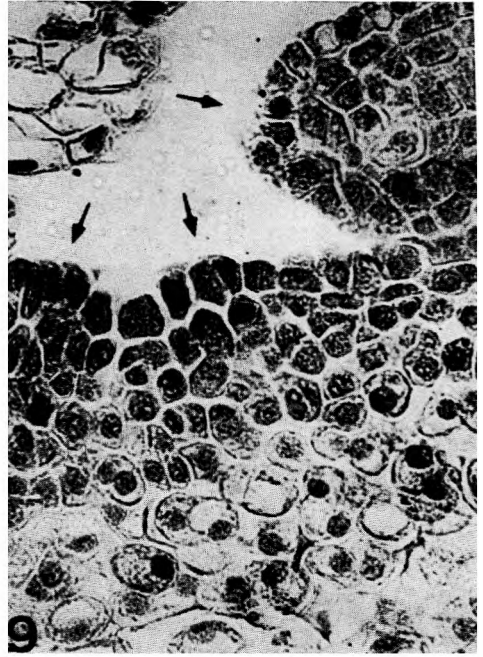
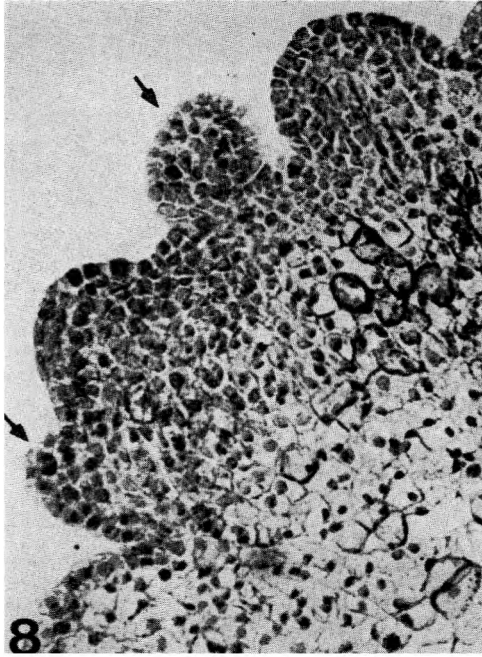
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Figs. 1—11. Morphology and histology of the development of adventitious buds in embryo culture of Black Pine (*Pinus nigra* Arn).

- Fig. 1. Explant at the end of the first week, with proliferations along the hypocotyl. A weak proliferation has occurred also along the cotyledons and round the meristem of the shoot tip.
- Fig. 2. Explant at the end of the 4<sup>th</sup> week. Round the apical meristem new formations have appeared.
- Fig. 3. Explant at the end of the 4<sup>th</sup> week with fully developed adventitious buds located on the border of the cotyledons and around the shoot tip.
- Fig. 4. Longitudinal section of a 4 days old explant. At the border of the cotyledon there are 4 daughter cells originating from successive longitudinal (periclinal) divisions of a hypodermal cell. 203 : 1
- Fig. 5. Longitudinal section of a 5 days old explant. On the border of the cotyledon some epidermal cells are anticlinally dividing. 172 : 1
- Fig. 6. Longitudinal section of a 12 days old explant. On the cotyledon a clearly limited meristemoid is visible. 110 : 1
- Fig. 7. Cross section of a cotyledon of a 16 days old explant. The primordial bud is located on its border. 10.5 : 1
- Fig. 8. Longitudinal section of 18 days old explant. On the border of the cotyledon there is a bud with laterally developed primordial leaves which cover it thoroughly by their length. 41 : 1
- Fig. 9. Longitudinal section of a 21 days old explant on whose cotyledon an adventitious bud has developed. 92 : 1
- Fig. 10. Longitudinal section of a 24 days old explant on whose cotyledon an adventitious bud has developed. 37,3 : 1
- Fig. 11. Adventitious bud at the border of a cotyledon of a 27 days old explant. 35,7 : 1



Figs. 1—7.



Figs. 8—11.

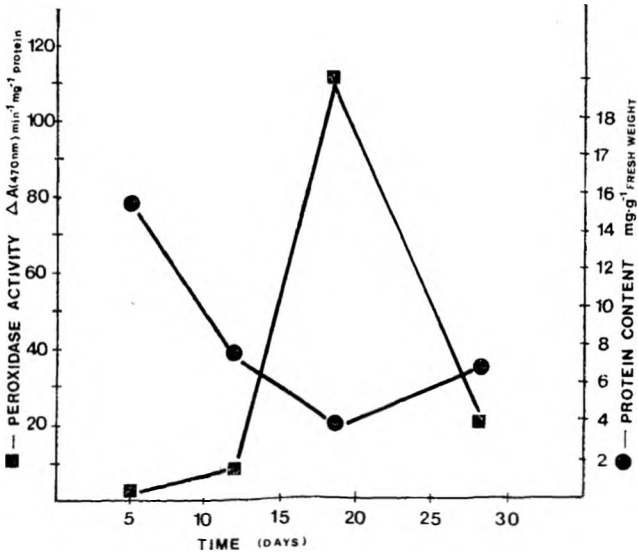


Fig. 12. Changes in the content of soluble proteins and in the total peroxidase activity during the development of the mature embryo.

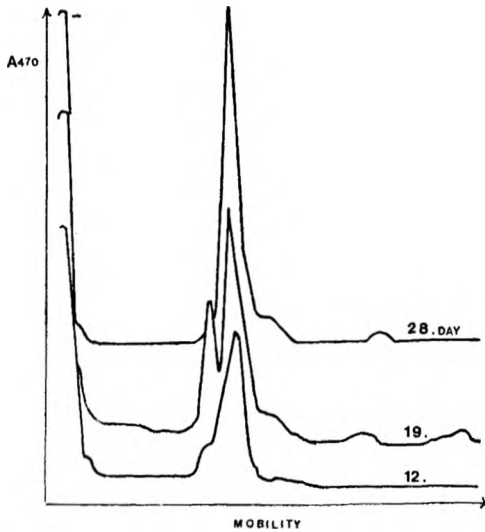


Fig. 13. Densitometry of the electrophoretic pattern of peroxidases in the explants.

*Adventitious buds*

The analysis of section series of explants, which have grown in culture for 3 weeks or longer, has shown that from primordial buds, in so far as they continue their development, adventitious buds are formed (Figs. 10 and 11). The cells, from which the adventitious bud is formed, differ in the same way as in the intact plant. This is in accordance with the data of Cheah and Cheng (1978), who stated that in *Pseudotsuga menziesii* the adventitious buds, induced in vitro, do not differ from the natural ones, being identical in anatomic structure. This concerns the adventitious shoots too.

As shown many authors have presented histological analyses of adventitious buds induced in vitro. Developmental sequences described in the present work are in accordance with the generally accepted view that these sequences have to be considered as normal in the processes of the in vitro developed adventitious buds (Thorpe 1980). By giving here for the first time a complete analysis of the time sequence of all histological changes which occur during the induction and development of adventitious buds in the embryo culture of Black Pine, we hope to have contributed to a better understanding of these processes in conifers.

*Peroxidases*

In the initial explants the peroxidase activity is low, and then it increases gradually till the 12<sup>th</sup> day, while the protein content is dropping at the same time. Between the 12<sup>th</sup> and the 19<sup>th</sup> day a considerable increase of peroxidase activity could be measured, the maximum of peroxidase activity being in accordance with the minimal protein content. Between the 19<sup>th</sup> and the 28<sup>th</sup> day of culturing the activity of the peroxidase is decreasing (Fig. 12). The electrophoretic pattern of isoperoxidases shows a characteristic separation of two isoenzymes which occurs between the 12<sup>th</sup> and the 19<sup>th</sup> day (Fig. 13), and disappears on the 28<sup>th</sup> day. A weak enzymatic reaction appears on the anodic side of the gel on the 19<sup>th</sup> and 28<sup>th</sup> day.

The induction of the adventitious buds in culture depends on the interaction of auxins and cytokinins (Reinert and Bajaj 1977, Kolevska-Pletikapić 1981). Since the activity of IAA-oxidase is ascribed to peroxidase activity (Grambov and Langenbeck-Schwich 1983), this enzyme could have an influence on the content of the free heteroauxin in the cell, and thus also on the direction of morphogenesis. The changes observed in the isoperoxidase pattern and the increase in the activity of peroxidases are probably reflected in the metabolic changes which finally lead to the formation of adventive buds. It has been perceived that histological events are accompanied by changes in peroxidase activity and in the electrophoretic pattern of its isoenzymes. It would be necessary, however, to determine the time sequence of these events by more sensitive biochemical and histochemical methods.

**Conclusion**

The development of adventive buds in the culture of mature embryos of Black Pine (*Pinus nigra* Arn.) has been analysed histologically and on the basis of the total activity and electrophoretic pattern of isoperoxidases. On the basis of these analyses the following has been stated:



1. The development of adventitious buds begins on the 4<sup>th</sup>, and terminates on the 21<sup>st</sup> day of culturing. The 4 sequences, in which the development of the bud has been subdivided, occur in the following way: the formation of meristemoids terminates on the 12<sup>th</sup>, of primordial buds on the 16<sup>th</sup>, of primordial leaves on the 19<sup>th</sup> and of complete adventitious buds on the 21<sup>st</sup> day of culturing. The adventitious buds grow then as long as the culturing is prolonged.

2. During the differentiation of adventive buds the total peroxidase activity is growing. Between the 12<sup>th</sup> and the 19<sup>th</sup> day a considerable increase in its activity has been measured. At the same time a change in the electrophoretic pattern of the investigated isoenzyme group occurs.

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## SAŽETAK

ANALIZA NASTANKA ADVENTIVNIH PUPOVA U KULTURI EMBRIJA CRNOG BORA  
(*PINUS NIGRA* ARN.)*Branka Kolevska-Pletikapić i Marijana Kršnik-Rasol*

(Botanički zavod Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu)

Razvitak adventivnih pupova u kulturi embrija crnog bora praćen je histološki i na temelju promjena u aktivnosti i elektroforetskoj slici izoperksidaza. Histološke analize pokazale su da su se adventivni pupovi počeli razvijati oko 4. dana, a njihovo formiranje je bilo okončano oko 21. dana trajanja kulture. Vremenski slijed četiriju sekvencija na koje je razvitak adventivnih pupova bio raščlanjen izgledao je ovako: nastajanje meristemoida bilo je završeno oko 12. dana, primordijalnih pupova oko 16. dana, a primordijalnih listova oko 19. dana nakon čega su rasli sve do kraja kulture. Praćenjem promjena u aktivnosti i elektroforetskoj slici izoperksidaza utvrđeno je da je ukupna aktivnost peroksidaza u embrijima crnog bora tijekom njihove kulture rasla tako da je između 12. i 19. dana došlo do naglog porasta aktivnosti. Slika izoperksidaza dobivena anodnim razdvajanjem pokazala je karakteristično razdvajanje na dva izoenzima također između 12. i 19. dana.

*Dr. Branka Kolevska-Pletikapić*  
*Dr. Marijana Kršnik-Rasol*  
Botanički zavod IV  
Prirodoslovno-matematičkog fakulteta  
Sveučilišta u Zagrebu  
Rooseveltov trg 6/III  
YU-41000 Zagreb (Jugoslavija)