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Peroxidases during the course of callusing and organ differentiation from root explants of *Cichorium intybus*

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Abstract

Growth of *Cichorium intybus* root explants was accompanied by an important increase of fresh mass during the course of callusing and rooting. The absence of glucose in callus forming medium was compensated for by hydrolysis of storage carbohydrates of the tissues, inducing a decrease in dry mass. Protein content showed similar slight variations in explants during the course of budding and callusing, whereas an important increase of protein content was found during the first 48 h in explants cultured on root forming medium.

Specific increase of soluble peroxidase activity during bud and root neoformation was found. A peak of peroxidase activity, preceeding the organ emergence, was always observed. This peak occurred earlier in bud forming than in root forming explants. Conversely, during callusing, the bulk peroxidase activity showed only weak variations, and no peak was detectable. Moreover one basic isoperoxidase was missing in these explants after a 6-d culture, whereas in both root and bud forming explants the isoperoxidase patterns were very similar. When explants growing on differentiation media were in darkness a supplementary basic isoperoxidase with the highest electrophoretic mobility was revealed.

The data obtained show that isoperoxidase patterns cannot be correlated to a particular organ differentiation process, but that peroxidase activity and especially the moment of peak emergence can be a reliable marker of the differentiation process. Moreover, light seems to inhibit the expression of a basic peroxidase in explants growing in media promoting organ differentiation.

Introduction

Organogenesis in higher plant tissue cultured *in vitro* is under the control of carbohydrate nutrition, environmental factors, *e.g.* light (Legrand and Bouazza

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Abbreviations: DM - dry mass; FM - fresh mass; RFM - root forming medium; BFM - bud forming medium; CFM - callus forming medium.
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1991) and of interactions among exogenous and endogenous phytohormones (Tran Thanh Van 1981). Moreover there is considerable evidence that a number of environmental influences are mediated through the effects on the level and distribution of plant regulators.

In this regard, by its participation in the oxidative degradation of indoleacetic acid, peroxidase (EC 1.11.1.7.) is an enzyme of particular interest, as it may modulate morphogenesis in plants (Gaspar *et al.* 1982). Therefore analysis of peroxidase activity and isoperoxidase patterns at different stages of the differentiation process may contribute to our understanding of the physiological and biochemical changes underlying and determining the process of morphogenesis.

In the present work possible relationship between variation in peroxidase activities, isoperoxidase patterns and specific morphogenetic events occurring in chicory explants cultured *in vitro* was studied. Witloof chicory, a biennal plant, produces a tuberous taproot at the end of the first year, the tissues of which have high regeneration capacity. Modification of the culture medium composition allows the differentiation of either roots (Vasseur and Sene 1984) or buds (Lefebvre 1979) or results in the proliferation of callus without organ neoformation (Vasseur *et al.* 1986). We here report results concerning growth, soluble peroxidase activity and isoperoxidase patterns in the course of callus, root and bud formation by small root explants of *Cichorium intybus* cultured *in vitro* under light or in darkness.

Material and methods

Cichorium intybus (var. Witloof cv. Flash) mature roots, harvested in fields, were used as a source of genetically uniform material. After surface sterilization of roots by calcium hypochlorite, explants (6 mm diameter, 2 mm height) were removed from vascular cambium and cultured on three different culture media. A medium containing glucose (0.01 M) in addition to the macro- and microelements of the Heller medium (1953), solidified by agar (6 g l^{-1}) was used as the bud forming medium (BFM). BFM, without glucose, supplemented with naphthalene acetic acid (NAA, 5×10^{-5} M), kinetin (10^{-7} M), inositol (100 mg l^{-1}), glutamine (250 mg l^{-1}) and the vitamins from Murashige and Skoog (1962) served as callus forming medium (CFM). For root production (RFM) we used BFM complemented with NAA (10^{-6} M), kinetin (10^{-7} M), gibberellic acid (10^{-5} M) and glucose (0.055 M). Cultures were grown in controlled environment cabinets at constant temperature (23 °C) in continuous light (10 W m^{-2}) or in full darkness. Sixty explants (three dishes each containing 20 explants) were harvested in each conditions every two days, individually weighed, frozen in liquid nitrogen and kept at -25 °C till determination of peroxidase activity. Thirty of them were lyophilized to determine dry mass and protein content. Water content was expressed as [(FM - DM) / FM] × 100. When the explants were grown on the BFM and RFM media, the capacity for organ regeneration was estimated 25 d after the onset of the culture. Therefore, peroxidase activity and isoperoxidase patterns were assayed before the organs became visible.

Extraction of soluble peroxidase was carried out as previously described (Legrand and Bouazza 1991). Protein content of the extracts was measured by the Bradford (1976) method using the *Bio Rad* microassay and bovine serum albumin as standard. Peroxidase activities were determined using guaiacol as an electron donor, by measuring changes in absorbance at 420 nm after five minutes at 25 °C and expressed as ΔA_{420} g^{-1}(FM) (POX), and specific peroxidase activites (SPOX) represented ΔA_{420} mg^{-1}(protein).

Vertical starch gel electrophoresis of isoperoxidases and staining with benzidine was done according to Darimont and Gaspar (1972).

Proteins of lyophilized explants were extracted by precipitation in 10 % trichloracetic acid (TCA) for 12 h at 4 °C. The precipitate was centrifuged and the pellet was submitted to serial washings in 5 % TCA; ethanol saturated with Na-acetate; absolute ethanol; absolute ethanol + diethyl ether + chloroform (3/2/1, v/v/v); and diethyl ether. After drying the pellet, the proteins were solubilized in 0.1 M NaOH for 12 h at 25 °C. After centrifugation the protein content of the supernatant was measured by the *Bio Rad* microassay.

Enzyme activities and protein contents were measured three times using three different extracts.

Results and discussion

Morphogenesis: Regardless the composition of the growth medium, explants produce callus which was detectable four days after the culture was initiated. However, the

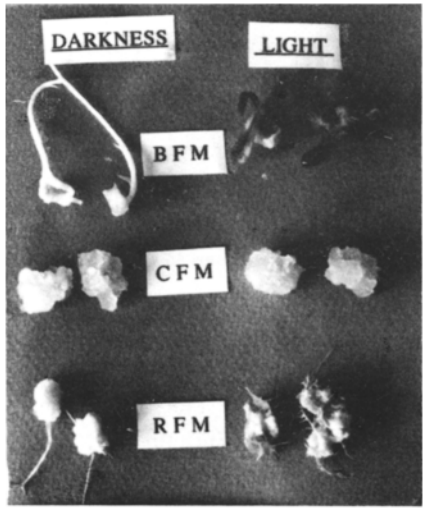


Fig. 1. *Cichorium intybus* root explants, after 25 d on callus forming medium (CFM), bud forming medium (BFM) or root forming medium (RFM) under light or in darkness.

colour and texture of the calli varied according to the growth conditions. In light the calli developed on explants cultured on BFM were green, compact and small, whilst, on RFM or CFM they were yellow-green and yellow respectively, smooth and well developped. In darkness the callus texture was identical as described above, all the calli were white (Fig. 1).

In CFM condition, only cellular proliferation was observed without any organ differentiation. On RFM roots emerged on day 16 and the rooting rate reached 64 % and 58 % in light and dark grown explants, respectively. These results are not in agreement with those obtained on *Malus* shoots (Druart *et al.* 1982). These conflicting results may be ascribed to the difference in the origin of the explants. Root tissues probably react in a different way than woody shoot cuttings.

On BFM, emergence of buds was observed between the 12th and 14th day. In the dark, the bud forming capacity was diminished from 100 % in light to 70 % in darkness.

Growth and protein content: Growth was characterized by a linear increment of fresh mass during the time of culture for each medium (Fig. 2). This increment was higher in light than in darkness.

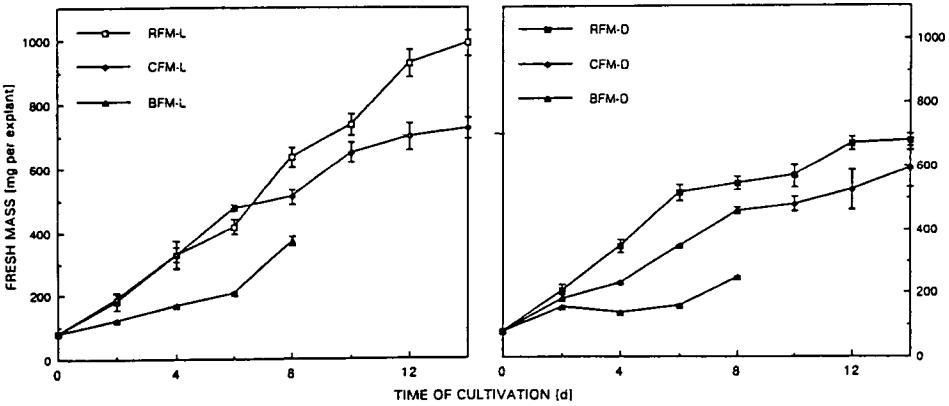


Fig. 2. Fresh mass variation of root explants of *Cichorium intybus* cultured in light (*L*) or in darkness (*D*). Bars indicate standard errors. Other abbreviations as in Fig. 1.

When expressed on a dry mass basis (data not shown), growth was characterized by a slight increase of dry matter when explants were cultured on RFM of BFM and a decrease of dry mass on CFM. The latter medium was lacking glucose and the loss of dry matter could be explained by hydrolysis of fructosans which are essential storage carbohydrates (about 15 % of the fresh matter) in *Cichorium intybus* taproots. At the onset of the culture the water content of the explant was 71.4 % of the fresh mass. On day 6, increase of FM essentially resulted from the water uptake which reached about 20 % in explants cultivated on CFM and RFM, and only 10 % on BFM (Fig. 3). These differences may probably be ascribed to an enhanced water

uptake on the high NAA content of CFM and RFM media (Hackett and Thimann 1952, Ketelapper 1953).

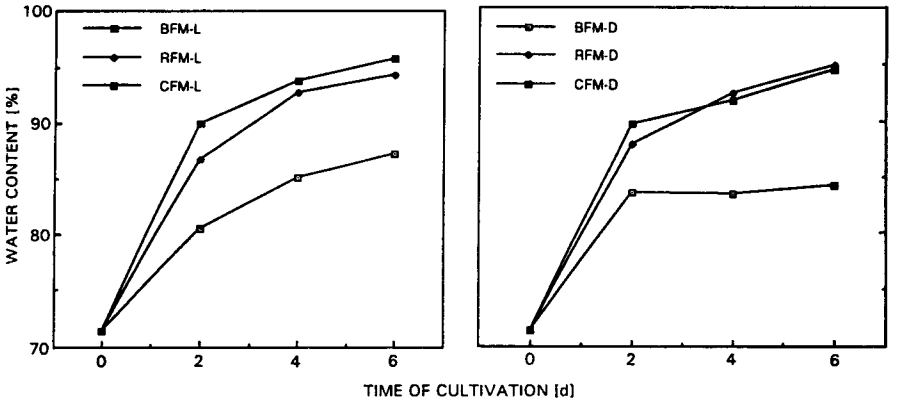


Fig. 3. Water content [%] in root explants of *Cichorium intybus* cultured in light (L) or in darkness.(D) For abbreviations see Fig. 1.

An increase in protein content during the first four days, followed by a slight and slow decrease was found in explants cultured on CFM; the same evolution at an lower level, was found for explants in darkness (Fig. 4). Similar changes were observed on BFM; in this case not influenced by the light regime. On RFM, variation of protein content was characterized both in light and in darkness by two peaks, the first one after 2 d, and the second one after 8 d.

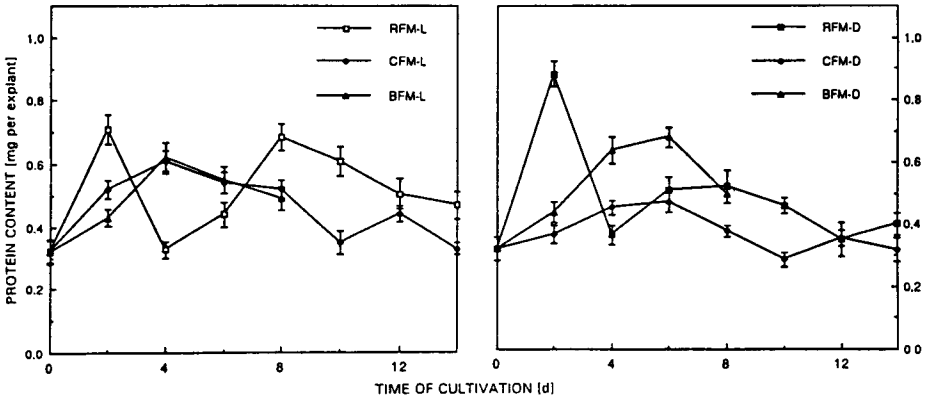
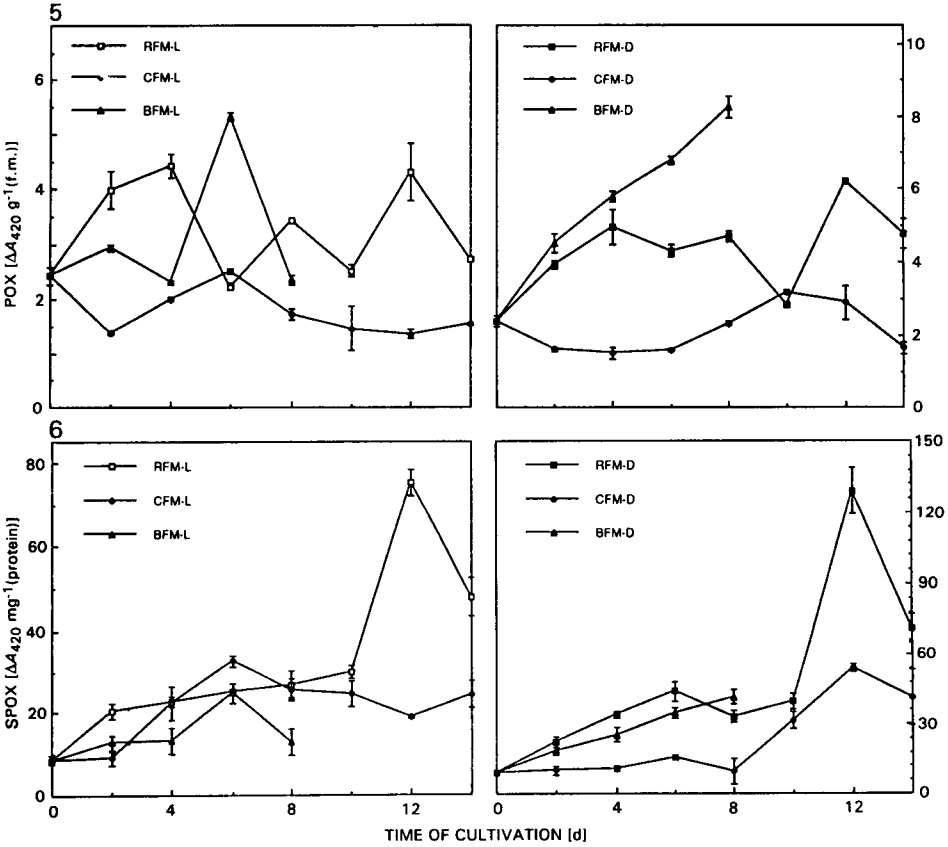


Fig. 4. Changes of protein content per explant of *Cichorium intybus* cultured in light (L) or in darkness (D). Explanations are given in Fig. 1.

Peroxidase activity and isoperoxidases: Peroxidase activity expressed on fresh mass basis (POX) of explants cultured on BFM medium in darkness increased continuously during 8 d. Under light peroxidase activity reached a maximum at day 6, and dropped later on with a high slope (Fig. 5). These results are in agreement with other reported bud initiation systems (Thorpe *et al.* 1978, Von Arnold and Gronroos 1986). Moreover, we have previously reported (Legrand and Bouazza 1991) that the slope of the drop was weak when peroxidase activity was expressed on dry mass basis.



Figs. 5 and 6. Peroxidase activity (POX - Fig. 5) and specific peroxidase activity (SPOX - Fig. 6) in root explants of *Cichorium intybus* cultured in light (L) or in darkness (D). Explanations are given in Fig. 1.

When explants were cultured on RFM in light, POX increased rapidly during the first 4 d, it subsequently decreased markedly till day 6 to increase again with a peak at day 12 (Fig. 5). A twelfth day peak was also found in explant cutured in darkness but no significant variations were seen between day 2 and 8, as is the case with SPOX (Fig. 6). Rooting has been widely studied on cuttings, and Gaspar (1981) has developed a model system showing that root formation occurs after the cutting has

reached and passed a peak of maximum peroxidase activity. It is interesting to note that with explants exhibiting a very different histological organisation, we found the same evolution of peroxidase activity during rhizogenesis.

In the course of callusing, POX remained nearly equal to the initial level, which was lower than those found in organogenetic explants.

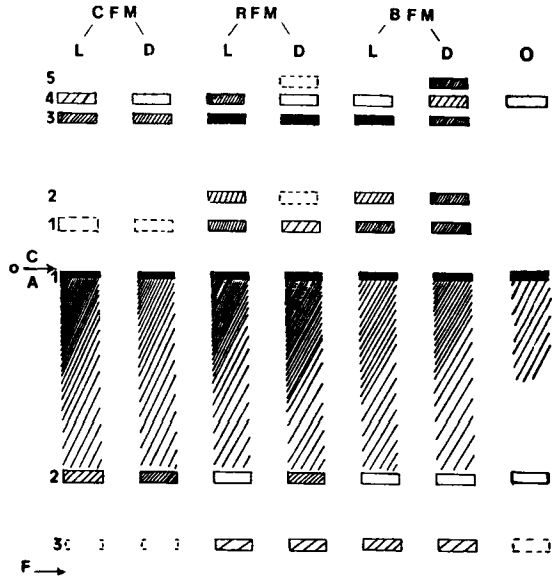


Fig. 7. Isoperoxidase zymogram of root explants of *Cichorium intybus* cultured in light (L) and in darkness (D) during 6 d. Comparative band intensity is indicated by the degree of shading

Fig. 7 shows the isoenzyme patterns in the initial explant and in 6-d-old explants cultured on different media under light and in darkness. No clear cut conclusions could be drawn from the changes in activity of the anodic bands. Among the cathodic bands, C_1, C_2 and C_4, were common for the different conditions of the culture. In complete darkness, one supplementary band, C_5 appeared, its intensity was very high on BFM and faint on RFM. In the Gaspar's model activity of both basic and acidic peroxidases increased during the induction of rooting and the activity of basic isoenzymes diminishes whilst the acidic peroxidases increased during subsequent culture of explants. Our results agree with this model if we considered only the basic peroxidases. Conversely in *Cichorium intybus* explants staining of acid peroxidases, which were present in low levels, did not change during the culture. On CFM, bands C_2 and C_5 were undetectable whatever the light conditions. Thus the number of isoperoxidases was lowest when there was no organogenesis as has been already demonstrated by Kevers *et al.* (1981).

In conclusion, the callus formation on *Cichorium intybus* root explants, before the emergence of organs, was quite different according to the growth medium. These differences are to be ascribed to the growth regulator composition of the medium as well as the carbohydrate concentrations. The bulk protein content increased or decreased before the increase or the decrease in peroxidase activity.

Peroxidase activities have been associated with changes in growth and development in numerous plants. Although there is some evidence indicating that peroxidases are essential by their involvement in indoleacetic acid metabolism, modifying thus the hormonal balance in plants and modulating morphogenesis, their role in organ induction remains ambiguous.

During cellular proliferation which produced callus without organ neoformation, the peroxidase activity showed weak variations, on the other hand when adventitious roots or buds were differentiated, peroxidase activity peaked; the peak arose before the emergence of organs.

Comparison of isoperoxidases in explant cultured in media inducing organ differentiation or preventing organ differentiation revealed that the number of peroxidase isoenzymes was larger in the former case. No difference in isoenzyme patterns could be found between caulogenesis and rhizogenesis.

When explants which differentiated organs were cultured in darkness a new basic peroxidase was revealed. Thus, light may repress the activity of a basic peroxidase in explants grown on media permitting the induction of bud or root differentiation. Since this particular isozyme remains undetecteble in the presence of a high concentration of exogenous NAA in explants grown in darkness, one must assume that the "light effect" may be mimicked in callus induction conditions.

The results presented here demonstrate that changes in peroxidase activities can be well distinguished during several different morphogenetic processes. But as the culture media are quite different we cannot conclude that typical variations of peroxidase activity are due to the morphogenetic processes rather than to the effects of medium composition.

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