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ISOZYMES OF PEROXIDASE IN CYTOKININ TREATED SEEDLINGS OF *CORYLUS AVELLANA* L.

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RESUMEN

ISOENZIMAS DE PEROXIDASA EN PLANTULAS DE *CORYLUS AVELLANA* L. TRATADAS CON CITOQUININA

El efecto de BAP sobre la proliferación de tallos, actividad peroxidasa y sus isoenzimas; se estudió en semillas y plántulas de avellano cultivadas "in vitro" en presencia o ausencia de esta citoquinina. Los resultados indican que el tratamiento con BAP favorece el aumento de la actividad peroxidasa paralelo a un descenso, consecuencia del proceso de germinación del contenido total de proteína. La separación mediante electroforesis de poliacrilamida de peroxidasa purificada demuestra la existencia de siete bandas específicas en las plántulas tratadas así como un aumento de la actividad de los isoenzimas preexistentes.

Palabras clave: BAP. 6-benzylaminopurina; PO peroxidasa.

SUMMARY

This paper deals with the effects of cytokinins on treated tissues. In trying to deal with the effects of BAP on shoot proliferation linked to peroxidase activity and PO isozyme changes, shoot clusters from seedlings maintained for 30 days in the presence of 40 μM BAP, seedlings grown without BAP and ungerminated seeds were analyzed.

An eightfold increase in peroxidase activity as a consequence of BAP treatment and lower protein content as a consequence of germination were observed when dry seeds, untreated seedlings and treated seedlings were compared.

Polyacrylamide gel electrophoresis showed seven bands specific to shoot clusters, besides four bands common to treated and untreated seedlings. Two of these common bands also appeared in dry seeds.

Key words: BAP, 6-benzylaminopurine. PO, peroxidase.

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INTRODUCTION

In micropropagation procedures of woody species, the proliferation phase may be the slowest step, and therefore a limitating factor for the whole process (Pérez *et al.*, 1983; Rodríguez, 1982a; Rodríguez, 1982b; Rodríguez, 1982c).

Proliferation is usually induced by addition of cytokinin to the culture medium, this strongly decreases the endogenous auxin/cytokinin ratio of the explants and favours the development of inhibited buds (Thiman, 1977) as well as the neoformation of embryo-like structures (Pérez *et al.*, 1983), tracheids (Minocha, 1984), nodules and shoots (Albuerne *et al.*, 1982). Cytokinin is strongly needed and the requirement seems to increase with age as it was proved. (Díaz-Sala *et al.*, 1990).

A detailed knowledge of the intermediate steps of the pathway leading from the first effector -cytokinin in the culture medium- to the last effect -multiple shoot bud proliferation- would greatly simplify the selection of the precise hormonal stimuli able to yield the desired response.

The study of peroxidase activity in these processes was chosen because of its close relationship to morphogenesis and differentiation (Kulaeva, 1980) as well as to the interesting results reviewed by Gaspar *et al.* (1982) concerning this system in relation to several exogenous stimuli.

This paper is a first approach to the understanding of the dramatic changes which occur on the explants during shoot bud induction. It focuses on the changes found in peroxidase in the presence of cytokinin, using as a control seedlings germinated and grown on a basal medium deprived of the effector.

MATERIAL AND METHODS

PLANT MATERIAL AND CULTURE

Corylus avellana L. seedlings treated with BAP (40 μM) were used as experimental material and compared with controls.

Genetic variability was reduced by using seeds from defined trees which were selected according to established phenotypic traits.

Seeds were surface sterilized by immersion in 85 % ethanol for 5 min and then in 25 g L^{-1} alconox (Inc. N.Y. 10003) as a moistening agent, and rinsed three times with sterile distilled water before being germinated in culture flasks with 25 mL of halfs-trength K(h) culture medium (Cheng, 1975) supplemented or not with 40 μM BAP.

As previously reported (Rodríguez *et al.*, 1984), the culture period was defined as 30 days because by this time shoot proliferation was satisfactory, thus seedlings treated or not with 40 μM BAP were used for biochemical analysis.

Media and culture conditions have been already published (Pérez *et al.*, 1983).

Growth room was kept at 25 ± 2 °C under 16-8 h photoperiod.

ENZYME EXTRACTION AND PARTIAL PURIFICATION

Twenty seeds or seedlings (13-14 g for seeds and 40-46 g for seedlings) were ground with extraction buffer (citrate-phosphate 0.04 M, pH 5) in a 1:5 (w/v) ratio. After being filtered through a double layer of cheesecloth, the homogenate was centrifuged at 50000 g for 1 h and the supernatant designated the crude extract, which was concentrated by reversed dialysis against polyethyleneglycol (aprox. M 20000) until a volume of 2 mL was reached and then applied to a Sephadex G-150 gel filtration column.

Fractions with peroxidase activity with over a quarter of one presenting maximum activity were pooled together and used as a partially purified extract.

All extraction and purification steps were carried out at 4 °C.

PEROXIDASE ASSAY

The Maehly and Chance (1954) method, based on the oxidation of guaiacol, was used with slight modifications.

The assay mixture contained 250 μL of citrate-phosphate buffer of suitable pH and concentration (Tabla 1), 250 μL of guaiacol, 40 μL and 125 μL of enzyme extract diluted when necessary with extraction buffer.

The reaction was started by the addition of 250 μL of optimum (Table 1) concentration H_2O_2 and measured at 470 nm Peroxidase activity expressed as μk L^{-1} (microkatals per liter).

PROTEIN CONTENT

Protein content was determined by the Bio-Rad protein assay method (Bio-Rad laboratories, 1979). Bovin serumalbumin was used as a protein standard. Protein content was expressed as $\mu g\ mL^{-1}$.

POLYACRILAMIDE GEL ELECTROPHORESIS

Partially purified extracts were concentrated by reverse dialysis against polyethyleneglycol until a protein content of at least 20 μg 100 μL^{-1} was reached.

Anodic slab gel electrophoresis was carried out in a Studier-type (Hames, 1981) apparatus following the Ornstein-Davis discontinuous system (Hames, 1981) with a 15 % acrylamide concentration.

Slabs were stained by immersion in a solution of equal volumes of guaiacol 0.44 %, H_2O_2 50 mM and citrate-phosphate buffer 5 mM pH 4.4, at 35 °C until band development was completed.

RESULTS AND DISCUSSION

In preliminary experiments we observed significant differences among the characteristic of the enzymatic systems. Therefore plots of PO activity versus pH, ionic strength of reaction buffer, temperature and substrate concentration (Fig. 1) were examined. These showed drastic

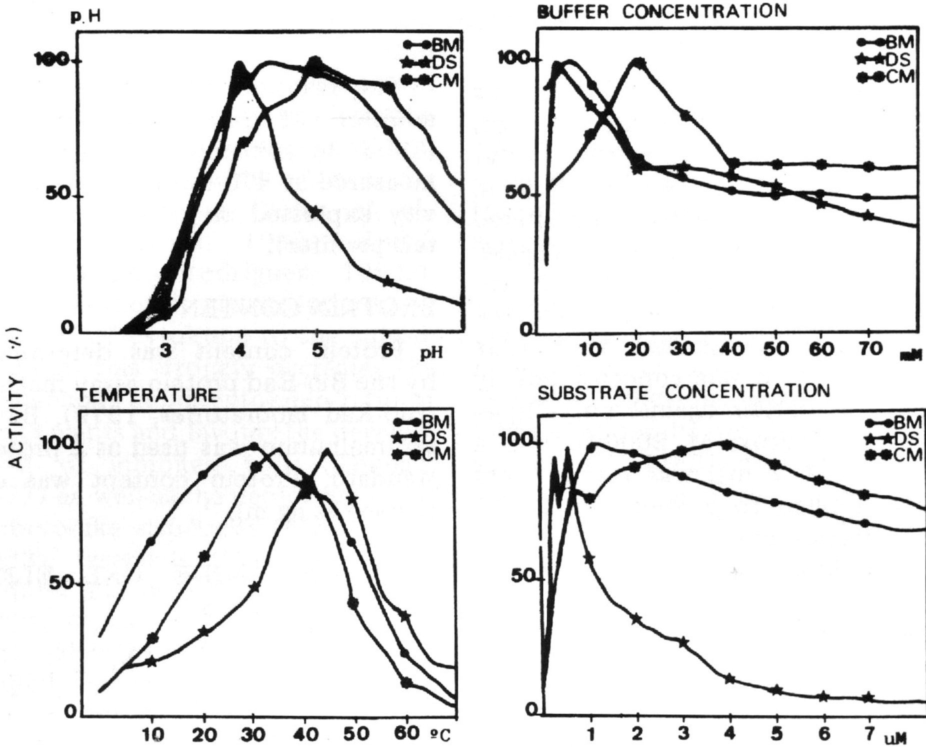

FIG. 1.—*Characterization assays of the extracts. DS, dry seeds; CM, seedlings cultured in basal medium supplemented with 40 µM BAP; BM, seedlings culture in basal medium.*

differences among the PO systems of the three types of extracts suggesting the release of new isozymes as a consequence of treatment.

Once the optimum conditions of PO reaction "in vitro" were determined for each material (Table 1), activity could be accunately quantified.

These data, as well as those of extracted protein content are shown in Table 2, where the influence of BAP on PO activity becomes clear: an eight fold increase of PO activity per unit fresh weight and of specific PO activity is observed when germination occurs in presence of BAP, compared to untreated seedlings.

When refering to protein content, catabolic processes seem more important than anabolic ones both in treated seedlings and in controls, although protein levels remained slightly higher in the latter, perhaps due to a stronger consumption of storage materials as a consequence of the higher biological activity of cytokinin treated tissues (Kuleava, 1980).

So, it seems clear that the addition of cytokinin to the culture medium of germinating seeds of *Corylus avellana* L. is followed by an enhancement of peroxidase levels.

So, we tried to separate the PO isozymes by their charge in polyacri-

TABLE 1

Conditions in which PO reaction was carried out for each material.

	Dry seed	Control seedling	Treated seedling
pH	4	4.4	4.9
Buffer conc. (mM).	2.5	5	20
Temperature (°C)	40	30	35
Substr. conc. (mM)	.6	1.2	4.2
Time (min).	10	10	10
Extract Dilution (v/v)	1/20	1/20	1/100

lamide gel electrophoresis and we found quite a different isoenzymatic composition for the PO system in each type of extract (Fig. 2). The composition was simplest for dry seeds with only two bands (DS-1 and DS-2) that also appeared in treated and untreated seedlings. In the untreated seedlings, two more bands were observed (BM-1 and BM-2) while in the shoot clusters, besides the four bands present in seedlings, seven faster bands (CM-1 to 7) could be detected.

The different isoenzymatic composition revealed by electrophoresis indicates modifications in the PO system leading either to new conditions of PO mediated reactions -e.g. substrate availability, higher velocity...- or to catalyze new ones, both possible consequences of BAP presence in the culture medium.

TABLE 2

Peroxidase activity and protein content of extracts.

	Dry seed	Control seedling	Treated seedling
μKat unit^{-1}	.022 ± 7.6x10^{-3}	.24 ± .015	1.7 ± .15
μKat g fresh weight^{-1}	.033 ± 1.7x10^{-3}	.11 ± .014	.85 ± .13
μg protein unit^{-1}	400 ± 15	75 ± 4.8	64 ± 5.6
μg protein g^{-1} fresh weight	600 ± 22	36 ± 2.3	32 ± 3.4
mkat g protein^{-1}.	.055 ± 4.1x10^{-3}	3.14 ± 0.4	27 ± 4.7

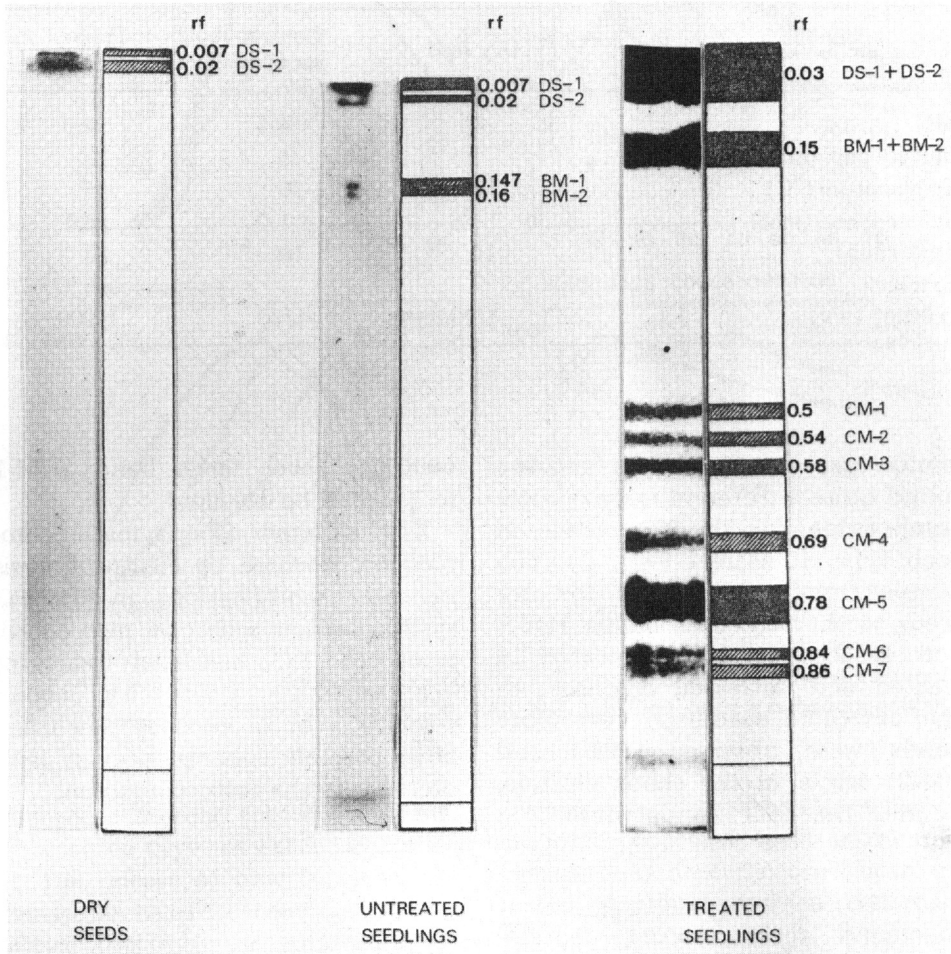

FIG. 2.–*Isozymes of PO in the three types of extract.*

CONCLUSIONS

Results demonstrate that it is possible to associate changes in the peroxidase activity to the germination process and plantlet development.

These changes are more remarkable in BAP treated seedlings in which the increase of peroxidase activity is a consequence of both an increase of the pre-existent isozymes and the

appearance of the new synthesized ones. Thus, we can conclude that peroxidase changes could be due to a particular physiological state induced by the growth regulator in the seedling in which the stimulation of the molecular events for peroxidase synthesis by BAP cannot be discarded. These results are directly related to multiple shoot formation induced by BAP.

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