BIOLOGIA PLANTARUM 39 (3): 337-347, 1997

Phenol content, acidic peroxidase and IAA-oxidase during somatic embryogenesis in *Theobroma cacao* L.

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Abstract

Calli were induced in cacao cotyledon explants on a half-strength Murashige and Skoog medium containing 6×10^{-2} g m^{-3} saccharose and various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) with kinetin (kin), benzylaminopurine (BAP) or 2-isopentenylphosphate (2-iP). Experiments were carried out on two clones of cacao differing in their susceptibility to black pod disease. The highest percentage of explants forming callus and the most rapid callus development were obtained with 10^{-6} g m^{-3} 2,4-D and 0.5×10^{-6} g m^{-3} kin. Somatic embryogenesis and rhizogenesis were induced by transferring 3-week-old callus in a half strength Murashige and Skoog medium containing 3×10^{-2} g m^{-3} saccharose and NAA or IBA in the 0 to 5×10^{-6} g m^{-3} concentration range. No differentiation could be observed when the medium was supplemented with kin or BAP. The conversion of callus into somatic embryos and roots was accompanied by a drop in phenol content and an increase in peroxidase and IAA-oxidase activities. Moreover, cell differentiation was characterized by the persistence in the callus of one acidic soluble isoperoxidase which was not detected in nondifferentiating callus. Although some differences were noticed between the clones, alterations responsible for cell differentiation were the same in both genotypes.

Additional key words: cacao, cell differentiation, oxidative enzymes, phytohormones.

Introduction

Cacao (*Theobroma cacao* L.) like most tropical trees is recalcitrant in tissue culture. Somatic embryogenesis has been reported from a number of tissues of cacao *e.g.* zygotic embryo axis (Pence 1991, Kononowicz and Janick 1984), nucellar tissue

Received 21 March 1996, *accepted* 10 June 1996.

Abbreviations: BAP - benzylaminopurine; BM - basal medium; CIM - callus inducing medium; 2,4-D - 2,4-dichlorophenoxyacetic acid; f.m. - fresh mass; IAA - indolylacetic acid; IBA - indolbutyric acid; 2-iP - 2-isopentenylphosphate; kin - kinetin; MS - Murashige and Skoog's medium; NAA - naphthalene acetic acid; SERIM - somatic embryo and root inducing medium.

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(Chatelet *et al.* 1992, Figueira and Janick 1993) and young bud petals (Söndahl *et al.* 1993, Lopez-Baez *et al.* 1993). Somatic embryogenesis of cacao can be induced in a half-strength Murashige and Skoog (1962, MS) medium with a high concentration of auxin and low concentration of cytokinin. Generally, embryos resulting from these cultures have a reduced capacity of development. Figueira *et al.* (1991) and Figueira and Janick (1995) induced conversion of nucellar somatic embryos into seedlings under elevated CO_2 and light conditions.

Peroxidase and IAA-oxidase activities are involved in numerous physiological mechanisms and have been considered as markers of embryogenic potentials in *Citrus* (Kohlenbach 1978), *Hevea* (El Hadrami and D'Auzac 1992), alfalfa (Hrubcová *et al.* 1994) and date palm (Baaziz *et al.* 1994). In cacao tissue culture, information on biochemical alterations during cell differentiation is rather scarce. Generally, calli from this species accumulate high amounts of phenolic compounds which probably impair further development. Phenols are known to be potent modifiers of peroxidase and IAA-oxidase activities (Galston *et al.* 1968).

The present work was undertaken to investigate phenol content and soluble peroxidase and IAA-oxidase activities as well as acidic soluble peroxidase electrophoretic pattern in cacao calli. The experiments were carried out on two cacao clones belonging to the Trinitario group but differing in their susceptibility to black pod disease: SNK 10 (high susceptible) and SNK413 (low susceptible). Our objective was to analyse these biochemical parameters during the conversion of calli into somatic embryo and root following treatment with various combinations of auxins and cytokinins.

Materials and methods

Plants: Immature pods (about 120-d-old) of *Theobroma cacao* L. belonging to the Trinitario group were harvested in the field of the Institute of Agronomic Research Station at Nkolbisson (Yaoundé, Cameroon). The clones used were SNK10 (high susceptible) and SNK413 (low susceptible to black pod disease). Pods were washed thoroughly with tap water and blotted dry with *Whatman No. 3* paper. They were then alcohol-flamed and seeds extracted in a laminar flow cabinet. The seeds were soaked in 10 % (v/v) mercurobutol (Mercyl Laurylé) for 20 min, rinsed in sterile distilled water and surface sterilized in 25 % (v/v) sodium hypochlorite followed by three rinses in sterile distilled water. Seed teguments and embryo axis were removed. The cotyledons were then cut into fragments (about 30 mm^3) which were used as explants.

Callus induction and embryo and root differentiation: Five explants were inoculated in 6 cm-diameter Petri dishes filled with 10 cm^3 of medium and sealed with parafilm. The basal medium (BM) used throughout consisted of half-strength MS inorganic salts, 0.7 % *Difco Bacto* agar and Morel's vitamin cocktail. The pH of the different media was adjusted to 5.8 before the addition of agar and autoclaved at 120 °C for 20 min.

Each experiment (10 to 15 Petri dishes per medium) was repeated twice. Calli were induced in a callus inducing medium (CIM) consisting of BM supplemented with 6×10^{-2} g m^{-3} saccharose and various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) with benzylaminopurine (BAP), kinetin (kin) or 2-isopentenylphosphate (2-iP). Cultures were placed in the dark at 25 ± 1 °C for 3 weeks. The best development of calli was obtained in the medium supplemented with 10^{-6} g m^{-3} 2,4-D and 0.5×10^{-6} g m^{-3} kin. Therefore these calli were used for somatic embryo and root induction.

Somatic embryogenesis and rhizogenesis were obtained by transferring the above calli in 6 cm-diameter Petri dishes containing 10 cm^3 of somatic embryo and root induction medium (SERIM) consisting of BM supplemented with 3×10^{-2} g m^{-3} saccharose and various combinations of indolbutyric acid (IBA), naphthaleneacetic acid (NAA), kin and BAP. Cultures were placed in a growth chamber at 25 ± 1° C, a 16 h photoperiod (irradiance of 10 µmol m^{-2} s^{-1}). Embryo and root differentiation started about 2 weeks later and was completed after 4 weeks. Calli were collected at this time for cytological and biochemical analyses.

Microscopic analysis: Callus and somatic embryo were observed with an *Olympus B061* lens equipped with a camera (model *OM 101, Type Power-Focus*, Japan).

For histochemical studies, samples were fixed for 24 h in ethanol/formalin/acetic acid (90/5/5, v/v/v), dehydrated in a graded ethanol series and embedded in paraffin wax. Longitudinal sections (10 µm thick) were double stained in Safranin-Fast Green and haematoxylin. Preparations were viewed in a microscope (*Nikon 233729*, Japan) equipped with a camera.

Extraction and measurement of soluble phenols and enzymes: Phenol content, peroxidase and IAA-oxidase activities were measured in the soluble fraction from 7-week-old callus. To do this, calli were induced for 3 weeks in CIM supplemented with 10^{-6} g m^{-3} 2,4-D and 0.5×10^{-6} g m^{-3} kin and then transferred for 4 weeks in SERIM supplemented with NAA, IBA, kin or BAP in the 0 to 5×10^{-6} g m^{-3} concentration range. The callus tissue together with the embryos formed were analysed. Soluble phenols and soluble peroxidase were extracted and estimated as previously described (Omokolo *et al.* 1995). The standard curve for phenol measurement was established using chlorogenic acid and all the concentrations were expressed in µg of this compound. The peroxidase activities were expressed as the difference in absorbance per mg fresh mass per second.

IAA-oxidase activity was estimated in the same extract as peroxidase. The reaction mixture (0.06 M phosphate buffer pH 6 containing 2 cm^3 of 175×10^{-12} g m^{-3} IAA + 1 cm^3 of 1 mM $MnCl_2$ + 1 cm^3 of 1 mM 2,4-dichlorophenol + 0.02 cm^3 of the enzyme extract) was incubated at 25 °C for 1 h in the dark. The remaining IAA in the reaction mixture was then estimated by incubating 2 cm^3 of the above reaction mixture in 8 cm^3 of Salkowsky reagent (3 cm^3 of 1.5 M $FeCl_2$ + 6 cm^3 fuming H_2SO_4 + 100 cm^3 H_2O). The absorbance was read after 5 min at 555 nm (spectrophotometer *Beckman DU 68*) and the remaining IAA calculated after Pilet (1957). IAA-oxidase activity was expressed in µg IAA destroyed after 1 h per mg

fresh mass. Data are means ± SE of 6 measurements from 2 extracts (3 replicates per extract).

Electrophoresis: Acidic soluble peroxidase isozymes were separated by non-denaturing polyacrylamide gel electrophoresis in a high pH system adapted from McDougall (1991). The resolving gel (1.5 mm thick) had a final composition of 12 % (m/v) acrylamide, 0.4 % (m/v) bisacrylamide and 0.5 % (v/v) tetramethylethylenediamine (TEMED). The stacking gel was 6 % (m/v) acrylamide, 0.4 % (m/v) bisacrylamide and 0.5 % (v/v) TEMED. Basic gel buffer was 0.4 M Tris[hydroxymethyl]aminomethane, pH 8.8, and the electrode buffer 0.03 M Tris and 0.2 M glycine, pH 8.6. Samples contained about 20 µg proteins and 10 % (v/v) glycerol. Gels were run at 6 °C at a constant voltage (70 V) for 18 h. After electrophoresis, the gels were stained in a solution containing 0.06 % (v/v) H_2O_2, 0.1 % (m/v) benzidine and 0.1 % (v/v) acetic acid. The drawings of the gels were made immediately after staining.

Results

Cacao cotyledon explants cultured on CIM containing a relatively high concentration of saccharose (6×10^{-2} g m^{-3}) and various combinations of growth regulators (2,4-D + kin, 2,4-D + 2-iP or 2,4-D + BAP) produced calli within 3 weeks. Callus

Table 1. Callus induction and development from cotyledon explants of *Theobroma cacao* L. (clones SNK10 and SNK413) after 3 weeks of culture in CIM supplemented with various combinations of 2,4-D/kin, 2,4D/2-iP and 2,4-D/BAP.

Hormones [$\times 10^{-6}$ g m^{-3}]	Number of explants cultured		Explants producing callus [%]		Callus formation	
	SNK10	SNK413	SNK10	SNK413	SNK10	SNK413
2,4-D/kin						
2.0/0.0	54	52	60	40	+	+
1.0/0.1	52	54	60	80	+	++
2.0/0.1	58	58	38	80	+	++
1.0/0.5	72	55	94	90	+++	+++
2.0/0.5	55	70	44	80	++	++
2,4-D/2-iP						
1.0/0.1	58	55	85	60	+++	+
2.0/0.1	55	55	85	44	+++	+
1.0/0.5	60	55	90	80	+++	+++
2.0/0.5	60	60	90	80	+++	+++
2,4-D/BAP						
1.0/0.1	49	56	70	70	+	++
2.0/0.1	55	50	45	64	+	+
1.0/0.5	61	58	55	95	+	++
2.0/0.5	55	49	45	75	+	++

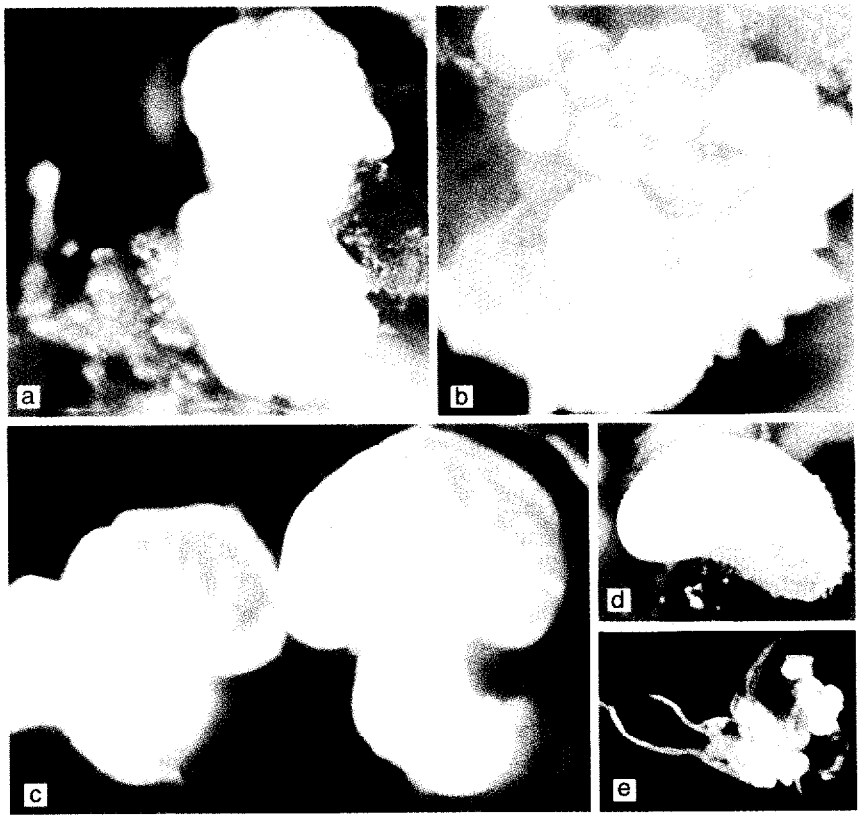

Fig. 1. Somatic embryogenesis and rhizogenesis from cotyledon explants of *Theobroma cacao* L. *A* - 3-week-old undifferentiated callus (× 10); *B* - *D*: embryo-like structures: *B* - globular (× 10), *C* - torpedo-shaped (× 10), *D* - cotyledon-shaped (× 7.5), *E* - 7-week-old rooting callus.

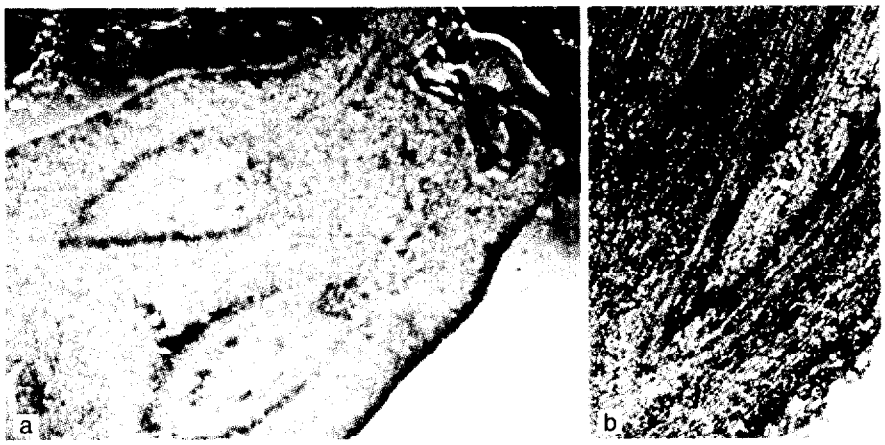

Fig. 2. Longitudinal sections of somatic embryos from 7-week-old callus from cotyledon explants of *Theobroma cacao* L. *A* - root meristem (× 400), *B* - shoot tip with an axillary meristem and well-formed procambial bundles (× 400).

appeared on the wounded part of the explant (Table 1). Calli were white and compact (Fig. 1*A*). The highest percentage of explants producing callus and the highest callus development for both clones were observed with 10^{-6} g m^{-3} 2,4-D and 0.5×10^{-6} g m^{-3} kin. Therefore calli from this medium were transferred in SERIM supplemented with IBA, NAA, kin, BAP or various combinations of IBA/kin for cell differentiation (Table 2). For both clones, differentiation was poor in media supplemented with kin,

Table 2. Effect of IBA and NAA on the conversion of callus into somatic embryos and roots. Callus was induced in CIM supplemented with 10^{-6} g m^{-3} 2,4-D and 0.5×10^{-6} g m^{-3} kin for 3 weeks from *Theobroma cacao* L. (clones SNK10 and SNK413) cotyledon explants and then transferred in SERIM supplemented with various concentrations of IBA or NAA. Data are means of 50 to 60 explants.

Phytoormones [$\times 10^{-6}$ g m^{-3}]	Callus forming embryos [%]		Number of embryos per callus		Callus forming roots [%]	
	SNK10	SNK413	SNK10	SNK413	SNK10	SNK413
IBA						
0.0	7	9	2 - 5	1	16	18
1.0	0	3	-	2	26	13
2.0	18	6	2 - 10	2 - 6	32	41
5.0	8	6	1 - 3	2	22	41
NAA						
1.0	16	3	2 - 3	1	38	60
2.0	20	15	2 - 4	2 - 3	50	51
5.0	3	7	1 - 2	1 - 2	30	15

BAP or IBA + kin (data not shown). Except for the clone SNK10 at 10^{-6} g m^{-3} IBA, somatic embryogenesis and rhizogenesis occurred on media supplemented with IBA and NAA. Somatic embryos formed either individually or in groups of 2 to 10 in the clone SNK10 and 2 to 6 in the clone SNK413. Different embryo-like structures were observed: globular, torpedo-shaped and cotyledon-shaped (Fig. 1*B-D*). Histological examination of longitudinal sections of these embryos showed the presence of root meristem (Fig. 2*A*) and a bipolarity was often noticed as a result of shoot meristem formation (Fig. 2*B*). Rhizogenesis (Fig. 1*E*) appeared only when SERIM was supplemented with IBA or NAA. The highest percentage of calli developing roots was obtained with 2×10^{-6} g m^{-3} NAA (Table 2), while the highest number of roots per explant was obtained with 5×10^{-6} g m^{-3} NAA (7 roots $explant^{-1}$) and with 2×10^{-6} g m^{-3} IAA (5 roots $explant^{-1}$) for clones SNK10 and SNK413, respectively (Fig. 3).

For both clones, the soluble phenol content decreased as the level of NAA or IBA in the medium increased (Fig. 4*A*). The decrease was about 50 % in 5×10^{-6} g m^{-3} auxin. On the other hand, the level of soluble phenols in the calli increased dramatically when cultured in media supplemented with kin or BAP (Fig. 4*B*). The magnitude of the increase was dependent on the concentration of the phytohormone in the medium. For both clones, the content of phenols was almost 20 times higher in

calli cultured in SERIM supplemented with 10^{-6} g m^{-3} kin than in those cultured in SERIM without phytohormones.

In the clone SNK10, peroxidase activity in the soluble fractions increased as the level of NAA or IBA increased in the culture medium. In the clone SNK413 the activity also increased with increasing NAA levels but remained almost constant at different levels of IBA (Fig. 5*A*). The increase was much higher in the clone SNK10 than in the clone SNK413. On the other hand, peroxidase activity increased with 10^{-6} g m^{-3} kin or BAP and then decreased at higher levels of both phytohormones (Fig. 5*B*).

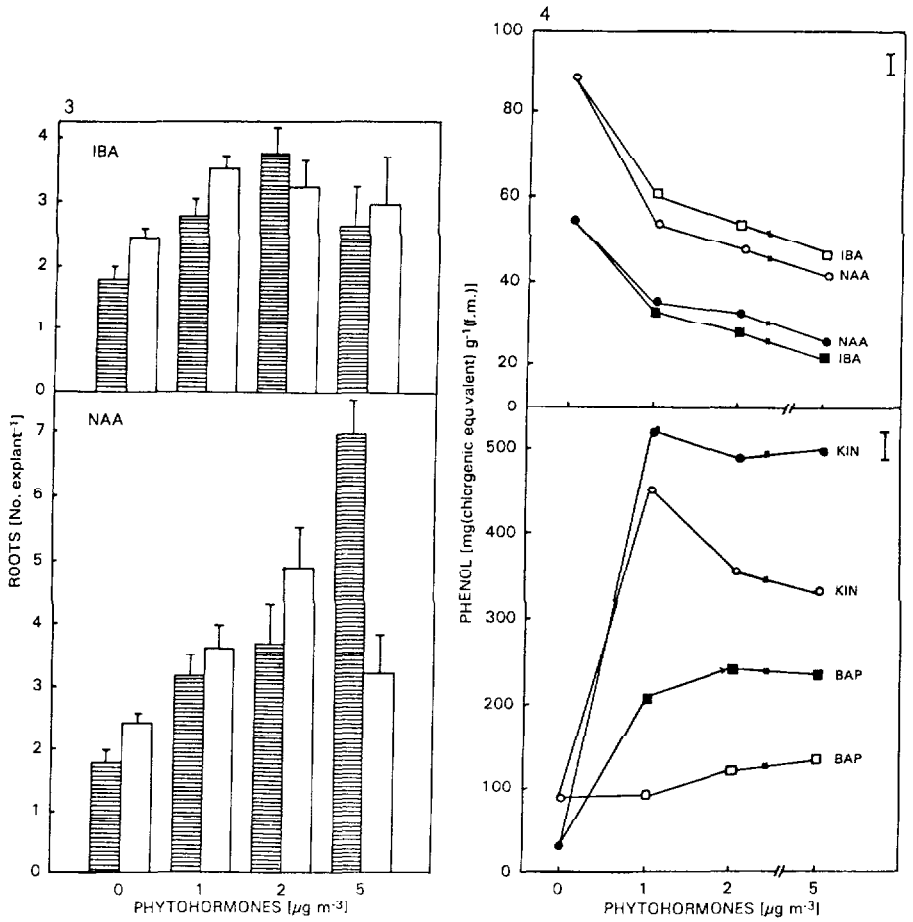

Fig. 3. Effect of IBA and NAA on root formation from 7-week-old callus of *Theobroma cacao* L. clone SNK10 (*shaded bars*) and clone SNK413 (*open bars*). Data are means ± SE, n = 50 to 60.

Fig. 4. Phenol content in 7-week-old callus from cotyledon explants of *Theobroma cacao* L.: clone SNK10 (*open symbols*) and SNK413 (*closed symbols*). Calli were grown for 3 weeks in CIM supplemented with 10^{-6} g m^{-3} 2,4-D and 0.5 × 10^{-6} g m-3 kin and then transferred for 4 weeks in SERIM supplemented with various concentrations of IBA or NAA (*A*) and kin or BAP (*B*). Data are means ± SE (n = 6).

In the clone SNK413, IAA-oxidase increased when the concentration of NAA or IBA increased in the culture medium (Fig. 6*A*). In the clone SNK10, IAA-oxidase increased as the concentration of NAA increased in the culture medium, while the activity remained almost constant with IBA. In the clone SNK413 IAA-oxidase activity increased as the level of kin increased in the culture medium, but remained almost constant when cultured in the presence of BAP (Fig. 6*B*). In the clone SNK10 IAA-oxidase activity increased as the concentration of BAP increased in the culture medium; meanwhile it remained almost constant in 10^{-6} and 2×10^{-6} g m^{-3} kin and dropped in 5×10^{-6} g m^{-3} kin.

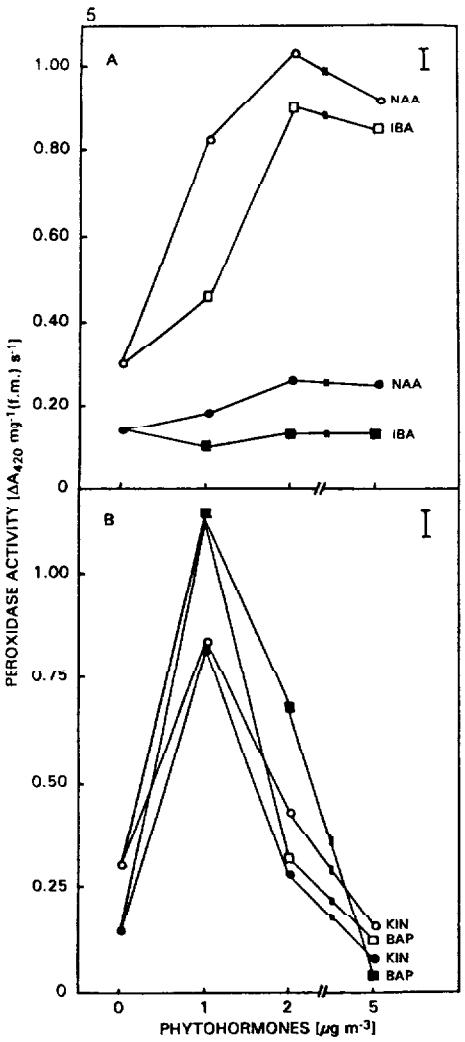

Fig. 5. Soluble peroxidase activity in 7-week-old callus from cotyledonary explants of *Theobroma cacao* L. Otherwise as in Fig. 4.

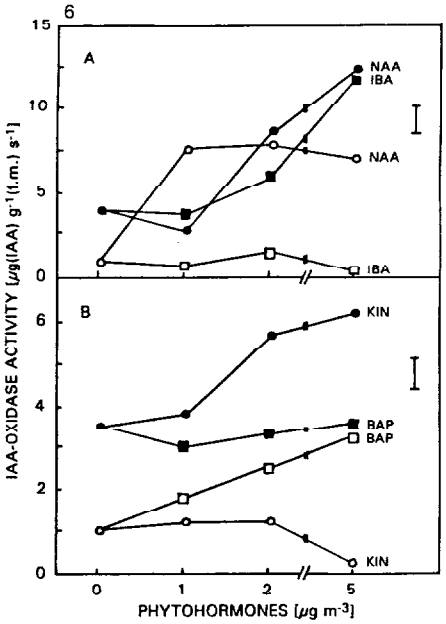

Fig. 6. IAA-oxidase activity in 7-week-old callus from cotyledonary explants of *Theobroma cacao* L. Otherwise as in Fig. 4.

Acidic soluble isoperoxidase pattern in calli from both clones are presented in Fig. 7. In the clone SNK10 4 bands were obtained from calli induced in CIM; meanwhile 3 bands were obtained with the clone SNK413 (band A2 with a relatively low electrophoretic mobility was not detected in this clone). When these calli were transferred in SERIM supplemented with IBA, NAA kin or BAP within the 0 to 5 × 10^{-6} g m^{-3} concentration range, the band A4 with the highest electrophoretic mobility persisted in the media where somatic embryogenesis and rhizogenesis occurred (media containing IBA or NAA). On the other hand, the band A4 disappeared when these calli were transferred in media where differentiation did not occur (SERIM supplemented with kin or BAP).

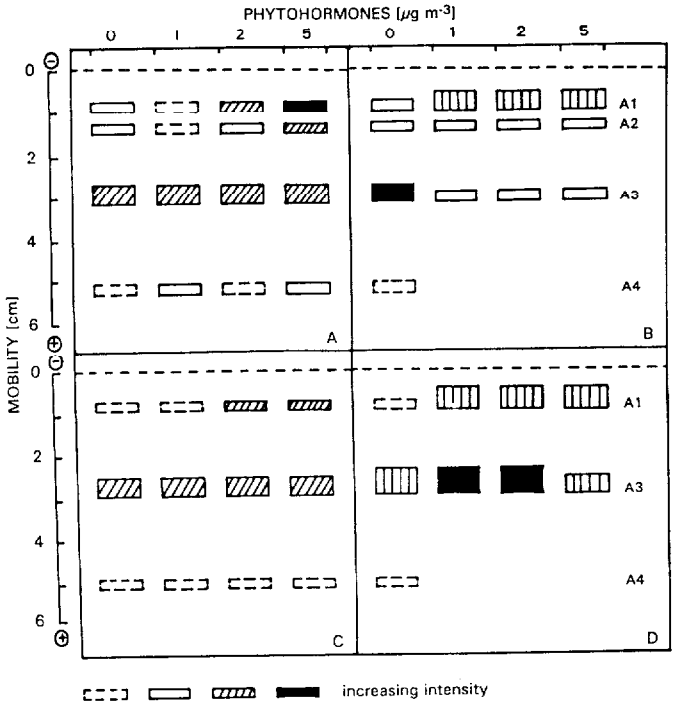

Fig. 7. Native polyacrylamide gel electrophoresis of acidic soluble isoperoxidases from callus of *Theobroma cacao* L.: clone SNK10 (*A*, *B*) and clone SNK413 (*C*, *D*). Calli were induced for 3 weeks in CIM supplemented with 10^{-6} g m^{-3} 2,4D and 0.5 × 10^{-6} g m^{-3} kin and then transferred for 4 weeks either in SERIM supplemented with various concentrations of IBA or ANA (*A*, *C*) for their conversion into somatic embryos and roots or in SERIM supplemented with kin or BAP (*B*, *D*) where no cell differentiation occurred.

Discussion

Basal medium supplemented with 2,4-D induced callus formation from cotyledon explants from both cacao clones SNK10 and SNK413. Nevertheless a combination of

2,4-D with a cytokinin (2-iP or BAP) was necessary for callus growth. Somatic embryos and rhizogenesis were induced with an auxin alone (NAA or IBA). Our results differ from those of Figueira and Janick (1995), and those of Chatelet *et al.* (1992) who obtained direct somatic embryogenesis with petal and nucellar explants from undefined genotypes of cacao. These authors could induce somatic embryos on a half-strength Murashige and Skoog (1962) inorganic elements supplemented with a high concentration of 2,4-D and a low concentration of BAP or kin. These studies show that the frequency of somatic embryo induction in cacao depends on the nature of the explant and probably on the genotype. In our experiments, somatic embryogenesis was generally accompanied by root formation.

Embryo differentiation and root growth were characterized by a drop in the level of phenolic compounds in the callus while peroxidase and IAA-oxidase activities generally increased. At the same time, the lack of differentiation (in SERIM supplemented with kin or BAP) was characterized by the disappearance of one acidic isoperoxidase band A4. These results demonstrate that the developmental alterations produced in calli due to different hormonal treatments are due, at least in part, to enzymes that catalyze oxidative reactions. Both peroxidase and IAA-oxidase are oxidative enzymes. A lot of work demonstrates that during callus differentiation, changes in isozymes are often dramatic (Wang *et al.* 1991, Calderon *et al.* 1994, Siminis *et al.* 1994). In this process, phenolic compounds influence oxidative enzymes both as inhibitors and stimulators. Our data show a relationship between phenolic content, peroxidase and IAA-oxidase activities, and the conversion potential of cacao callus. Callus convertion into somatic embryos and roots is characterized by a drop in phenol content, the persistence of one acidic soluble isoperoxidase band A4, and maximum peroxidase and IAA-oxidase activities. The results presented are in agreement with those reported on two other tropical trees: *Sequoiadendron giganteum* (Berthon *et al.* 1993) and *Eucalyptus globulus* (Pacheco *et al.* 1995). It is suggested that phenols as antioxidants would represent a better substrate for oxidative enzymes, leaving the auxins free to promote embryo differentiation and root growth. Genotypic differences were noted in the phenol content, peroxidase and IAA-oxidase activities and the number of acidic soluble peroxidase electrophoretic bands. Nevertheless, the observed biochemical alterations due to hormonal treatment were the same in both cacao genotypes. Further screening of other biochemical indicators (*e.g.* basic peroxidases and the analysis of different types of phenols) will help in predicting developmental events in cacao tissue culture.

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