Localization and Identification of Phenolic Compounds in *Theobroma cacao* L.
Somatic Embryogenesis

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Cocoa breeders and growers continue to face the problem of high heterogeneity between individuals derived from one progeny. Vegetative propagation by somatic embryogenesis could be a way to increase genetic gains in the field. Somatic embryogenesis in cocoa is difficult and this species is considered as recalcitrant. This study was conducted to investigate the phenolic composition of cocoa flowers (the explants used to achieve somatic embryogenesis) and how it changes during the process, by means of histochemistry and conventional chemical techniques. In flowers, all parts contained polyphenolics but their locations were specific to the organ considered. After placing floral explants *in vitro*, the polyphenolic content was qualitatively modified and maintained in the calli throughout the culture process. Among the new polyphenolics, the three most abundant were isolated and characterized by 1H- and 13C-NMR. They were hydroxycinnamic acid amides: *N*-trans-caffeoyl-L-DOPA or clovamide, *N*-trans-p-coumaroyl-L-tyrosine or deoxiclovamide, and *N*-trans-caffeoyl-L-tyrosine. The same compounds were found also in fresh, unfermented cocoa beans. The synthesis kinetics for these compounds in calli, under different somatic embryogenesis conditions, revealed a higher concentration under non-embryogenic conditions. Given the antioxidant nature of these compounds, they could reflect the stress status of the tissues.


INTRODUCTION

Cocoa provides a substantial income for smallholders in the Tropics. Cocoa breeders continue to face the problem of high heterogeneity between individuals derived from one cross, and heterogeneous transmission of genetic traits to the progeny. In this context, the use of somatic embryogenesis, a generally efficient micropropagation technique to multiply elite material, would constitute an important step. A few papers report cocoa somatic embryogenesis from floral parts (Lopez-Baez et al., 1993; Alemanno et al., 1996, 1997; Li et al., 1998). It is now possible to produce plantlets from numerous genotypes (Li et al., 1998). However, cocoa remains a generally recalcitrant species, and the results are inadequate for commercial production of elite material. Recalcitrance is defined as the inability of plant cells, tissues and organs to respond readily to tissue culture (Benson, 2000). One of the factors often considered as a component of *in vitro* recalcitrance is a high phenolic content and oxidation of these compounds. Naturally, cocoa contains large amounts of polyphenolics (Griffiths, 1958; Kim and Keeny, 1983). Their oxidation could be a limiting factor preventing proper tissue multiplication and maintenance. The objectives of the reported work were to investigate, by histochemical means, the composition and location of phenolic compounds in cocoa flowers, and their evolution throughout the somatic embryogenesis process. This paper reports on the qualitative study of this chemical change, focusing on the isolation and characterization of three hydroxycinnamic acid amides never before identified in cocoa callus cultures. Embryogenic and non-embryogenic situations were also compared.

MATERIALS AND METHODS

Plant material and *in vitro* culture

Two genotypes, belonging to two cocoa tree groups, were used: NA 79 (Forastero) and R 106 (Criollo). Flower buds (6–8 mm long) from *Theobroma cacao* L. were collected from trees grown in the glasshouse and surface-sterilized for 20 min in a 10% sodium hypochlorite solution. After dissecting the buds, the staminodes and anthers were cultured on a calllogenesis medium (CM) consisting of Murashige and Skoog’s medium (Murashige and Skoog, 1962) supplemented with glycine (3 mg l−1), lysine (0.4 mg l−1), leucine (0.4 mg l−1), arginine (0.4 mg l−1), tryptophan (0.2 mg l−1), 2,4-dichlorophenoxyacetic acid (2 mg l−1), kinetin (0.25 mg l−1), coconut water (50 ml l−1) and sucrose (40 g l−1) as described previously (Lopez-Baez et al., 1993). After 3 weeks on CM medium, the tissues were transferred onto a hormone-free expression medium (EM), supplemented with glycine (1 mg l−1), lysine (0.2 mg l−1), leucine (0.2 mg l−1), arginine (0.2 mg l−1), tryptophan (0.1 mg l−1), coconut water (100 ml l−1) and sucrose (40 g l−1). On this medium, somatic embryos regenerated from the calli.

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**Histochemistry**

Histochemical localization of polyphenolics was carried out on fresh flower buds and calli derived from staminodes/ anthers (7, 21 and 42 d after culture initiation) of NA 79 (Forastero) and R 106 (Criollo) genotypes. After adding the material to an 8% agar solution, 50-μm-thick sections were cut with a vibration microtome (Micro-cut H1200; Bio-rad; frequency 4 and speed 4). Fresh sections were immediately mounted on microscope slides with different chemicals depending on the polyphenolic compound being sought. Naturally coloured anthocyanins were directly observed with a photonic microscope in visible light on sections mounted in 15% glycerine water. For red-staining tannins, sections were placed for a few minutes in chlorhydric vanillin and mounted in a drop of this reagent (Dai et al., 1995). Observations were made in visible light. For hydroxycinnamic derivatives, sections were placed for a few minutes in Neu’s reagent [1% 2-aminoethyl diphenyl borinate (Fluka, Chemie AG, Buchs, Switzerland) in absolute methanol] and mounted in 15% glycerine water (Dai et al., 1995). Observations were carried out using a light microscope (Nikon Optiphot) with two filter sets: a UV filter set with 365 nm excitation and a 400 nm barrier filter, and a blue filter with 420 nm excitation and a 515-560 nm barrier filter. Several sections were used with five replicates for each treatment.

**Extraction of polyphenolics**

Extraction was identical for both qualitative and quantitative studies. Twenty milligrams of freeze-dried material were ground in liquid N2 and extracted with 15 ml MeOH–H2O (8 : 2) for 15 min at 4 °C. After adding the material to an 8% agar solution, 50-μm-thick sections were cut with a vibration microtome (Micro-cut H1200; Bio-rad; frequency 4 and speed 4). Fresh sections were immediately mounted on microscope slides with different chemicals depending on the polyphenolic compound being sought. Naturally coloured anthocyanins were directly observed with a photonic microscope in visible light on sections mounted in 15% glycerine water. For red-staining tannins, sections were placed for a few minutes in chlorhydric vanillin and mounted in a drop of this reagent (Dai et al., 1995). Observations were made in visible light. For hydroxycinnamic derivatives, sections were placed for a few minutes in Neu’s reagent [1% 2-aminoethyl diphenyl borinate (Fluka, Chemie AG, Buchs, Switzerland) in absolute methanol] and mounted in 15% glycerine water (Dai et al., 1995). Observations were carried out using a light microscope (Nikon Optiphot) with two filter sets: a UV filter set with 365 nm excitation and a 400 nm barrier filter, and a blue filter with 420 nm excitation and a 515-560 nm barrier filter. Several sections were used with five replicates for each treatment.

**Two-dimensional thin layer chromatography**

The concentrated extracts were separated on cellulose layers (Merck, ref. 5552 KGaA, Darmstadt, Germany) by two-dimensional thin layer chromatography (2D TLC), using BuOH-HOAc-H2O (4 : 1 : 2:2) (solvent I) in one direction then 2% OHAc (solvent II) in the other.

**Analytical HPLC**

This was performed on a 250 mm × 4-6 mm Kromasil 5 μm C18 analytical column at 22 °C. A linear gradient from 20–65% acetonitrile in H2O, pH 2-6 for 20 min was used. The flow rate was 1 ml min⁻¹. Detection was at 330 nm.

**Purification and isolation for NMR studies**

This was done with an XAD 761 Amberlite column resin (50 cm × 2 cm) which is an absorbent duolite. The fractions containing the hydroxycinnamic acid amides were eluted in water and then purified on a Sephadex LH 20 column (50 cm × 2 cm) with water as the elution solvent. Final purification of the three hydroxycinnamic acid amides was performed with semi-preparative HPLC on a 250 mm × 10 mm Kromasil 10 μm C18 analytical column at 22 °C. A linear gradient from 30–48% MeOH in H2O and a flow rate of 20 ml min⁻¹ was used.

**NMR studies**

NMR spectra were measured at 360-13 MHz for 1H-NMR and for 90-53 MHz for 13C-NMR (Brucker AMX 360 apparatus). Chemicals were dissolved in DMSO-d6 and the chemical shifts were given on the parts per million scale with TMS as the internal standard. The location of 1H and 13C was determined using COSY (correlation spectroscopy) two-dimensional experiments, NOESY (nuclear overhauser effect spectroscopy), HMQC (heteronuclear multi-quanta correlation) and HMBC (heteronuclear multi-bond correlation).

**Biochemical measurements**

Total phenolics were measured with Folin-Ciocalteu reagent (Dai et al., 1994). Twenty-five microlitres of the extracts were mixed with 110 μL Folin-Ciocalteu reagent, 200 μL 20% sodium carbonate and 1-9 ml distilled water, and placed at 60 °C for 30 min. Optical density was measured with a spectrophotometer at 750 nm. A standard curve was established beforehand with chlorogenic acid.

Condensed tannins were measured with DMCA (Dai et al., 1995). Ten microlitres of extract were mixed with 200 μL DMCA reagent (100 mg DMCA + 10 mL 12 N hydrochloric acid + 90 mL methanol) and 1 mL methanol. Optical density was measured with a spectrophotometer at 640 nm, after 10 min at room temperature. A standard curve was established beforehand with (-)-epicatechin. Three hydroxycinnamic derivatives were analysed by HPLC as previously described (Analytical HPLC). For determination of peak surfaces, three standard curves were established beforehand. Two measurements were taken per replicate.

Measurements of total phenolics, condensed tannins, and the three hydroxycinnamic derivatives were taken on three replicate replicates for each treatment (genotype and 2,4-D concentrations). Measurements were taken on days 0, 21, 32 and 42 of the somatic embryogenesis process. The data sets were processed by the Newmann–Keuls test, at the 5% level.

**RESULTS**

**Histo-localization of polyphenolics**

In cocoa flowers. The cocoa flower has five free sepals, five free petals, five staminodes, five stamens (Fig. 1A) and an ovary of five united carpels. The petals comprise two distinct parts: a flat yellow ligule and a cup-shaped white and purple pouch. Several polyphenolics were found in cocoa flowers. Hydroxycinnamic acids and flavonoids were
revealed under UV and blue light with Neu's reagent reactive; tannins were revealed under white light with chlorhydric vanillin; and anthocyanins were naturally stained red. All floral parts contained polyphenolics but their location and grouping were specific to the organ studied (Table 1; Fig. 1B–M). Generally, hydroxycinnamonic acids were located in the epidermis of sporophytic organs: sepals (Fig. 1B), petals (Fig. 1C, F and G), staminodes and stamen filaments (Fig. 1I). In the ovary, these compounds were present in all tissues except the epidermis (Fig. 1L). Tannins were often present in all tissues, both epidermal and parenchymal (Fig. 1E, H and K). Anthocyanins were present in the epidermis and parenchyma of specific parts of the petals (Fig. 1D) and staminodes (Fig. 1J). No difference was detected between the two genotypes NA 79 (embryogenic) and R 106 (non-embryogenic).
In staminodes, during the somatic embryogenesis process. At culture initiation, hydroxycinnamic derivatives were located only in the epidermis of staminodes (Fig. 1I). Seven days after culture initiation, most parenchyma cells of the staminodes contained hydroxycinnamic derivatives, which were probably different from those present at culture initiation (Fig. 2A). Tannins and anthocyanins tended to disappear. At day 21, the embryogenic genotype NA 79 consisted of meristematic nodules (Alemanno et al., 1996) containing hydroxycinnamic derivatives (Fig. 2B). Tannins were exclusively located in cells situated around the meristematic nodules (Fig. 2C). In the second phase of the culture, the same observations were made. When somatic embryos regenerated, they were free of tannins (Fig. 2D) and had very small amounts of hydroxycinnamic derivatives. For the non-embryogenic genotype R 106 at day 21, calli were compact and non-meristematic (Alemanno et al., 1996). Such structures contained hydroxycinnamic derivatives distributed in a uniform manner (Fig. 2E). The location of tannins differed from that in embryogenic callus, being distributed in a random manner all over the callus (Fig. 2F). Of these molecules, a marker to distinguish embryogenic from non-embryogenic calli could be a marker to distinguish embryogenic from non-embryogenic calli (Table 3). There was no qualitative difference between the tannins present in floral explants and the derived calli (data not shown).

HPLC studies. Hydroxycinnamic acid derivatives of staminodes/anthers and derived calli at day 21 were separated by HPLC (Fig. 3C). A comparison of both profiles confirmed the results obtained by 2D TLC. The two peaks, a and b, which may be minor caffeic acid derivatives, were common to both profiles. Five other peaks, c–g, were present only in calli. Of these, e–g were the most abundant and were more precisely identified. Correspondence between 2D TLC spots and HPLC peaks was established (Fig. 3H). A comparison with fresh bean extracts showed clearly the presence of these three compounds in the beans (Fig. 3D).

Identification of three hydroxycinnamic amides. UV spectra for these three compounds (Fig. 3E–G) were characteristic of hydroxycinnamic derivatives. Peaks 1 and 2 with respective maximum absorption (290 nm and 323 nm, Fig. 3E; 296 nm and 322 nm, Fig. 3F) were derivatives of caffeic acid, and peak 3 (292 nm and 310 nm, Fig. 3G) a derivative of p-coumaric acid. These data confirmed the previous 2D TLC study. Alkaline hydrolysis experiments confirmed the presence of caffeic or p-coumaric acid in these molecules. A second component of these molecules was identified as an amide or an amino acid.

Final identification was carried out using NMR studies (Table 4). Peak 1 was defined as N-trans-cafeoyl-L-DOPA, peak 2 as N-trans-cafeoyl-L-tyrosine and peak 3 as N-trans-p-coumaroyl-L-tyrosine. These compounds differed in the degree of hydroxylation and could derive from one another (Fig. 3H).

Quantitative studies of variations in phenolics during somatic embryogenesis. Total phenolics, condensed tannins, N-trans-cafeoyl-L-DOPA, N-trans-cafeoyl-L-tyrosine and N-trans-p-coumaroyl-L-tyrosine were measured during tissue culture on two genotypes differing in their embryogenic capacity (Fig. 4). R 106 was non-embryogenic (Table 5) and

<table>
<thead>
<tr>
<th>Location</th>
<th>Compound Class</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepal</td>
<td>Hydroxycinnamic acids</td>
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</tr>
<tr>
<td>Petal</td>
<td></td>
<td>Epidermal hair (Fig. 1B)</td>
</tr>
<tr>
<td>Ligule</td>
<td></td>
<td>Sub-epidermis and parenchyma</td>
</tr>
<tr>
<td>Pouch</td>
<td></td>
<td>Ornamental parenchyma (Fig. 1D)</td>
</tr>
<tr>
<td>Staminode</td>
<td></td>
<td>Epidermis and parenchyma (Fig. 1E)</td>
</tr>
<tr>
<td>Anther filament</td>
<td></td>
<td>Epidermis and parenchyma (Fig. 1K)</td>
</tr>
<tr>
<td>Ovary</td>
<td></td>
<td>Epidermis and parenchyma (Fig. 1M)</td>
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</tbody>
</table>

TABLE 1. Location of different classes of polyphenolics in cocoa flowers

Biochemical characterization of some phenolic compounds, particularly hydroxycinnamic acid derivatives, found in staminodes/anthers and derived calli

Thin layer chromatography (TLC) studies. Two-dimensional TLC was performed on staminodes/anthers (Fig. 3A) and derived calli at day 21 (Fig. 3B). The polyphenolic profiles of these two explants were significantly different. The floral organs contained two anthocyanins as well as three phenolic acids or derivatives. These were not studied in detail, but their response to UV light and to a few spray reagents revealed their chemical nature (Table 2). The profiles were qualitatively identical whichever genotype was analysed. The 2D TLC callus profile was far more complex (Fig. 3B) than that of the original explants (Fig. 3A). Seven hydroxycinnamic acid derivatives were revealed, all differing from those contained in the floral explants. They were identified as phenolic acid derivatives (Table 3).
NA 79 was non-embryogenic or embryogenic, depending on the 2,4-D concentration of the callus medium: 0 or 2 mg l⁻¹ (Table 5).

Total phenolics and tannins evolved in a similar way during tissue culture, irrespective of genotype and embryogenic capacity (Fig. 4A and B). They were abundant in flowers, but their concentrations decreased during the process. However, from day 21 for tannins and day 32 for total phenolics, significant concentrations were observed in different callus, with non-embryogenic R 106 calli containing the smallest amount of phenolics and tannins. These compounds were immediately concentrated in embryogenic genotype NA 79 cultured under conditions in which embryogenic capacity was expressed. Conversely, total phenolics and tannins were significantly higher in NA 79 explants placed under conditions in which embryogenic capacity could not be expressed (no 2,4-D in the medium).

Under these conditions, a histological study showed that cells of the staminodes/anthers hardly divided, accumulated starch and phenolics, and finally degenerated (data not shown).

The kinetics of the three identified hydroxycinnamic amides were different from those of total phenolics and tannins (Fig. 4C–E). They were absent from flowers, but their synthesis started and increased with tissue culture until approx. day 21. In the second phase of the culture, these compounds decreased except in explants of NA 79 on 2,4-D-free medium where they continued to increase. The clearest example is that of clovamide or N-trans-caffeoyl-L-DOPA, the most abundant hydroxycinnamic amide. In the second phase of the culture, it was significantly higher in NA 79 calli on 2,4-D free medium. Between R 106 and embryogenic NA 79, this compound was significantly higher in non-embryogenic R 106. This
Alemanno et al. — Phenolic Compounds in Theobroma cacao L.
difference disappeared at the end of the culture process. The same tendency was noticed also for the other two hydroxy-
cinnamic amides.

DISCUSSION

Cocoa flowers contained different polyphenolics: hydro-
xycinnamic acids were located on the periphery of organs, except in the ovary where they were present in parenchyma
cells and ovules; tannins and anthocyanins were located both in the epidermis of different floral parts and also internally; anthocyanins were restricted to ornamentation in petals and staminodes. This mainly external positioning of polyphenolics has been observed in other plants. Tannins are present in fruit pericarp and in the root and stem cortex of *Struthanthus vulgaris* (Salatino *et al.*, 1993). In the radicle of germinating *Brassica napus* zygotic embryos, phenolic compounds are located outside the epidermis (Zobel *et al.*, 1989). This external location might suggest a protective chemical-barrier role for phenolics against
damaging environmental factors.

A comparison between cultured floral explants and
derived calli, by both histochemistry and chromatography,
revealed clearly the synthesis of new phenolic compounds
in the calli, including four caffeic derivatives and three
hydroxycinnamic amides. The same profile was observed
irrespective of the callus stage and genotype. Synthesis of
new molecules in cultured tissues compared with the mother
plant is sometimes observed. In *Eucalyptus robusta*, calli
derived from seedling hypocotyls had the same qualitative
phenolic content as wood and bark, although quantitative
differences were observed (Yamaguchi *et al.*, 1986). In
*Crataegus* calli, phenolic composition was simpler than in
floral buds (Bahorun *et al.*, 1994). Conversely, on a different
original explant of the same species, shoot tips indicated
synthesis of new flavonoids, and several benzoic and

![Chemical formulae of phenolic compounds.](image)

Fig. 3. Characterization of phenolics induced by *in vitro* culture conditions. A, 2D TLC of NA 79 staminodes with corresponding diagram showing anthocyanins (An) and hydroxycinnamic acid derivatives (A, A', B and C). B, 2D TLC of NA 79 calli derived from staminodes/anthers 21 d after


hydroxycinnamic acid amides in the calli (Kartnig et al., 1993). Tahrouch-Skouri et al. (1993) showed one newly synthesized caffeic acid heterosidic ester in leaf calli. Surprisingly, the phenolic composition of cocoa calli was closer to that of cocoa beans than to that of the staminodes from which they were derived. The synthesis of new compounds could be due to cellular dedifferentiation and/or to a response to wounding and stress, induced by in vitro culture conditions.

Of the new synthesized phenolics, the three most abundant were identified as hydroxycinnamic amides. Both N-trans-cafeoyl-L-DOPA, or clovamide, and N-trans-p-coumaroyl-L-tyrosine, a deoxy analogue of clovamide: deoxyclovamide, have already been identified in the tropical tree Dalbergia melanoxylon (Van Heerden et al., 1980) and more recently in cocoa liquor (Sanbongi et al., 1998). In this paper, the antioxidant effect of these compounds in vitro has been demonstrated. Clovamide

### Table 2. Characteristics of phenolic compounds in staminodes/anthers separated by 2D TLC

<table>
<thead>
<tr>
<th>Spot</th>
<th>Observations</th>
<th>Conclusions</th>
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</thead>
<tbody>
<tr>
<td>A–A’</td>
<td>Fluorescence after spraying with Benedict’s reagent</td>
<td>Derivative of caffeic acid</td>
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<tr>
<td>B</td>
<td>Non-extinction of blue fluorescence after spraying with Benedict’s reagent</td>
<td>Hydroxycinnamic derivative</td>
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<tr>
<td>C</td>
<td>Non-extinction of blue fluorescence after spraying with Benedict’s reagent</td>
<td>Sinapic or ferulic derivative</td>
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</table>

Benedict’s reagent induces fluorescence of mono-hydroxylated phenolics and extinction of fluorescence of di-hydroxylated phenolics.

### Table 3. Characteristics of phenolic compounds in calli separated by 2D TLC

<table>
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<th>Spots</th>
<th>Observations</th>
<th>Conclusions</th>
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<tr>
<td>1–1’, 2–2’, 5–5’, 6–6’</td>
<td>Extinction of fluorescence after spraying with Benedict’s reagent</td>
<td>Derivative of caffeic acid</td>
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<tr>
<td>3–3’</td>
<td>Separation by an aqueous solvent: 2 % acetic acid</td>
<td>Isomers</td>
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<td>4–4’</td>
<td>Intensification of dark fluorescence under UV light after ammonia spraying</td>
<td>Derivative of para-coumaric acid</td>
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<tr>
<td>7</td>
<td>Non-extinction of fluorescence after spraying with Benedict’s reagent</td>
<td>Non-derivative of caffeic acid</td>
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Benedict’s reagent induces fluorescence of mono-hydroxylated phenolics and extinction of fluorescence of di-hydroxylated phenolics.

### Table 4. $^1$H- and $^{13}$C-NMR spectrum of the compounds

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<tr>
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<th>$^1$H 1</th>
<th>$^1$H 2</th>
<th>$^1$H 3</th>
<th>$^1$C 1</th>
<th>$^1$C 2</th>
<th>$^1$C 3</th>
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<td>Caffeic acid (1 and 2)</td>
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<td>126-1</td>
<td>125-9</td>
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<td>$p$-Coumaric acid (3)</td>
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<td>6-93 (d; 2-0)</td>
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<td>2-80 (dd; 7-9; 13-4)</td>
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<td>9-9’</td>
<td>4-21 (m)</td>
<td>4-41 (m)</td>
<td>4-35 (m)</td>
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<td>NH 8-04 (broad singulet)</td>
<td>NH 7-79</td>
<td>9-174-7</td>
<td>174-3</td>
<td>174-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4’ OH 9-14</td>
<td>4’ OH 9-12</td>
<td></td>
</tr>
</tbody>
</table>

* Values shown in brackets are shifts and J values (Hz).
was first reported in *Trifolium pratense* stems and leaves (Yoshihara et al., 1974). The presence of these two compounds has been demonstrated in cocoa beans and, for the first time, in calli. This is also the first report of N\(^{\text{trans}}\)\(-\)caffeoyl-L-tryosine in cocoa beans and calli, although it has already been identified in Angola green coffee beans (Clifford et al., 1989). The physiological roles of hydroxycinnamic amides are not clear. Hydroxycinnamic amides with aliphatic amines (spermidine, putrescine) are widely distributed in flowering plants (Martin-Tanguy et al., 1978) and are often characteristic of ovaries and anthers (Ponchet et al., 1982; Leubner-Metzger and Amrhein, 1993) as well as of maize embryos and endosperm tissues where they accumulate (Martin-Tanguy et al., 1982). Hydroxycinnamic amides are not clear. Hydroxycinnamic amides with aliphatic amines (spermidine, putrescine) are widely distributed in flowering plants (Martin-Tanguy et al., 1978) and are often characteristic of ovaries and anthers (Ponchet et al., 1982; Leubner-Metzger and Amrhein, 1993) as well as of maize embryos and endosperm tissues where they accumulate (Martin-Tanguy et al., 1982). Hydroxycinnamic...
amides containing an amino acid have been found more rarely, and only in tissue cultures, Arabidopsis thaliana cell suspensions synthesized coumaroylaspartate (Mock et al., 1993). Feruloylaspartate was identified in Beta vulgaris (Bokern et al., 1991a) and in Chenopodium rubrum (Bokern et al., 1991b) cell cultures. No physiological role was suggested. In Ephedra distachya, as the production of \( p \)-coumaroylglycine and \( p \)-coumaroylalanine by cell suspensions was induced after adding yeast extracts to the medium, Song et al. (1992) suggested that these compounds might be produced as phytoalexins in intact plants. In a review on hydroxycinnamates and their conjugates, Kroon et al. (1999) focused on their potent antioxidant activity. Pearce et al. (1998) demonstrated the synthesis of \( E \)-feruloyltyramine and \( E \)-p-coumaroyltyramine in response to wounding in tomato leaves. In cocoa beans and calli, because of the antioxidant nature of \( N \)-trans-\( p \)-coumaroyl-L-tyrosine and \( N \)-trans-caffeoyl-L-DOPA, or clovamide (Sanbongi et al., 1998), the two most abundant hydroxycinnamic amides in calli, the synthesis of these compounds could be a protective reaction against stress.

The purpose of the final experiments described here was to identify a possible relationship between phenolic composition and embryogenic capacity. At the flower level, no significant differences were observed. Under embryogenic conditions (\( 2 \), 106, 24-D at 2 mg \( L^{-1} \)), the concentrations of total phenols and tannins were intermediate compared with their lower concentrations under non-embryogenic conditions (\( R \) 106, 24-D at 2 mg \( L^{-1} \)) and to their higher concentrations under non-embryogenic condition (NA 79, 24-D at 0 mg \( L^{-1} \)). Embryogenic capacity, therefore, seemed to be associated with a balanced concentration of phenolics. High concentrations of hydroxycinnamic acid amides were associated with the non-embryogenic response. Owing to the antioxidant nature of these molecules (Pearce et al., 1998; Sanbongi et al., 1998; Kroon and Williamson, 1999) they are probably more related to stress conditions than to the embryogenic status itself. The more the tissues were stressed, the more of these substances were synthesized.

Somatic embryogenesis could be expressed under less stressful conditions. Apparently, this reaction against stressful conditions was not sufficient to protect the tissues. It is planned to carry out a more detailed study of the different protective systems that protect cocoa tissues against stress in \( \text{vitro} \) (e.g. detoxification). This should help to define recalcitrance.

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