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Phenolic compounds in hormone-dependent and independent sugarbeet callus lines compared to donor-plants

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ABSTRACT

ENGELMANN, I., J.-J. MACHEIX & Th. GASPAR (1993). Phenolic compounds in hormone-dependent and independent sugarbeet callus lines compared to donor-plants. *Saussurea* 24: 15-21. In English, English and French abstracts.

Phenolic compounds were extracted with cold ethanol (80%) from a normal, auxin- and cytokinin-dependent, sugarbeet callus and from two habituated, hormone-independent, callus lines, to be compared with phenolics of leaves from the donor-plant. After purification, they were separated by HPLC and analysed spectrophotometrically. A major -but different- compound was HPLC separated from each the donor-plant and the habituated shoot-producing callus. These compounds were spectrophotometrically characterized as being of the flavonoid family. The HPLC profile from the normal callus was more heterogeneous with a main component identified as a ferulate derivative. A major benzoic acid derivative characterized the habituated non-organogenetic callus.The respective role of these phenolic compounds is briefly discussed but assessed with difficulty at the time being.

RÉSUMÉ

ENGELMANN, I., J.-J. MACHEIX & Th. GASPAR (1993). Composés phénoliques comparés dans des feuilles et des cals, normaux et habitués, de betterave sucrière. *Saussurea* 24: 15-21. En anglais, résumés anglais et français.

Les composés phénoliques ont été extraits à l'éthanol froid (80%) d'un cal de betterave normal, auxine- et cytokinine-dépendant et de deux lignées de cals habitués, hormone-indépendants, et comparés avec les composés phénoliques extraits de feuilles de la plante-mère. Après purification, ils ont été séparés par HPLC et analysés par spectrophotométrie. Un composé majoritaire différent a été isolé par HPLC chez la plante-mère et le cal habitué organogène. Ces composés ont été caractérisés par spectrophotométrie comme appartenant à la famille des flavonoïdes. Le profil HPLC du cal normal était plus hétérogène, avec comme composé majoritaire un dérivé d'acide férulique. Un dérivé de l'acide benzoïque majoritaire caractérisait le cal habitué non organogène. Les rôles respectifs de ces composés phénoliques sont discutés brièvement mais ne peuvent à l'heure actuelle qu'être imparfaitement déterminés.

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Introduction

Three callus lines differing by their hormone dependence and organogenetic capacities have been initiated from sugarbeet leaf pieces in 1979 at the University of Brussels (DE GREEF & JACOBS, 1979). They were kept through regular subcultures from that time at the University of Liège, without loosing their main characteristics. A normal auxin- and cytokinin-dependent callus line is green and compact, produces and releases much peroxidase and ethylene and differentiates unorganized xylem cells (KEVERS & al., 1981a; GASPAR & al., 1988, 1991a; CRÈVECOEUR & al., 1987; HAGÈGE & al., 1991). A green fully habituated (auxin- and cytokinin-independent) callus line is regenerating shoots automatically, without special treatment (KEVERS & al., 1981b; PENEL & al., 1984; GASPAR & al., 1988). The third one, a fully habituated one completely differs from the two preceding in that it is white with very few chlorophylls, looks hyperhydric, produces and releases very few peroxidase activity and ethylene, and does not differentiate xylem cells (KEVERS & al., 1981a; GASPAR & al., 1983, 1988; CRÈVECOEUR & al., 1987; HAGÈGE & al., 1991). Cells from the latter show many characteristics (among others abnormal polyploid and aneuploid big nuclei) of cancerous cells (GASPAR & al., 1991a; HAGÈGE & al., 1992a; CRÈVECOEUR & al., 1992).

The present study aims at analysing the phenolic contents of three calli, compared with the donor mother-plant. Phenolic compounds indeed are obligatory intermediates in lignin formation thus in cell differentiation, partly mediated by peroxidases (GASPAR & al., 1991b). Phenolic compounds are also effectors of auxin catabolism (PILET & GASPAR, 1968) and have been involved in auxin autotrophy (STONIER, 1970). Phenolic compounds can also be considered as reductants (MACHEIX & al., 1990). The habituated non-organogenetic callus precisely has been shown to be well protected against activated forms of oxygen through superior activities of the so-called protective enzymes superoxide dismutase, catalase, ascorbate peroxidase (HAGÈGE & al., 1992b), and through deficiency of tetrapyrrole-containing compounds generating such oxygen species (HAGÈGE & al., 1992c).

Materials and methods

Plant material and culture conditions

Experimental conditions for obtaining normal (auxin- and cytokinin-requiring) and habituated (auxin- and cytokinin-independent) calli of sugarbeet (*Beta vulgaris* var. *altissima*) and for maintaining these tissues in stock solid cultures have been reported elsewhere (DE GREEF & JACOBS, 1979; KEVERS & al., 1981). Such calli, when subcultured every three weeks on their respective medium (basal medium without plant growth regulators in the case of the habituated line, but supplemented with 0.1 mg/l 2,4-D and 0.1 mg/l BAP in the case of the normal one) have shown constant cytological and ultrastructural characteristics during at least one year when taken for the present analyses.

Phenol extraction

Soluble phenolic compounds of the three callus lines and leaves from the donor plant were extracted with cold aqueous ethanol 80% (4°C) until the solid residue was exhausted. After elimination of pigments by petrol ether, polyphenols were purified by ethyl acetate then put back in methanol according to a method previously established (MACHEIX &

al., 1990). The extracts thus obtained were used for separation and identification of soluble phenolic compounds.

Phenol analysis

The extracts were analysed by HPLC (Waters 990, equiped with a photodiode detector 600 E). This apparatus was connected with a computerized integrator NEC APC VI (Millipore software). It was equiped with a silice reversed phase column and precolumn (Macherey and Nagel C 18). The gradient programme was set as indicated in Table 1.

Spectrophotometric analyses of the global phenolic extracts were performed using a DMS 200 spectrophotometer. The absorption spectrum of each individual compound was obtained by coupling the spectrophotometer with the HPLC Waters 990.

Time (min)	0	20	28	40	45	47	50	52	55
Acetonitrile (%)	10	20	23	27	29	33	80	80	10

Table 1. – Gradient programme for HPLC analysis of methanolic extracts.

Alkaline hydrolysis

A fraction of the methanolic extracts (200 µl) of the three callus lines and leaves from the donor plant were evaporated and then suspended in 1 ml of 2N NaOH and shaken for 4 hrs at 25°C under nitrogen atmosphere. The ester bounds are easily broken under these conditions. The solutions were acidified to pH 2 with concentrated chlorhydric acid and extracted with four volumes of ethyl acetate. The ethyl acetate was evaporated under a stream of nitrogen and the residue taken up in 500 µl of methanol. The resultant solution was subjected to HPLC and co-analysed by TLC.

Thin-layer chromatography (TLC)

Thin layer chromatography was performed on silica plates with a solvent system of benzene, acetic acid, water (6-7-3).

Results

HPLC profiles

HPLC profiles of methanolic extracts from whole leaves of sugarbeet mother-plant, normal callus, habituated organogenetic and non-organogenetic calli are presented in Fig. 1 A, B, C, D, respectively. The most resembling profiles are those from the mother-plant (Fig. 1 A) and the habituated shoot-producing callus (Fig. 1 C) with peaks called a1, a2 hereafter characterized . The normal callus (Fig. 1 B) reveals a typical b peak, represented at a quite lower level in the habituated non-organogenetic callus (Fig. 1 D). The latter additionnally exhibits a peak (c) not found in any of the other materials.

Characterization of compounds corresponding to peaks a1, a2, b, c

Spectra of peaks called a1, b, a2, c from sugarbeet mother-plant leaves, normal callus, habituated organogenetic, non-organogenetic calli are presented in Fig. 2, A, B, C, D, respectively.

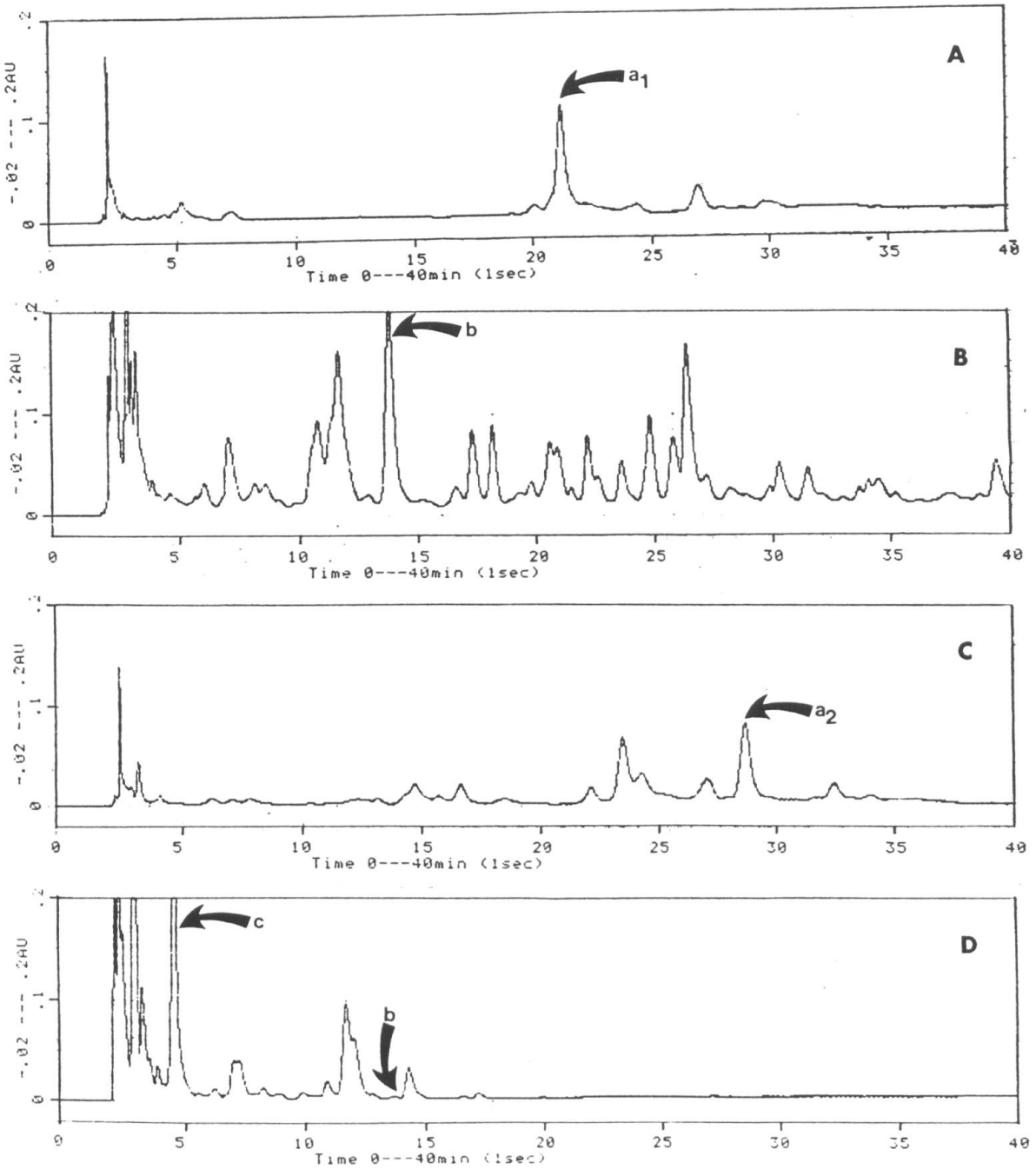

Fig. 1. — HPLC profiles of phenolic compounds separated from sugarbeet donor-plant leaves **(A)**, normal callus **(B)**, habituated organogenic **(C)** and non-organogenic **(D)** calli.

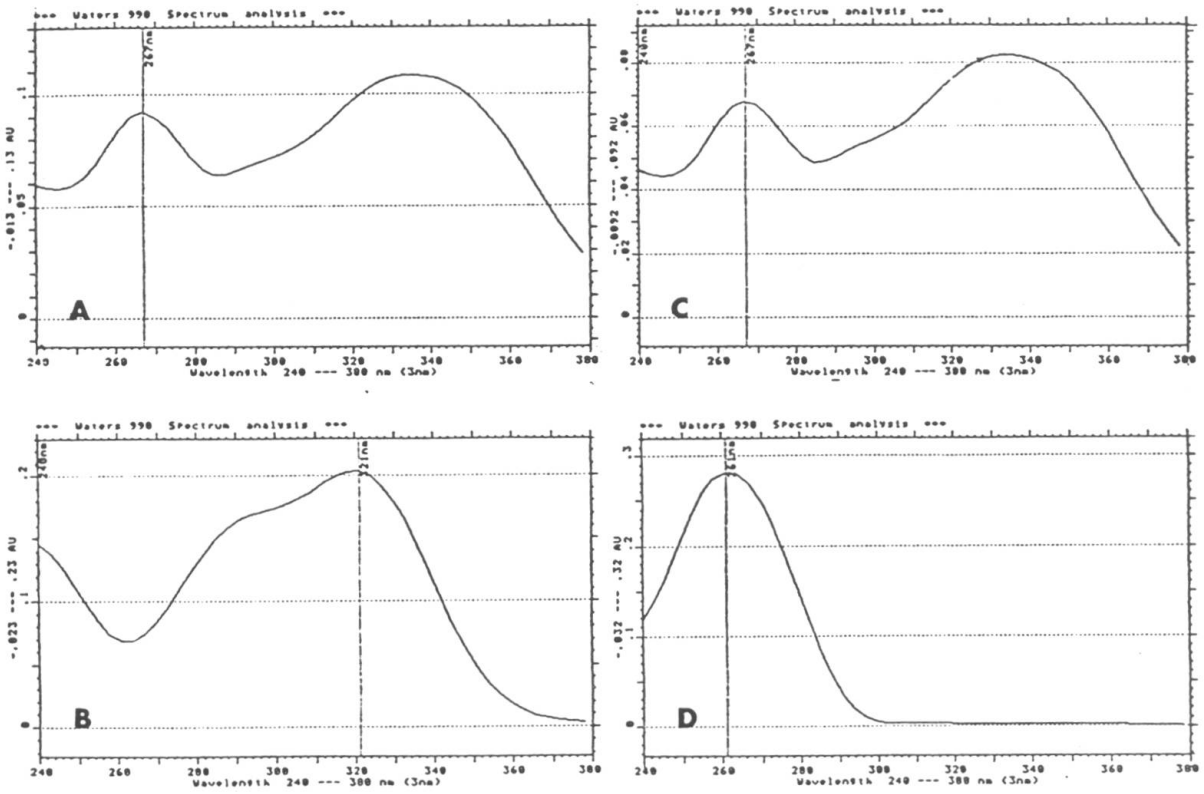

Fig. 2. — Spectra of the main phenolic compounds a1 **(A)**, b **(B)**, a2 **(C)** and c **(D)** separated by HPLC from respectively leaves of donor-plant, normal callus, habituated organogenic and habituated nonorganogenic callus.

Spectra of peaks a1 and a2 from mother-plant leaves (Fig. 2 A) and shoot-producing habituated callus (Fig. 2 C) respectively were similar with a maximal absorption at 267 nm and a shoulder around 330-340 nm, characterized as being from the flavonoid family. The normal callus (Fig. 2 B) did not exhibit the maximal absorption at 267 nm but at 321 nm with a shoulder at 290 nm, characterized as a ferulate derivative by alkaline hydrolysis which gave ferulic acid identified by co-TLC and co-HPLC. The habituated non-organogenetic callus (Fig. 2 D) presented a unique peak at 261 nm, characterized as being benzoic acid derivative but not identified. However, alkaline hydrolysis gave p-hydroxybenzoic acid identified by co-TLC and co-HPLC.

Discussion

Table 2 shows the repartition of the main phenolic compounds/families identified in the three sugarbeet calli compared with leaves from a mother-plant. There was a great resemblance between the mother-plant and the organogenetic callus, both containing the phenolic compounds a, probably because of the presence of many leaved shoots in the latter. The normal and the habituated non-organogenetic calli did not contain any of the phenolics a but specifically compounds of the b (ferulate derivatives) and c (benzoic acid derivatives) families respectively.

Ferulate derivatives of the b family, as found specifically in the normal callus also have been found in other heterotrophic unorganized systems such as cell-suspension

Compounds	Mother-plant	N callus	HO callus	HNO callus
a	+		+	
b		+		(+)
c				+

Table 2. – Distribution of the main phenolic compounds families (a: flavonoid; b: ferulate derivative; c: benzoic acid derivative) in sugarbeet mother-plant, normal (N), habituated organogenetic (HO) and habituated non-organogenetic (HNO) calli.

cultures (BOKERN & al., 1991a, b). The significance of their presence specifically in unorganized normal auxin- and cytokinin-dependent callus is not known. Might they be involved in the differentiation of unorganized tracheary elements in such cultures?

The habituated non-organogenetic callus specifically contains benzoic acid derivatives. In the course of neoplastic progression (GASPAR & al., 1991) of that callus type, these compounds can be formed by β-oxidation of the cinnamoyl-coA which can originate directly from hydroxycinnamic acids activated by coA through hydroxycinnamoyl coA:ligase activity (RHODES & WOOLTORTON, 1976).

The functions of these benzoic acid derivatives in such a fully habituated callus are not known. They might simply act as reductants in that callus which has been shown to be particularly protected against active forms of oxygen (HAGÈGE & al., 1992b). They also might act as auxin-protectors against the auxin-oxidase system as suggested by STONIER (1970) to explain the auxin-autonomy. Such auxin-protectors have also been found in other autonomous callus or crown gall cultures (ATSUMI & HAYASHI, 1978; SYONO, 1979). Further investigations will aim also at looking whether some of the analyzed phenolic compounds might substitute for auxin and/or cytokinins as cell-division promoting factors as shown by TEUTONICO & al. (1991) in autonomous crown gall tumors.

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