Pergamon

PII: S0031-9422(98)00210-6

Phytochemistry, Vol. 49, No. 3, pp. 697–702, 1998

Printed in Great Britain
0031-9422/98/$—see front matter

EFFECTS OF CINNAMIC ACID ON POLYPHENOL PRODUCTION IN *PLANTAGO LANCEOLATA*

FRANÇOISE FONS,† ANNICK GARGADENNEC,† ALAIN GUEIFFIER,‡ JEAN LOUIS ROUSSEL† and CLAUDE ANDARY*†

†Laboratoire de Botanique, Phytochimie et Mycologie, Faculté de Pharmacie, 15 Avenue C. Flahault 34060 Montpellier, Cedex 2 France and ‡Laboratoire de Chimie Thérapeutique, Faculté de Pharmacie, 31 Avenue Monge 37200 Tours, Cedex France

(*Received in revised form* 4 *March* 1998)

Key Word Index—*Plantago lanceolata*; Plantaginaceae; *in vitro* culture; roots; caffeic acid glycoside esters; plantamoside; verbascoside; (*E*)-cinnamic acid.

Abstract—Ribwort (*Plantago lanceolata*) contains two main caffeic acid glycoside esters, plantamoside and verbascoside. These two polyphenols were investigated in the aerial and underground parts of *in vitro* cultured ribworts. For the first time, it is reported that, whatever the age of this plant, plantamoside and verbascoside are concentrated in the roots with plantamoside levels double those of verbascoside. When *P. lanceolata* was transferred into a medium containing 10^{-3} M (*E*)-cinnamic acid, this chemical stress induced a slow degeneration of the initial roots. These were superseded by neoroots whose morphology was atypical during the first eight days following their appearance. In the initial roots, (*E*)-cinnamic acid induced a temporary appearance of two cinnamic acid derivatives (NCD), but did not change the plantamoside and verbascoside levels. In the neoroots, high NCD levels were detected for only eight days. After the large decrease of these NCD, plantamoside and verbascoside appeared and increased. These NCDs have been identified as glucoside esters of ferulic and *p*-coumaric acids. These two compounds, which are absent from the traditional chemical profile of ribwort, probably arose from a (*E*)-cinnamic acid detoxification pathway.

INTRODUCTION

In folk medicine, the aerial parts of *Plantago lanceolata* are used as an anti-inflammatory, antibacterial, healing, diuretic and anti-asthmatic remedy without toxicity [1–5]. For this reason, many authors have studied the chemical composition of *P. lanceolata* leaves and isolated several active components that could explain the numerous folk uses of this plant. *P. lanceolata* contains iridoids (catalpol, aucubin, asperuloside) with laxative and diuretic activities [6–10] and flavonoids (apigenin 7-*O*-glucoside, scutellarein) that are anti-inflammatory [9–11]. Caffeic acid glycoside esters (CGEs), i.e. plantamoside (=plantamajoside) and verbascoside, as well as the minor lavandulifolioside, and isoverbascoside [12, 13] have antibacterial [14, 15], antifungal [16, 17], antiviral [18] and antioxidant [17, 19–21] activities and are selective inhibitors of aldose reductase [17, 22], 5-lipoxygenase [18] and protein kinase C [23, 24]. However, *in vitro* culture has never been used to modify the CGE profile of *P. lanceolata*. Therefore, in this study, the time course of verbascoside and plantamoside content was observed in the leaves and roots of *in vitro* cultured *P. lanceolata*. In order to obtain new pharmacologically active compounds, this plant was cultured in a medium containing (*E*)-cinnamic acid which has been shown to be integrated into the polyphenol pathway [25, 26]. Plantamoside and verbascoside contents were then investigated. The temporary changes in the *P. lanceolata* chemical profile are reported and discussed below.

RESULTS AND DISCUSSION

Time course of plantamoside and verbascoside contents in the normal plant

Dried aerial and underground parts of *P. lanceolata* were investigated for caffeic acid derivatives using TLC, every eight days from day 21 to day 70. Quantitative evaluation of plantamoside (P) and verbascoside (V) was carried out using HPLC.

Whatever the age of the plants, the quantities of P in the leaves were negligible (Fig. 1), never exceeding

*Author to whom correspondence should be addressed

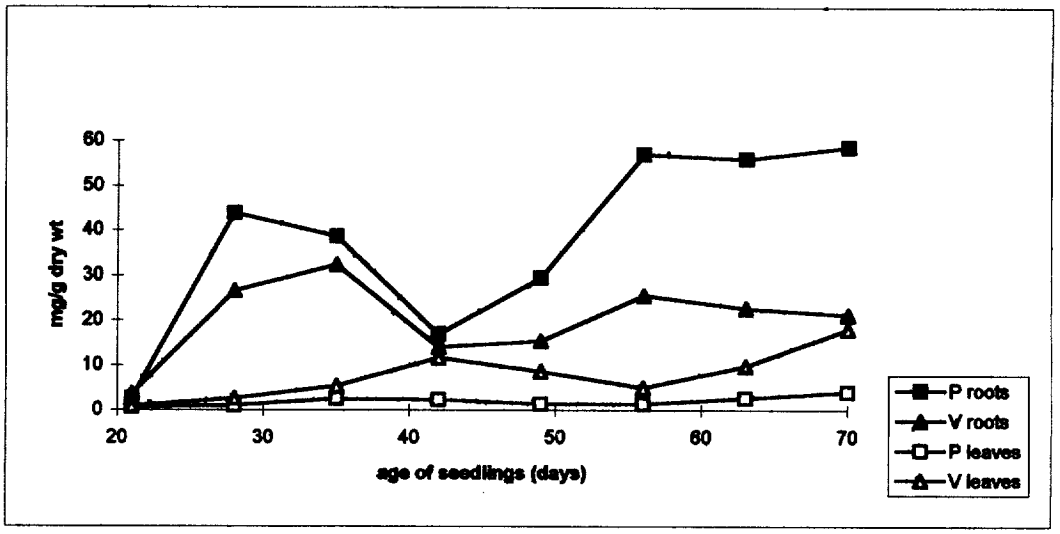

Fig. 1. Time course of P and V contents in the roots and leaves of *Plantago lanceolata*.

4.04 mg g^{-1} dry weight. The levels of V were higher than those of P and oscillated around an average value of 8.71 mg g^{-1} dry weight from day 21 to day 70. P content in the roots (Fig. 1) was always higher than V content and the two curves fluctuated in the same way, although the variations of the first component were more marked. P and V levels reached a first maximum around day 30 (43.9 ± 2.5 and 32.3 ± 2.2 mg g^{-1} dry weight, respectively) and a second at day 56 (57.0 ± 25.3 and 25.4 ± 1.5 mg g^{-1} dry weight, respectively). In general, levels oscillated around average values of 42.9 and 22.6 mg g^{-1} dry weight, respectively. Cumulated P and V content was always low in the leaves (average value, 9.74 mg g^{-1} dry weight). On the other hand, cumulated P and V content in the roots was age-dependent and much higher than in the leaves (30.9 ± 2.4 to 82.5 ± 26.7 mg g^{-1} dry weight on day 42 and day 70 respectively, average value 58.0 mg g^{-1} dry weight).

From day 21 until the end of the experiment (day 71), the average ratio P (roots)/P (leaves + roots) was close to 0.93 (Fig. 2), which confirmed that P was heavily stocked in the roots and that only very small amounts are found in the aerial parts. The average ratio V (roots)/V (leaves + roots) was 0.73 and showed that the level of V in the roots was always higher than in the leaves, although the values were more dispersed than the P values, oscillating between 0.55 and 0.90 from day 21 to day 71. All these results indicate that P is the major CGE in the roots of ribwort and they highlight the importance of P and V storage in the roots of *P. lanceolata*, which has not previously been

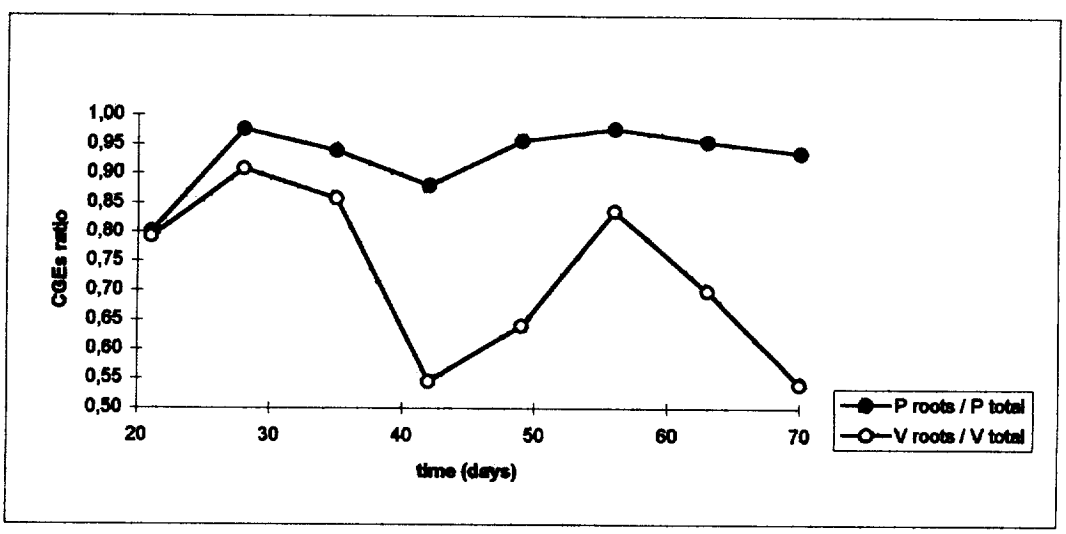

Fig. 2. Ratio: CGEs (roots)/CGEs (leaves + roots).

reported. For this reason, only the roots containing much higher levels of CGEs than the leaves were analysed during the experiment with (*E*)-cinnamic acid.

Time course of P and V contents in the roots of P. lanceolata *fed with* (E)*-cinnamic acid.*

Plants cultivated *in vitro* for 75 days following germination were transferred into MS culture medium containing (*E*)-cinnamic acid. P and V levels in the roots of these plants were then evaluated over 41 days and compared with the control. (*E*)-cinnamic acid was added at the minimum toxic concentration for the plants (10^{-3} M on day 0). This concentration was determined during a preliminary toxicity study on whole plants (F. Fons, unpublished results). It was chosen because it did not prevent the plant from living and growing throughout the experiment, but induced morphological and chemical profile modifications in the roots. Although this acid was only ever detected at negligible concentrations in these plants, their roots gradually degenerated, darkened and, 41 days later, withered completely. At the same time, new roots (neoroots) appeared at day 8 and gradually superseded the initial ones. From day 8 to day 16, the neoroots showed an atypical morphology while being anisodiametric and thicker than the usual roots. Thereafter, the neoroots became morphologically normal (isodiametic and thin). Hence the interest in comparing their chemical profiles with the control throughout this period.

P and V contents were 63.6 ± 13.3 and 29.3 ± 12.5 mg g^{-1} dry weight respectively in the control roots on day 0 (Fig. 3). Both levels decreased slightly after the plants were transferred, but no major differences were noted between the contents of stressed and control plants. Our results showed that (*E*)-cinnamic acid was not integrated into the P and V pathway.

It was also of considerable interest to study the P and V contents in the neoroots, which appeared at day 8 and which constituted almost the total mass of the roots on day 41 in plants fed with (*E*)-cinnamic acid. These neoroots were analysed from day 13 because, until this day, their biomass was too low. P and V were absent in the neoroots from day 13 to day 19 and then increased up to 14.2 ± 5.2 and 20.5 ± 8.3 mg g^{-1} dry weight respectively on day 41 (Fig. 3).

New cinnamic derivatives.

The most striking chemical modifications detected during the course of this experiment involved the temporary appearance of two cinnamic acid derivatives detected by TLC and HPLC analysis. Their concentrations from day 0 to day 41 were estimated from peak areas as a fraction of their maximum concentrations, attained on day 11 (Fig. 4). These NCD, which appeared on day 2 in the initial roots of *P. lanceolata* fed with (*E*)-cinnamic acid, were completely absent from the roots of the control plants. The cumulated ratio of NCD increased in the initial roots during the first week of the experiment (0.27 ± 0.00 on day 2, 0.87 ± 0.17 on day 6) to reach high concentrations throughout the second week (highest value, 1.00 ± 0.19 on day 11) and then decreased from the third week to the end of the experiment (0.13 ± 0.10 on day 41). These NCD were also detected in neoroots at high levels from day 13 (0.75 ± 0.53 and 0.95 ± 0.59 on day 13 and 16 respectively) but levels fell suddenly on day 19 (0.13 ± 0.07). From day 19 to the end of the experiment, low levels were detected (0.04 on day 41). It is very interesting to note that from day 19, when NCD levels in the roots fell dramatically, P and V appeared and attained the levels found in the controls. At the same time, the morphology of these roots became normal.

The NCD were isolated and purified in order to determine their structure. Total acid hydrolysis of both components released a sugar which was identified as glucose by TLC and Clinistix® test (Ames). Alkaline hydrolysis of both esters produced two molecules identified, using TLC and HPLC, as (*E*)-*p*-coumaric acid and (*E*)-ferulic acid. Traces of the two (*Z*)-isomers of each compound were also detected in the crude extracts of plant roots using HPLC after UV-exposure. The amounts of these two NCDs were too small to allow full structural determination. The (*E*)-cinnamic acid introduced into the culture medium of *P. lanceolata* at 10^{-3} M can be considered as constituting a toxic chemical stress for this plant. This acid induced a progressive withering of the initial roots, which were superseded by neoroots. These neoroots showed disturbed morphology and chemical profile for eight days (no CGEs and high levels of NCD). We can suppose that both cinnamic derivatives (NCD), appearing temporarily during the experiment, arose from a short detoxication pathway which was the most efficient way to protect *P. lanceolata* from high levels of cinnamic acid. Following this, the disappearance of these detoxication molecules allowed the normal chemical and morphological root profiles to be re-established.

EXPERIMENTAL

Plant material and growth conditions.

Seeds of *P. lanceolata* L. (a gift from Dr. S. Puech, Institut de Botanique, Montpellier, France) were sterilized in 70% EtOH for 30 sec, then in a commercial solution of NaClO (3.6%) for 20 min. The seeds were then rinsed ($\times 3$) in sterile H_2O and cultured in Murashige and Skoog's (MS) medium [27] with 10 g l^{-1} agar under alternating 12 hr light/dark. During the growth experiments, the seedlings were grown without transfer to fresh medium, in order to avoid stress-induced variations in CGE content and were analysed from day 21 to day 70. For the experiment with cin-

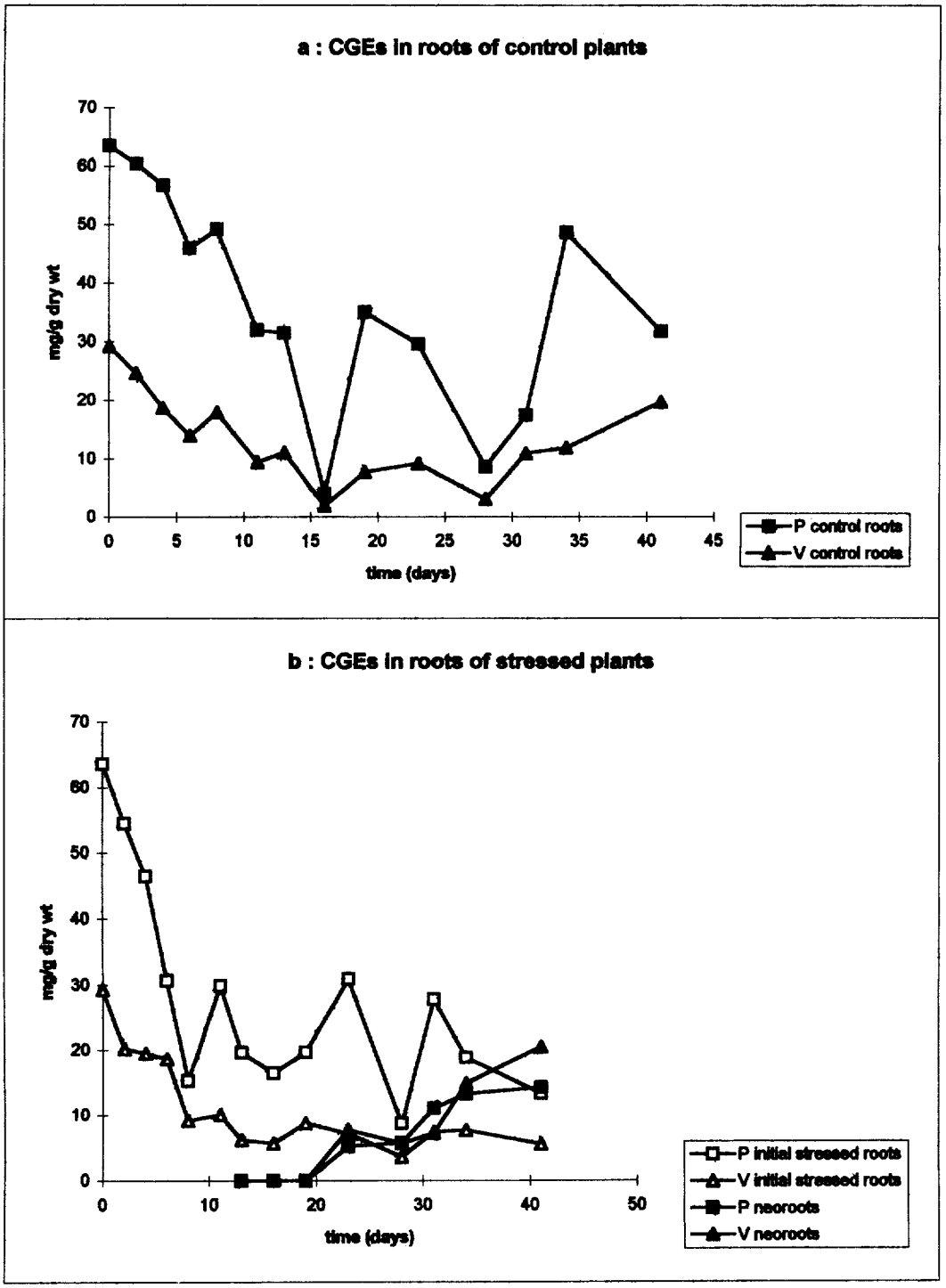

Fig. 3. Time course of root CGEs contents during the experiment with *trans*-cinnamic acid, (a) CGEs contents in the roots of control plants, (b) CGEs contents in the roots of stressed plants.

namic acid, after a first transfer into MS medium at day 35, 75-day-old plants were subcultured in MS medium containing 10^{-3}M (*E*)-cinnamic acid dissolved in 0.1% DMSO and the experiment run for a further 41 days.

CGEs extraction was carried out under the same conditions for both experiments. 50 seedlings were used for each extraction. Each experiment was carried out in triplicate and the average value calculated. Error values were not integrated into graphs to sim-

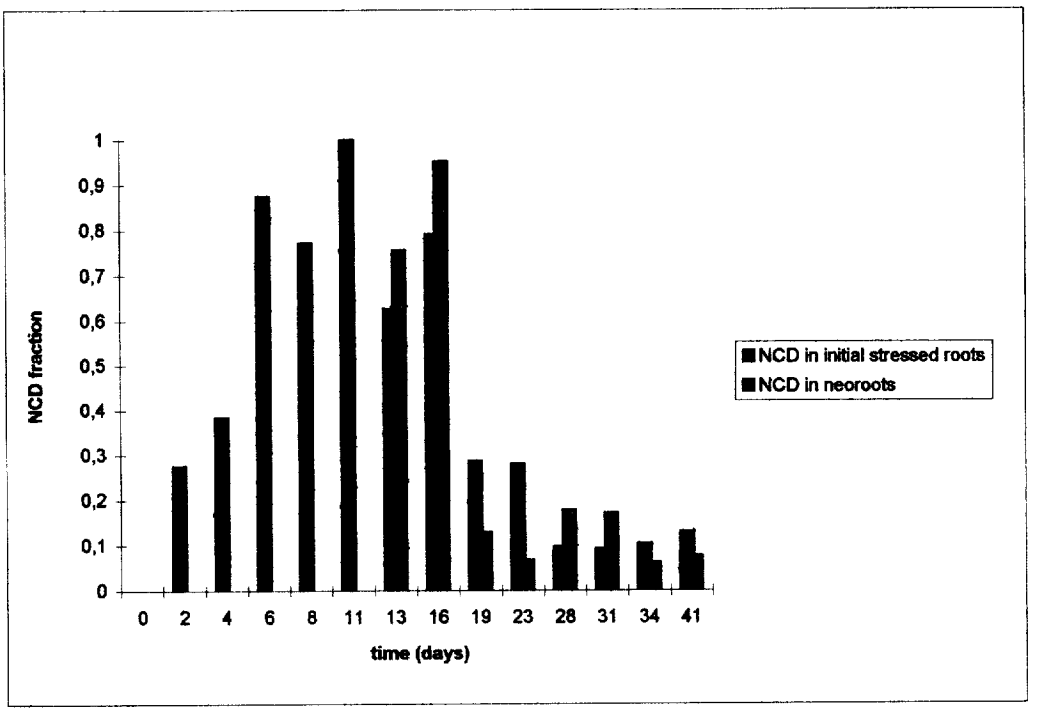

Fig. 4. Time course of NCD ratio in both initial roots and neoroots during the experiment with (*E*)-cinnamic acid.

plify their appearance. Dried tissues (roots, leaves and further neoroots) were separately weighed, ground in a mortar, extracted at room temperature in 70% MeOH (3 × 50 ml/g dry weight) with magnetic agitation (15 min), sonication (15 min) and then filtered. The combined extracts were concd. to dryness under vacuum and then analysed using TLC and HPLC. The crude extracts were taken up into MeOH (1 ml/100 mg of dry organ) and submitted to TLC on cellulose plates using: EtOAc-MeOH-H_2O-C_7H_{16} (10:2:1.5:1) or 2% HOAc. CGEs were detected by UV fluorescence after spraying with 1% 2-aminoethylphenylborinate in MeOH. The MeOH extracts of roots, leaves and neoroots used for TLC were diluted 10 times in 70% MeOH, filtered with a Millipore filter (0.45 μm) and injected (20 μl) into an HPLC system comprising a Kromasil RP-18 column (250 × 4.6 mm, 5 μm); mobile phase: MeCN-H_3PO_4 (1:4) (pH 2.6); flow rate 1 ml min^{-1}. Quantitative analysis was performed at 335 nm and compared with plantamoside (Rt:6.6 min) and verbascoside (Rt:8.8 min) standard solutions isolated from *P. lanceolata* [13].

Extraction and identification of NCD

Dried roots of *P. lanceolata* fed with cinnamic acid were extracted twice with 70% MeOH for 90 min at room temperature. The crude extract was concd under vacuum and subjected to CC on polyamide with 30% EtOH as eluent. A second purification was carried out on a cellulose column with H_2O to yield two NCDs. Total acid hydrolysis of NCD: each NCD was dissolved in H_2O, 3M HCl was added (1:1) and the mixture heated to 100° maintained for 5 hr. The cooled solution was extracted 3 times with an equal volume of EtOAc. The aq. layer was evaporated to dryness and the residue taken up in 50% MeOH. The solution was analysed using Clinistix (Ames) or precoated silica gel TLC plates, with two solvent systems: EtOAc-H_2O-MeOH-HOAc (13:3:3:5) and EtOAc-*iso*-PrOH-H_2O (2:7:1). Developed plates were sprayed with naphthoresorcine-H_3PO_4, anisaldehyde-HOAc or triphenyltetrazolium reagent and heated to 100°. D-glucose was identified by direct comparison with a standard. Alkaline hydrolysis was carried out on an aq. solution of NCD and 1 M NaOH (1:1) heated for 45 min at 50°. The solution was then acidified with Dowex resin 50W, filtered and then analysed using TLC (cellulose plates developed with 2% HOAc using 1% 2-aminoethyldiphenylborinate in MeOH or diazotized *p*-nitroaniline acid as reagents). The same solution was analysed using HPLC (conditions were the same as those used for CGE analysis, see above) and compared with standards of *p*-coumaric acid (Rt:10.3 min) and ferulic acid (Rt:12.0 min).

Acknowledgements—We are very grateful to Dr S. Rapior and Dr M. P. Bonzom for their advice during this work. We would like to thank Mr N. Wynn for editorial assistance.

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