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Accumulation of *p*-hydroxybenzoic acid in hairy roots of *Daucus carota*

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Summary

We report here the accumulation of *p*-hydroxybenzoic acid in *Agrobacterium rhizogenes*-induced hairy root cultures of *Daucus carota*. This phenolic acid finds application in food, pharmaceutical and polymer industries. Metabolic profiling of phenolics by HPLC/ESI–MS from these hairy roots showed a considerable amount of *p*-hydroxybenzoic acid accumulation both in cytosol and in the cell wall. Analyses of HCl and NaOH treated soluble phenolic fractions resulted in the elution of peaks with same retention time and similar UV-absorption spectra as observed with *p*-hydroxybenzoic acid standard. This suggests that *p*-hydroxybenzoic acid is present in the cytosol as free-form (unconjugated). A correlation has been drawn between the accumulation of soluble and wall-bound phenolic acids on a time-course basis. An apparent absence of any *p*-hydroxybenzoic acid-glucoside supports this observation, which in turn encourages the idea of its incorporation in the cell wall in an alkaline-labile form.

Introduction

The major bulk phenylpropanoid derivatives of plant cell wall are hydroxybenzoates and hydroxycinnamates. Hydroxycinnamates (C_6–C_3) are synthesized via plant phenylpropanoid pathway (Dixon and Paiva, 1995). Hydroxybenzoates (C_6–C_1) are usually formed by cleavage of a C_2 fragment from the hydroxycinnamates (Hertweck et al., 2001). Among hydroxybenzoates, *p*-hydroxybenzoic acid has received considerable attention because of its application in food (Tomas-Barberan and Clifford, 2000), medicine (Cho et al., 1998; Liu et al., 2002), cosmetics and polymer industries (Tullo, 1999; McQualter et al., 2005). This phenolic

Abbreviations: ESI–MS, Electro-spray ionization–mass spectrometry; HPLC, High performance liquid chromatography; UV, Ultra violet

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acid also serves as the key intermediate in the biosynthesis of ubiquinones and naphthaquinone pigments such as, shikonin (Löscher and Heide, 1994). Although the occurrence of *p*-hydroxybenzoic acid is nearly ubiquitous in plant kingdom, the biosynthetic route to benzoate and hydroxybenzoate is still elusive (Abd El-Mawla and Beerhues, 2002).

Daucus carota (carrot) roots are known to accumulate a considerable amount of *p*-hydroxybenzoic acid in their cell wall (Hartley and Harris, 1981). In the past, elicited cell suspension culture of *D. carota* was explored for producing *p*-hydroxybenzoic acid (Schnitzler et al., 1992). However, not much success was achieved in resolving *p*-hydroxybenzoic acid biosynthesis. While examining these reports on *p*-hydroxybenzoic acid accumulation in the cell wall of disorganized culture of carrot, we anticipated that perhaps an organized culture, in particular, hairy root culture could be a suitable system to explore *p*-hydroxybenzoic acid accumulation in carrot cell wall. This rationale is based on the fundamental question at the differences in whole pathway enzyme expression between disorganized and organized culture (Walton et al., 1999). Besides, there is scant information in literature concerning phenylpropanoid metabolism in roots of any kind (Hemm et al., 2004; Rhodes et al., 1997). Moreover, hairy root are robust in culture and may be maintained for extended periods without detectable changes in their growth and biosynthetic capabilities (Hamill et al., 1987).

In this paper, we report on the substantial accumulation of *p*-hydroxybenzoic acid in *Agrobacterium rhizogenes*-induced hairy root culture of *D. carota*. A correlation has been drawn between the accumulation of major soluble and wall-bound phenolic acids on a time-course basis. We also comment on the formation of free *p*-hydroxybenzoic acid in the context of its incorporation in the cell wall as an alkaline-labile form.

Materials and methods

Plant material and chemicals

Daucus carota L. were grown in pots on pesticide free sandy soil. Two months old carrot roots were taken for the induction of hairy roots. Analytical grade chemicals were used in sample preparation and all solvents used for analysis were of HPLC grade. Deionized water for all the experiments was obtained from a Barnstead/Thermolyne water purification system (Dubuque, USA). All authentic standards were procured from Sigma-Aldrich Chemical Co. Ltd (New Delhi, India).

Induction and maintenance of hairy root cultures

Hairy root cultures of *D. carota* were induced by infecting carrot discs with *A. rhizogenes* (LBA 9402) as described earlier by de Araujo et al. (2002). About 15–20 days after infection, several hairy roots appeared at the site of infection. Hairy roots were then transferred into liquid B_5 medium, supplemented with 250 mg/L cefotaxime antibiotic and incubated in a rotary shaker in the dark at $25 \pm 1\,^{\circ}C$ under constant agitation (100 rpm) with regular subculturing after every 15 days. Antibiotic dose was progressively decreased during subculturing and eliminated completely after 6–7 subculturing.

Extraction of cell wall bound and methanol soluble phenolic compounds

1 g fresh mass of hairy root tissue was crushed in liquid nitrogen and then extracted with 5 mL of 50% (v/v) methanol at room temperature (28 °C). The suspension was homogenized for 1 min and then centrifuged at 5000 rpm for 15 min. The supernatant was collected and used in all further experiments as the source of soluble phenolics. This supernatant was later concentrated under reduced pressure in a rotary evaporator at 40 °C and aliquoted into three micro-centrifuge tubes (0.5 mL per tube) in order to determine total phenolics, methanol soluble ester-bound phenolics and methanol soluble glycoside-bound phenolics as essentially described by De Ascensao and Dubery (2003). The remaining precipitate was dried for 70 °C for 24 h for the preparation of cell wall material, to be used for the saponification of wall-bound phenolics (Parr et al., 1996).

For determining wall-bound phenolics, the centrifuged pellet was treated with 1.5% (w/v) SDS and 5 mM sodium thiosulphate for 20 min at 26 °C and then centrifuged at 5000 rpm for 15 min. The supernatant was discarded and the pellet was again treated with 0.5% (w/v) SDS and 3 mM sodium thiosulphate for 2 h, at 26 °C. This was further centrifuged to collect the pellet. The pellet was extracted with boiling ethanol to remove any alcohol soluble phenolics, and subsequently washed thrice with acetone and finally air dried at room temperature. Approximately 25–30 mg of air-dried cell-wall-material was obtained per gram fresh mass of hairy root tissue. This cell wall material