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Characterization of Two Non Constitutive Hydroxycinnamic Acid Derivatives in Date Palm (*Phoenix dactylifera* L.) Callus in Relation with Tissue Browning

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Abstract: Two new phenolic compounds were isolated from date palm (*Phoenix dactylifera* L.) callus and have been identified to be apolar hydroxycinnamic acid derivatives (DHC3 and DHC4) by spectrophotometry, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) separations. Their accumulation is being in relation with tissue browning that occurs during *in vitro* culture and is cultivar dependent. A higher accumulation of these two DHCs was observed in BSTN and BFG cultivars ('susceptibles' to browning) compared to JHL and IKL cultivars (less 'susceptibles' to tissue browning). These compounds were completely degraded with 100 U mL^{-1} of exogenous peroxidase. Their formation doesn't occur when enzymatic extract was added to phenolic acids demonstrating that DHC3 and DHC4 constitute a substrate and not a product of enzymes implicated in tissue browning. The possible relationship between DHC3 and DHC4 and abiotic stress leading to tissue browning which affects somatic embryogenesis in date palm is discussed.

Key words: *Phoenix dactylifera* L., phenolics, hydroxycinnamic acid derivatives, tissue browning, somatic embryogenesis

INTRODUCTION

The multiplication of date palm (*Phoenix dactylifera* L.) a dioecious monocotyledonous species, has been achieved for a long time only by seeds and off-shoots. This method is not sufficient to rehabilitate the date palm groves and can be inadvertently spreads diseases such as bayoud, a tracheomycosis caused by *Fusarium oxysporum f.* sp *albedinis* (F.o.a).

Tissue culture appears to be a viable option for cloning this arborescent monocots which has an important socio-economic interest. Successful micropropagation of date palm has been achieved *via* somatic embryogenesis[1]. This technology can provide a rapid *in vitro* method for date palm mass propagation. However, the acquisition of embryogenic capacity in several cultivars remains difficult because calli, obtained after 6 to 12 months of culture, frequently displayed browning leading to a rapid tissue degeneration.

Date palm callus was characterized during embryogenesis by a change in the biosynthesis and accumulation of some phenolics. During the first month of culture, callus turned brown due to oxidation of its high level of caffeoylshikimic acids and accumulation of a high content of methoxy substituted phenols such as sinapic derivatives[2,3]. Whereas, the embryogenic callus, characterized by a low browning potential, accumulated lower phenolic levels than explants and the transitory accumulation of some flavonoids such as luteolin and isorhamnetin derivatives in callus may be associated with acquirement of embryogenesis competency[2]. Recently, a high level of phenolic esters incorporated into the walls of embryogenic cells was found to correlate with the activity of p-coumaroyl and feruloyl oxidases[4]. In alfalfa, Cvikrova *et al.*[5] showed that lower levels of ethylene and phenolic acids were involved in the complex of biochemical changes associated with somatic embryogenesis. A high level of phenolic esters incorporated into the embryogenic cell walls in alfalfa was found to correlate with the activity of ionically bound peroxidase[6]. Recently, it has been described that the phenylalanine ammonia lyase activity was in embryogenic callus twice as high as the activity determined in non embryogenic callus[7]. A higher proportion of cell wall bound phenolic acids was observed in embryogenic cells (about 10%) compared with both non embryogenic and primary callus cells (5-6%).

The aim of this work was to characterize two other non constitutive hydroxycinnamic acid derivatives which appear during *in vitro* culture and to establish their

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possible relationship with the callus tissue browning that affects negatively the acquisition of embryogenic potential in date palm.

MATERIALS AND METHODS

Plant material: Shoot tips of 4 cultivars of *Phoenix dactylifera* L. were surface sterilized for 20 min with 1% Desogerm, following 20 min immersion in commercial hypochlorite (ACE) containing 300 mg L^{-1} of potassium permanganate. Callus induction were conducted according to El Hadrami *et al.*[8]. The explants were placed on callogenesis induction medium containing Murashige and Skoog[9] salt, Fossard vitamins, 30 g L^{-1} sucrose, 150 mg L^{-1} activated charcoal, 6.8 g L^{-1} of carrageenan, 5 mg L^{-1} of 6-benzylamino-purine (BAP) and 5 mg L^{-1} of dichlorophenoxyacetic acid (2, 4 D). Tissue were then incubated at 25±2°C in the dark and transferred to freshly medium every 6 weeks.

Plant material in gras: Phenolic compounds were extracted and analyzed as described earlier[3,10-12]. Briefly, tissues of calli were extracted with 80% aq methanol at 4°C with continuous stirring. The extracts were purified and finally dissolved in methanol (HPLC grade) after evaporation in vacuo at 32-35°C. Phenolics analysis was performed using spectrophotometric, thin layer (TLC) and high performance liquid (HPLC) chromatographic methods.

TLC was conducted on microcrystalline cellulose in the upper phase of BAW (n-BuOH-HOAc-H_2O, 4:1:5). UV light (256-336 nm) was used with or without ammoniac vapor as well as other specific phenol reagents such as Benedikt and diphenyl boric acid 2-aminoethyl ester (NEU) reagents.

Quantitative and qualitative analysis were carried out with a waters 600 E liquid chromatograph equipped with waters 990 photodiode array detector (280, 320 and 360 nm) and a Licrosorb C-18 reversed phase column.

Alkaline hydrolysis: After their purification on TLC plates, the hydroxycinnamic derivatives (DHC3 and DHC4) were treated at room temperature for 2 h with NaOH 2 N. Nitrogen was immediately bubbled through the sample after NaOH addition to minimize the oxidation of phenolic acids[7]. Hydrolysis products were extracted two times with ethyl acetate after acidification with HCl 6N, evaporated to dryness and finally dissolved in methanol for HPLC analysis.

Effect of peroxidases on DHC3 and DHC4 oxidation: The effect of peroxidase (HRP) (Horseradish, Sigma) was determined as following: 100 µl of phenolic extract rich in DHC3 and DHC4 was evaporated to dryness and redissolved in phosphate buffer (0.1 M pH 6) with four concentrations of peroxidase (0, 1, 10 and 100 U mL^{-1}). The reaction was started by adding 20 µl of H_2O_2 1%. After one min of incubation at room temperature, the oxidation of phenolic compounds was stopped with 20 µl HCl 2 N. The products of reaction were extracted three times with ethyl acetate, evaporated to dryness and finally dissolved in methanol and analyzed by HPLC.

RESULTS AND DISCUSSION

In date palm tissue culture, only caffeoylshikimic acids, flavans and hydroxycinnamic acid derivatives were up to date identified as major phenolic compounds associated with tissue browning[2-3].

The analysis of HPLC chromatograms of the phenolic substances extracted from calli cultivated on culture medium containing 150 mg L^{-1} activated charcoal, showed the induction and accumulation of two other phenolic compounds (DHC3 and DHC4) most representative after 2-3 months of culture (Fig. 1).

Chromatographic and spectrophotometric methods revealed that purified DHC3 and DHC4 were apolar hydroxycinnamic acid derivatives; their HPLC retention times were 54 and 57 min for DHC3 and DHC4, respectively; Rf on microcrystalline cellulose in BAW=0.99; UV spectrum with an absorption maximum at 325 nm (for both DHC3 and DHC4), blue color under UV-366/254 nm and blue green under ammoniac.

HPLC analysis of ethyl acetate extract after alkaline hydrolysis of purified DHC3 yielded three peaks (Fig. 2) that were identified as p-coumaric, ferulic and sinapic acids on the basis of their retention time and TLC analysis. The same results were obtained for DHC4.

The apolar nature of these phenolics suggests the possible linking of sinapic, p-coumaric and/or ferulic acids with apolar compounds like fatty acids. In this way, it has been shown in *Solanum tuberosum* that the response to wounding is being in relation with changes in phenolic metabolism and particularly at the level of hydroxypalmitic acid-o-hydroxycinnamoyl transferase involved in the biosynthesis of hydroxycinnamoyl oxypalmitic esters of p-coumaric, ferulic and sinapic acids[11].

The involvement of the phenolic metabolism in resistance of plant to different stress (abiotic or biotic) was in general characterized by an increase in total phenolics content caused by an accumulation of compounds present before the stress and/or the appearance of compounds which had not previously been detected[12,13]. DHC3 and DHC4 may be considered as a response of tissue to stress. The time-course of DHC3

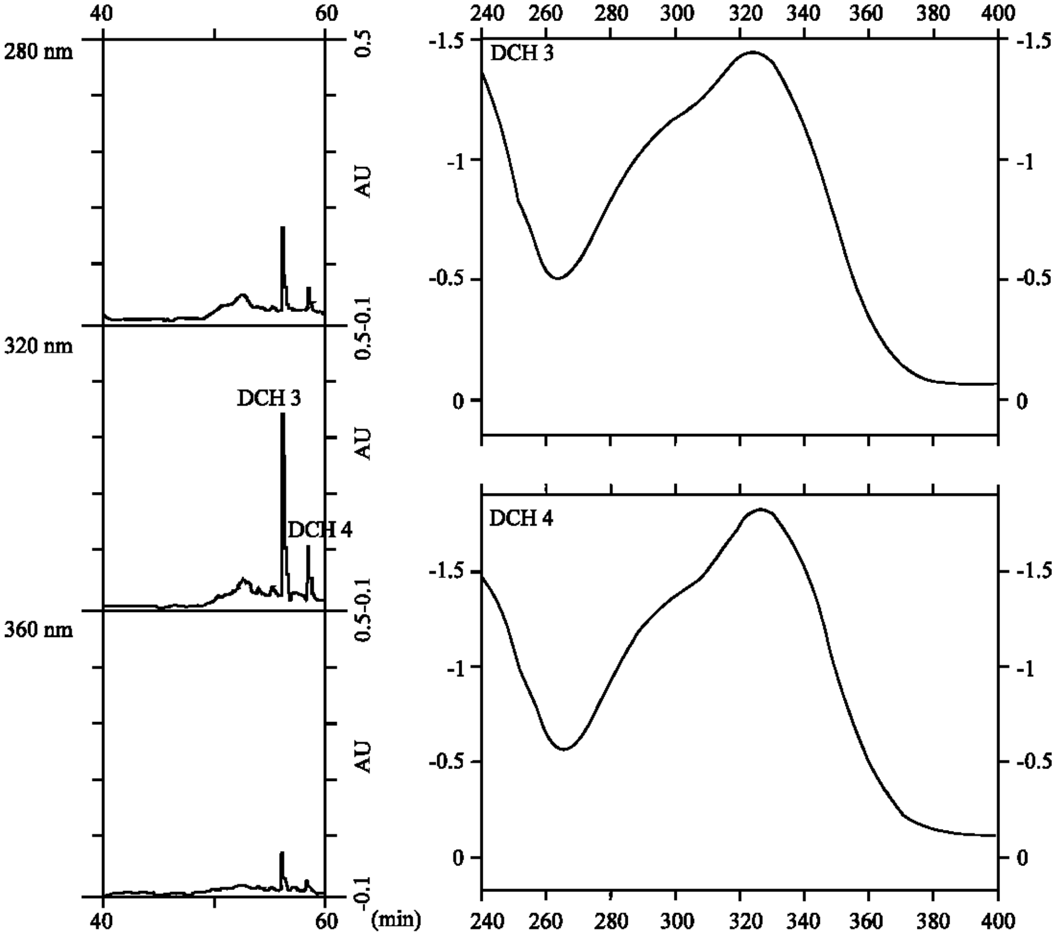

Fig. 1: HPLC chromatograms and spectra of hydroxycinnamic acid derivatives DHC3 and DHC4.

showed a higher increase until day 80 (Fig. 3a), reaching 32 and 35 µg eq chlorogenic acid/g FW, respectively in BSTN and BFG (two cultivars exhibiting a higher browning potential) and only 6 and 8 µg eq chlorogenic acid/g FW, respectively in JHL and IKL (two cultivars showing a weaker tissue browning potential).

The IKL and JHL cultivars accumulated low levels of DHC4 (2-6 µg eq chlorogenic acid/g FW between 40 and 80 days of culture) in comparison with BSTN and BFG cultivars, exhibiting a higher browning potential accompanied by a higher accumulation of this compound (19-20 µg eq chlorogenic acid/g FW between 40 and 80 days of culture) (Fig. 3b).

It seems to be clear that the behaviour of the two hydroxycinnamic acid derivatives DHC3 and DHC4 during the first weeks of culture on the callogenesis medium, indicated a stress response of tissue that is probably caused by the non adaptation of culture medium depending on the cultivar. These findings are in agreement with those reported by other workers who showed that phenolic metabolism was strongly activated in stressed cell-suspension culture of alfalfa[14]. Also, it has been shown that the intensification of tissue browning that limit somatic embryogenesis in *Hevea brasiliensis*, is accompanied by an increase of peroxidases, polyphenoloxidase and phenolic levels[15,16].

The tissue browning is generally caused by enzymatic oxidation of phenolic compounds by polyphenoloxidases and peroxidases[12]. The HPLC chromatograms of the extracts treated with peroxidases revealed that hydroxycinnamic acid derivatives were moderately degraded with 1 U mL^{-1} (HPR) and completely degraded with 100 U mL^{-1} (HPR) (Fig. 4). In addition, their formation *in vitro* doesn't occur when crude enzymatic

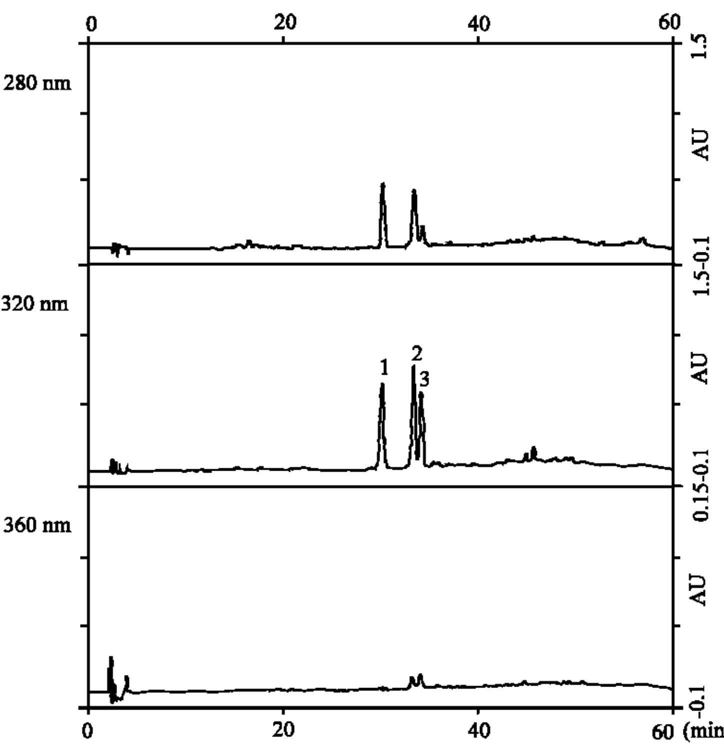

Fig. 2: HPLC chromatogram of phenolic acids yielded after alkaline hydrolysis of DHC3 or DHC4 (1: p-coumaric acid; 2: ferulic acid; 3: sinapic acid)

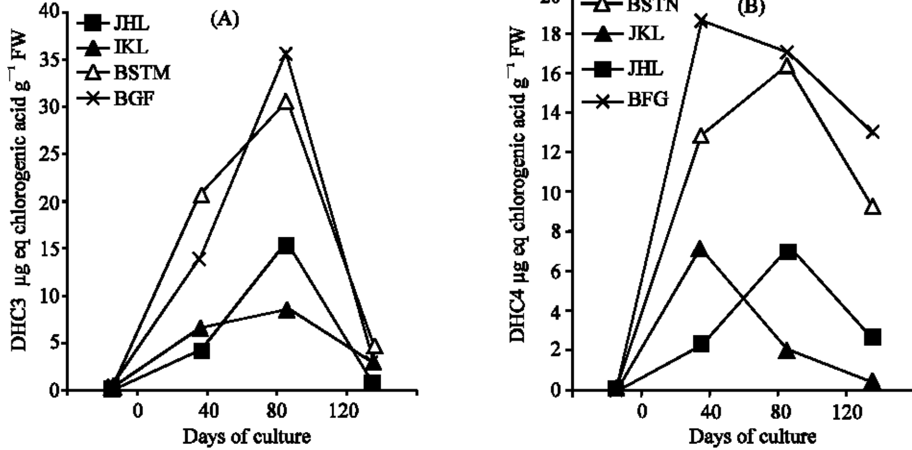

Fig. 3: Comparison of hydroxycinnamic acid derivatives DHC3 (A) and DHC4 (B) accumulation during the first weeks of culture in callus of BSTN, BFG, IKL and JHL date palm cultivars

extracts were added to phenolic acids (p-coumaric, ferulic and sinapic acid) (data not shown). These results demonstrate that DHC3 and DHC4 constitute a substrate and not a product of enzymes implicated in tissue browning such as peroxidases. The preferential accumulation of DHC3 and DHC4 in calli of cultivars exhibiting a higher browning potential such as BSTN and BFG suggest their potential implication in tissue browning.

These phenolic compounds are not constitutive in date palm tissue. Their induction occurs under abiotic stress such as the non adaptation of culture media used for somatic embryogenesis. They can serve as bio-indicators of stressed tissue and may represent a

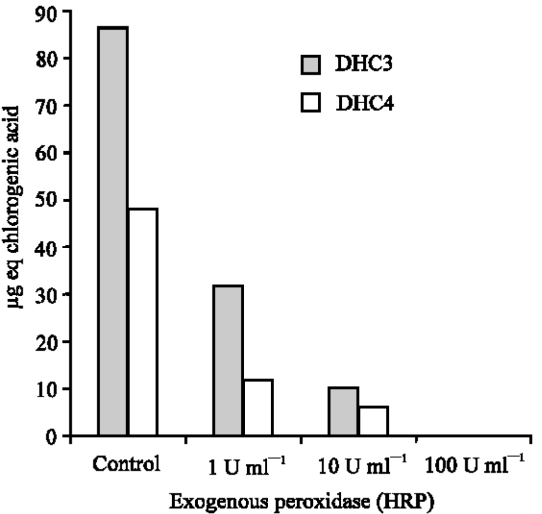

Fig. 4: Effect of peroxidases (HRP) on hydroxycinnamic acid derivatives DHC3 and DHC4

cellular protectors resulting from the expression of phenylpropanoid genes induced under stress[17].

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