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Accumulation of phenylpropanoid derivatives in chitosan-induced cell suspension culture of *Cocos nucifera*

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Summary

Chitosan-induced elicitation responses of dark-incubated *Cocos nucifera* (coconut) endosperm cell suspension cultures led to the rapid formation of phenylpropanoid derivatives, which essentially mimics the defense-induced biochemical changes in coconut palm as observed under *in vivo* conditions. An enhanced accumulation of *p*-hydroxybenzoic acid as the major wall-bound phenolics was evident. This was followed by *p*-coumaric acid and ferulic acid. Along with enhanced peroxidases activities in elicited lines, the increase in activities of the early phenylpropanoid pathway enzymes such as, phenylalanine ammonia lyase (PAL), *p*-coumaroyl-CoA ligase (4CL) and *p*-hydroxybenzaldehyde dehydrogenase (HBD) in elicited cell cultures were also observed. Furthermore, supplementation of specific inhibitors of PAL, C4H and 4CL in elicited cell cultures led to suppressed accumulation of *p*-hydroxybenzoic acid, which opens up interesting questions regarding the probable route of the biosynthesis of this phenolic acid in *C. nucifera*.
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Introduction

Plant cell cultures can often serve as model systems to study the biochemical changes in relation to plant defense responses against pathogens in those plants where conducting *in vivo* studies are apparently difficult (Kneer et al., 1999). Cell cultures not only

Abbreviations: AOAA, aminooxyacetic acid; 4CL, *p*-coumaroyl-CoA ligase; *p*-HBA, *p*-hydroxybenzoic acid; HBD, *p*-hydroxybenzaldehyde dehydrogenase; MDCA, 3, 4-methylenedioxycinnamic acid; PAL, phenylalanine ammonia-lyase; PIP, piperonylic acid.

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have a higher rate of metabolism than differentiated plants, but also have the biosynthetic cycles compressed into shorter time periods (Zenk, 1991). As cell culture systems are relatively easy to manipulate by empirical means, these can provide a better control of external factors that can interfere with the metabolic activities, and thus advantageous over studies on *in vivo* plant–pathogen interactions (Stratmann et al., 2000). In the recent past, many elicitor-induced cell cultures of different plant species were used as model systems to understand plant–pathogen interactions both at biochemical and molecular levels (Lizama-Uc et al., 2007; Shein et al., 2003).

Plant cell and organ cultures grown *in vitro* usually exhibit changes in physiological and biochemical responses upon exposure to biotic and abiotic elicitors (Sircar and Mitra, 2008). Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plant cells to ensure their survival, persistence and competitiveness. Chitosan, the deacetylated form of chitin is a well-known elicitor for plant biochemical experiments using either cell and organ cultures or excised *in vivo* plant organs (Agrawal et al., 2002; Chakraborty et al., 2008; Kohle et al., 1984). It is the main component of the cell walls of some fungal species. As a natural, biocompatible, cationic biopolymer, chitosan mimics the effects of some pathogenic microorganisms to stimulate plants to synthesize defense-related secondary metabolites. Therefore, chitosan is widely applied as a potent elicitor for various disease-resistance responses in several plant species (Benhamou and Thériault, 1992).

Coconut (*Cocos nucifera*) is a woody species of Arecaceae (Palmae) family, and a valuable industrial crop of the tropical countries (Peter, 2002). Every year, a large number of coconut crops are attacked by several pathogens. Even though many epidemiological studies for coconut diseases were conducted, the biochemical basis of disease resistance upon coconut–pathogen interactions is still unclear. Because of long life cycle and large size, *in vivo* studies with this palm appear to be difficult to carry out. These necessitate the exploration of an efficient *in vitro* system for such type of work. Here we report the outcome of chitosan-induced elicitation responses in cell suspension culture of *C. nucifera*, in particular on the level of accumulation of phenylpropanoid derivatives. This approach should in principle mimic the defense-induced biochemical changes in coconut palm as observed under *in vivo* conditions (Karthikeyan et al., 2006). An enhanced accumulation of *p*-hydroxybenzoic acid as the major wall-bound phenolics was evident. This was followed by *p*-coumaric acid and ferulic acid. Along with enhanced peroxidases activities in elicited lines, we also describe the increase in activities of some biosynthetic enzymes such as, phenylalanine ammonia lyase (PAL), *p*-coumaroyl-CoA ligase (4CL) and *p*-hydroxybenzaldehyde dehydrogenase (HBD) in elicited cell cultures catalyzing the formation of *p*-hydroxybenzoic acid. We further studied the effect of supplementing specific inhibitors of some of the early enzymes of phenylpropanoid metabolism on the accumulation of *p*-hydroxybenzoic acid, and comment on the probable route to the biosynthesis of this phenolic acid in *C. nucifera*.

Materials and methods

Plant material and cell suspension culture

Endosperm callus cultures of coconut (*C. nucifera* L.) were established in Y3 medium (Eewens, 1976) from *in vitro* culture of coconut endosperm, as described by Ceniza et al. (1992). Callus cultures were maintained in the same medium supplemented with charcoal and sucrose (3% w/v). Cell suspension cultures were established from callus cultures. Briefly, 0.5 g of callus was suspended in 10 mL of Y3 medium and incubated in a rotary shaker under dark condition. After 3 weeks, 1 mL of liquid suspension culture was inoculated in 10 mL of fresh Y3 medium and incubated under same condition mentioned before. Seven-day-old dark brown cell suspension cultures (white in color) of *C. nucifera* were used for the experiments.

Elicitor and inhibitor(s) treatment

The method of *in vivo* elicitation with chitosan was adopted according to a published procedure (Khan et al., 2003). Chitosan was dissolved in dilute HCl by gentle heating. The pH of this solution was adjusted to 5.0 with NaOH and autoclaved for 1 h which was later added to the medium under sterile condition. The elicitor was added at a final concentration of 200 mg/L in a 7-day-old suspension cultures and incubated for a further period of 48 h. Control cultures were treated with sterile distilled water. In those experiments where specific inhibitors were used to block the elicitor effect, each enzymatic inhibitor was added separately at defined concentrations along with chitosan in the liquid medium. Filter-sterilized aminooxyacetic acid (AOAA), piperonylic acid (PIP) and 3,4-methylenedioxycinnamic acid (MDCA) were added individually into the culture medium at three different concentrations (25, 50 and 100 μg/mL). Inhibitor-treated cells were harvested after 36 h for analyzing the phenolic acid contents by HPLC.

Assay of total phenols

The total phenolic content was determined as described by Singleton et al. (1999). The reaction mixture

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contained 100 μL of methanolic extract, 200 μL distilled water with 500 μL of Folin–Ciocalteu reagent (Sigma). After 5 min, 800 μL of 20% Na_2CO_3 was added and after 1 h of incubation, the absorbance was measured at 254 nm in a Systronics UV–VIS scanning spectrophotometer (Ahmedabad, India). Standard curve was prepared with *p*-hydroxybenzoic acid in 50% (v/v) methanol. The total phenolic content was expressed as micrograms of *p*-hydroxybenzoic acid equivalent per gram fresh mass of harvested cell biomass.

Extraction and analysis of wall-bound phenolics

Suspended cell biomass was harvested by filtration. Cell biomass (0.5 g fresh mass) was crushed in liquid nitrogen and then extracted with 3 mL of 50% (v/v) methanol at room temperature (28 °C). The suspension was homogenized by vortex-mixing for 1 min and then centrifuged at 5000*g* for 15 min. The supernatant was discarded. For extracting wall-bound phenolics, the centrifuged pellet was treated with 2 M NaOH in dark for 24 h (Parr et al., 1996). This saponified extract was acidified with HCl (pH 1–2), and subsequently extracted with an equal volume of ethyl acetate. The ethyl acetate fraction was evaporated to dryness under reduced pressure, and the residue was re-dissolved in 0.5 mL of aqueous MeOH (50% *v/v*) for separation and quantification of wall-bound phenolics by HPLC. Phenolic acids were separated on a Phenomenex™ (Torrance, USA) C_{18} column (RP-Hydro, 4 μm, 250 × 4.6 mm^2) using a Waters HPLC system (Milford, USA) in an isocratic mode comprising of aqueous trifluoroacetic acid (TFA) (68%) and methanol (32%) as established earlier by our group (Sachan et al., 2004).

Alternatively, the wall-bound phenolics were determined by phloroglucinol–HCl staining as reported in the literature (Peltonen et al., 1997). Briefly, after methanol extraction, the cell pellet was separated by centrifugation at 15 000*g* for 5 min. The pellet was then dissolved in DMSO by incubating in an ultrasonic bath for 5 min. This was followed by the addition of 0.1 mL of 4% phloroglucinol/ethanol (w/v). Subsequently in the mixture, 0.7 mL of 37% HCl was added. After incubating for 30 s, the absorbance was measured at 555 nm.

Extraction of enzymes and determination of total protein content

Both treated and untreated cell biomass was grinded in liquid N_2 and extracted in Tris–HCl buffer as described recently in the literature (Chakraborty et al., 2008). Total protein content was determined by the method of Bradford (1976), using bovine serum albumin as standard.

Assay of phenylalanine ammonia lyase (PAL)

PAL activity was assayed as described recently by Chakraborty et al. (2008).

Assay of 4-coumaroyl-CoA ligase (4CL)

The activity of 4CL was assayed as described by Knobloch and Hahlbrock (1975) with minor modification. The reaction mixture (volume 1 mL) contained 0.1 mL crude protein extract, 5 mM *p*-coumaric acid, 10 mM ATP, 1 mM CoASH (coenzyme A-lithium salt), 5 mM $MgCl_2$, 10 mM DTT and 0.4 mL of 100 mM Tris–HCl buffer (pH 7.5). The mixture after incubating for 1 h at 34 °C was stopped by addition of 0.1 mL of 6 M HCl. The stopped reaction mixture was then centrifuged for 10 min at 10,000*g* to pellet-down the denatured protein. The absorbance of the supernatant was measured at 333 nm before (at 0 min) and after incubation. Blank reaction contained boiled enzyme extract. One unit of 4CL activity was defined as change in absorbance value by 0.01/h, and expressed as change in absorbance/mg protein/min.

Assay of hydroxybenzaldehyde dehydrogenase (HBD)

HBD activity was assayed as described by Sircar and Mitra (2008).

Peroxidase (POD) assay

The enzyme extract was prepared in the same way as that of PAL assay except here we use 0.1 mM sodium acetate buffer (pH 7.0). The assay mixture (total volume 3 mL) contained 20 mM guaiacol (0.5 mL), 0.1 mM acetate buffer (2.1 mL) 40 mM H_2O_2 (0.2 mL) and crude protein extract (0.2 mL). Oxidation of guaicol was measured by the increase in absorbance at 470 nm for 30 s at an interval of 5 s as essentially described by Deepa and Arumughan (2002). The assay mixture without enzyme was taken as blank. Extracellular POD was determined from the medium of cell suspension. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.1/min. Specific activity was expressed as U/mg protein.

Results and discussion

Enhanced *p*-hydroxybenzoic acid accumulation by chitosan treatment

In control cell suspension cultures (without chitosan treatment), the level of total soluble phenolics remained more or less constant. However, in response to chitosan treatment, an increase in the phenolic content was observed 12 h after elicitation. A maximum amount of 1.3 mg/g fresh mass of total phenols was observed at 36 h of incubation, and thereafter, a marked decrease in the content was noted (Figure 1). HPLC analysis of wall-bound phenolics revealed that there was a considerable increase in the content of *p*-coumaric acid accumulation in the cell wall

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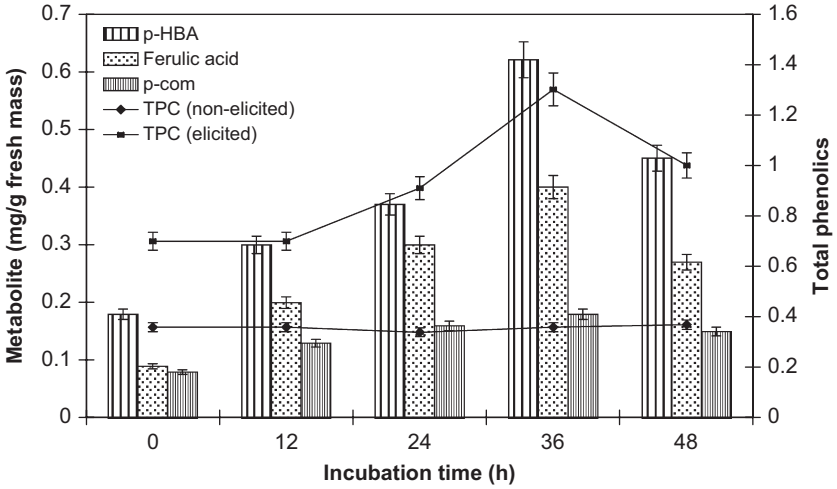

Figure 1. Changes in the contents of total phenolics (mg/g fresh mass) and cell wall-bound phenolic acids in chitosan-elicited cell suspension cultures of *C. nucifera*. The concentration of chitosan used in this experiment was 10 μg/mL. Data consists of the mean of two independent experiments ± SD from at least three different cell lines.

during first 36 h following elicitation. Beyond 36 h, a decline in the content was observed. In addition to these changes in the content of *p*-coumaric acid, a substantial increase of *p*-hydroxybenzoic acid accumulation in elicited cultures was noticed, which also followed more or less the same trend on a time-course accumulation pattern. HPLC analyses further confirmed a marked increase in the concentration of ferulic acid in the cell wall as compared to control cultures. A similar trend of wall-bound phenolic acid accumulation was observed in elicitor-treated cell suspension cultures of *Solanum tuberosum*, where increased incorporation of *p*-hydroxybenzoic acid into the cell was evidenced (Schmidt et al., 1998). More recently, methyl-jasmonate-elicited hairy root cultures of *Daucus carota* were shown to accumulate a substantial amount of *p*-hydroxybenzoic acid in the cell wall as compared to untreated ones (Sircar and Mitra, 2008). Furthermore, as elicitors are known to mimic in particular, the effects of several pathogenic fungi in stimulating defense-related secondary metabolites in resistant plants, a correlation can also be recognized from the findings with date palm. The *Fusarium oxysporum*-resistant cultivars of date palm were shown to accumulate *p*-hydroxybenzoic acid and *p*-coumaric acid in the root cell wall by 8- and 2-fold, respectively, as compared to the susceptible cultivars (Modafar and Boustani, 2001). This indicates that the enhanced accumulation of cell wall-bound phenols in the resistant cultivars not only reduced strongly the mycelial growth, but also made polysaccharides attached to the cell wall less sensitive to the cell wall degrading enzymes of pathogens (Ikegawa et al., 1996).

In addition to HPLC analysis of saponified cell wall materials, phloroglucinol–HCl staining of DMSO-soluble sonicated cell mass also revealed the presence of cell wall-bound phenolics (figure not shown). This material was perhaps composed of low-molecular weight cell wall-bound phenolics that are usually attached to lignin. However, leaching of wall-bound phenolics into the medium was not observed. Increase in absorbance of phloroglucinol-stained substances up to 72 h post-elicitation indicated the accumulation of wall-bound phenolic compounds. A similar trend was observed in barley cell suspension cultures upon elicitor treatment (Peltonen et al., 1997).

Elicitation of peroxidases activities upon chitosan treatment

It was observed that prolonged elicitation often turned the suspension culture brown. The brown pigments originate from the oxidative polymerization of phenolic compounds (Mayer and Harel, 1979) and this process is usually accompanied by induced phenolic biosynthesis and increased peroxidase activity (Legrand et al., 1976; Van Loon and Geelem, 1971). Peroxidases present in the intracellular spaces and peroxidases ionically bound to the cell walls are involved in the polymerization of phenylpropanoid (Lewis and Yamamoto, 1990). Therefore, the pattern of peroxidase induction in the elicited suspension culture was studied in elicited and control lines of *C. nucifera*. Peroxidases activities of the suspension cells were also affected by chitosan treatment. Both cellular and extracellular peroxidase activities were detected

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in cell cultures of *C. nucifera*. A sharp increase in cellular peroxidase activities was observed in chitosan-elicited cell lines as compared to control. A maximum of 25-fold increase in the activity was observed after 45 h of elicitation (Figure 2a). While the cellular activity of peroxidase gradually increased after elicitation, extracellular peroxidase activity rapidly declined to a significantly lower level within 45 h of elicitation (Figure 2b). This phenomenon occurred due to the inactivation of this particular peroxidase isoenzymes by the enhanced production of hydrogen peroxide (Ferrer and Barcelo Ros, 1994), which was also a part of elicitation response. This decrease in the extracellular activity was also reflected in the wall-bound phenolic content. Possibly this could be one of the factors because of which there was a decrease in the wall-bound phenolics after 36 h of elicitation. A similar trend was also noticed in spruce (Messner and Boll, 1991), where enhancements of peroxidase activities were observed in cell cultures of *Picea abies* upon elicitation.

Elicitation of phenylpropanoid enzyme activities upon chitosan treatment

The induction of PAL by chitosan treatment is shown in Figure 3a. A sharp rise in PAL activity was observed within 8 h of incubation with chitosan followed by a gradual decline for up to another 36 h after which no activity was detected. However, no appreciable level of PAL activity was detected in un-elicited cell lines. A 4-fold increase of PAL activity was observed in chitosan-stimulated cell cultures at 24 h post-elicitation, and thereafter, a reduction in the activity was noted. An analogous situation was also observed in date palm (*Phoenix dactylifera*); when seedling roots of *P. dactylifera* were inoculated with pathogenic fungi *Fusarium oxysporum* f. sp. *albedinis*, an increase in PAL activity was observed which was 4.6-fold higher than control (Modafar et al., 2006).

Besides PAL, the activity of 4CL also showed (Figure 3b) more or less the same trend; a 7-fold increase in 4CL activity was observed in elicited cultures after 24 h of chitosan treatment as compared to control. Chitosan treatment of cell suspension culture of *Vanilla planifolia* was shown to enhance 4CL activity in a similar range as observed in chitosan-treated *C. nucifera* suspension cell cultures. The 4CL activity in untreated cells remained low throughout the incubation (Funk and Brodelius, 1990a). In addition to PAL and 4CL, an enhanced activity of HBD was also detected in chitosan-stimulated cell cultures. The HBD activity reached its maximum (Figure 3c) (0.46 pkat/mg protein) after 24 h of elicitation, which appeared to be 2.5-fold higher than the control cell lines (without chitosan treatment). A similar situation

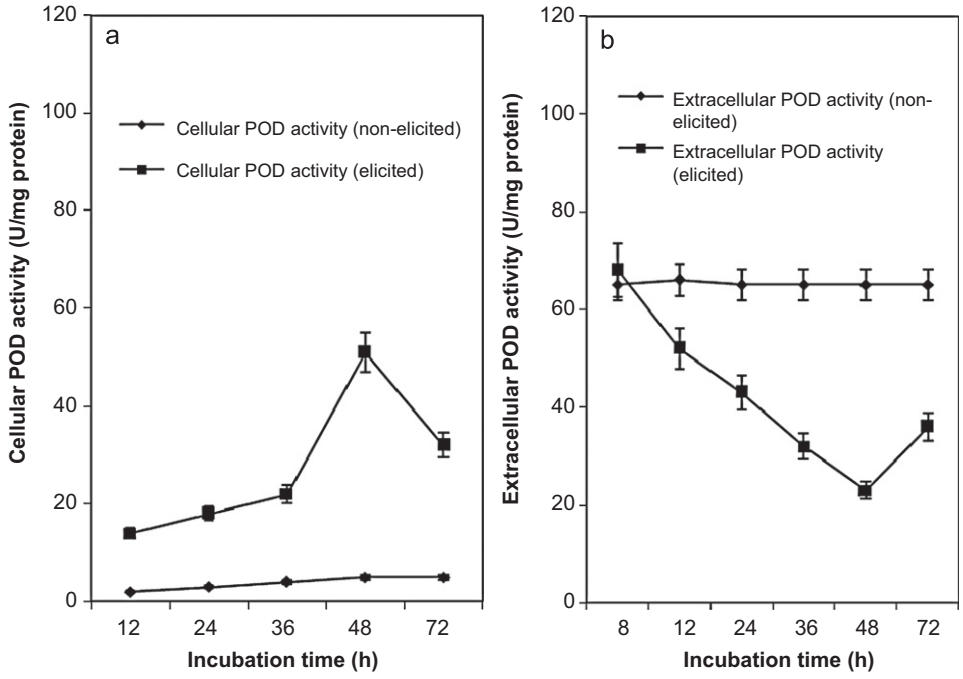

Figure 2. Time course changes in cellular peroxidase activities (a) and extracellular peroxidase activities (b) in the chitosan-elicited *C. nucifera* cell suspension cultures. Each value is the mean of two independent experiments ± SD from at least three extractions.

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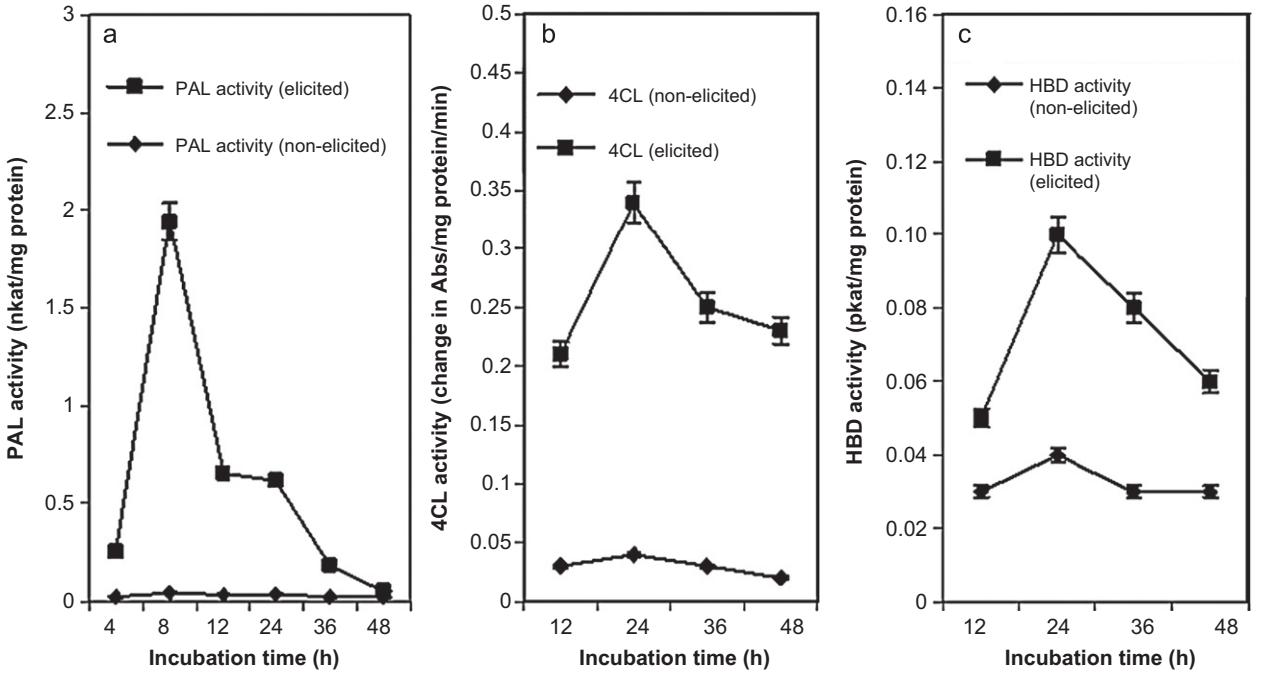

Figure 3. Changes in the PAL (a), 4CL (b) and HBD (c) activities in chitosan-elicited cell suspension cultures of *C. nucifera* on a time-course basis. Each value is the mean ± SD from at least three independent extractions/protein preparations.

was also observed with elicited cell suspension cultures of *D. carota*, where the activity of HBD was moderately induced by the elicitor compared with the samples from untreated cultures (Schnitzler et al., 1992). Moreover, an analogous benzaldehyde dehydrogenase activity was also found to be stimulated by methyl-jasmonate elicitation in cell suspension cultures of *Hypericum androsaemum*, indicating a similar upliftment of a phenolic-secondary metabolism-specific dehydrogenase function (Abd-El-Mawla and Beerhues, 2002).

Supplementation of phenylpropanoid enzyme inhibitors suppressed the accumulation of *p*-hydroxybenzoic acid in chitosan-treated cell cultures

In order to get an insight on the accumulation of *p*-hydroxybenzoic acid in elicited cell cultures of *C. nucifera*, effect of supplementation of phenylpropanoid enzyme inhibitors were studied (Figure 4). Three selective enzyme inhibitors of plant phenylpropanoid pathway namely, AOAA, PIP and MDCA were chosen which are known to inhibit PAL, C4H and 4CL, respectively. It was anticipated that by selectively inhibiting these enzymes under *in vivo* condition a possible metabolic route of *p*-hydroxybenzoic acid formation could be established. AOAA is a competitive inhibitor of PAL (Amrhein et al., 1976). Supplementation of AOAA with chitosan led to a decreased accumulation of *p*-hydroxybenzoic acid in the cell wall. A similar decrease in the accumulation of methoxybenzaldehyde upon the increase in concentration of AOAA was observed in excised roots of *Hemidesmus indicus* when co-incubated with chitosan (Chakraborty et al., 2008). Another upstream part enzyme of phenylpropanoid metabolism is C4H, a cytochrome P450 enzyme that converts *trans*-cinnamic acid to *p*-coumaric acid. PIP is a partially reversible inhibitor of C4H. Supplementation of PIP in elicited tobacco cell had led to the decreased formation of *p*-coumaric acid and the consecutive accumulation of the 7-hydroxylated coumarin scopoletin (Schalk et al., 1998). Selective inhibition of C4H by PA (100–800 μM) and PAL by AOAA (200 μM–1 mM) resulted in decreased accumulation of *p*-hydroxybenzoic acid in the cell wall. MDCA is a competitive inhibitor of hydroxycinnamate CoA-ligase (4CL) enzyme that converts hydroxycinnamic acid to hydroxycinnamoyl-CoA thioesters. It was evident here that by supplementing increasing concentrations of MDCA in a concentration-dependent manner, the accumulation of *p*-hydroxybenzoic acid was markedly reduced. This was due to the 4CL inactivation (Figure 4) by MDCA thus suggestive of a CoA-dependent *p*-hydroxybenzoic acid biosynthesis in

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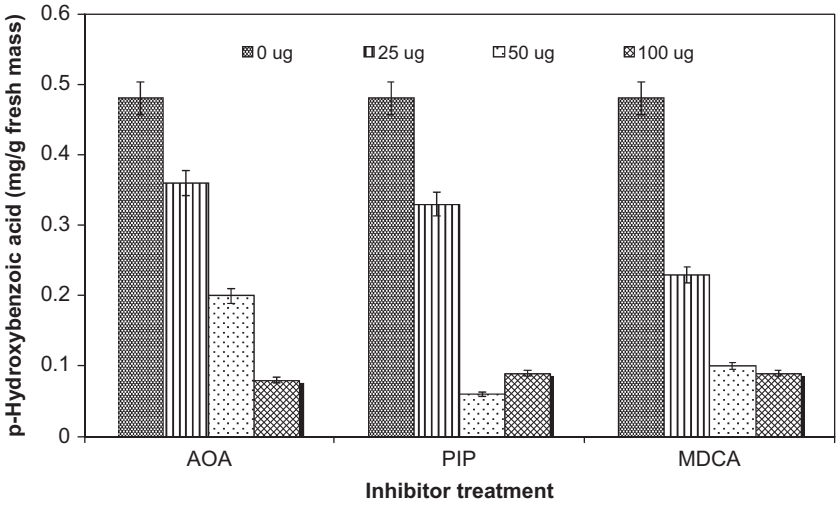

Figure 4. Inhibition of chitosan-induced *p*-hydroxybenzoic acid accumulation by supplementing individually AOAA, PIP and MDCA, the specific inhibitors of PAL, C4H and 4CL respectively in the cell suspension cultures of *C. nucifera*. All values are mean $\pm$ SD from at least three independent experiments.

C. nucifera. A similar observation was also evidenced with cell suspension culture of *V. planifolia*; addition of MDCA resulted in a reduced biosynthesis of ligneous material (Funk and Brodelius, 1990b).

In conclusion, an enhanced accumulation of cell wall-bound *p*-hydroxybenzoic acid was observed in chitosan-treated cell suspension cultures of *C. nucifera*. Addition of MDCA (a potent inhibitor of 4CL) in the elicited cell cultures resulted in the decreased accumulation of this phenolic acid. This suggests that *p*-hydroxybenzoic acid formation in *C. nucifera* appears to be a CoA-dependent. Incubation of cell-free extract with *p*-coumaric acid and other cofactors (ATP, CoASH and NAD^+) failed to detect any product formation under *in vitro* conditions. Thus at this moment it is not possible to conclude if this CoA-dependent route is β-oxidative or non-β-oxidative. However, cell-free extract when incubated with *p*-hydroxybenzaldehyde and NAD^+, formation of *p*-hydroxybenzoic acid was observed. This suggests the apparent presence of a NAD-linked aromatic aldehyde dehydrogenase function (Abd-El-Mawla and Beerhues, 2002). Thus it is plausible that this *p*-hydroxybenzaldehyde, the first chain-shortened C_6–C_1 product might have formed from *p*-coumaroyl-CoA through a non-β-oxidative route via retro-aldol cleavage (Mitra et al., 2002). However, formation of *p*-hydroxybenzoic acid from *p*-coumaroyl-CoA via benzoyl-CoA cannot be ruled out (Löscher and Heide, 1994). Future work should bring us closer to resolving this puzzle, which remains unresolved now for several decades (Wildermuth, 2006).

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