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Research article

Modulation of cell-wall bound phenolics accumulation by shikimate pathway in yeast extract elicited fragrant roots of *Hemidesmus indicus*

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Abstract: Plant cell walls contain various phenolic compounds, originated from phenylpropanoid pathway. *Hemidesmus indicus* is a well-known tropical Indian medicinal plant, traditionally used as herbal medicine for different ailments. Several reports have been published on its soluble phenolic constituents so far. Recently, shikimate pathway modulated biosynthesis of 2-hydroxy-4-mrthoxybenzaldehyde, a major soluble phenolic compound of *H. indicus*, has been demonstrated. Here we have reported three major cell-wall bound phenolic compounds (4-hydroxybenzoic acid, 4-coumaric acid and ferulic acid) in the fragrant *Hemidesmus indicus* root. We described their accumulation pattern in quantitative manner by high performance liquid chromatographic analysis before and after elicitation with yeast extract and correlation of their accumulation with shikimate pathway by inhibition with glyphosate treatment.

Keywords: Phenylpropanoid pathway - Glyphosate - Shikimic acid.

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INTRODUCTION

Hemidesmus indicus, commonly known as 'Indian Sarsaparilla' is an endemic medicinal plant of Indian subcontinent that accumulates a fragrant compound, 2-hydroxy-4-methoxybenzaldehyde in the woody rootstocks only as a constitutive secondary metabolite (Sreekumar *et al*. 2000).These fragrant roots are used as flavoring agent in '*sherbets*' (sweet-drinks) mainly in south India. This phenolic aldehyde was detected in the soluble fraction of the methanolic extract of the root part of *H. indicus* (Sircar *et al.* 2007a). Though, in Indian traditional medicine system this plant drew attention since ancient era, limited information is available on the biosynthesis of soluble as well as cell-wall bound phenolics in this plant (Chakraborty *et al*. 2008). In recent past, we made some attempts to demonstrate the biosynthesis of the major soluble methoxybenzaldehyde in elicitor treated *H. indicus* roots (Chakraborty *et al*. 2008, Kundu *et al*. 2012) and it was observed that elicited roots accumulated enhanced amount of this methoxybenzaldehyde when compared with the untreated one. It was also evident that, enzymes of shikimate and phenylpropanoid pathway were induced after elicitor treatment that leads to overproduction of the soluble methoxybenzaldehyde and blocking of shikimate pathway resulted in reduction of its accumulation (Kundu *et al*. 2012). This incident have raised a question if there is any significant effect of shikimate pathway on the cell-wall bound phenolics accumulation in elicited *H. indicus* root, as lignin and related cell-wall bound phenolic biosynthesis follow the phenylpropanoid pathway (Nair *et al.* 2004). In this communication we report the accumulation pattern of three major phenolic compounds in cell wall bound fraction of both elicited and non-elicited *H. indicus* root for the first time. These are 4-hydroxybenzoic acid (4-HBA), *trans*-ferulic acid (*t*-fer A) and 4-coumaric acid (4-com A). All these three have significant importance in pharmaceutical and industrial aspects. For example, *4*-HBA can be used as preservatives in pharmaceuticals, cosmetics, foods, and industrial products as well as its esters showed bioactivity against fungi and gram positive bacteria (Aalto *et al.* 2006). On the other hand, 4-comA has strong antioxidant properties (Kannan *et al*. 2013)

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and bactericidal activity which act on bacteria by dual damage mechanism (Lou *et al*. 2012). It has been reported that *t*-fer A could be used as antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, anti-carcinogen, antiviral, agent with vasodilatory effect, antithrombotic, and inducing agent of the viability of sperms (Ou & Kwok 2004, Kumar & Pruthi 2014). We also demonstrated a probable modulation of their accumulation pattern via shikimate pathway where inhibiting shikimate pathway with glyphosate (an inhibitor of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme) treatment decreased the accumulation of these phenolic compounds but uplifted shikimic acid in significant manner. Surprisingly, inhibition of shikimate pathway with glyphosate also resulted in decrement of shikimate dehydrogenase (SKDH) activity which might be due to over accumulation of substrate and feed-back inhibition.

MATERIALS AND METHODS

Plant material

All root samples of *H. indicus* were from same ecotype (from experimental garden of IIT Kharagpur campus, India) and more or less of same age. The plant was identified on the basis of reproductive and vegetative morphology by Pranjit Sarma, a former professor of botany at the University of Burdwan, India.

Chemicals

All solvents and chemicals were of analytical or high performance liquid chromatography (HPLC) grade unless specified otherwise. Glyphosate (*N*-(phosphonomethyl) glycine), 4-hydroxybenzoic acid (4-HBA), 4-coumaric acid (4-com A) and *trans* ferulic acid (*t*-fer A) were purchased from Sigma-Aldrich Chemical Co. Ltd. (New Delhi); The chemicals purchased were used as received. Deionized water was used in all the experiments and obtained from Barnstead/Thermolyne Diamond Nanopure™ water purification system (Dubuque, USA).

Preparation of elicitor and its application to excised roots

Yeast extract solution was prepared and used for elicitation according to a published method (Kundu *et al*. 2012).

Extraction of cell-wall bound phenolic compounds

Cell wall bound phenolic compounds were extracted according to a published method (Sircar *et al*. 2007b).

Detection of cell-wall bound phenolic compounds

Cell wall bound phenolic acids were analyzed in a Waters HPLC system (Milford, USA) in isocratic mode according to the method developed by Sachan *et al*. (2004) for separation of hydroxycinnamates and hydroxybenzoates.

Glyphosate treatment and shikimic acid quantification

A stock solution of 5 mM glyphosate was prepared in deionized water in sterile environment and added as required volume in the yeast extract solution. Shikimic acid was quantified by isocratic method through Waters®RP-HPLC using RP-Hydro C_{18} column and a mobile phase containing trifluoroacetic acid (1 mM) added water as solvent A (68%) and HPLC grade methanol as solvent B (32%). Injection volume was 50 µl and detection was done at 194 nm.

Cell free extract preparation and SKDH assay

Cell free extract was prepared as described by Kundu *et al*. (2012). SKDH assay was done according to a published method (Díaz *et al*. 2001) with the prepared cell free extract before and after elicitation, and after 18 h of 1.5 mM ‘glyphosate’ treatment upon the elicited roots.

RESULTS

Enhancement of cell-wall bound phenolics upon elicitation with yeast extract

In this study, the profile of major cell wall-bound phenolic acids were studied in *H. indicus* excised roots, both before and after 18 h of yeast extract treatment, aiming at finding any quantitative and qualitative differences upon elicitation. This ‘18 h’ time point was selected on the basis of the report by Kundu *et al*. (2012), where maximum elicitation was observed in *H. indicus* root after 18 h of incubation in yeast extract solution. Three phenolic compounds were identified through RP-HPLC analysis in both elicited and control root samples. Those were 4-HBA, 4-com A and *t*-fer A (Fig. 1A, 1B). Identification of these phenolic acids were confirmed by comparing their retention times and UV spectral properties with their authentic standards.

Quantification of these three compounds was done by standard chromatographic quantification method with authentic standard of each of three compounds. Comparison of HPLC chromatograms of both control and elicited samples clearly showed the increment of the phenolic accumulation (data not shown). The contents of 4-HBA, 4-comA and *t*-ferA in elicited roots were increased five, four and three-folds, respectively, than that of controls (Fig. 2A).

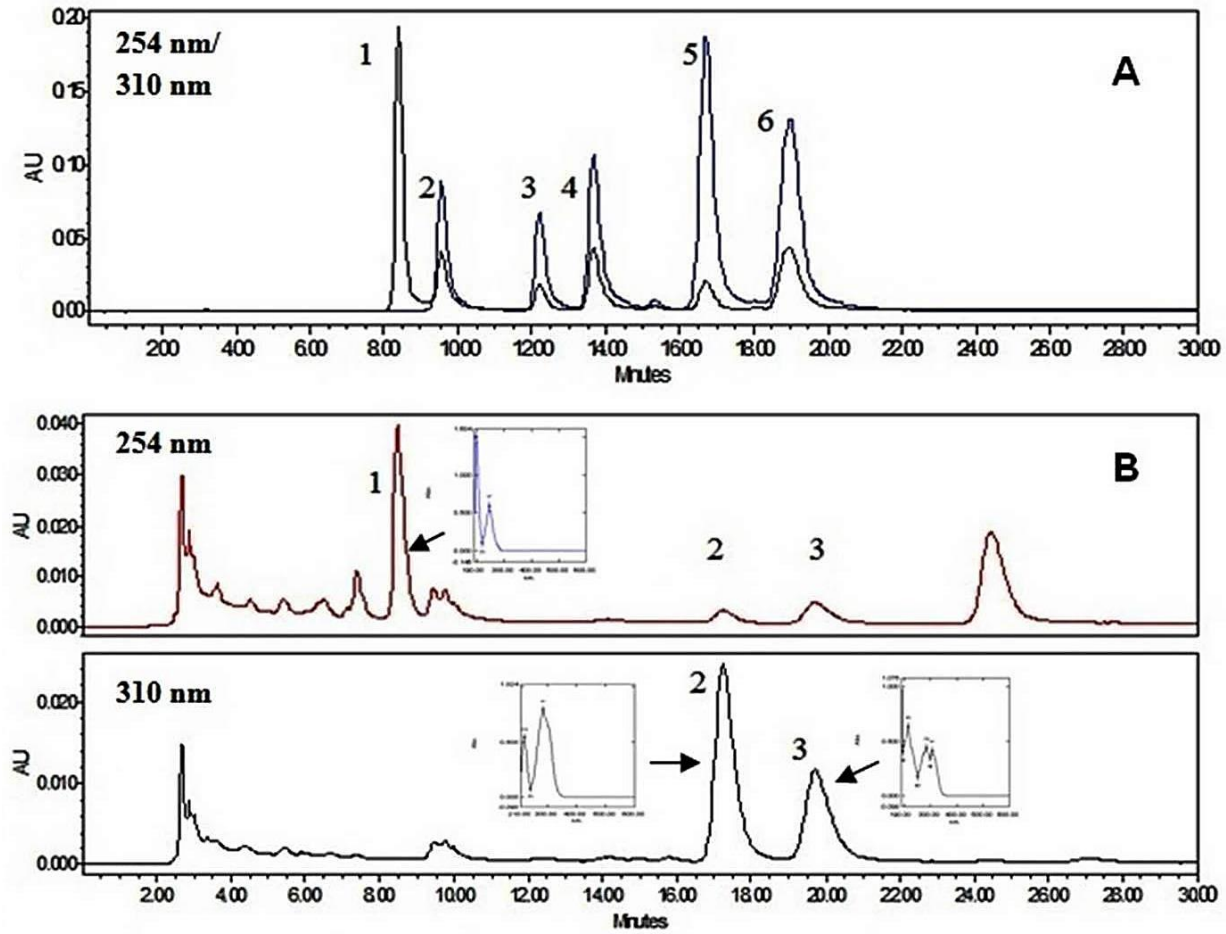

Figure 1. Chromatographic analysis: **A,** RP-HPLC chromatograms of authentic standards of different phenolic compounds. Peak identity: 1. 4-Hydroxybenzoic acid, 2.vanillic acid, 3.vanillin, 4. 4-hydroxybenzaldehyde, 5.4-coumaric acid, 6. *trans*-ferulic acid; **B,** RP-HPLC chromatograms of cell-wall bound fractions. Peak identity: 1. 4-hydroxybenzoic acid, 2. 4-coumaric acid, 3.ferulic acid. UV spectra are shown in inset.

Effect of glyphosate on cell-wall bound phenolics

Roots were harvested and subsequently analyzed to check the wall bound phenolic contents after glyphosate treatment in three different concentrations (0.5 mM, 1 mM, and 1.5 mM) for 18 h along with elicitor. This time point was selected HPLC analysis of wall bound fractions of each set revealed that 4-hydroxybenzoic acid accumulation decreased about five times by 0.5 mM glyphosate but decrement was more or less same with further increment of glyphosate in compare with the positive control (only yeast extract treated roots). In a similar way 4-coumaric acid accumulation was diminished eight times but higher concentration of glyphosate did not show further reduction. In case of ferulic acid a clear decrement was found with increasing glyphosate concentrations. Specifically, after increment of glyphosate from 0.5 mM to 1 mM a drastic fall in ferulic acid accumulation was found (3 folds) (Fig. 2B).

Accumulation of shikimic acid upon glyphosate treatment

After 18 h of treatment with 1.5 mM 'Glyphosate', shikimic acid content in the *H. indicus* root extract was quantified through HPLC analysis to check whether the shikimic acid pool was changed or not. Detection of shikimic acid was done by comparison with authentic standard, co-chromatography and spectral analysis. It was observed that inhibition of EPSPS enzyme resulted in increment of shikimic acid accumulation about four folds (Fig. 2C). Therefore, the maximum amount of shikimic acid quantified upon glyphosate treatment on elicited *H. indicus* root was 6.17±0.4 mg g^{-1} fresh mass.

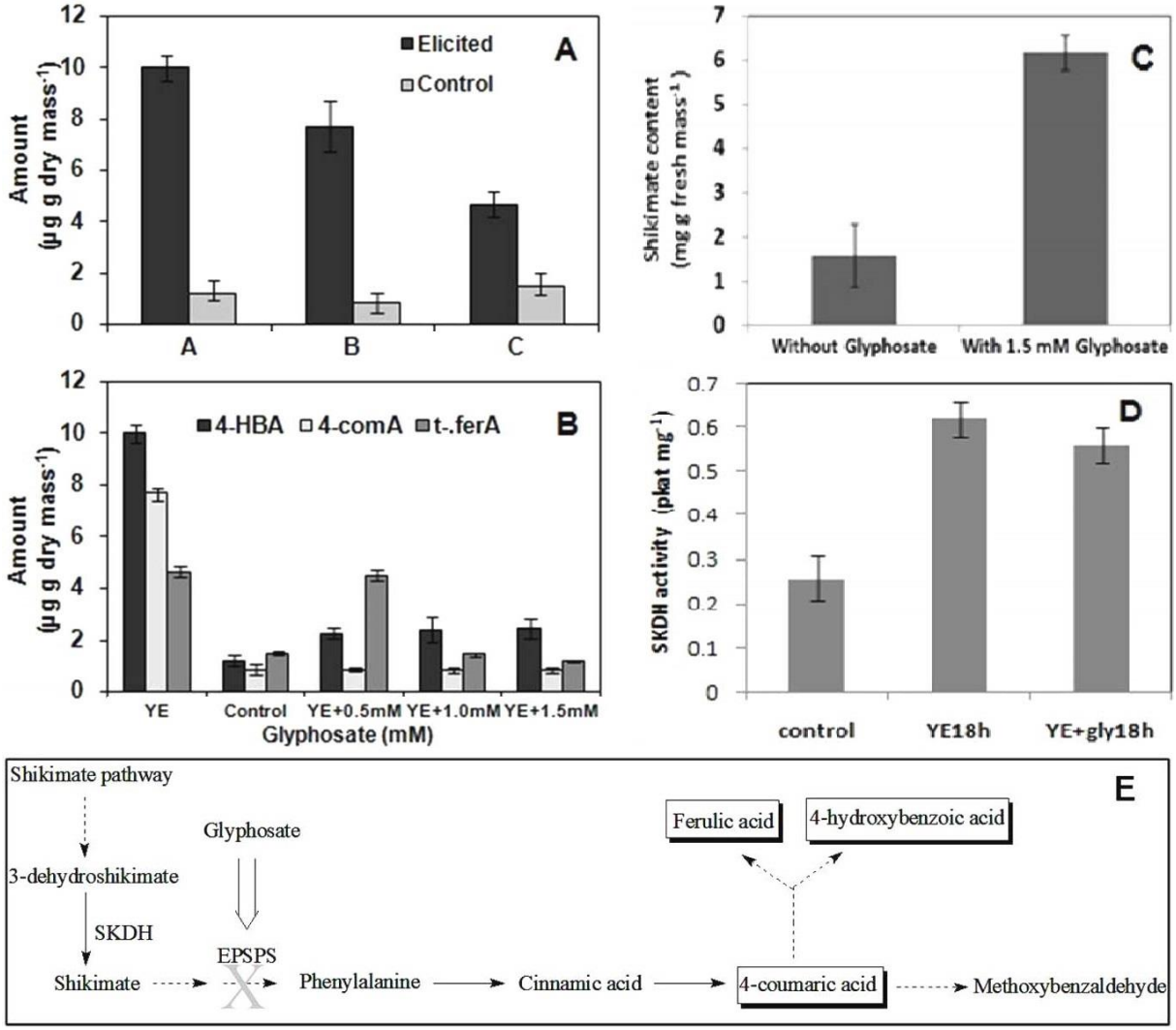

Figure 2. Effect of glyphosate treatment on elicited *H. indicus* root's cell-wall bound phenolics, shikimic acid accumulation and SKDH activity: **A,** Major cell-wall bound phenolic acids detected in excised roots of *H. indicus*. Key to histograms: A - 4-hydroxybenzoic acid, B - 4-coumaric acid and C - *trans*-ferulic acid. The black and grey bars denote contents of the above phenolic acids in elicited and control roots, respectively, after 18 h of treatments. Deionized water was used for treatment of root in case of control sets; **B,** Suppression of cell-wall bound phenolics after treatment with different concentrations of glyphosate (0.5. 1.0 and 1.5 mM) for 18 h in elicited *H. indicus* root; **C,** Enhanced shikimic acid accumulation upon glyphosate treatment for 18 h; **D,** Effect of elicitation and glyphosate treatment on SKDH activity; **E,** Schematic representation of the co-relation of shikimate pathway with cell wall bound phenolics biosynthesis as well as the route of glyphosate's effect on their accumulation. Discontinuous arrows represent multiple enzymatic reactions. All values are mean ± SD of three individual extractions.

Effect of glyphosate on SKDH activity

SKDH activity was found to be enhanced more than two folds upon 18 h of elicitation with yeast extract but this enhancement suppressed a little when 'Glyphosate' was used together with yeast extract (Fig. 2D). Effect of 'Glyphosate' on SKDH was investigated as this enzyme is one of the key enzymes of shikimate pathway.

DISCUSSION

Detection and quantification of phenolic compounds in cell-wall bound fraction have been done first time for *H. indicus* root. Three compounds, 4-HBA, 4-com A and *t*-fer A were confirmedly picked out through HPLC and UV-VIS spectral analysis. It was found that 4-HBA is the most abundant in total phenolic acid fraction of cell-wall that supported the earlier reports (Parr *et al*. 1997, Kang *et al*. 2008).We have previously reported that elicitation experiments with yeast extract increased the accumulation of the soluble phenolic content, which distinctly suggested yeast extract as potent elicitor for this system (Kundu *et al*. 2012). In the present investigation, increment of each individual phenolic acid was checked that clearly showed the rise in 4-HBA

content about 10 folds and it was maximum among three major phenolic acids. An enhanced accumulation of 4-HBA upon elicitor treatment supports the previous reports (Abd-El-Mawla & Beerhues 2002, Gaid *et al*. 2009).

Lignin and related wall bound phenolic acids were synthesized from the phenylpropanoid pathway (Nair *et al*. 2004); thus, interrogation of the effect of glyphosate on cell-wall bound phenolic fraction was carried out. In our previous report, we found that methoxybenzaldehyde accumulation was decreased upon blocking shikimate pathway with glyphosate treatment (Kundu *et al*. 2012). Here, glyphosate supplementation in elicited root exhibited reduced accumulation of cell-wall bound phenolics. Shikimic acid accumulation was studied as a confirmation of blocking of shikimate pathway. It was very significant that shikimic acid accumulation increased 4 folds (upto 6.1± 0.4 mg g^{-1} fresh mass) upon glyphosate treatment which could make *H. indicus* root as a useful bio-resource of shikimic acid, as shikimic acid has several medicinal importance (Bochkov *et al*. 2011).

Reduction in the cell-wall bound phenolics accumulation has confirmed the correlation between shikimate pathway and the phenylpropanoid mediated biosynthesis of cell-wall bound phenolics. Channeling of the product formation is directed from shikimate pathway to phenylpropanoid metabolism where a probable bifurcation results in biosynthesis of both methoxybenzaldehyde and cell-wall bound phenolics; thus blocking shikimate pathway by glyphosate showed effect on both soluble and cell-wall bound phenolic contents (Fig. 2E).

CONCLUSION

Being a very well-known Indian medicinal plant *Hemidesmus indicus* had been studied from decades to evaluate its medicinal properties. Though several investigations were done on its medicinal properties but least is known about its secondary metabolism. This work has demonstrated an idea of the accumulation of major cell-wall bound phenolic constituents in *H. indicus* roots, which have been suggested to be modulated via shikimate pathway. This finding indicates a platform of future investigation to identify the enzymes and genes, which are involved in the co-ordinate modulation of both soluble and cell-wall bound phenolic contents as well as the shikimate pathway-derived benzoates in *H. indicus*.

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