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Influence of *Agrobacterium rhizogenes* strains and elicitation on hairy root induction and Glycyrrhizin production from *Abrus precatorius*

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Abstract

Agrobacterium rhizogenes is the key step toward an *in vitro* culturing method for the mass production of secondary metabolites. We aimed to develop a protocol for development of hairy root culture and to enhance glycyrrhizin level in *Abrus precatorius*. In the present study influence of two *Agrobacterium rhizogenes* strains MTCC 532 and MTCC 2364 was evaluated in terms of transformation frequency, number of hairy roots formed and glycyrrhizin production in *A. precatorius*. Even though same source of *Abrus* explants (*in vitro* germinated seedlings) were used to transform with two strains, it was observed that there was significant variation in all the parameters assessed. Out of the two strains, MTCC 532 proved better than MTCC 2364 in terms of Transformation efficiency, number of hairy roots as well as higher glycyrrhizin content. TLC and HPLC analysis of *Abrus* hairy roots confirmed the presence of glycyrrhizin.

Keywords: *Abrus precatorius*, *Agrobacterium rhizogenes*,Hairy roots, glycyrrhizin, HPLC.

INTRODUCTION

Abrus precatorius L., locally known as Indian liquorice because of the presence of glycyrrhizin belonging to the family Fabaceae.Roots are the sites of synthesis and/or storage of many phytochemicals of pharmaceutical importance and hence seem to be most suitable organ for large scale cultivation/production.Hairy roots are the adventurous roots derived from cells transformed by the root inducing plasmid of *A.rhizogenes* and they grow in the absence of phyohormone.*A.rhizogenes* mediated hairy root formation is the voluble tool for the biosynthesis of secondary metabolites and biotechnological production of rootderived compounds. Glycyrrhizin, isthe sweet component present in the leaves and roots of *Abrus precatorius* [1,2]. Glycyrrhizin is an important phytoconstituent which is widely used in the pharmaceutical and food industry.Glycyrrhizin is used as anti-ulcerative, and anti-inflammatory preparations [3]. Glycyrrhizin is reported to exhibit antiviral [4], anti-HIV [5] .Treatment with elicitors, lead to an array of defense reactions, including the accumulation of a range of plant defensive secondary metabolites in plants or in cell cultures [6].The present study is aimed to induce hairy rootsfrom *Abrus precatorius* explants using two different strains of *A.rhizogenes*. Further, a comparative study was carried out to study the effect of two strains of *Agrobacterium rhizogenes* on hairy root induction using methyl jasmonate as elicitor towards enhancing glycyrrhizin production. Elicitation by methyl jasmonate and salicylic acid has increased the production of glycyrrhizin in whole plant cultures of G. glabra [7]. Currently hairy root culture coupled with elicitation tech-nique was found to be immensely helpful in the augmentation of valuable secondary metabolites in vitro. Methyl jasmonate is the extensively used elicitor due to its outstanding role in signal transduction pathway and has often warranted high quantity of desired products in hairy roots of several medicinal species including Centella asiatica (L) Urban [8], Salvia sclarea [9], Glycyrrhiza inflata [10], Plumbagoindica L. [11] and Withania somnifera (L.) Dunal [12]

MATERIALS AND METHODS

Plant Material and Extraction

The dried roots of *Abrus precatorius*were collected from Ghatkesar, Hyderabad, powdered separately and stored in air tight containers. The dried powder of *Abrus* (10 g) was extracted by soxhlet method with methanol as solvent for 4 hours. The extract was filtered with Whatman filter paper no 1 and the filtrate was concentrated under reduced pressure by rotary flash evaporator. The extract obtained was then dried in a desiccator and stored in air tight container [13]. Qualitative analysis of the root extract was carried out to detect the presence of various phytochemicals [14].

Hairy root production

The commonly cultivated *Abrus precatorius* red seeds wereused as an experimental material. Collected seeds were washed with tap water for 10 - 15 min followed by immersion in liquid detergent solution for 2 min. After washing with distilled water, the seeds were soaked in distilled water for two hours. Then seeds were washed with 70% alcohol for few seconds and rinsed two times with distilled water. The explants were brought to the inoculation chamber and surface sterilized with 0.1% $HgCl_2$ for 2 min and again rinsed with sterile distilled water several times. The seeds were then inoculated on MS [15] medium containing 3% sucrose (w/v) at pH 5.7 for *in vitro* germination.

Preparation of *Agrobacterium rhizogenes*:

A. rhizogenes MTCC 532 and 2364 strains were obtained from Microbial Type Culture Collection Centre (MTCC), Chandigarh, India. Hairy roots were induced by infecting the shoot nodal explants with two *Agrobacterium rhizogenes* strains (Table 1). During transformation, the

insertion of T-DNA of the Ri plasmid, present in *A. rhizogenes,* causes growth of very fine roots, known as hairy roots. For the preparation of pre inoculum, one loop full of fresh culture grown on solid YEB medium was added to 25 ml liquid YEB medium and was kept on shaker rotating at 120 rpm and 28±1°C for 24 hr for activation. Optical density of activated bacterial suspension was measured at 600 nm for identifying the log phase. This activated culture with an absorbance of 0.8 was then centrifuged at 6000 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in the fresh MS liquid medium to be used for infection.

Effect of infection and co-cultivation periods on hairy root induction

In vitro grown seedling segments of *Abrus* were injured all over the surface to facilitate the infection process before treatment with *A. rhizogenes.* The explants were infected with 532 and 2364 strains of *A. rhizogenes* suspension in an Erlenmeyer flask and kept on a shaker for 60 and 120 min. The infected explants were blotted dry on sterile filter-paper to remove excess bacteria and incubated in the dark at 28^{o}C in hormone-free MS medium. Uninfected explants (control) were cultured under the same conditions. After 24 hours the explants were transferred to hormone-free MS medium containing 100, 200, 300 mg/L cefotaxime to eliminate bacteria and then incubated in the dark. Hairy roots emerged from the wound sites of *Aburus* seedlingsfrom 5th day onwards. Fully grown long hairy roots were obtained after two weeks of culture. These hairy roots were separated from the explants tissues and sub-cultured in dark at 25°C on liquid hormone-free MS medium on a rotary shaker at 100 rpm. These hairy roots were further used for growth studies in terms of fresh / dry weight and for glycyrrhizin analysis.Hairy root induction was measured by calculating the hairy root transformation frequency as:

$$\% \text{ transformation frequency} = \frac{\text{Number of explants inducing hairy roots}}{\text{Total no. of explants infected with } A. \text{ rhizogenes}} \times 100$$

The fresh and dry hairy roots of *Abrus* were crushed in 10 ml of methanol and filtered through the muslin cloth. The extracts were centrifuged at 8,000 rpm for 5 minutes and supernatant was collected. The extracts were condensed on the rotary evaporator and stored at 4^{0}C for further use.

Elicitation of hairy roots with methyl jasmonate

Elicitors are compounds that stimulate plant cell secondary metabolism and are usually derived from components of fungal or plant cell walls. Feeding of elicitors has been proven to be an effective way to enhance secondary metabolites in plant cell cultures. Elicitors like methyl jasmonate enhance hypericin production in *Hypericum perforatum*L. [16,17]. Elicitation in *Abrus precatorius*was carried out with methyl jasmonate (MJ).A methyl jasmonate (10 mM) stock solution was prepared in 40% (v/v) ethanol and then filter-sterilized.Hairy roots were subcultured into a 125-ml flask containing 50 ml of MS liquid medium, pH 5.5, and grown at 25 °C under agitation (100 rpm).After 10 days of culture, various concentrations of methyl jasmonate (50, 100,150 and 200 µM), were added to the cultures. Hairy roots were then harvested after 2, 4, 6 or 8 d of elicitor treatment. All treatments were performed in triplicate.

Isolation of Glycyrrhizin from *Abrus* hairy roots

The methanol hairy root extract is treated with ammonia for precipitating glycyrrhizic acid. Glycyrrhizic acid thus precipitated is filtered and treated with $H_2SO_4$to adjust pH.Precipitated glycyrrhizin is then washed with water and filtered. The filtrate is dried used for TLC and HPLC.

Phytochemical and Chromatographic analysis of hairy roots

*Abrus*Hairy roots were dried in a hot air oven at 50 °C for 48 h and ground to powder. Dried powder samples (50 mg) were extracted five times with 0.5 ml methanol. Methanolic hairy root extract of two strains of *A.rhizogenes* solution (0.2 mg /ml) was then subjected to preliminary phytochemical analysis for saponin and thin layer chromatography (TLC) for the detection of glycyrrhizin using a mixture of n-Butanol: acetic acid: Water (70:10:20) The plate was allowed to dry for 10 minutes, then it was sprayed with Anisaldehyde/sulphuric acid reagent .

HPLC Profiling of *Abrus* hairy roots for glycyrrhizin

HPLC chromatograms were obtained using Shimadzu HPLC system with a 20 µl sample. The HPLC analysis was completed using a C-18 reversed phase column (250 x 4.6 mm, 5 µm) , LC-2010 pump and Column temperature 40^{0}C .The column effluent was monitored with a variable wavelength photodiode-array detector (UV/VIS detector), which has the ability to scan from 200-800 nm. The detector was connected to a computer and the data were analyzed by LC-Solution software version 1.25.Determination of glycyrrhizin was done by using mobile phase acetonotrile: acetic acid:water (41: 02: 57) at flow rate of one ml/min . The detector wavelength was 248 nm.

RESULTS AND DISCUSSION

Establishment of Hairy Root Cultures

In this study, *in vitro* germinated *Abrus precatorius* seedlings were used as explants. Hairy roots appeared at the wound sites of explants 5th day after infection with *Agrobacterium* MTCC 532 and after 9 days with MTCC 2364 (Fig 1 & 2). Wild type roots (control) also grew in a hormone- free medium. However, the growth rate of these roots was significantly lower than that of hairy roots.When hairy roots were inoculated on MS medium containing cefotaxime (300 mg/l), strain 532 showed maximum transformation frequency of 58.67 ± 0.20% (Table 1) than Strain 2364 (maximum transformation frequency of 50.57 ± 0.10 %). The glycyrrhizin production in *Abrus precatorius* hairy roots (MTCC 532 and MTCC 2364 ,without elicitation) shows gradually increased with time course until it reached the maximum level in the fifth week of culture (31.0 µg/g dry wt and 23.0 µg/g dry wt) and there after the production slightly decreased (Table. 2 and Fig 6).

Figure 1 Abrus precatorius hairy roots (MTCC 532)

Figure 2 Abrus precatorius hairy roots (MTCC 2364)

Table 1: *Agrobacterium rhizogenes* mediated genetic transformation in *Abrus precatorius*

Experiment No.	Number of explants inoculated	No. of explants forming hairy roots	Percent hairy root induction MTCC 532	Percent hairy root induction MTCC 2364
1	10	5	42.50 ± 0.12	40.30 ± 0.11
2	10	6	51.30± 0.09	47.20 ± 0.06
3	**10**	**8**	**58.67 ± 0.20**	**50.57 ± 0.10**
4	10	6	52.30 ± 0.09	46.30 ± 0.08
5	10	5	41.7 ± 0.13	41.60 ± 0.13

Table 2: Effect of different strains of *A. rhizogenes* on growth and glycyrrhizin production in *A. precatorius* hairy root cultures (Before Elicitation)

Time Taken(Weeks)	Strain MTCC 532		Strain MTCC 2364	
	Dry weight g/flask	Glycyrrhizin content (µg/g dry weight)	Dry weight g/ flask	Glycyrrhizin content (µg/g dry weight)
1	0.04	8	0.04	6
2	0.09	13	0.07	11
3	0.12	21	0.11	19
4	0.15	27	0.13	21
5	0.22	**31**	0.17	**23**
6	**0.25**	28	**0.20**	20
7	0.19	25	0.16	18
8	0.17	23	0.12	16

Phytochemical and Chromatographic analysis of *Abrus* hairy roots

Phytochemical analysis of methanolic extract of field grown roots showed the presence of alkaloids, flavonoids, phenols, saponins and triterpinoids. Thin layer chromatography of methanolic hairy extract of *Abrus* showed presence of glycyrrhizin. When the developed plates were sprayed with Anisaldehyde sulfuric acid, spots were visualized and coincided with that of the standard reference glycyrrhizin. HPLC spectra of standard glycyrrhizin shows peak at retention time of 8.75 in mobile phase with acetonitrile: acetic acid:water (41: 02:

57).HPLC spectra of methanolic hairy root extracts (MTCC 532 and MTCC 2364) of *Abrus precatorius* shows peak at the same retention time as compared to standard glycyrrhizin. (Fig 3,4,& 5)

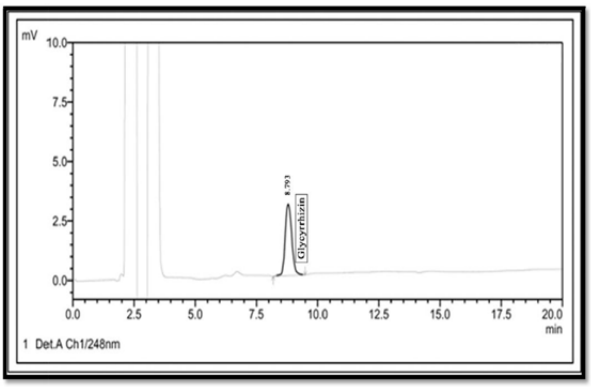

Figure 3. Standard Glycyrrhizin

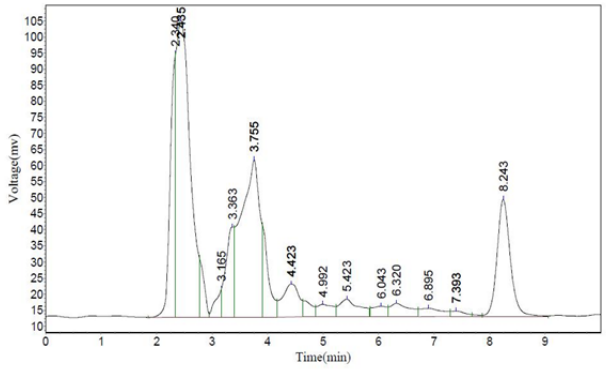

Figure 4 .Chromatogram HR532

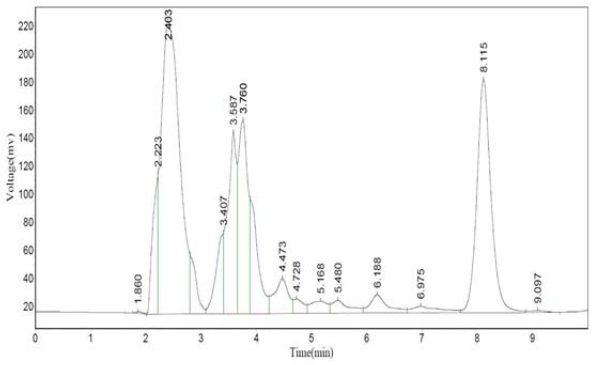

Figure 5 .Chromatogram HR 2364

Elicitation of hairy roots with methyl jasmonate

Figure 7 shows the effect of methyl jasmonate on glycyrrhizin production in hairy roots. Glycyrrhizin production increased with the duration of the treatment (1 – 8 days) with 100 and 200 µM of methyl jasmonate. Hairy roots of strain MTCC 532 shows highest glycyrrhizin content (80.01 µg/g dry wt) after 4 days in samples treated with 100 µM methyl jasmonate. While Hairy roots of strain MTCC 2364 shows highest glycyrrhizin content (72.01 µg/g dry wt) after 4 days in samples treated with 100 µM methyl jasmonate. Thus, hairy roots treated with Methyl jasmonate produced 2.5 times higher glycyrrhizin content than the hairy roots without elicitor. [18] reported that yeast extract (50 mg/l) added to *Abrus precatorius* cell cultures significantly stimulated the glycyrrhizin accumulation after treatment for 2 days.The literature survey showed that MTCC532 has been successfully used to obtain hairy roots of Solanaceace members including Solanum surattense Burm f., W. somnifera (L) dunal [19] and Solanum xanthocarpum [20].

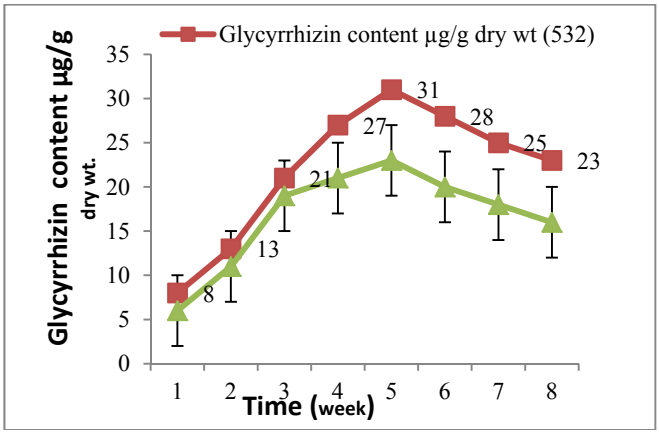

Figure 6.Glycyrrhizin content before elicitation

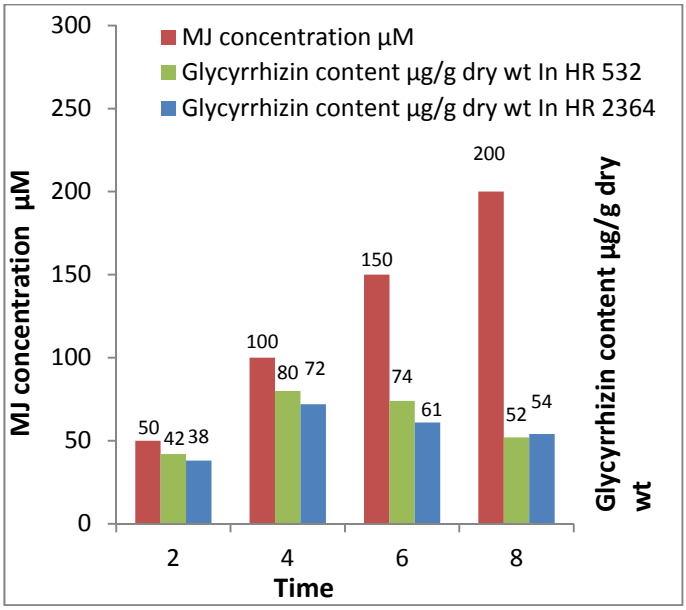

Fig 7 Glycyrrhizin content after elicitation with Methyl Jasmonate

CONCLUSION

Hairy roots were successfully induced in *Abrus precatorius*. The results show that maximum glycyrrhizin production after elicitation with methyl jasmonate was found in hairy roots transformed with strain 532 than strain 2364.Our results suggest that methyl jasmonate is an effective elicitor for the enhancement of glycyrrhizin in hairy roots cultures of *A. precatorius*. In summary, our study has resulted in producing 2.5 times higher glycyrrhizin content from *Abrus* hairy roots when treated with methyl jasmonate as elicitor.

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