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RESEARCH ARTICLE

AGROBACTIRIUM RHIZOGENES INDUCED HAIRY ROOT DEVELOPMENT AND ITS EFFECT ON PRODUCTION OF GLYCYRRHIZIN IN *Abrus precatorius* (L).

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ABSTRACT

There is a strong correlation between secondary metabolite production and morphological differentiation, which gives more impetus to application of organized cell culture for large-scale production of phytopharmaceuticals. The roots are the sites of synthesis and /or storage of plant metabolites; they are important organs for the production of valuable phytochemical. In Ayurvedic system of medicine A. precatorius is used as important drug for various ailments as Roots are used as diuretic, tonic, emetic, alexitric and as substitute of liquorices and have antifertility activity. Roots contain about 5% glycyrrhizin (pentacyclic triterpene glycoside) and 8% of an acid resin. Therefore, an attempt had been made to study the inoculation effect of Agrobacterium rhizogenes on development and production of secondary metabolites in A. precatorius. Significant increment in fresh weight in hairy roots cultured in solidified media was observed. The data revealed 5.25 times increment in fresh weight from initial fresh weights. 700 mg of glycyrrhizin was obtained from 20g of roots of field-grown plant giving the concentration of glycyrrhizin - 35mg/g d.w.b. Absorbance of hairy roots extract at 248nm is 6.615 with the help of regression equation (for standard curve of glycyrrhizin) concentration of glycyrrhizin was found to be 71.35mg/g d.w.b.

INTRODUCTION

Amongst new approaches under investigation one of the most promising is the "Plant organ culture". Tissue culture has played an important role in the areas of mass propagation and secondary metabolite production for certain specific compounds of interest. Besides known compounds that are produced from plants, like pharmaceuticals (alkaloids, steroids, terpenoids, flavonoids, liganans, enzymes), food additives (carotenoids, anthocyanins, betalains, vanilla, rose, lavender), perfumes (Sandal wood oil, agarwood oil, artemisia oil) and biopesticides, this technique serve as a unique source for altogether new compounds which have not been found in the mother plant eg. Rutacultin (synthatic) from cell cultures of *Ruta graveolens* (Neckell, 1980). So keeping these points all together production of these so called secondary metabolities using plant cells in culture as an alternative to extraction of plant tissue has long been identified as a worthwhile objective (Tabla and Fuijita, 1985).

However, there are few problems/limitations associated with tissue and cell culture such as increased biomass causes serious problems in the seperation of biomass from the culture media, utilization limitation of cells, heterogeneity of cell stages, damage and production of inhibitory products etc. (Rhodes *et al.,* 1987; Kamada *et al.,* 1986).

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To overcome these, attention was focused towards *in vitro* culture of organized tissue particularly the roots. The classical functions associated with roots are mechanical support and the uptake of water and mineral nutrients. Since the roots also are the sites of synthesis and /or storage of plant metabolites, they are important organs for the production of valuable phyto and physiochemicals (Duke *et al*., 1985). The strong correlation between secondary metabolite production and morphological differentiation laid more emphasis on plant organ culture as one of the alternate approaches (Flores, 1992).

A plant root seems to be most suitable for large scale cultivation since the roots are the sites of synthesis and/or storage of certain chemicals of pharmaceutical importance. Slow growth rate due to highly organized nature of normal roots posed another serious limitation in commercialization of technology using root biomass as a source for secondary metabolite production. Recently root-cultures has been redeveloped as an experimental tool making use of natural ability of soil bacterium *Agrobacterium rhizogenes* to transfer genes into the host plant genome. *A. rhizogenes*, a gram negative soil bacterium, infects a wide range of plant species and causes the neoplastic plant disease syndrome known as 'hairy root disease'. Hairy roots are fast growing, initiating highly branched fine adventitious at the site of infection and can grow even in a hormone free liquid culture medium. Attention is now being focused on these fast growing genetically transformed hairy roots for

commercial production of secondary metabolites. The name 'hairy root' was first mentioned in the literature by Steward in 1900. Ricker *et al.* (1930) described and named the hairy root causing organism as *Phytomonas rhizogenes* which was later renamed as *Agrobacterium rhizogenes*. The first directed transformation of higher plants using A. rhizogenes was made by Ackermann (1977). These plants had a heritable abnormal phenotype. Based on this he proposed that those plants were genetically transformed and confirmed the presence of Ri-TDNA by molecular hybridization. Agrobacterium rhizogenes brings about transformation in plant cells by transfer of the T-DNA of the Ri-plasmid to the host genome and here the genes contained in the T-DNA are expressed. (Rhodes, 1989; Toivonen, 1993; Costacurta and Varaderlayden, 1995). Genes in Agrobacterium that are involved in the transfer of the T-region to the plant cells are called "Vir" genes and are located in a 40-kbp region of the Ri-plasmid called the virulence (vir) region. The genes in the 'vir' region are only expressed in presence of acetosyryngone. Certain genes in Agrobacterium genome produce proteins which help bacterial attachment to plant cell wall and microfibril formation(Chilton *et al*., 1982). Conformation that a plant cell is transformed on infection with *A. rhizogenes* can be obtained by transformed root morphology exhibited by hairy root cultures and their transformed regenerants. The hairy roots have altered phenotype and these roots show high degree of lateral branching, profusion of root hairs and lack of geotropism (Tepfer, 1982, 1990; Tepfer and Tempe, 1981).

One of the most important characteristics of the transformed roots is their capability of synthesis of secondary metabolites specifies to that plant species from which they have been developed (Hamill *et al*., 1986, Doran, 1989). Growth kinetics and secondary metabolite production by hairy roots is highly stable (Aird *et al*., 1988; Flores, 1986) as compared to cell cultures. Effect of cytokinins and auxins on growth and morphogenesis of hairy root have indicating that IAA plays an important role in the hairy root growth. The sensitivity of hairy root tips to exogenous auxin was found to be 100-1000 times higher than that of untransformed material (Ohkawa *et al*., 1989). Advantages of hairy root culture system over cell culture system have been discussed by Wilson *et al*., 1987. Viral protection, insect resistance, herbicide resistance, increases in the starch content, improved seed storage, protein and altered ripening are some of the characters which are transferred. Efforts are also on to transfer genes coding for secondary metabolites of pharmaceutical importance to make the process more advantageous and economical (Mascarenhas, 1997).

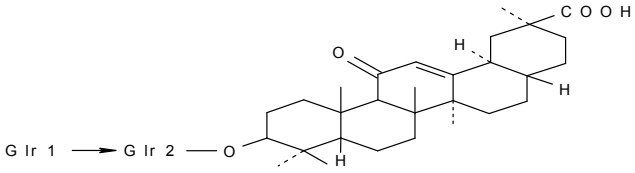
Abrus precatorius Linn.

A. precatorius Linn. commonly known as Indian liquorice; Rosary pea; Red beadvine; Prayer beads; Coral bead plant; Crab's eye; Love bean; Licorice vine; Lucky bean; Minnie-minnies or Weather plant belong to family Leguminosae (Fabaceae). In Hindi it is called Gunchi, Rati; in Sanskrit as Gunja and in English it is called Jequirity. *A. precatorius* is a perennial plant with multiple branches, deciduous climber with a maximum height of 4 meter. Flowers are purple in colour. Seeds are scarlet and black. (Photo - 1)

Phytoconstituents

Roots contain about 5% glycyrrhizin (pentacyclic triterpene glycoside) and 8% of an acid resin. Leaves contain glycyrrhizin, the principal constituent of liquorice. Four sweet tasting terpeneglycoside isolated from methanolic extract of leaves, are abrusissiodes A, B, C, D. Methanolic extract concentrate is 100 times sweeter than sugar (Chaudhri, 1996). Structure of glycyrrhizinic acid shown below:

Glycyrrhizinic acid -



Glr → Galacturonic acid

Glycyrrhizin = mixture of calcium and sodium salts of glycyrrhizinic acid

Seeds are poisonous and principal poisonous constituent is abrin, a toxalbumin similar to ricin from *Ricinus communis* seeds. It contains two fractions - a globulin and an albumin, the former being more powerful. Seeds contain a fat splitting enzyme, a glucoside, abrussic acid, haemagglutinin and a quantity of urease (Chopra, 1986).

Therapeutic uses

Roots are used as diuretic, tonic, emetic, alexitric and as substitute of liquorice. They have antifertility activity (Milhet *et al*., 1978). Leaves tea is used to treat cough, cold and fever. It's juice mixed with oil is applied on painful swelling of the body (Seaforth, 1981). Seeds are purgative, emetic, tonic, aphrodisiac, anti-ophthalmic, anti-phlogistic, used in nervous disorder and cattle poisoning. Paste of seeds is applied locally in sciatica, stiffness of shoulder, joints and paralysis. Abrin suppresses Ehrlich ascites tumor growth in mice. Protein extract of seeds exhibits anti-tumor activity on Yeshida sarcoma in rats and mice. Poultice of seeds is used as suppositories to bring about abortion. Bruised seeds have been used criminally for poisoning cattle and for homicidal purpose (Kashyapa *et al.*, 1986).

MATERIAL AND METHODS

Nutrient Medium for Hairy Roots

Murashige and Skoog's medium (MS) is mainly used for the induction and maintenance of hairy roots. However, enhanced production of metabolites has been achieved in specific root culture medium (RC), Linshmier and Skoog's (LS), B_5 medium and woody plant media. Simple media used for normal (untransformed) root culture such as Nitch's Hoagland's solution and white's root culture media need reassessment for their efficacy in enhancing secondary product synthesis in hairy roots.

Viability Studies of Seeds Seeds of *A. precatorius* were collected from Botanical garden of Dr. H.S. Gour University, Sagar, M.P. The viability testing of seeds was carried out by observing germination. The seeds were taken in a beaker and kept under running tap water. The tap water was passed through a strainer kept over the beaker. The strainer prevented the overflowing of seeds

while washing. The seeds were then washed with unspecialized double distilled water. The washed seeds were transferred with the help of a clean forceps in Petri dishes containing pad of absorbent cotton wool covered with a filter paper. The pad was moistened with double distilled water and sufficient space was maintained in between the seeds to provide sufficient area for germination. The Petri dishes were covered and kept for germination in dark at 25±2°C. The above experiment was carried out under normal laboratory conditions.

Culture Medium for Agrobacterium rhizogenes

The strain of *A. rhizogenes* used was MTCC- 532, which was obtained from Institute of Microbial Technology, Chandigarh. It was maintained on nutrient agar medium in Petri dish having following composition:

Beef Extract	-	1g
Yeast Extract	-	2g
Peptone	-	5g
NaCl	-	5g
Agar	-	15g
D.W.	-	1 liter

As per requirement, the temperature of bacterial culture was maintained at 26°C. The culture was sub-cultured at regular intervals of 30 days. Developed cultures of *A. rhizogenes* stain 532 have been shown in Photo – 2.

Inoculation of Bacteria

Plantlets were cut at stem portion just above the crown (attachment point of the roots and stem) and were infected with *A. rhizogenes* using a sterile needle under laminar flow hood in presence of two spirit lamps to overcome the chances of contamination (Wei *et al*., 1986). Both control and inoculated plantlets were maintained at room temperature till 45 days. Control and inoculated roots of *A. precatorius* have been shown in Photo – 4

Determination of Fresh and Dry Weights

The weight of the beaker with roots and medium minus the weight of pre-weighed beaker with medium provided "apparent" fresh weight of roots. Finally 45days old hairy roots were withdrawn, weighed for getting final fresh weight and dried at 60 to 80°C until constant weight was obtained. Fresh and dry weights have been shown in Table 1.

Analysis of Glycyrrhizin in Abrus Precatorius

The glycyrrhizin was extracted from field grown plants of *Abrus precatorius*. 20g roots were taken, dried in shade and powdered (A 40-mesh screen was used to screen the powdered mass), mixed thoroughly with 50 ml acetone and 20 ml dilute HNO_3. The flask were corked and macerated for 2h with occasional shaking and the contents were filtered. To the marc 20 ml of acetone was added and maceration was done on water bath and filtered. Both the filtrates were combined and concentrated. To the combined extracts, sufficient quantity of dilute ammonia solution was added for precipitation of ammonium glycyrrhizinate. The precipitate was separated by filtration. It was washed with 5 ml of acetone twice and acetone fraction was dried. The concentrated form was used for qualitative and quantitative estimation (Kokate, 1994). One gram of powdered hairy roots was taken and extracted same as the procedure described above.

Qualitative Estimation of glycyrrhizin

Five mg hairy root extract and 5mg of extract of field grown material was dissolved in 1ml of chloroform separately and 1ml of acetic anhydride was added following the addition of 2ml of concentrated sulphuric acid from the side of test tube.

Formation of reddish violet colour at the junction indicates the presence of triterpenglycoside- glycyrrhizin (Hawk *et al*., 1954).

Qualitative Estimation of Glycyrrhizin by TLC

Silica gel G was used as stationary phase. Aqueous slurry with1 part silica gel G and 3 parts of water was prepared and spread over the clean glass plates (18 cm) in a way to achieve fine layer of slurry on the glass plate up to the height of 15 cm. The prepared TLC plates were dried in air and then activated for 30 minutes at 110°C in an oven. Two mg glycyrrhizin (obtained from field grown plant extract) was dissolved in 1ml 50% v/v methanol to prepare reference solution. Hairy root extract of *Abrus precatorius* was used as sample solution.

Solvent system was prepared using chloroform 64ml, methanol 50ml and water 10ml. The spots of sample and reference solution were applied on TLC plates and the plates were placed in airtight chromatographic chamber, previously saturated with the solvent system being used for TLC. The mobile phase was allowed to run up to 3/4 of the height of the TLC plates. The plates were taken out, air dried and sprayed with anisaldehyde-sulphuric acid reagent (prepared by mixing 0.5 ml anisaldehyde with 10 ml glacial acetic acid, followed by 85ml methanol and 5ml concentrated sulphuric acid), heated at 100°C for 5 min then observed under in UV-under at 365nm (Wagner, 1996). TLC plate has been shown in Photo -6 .

Quantitative Estimation of Glycyrrhizin

Preparation of standard curve

Hundred mg of glycyrrhizin (obtained from extraction of field grown roots of *Abrus precatorius*) was weighed, dissolved in absolute alcohol and aliquots of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µg/ml were prepared. Absorbencies of these were taken at 248nm on Cintra-10 spectrophotometer (Bruneton, 1995) and data were tabulated in Table 2.

Absorbance of hairy root extract

1ml of hairy root extract of *A. precatorius* was diluted to 100ml with absolute alcohol and absorbance of these was taken at 248nm.

RESULT AND DISCUSSION

The germination of seeds of *A. precatorius* started on 3 day, sixty percent germination was observed on 10th day and after 12th day the germinated seedling had two distinct cotyledons. (Photo 3)

Table 1. Fresh and dry weights of hairy roots of experimental plants.

	Abrus precatorius	
	Apparent fresh weight (g)	Final dry weight (g)
Initial	0.4	-
45 days old	2.1	0.44

Increment in fresh weight in hairy roots cultured in solidified media was observed (Ph - 4). The data revealed 5.25 times increment with *A. precatorius* in fresh weight

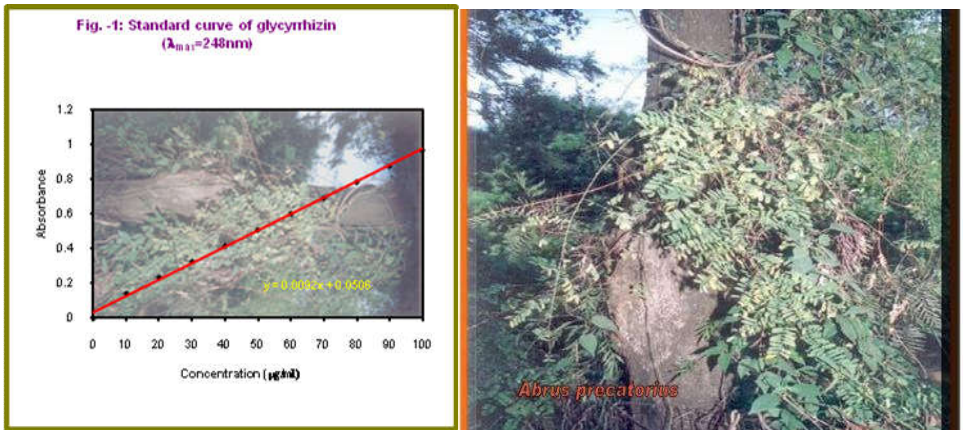


Photo 1. Morphological characters of Abrus precatorius

Photo 2. Developed culture of *Agrobacterium rhizogenes* (Strain 532)
Developed culture of *Agrobacterium rhizogenes* (Strain 532)

Photo3. Aseptically developed plantlets from seeds of *Abrus precatorius*

Photo 4: Aseptically developed roots of *Abrus precatorius* under control and hairy roots after inoculated with *Agrobacterium rhizogenes*

Photo 5. T.S. of *in-vitro Agrobacterium rhizogenes* inoculated root of *Abrus precatorius.* Arrows sowing initiation of hairy roots.

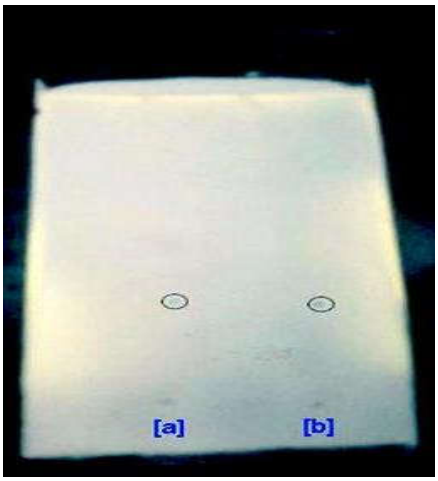


Photo 6. Co TLC of Standard(s):
[a] – Field grown plant extract [b] – *A. rhizogenes* inoculated hairy root extract

from initial fresh weights. (Table 1). Transverse sections of both inoculated and control roots of *A. precatorius* were cut processed and stained with saffranin (1g of saffranin was dissolved in 50ml of alcohol and volume made up to 100ml with water) and observed using microscope (at 100 x magnification). Transverse sections have been shown in Photo 5. Same histological features were observed in control roots and roots infected with *A. rhizogenes*. (Photo 5) In inoculated roots initiation of hairy roots was observed.

Characteristics of glycyrrhizin-

Colour - Yellowish brown
Solubility - soluble in water, methanol and ethyl alcohol

Sample and reference solution showed same chromatographic pattern i.e. spot of similar Rf value and color which indicate the presence of same constituent i.e. glycyrrhizin in the hairy roots. (Photo- 6) 700 mg of glycyrrhizin was obtained from 20g of roots of field-grown plant giving the concentration of glycyrrhizin - 35mg/g d.w.b. Absorbance of hairy roots extract at 248nm is 6.615 with the help of regression equation (for standard curve of glycyrrhizin) (Fig.1)concentration of glycyrrhizin was found to be 71.35mg/g d.w.b.

Table 2. Data for Standard curve of Glycyrrhizin at λ_{max} 248 nm

S. No.	Con. (µg/ml)	Absorbance	Regressed value	Statistical Analysis
1.	10	0.0906	0.1425	**Correlation coefficient**
2.	20	0.1925	0.2344	$r^2 = 0.971769$
3.	30	0.3961	0.3263	**Standard error**
4.	40	0.4875	0.4182	SE=0.054767
5.	50	0.5775	0.5101	**Linear Equation**
6.	60	0.6308	0.6020	y = 0.0092x + 0.0506
7.	70	0.6885	0.6939	**Intersect**
8.	80	0.7973	0.7858	m = 0.0092
9.	90	0.8225	0.8778	**Slope**
10.	100	0.9280	0.9697	c = 0.0506

Absorbance of hairy root extract - 6.615

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