

***Agrobacterium rhizogenes* MTCC 532 Induced Hairy Root Development in *Tephrosia purpurea* (L) and its Effect on Production of Tephrosin**

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Abstract

In Ayurvedic system of medicine *T. purpurea* is used as important drug for various ailments as tonic, laxative, anthelmintic, purifier of blood, a cordial, cough, asthma, tympanitis, dyspepsia, snake bite and in bilious febrile attacks enlargement and obstruction of the liver, spleen and kidneys. The roots are the sites of synthesis and/or storage of many plant metabolites and also an important organ for the production of valuable phytochemical. Roots of *T. purpurea* contain tephrosin, deguelin, isotephrosin and maxima substances which makes it important drug plant. Therefore, an attempt had been made to study the inoculation effect of *Agrobacterium rhizogenes* (MTCC- 532) on growth, development of hairy roots and its effect on production of secondary metabolites in *T. purpurea* roots. After transformation of *A. rhizogene* (MTCC-532) significant increment in fresh weight in hairy roots cultured in solidified media was observed, the data revealed 7.6 times more fresh weight from initial fresh weights. In inoculated roots fast initiation of hairy roots was observed. 1.528g tephrosin was obtained from 40g of roots of field grown plants, giving the concentration of tephrosin in field grown plants roots to 38.2 mg /g d.w.b.

Keywords: Hairy roots; *A. rhizogenes*; *T. purpurea*; Tephrosin.

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1. Introduction

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 have 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

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soil bacterium, infects a wide range of plant species and causes the neoplastic plant disease syndrome known as 'hairy root disease'.

In *Agrobacterium rhizogenes* infected roots the level of secondary metabolite production has been observed higher than that of non-transformed roots of control plants. Ravishanker *et al.* [1] observed that the hairy roots producing secondary metabolites of interest at levels equal or higher to those of field grown plants and producing novel secondary metabolites not known to be present in the control tissues.

Growth kinetics and secondary metabolite production by hairy roots is highly stable [2, 3] as compared to cell cultures though the kinetics of secondary metabolite production in transformed roots is poorly understood. Integration of Ri-T-DNA plasmid into host plant genome is stable which accounts for genetic stability of transformed roots cultures. The T-DNA region in Ri-plasmid is a minor part of the large (>200 kbp) plasmid called the T-Ri (Root inducing) plasmid. The Ri-plasmid transfer two regions of its genome namely T_L and T_R regions. These two DNA regions code for different mechanisms each of which can individually induce root formation on host plant organs. Cytological investigations of hairy root lines exhibit the expected euploid chromosome number specific to that of parent plant.

Effect of cytokinins and auxins on growth and morphogenesis of hairy root has been examined. IAA synthesizing genes are present on TR-DNA of the agropine type Ri-plasmid and treatment with antiauxins inhibited the hairy root growth sensitivity of hairy root tips to exogenous auxin was found to be 100-1000 times higher than that of untransformed material [4, 5].

Tephrosia purpurea Linn. commonly known as *Wild indigo* or *Galegu purpurea*, belongs to family Leguminosae (subfamily-Papilionaceae) (see Ph-1). In Hindi it is called Sarphonka or Dhamasia and in Sanskrit it is called Sharapunkha. It is distributed throughout the tropics and sub-tropics and has been introduced elsewhere for green manure and soil conservation. It is highly branched sub erect herbaceous perennial herb usually 30-60 cm in high; with thin firm glabrous branches. Leaves are 6.5-12.5cm long, short petiolated, with 13-14 narrowly oblanceolate leaflets, green and glabrescent above and obscurely silky beneath. Flowers-red or purple, pods slightly recurved, pedicels short and bracteoles minute [6].

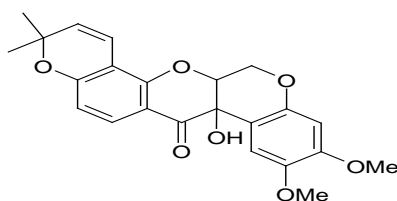


Ph-1. Morphological characters of *Tephrosia purpurea*.

Ayurvedic literature mentions two species of Tephrosia, one with red or purple flowers-Rakta sharpunkha which is *Tephrosia purpurea* Perse, and other one with white flowers-swetha sharpunkha which is *T. villosa* Perse [7].

Plant contains alkaloid 4.5% Rutin (by Flavonoids Assay method), gum, and a trace of albumin and coloring matter, brown resin and chlorophyll. Ash contains a trace of Mg, Ca [8]. Roots contain- tephrosin(rotenoid), deguelin, isotephrosin, maxima substance A, B and C in concentration of 0.8%, 0.1% and 0.8% respectively. Maxima substance C shows isoflavone structure chemically related to rotenone [9]. Leaves contain 2.5% of rutin. Pods give purpurin A', purpurin B' and maximin.

Tephrosin is a rotenoids, which is a class of isoflavonoid characterized by the presence of an extra carbon atom in an additional heterocyclic ring [10] as shown below.



Tephrosin

Therapeutic uses

Plants is used as tonic, laxative anthelmintic for children; used internally as a purifier of blood and considered as a cordial. Drug is used in cough, asthma and tightness of chest [11].

Roots are bitter they are given in tympanitis, dyspepsia and used as fish poison and as antidote to snake bite. Its paste with turmeric and rice water or cow's milk is applied to scrofulous glands. It's decoction with Pepper powder is given in bilious febrile attacks enlargement and obstruction of the liver spleen and kidneys. In hepatic dropsy roots ground in buttermilk are used.

A powder of root taken with water for about a month cures enlarged scrotum. Powdered root mixed with honey is applied to ulcers. Root grounds in curds are used as a remedy for bleeding piles and with black pepper it acts as diuretic in gonorrhoea. Roots powder with honey is applied to ulcers. Fresh root bark is used in obstinate colic. A liniment prepared from roots is smoked for relief from asthma and cough. Roots are also recommended for boils, pimples, abscesses especially carbuncles on the back, as tonic, laxative and as blood purifier. Oil of seeds is used for itch, scabies, eczema and other skin eruptions. Decoction of pods is used as a vermifuge and to stop vomiting. Ashes of plant mixed with powder of *Chebulic myrobalan* is used in treatment of tumours [8].

The plant shows hepatoprotective activity against carbon tetra chloride. It has undergone clinical trials in viral hepatitis and is claimed to improve liver function [12-16]. Drug acts on specific area in the brain and modifies the effects of some CNS depressants [16]. In addition, the roots are reported to be effective in the treatment of leprous lesions

while their juice is used to treat skin eruptions. An extract of the pods is effective for the treatment of pain, inflammation and their decoction is used to combat vomiting [17].

2. Material and Methods

Surface sterilization and germination of seeds

Seeds of *T. purpurea* were obtained from Botanical garden, Department of Botany, University of Saugor. Seeds were surface sterilized with alcohol (70% v/v) for 1 minute and sodium hypochlorite solution (4% v/v) for 10 minutes and finally washed with sterilized water for 3 times. Seeds were ready for aseptic transfer on to the sterilized Petri dishes containing moistened cotton pad and layers of filter paper. After germination they were planted on the surface of agar solidified MS media in Petri dishes.

Preparation of culture media for bacterium

The strain of *A. rhizogenes* used was MTCC-532, which was obtained from Institute of Microbial Technology, Chandigarh (see Ph-2). 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.



Ph-2. Culture of *Agrobacterium rhizogenes* (strain 532).

Preparation of culture media for hairy roots

Murashige and Skoog's medium (MS) is mainly used for the induction and maintenance of hairy roots [18]. However, enhanced production of metabolites has been achieved in specific root culture medium (RC), Linshmier and Skoog's (LS), B₅ 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. 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 [19]. Both control and inoculated plantlets were maintained at room temperature till 45 days.

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 45 days 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.

Table 1. Fresh and dry weights of hairy roots in *Tephrosia purpurea*.

	Apparent fresh weight (g)	Final dry weight (g)
Initial	0.3	-
45 days old	2.3	0.50

Extraction of tephrosin

40g of root powder (A 40-mesh screen was used to screen the powdered mass), of *Tephrosia purpurea* was macerated with 400ml of alcohol for five days at room temperature. It was filtered and the marc was again macerated with fresh 200ml alcohol for another 5 days. First and second macerate were mixed, the alcohol was recovered until syrupy consistency of residue was obtained. It was then dried in air and subsequently at temperature below 50°C. This alcoholic extract was mixed thoroughly with about 50g of silica gel. This mass was dried and successively extracted with petroleum ether (60-80°C), chloroform, acetone and methanol to obtain different fractions. After each fractionation, the evaporation test was performed to assure the complete extraction. The completion of extraction was confirmed by evaporating a few drops of the extract on a watch glass and ensuring that no residue remains after evaporating the solvent. Every time the marc was air-dried before extracting with the next solvent. The various fractions were tested for the presence of Tephrosin. The methanolic fractionation of alcoholic extract contains tephrosin [20].

One gram of hairy root powder of *Tephrosia purpurea* were macerated with 20 ml of alcohol for five days at room temperature. It was filtered and the marc was again macerated with fresh 10 ml alcohol for another 5 days. First and second macerate were

mixed. Residue was air-dried. This extract was extracted with methanol and the extract obtained was used for qualitative and quantitative estimations.

Qualitative estimation and quantitative estimation

5ml each of extract (Hairy root extract and root extract from field grown plant) was hydrolyzed with 10% v/v sulphuric acid, cooled and was extracted with diethyl ether and divided into 3 portions in three separate test tubes 1ml of dilute ammonia, 1ml of diluted sodium carbonate and 1ml of 0.1N sodium hydroxide was added to the first, second and third test tubes, respectively. Formation of yellow colour in each test tube indicated the presence of tephrosin [20].

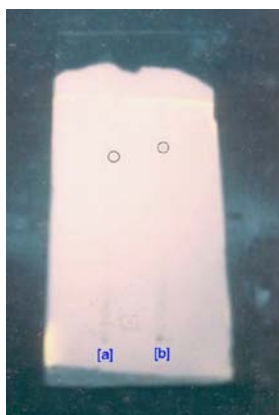
Silica gel G was used as stationary phase and plates were prepared for TLC studies. Chromatographic chamber was saturated with solvent system, which was prepared mixing 100ml ethyl acetate, 11 ml formic acid, 11 ml glacial acetic acid and 26 ml distilled water. The spots of reference solution (tephrosin extract from field shown plant) and sample solution (hairy root extract of *T. purpurea*) were applied on TLC plates and plates were placed in chromatographic chamber. The mobile phase was allowed to run by 3/4 of the height of TLC plates. The plates were taken out air dried and sprayed with folin-ciocalteu reagent [21, 22]. Plates were sprayed with spraying agent – I and II [23].

Preparation of standard curve of Tephrosin→100mg tephrosin was accurately weighed dissolved in water and aliquots of 10 to 100µg/ml were prepared. Standard curve was prepared by taking absorbance at 213.19 nm on Cintra-10 spectrophotometer.

3. Result and Discussion

Hairy roots induced by *A. rhizogenesis* MTCC 532 in *Tephrosia purpurea* were confirmed to have rol C gene in T-DNA. These hairy roots had a typical growth curve. Increment in fresh weight in hairy roots cultured in solidified media was observed. The data revealed 7.6 times with *T. purpurea* in fresh weight from initial fresh weights. High level of lateral branching of hairy roots due to presence of many lateral root tips result in high growth rate in cultures exceeding that of non-transformed root and cell suspension cultures. Measurement of root growth in terms of dry weight increase reflects mainly the involvement of cell enlargement rather than increase in cell number. The growth rate and extent of branching vary between hairy roots of a plants species, different species of a genus and among the hairy roots of different genus and with the culture conditions [24, 25].

Thin layer chromatographic studies showed the same chromatographic pattern under UV light at 365 nm, pattern i.e. spot of similar Rf value and color which indicate the presence of same constituent, i.e. tephrosin in the hairy roots (see Ph-3a). Same histological features were observed in control roots and roots infected with *A. rhizogenes* (see Ph-3b). In inoculated roots initiation of hairy roots was observed. The colour of tephrosin is observed as dark brown and is found soluble in water and alcohol.



Ph-3. Co TLC of standard(s): (a) Field grown plant extract, (b) *A. rhizogenes* inoculated hairy root extract.

Absorbance of hairy root extract

1ml of hairy root extract of *T. purpurea* was diluted to 10ml. 1ml of this was further diluted to 100ml. Absorbance of this was taken at λ_{\max} -213.19nm and absorbance of hairy root extract nm was 0.6447; with the help of regression equation the concentration of tephrosin was found to be 64.796 mg/g d.w.b. Quantitatively 1.528g tephrosin was obtained from 40g of roots of field grown plants, giving the concentration of tephrosin in field grown plants roots to 38.2 mg /g d.w.b.

Table 2. Data for standard curve of tephrosin (λ_{\max} -213.19nm).

Sl. no.	Concentration ($\mu\text{g/ml}$)	Absorbance	Regressed value	Statistical analysis
1.	10	0.1478	0.1077	Correlation coefficient
2.	20	0.2417	0.2058	$r^2 = 0.989398$
3.	30	0.2794	0.3039	Standard error
4.	40	0.3551	0.4020	SE = 0.035502
5.	50	0.4682	0.5001	Linear equation
6.	60	0.5890	0.5982	$y = 0.0098x + 0.0097$
7.	70	0.7457	0.6963	Intersect
8.	80	0.7833	0.7944	$m = 0.0098$
9.	90	0.8662	0.8925	Slope
10.	100	1.0252	0.9906	$c = 0.0097$

4. Conclusion

Hairy roots induced by *A. rhizogenes* offer a unique combination of physical and biological properties that must be accommodated in large scale synthesis of bioactive compounds. The study suggests that Ti mediated transformation of *Tephrosia purpurea* led to increase in tephrosin content more than the control hairy root cultures. Hairy roots cultures often exhibit greater biosynthetic capacity for secondary metabolite production compared to their mother plants. The differentiated characteristics of the hairy roots have offered the potential for the alternative production of various pharmaceutical alkaloids.

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