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Micropropagation and *Agrobacterium rhizogenes* mediated transformation studies in *Mucuna pruriens* (L.) DC.

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An efficient protocol for micropropagation and *Agrobacterium rhizogenes* mediated transformation has been standardized for *Mucuna pruriens* (L.) DC. For micropropagation, shoot tips from aseptically germinated seedlings were inoculated on MS medium supplemented with different concentrations (2–20 µM) of phytohormones. Of the various phytohormones tried, kinetin (14 µM) resulted in 4 shoots/explant after four weeks of culture. Rooting was best obtained on half-strength MS medium supplemented with 6 µM concentration of NAA. Regenerated plants were hardened with 75 % of survival frequency. For hairy root induction, different explants were infected by two strains of *Agrobacterium rhizogenes* (MTCC 2364 and MTCC 532) and effect of manual wounding and acetosyringone was studied. Of the two strains tried, MTCC 2364 showed hairy roots induction in leaf explant only by manual wounding method. While in case of acetosyringone (50-250 µM), mediated method hairy root induction was observed in all the explants studied except leaf. Out of the two strains used, MTCC 2364 showed better results in terms of roots emerged/explant and number of days required for root induction as compared to the strain MTCC 532.

Keywords: Acetosyringone, *Agrobacterium rhizogenes,* Hairy root, Manual wounding, Micropropagation, *Mucuna pruriens* (L.) DC.

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Introduction

Mucuna pruriens (L.) DC., commonly known as velvet bean plant, is an annual climber endemic in India and other parts of the tropics including Central and South America. It is an important tropical legume found in bushes and hedges and dry deciduous low forests in the plains of India[1]. It is one of the most popular crops used for green manure in sustainable agricultural systems[2]. All parts of *M. pruriens* have medicinal properties and there is a heavy demand for it in the Indian drug market as it is a constituent of more than 200 indigenous drug formulations[3]. Traditionally, *M. pruriens* is used to treat Parkinson's disease[4]. It is an important source of L-DOPA (L-3, 4-dihydroxyphenylalanine), a non-protein amino acid that acts as a precursor for the neurotransmitter dopamine[5]. All parts of the plant are known to contain L-DOPA. It is not only a medicinally important plant but because of its properties like high nitrogen fixing capability, aggressive growth habit and high productivity of vegetative nature make it an excellent soil improving manure cover crop as well[6]. In nature, *M. pruriens* propagates only through seeds, which is plagued by bottleneck like high allergic properties of pods, low germinability and poor viability of seeds. An efficient *in vitro* plant regeneration system not only provides high quality planting material, but also improves and complements the conventional seed legume breeding programme[7]. Plant tissue culture techniques are intensively used for the propagation of medicinally important plants and have opened new avenues for large scale multiplication and conservation of many of them using different explants[8,9]. *In vitro* culture of plant is an important step in many experiments such as micropropagation, production of virus free plant, and for genetic transformation[10]. Micropropagation is also an important tool for recovery and conservation of germplasm[11].

Field grown plants have been an economical source for extraction of secondary metabolites[12], but harvesting the plants is a destructive process. Establishment of *Agrobacterium rhizogenes* mediated hairy roots for production of secondary metabolites in *in vitro* may circumvent this[13]. *A. rhizogenes*, a soil borne bacterium, causes formation of hairy roots at a wounding site. It transfers T-DNA from Ri plasmid into the genome of plant[14] where it becomes stably

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integrated. The expression of T-DNA genes in the host cell's genome leads to the transformed phenotype. *In vitro* hairy root culture using *A. rhizogenes* gained considerable attention because of their fast growth, without any plant growth regulator, and ability to synthesize secondary metabolites above the level that is found in roots of intact plants[15]. The present paper describes the successful micropropagation and *A. rhizogenes* mediated hairy root induction in this medicinally important plant.

Materials and Methods

Plant material and preparation of explant

For micropropagation using shoot tip culture of *M. pruriens*, its seeds were collected from Eklagna village near Jalgaon, Maharashtra during December, 2012. The plant species was identified and authenticated by Dr. J. Jayanthi, Botanical Survey of India, Western Regional Centre, Pune and a voucher specimen (031844) was deposited in the School of Life Sciences, North Maharashtra University, Jalgaon and Agharkar Research Institute, Pune (Coll. No. 031844-JL). A protocol was standardized for sterilization and aseptic germination of seeds of *M. pruriens*. Seeds were washed under running tap water for 30 min, followed by soaking in 1 % (w/v) chilled ascorbic acid in distilled water for three hours. The seeds were then washed with 5 % (v/v) Tween 20 (Hi-Media Chemicals) followed by rinsing with sterile distilled water. Further surface sterilization of seeds was done with 70 % ethyl alcohol (v/v) for 3 min followed by 0.1 % (w/v) mercuric chloride for 1 min followed by rinsing with sterile distilled water 4-5 times. The seeds were aseptically inoculated on Murashige and Skoog (MS) medium for germination and kept in dark. From these aseptically germinated 15 days old seedlings, healthy shoots (1.5–2 cm in height) served as the source of explant. These shoots were trimmed to obtain shoot tip and inoculated on MS medium solidified with 0.4 % ClariGel (Hi-Media Chemicals) and fortified with different concentrations (2–20 μM) of phytohormones namely, 6-benzyl adenine (BAP), 2–isopentenyl-adenine (2-ip), kinetin, and zeatin.

The multiple shoots were separated and transferred for rooting. The medium used for rooting was half strength MS medium containing 2 % sucrose and augmented with different concentrations (2–20 μM) of α-naphthalene acetic acid (NAA).

Strains of *A. rhizogenes*

Two strains of *A. rhizogenes,* MTCC 2364 and MTCC 532, obtained from Microbial Type Culture Collection, IMTECH, Chandigarh were used for transformation. The cultures obtained were revived and sub cultured three times on nutrient medium (beef extract 1.0 g/L, yeast extract 2.0 g/L, peptone 5.0 g/L and sodium chloride 5.0 g/L). For preparation of suspension, a loop full of microorganism was inoculated into 50 mL of nutrient broth and kept in dark at 28 ºC for 48 h on orbital shaker. This suspension was used for transformation as per the earlier protocol[16] by Kumar *et al*. Effect of manual wounding and treatment with acetosyringone on induction of hairy roots was studied with cotyledon, node, leaf, and stem explants, obtained from 15 days old seedling of aseptically grown *M. pruriens*.

In the manual wounding method, segments (10 mm) were cut aseptically and kept in the 48 h old suspension of *Agrobacteria* followed by incubation in dark for 40 min on shaker at 70 rpm. After incubation, the explants were blotted on sterile filter papers and incubated on MS medium for 72 h in dark at 25 ºC. The explants after incubation were transferred to MS medium containing 250 mg/L kanamycin (Hi-Media Chemicals) for disinfection, followed by sub culturing the explants on same medium after every two weeks. In case of acetosyringone mediated method, acetosyringone (Hi-Media Chemicals) at 50-250 μM was incorporated in the bacterial suspension 1 h before infection. Acetosyringone (50 μM) was also added in co-cultivation medium. The explants were dipped in *A. rhizogenes* suspension containing acetosyringone followed by incubation on a shaker at 70 rpm for 30 min in dark. The explants were blot dried using sterile filter paper and inoculated on co-cultivation medium for 48 h at 25 ºC in dark. After incubation for 48 h, the explants were transferred on MS medium containing 250 mg/L kanamycin for disinfection. The explants were sub cultured on the same medium after every two weeks.

Results and Discussion

Micropropagation using shoot tip culture

For multiple shoot induction, 0-20 μM concentration each of BAP, 2ip, kinetin, and zeatin alone were tried. The cultures were kept at 25±2 °C and 16⁄8 h (light/dark) photoperiod. Shoot tips cultured on MS medium without phytohormone failed to induce shoot formation. Of the various phytohormones tried, kinetin was found to be better for shoot proliferation and 4 shoots/explant were obtained on MS medium supplemented with its 14 μM concentration after four

weeks of culture (Table 1). The shoots elongated rapidly and developed leaves in the same medium (Fig. 1a-c). The elongated shoots with leaves were separated and transferred to rooting medium individually. The rooting medium comprised half-strength MS medium containing 2 % sucrose, 0.4 % ClariGel (Hi-Media) supplemented with 2-20 µM concentration of NAA and the frequency of rooting, was recorded after four weeks of culture. It was found to be the highest (70 %) in medium supplemented with 6 µM concentration of naphthalene acetic acid (NAA) (Fig. 1d and Table 2).

Table 1 — Micropropagation using shoot tip culture in *Mucuna pruriens* as a function of different concentrations of phytohormones

Concentration (µM)	Phytohormones Number of shoot formation			
	Zeatin	2 ip	BAP	Kinetin
2	1.04±0.12	1.15±0.18	1.64±0.19	1.78±0.21
4	1.02±0.10	1.15±0.18	3.21±0.35	1.82±0.23
6	1.15±0.12	1.19±0.15	2.56±0.21	3.48±0.41
8	1.17±0.13	1.21±0.17	1.86±0.18	3.61±0.36
10	1.18±0.13	1.23±0.18	1.64±0.17	3.79±0.38
12	1.18±0.12	1.23±0.17	1.52±0.16	4.12±0.42
14	1.21±0.14	1.25±0.16	1.48±0.13	4.56±0.51
16	1.23±0.16	1.31±0.15	1.23±0.13	4.23±0.47
18	2.26±0.14	1.03±0.11	1.12±0.12	2.87±0.41
20	1.21±0.12	1.01±0.10	1.07±0.13	1.56±0.19

Data recorded after 4 weeks, each concentration was tried with 10 explants (n=10), values are expressed as mean±SD

Plantlets with well-developed root and shoot systems were hardened in polythene covered thermocol cups (Fig. 1e). The hardened plants were transferred to the fields with a survival frequency of about 75 % (Fig. 1f). The hardened plants did not show any detectable variation in morphology or growth characteristics with respect to the donor plant.

A. rhizogenes mediated transformation for establishment of hairy roots

Cotyledon, node, and stem explants from aseptically germinated 15 days old seedlings of *M. pruriens* were excised and used for transformation studies using the two stains of *A. rhizogenes*. In case of manual wounding,

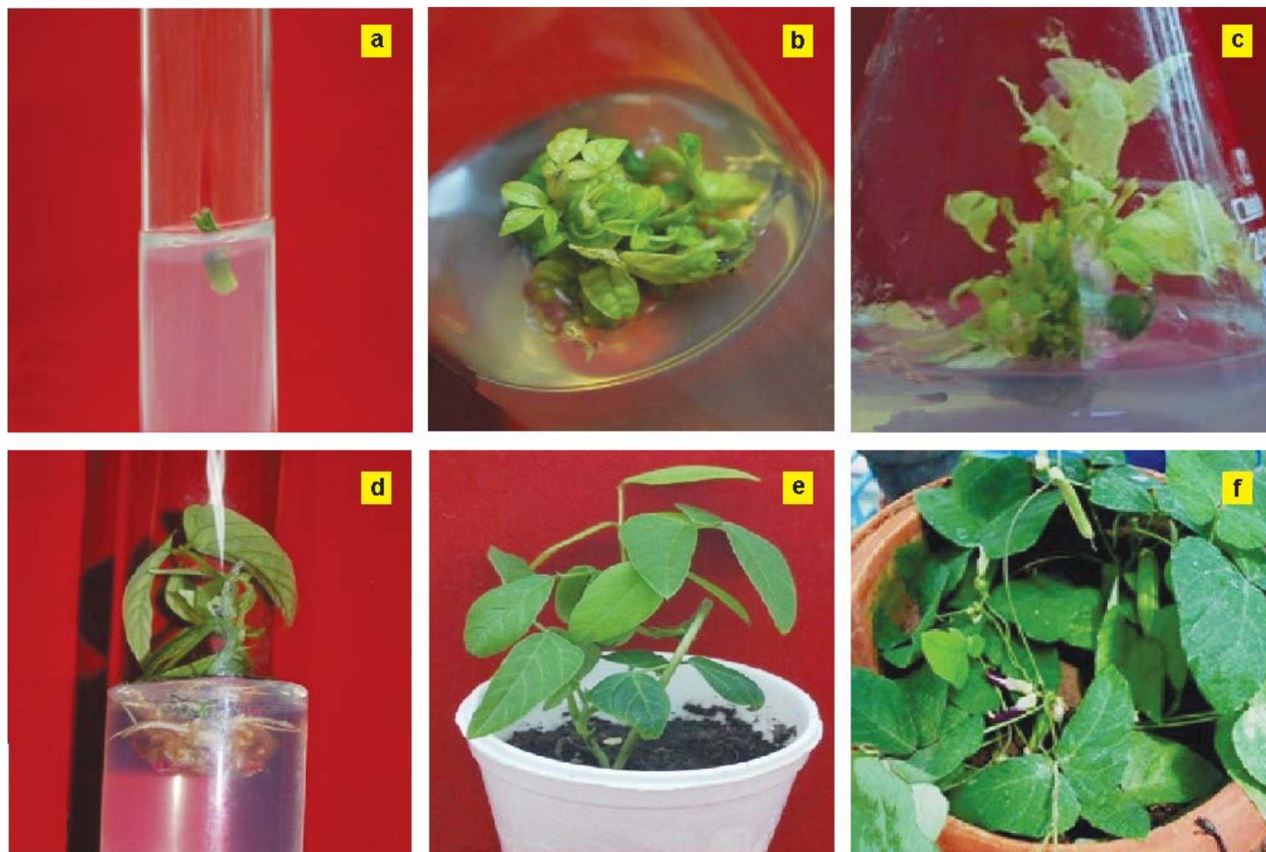

Fig. 1 — Shoot tip culture in *M. pruriens*, a) Shoot tip on MS basal medium with 14 µM Kinetin, b) Multiple shoot formation after four weeks, c) Elongation of shoots in a same medium after six weeks, d) Root induction and growth in MS basal medium containing 6 µM NAA, and e-f) Hardened plantlet in cup and pot.

Table 2 — Root induction in cultured shoots of *Mucuna pruriens* as a function of NAA concentration

NAA Concentration (µM)	Rhizogenesis frequency (%)
0	00
2	00
4	30
6	70
8	30
10	20
12	20
14	20
16	20
18	00
20	00

Data recorded after 4 weeks, each concentration was tried with 10 explants (n=10)

out of the two strains tried, MTCC 2364 showed hairy root induction in leaf explants only (Table 3, Fig. 2a). Root that emerged from explant showed an active branching pattern on hormone free medium. Higher transformation frequency, as compared to manual wounding, was observed in case of infection using both the strains of *A. rhizogenes* when acetosyringone was added to the medium. Both strains showed hairy root induction in all the explant types, except leaf, in presence of acetosyringone after 7-9 days. However, strain MTCC 2364 showed better results in terms of number of roots emerged/explant and number of days required for root induction compared to strain MTCC 532 (Table 4, Fig. 2b-f).

Table 3 — Effect of manual wounding on *Agrobacterium rhizogenes* mediated transformation in *Mucuna pruriens*

Explant Type	*Agrobacterium rhizogenes*					
	Strain MTCC 2364			Strain MTCC 532		
	Trans-formation	Days of emergence of roots	Roots emerged/ explant	Trans- formation	Days of emergence of roots	Roots emerged/ explant
Cotyledon	No	-	0	No	-	0
Node	No	-	0	No	-	0
Leaf	Yes	12	1	No	-	0
Stem	No	-	0	No	-	0

Each experiment was tried with 10 explants (n=10)

Fig. 2 — Induction of hairy roots by *A. rhizogenes* in *M. pruriens*, a) By manual wounding in leaf segment and by acetosyringone treatment in b) stem segment, c-d) node, e)cotyledon, and f) Growth and proliferation of induced hairy roots in cotyledon.

Table 4—Effect of acetosyringone treatment on *Agrobacterium rhizogenes* mediated transformation in *M. Pruriens*

Acetosyringone Concentration (μM)	Explant type	*Agrobacterium rhizogenes* Strain MTCC 2364			*Agrobacterium rhizogenes* Strain MTCC 532		
		Trans-formation	Days of root emergence	Roots emerged/ explant	Trans-formation	Days of root emergence	Roots emerged/ explant
0	Cotyledon	No	-	0	No	-	0
	Node	No	-	0	No	-	0
	Leaf	No	-	0	No	-	0
	Stem	No	-	0	No	-	0
50	Cotyledon	No	-	0	No	-	0
	Node	No	-	0	No	-	0
	Leaf	No	-	0	No	-	0
	Stem	No	-	0	No	-	0
100	Cotyledon	No	-	0	Yes	13	2.18±0.98
	Node	No	-	0	Yes	12	3.87±1.87
	Leaf	No	-	0	No	-	0
	Stem	No	-	0	Yes	12	2.49±1.23
150	Cotyledon	Yes	9	3.45±1.21	Yes	13	2.98±1.45
	Node	Yes	7	3.87±1.37	Yes	12	4.35±1.87
	Leaf	No	-	0	No	-	0
	Stem	Yes	7	3.91±1.68	Yes	12	3.25±1.49
200	Cotyledon	Yes	9	5.21±2.39	Yes	13	2.36±1.36
	Node	Yes	7	4.56±1.96	Yes	12	2.87±1.51
	Leaf	No	-	0	No	-	0
	Stem	Yes	7	3.68±1.59	Yes	12	2.03±1.05
250	Cotyledon	Yes	9	4.17±1.74	No	-	0
	Node	Yes	7	3.81±1.56	No	-	0
	Leaf	No	-	0	No	-	0
	Stem	Yes	7	2.59±1.23	No	-	0

Each experiment was tried with 10 explants (n=10), values are expressed as mean±SD

The plant regeneration in *Mucuna* has been successfully reported using cotyledonary node[17] (17 shoots/explant), nodal segment[18], axillary bud[19] (6.70±1.15 shoots/explant) and somatic embryogenesis[20]. Medium type, strength, and pH have been found to affect multiple shoot induction from various explants[17]. Of the cytokinins, either BAP alone or combinations of BAP and NAA have shown good results[17,19]. Using hypocotyl explants with cotyledons from one week old aseptically grown seedling, Chattopadhyay *et al*.[17] demonstrated successful regeneration of the plants (4-5 shoots/explant) on revised tobacco (RT) medium supplemented with NAA and 2ip. They also reported marked influence of the seedling age on multiple shoot induction. Six to seven days old seedlings were found to be the best for multiple shoot induction in their study. Similarly, half strength MS medium containing Indole-3-butyric acid[17] or NAA[19], or half strength RT medium supplemented with NAA have been reported for root induction.

Plants have been a valuable source of commercially and medicinally important compounds[21]. Majority of these valuable compounds from plants are classified as secondary metabolites that are not essential for plant growth and development, are produced in small quantities, often accumulate in specialized tissues, and may have complicated structures and/or exhibit chirality[22]. These attributes make their laboratory synthesis difficult and expensive and extraction from field grown plant is the only way to obtain them commercially[23]. Traditionally, it may require months to years to obtain a crop in agricultural field[24]. Moreover, many factors like climate, age of plant, pathogen attack, etc. may contribute to the accumulation of secondary metabolites in plants.

Though, plant cell suspension culture has been considered an alternative to farm mediated production of valuable secondary metabolites and there are a few success stories as well[25,24], differentiation of cells to specialized cell type in suspension culture has remained a major bottleneck[26]. Hairy roots have

attractive properties for secondary metabolites production. They exhibit high growth rate in hormone free media and genetic stability[27]. Their biosynthetic capacity for secondary metabolite production is often comparable or greater than their mother plant[28]. Moreover, even in cases where the secondary metabolite of choice is known to accumulate in the aerial parts of intact plant, hairy root cultures have been shown to synthesize metabolites[29]. *A. rhizogenes* mediated gene transfer is used widely to obtain hairy root lines with large production of useful metabolites. Hairy root cultures have been investigated and established for more than 200 species of higher plants including medicinal plants[30,27].

This is the first report for efficient *A. rhizogenes* mediated hairy root induction in *M. pruriens*. Actively dividing host tissues like nodes and internodes have been proposed as good target sites for successful transformation by *Agrobacteria*[31]. Hairy root induction in *M. pruriens* with different explant types was observed by using commercially obtained strains of *A. rhizogenes* following manual wounding as well as acetosyringone treatment.

It was found that addition of 200 µM acetosyringone to MS medium increases the number of roots emerged per explant as compared to manual wounding. There is relatively less difference between the roots emerged from explant treated with or without acetosyringone, which suggests that acetosyringone alone was not responsible as a key factor in the transformation of *M. pruriens*. It may be due to the presence of high level of phenolic content in the explant. The frequency and growth of hairy root induction was marginally better in explants treated with acetosyringone in our studies, in agreement with an earlier report[16]. The marginal increase in transformation frequency by acetosyringone treatment in our studies could be attributed to its ability to active *vir* genes[16]. Acetosyringone mediated activation of *vir* genes has been known to enhance transformation efficiency of *A. tumefaciens*[32]. Moreover, transformation efficiency was assessed and found to be enhanced under the influence of sonication, calcium treatment, application of acetosyringone, and macerating enzymes in a suitable combination in tobacco as a model system[16].

Conclusion

The study provides a successful protocol for micropropagation of *M. pruriens* with good survival frequency. It also describes induction and establishment of *A. rhizogenes* mediated hairy roots from different explants, which may serve as a first step in their meaningful utilization as a source of medicinally important secondary metabolite(s) of the plant.

Conflict of interest

The authors declare that they have no conflict of interest.

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