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Agrobacterium rhizogenes mediated hairy root induction in endangered *Nothapodytes foetida*

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Abstract
Nothapodytes foetida is a member of family Icacinaceae and yields an anti-cancer drug: Camptothecin (CPT) which has a huge global demand. An alternative method for the production of camptothecin is highly desirable and can be attained by the application of hairy root culture. The present study was carried out to establish an efficient protocol for hairy roots in *N. foetida* using five different strains of *Agrobacterium rhizogenes* (A4, LBA 9204, MTCC 532, MTCC 2364 and NCIM 5140). *In vitro* grown seedlings were used for the transformation experiment. Young seedlings were used as explants for the induction of hairy roots. Explants were infected with different *A. rhizogenes* strains. Then the explants were co-cultivated with the respective strains in Murashige and Skoog media and subsequently transferred to MS media containing antibiotics. The strain A_4 was more effective than the other four strains in hairy root induction in *N. foetida.*

Keywords: Camptothecin, hairy roots, *Nothapodytes foetida*, *Agrobacterium rhizogenes*

1. Introduction
Secondary metabolite production from the plants can be achieved by the application of tissue culture combined with genetic transformation. Root derived phytochemicals can be obtained from hairy root culture (Giri and Narasu, 2000) [7]. *Agrobacterium rhizogenes* causes hairy roots at the site of infection. It has been reported that hairy roots yields higher amounts of secondary metabolites in comparison to intact plant roots and cell suspension cultures (Allan *et al.,* 2002; Hamill *et al*., 1995; Hashimoto and Yamada, 1983; Hartmann *et al.,* 1986) [2, 9, 11, 10].
Nothapodytes foetida (Grah.) Mabb. (Syn. *N. nimmoniana*, *Mappia foetida*), which yields camptothecin (CPT) used in anticancer drug formulation. This tree immediately needs conservation attention because it is the most convenient source for large-scale production of CPT. *N. foetida* has become endangered and is now confined only to the remnant of forest pockets. Due to loss of its habitat and over exploitation, the population of this species has declined by 50-80% (Singh *et al.,* 2010) [17]. RET (Rare, Endangered and Threatened) list of medicinal plants made in accordance to criteria given by International Union for Conservation of Nature and Nature Resources (IUCN), given by ENVIS also includes *N. foetida* in endangered categoery. As it has been reported that maximum concentration of CPT is found in the roots of *N. foetida*, therefore hairy root culture could be better option for the large scale production of CPT (Namdeo *et al.,* 2012) [13].
Importance of N. foetida in cancer treatment creates a huge necessity for the development of a protocol so that the production of camptothecin could be enhanced. Thus, the present study was undertaken to establish a proficient protocol to increase the camptothecin yield. This would lead to decline in burden on the plant species in its natural home range area, there upon halting the over harvesting of N. foetida in the wild.

2. Material and Method
2.1 Plant material
Seedlings grown in vitro were used as the explant (leaves, nodal segment, hypocotyl and radical) for the induction of hairy roots. The explants were excised and used for transformation studies.

2.3 Bacterial strains
Following *Agrobacterium rhizogenes* strains were used for transformation:

Table 1: Bacterial strains used for infection

S. No	Bacterial strains	Growth medium
1	Agrobacterium rhizogenes NCIM 5140	YEB
2	Agrobacterium rhizogenes A4	YMB
3	Agrobacterium rhizogenes MTCC 532	YEB
4	Agrobacterium rhizogenes MTCC 2364	Xanthomonas
5	Agrobacterium rhizogenes LBA 9204	YMB

Culture conditions and media

Transformation frequency was calculated in all the experiments using following formula:

$$\text{Transformation frequency} = \frac{\text{Number of explants forming hairy roots}}{\text{Total number of explants taken}} \text{ X } 100$$

Co-cultivation medium: MS basal medium without PGRs
Growth medium: MS basal medium with antibiotics
Proliferation medium: MS basal liquid medium without PGRs.

2.4 Transformation

2.4.1 Preliminary transformation experiments

For the preliminary studies standard protocol given in the literature was followed. Standard protocol is discussed as follows:

1. First of all bacterial culture for all the five strains were initiated on the respective medium. Single colony of *A. rhizogenes* was picked from 18 hrs old culture and was inoculated in suitable liquid bacterial medium (shaker incubate at 28°C (200rpm) overnight).
2. After this, aliquot of the culture was taken at different time interval and optical density (OD) at 600 nm was taken (0.6, 0.8, 1, 1.2, 1.4, and 1.6).
3. On the day of experiment bacterial cultures were centrifuged at 6000 x g for 8 minutes and the pellet was resuspended in liquid plant MS medium.
4. Then the explants (leaves, petiole, nodal segment and radical) from in vitro grown seedlings were cut into small pieces of around 1-1.5 cm.
5. Two methods were employed for the infection. Explants were either pricked with needle of a syringe containing *Agrobacterium rhizogenes* culture or cut and submerged in the bacterial suspension (10-15 explants/5ml of media) for 5-35 minutes of infection time.
6. Excess of bacterial suspension was removed by blotting them on a sterile filter paper, after the completion of infection period.
7. The explants were then transferred to MS basal media without PGRs for co-cultivation (24-96 hours).
8. Temperature for co-cultivation was varied from 18ºC to 26ºC.
9. After co-cultivation, the infected explants were washed three times with sterile distilled water, followed by washing with antibiotic solution (50mg/L) with vigorous stirring using sterile forceps and then sterile filter paper was used for blotted drying.
10. Further explants were transferred to MS medium without PGRs and supplemented with different concentration and type of antibiotics.
11. Subsequent sub culturing of the infected explants was done under same incubation conditions on solid medium containing different concentration and type of antibiotics to prevent the growth of bacteria.

3. Results

3.1 Effect of different *Agrobacterium rhizogenes* strains on transformation frequency

Leaf explant was chosen first among all the explants to transform with various *A. rhizogenes* strains as leaf explant has shown best results in other plant species. In the explants infected with *A. rhizogenes*, roots emerged directly from the wounded regions. In some explants, swellings were observed along the infected sites and in some cases callus like structure was also observed, from most of which roots also developed. Aerial roots were also obtained due to lack of positive geotropism. These roots displayed Plagiotropism (Growing parallel to the culture medium).

When the roots were further sub cultured onto fresh medium, most of the roots showed lateral growth. After 20 days of culture a closely interwoven masses was obtained both over the surface of the medium and over the upper side of the petri dish. Whereas after 25-30 days, hairy roots changed their color from white to brownish white.

The control explants, deprived of bacterial infection, did not produce roots at all. Only, swelling of the explants was observed and prolonged culture lead to browning of the explants and ultimately causing death of the explants. The developed hairy root clones were detached from the mother explants and cultured on MS basal medium with antibiotic showed a normal growth.

Initially preliminary studies were carried out following the standard protocol using all the five strains (A4, LBA 9204, MTCC 532, MTCC 2364 and NCIM 5204) of *A. rhizogenes* with leaf as an explant source. Optical density, infection time, c0-cultivation period, co-cultivation temperature, acetosyringone concentration were checked for the induction of hairy roots. Values were not determined due to the overgrowth of the *A. rhizogenes* (Table. II).

Optical density (OD) of 1.0 was found to be optimum, as maximum strains (A4, LBA 9204 and MTCC 532) of *A. rhizogenes* responded to this OD. Two other *A. rhizogenes* strains (MTCC 2364 and NCIM 5204) did not responded to different OD as no hairy roots formation was observed when the explants were infected with these two strains.

Infection times of 5, 10, 15, 20, 25, 30 and 35 minutes were tested for the induction of hairy roots. Again no root formation was observed with MTCC 2364 and NCIM. With infection time of 20 and 25 minutes, all the three strains (A4, LBA 9204 and MTCC 532) produced hairy roots. When the infection time was increased to 30 and 35 minutes, contamination due to overgrowth of *A. rhizogenes* was observed.

Explants were co-cultivated for 24, 48, 72, and 96 hours with bacterial suspension to check the effect of co-cultivation duration on hairy root induction. At 48 hours of co-cultivation, three strains (A4, LBA 9204 and MTCC 532) produced hairy roots. MTCC 2364 and NCIM didn't produced hairy roots at all. Overgrowth of *A. rhizogenes* was observed when the co-cultivation period was extended beyond 72 hours.

Different co-cultivation temperature (18, 20, 22, 26 and 28 º C) was checked for the induction of hairy roots. At a temperature of 22 º C maximum of 3 strains (A4, LBA 9204 and MTCC 532) induced hairy roots, but other two strains (MTCC 2364 and NCIM) did not responded at all. *A. rhizogenes* strain A4 induced hairy roots in *N. foetida* explants at a wide range of temperature (20-28 ºC).

Table 2: Preliminary studies on hairy root induction in *N. foetida*

Parameters	Strains	MTCC 532	A4	LBA 9204	MTCC 2364	NCIM
Optical density (OD)	0.6	-	+	-	-	-
	0.8	-	+	+	-	-
	1.0	+	+	+	-	-
	1.2	+	+	-	-	-
	1.4	-	-	-	-	-
	1.6	-	-	-	-	-
Infection time (minutes)	5	-	-	-	-	-
	10	-	-	-	-	-
	15	-	+	-	-	-
	20	+	+	+	-	-
	25	+	+	+	-	-
	30	-	-	-	-	-
	35	-	-	-	-	-
Co-cultivation period (hours)	24	-	+	-	-	-
	48	+	+	+	-	-
	72	-	+	+	-	-
	96	-	-	-	-	-
Co-cultivation temperature (°C)	18	-	-	-	-	-
	20	+	+	-	-	-
	22	+	+	+	-	-
	24	-	+	+	-	-
	26	-	+	-	-	-
	28	-	+	-	-	-
Acetosyringone conc. (µM)	0	-	-	-	-	-
	100	+	+	+	+	+
	150	+	+	+	+	+

+ means induction and – means no induction of hairy roots

Hairy root induction is influenced by different A. rhizogenes strains, which has been reported in several plant species (Sujatha *et al.,* 2013) [19]. In present investigation, among all the three *A. rhizogenes* strains used, A4 was found to be most virulent with respect to transformation frequency and initiation of hairy root formation. A4 gave a maximum of 80.7 percent transformation frequency followed by LBA 9402 which gave 75.7 percent transformation frequency and least transformation frequency of 60.0 percent was observed in MTCC 532.

Table 3: Transformation frequency using different *Agrobacterium* strains

Co-cultivation period (hrs)	Infection Time (minutes)	Transformation frequency (%)
MTCC 532		
24	15	0
	20	0
	25	0
48	15	0
	20	39.7±1.5 g
	25	60.0±1.7 c d
LBA 9402		
24	15	0
	20	0
	25	0
48	15	0
	20	45.3±0.9 f
	25	75.7±1.9 b
A4		
24	15	49.0±1.2 f
	20	56.3±1.2 d e
	25	62.3±1.5 c
48	15	53.3±1.7 e
	20	65.0±1.5 c
	25	80.7±1.2 a
72	15	-
	20	-
	25	-

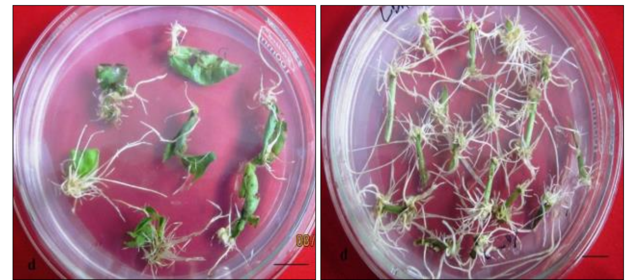

Fig 1: Induction of Hairy root in leaf explants and nodal segment in *Nothapodytes foetida*

3.2 Confirmation of transformation by PCR analysis

Transgenic status of the tissue was confirmed by PCR amplification of the DNA obtained from hairy roots, untransformed roots and A. rhizogenes using the forward and reverse primers of rol B genes. Positive control was provided by A. rhizogenes and negative control was provided by untransformed roots.

Transformed hairy root samples were found to be positive for *rol* B genes. The product of the PCR were of expected size (750 bp) and were corresponding with the positive control (*A. rhizogenes* LBA 9402). The untransformed roots (negative control) were negative for *rol* B genes. Presence of the amplified products of the expected size in positive control and hairy root samples, confirmed the identity of this amplification product and the transgenic nature of hairy roots. The results demonstrate that the *rol* B genes were integrated successfully into the genome of *N. foetida* hairy roots from the Ri plasmid of *A. rhizogenes*.

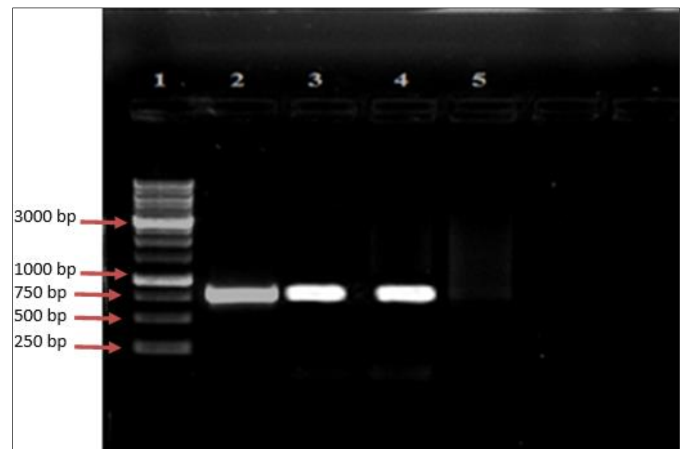

Fig 2: Confirmation of transformation by PCR analysis. Lane 1: molecular weight marker (1 kb DNA ladder), lane 2: *Agrobacterium rhizogenes* DNA (positive control), lane 3 & 4: DNA from transformed hairy root, lane 5:DNA from untransformed root (negative control).

4. Discussion

Frequency of hairy root production was affected accordingly due to the virulency of A. rhizogenes (Giri *et al*., 2001) [8]. A. rhizogenes mediated transformation is the most common and efficient method for genetically modifying the plants. That is why A. rhizogenes is also known as natural genetic engineer. Virulence of different A. rhizogenes strains is responsible for variation in hairy root induction (Porte 1991; Thiruvengadam 2014) [15, 21]. Plasmid harbored by bacterial strains could be one of the reasons of the differences in virulence (Batra *et al.,* 2004; Chaudhuri *et al*., 2005 Bansal *et al.,* 2014; Nourozi *et al.,* 2014) [4, 5, 3, 2].

The results presented in the paper shows that three strains (A4, MTCC532 and LBA 9204) were found to induce hairy roots in *N. foetida.* Other two strains (MTCC 2364 and NCIM) did not respond to the transformation experiment. This could be explained due to the differential expression of T-DNA genes present in the explants and effect of integration at different positions of T-DNA in the genome of host (Cho *et al.,* 1998) [6]. Moreover, compatibility between *A. rhizogenes* and host plant tissue, phytohormones production and juvenility of host tissue are contributing factors for efficient production of hairy roots (Huang *et al*., 1991) [12].

The superiority of A4 strain over other strains is due to its wild origin (Bansal *et al*., 2014; Nourozi *et al*., 2014; Srivastav *et al*., 2016) [3, 14, 18] and A4 strain has been found to induce hairy roots in many other plant species like *Catharanthus roseus* (Batra *et al*., 2004) [4], *Piccorhiza kurroa* (Verma *et al*., 2007; Rawat *et al.,* 2016) [23., 16], *Hyoscymus sp*. (Akramian *et al.,* 2008), *Clitoria ternatae* (Swain *et al.,* 2012) [20], *Monirelica charantia* (Thiruvengadam, 2014) [21], *O. basilicum* (Srivastav *et al.,* 2016) [18].

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