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Original Article

Agrobacterium Rhizogenes-mediated Transformation of *Peganum multisectum* (Maxim) Bobrov and Harmine Production in Hairy Roots

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Abstract

Using *Agrobacterium rhizogenes* due to create hairy roots is a useful method to product secondary metabolites in many medicinal plants. The transgenic hairy roots were induced from *Peganum multisectum* (Maxim) Bobrov a medicinally important species, by infecting leaf and stem explant with wild type *Agrobacterium rhizogenes* strain ATTCC 15834, which led to the induction of hairy roots from 19% of the explants. Polymerase chain reaction with primers for *rol* A confirmed the integration of T-DNA fragment of Ri plasmid of *A. rhizogenesis* into the genome of hairy roots obtained after transformation. Four transformed lines of hairy roots were established. Doubling time of the faster growing hairy root lines was about 11 days and these cultures showed about 12-fold increase in biomass at the end of 6 weeks as compared to non-transformed roots. The hairy roots showed an ability to synthesize harmine, a β-carboline alkaloid of medicinal value. The effect of the initial sucrose and ammonium nitrate concentration in biomass and harmine production of the liquid MS cultural medium cultures was studied. The highest values for harmine accumulation and fresh weight were obtained between 30-45 g l^{-1} of sucrose. The results also showed that the addition of extra ammonium nitrate up to optimum level (2475 mg l^{-1}), as a source of nitrogen was significantly effective than standard ammonium nitrate in MS basal medium for growth of hairy roots and harmine production. This is the first report on the induction of hairy roots in *P. multisectum* (Maxim) Bobrov.

Keywords: *Peganum multisectum*, *Agrobacterium rhizogenesis*, Hairy roots, Harmine

Introduction

The family of Zygophyllaceae, contains more than 285 species within 22 genera. The genus of *Peganum* L. has six species such as *Peganum harmala* L., *P. mexicanum* A. Gray, *P. nigellastrum* Bunge, *P. rothschildianum* Buxb., *P. texanum* M. E. Jones and *P. multisectum* (Maxim) Bobrov, which occur in the regions of warm and subtropical temperatures in the world [1]. Species of *Peganum* can be widely found across North Africa, Pakistan, India and Southern part of Iran and have been introduced in Southern and Northern America, Australia, Mexico, Tunisia, China and Mongolia [2]. The seeds of *Peganum* are used as an anti-hemorrhoids and central nervous system stimulating agent in folk medicine [3,4]. In traditional medicine, seeds of *Peganum* were used as powder, decoction, maceration or infusion for fever, diarrhea, abortion and subcutaneous tumors. Some of reported pharmacological effects of *Peganum* may be attributed to its β-carboline alkaloids, mostly harmine, as well as harmaline, harmalol and harman [5,6] that have a wide spectrum of pharmacological applications in various areas.

These β-Carboline alkaloids consist of anti-spasmodic, anti-pyretic [7,8], anti-cancerous and anti-tumor [9,10], central nervous system effects [11], Cardiovascular actions [12], hallucinogenic [13], central monoamine oxidase inhibition [14], binding to various receptors including 5-HT and the benzodiazepine binding receptors [15], platelet

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aggregation inhibitory [16] and immunomodulatory effects [17]. β-Carboline alkaloids also affect a number of molecular targets, ranging from DNA (intercalation) via neuroreceptors to monoamine oxidase3 and therefore figure as potent defense compounds of *Peganum*. DNA intercalation, mutagenic, genotoxic and cytotoxic have been investigated [18-20]. They suggested that the antibiotic and toxic effects of β-carboline could be a function of DNA intercalation and resulting mutations. These give strong indications for the validity of harmine as an *in vivo* tracer for the assessment of Monoamine oxidase A (MAO-A enzyme) binding in the brain [21]. Trypanosomicidal activity of several β-carboline alkaloids [22] and larvicidal against the larvae of the cotton leaf worm [23] have stated that the biological activity of β-carboline alkaloids and a highly toxic effect reasonably underlie the neurotoxicity [24]. In previous studies, the alkaloids including harmine, evodiamine, vasicine, vasicinone, deoxyvasicinone and fagomine were reported from *Peganum multisectum* (Maxim) Bobrov [1, 25]. Beline *et al*., [26] and Zayed [27] had found that harmine was the main β-carboline alkaloid in root cultures under the tested conditions in *P. harmala*. Furthermore, biological and pharmacological activities of harmine found from *P. multisectum* (Maxim) Bobrov (*P. multisectum*) such as antitumor activity [9], antioxidative activity [25], cytotoxicity [28], antimicrobial activities [29] and antileishmanial activity [30] were reported.

Genetic transformation of plants using the natural vector system of *Agrobacterium rhizogenes*, the causative agent of hairy root disease in several plants, has emerged as an important alternative to intact plants as well as cell suspension cultures for the production of secondary metabolites [31,32]. Hairy roots have been reported to yield higher amounts of secondary metabolites than cell suspension cultures and in some cases, intact plant roots [33]. *A. rhizogenes*–mediated hairy root production is a valuable tool for the biosynthesis of secondary metabolites and metabolic engineering studies. It is also considered for biotechnological production of root-derived compounds [34]. The hairy roots constitute a very good system for the continuous synthesis of these metabolites in a germ-free condition in the absence of expensive phytohormones in the culture medium. Growth of hairy roots can be scaled up using bioreactors and hence they can be exploited for commercial production of secondary metabolites [35]. Hence, the present study was designed to develop an efficient *in vitro* procedure to enhance the biosynthesis of harmine in biomass of transformed hairy root cultures of *P. multisectum*.

Material and methods

Plant Material

Mature seeds of *P. multisectum* (maximum 6-month old) were obtained from Dr. Jamir, University of Pune, India. The plant specimens were identified from the Botanical Survey of India, Regional Office, Pune, 411001 (specimen voucher number-MAECB1). The seeds were surface-sterilized in 1% sodium hypochlorite solution for 4–5 min then they were rinsed thrice with sterile distilled water. Afterwards the seeds were treated with EtOH 70% for 2 min and rinsed thrice with sterile distilled water. The seeds were left overnight in sterile water and then incubated on agar plates until germination. The germinated seeds were transferred to hormone-free MS solid medium [36] at a temperature of 20–22 °C and illumination of 35 µmol $m^{-2}s^{-1}$ with photoperiod of 16-h light:8-h dark to produce the seedlings.

Bacterial Strain and Cculture Conditions

Wild type *A. rhizogenes* ATCC 15834 (harboring pRi 15834) were used for transformation of *P. multisectum.* ATCC 15834 strain is a rifampicin resistant strain possessing an agropine-type Ri plasmid pRi 15834. *A. rhizogenes* ATCC 15834 raised and maintained on nutrient medium (peptone 5 g l^{-1}, beef extract 1.5 g l^{-1}, NaCl 5 g l^{-1}, yeast extract 1.5 g l^{-1}, 50 mg l^{-1} rifampicin and agar 15 g l^{-1}). A single bacterial colony was inoculated in 5 ml of nutrient broth medium and the culture was placed on rotary shaker (80 rpm) at 26°C for 16 hours until the OD_{600} was about 0.5. The bacterial suspension was centrifuged at 8000 rpm for 10 min and the pellet was then resuspended in 5 ml MS liquid medium for subsequent inoculation step.

Establishment of Hairy Root Cultures

The 30-40 days *in vitro* grown plants were cut (leaves into 0.5 cm^2 pieces and stem into 1 cm length, approximately) and used for inoculation with *A. rhizogenes*. The explants placed for 30 min in conical flacks containing the bacterial suspension, after which they were blotted by sterile blotting paper and transferred to the half strength

MS medium [consist of half strength of macro element without NH_4NO_3, full strength of $CaCl_2.H_2O$ minor salts and organic supplements and 1.5% (w/v) sucrose]. After two days' co-culture with *A. rhizogenes* on hormone-free MS basal media, the explants were washed five times with sterile water to remove superficial bacteria and then were transferred to MS medium containing 300 mg l^{-1} cefotaxime so as to kill the residual *Agrobacterium*. The antibiotic was dissolved in sterile distilled water and added in autoclaved medium at temperature near to 50 ^oC. Controls consisted of explants treated similarly except that they were not co-cultivated with *A. rhizogenes.* The cefotaxime concentration in medium was then reduced every week from 300 to 50 mg l^{-1} [63]. Finally, the cultures were made free of *A. rhizogenes.* The bacterial free explants were transferred to the agar-solidified MS medium without phytohormones and observed for induction of hairy roots. Hairy roots (HRs), which arose mainly from the cut surfaces of the explants, were separated from the explants, (when they attained a length of 4-5 cm) and placed on MS liquid medium without antibiotic for further growth. All the cultures were maintained in 35 µmol m^{-2} s^{-1} with 8/16 hours of light/dark photoperiod and 25±2^{o}C temperature. Excised roots of *in vitro* explants were cultured similarly and served as controls. After 10-12 days HRs started to appear. These were maintained by subculture of 3–4 cm long pieces on MS liquid medium containing 50 mg l^{-1} cefotaxime. The hairy root cultures (HRC) were also inoculated into liquid B5 medium in 250 ml flask conical and incubated in 35 µmol m^{-2} s^{-1}, 16/8 hours of photoperiod, 25±2^{o}C for 6 weeks, at the end of which, growth and harmine production was analyzed. HRC have a different morphology as compared to RC. They have many root hairs and much more branches and the most overall important advantage of the transformed root cultures is their long-term stability.

Detection of Ri T-DNA Integration

The polymerase chain reaction (PCR) was used to detect the Ri T-DNA integration into the plant genome. The bacteria-free roots grown in MS basal medium were removed, dried on sterile filter paper, and quickly frozen in liquid N_2. Thereafter, genomic DNA from putative transformed and normal roots was extracted by the method of CTAB [37, 38]. PCR was performed to detect the *rol* A gene using a set of *rol*A–specific primer pair. A 308-bp *rol*A gene fragment was amplified by using the following primer set: forward, 5´AGA ATG GAA TTA GCC GGA CTA 3´, and reverse, 5´ GTA TTA ATC CCG TAG GTT TGT TT 3´. The PCR mixture (25 µL) contained 50 ng of DNA as the template prepared from non-transformed root and HRs, 1×PCR buffer, 25 pmole of each primer, 2.5 mM of dNTPs, and one unit of Taq DNA polymerase (MBI fermentas). PCR for *rol*A was carried out by amplifying with initial denaturation at 94 °C for 5 min followed by 35 cycles of 1-min denaturation at 94 °C, 1-min annealing at 55 °C and 1-min extension at 72 °C with a final extension of 72 °C for 10 min using a thermal cycler (MWG Biotech, Germany). The PCR obtained products were separated on 1% agarose gel, stained with ethidium bromide, observed, and documented using a trans-illuminator equipped with a gel documentation system (Herolab GMBH, Germany).

Classification of Hairy Root Lines and the Measurement of Growth Index.

After 10-12 days HRs started to appear on the stem explants and after 11-15 days on the leaf explants of *P. multisectum*. Four different hairy root (HR) lines were chosen, each line representing the occurrence of an independent transformation event. These were maintained on MS medium without phytohormones, as separate root lines and were named as line S1, S2 (obtained from stem) and line L1 and L2 (obtained from leaf). Individual root lines were maintained separately and were subcultured by using 3-4 cm long pieces on MS medium. The different primary transformed root lines were observed and studied for growth kinetics and harmine production. Growth of HR was expressed as fresh growth index (FGI) as suggested by Kittipongpatana *et al.*, [39].

HRs (100 mg in 50 ml of MS liquid medium in 250 ml Erlenmeyer flasks) were cultivated for eight weeks at 100 rpm, 25^{o}C, 35 µmol m^{-2} s^{-1} of light with a 14-h photoperiod for determine of growth and total harmine content. Doubling time was calculated by plotting a graph of $\log_2$ fresh weight (g) versus time (days) and calculating the inverse of slope for the linear part of the curve [40,41].

$$FGI = \frac{\text{Final fresh weight of biomass - Initial fresh weight of inoculum}}{\text{Initial fresh weight of inoculum}}$$

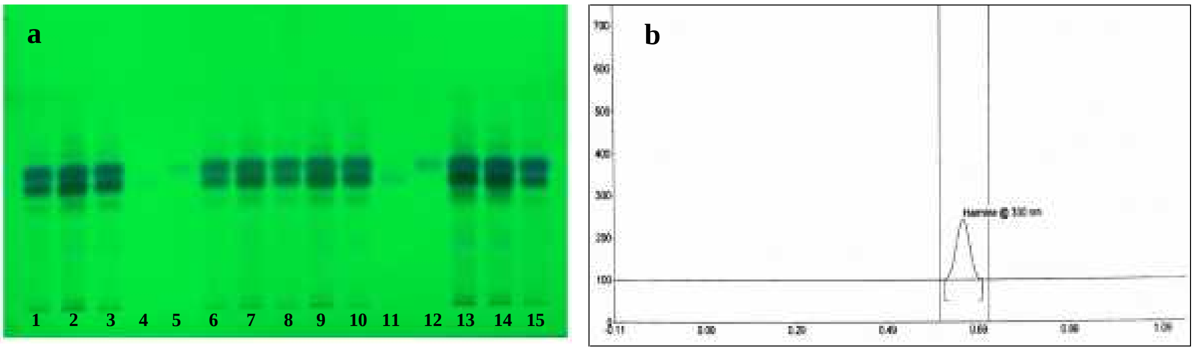

Fig. 1 HPTLC fingerprint profile for the quantitative analysis of harmine from nontransformed root and HRC of *Peganum multisectum* (Maxim) Bobrov on hormone-free MS medium at 245 nm (Lanes: No.1: non-transformed root, No.2, 8, 15: HRC at 6th week after culturing in hormone-free MS medium, No.3: HRC at 8th week after culturing in hormone-free MS medium, No.5, 12: Standard of harmine, No.6: HR cultured in hormone-free B5 medium, No.7, 9, 10: HR cultured in MS+15, 45, 90 g l^{-1} sucrose, respectively, No. 13, 14: HR cultured in MS+2475, 3300 mg l^{-1} of NH_4NO_3, respectively; b: Harmine in HRC of *Peganum multisectum* (Maxim) Bobrov

Secondary Metabolites Extraction

The dried powdered HRC and seeds of *P. multisectum* were used for obtaining the crude extract by soaking 1.0 g of the dried biomass in 50 ml methanol at 50 ^{o}C in water bath for 1 h. The extracts were combined and evaporated to dry. The residue was dissolved in 50 ml HCl (2%) and filtered through Whatman No. 1 filter paper. The filtrate was extracted two times with 20 ml petroleum ether. The aqueous acid layer was basified (pH:10) with NH_4OH and extracted four times with 50 ml chloroform. The chloroform layer was combined and evaporated to dry, and then the residues were dissolved in 25 ml methanol [42]. The solution of alkaloid extract was passed through 0.45 mm filter and 0.2 µl extract was directly injected into the HPTLC[1]. For estimation of harmine, CAMAG analytical HPTLC system was used. The results were obtained as a mean value of three separate injections. Harmine (Sigma; H-8646) was obtained from Sigma chemicals and were used as standards. Aluminum sheets of silica gel $60F_{254}$ (Merck) were also been applied. The chromatograms were developed in the mobile phase chloroform: methanol: 25% ammonia (5:4:1) dried and sprayed. Harmine was analyzed by using CAMG TLC Scanner 3 in UV-254 and UV-366 nm (Fig. 1 a, b). The peaks corresponding to harmine were confirmed by comparison with the commercial standard of the crude extract samples. The alkaloids content in the crude extract was determined by comparing the peak areas with those of standard harmine.

[1] High Performance Thin Layer Chromatography

Effects of MS and B5 Media

HRs culture of *P. multisectum* was grown separately on two different liquid media to determine the medium for optimal growth. MS (containing iron chelated to the di-sodium salt of EDTA, 100 mg l^{-1} inositol, 3% sucrose, pH=5.8) and B5 [43] (containing iron chelated to the mono-sodium salt of EDTA, 100 mg l^{-1} inositol 3% sucrose, pH=5.8) media for 4 weeks after which FGI and harmine production were analyzed. The HRC were incubated at 25^{o}C, 35 µmol m^{-2} s^{-1} of light with a 14-h photoperiod.

Effects of Different Concentrations of Ammonium Nitrate and Sucrose

HRs lines S2 and L2 were used for this experiment. Four levels of NH_4NO_3 in MS basal medium (825, 1650, 2475 and 3300 mg l^{-1}) were tested. MS liquid medium (1650 mg l^{-1} NH_4NO_3) was taken as reference. Also, the effect of initial sucrose concentrations (15, 30, 45, 60 g l^{-1}) on growth and secondary metabolites content of HR lines S2 and L2 were examined by using basal MS liquid medium.

Statistical Analysis

All transformation experiments were set up in a randomized design. All experiments were repeated at least twice. Data were analyzed by analysis of variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using the Duncan multiple range test (DMRT) at the 5% probability level by using SPSS software. Variability around the mean was represented as the standard deviation.

Results and discussion

Induction of Hairy Root Cultures

To establish a productive strain of root cultures of plants, it must be selected the best producing strain and optimize the culture conditions for growth and production of secondary compounds. Different media (MS, MS+0.5 mg l^{-1} 2,4-D, MS+0.5 mg l^{-1} IBA, B5, B5+0.5 mg l^{-1} 2,4-D, B5+0.5 mg l^{-1} IBA and MS+0.5 mg l^{-1} kinetin) and different conditions (e.g. light and dark) were applied, but each time the root cultures were easily broken. After a certain time, normal root cultures acquired brownish color and more over the medium. At the end of third week, the growth of cultures stopped and they finally died. Zayed [44] has reported the same results on *P. harmala* as well. While looking for a suitable medium for the root cultures, MS liquid and solid media proved to be optimal for growth and maintenance of *P. multisectum* cultures as well as for secondary metabolite accumulation. HRs was obtained after transformation of *P. multisectum* with *A. rhizogenes* strain ATCC 15834. Different *in vitro* explants such as stem and leaf showed a varying of root induction after co-cultivation with *A. rhizogenesis* (Table 1). Root differentiation was noted from the injured portion of explants. Maximum root induction frequency of 19.2% of cultured *P. multisectum* was observed using stem explants and the HR induction in leaf explant was 7.3%. The time period required for HR induction was two to three weeks. The same results were reported by Berlin *et al.*, [26] and Zayed [27] on *P. harmala* as well. Therefore, the stem of *P. multisectum* was highly suitable for transformation. Wounding and co-cultivation was seen as an efficient method of transformation. HRC were maintained as stable root cultures in hormone-free MS liquid medium. The most important advantage of the transformed root cultures is their stability, which gives suitable chance for further investigations.

Confirmation of Transgenic Status of HRs

PCR analysis using the *rol*A primers provided the molecular evidence supporting the transgenic nature of the HRC. *A. rhizogenes* (colony PCR) served as the positive control and DNA from the non-transformed roots served as the negative control. Primers for *rol*A gene were expected to produce a fragment of 308 bp with *rol*A primers; the expected fragment size was obtained in lanes containing DNA from HRC, whereas no bands were observed in lanes containing DNA from untransformed roots (Fig. 2).

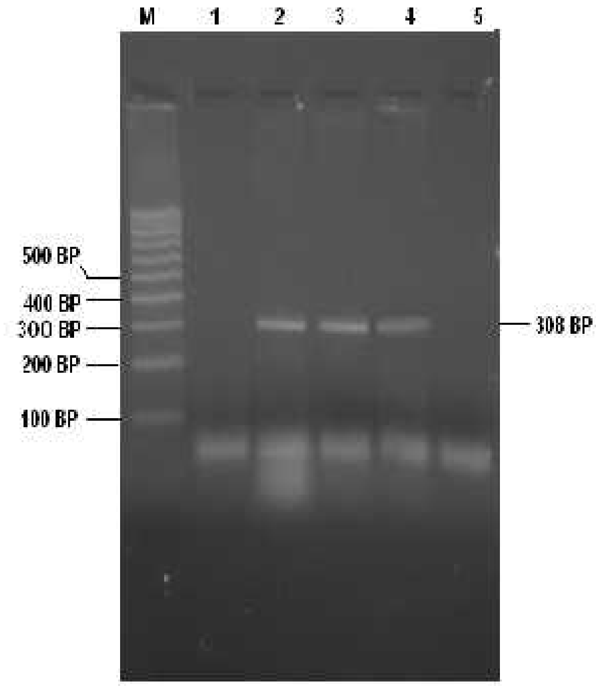

Fig. 2 PCR amplification of a 308 bp fragment of the *rol*A gene using HR derived DNA. Lane M molecular weight marker (100 bp ladder); Lane 1 and 5: DNA from non-transformed roots (negative control); Lane 2: *Agrobacterium rhizogenes* DNA (positive control); Lane 3 and4: T-DNA from *P. multisectum* HR.

Growth and Harmine Production in HRC

An efficient transformation system for *P. multisectum* was developed in this investigation. The roots showed the typical HR syndrome and grew on hormone free MS medium (Fig. 3). Four lines from different primary transformed roots were chosen. The different lines showed varying growth and harmine content (Table 2). The growth of HRs (FGI) showed an exponential pattern with doubling times ranging from 11-14 days (Table 2) but doubling time was observed in control (non-transformed) after 30 days. Line S2 showed fast growth and a large number of lateral roots. This line also had the highest FGI (11.1±0.9) and showed the highest secondary metabolites accumulation (0.42±0.02 mg g^{-1} DW) at the end of six weeks with a doubling time of 11.4 days. Line S1 and L2 showed lesser biomass accumulation whereas L1 showed the least. The result showed that obtained HRC from root explant had more potential for accumulation of biomass.

Table 1 Induction of HRs from different explants of *Peganum multisectum* (Maxim) Bobrov.

Explant	Number of control cultures (non-transformed)	Number of cultures used for genetic transformation	Number of cultures showing HR induction	% HR induction
Stem	37	99	19	19.2
Leaf	41	149	11	7.3

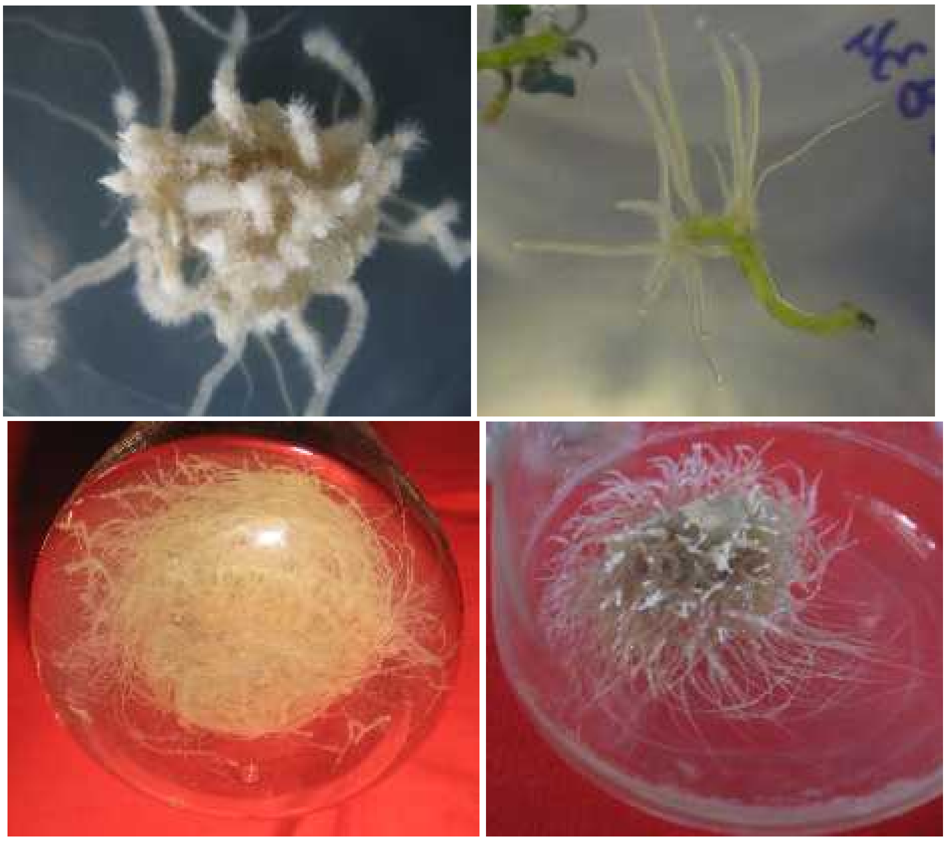

Fig. 3 (a) Induction of HRs from leaf explants of *Peganum multisectum* (Maxim) Bobrov; (b) Induction of HRs from stem explants of *Peganum multisectum* (Maxim) Bobrov; (c) growth of HRC in MS liquid medium after 2 weeks of culture. (d): growth of HRC in MS liquid medium after 4 weeks of culture.

Table 2 Growth and harmine content of different lines of root of *Peganum multisectum* (Maxim) Bobrov MS liquid medium.

Root lines	Doubling time (days)	FGI after 6 weeks	harmine content after (mg g^{-1} DW) 6 weeks
NR	30.2±3.8	0.9±0.3	0.29±0.01
HRS	11.4±1.02	11.1±0.9	0.42±0.02
HRL	14.1±3.13	8.7±0.9	0.39±0.02

Values for growth parameters represent mean and standard deviation of four replicates. Weight of the initial inoculum of roots was 0.5±0.05 g (NR: non-transformed; HRS and HRL: hairy root cultures obtained from stem and leaf, respectively)

The growth curve of *P. multisectum* showed a lag phase of two weeks (on MS medium) (Fig. 4). The linear growth phase began on third week and continued to grow rapidly up to sixth week. The FGI was 11.1±0.9 at this time course growth. The growth of HR slightly increased during 7th week and showed the highest FGI of HR (12.4±0.8). Stationary phase started after sixth week and the fresh weight slightly declined after seventh week. The highest harmine content (0.42±0.02 mg g^{-1} DW) was detected at the stationary phase (6th

week) in HR cultured of *P. multisectum* which was about four time more than harmine content at the first week. Although, the highest FGI was observed at the seventh week but obtained HRs at the sixth week were fresher than those from the seventh and the eighth week and with the highest content of harmine (0.42±0.02 mg g^{-1} DW) in line S2. An inverse relation between FGI and harmine contented was observed in both MS and B5 media. It can be suggested that the best time for harvesting HRC of *P. multisectum* with high biomass and alkaloid content is 6^{th} week after culturing.

Comparison between MS and B5 in Hairy Root Culture

In order to understand the effect of MS and B5 media on growth and harmine production of HRC, it was important to conduct time course study. Figure 5 shows growth of HRC (Line S2) on MS and B5 media after six weeks. The culture medium strongly affected growth of HRs. The time course of the HRs in both MS and B5 nutrient media (with 3% sucrose) without phytohormones followed a sigmoidal curve. The linear growth phase was from the second week and stationary phase began after sixth week. The maximum growth of HRs (based on fresh growth index) was observed at sixth week in both media. MS basal medium was more effective for biomass production than B5. The maximum biomass production (FGI) was obtained in MS medium (11.1±0.9) which was about 6% more than obtained FGI in B5 medium after six weeks (10.4±0.7). The results showed that MS medium without phytohormone was more effective for biomass production of HRC of *P. multisectum*.

The harmine content in the cultured HRs in both MS and B5 nutrient media increased at the lag phase (till the second week). However, it increased exceptionally more during the linear growth phase, but towards the end of linear growth phase, the harmine content reached the maximum value. The harmine production reached an optimum level during the stationary phase of the culture cycle in both the media. The maximum harmine content (0.463±0.03 mg g^{-1} DW) was obtained in B5 medium which was 7% more than harmine content of cultured HR in MS medium (Fig. 5). The results showed that after 6 weeks in culture, HRC of *P. multisectum* showed increased growth in MS medium, while B5 medium showed slightly higher accumulation of harmine.

B5 vitamins differed from the vitamins of MS in having a high concentration of thiamine. Thiamine is reported to be involved in cell biosynthesis and metabolism [45]. The results on effect of B5 and MS showed growth rates comparable to those observed in *Linum flavum* L. [46], *Gentiana macrophylla* Pall. [47]; *Atropa belladonna* L. [48] and *Solanum khasianum* C.B.Clarke [49].

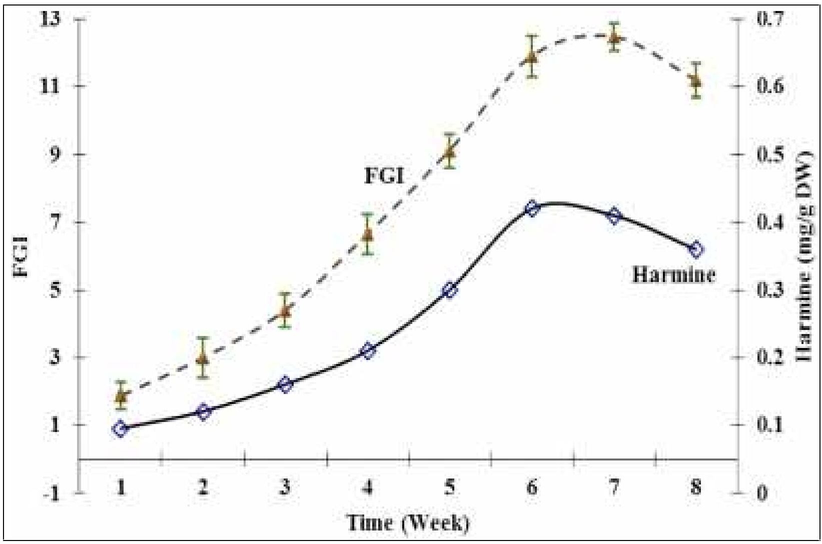

Fig. 4 Time course of FGI and accumulation of harmine in HRC (line S2) of *Peganum multisectum* (Maxim) Bobrov.

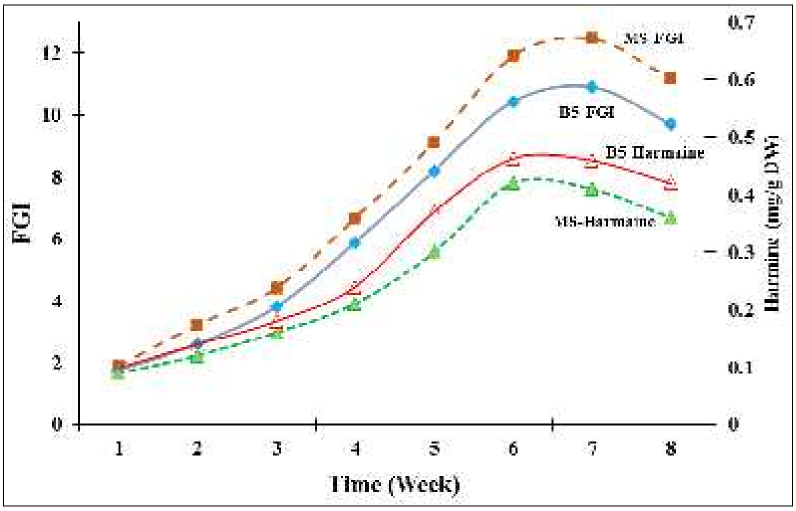

Fig. 5 Growth of HRs and accumulation of harmine in HRC of *Peganum multisectum* (Maxim) Bobrov (line S2) on MS and B5 medium.

The finding by Kuzovkina *et al*., [50] and Zayed & Winka [44] proved that MS medium was optimal medium for growth and higher alkaloid yield in HRC of *P.harmala*. Manipulation of MS and B5 components for enhancement of growth and solasodine production in HRC of *S. khasianum* Clarke was investigated by Jacob and Malpathak [64]. They reported that tissue growth and solasodine production are strongly affected by the culture medium. Also, the results showed that in part the effect of each component of B5 and MS is dependent on the growth phase of the HRC [64].

Effect of Sucrose

The biosynthesis of secondary metabolite in transformed roots is influenced by nutritional and environmental factors [62]. Sucrose is the best source of carbon and is hydrolyzed into glucose and fructose by plant cells during assimilation; its rate of uptake varies in different plant cells [51]. Sucrose concentration is known to affect a range of culture parameters such as growth, primary metabolism and yield of secondary products. Sucrose has been reported to have a significant effect on growth and steroidal content in transformed roots of *Solanum aviculare* G. Forst [52]. It also has a physical role as an osmotically active solute, osmotic stress being known to exert considerable influence on productivity of cultured plant cells [52].

Sucrose with increasing concentration (15, 30 and 45 g l^{-1}) significantly improved the growth of HRs in terms of FGI (Fig. 6). The highest FGI of HRs were obtained in media supplemented with sucrose concentration of 45 g l^{-1}. However, considerable biomass was also noted at 30 g l^{-1} of sucrose. The addition of the sucrose also improved the alkaloid content of HRC (Fig. 6). The higher harmine content in roots was detected at 45 g l^{-1} sucrose in medium but less or more harmine content was obtained by using 30 g l^{-1} sucrose. The result showed that MS medium supplemented with 30 and 45 g l^{-1} sucrose was the best for growth of HRs of *P. multisectum*.

Lourenço *et al*., [53] have studied the effect of initial sucrose concentrations on biomass and proteinase production in HRs of *Centaurea calcitrapa* L.. The highest values for both proteolytic activity and fresh weight were attained when sucrose was used at 30 and 50 g l^{-1} of medium [53]. Yu *et al*., [52] found that sucrose concentration affects the growth of *S. aviculare* HRs in a similar manner. The influence of sucrose concentration on HRs growth seems to be quite important but its effect as a metabolic substrate must be distinguished from its physical role as an osmotic active solute in the medium and as a modulator of gene expression [52, 54]. We have observed that growth and harmine production improved at 30 or 45 g l^{-1} sucrose and at low concentration of sucrose (15 g l^{-1}) and high concentration (60 g l^{-1}), inhibited the growth and harmine production.

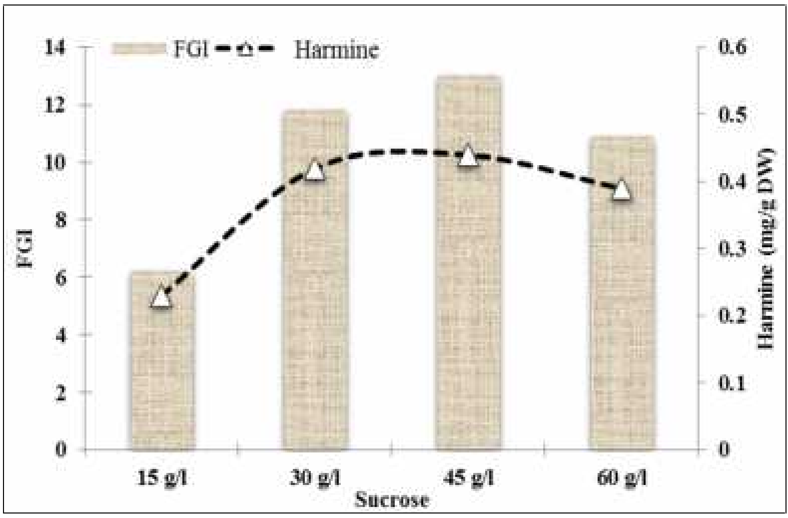

Fig. 6 Effect of varying sucrose concentration on growth of HRs and accumulation of harmine in HRC of *Peganum multisectum* (Maxim) Bobrov.

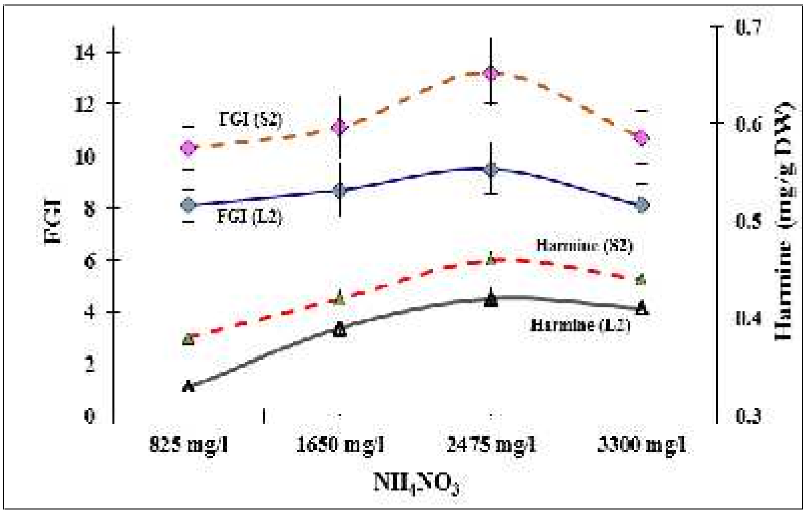

Fig. 7 Influence of different concentrations of ammonium nitrate on growth and harmine in HRC of *Peganum multisectum* (Maxim) Bobrov. Results represent mean of five replicates. Error bars represent standard deviation.

Effect of Ammonium Nitrate

Medium optimization studies showed that nitrogen concentration in the medium triggers the metabolism from primary to secondary [55]. The nitrogen concentration and the carbon/nitrogen ratio of the culture medium often influence the synthesis of alkaloids [56,57]. An increase in FGI was observed in both S2 and L2 HR lines when ammonium nitrate concentration was raised in the medium to 2475 mg l^{-1}. The highest FGI (13.2±0.8) was obtained with the increase in NH_4NO_3 concentration to 2475 mg l^{-1} in the MS medium. In these conditions, the FGI of line L2 and line S2 were about 10-18% more than FGI in MS basal medium (1650 mg l^{-1} of NH_4NO_3). The results showed that addition of extra ammonium nitrate upto optimum level (2475 mg l^{-1}), as a source of nitrogen was more effective than standard ammonium nitrate in MS basal medium for growth of HRs. Addition of high level of ammonium nitrate (3300 mg l^{-1}) declined FGI of HRs as compare to FGI of MS medium. Lower concentration of NH_4NO_3 (825 mg l^{-1}) in the medium resulted in considerably decreased harmine content of HRs. The maximum harmine content (0.46±0.04 mg g^{-1} DW) in the HRs was obtained with addition of extra level of ammonium nitrate (2475 mg l^{-1}) in the culture medium. In line S2, addition of 2475 mg l^{-1} ammonium nitrate in MS medium increased harmine content to 0.46 mg

g^{-1} DW, which was about 11% more than MS basal medium (Fig. 7).

The current results correspond to the assumption that higher nitrogen supply to a plant will increase alkaloid production [61] as in *Hyoscyamus muticus* L. [58]. Nutritive factors like nitrogen are important parameters that influence alkaloid production [26]. The identity and quantity of nitrogen in the culture medium affected both growth and proteolytic enzyme production of *C. calcitrapa* HR and ammonium, as the sole nitrogen source did not support growth to a visible extent [65]. This fact has been previously described for *H. muticus* HRs [58]. Wang and Tan [59] reported that nitrogen source was an essential factor for the growth and biosynthesis of artemisinin in *Artemisia annua* Pall. HRs. An increase of the ammonium nitrate concentration (upto 2475 mg l^{-1}) in the culture medium led to increased growth and accumulation of harmine contents in the HRs of *P. multisectum*.

The first important factor which switched our investigation with *P. multisectum*, was the production of long-stable hairy root culture using *A. rhizogenesis*. After establishment of hairy root, it has been tried to come up with a suitable media (MS & B5 solid and liquid medium) and the conditions for maintenance of the cultures and also for the production of secondary compounds [60]. Our results showed that *in vitro* harmine production by HRs culture could be influenced at some extent by nitrogen and sucrose concentrations.

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