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Full Length Research Paper

Induction and establishment of adventitious and hairy root cultures of *Plumbago zeylanica* L.

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This study was investigated to develop efficient protocols for adventitious and hairy root cultures of *Plumbago zeylanica*. Adventitious roots were initiated from leaf and stem explants cultured on MS medium with different concentrations and combinations of auxins. The highest number of roots was obtained when the explants was cultured on MS medium with 1.0 mg·L^{-1} IBA and 0.5 mg·L^{-1} NAA. Hairy root culture of *P. zeylanica* was obtained by infecting leaf explants cultured *in vitro* with *Agrobacterium rhizogenes* MTCC 532. The highest frequency of explant transformation was about 93%. The developed culture exhibited fast growth and high lateral branching on growth regulator free MS medium. The root cultures obtained were inoculated into B_5, MS and SH media supplemented with different carbon sources with or without auxins and were placed on a rotary shaker 80 rpm for 35 days under dark or light conditions. Of the three media tested, MS medium sustained better root growth than others and sucrose proved to be the best carbon source. The biomass in hairy root culture was higher than in non-transformed root culture.

Key words: *Agrobacterium rhizogenes*, auxins, carbon source, MS medium, root culture, *Plumbago zeylanica*

INTRODUCTION

Roots of numerous plant families are the site for biosynthesis or accumulation of major secondary metabolites including alkaloids, polyacetylene, sesquiterpenes and napthoquinones. These compounds could be similarly synthesized in hairy roots. *Agrobacterium rhizogenes* is able to induce hairy root disease on a large variety of plant species. These hairy roots can be excised and cultivated indefinitely under sterile conditions. In such cultures, secondary metabolite production is stable over successive generations due to their genetic stability. *Plumbago zeylanica* L. is an important medicinal plant widely used in treatments of various diseases such as rheumatism, piles, diarrhea, leprosy and anasarca (Anonymous, 1989). Its roots are the main source of plumbagin (2-methoxy-5hydroxy-1,4-napthoquinone), a natural napthoquinone possessing various pharmaceutical activities such as anticancer (Aziz et al., 2008), antioxidant (Tilak et al., 2004), antimicrobial (Paiva et al., 2003), radiosensitizing (Prasad et al., 1996) and antifertility (Bhargava, 1984). Multiple use of this plant species have necessitated its large scale collection as raw material to the pharmaceutical industry, leading to over exploitation and disappearing in the wild. The conventional propagation of this species is difficult therefore *in vitro* propagation methods developed by many researchers, however, this plant grow quite slowly and take long time until the roots are suitable to use (Kitanov and Pashankov, 1994). Hence, an alternative source needed in order to meet the demand. *In vitro* root culture has become an alternative method for the production of valuable secondary metabolites on commercial scale. Adventitious roots induced by *in vitro* methods showed high rate of proliferation and active secondary metabolism (Hahn et al., 2003; Yu et al., 2005). Adventitious roots are natural, grown vigorously in phytohormone supplemented medium and have shown tremendous potentialities of accumulation of valuable secondary metabolites (Murthy et al., 2008). Hairy root culture have been recognized as potential alternative source of many secondary compounds with comparable or even greater

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level of metabolites production within shorter time than is commonly expected from naturally grown plants (Giri and Narasu, 2000). Verma et al. (2002) reported production of plumbagin from hairy root culture of *P. zeylanica*. However, the transformation frequency is low, further, media manipulation and culture conditions are required for root cultures. Panichayupakaranant and Tewtrakul (2002) reported plumbagin production by root cultures of *P. rosea*. However, no reports are available for plumbagin production from adventitious roots of *P. zeylanica*. Hence, development of efficient method is needed for induction and establishment of adventitious root cultures of *P. zeylanica*. In this study, we investigated the effect of explants, PGRs and media on induction and establishment of adventitious and hairy root cultures of *P. zeylanica*.

MATERIALS AND METHODS

Adventitious root culture (non-transformed roots)

Leaves which were excised from *in vitro* grown plants (Sivanesan, 2007), cut into small segments (0.5-1.0 cm) and cultured on MS medium (Murashige and Skoog, 1962) supplemented with IAA, IBA and NAA 0.1-1.0 mg·L^{-1} alone or combinations of growth regulators, IAA (0.5 mg·L^{-1}) + NAA (0.5 mg·L^{-1}) and IBA (1.0 mg·L^{-1}) + NAA (0.5 mg·L^{-1}). After 2 weeks, the emerging roots were transferred to 250 ml Erlenmeyer flasks containing 50 ml of B_5 (Gamborg et al., 1968), MS and SH (Schenk and Hildebrandt, 1972) media fortified with 1.0 mg·L^{-1} IBA and 0.5 mg·L^{-1} NAA. The flasks were placed on a rotary shaker 80 rpm in dark or light conditions (a 12 h photoperiod with 10 µmol m^{-2} s^{-1}) for 35 days. For one experiment, 25 leaves were used and the experiment was repeated thrice in the same environment. The growth rate was measured by taking into account, the fresh and dry weight of the roots at every 7 days interval, upto 35 days. Fresh weight was determined after completely removing the medium by using tissue paper and was placed on pre-weighed aluminum foil and weighed and then kept in an oven at 50°C for 24 h and weighed again to obtain the dry weight.

Hairy root culture

Agrobacterium rhizogenes 532 (MTCC) was used for hairy root induction in *P. zeylanica*. The bacterium was maintained on YEB agar medium containing 1 g·L^{-1} mannitol, 0.1 g·L^{-1} NaCl, 0.5 mg·L^{-1} glutamine, 0.2 g·L^{-1} KH_2PO_4, 0.02 g·L^{-1} yeast extract and 15 g·L^{-1} agar. Prior to inoculation, the bacterium was sub-cultured on YEB liquid medium and cultured for 48 h in the dark on a rotary shaker at 80 rpm. Bacterial count of about10^8 cells/ml as measured by OD at 660 nm was used for inoculation. Young leaves was excised from *in vitro* grown shoots cut into small segments (1.0 cm) and were dipped in the bacterial suspension for an hour and then blotted dry on a sterile filter paper. Explants were co-cultivated with bacteria for 1 - 5 days on solid basal MS medium. The infected explants were rinsed with sterile distilled water (containing 500 mg·L^{-1} carbenicillin) three times and then transferred to full strength MS basal medium containing 250 mg·L^{-1} carbenicillin and was kept under darkness. Control explants were given the same treatment but were dipped in sterile YEB medium. After about 2 weeks, explants develop roots at the infected sites, they were then sub-cultured again in MS basal medium containing 250 mg·L^{-1} carbenicillin. Before transferring to the solid medium, the explants were rinsed with MS liquid medium containing 500 mg·L^{-1} carbenicillin. They were sub-cultured at every 14 days interval. Hairy roots was excised and transferred to YEB medium, absence of bacterial growth confirm the roots devoid of bacteria. The axenic hairy roots (approximately 0.2 g) were cut and inoculated in hormone free B_5, MS, and SH liquid media containing different carbon sources and kept in a rotary shaker at 80 rpm in dark or light conditions (12 h photoperiod with 10 µmol m^{-2} s^{-1}) for 35 days. For one experiment, 50 leaves were used and the experiment was repeated thrice. The cultures were harvested and three replicates were maintained. The growth of hairy root cultures was estimated by determining the fresh weight and dry weight of the roots.

Statistical analysis

All experiments were repeated thrice. Data were analyzed by analysis of variance (ANOVA) to detect significant differences between means using SAS computer package (SAS Institute Inc., Cary, NC, USA, Release 9.1). Means differing significantly were compared using the Duncan's multiple range tests at the 5% probability level.

RESULTS AND DISCUSSION

Adventitious root culture (non-transformed)

Leaf and stem explants were cultured on MS medium supplemented with various auxins such as IAA, IBA and NAA used for rot induction. The medium devoid of auxins did not induce root formation. Explants cultured on MS medium containing auxins induced roots from the cut ends and veins within 2 weeks. IAA, when supplemented into the medium at 0.5 mg·L^{-1} induced a mean of 9.2 and 5.6 roots from leaf and stem explants, respectively. Increasing concentration of IAA did not enhance root induction in both explants. When IBA was supplemented with the MS medium, the number of roots and percentage of root induction increased with an increase in the concentration from 0.1 to 1.0 mg·L^{-1}. Of the three auxins used, NAA gave cent percent root induction at 0.5 mg·L^{-1} and the number of roots obtained were 12 (leaf) and 9.6 (stem). Further increase in NAA concentration resulted callusing and callus mediated root formation. Panichayupakaranant and Tewtrakul (2002) reported that the combination of NAA and kinetin was found to be best for root growth in *P. rosea*. However, in the present study, a combination of auxins enhanced significantly number of roots in both explants. The highest number of roots was obtained when the explants cultured on MS medium with 1.0 mg·L^{-1} IBA and 0.5 mg·L^{-1} NAA (Table 1). The roots were separated from the solid medium and inoculated into MS medium containing 3% (w/v) sucrose, maintained under dark conditions and were served as an inoculums for further experiments.

Adventitious root culture of *P. zeylanica* was initiated by transferring 0.2 g of the inoculum into B_5, MS, and SH media fortified with 1.0 mg·L^{-1} IBA and 0.5 mg·L^{-1} NAA and 3% (w/v) sucrose. Of the three media tested, MS medium sustained better root growth followed by B_5 and SH media. When the cultures were maintained under light conditions, the growth rate was less than those that were kept under darkness (Figure 1 a and b). The stimulatory effect of

Table 1. Effect of auxins on root induction from leaf and stem explants of *P. zeylanica*.

Concentrations (mg·L^{-1})			Root induction (%)		Number of roots/explant	
IAA	IBA	NAA	Leaf	Stem	Leaf	Stem
0.1	0	0	46e	41g	3.4g	2.6f
0.5	0	0	87c	76e	9.2d	5.6de
1.0	0	0	84c	70f	8.0e	6.7d
0	0.1	0	74d	72f	6.2f	4.0e
0	0.5	0	85c	79d	8.0e	4.6e
0	1.0	0	96b	88c	8.7de	5.3de
0	0	0.1	94b	91b	7.8e	6.0d
0	0	0.5	100a	100a	12.0c	9.6c
0	0	1.0	C+R	C+R	C+R	C+R
0.5	0	0.5	100a	100a	16.4b	12.6b
0	1.0	0.5	100a	100a	19.2a	14.0a

Means followed by the same letters in column were not significantly different by DMRT test ($p \leq 0.05$). C+R, callus and root.

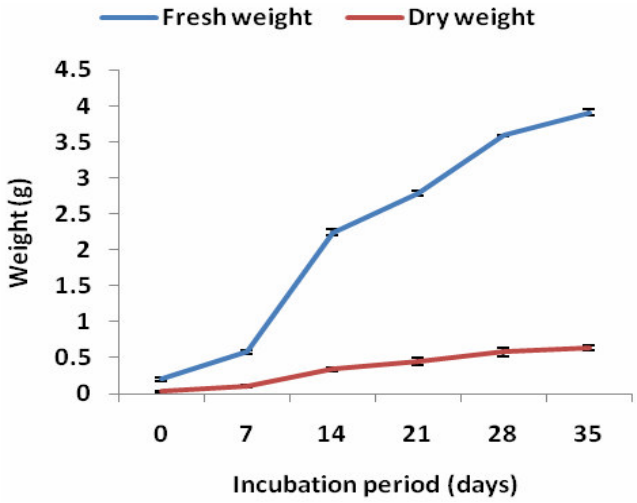

Figure 1a. Growth of non-transformed root culture of *P. zeylanica* using MS medium with 3% (w/v) sucrose under dark.

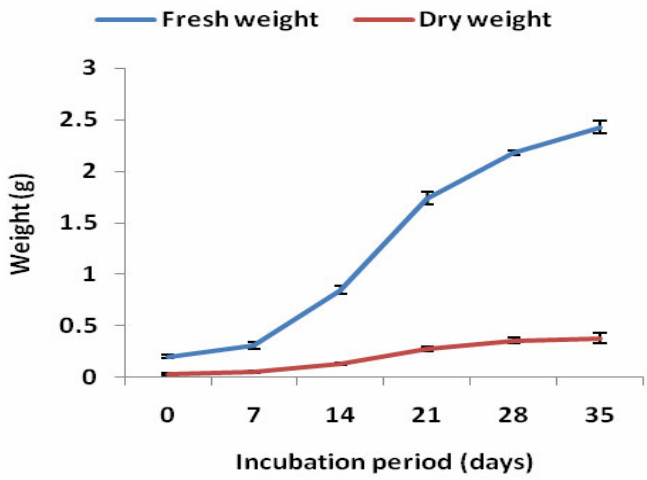

Figure 1b. Growth of non-transformed root culture of *P. zeylanica* using MS medium with 3% (w/v) sucrose under light, and Basal medium with 1.0 mg·L^{-1} IBA and 0.5 mg·L^{-1} NAA, under dark.

darkness and inhibitory effect of light condition on root induction have been well documented in other plant species (Sudha and Seeni, 2001; Seo et al., 2003). A maximum of 2.43 and 0.37 g of fresh and dry weights respectively was obtained when roots were cultured on MS medium without plant growth regulators (Figure 1a). No lateral roots were observed in roots cultured in the basal media without plant growth regulators; however, lateral roots were formed in the basal media supplemented with auxins. Therefore, addition of auxins to the culture media resulted in a high yield compared with basal media alone. The greatest fresh (3.91 g) and dry (0.64 g) weights were obtained when MS medium was supplemented with IBA and NAA (Figure 1c). There was an increase in the fresh and dry weight with an increase in the number of day of incubation. The highest fresh weight of roots obtained in B5 and SH media were 2.52 and 1.86 g, respectively (Figure 1d and e). These results indicate that MS medium was found to be the best for normal root culture and this method could be useful for the production of plumbagin in large scale. Moreover, root cultures can be used in many ways including studies of carbohydrate metabolism, mineral nutrient requirements, essentiality of vitamins and other growth regulators, differentiation of the root apex and gravitropism (Nandagopal and Ranjitha Kumari, 2007).

Hairy root culture

The explants were infected with the *A. rhizogenes* and co-

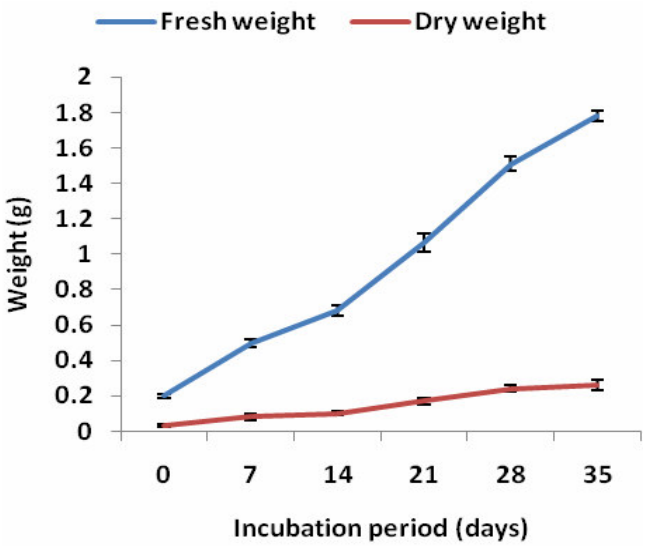

Figure 1c. Growth of non-transformed root culture of *P. zeylanica* using MS medium.

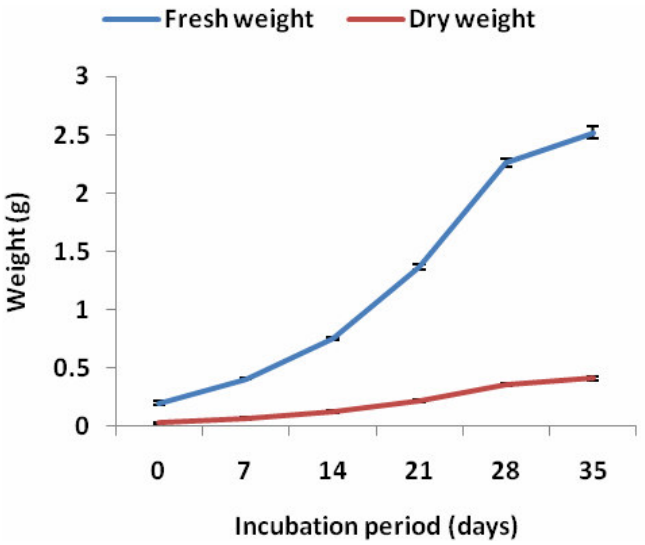

Figure 1e. Growth of non-transformed root culture of *P. zeylanica* using SH medium.

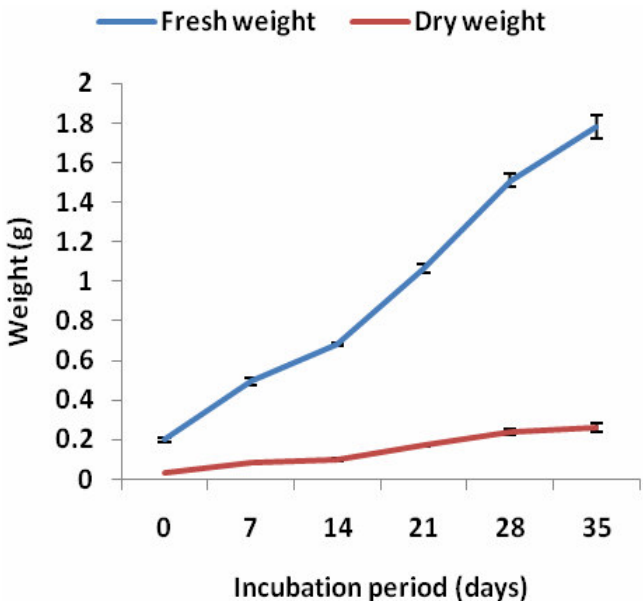

Figure 1d. Growth of non-transformed root culture of *P. zeylanica* using B_5 medium.

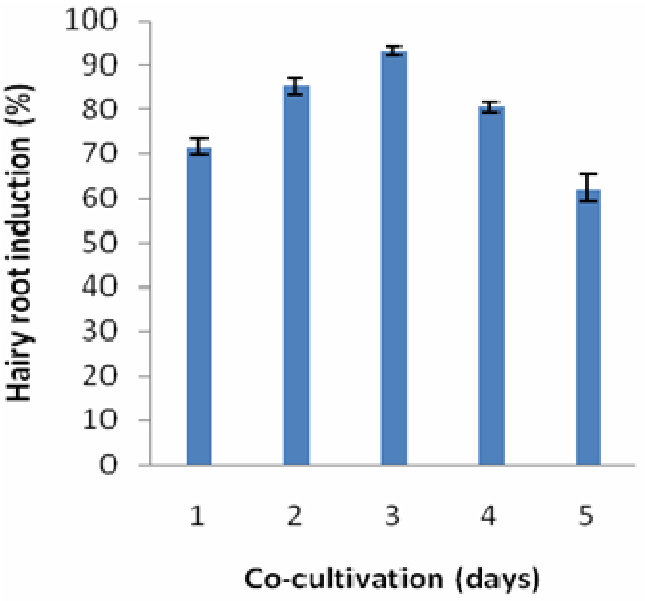

Figure 2a. Influence of co-cultivation period on hairy root induction.

cultured for 1-5 days on solid MS medium. A gradual increase in the hairy root induction was noticed with an increase in co-cultivation days from 1 to 3 (Figure 2a). However, further increase in co-cultivation days decrease hairy root induction due to over growth of bacterium and this may lead to browning of the explants. The highest percentage (93%) of hairy root induction was obtained when the explants were co-cultivated with the bacterium for 3 days. Verma et al. (2002) obtained hairy roots in *P. zeylanica* after 3 weeks of bacterial infection and the relative transformation frequency was 0.9 ± 0.05. In the present study, induction of hairy roots was noticed after 2 weeks of bacterial infection (Figure 3a). These results indicated that hairy root induction and frequency are strain specific (Sarma et al., 1997). When the roots were 5 -10 mm long, they were excised along with the stem segment bearing roots and it was placed in 25 ml of the MS medium containing 250 $mg \cdot L^{-1}$ carbencillin. The developed culture exhibited fast growth and high lateral branching on growth regulator free MS medium and root plagiotropism. Hairy root culture was initiated by transferring 0.2 g of axenic roots (as inoculum) into B_5, MS and SH media con-

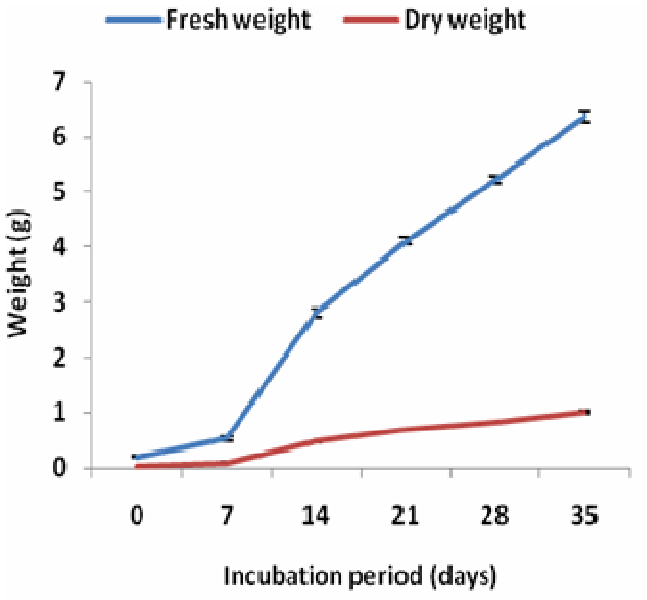

Figure 2b. Effect of MS medium on the growth of hairy root culture.

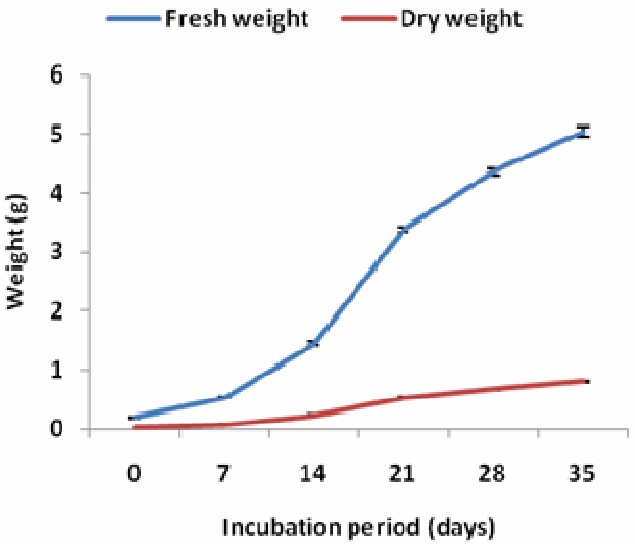

Figure 2c. Effect B_5 medium on the growth of hairy root culture.

taining 3% (w/v) sucrose and cultured under dark or light conditions. It was found that in all the three media used, growth increased with the increase in the incubation period. However, MS medium was found to be best for root growth followed by B_5 and SH media. The highest yield of roots obtained in MS, B_5 and SH media were 6.3, 0.99, 5.0, 8.0 and 4.6, 7.1 g, fresh and dry weights, respectively (Figure 2b-d). In the present study, the biomass in hairy root culture was higher than in non-transformed root culture. This is in agreement with previous studies (Verma et al., 2002; Gangopadhyay et

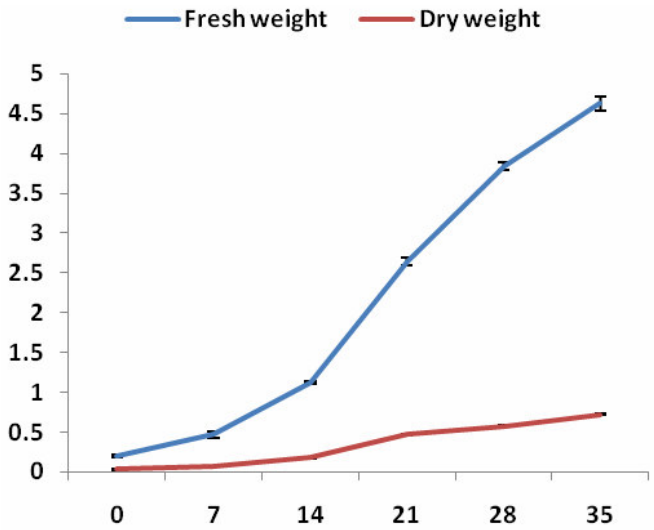

Figure 2d. Effect of SH medium on the growth of hairy root culture under dark conditions.

Figure 3a. Hairy root induction from leaf explants after co-cultured with *Agrobacterium rhizogens*.

al., 2008). In hairy roots, high growth rate was observed because of high frequency lateral root formation and consequent increase in the number of elongating tips (Mano et al. 1989). In the presence of numerous meristems which lead to high growth rate for hairy roots in the culture, exceeds over most non-transformed root culture (Quattrocchio et al. 1986). The growth of hairy roots was not significantly different when compared with dark conditions, however, dark condition yield more biomass (data not shown). Inhibition of growth of hairy roots of *P. indica* was observed when incubated under continuous irradiance (Gangopadhyay et al., 2008).

The effect of different carbon sources (fructose, glucose and sucrose) on growth of hairy roots was also tested.

Figure 3b. Hairy root grown under light condition.

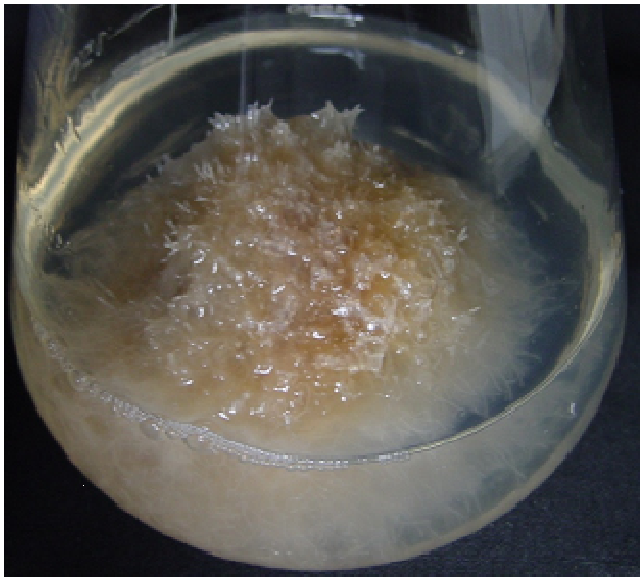

Figure 3c. Growth of hairy root in MS medium with 3% (w/v) sucrose after 35 days under dark condition.

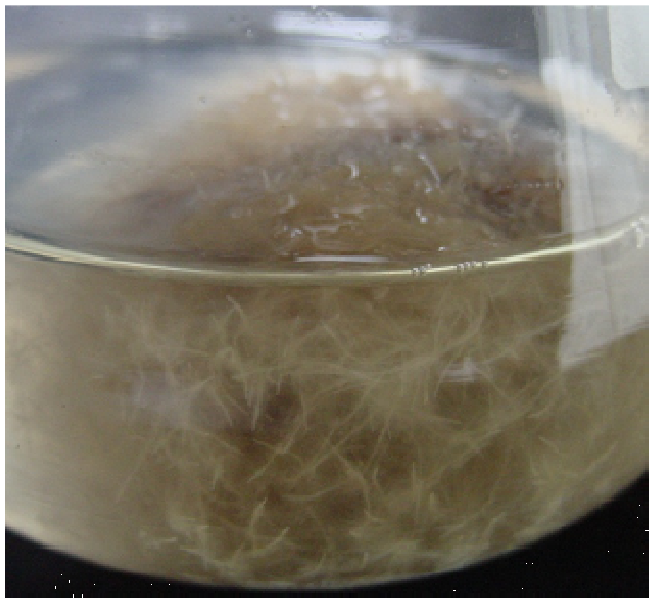

Figure 3d. A closer view of root growth.

When fructose or glucose (3%, w/v) was used instead of sucrose, the growth rate was comparatively less (data not shown). 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 (Srinivasan et al., 1995). In the present study, MS medium supplemented with 3% (w/v) sucrose gave maximum growth of roots (Figure 3b-d). In contrast, maximum biomass obtained in *P. indica* (Gangopadhyay et al., 2008) and in *P. zeylanica* (Verma et al., 2002) when the medium was supplemented with 1 and 4% (w/v) sucrose, respectively.

In conclusions, non-transformed and hairy roots were grown in different media such as B5, MS and SH with different carbon sources. Among them, MS medium sustained better growth than others and sucrose proved to be the best carbon source. The growth in glucose or fructose containing medium was remarkably slower than that of sucrose amended medium. Addition of plant growth regulators enhanced the growth of non-transformed root culture. The growth of hairy roots was not significantly different when compared with dark conditions. The biomass of hairy root culture was higher in non-transformed root culture. In many transformation studies selectable marker genes are used to identify genetic transformation. Use of selectable markers has raised questions of human health concerns when the target material is a functional food (Murthy et al., 2008). In the present study, we used wild strain of *A. rhizogens* which is marker free; hence, both normal and hairy root cultures could be useful for the production of plumbagin in large scale.

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