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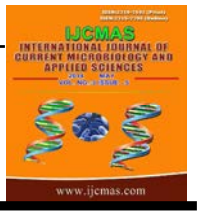

Original Research Article

A comparative study of Hairy Root Culture induction efficiency in four medicinally important plants using *Agrobacterium rhizogenes*

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A B S T R A C T

Hairy Root Culture is the new route for large scale secondary metabolite production because of their fast and plagiotropic growth, genetic and biochemical stability. Axenic explants of four medicinally important plants *Aegle marmelos, Boerhavia diffusa, Datura innoxia* and *Solanum xanthocarpum* were used for induction of Hairy Root Cultures using the *Agrobacterium rhizogenes* strain MTCC 532, by stabbing, co-culturing and cut end infection methods to establish cultures of transformed roots. We were able to induce Hairy Roots in three plants except *Aegle marmelos* by stabbing method with high transformation frequency (60-75%). In *Solanum xanthocarpum* transformation frequency was highest (75%) followed by *Datura innoxia* (62.8%) and *Boerhavia diffusa* (60%). Copious growth of *Solanum xanthocarpum* Hairy Roots was observed in ½ MS liquid medium without hormones with growth index of 45 with doubling time of 7 days. Further this study creates a paradigm for future scale up of hairy roots in bioreactors for large scale production of secondary metabolites & its increasing use as model system in metabolic engineering.

K e y w o r d s

Hairy root culture, Agrobacterium *rhizogenes, Aegle marmelos, Boerhavia diffusa, Datura innoxia, Solanum xanthocarpum,*

Introduction

Plants remains as indispensable source for a number of chemical substances like phytochemicals and secondary metabolites which find application in the pharmaceutical, food and flavor industries. As these are difficult to synthesize chemically owing to their complicated structures, Hairy Root Culture (*HRC*) was developed as the new route for large scale secondary metabolite production (Flores et al, 1987; Rhodes et al, 1987), production of phytochemicals (Shanks and Morgan, 1999). Thus this technique is of immense importance to develop large amount of roots and secondary metabolites in short period to maintain continuous supply of better quality products (Giri and Narasu, 2000).

Agrobacterium rhizogenes mediated hairy root production is a valuable tool for studies on the biosynthesis of secondary

metabolites and for exploitation in metabolic engineering. Hairy roots (HRs) are characterized by rapid growth and extensive branching in growth regulator-free medium. In general, they exhibit genetic stability and, in certain cases, they have the capability of synthesizing secondary metabolites normally present in roots and organs of the species of origin. For this reason, hairy roots have been induced in several medicinal and aromatic plants and cultured for the production of secondary compounds (Bonhomme et al, 2000; Murthy et al, 2008). Hairy root cultures from nearly 200 species of higher plants (at least 30 families), mostly dicots, have been reported as an established experimental system with a remarkable range of biosynthetic capabilities. In a recent investigation (Pawar and Maheshwari, 2004), extensive hairy roots were induced from leaf explants of *Solanum surratennes* and then established on MS basal medium independent of exogenous supply of phytohormones. Wu and coworkers investigated induction and culture conditions for *Solanum nigrum* hairy roots from the cut edges of leaf explants and its solasodine production (Wu et al, 2008).

In the present study four medicinal plants namely *Aegle marmelos, Boerhavia diffusa, Datura innoxia* and *Solanum xanthocarpum* were chosen to induce the hairy root cultures, as these have medicinal properties residing in the roots. *Aegle marmelos* (Rutaceae) commonly called Bael is a fruit-bearing tree indigenous to dry forests on hills and plains. The genus produces a wide variety of metabolites including alkaloids, terpenoids, coumarins and sterols, Limonene, γ-sitosterol are major constituents. *Boerhavia diffusa* (Punarnava) a member of *Nyctaginaceae* family, is a perennial creeping herb distributed in the tropical, subtropical, and temperate regions characterized by the presence of flavonoids, alkaloids, steroids and triterpenoids. The major active principle present in the roots is alkaloidal, known as punarnavine. *Datura innoxia*, an important member of *Solanaceae* family, has great medicinal importance. It is a shrub often grown as an annual in temperate zones characterized by the presence of alkaloids of the tropane class, namely atropine, hyoscyamine and scopolamine, commonly grouped under the headings of daturine. It is a potential commercial source of Scopolamine. *Solanum xanthocarpum* (*Solanaceae*) is a perennial herb growing in dry plains and low hills characterized by the presence of steroidal alkaloids, solasodine and flavonoids. Solasodine (SD) has reported antiandrogenic activity, is the main constituent isolated from the berries and roots of the plant. SD serves as an important intermediate in synthesis of steroidal hormones and is a potential alternative to diosgenin, a precursor in the synthesis of steroidal hormones. The objective of the study was to find the induction efficiency of hairy roots by *Agrobacterium rhizogenes* in all the four plants. The growth dynamics of cultured transformed root were investigated.

Materials and Methods

Explants Preparation

Plants were collected from Pharmacy Garden of Punjab University, Chandigarh. Axillary buds taken from young and healthy plants were washed thoroughly under running tap water and surface sterilized first with few drops of antiseptic solution (Savlon) in distilled water for 5-10 minutes to remove fungal

contamination, and then treated with 0.1% (w/v) $HgCl_2$ for 8 min, followed by several rinses in sterilized distilled water aseptically. Then disinfected with 70% ethanol for 30 seconds, and finally a rinsed with sterilized distilled water. The surface sterilized explants were inoculated on MS (Murshige and Skooge, 1962) basal media supplemented with 3% sucrose, 0.8% agar and optimized concentrations and combinations of plant growth regulators (PGRs) mainly IAA, IBA, BAP and FAP (kinetin). For S. xanthocarpum cultures 1% Activated Charcoal was used. After growth of explants in cultures, three subcultures at 20 days interval were made in the same media. The pH of all the media were adjusted to 5.6 before autoclaving at 121°C for 15 min. Cultures were incubated at 25 ± 2º C under fluorescent light with a 16 hour photoperiod. After 2 months, the in vitro grown shoots and leaves were used as explants for transformation.

Bacterial Culture Preparation

Agrobacterium rhizogenes strain MTCC 532 (Microbial Type Culture Collection, IMTECH, Chandigarh), stored in sterile glycerol at −70°C, was cultured on Yeast Extract Peptone Dextrose (YEPD) solid medium for activation of the strain and subcultured three times on the same medium (Mariya-John et al, 2009).

Induction and Establishment of Hairy Root Culture

The explants were infected with bacteria using three different methods, a) by stabbing the leaves with the bacteria, b) applying the bacteria at the cut-end, and c) by co-culturing the cut explants in 50 ml liquid MS medium along with 200µl of fresh bacterial culture. After infection explants were cultured in half strength MS solid medium supplemented with 3% sucrose in dark regime at 25ºC for 48 hrs. Elimination of bacteria was then done by transferring the stabbed explants to ½ MS solid medium with antibiotic ampicillin (0.5mg/ml) at 25ºC in dark. Hairy Roots that developed were transferred to antibiotic and hormone free ½ MS liquid medium at 25ºC at 100 rpm on orbital shaker under dark and sub-cultured in fresh medium every 15 days.

Inoculums of roots were transferred in aseptic conditions to 50 ml liquid MS medium, which corresponds to initial fresh weight (FW). The Growth Index (GI) was calculated using the ratio of final fresh weight to initial fresh weight. Doubling time was calculated by plotting a graph of $\log_2$ FW (g) v/s time (d) and then calculating the inverse of the slope for the linear part of the curve.

Results and Discussion

Culture Establishment

In the present study, axenic culture of *Aegle marmelos, Boerhavia diffusa, Datura innoxia* and *Solanum xanthocarpum* plants were established using meristematic axillary buds in MS medium supplemented with IAA, IBA, BAP and FAP (kinetin) at optimized concentration and combinations for each plant (Fig. 1). The optimum percentage growth and shootlets per explants in optimized PGR combination is presented (Table.1). Activated charcoal helped in absorbing the phenolic compounds in *S.xanthocarpum* and thereby stopped browning of the explant. As only leaves of axenic cultures were used as explants for the establishment of the *HRC*, which was the sole objective, data for rooting has not been presented.

Induction and Establishment of Hairy Root Culture

For establishment of the *HRC*, the source of explants must be a true to type of the plant and should not have any genotypic variation. Therefore to maintain the genetic and biochemical stability in the HRs, axillary buds were preferred as explants for axenic cultures, to obtain clonal explants than the callus culture in which somaclonal variation are more common leading to plants with genotypic difference. Various parts (cut leaves, cut stems) of these axenic cultures of all the four plants were used to initiate transformed root cultures by infecting with *A. rhizogenes* strain MTCC 532, and to establish cultures of transformed roots for the production of secondary compounds. Though, there are various reports on induction of transformed hairy roots from various plants of different genera and in *Datura innoxia* (Boitel-conti and Dechaux, 2005) and *Boerhavia diffusa* (Ratti and Verma, 2004; Jenifer et al, 2012). There are no reports on induction and establishment of hairy roots in *Solanum xanthocarpum* and *Aegle marmelos.*This is the first report on hairy roots development in *S. xanthocarpum*.

Most of the previous reports described co-culturing to be one of the best methods for developing hairy roots. However, in the present study *Agrobacterium rhizogenes* MTCC 532 strain used was capable of inducing hairy roots formation by stabbing with high transformation frequency (60-75%) and cut end method but no HRs developed by co-culture method in any of four plants, and stabbing method was found to be the best (Table.2). In all three plants the roots appeared within 10 to 15 days by stabbing method (Fig.2).

There was no HRs induced by any of three methods in the present study in *Aegle marmelos.* The possible explanations of this may be the absence of receptors for the *A. rhizogenes* on the plants cells and/or insusceptibility of *Aegle marmelos* to this particular strain of *A. rhizogenes* MTCC 532 used for transformation in the present work. Another probable reason which can be attributed for the non transformation of *Aegle marmelos* is that the plants of rutaceae family have been regarded as recalcitrant to *Agrobacterium* mediated transformation and give only low, in vitro regeneration efficiency which is highly genotype dependent. Genetic transformation with *Agrobacterium* in the *Citrus sinensis* (Rutaceae) species has been considered as recalcitrant due to the low transformation efficiency (Almeida et al, 2003). *Aegle marmelos* therefore might be recalcitrant to genetic transformation similar to citrus species as these are not natural hosts of *Agrobacterium* due to absence of specific phenolic compounds primarily acetosyringone which activates the virulence genes (Vir A protein) of the Ri plasmid to initiate the transfer of T-DNA and also there are problems in the selection and rooting of shoots from transformed cells.

In *Boerhavia diffusa* about 60 % transformation frequency was observed after 13 days of infection by stabbing method as compared to 50% after 15 days in cut end method (Fig. 2b, 3a). HRs when grown in ½ MS liquid medium at 100 rpm on an orbital shaker, the roots got contaminated after 7 days. In the earlier reports of HRs production in *Boerhavia diffusa* (Ratti and Verma 2004; Jenifer et al, 2012), they obtained numerous roots up to 32 per explant emerged from the proximal end and from the cut end of the leaf respectively by transformation of *Boerhavia diffusa* by *Agrobacterium rhizogenes* using co-culture method.

Table.1 *In vitro* response of axillary buds of four plants on MS medium with optimized concentration and combination of PGR's

Plants	MS Medium + PGR's	%Shooting (Mean±SE)	Shootlets/explants (Mean±SE)
Aegle marmelos	IBA (1.0 mg/l) + BAP (2.0 mg/l)	80.00±0.289	4.00±0.50
Boerhavia diffusa	IBA (1.0 mg/l) + BAP (2.0 mg/l)	70.00±0.346	1.66±0.09
Datura innoxia	IAA(0.25 mg/l) + BAP(0.50 mg/l)	72.11±0.375	1.70±0.14
Solanum xanthocarpum	IBA(0.25mg/l) + Kinetin(0.50mg/l)+ 1% charcoal	65.38±0.403	1.30±0.05

Table.2 Hairy roots induction time and efficiency in the four plants with stabbing and cut end method with *A. rhizogenes*

Plant	Method used	Transformation frequency (%)	Induction time (Days)
Aegle marmelos	Stabbing	No response	No response
	Cut end	No response	No response
Boerhavia diffusa	Stabbing	60.0	13
	Cut end	50.0	15
Datura innoxia	Stabbing	62.8	10
	Cut end	50.0	13
Solanum xanthocarpum	Stabbing	75.0	12
	Cut end	No response	No response

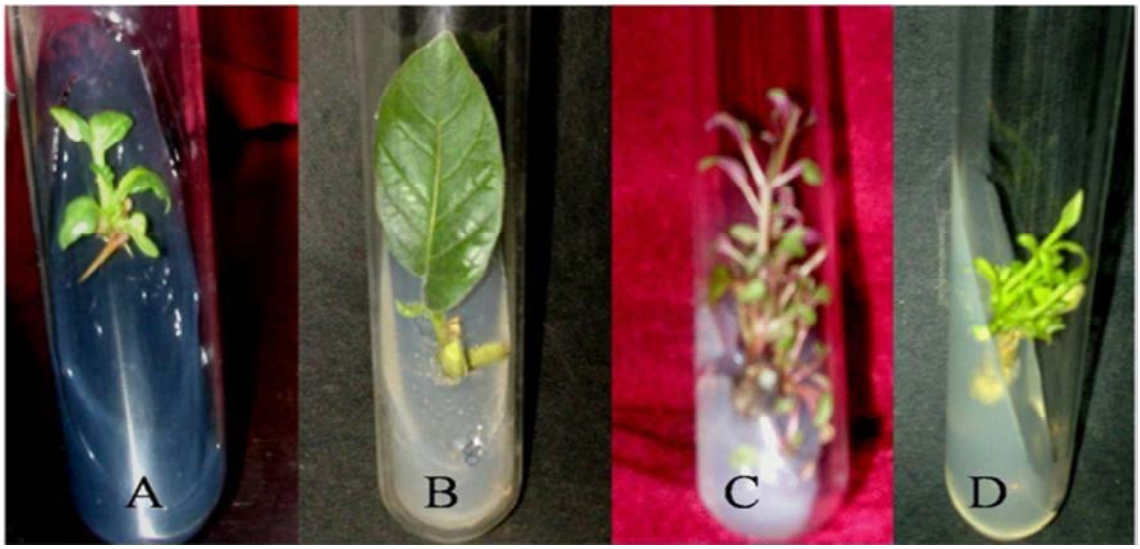

Fig.1 Picture showing *in vitro* response of axillary buds of four plants on MS medium with optimized concentration and combination of PGR's. **A.** *Solanum xanthocarpum* [IBA(0.25mg/l) + Kinetin(0.50mg/l)+1% charcoal]; **B**. *Datura innoxia* [IAA(0.25 mg/l) + BAP(0.50 mg/l)]; **C.** *Boerhavia diffusa* [IBA (1.0 mg/l) + BAP (2.0 mg/l)]; **D**. *Aegle marmelos* [IBA (1.0 mg/l) + BAP (2.0 mg/l)].

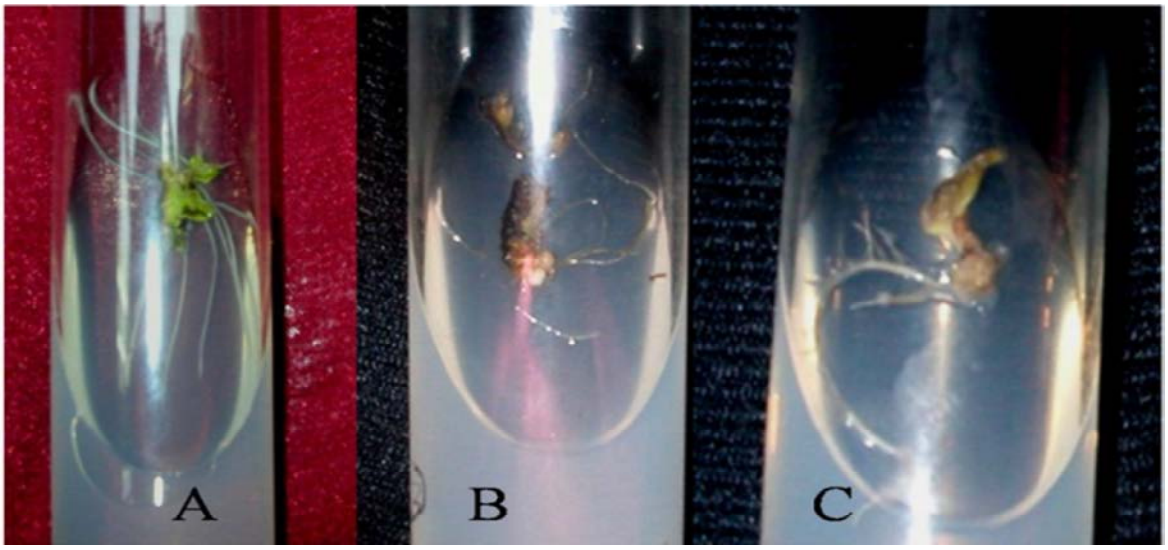

Fig.2 Hairy root induction by stabbing method using *A. rhizogenes* in *Solanum xanthocarpum* (A); *Boerhavia diffusa* (B) and *Datura innoxia* (C) cultured in half strength MS solid medium supplemented with 3% sucrose in dark regime at 25º C.

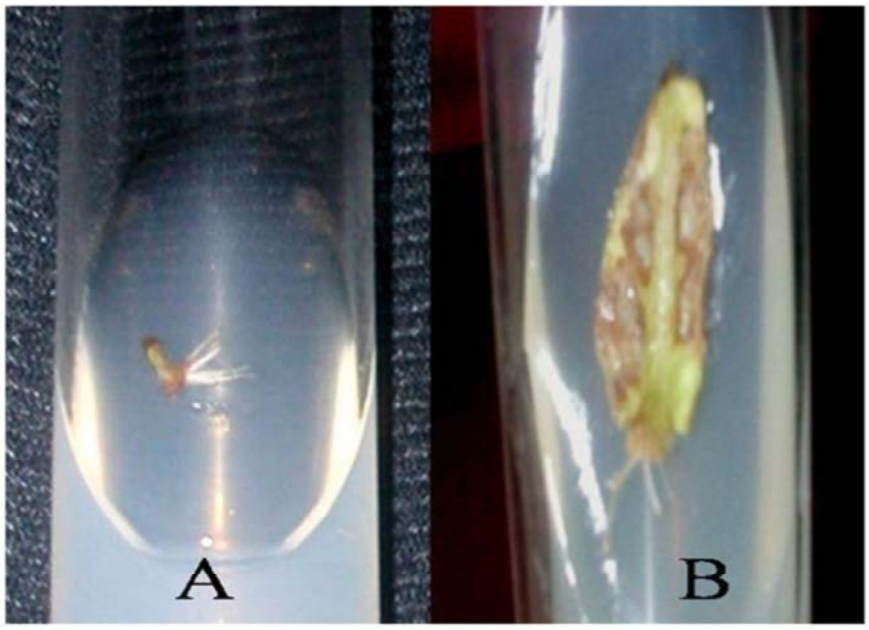

Fig. 3 Hairy root induction by cut end method using *A. rhizogenes* in *Boerhavia diffusa* (A) and *Datura innoxia* (B) cultured in half strength MS solid medium supplemented with 3% sucrose in dark regime at 25º C.

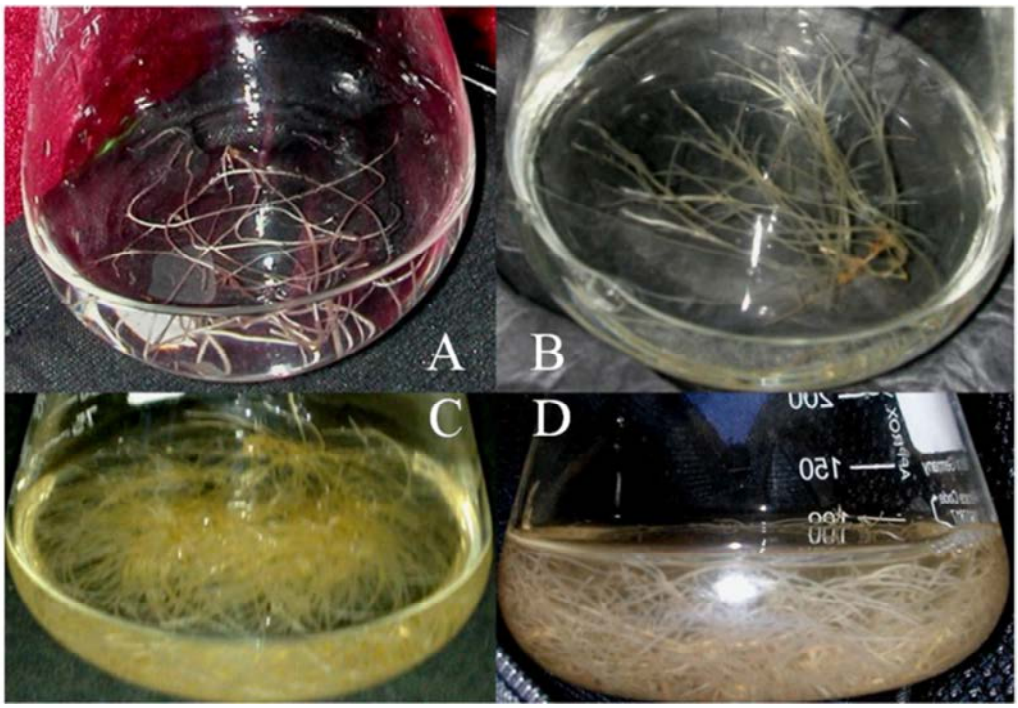

Fig.4 Copious growth of the Hairy root cultures of the *Solanum xanthocarpum* developed by stabbing method cultured in ½ MS liquid medium without hormones at 100 rpm on orbital shaker. A. after 5 days; B. after 15 days; C. after 26 days; D. after 57 days.

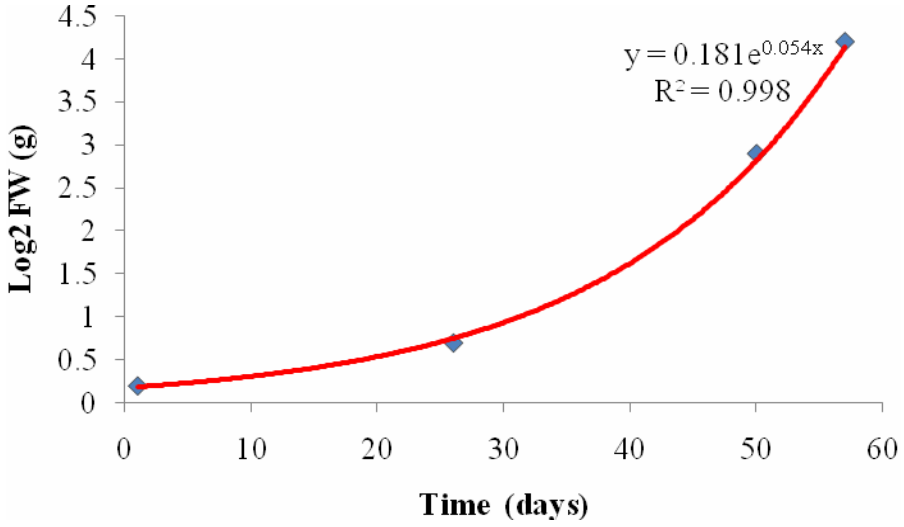

Fig. 5 Showing the graph of log_2FW(g) versus time (days) of the HRs of *Solanum xanthocarpum* cultured in ½ MS liquid medium at 100 rpm on orbital shaker.

In *Datura innoxia* about 62.8 % transformation frequency was observed after 10 days of infection by stabbing method as compared to 50% after 13 days in cut end method (Fig. 2c, 3b). HRs when grown in ½ MS liquid medium at 100 rpm on an orbital shaker, the copious roots developed after 10 days. The transformed roots were developed at the end of petiole close to the point of attachment to the stem axis. Boitel-Conti and Dechaux (2005) transformed *Datura innoxia* whole plant by *Agrobacterium rhizogenes*, and the obtained hairy roots were studied for their tropane alkaloid production.

In *Solanum xanthocarpum* about 75 % transformation frequency was observed after 12 days of infection by stabbing method which is very simple method comparing to other reports (Fig. 2a). Copious growth of HRs was observed in ½ MS liquid medium without hormones at 100 rpm on an orbital shaker, 15 days after the elimination of bacteria using ampicillin (0.5 g/l). These transformed roots have several properties, such as fast and plagiotropic growth, a high degree of lateral branching (Fig. 4). Inoculums of 0.2g roots were transferred in aseptic conditions to 50 ml liquid ½ MS medium, which corresponds to initial fresh weight (FW). After 57 d of culture the Growth Index was found to be about 45 and the doubling time was 7 days (Fig. 5).

Wu et al (2008) investigated induction and culture conditions for *Solanum nigrum* hairy roots and its solasodine production. The results showed that hairy roots could be initiated from the cut edges of leaf explants 5 days after inoculation with the strain of *A. rhizogenes* ATCC15834. Mariya-John (2009) reported that physiological factor, such as, hormone plays an important role in the formation of hairy roots and also indicated that source and concentration of carbon in the basal medium was one of the important factors for hairy root induction. But results of our studies suggest that these hairy roots were established on MS basal medium and their growth was observed to be independent of exogenous supply of phytohormones as also reported by Pawar and Maheshwari (2004).

This study demonstrated the transformation of *Boerhavia diffusa*, *Datura innoxia* and *Solanum xanthocarpum*, leaves with *A. rhizogenes* and establishment of hairy root cultures with highest transformation frequency of 75% in *Solanum xanthocarpum*. These established HRs produced need to be further investigated for their potential as source of enhanced production of commercially important secondary metabolites (Pawar et al, 2004; Araujo et al, 2006; Eapen et al, 2007) and also for phytoremediation (Suza et al, 2008; Datta et al, 2011). Further there is a ever growing demand of herbal medicines, so scale up of HRs of *S. xanthocarpum* in bioreactors will be a useful technique for large scale production of secondary metabolites in less time and at low cost.

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References

Almeida, W., Filho, F., Mendes, B., Pavan, A., Rodriguez, A., 2003. *Agrobacterium*-mediated

transformation of *Citrus sinensis* and *Citrus limonia* epicotyl segments. Science Agriculture Piracicaba Brazilian. 60: 23-29.

Araujo, S., Dec, J., Bollag, J., Pletsch, M., 2006. Uptake and transformation of phenol and chlorophenols by hairy root cultures of *Daucus carota, Ipomoea batatas* and *Solanum aviculare*. Chemosphere.63: 642-651.

Boitel-conti, M., Dechaux, C., 2005. A strategy for overaccumulation of scopolamine in *Datura innoxia* hairy root cultures. Acta Biologica Cracoviensia Series Botanica. 47: 101-107.

Bonhomme, V., Laurain-Mattar, D., Lacoux, J., Fliniaux, M., Jacquin-Dubreuil, A., 2000. Tropane alkaloid production by hairy roots of *Atropa belladonna* obtained after transformation with *Agrobacterium rhizogenes* 15834 and *Agrobacterium tumefaciens* containing *rol A, B, C* genes only. Journal of Biotechnology. 81:151-158.

Datta, K.A. and Rita, P., 2011. An updated overview on *Solanum xanthocarpum* Schred and Wendl. International Journal of Research in Ayurveda and Pharmacy. 2: 730-735.

Eapen, S., Singh, S., D'Souza, S., 2007. Advances in development of transgenic plants for remediation of xenobiotic pollutants. Biotechnology Advances.25: 442-451.

Flores, H., Hoy, M., Pickard, J., 1987. Secondary metabolites root cultures. Trends in Biotechnology. 5: 64-65.

Giri, A., Narasu, M., 2000. Transgenic hairy roots: recent trends and applications. Biotechnology Advances 18: 1-22.

Jenifer, U., Francina Cecilia, K., Ravindhran, R., 2012. *In vitro* adventitious root and hairy root cultures in *Boerhaavia diffusa L.* International Journal of Current Research. 4(1): 65-67.

Mariya-John, K., Sarvottam, D., Mandal, A., Ram, K., Raj, K., 2009. *Agrobacterium rhizogenes*-mediated hairy root production in tea leaves [*Camellia sinensis* (L.) O. Kuntze]. Indian Journal of Biotechnology. 8: 430-434.

Murshige, T., Skooge, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiology Plant. 15: 473-497.

Murthy, H., Dijkstra, C., Anthony, P., White, D., Davey, M., Power, J., Hahn, E., Paek, K., 2008. Establishment of *Withania somnifera* Hairy Root Cultures for the Production of Withanolide A. Journal of Integrative Plant Biology. 50: 975-981.

Pawar, P., Maheshwari, V., 2004. *Agrobacterium rhizogenes*-mediated hairy root induction in two medicinally important members of family *Solanaceae*. Indian Journal of Biotechnology. 3: 414-417.

Ratti, N., Verma, H., 2004. Transformation of *Boerhaavia diffusa* leaf by *Agrobacterium rhizogenes*. Journal of Mycology and Plant Pathology. 34: 136-138.

Rhodes, M., Payne, J., Hamill, J., Robins, R., 1987. Production of hyoscyamine by hairy root cultures of *Datura stramonium.* Planta Medica. 53: 474-478.

Shanks, J., Morgan, J., 1999. Plant 'hairy root' culture. Current Opinion Biotechnology. 10: 151-155.

Suza, W., Harris, R.S., Lorence, A., 2008. Hairy roots: from high-value metabolite production to phytoremediation. Electronic Journal of Integrative Biosciences. 3: 57-65.

Wu, X., Shi, H., Tsang, P., Keung, E., 2008. Induction and in vitro culture of hairy roots of *Solanum nigrum* L. var. pauciflorum Liou and its solasodine production. Journal of Molecular Cell Biology. 41: 183-191.