HAIRY ROOT PRODUCTION OF TRANSGENIC PLUMBAGO ZEYLANICA L. PLANTS WITH AGROBACTERIUM RHIZOGENES UNDER IN-VITRO CONDITIONS

ANAND S. P1, NANDAGOPALAN V2, DOSS A3 & JEYACHANDRAN R4

1,2,3PG & Research Department of Botany, National College (Autonomous & CPE), Tiruchirappalli, Tamil Nadu, India
4Department of Botany, St. Joseph’s College (Autonomous), Tiruchirappalli, Tamil Nadu, India

ABSTRACT

Plumbago zeylanica L. is an important medicinal plant and often used for treating the leprosy, diarrhoea and skin diseases. The in vitro studies on P. zeylanica showed high frequency of micro shoots production from the explants supplemented with various concentrations of BAP (6-Benzylaminopurine). A maximum number of shoots were obtained from shoot tip explants in the medium containing 0.5 mg/l BAP. The regenerated shoots were further elongated on MS medium supplemented with MS and BAP (1.5 mg/l). The factors affecting Agrobacterium rhizogenes mediated transformation on formation of hairy roots of P.zeylanica have also investigated in this study. The shoots were subjected to root induction medium and calculated their 20% of the inoculated shoots cultured on MS and NAA (2.0 mg/l) medium developed maximum number of roots (5-7) per shoot.

KEYWORDS : Hairy Roots, Agrobacterium rhizogens, MS Medium, Plumbago zeylanica, NAA

ABBREVIATIONS

BAP: 6-Benzylaminopurine; MS: Murashige and Skoog medium ; NAA: Naphthene Acetic acid; IAA : Indole Acetic acid

INTRODUCTION

Mass propagation of plant species through in vitro culture is one of the best and most successful examples of commercial application of plant tissue culture technology (Anand et al., 2011). Plumbago zeylanica L. commonly known as white chitrak (family: Plumbaginaceae) is a perennial herb that is grown in most parts of India and is used in the traditional system of Indian medicine against a number of ailments including skin diseases, diarrhea and leprosy (Kritikar and Basu, 1993). The pharmacological studies carried out by several workers indicate that Plumbago zeylanica L. possesses antibacterial, antifungal, anticarcinogenic (Krishnaswamy et al., 1980) and radio modifying properties (Bopaiah and Pradhan, 2001). It is also reported to have antitumor activity (Kavimani et al., 1996). The roots of this plant have been reported to be a powerful poison when given orally causes abortion (Azad Choudhary et al., 1982). A number of naphthoquinones, flavinoids, anthocyanins and beta sitosterol have been reported previously from this plant source (Dinda et al., 1995).

Metabolic engineering of this plant by genetic transformation may lead to enhancement of the production of specific secondary metabolites at the whole plants level (Choi et al., 2004). During the last two decades a significant increase in the number of reports on the successful application Agrobacterium rhizogenes-mediated genetic transformation of various plant species, variants and cultivars (Mohsen Zargar et al., 2010) that have been utilized for the production secondary compound in lab condition. Hairy roots are genetically and biochemically stable, have a rapid growth rate, and
synthesize natural compounds at levels comparable to intact plants (Srivastava and Srivastava, 2007). In this study, production and growth hairy roots of transgenic callus with *A. rhizogenes* was investigated.

**MATERIALS AND METHODS**

**Plant Material**

*Plumbago zeylanica* plants were obtained from the bank of river of Cauvery in Tiruchirappalli, Tamilnadu and the specimen sample was confirmed by Botanical Survey of India (Southern Circle), Coimbatore, Tamilnadu, India.

**Explants and Surface Sterilization**

Shoot tips were collected from the field grown five-day-old plants of *P. zeylanica* and washed repeatedly with distilled water and finally treated with HgCl$_2$ (0.1%) for 4 min in a laminar flow cabinet and washed three times with autoclaved distilled water to remove any trace of HgCl$_2$. After surface sterilization, shoot tips were excised at the base and divided into pieces as explants of size 25 - 30 mm.

**Culture Medium and Conditions for Plant Regeneration**

Under a laminar flow, cabinet explants were inoculated aseptically on MS (Murashige and Skoog, 1962) medium supplemented with various concentrations of Indole Acetic Acid (IAA) and Cytokinins [6-benzylaminopurine (BAP) and Kinetin]. All media were adjusted to pH 5.8, and 0.8% agar and 30 g/l-1 sucrose were added. About 15 ml of the medium were dispensed in each culture bottle and sealed with plastic cover before autoclaving at 121 ºC for 15 min under pressure of 15 Psi. The media were left to cool as slant in the culture room until use. All cultures were maintained at 16 hr light of 1000 lux using fluorescent lamps at 25 ± 2 ºC. Results were observed at regular intervals and data were collected from three independent experiments and presented as average ± standard error (SE).

**Transfer of the Explants**

Maximum precautions were taken during the time of transfer of the explants. The hands were thoroughly washed with detergents and wiped with spirit. The surface sterilized explants were cut into required size, and inoculated on pre-sterilized media in the presence of a spirit lamp. Non-absorbent cotton plug wrapped with white open-wove bandage cloth was used to plug the culture tubes.

**Induction and Establishment of Hairy Roots**

*A. rhizogenes* ATCC 15834 (that were cultured into LB agar medium) was used for inoculation with callus explants. The bacteria for growth and multiplication were cultured into LB agar medium. The suspension of the Agrobacterium strain was diluted with a liquid MS medium to obtain 1.0 OD (600 nm) concentrations (5x $10^8$ cells/ml). For co-cultivation the infected explants were transferred to hormone-free MS basal medium under light intensity, 16/8 hr photoperiod at 22 ± 2 ºC and 80% relative humidity. After 48 h of inoculation, explants were rinsed three times with liquid MS basal medium supplemented with 0.5 mg/l BAP, 300 mg/l cefatoxime and 50 mg/l Kanamycin. The regenerated shoots were transferred to MS medium fortified with 0.2 mg/l NAA, 300 mg/l cefatoxime and 50 mg/l Kanamycin for produce hairy roots.

**RESULTS AND DISCUSSIONS**

Tissue culture techniques are being increasingly exploited for clonal multiplication and *in-vitro* conservation of valuable indigenous germplasm especially threatened with extinction conditions. The increasing demand for plant based drugs leads to the rapid causes of valuable plants from their primary habitats. The *in vitro* studies on medicinal plants have
been used as the alternative source for conservation of rare species and also for the production of chemical compounds. The shoot tip of *P. zeylanica* were isolated aseptically and cultured on MS medium supplemented with BAP for initiating vegetative growth and inducing maximum number of plantlets. The shoot tip explants were inoculated on MS medium containing different concentrations of BAP for the production of multiple shoots (Table 1 & Fig 1&2). As a supplement of 0.5 mg/l BAP resulted in maximum proliferation was observed in shoot tip explants. The shoot tip explants produced the maximum number of shoots per culture with a mean length of 9.33 ± 0.47 cm.

These explants were capable of directly developing multiple shoots on MS medium containing different concentrations of MS and BAP. From the results, it is clear that a combination of MS and BAP (1.5 mg/l) at lower concentration was suitable for shoot elongation. In contrast to the above mentioned results, some researchers observed that the combination of BAP and IAA on MS medium favoured multiple shoot buds in *Capsicum annuum* (Sobhakumari and Lalithakumari, 2003) and *Acalypha wilkesiana* (Sharma *et al*., 2007). Combination of cytokinins also favoured multiple shoot proliferation in *Ocimum sanctum* (Girija *et al*., 2006) and *Amygdalus communis* (Akbas *et al*., 2009). These explants were capable of directly developing multiple shoots on MS medium containing different concentrations of BAP and IAA.

The establishment of transformed hairy root cultures offers significant scope for the higher yield of the compounds and production of high-volume plant derived compounds. The hairy roots so formed in-vitro are also morphogenetically active in-vitro in certain species and successful field establishment of hairy root-derived plants offers opportunity for plant improvement. Hairy roots represent a valuable source of phytochemicals useful as pharmaceuticals, cosmetics, and food additives (Srivastava and Srivastava, 2007).

Many medicinal plants have been transformed successfully by *A. rhizogenes* and the hairy roots induced show a relatively high productivity of secondary metabolites, which are potentially important pharmaceutical products. Sevon *et al*., (2002) as summarized the most important alkaloids produced by hairy roots, including *Atropa belladonna* L., *Catharanthus tricophyllus* L., and *Datura candida* L.

The bacterial inoculated shoots cultured on the MS based medium root development were observed in minimum percentage of these shoots around the 30th day but the roots were mostly unbranched. Emergence of the hairy root was rarely observed. In contrast, 20% of the inoculated shoots cultured on MS and NAA (2.0 mg/l) medium developed maximum number of roots (5-7) per shoot, 14 days after inoculation (Table 2 & Fig. 3).

The data indicates that the presence of NAA in the MS medium helps in better emergence and growth of the inoculated roots. The survival of the inoculated-rooted plantlets was higher compared to that of the control-rooted plantlets. These results establish the fact that efficient rooting can be achieved for production of hybrid *Plumbago zeylanica* plantlets through transformation with *A. rhizogenes*. Production of roots of a high frequency has been attributed to the presence of T-DNA genes (Cardarelli *et al*., 1987a; Cardarelli *et al*., 1987b).

*A. rhizogenes* to plant, because after trans-fragment T (TDNA) from plasmid of *Agrobacterium* to gene plant that plants derived from hairy roots retained the Ri Ti-DNA (Choi *et al*., 2004) and it makes phyto-hormones (auxin and cytokinin) itself and it does not need hormone, again.

Genetically engineered root cultures have been used as a model system to study various aspects of the metabolic and molecular regulation of several natural product pathways. The present study established that an efficient *Agrobacterium rhizogenes* mediated transformation protocol for the establishment of *Plumbago zeylanica* hairy root cultures, and a valuable alternative approach for the production of secondary metabolites from medicinal plants.
REFERENCES


Table 1: Multiple Shoot Induction through Shoot Tip Explants of Plumbago zeylanica L

<table>
<thead>
<tr>
<th>Explants</th>
<th>Hormone Concentration BAP (mg/l)</th>
<th>Number of Shoot/Explants Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot tip</td>
<td>0.1</td>
<td>3.67 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>8.67 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>9.33 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>7.00 ± 0.82</td>
</tr>
</tbody>
</table>

Table 2: Growth and Survival of Rooted Hybrid Plumbago zeylanica Plantlets after Incubation with Agrobacterium rhizogenes

<table>
<thead>
<tr>
<th>Shoots for Rooting</th>
<th>Culture Medium</th>
<th>Type of Roots</th>
<th>% of Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>MS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inoculated</td>
<td>MS</td>
<td>Simple and Unbranched roots</td>
<td>33</td>
</tr>
<tr>
<td>Control</td>
<td>MS + NAA</td>
<td>Simple and Unbranched roots</td>
<td>64</td>
</tr>
<tr>
<td>Inoculated</td>
<td>MS + NAA</td>
<td>Hairy roots</td>
<td>80</td>
</tr>
</tbody>
</table>

Figure 1: Micropropagation through Shoot Tip Explant of Plumbago zeylanica

- a - Multiple shoot initiation from shoot tip region with MS + BAP 0.5 mg/l.
- b - Shoot elongation on MS + BAP 1.5 mg/l.
- c - Root formation on MS + NAA 2.0 mg/l.
- d - Hardened plantlet
Figure 2: Micropropagation through Nodal Explant of *Plumbago zeylanica*

- a - Multiple shoot initiation from nodal portion with MS + BAP 0.3 mg/l + 0.2 mg/l.
- b – Node derived shoot elongation on MS + BAP 1.5 mg/l.
- c - Root formation on MS + NAA 2.0 mg/l.
- d - Hardened plantlet

Figure 3: Hairy Root Induction in *Plumbago zeylanica* through *Agrobacterium rhizogenes*

- a – Simple root formation from shoot tip region with MS + NAA 2.0 mg/l
- b – Hairy root formation from shoot tip region with MS + NAA 2.0 mg/l
- c - Simple root formation from nodal region with MS + NAA 2.0 mg/l
- d - Hairy root formation from nodal region with MS + NAA 2.0 mg/l