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IN VITRO REGENERATION OF AN IMPORTANT MEDICINAL PLANT *PLUMBAGO ZEYLANICA* L. FROM NODAL EXPLANTS

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ABSTRACT
The present study attempted to develop an efficient *in vitro* micropropagation protocol for *Plumbago zeylanica* L. using nodal explants in different concentrations and combination of plant growth regulators in order to produce multiple shoots. The results showed that the nodal segments cultured on Murashige and Skoog medium (MS) basal medium fortified with 2.0 mg/L 6-benzylaminopurine (BAP) + 0.5mg/L α-Napthalne acetic acid (NAA) facilitated favorable shoot induction. The well developed shoots were transferred on rooting medium (MS) supplemented with various concentration of Indole 3-butyric acid (IBA) and Indole 3-acetic acid (IAA). The medium supplemented with 1.0 mg/L of IAA, showed efficient root induction and further development of healthy roots. The well rooted plantlets were acclimatized and successfully established in field with 95% of survival.

KEY WORDS: *Plumbago zeylanica*, medicinal plant, micropropagation, nodal explants.

INTRODUCTION

Medicinal plants are the source of important therapeutic aid for alleviating human ailments (Dev 1997). Medicinal plants are richest source of drugs for traditional medicines, neutraceuticals, food supplement, folk medicines, pharmaceutical intermediates etc (Hammer *et al.,* 1999). *Plumbago zeylanica* L. is one of the most important medicinal plants, which are good source of traditional and synthetic medicines containing various types of organic compounds having therapeutic properties. This is a rare medicinal herb or under shrub commonly called as Ceylon leadwort and doctor bush which belongs to Plumbaginaceae family. Its root and milky juice of whole plant are used for medicinal purpose by tribal peoples as it contains two important alkaloids namely, napthoquinone and plumbagin (Ghani, 1998). Extract of the root of this plant is given internally or applied to the ostium uteri, causes abortion (Prema Kumari *et al.,* 1977; Bharghava, 1984). *P. zeylanica* has the antioxidant activity and phytochemical contents in *in vitro* grown callus and *in vivo* grown plants (Satyajit Kanungo *et al.,* 2012). The roots contain an alkaloid called plumbagin, a natural napthoquinone (5-hydroxy-2-methyl-1, 4-napthoquinone), possessing various pharmacological activities such as antimalarial, antioxidant activity (Didry *et al.,* 1994; Nahak *et al.,* 2011). The roots are used extensively in china and other Asian countries for the treatment of cancer, rheumatoid arthritis, dysmenorrheal and contusion of extremities (Atta-Ur-Rahman, 1988). Plant tissue culture techniques are being applied for germplasm conservation, mass propagation and disease free plant production of medicinal plants (Bajaj, 1995). Micropropagation has been used to conserve the germplasms of many rare and endangered species of medicinal plants (Mikulik, 1999). The present study is aimed to develop an appropriate and efficient micropropagation protocol from nodal explants of *P. zeylanica* for the large scale production of plants. The protocol reported here could be used for the conservation and production of disease free plants.

MATERIALS & METHODS

Collection of explants

The healthy mother plants of *Plumbago zeylanica* L. were collected from herbal nursery maintained by Forest department of Tamil Nadu at Pollachi, Coimbatore. The mother plants were authenticated by taxonomist Dr. S. Saravanan in the Department of Botany, Pachaiyappa's College. The collected mother plants were grown and maintained in green house of Pachaiyappa's College, Chennai. The nodal segments of the one year grown disease free plants were collected and used as explants.

Surface sterilization

The nodal explants excised from the mother plant (1.5-2.0 cm length) were washed with running tap water to remove the dust particles available on the surface of the explants. Then the explants were immersed in soap solution for 30 min and washed thoroughly with tap water and brought it to aseptic condition under laminar air flow chamber. Then the explants were finally treat with 0.1% of $HgCl_2$ (w/v) for five minutes and rinse thoroughly 3 to 4 times with sterile water to remove the traces of chemicals. Under the aseptic condition the nodal segment explants were resized to 1 to 1.5cm long and inoculated on to the culture medium. All the media used were autoclaved for 20 minutes at 121°C and 15 psi pressure.

Culture media

All the media used in this study were based on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 3% of sucrose and fortified with different concentration and combination of growth regulators such as 6-benzyl amino purine (BAP) 0.5-2.0

mg/L, α-Napthalne acetic acid (NAA) 0.5 mg/L , Indole 3-butyric acid (IBA) 0.2-1.00 mg/L and Indole 3-acetic acid (IAA) 0.2-1.00 mg/L to study their response on multiplication of shoots and rooting. All the chemicals used were of analytical grade. The pH of the media was adjusted by using 0.1N NaOH or 0.1N HCl to 5.8 after the addition of growth regulators.

Culture conditions

All the cultures were incubated under the temperature of 25 $\pm 2^0$C and the light intensity of 2000-4000 Lux. The cultures were maintained with the photoperiod regime of 16 hr light and 8 hr dark and the relative humidity was maintained between 50-60%.

Multiple shoot initiation

Nodal explants were inoculated vertically in each culture bottles containing MS medium supplemented with different combinations of plant growth regulators BAP (0.5-2.0 mg/L) along with NAA (0.5 mg/L) was used to study their effect on multiple shoot induction. MS medium without any growth regulators served as control.

Sub culturing

After thirty days of inoculation, the multiple shoots initiated were separated and sub cultured on fresh medium for further multiplication. The number and the length of shoots were recorded in 30 days old culture.

Rooting and hardening

Thirty days old well elongated shoots were transferred to the rooting media. MS medium supplemented with different combinations of plant growth regulators IBA (0.2-1.0 mg/L) along with IAA (0.2-1.0 mg/L) was used to study their effect on *in vitro* rooting. During this process, number and the length of roots were recorded to assess the best combination treatment. The well developed plants with roots were taken out and were washed thoroughly with running tap water to remove the traces of agar. The healthy plants were transferred to polythene containing the primary hardening mixtures of sand and vermiculite (1:1) under the lab conditions for 15 days. Then all the plants were transferred to large bags containing garden soil: compost in the ratio of 1:1 to achieve maximum survival rate and kept in green house conditions. It was periodically recorded to check the surviving rate.

Statistical Analysis

The collected data were analyzed by analysis of variance (ANOVA) followed by Tuky's HSD test values to compare the significant differences among means at 5 % level of significance. All the data were expressed in the Mean ±Standard Deviation.

RESULTS

The axillary buds of *P. zeylanica* were cultured on the MS basal medium fortified with different concentrations of BAP (0.5-2.0 mg/L) alone and also in combination with NAA (0.5mg/L). Multiplication of shoots occurred in all concentration and combinations of growth regulators, but the number and the length of shoots per explants varied.

TABLE 1: Effect of growth regulators on shoot induction and multiplication

Growth regulators		Number of shoots	Length of shoots(cm)
BAP (mg/L)	NAA (mg/L)	Mean ±SD	Mean ±SD
0.00	0.00	02.70 ± 0.67^{a}	2.65 ± 0.78^{a}
0.50	0.00	03.30 ± 0.94^{ab}	4.06 ± 1.13^{b}
0.50	0.50	05.40 ± 1.26^{bc}	5.79 ± 0.91^{de}
1.00	0.00	05.10 ± 0.87^{bc}	3.93 ± 0.84^{b}
1.00	0.50	08.00 ± 1.76^{d}	5.38 ± 0.78^{cd}
1.50	0.00	06.90 ± 1.37^{cd}	4.28 ± 0.83^{bc}
1.50	0.50	13.30 ± 2.45^{e}	6.35 ± 0.88^{de}
2.00	0.00	11.50 ± 1.64^{e}	4.58 ± 0.73^{bc}
2.00	0.50	15.60 ± 3.59^{f}	6.73 ± 1.19^{e}

The values represent the Mean±SD of ten replicates and all experiments were repeated thrice, mean difference of significant is at the 0.05 level. Means with different letter within column are significantly different from each other at $P \leq 0.05$.

FIGURE 1: *In vitro* regeneration of *Plumbago zeylanica*. **a)** Shoot initiation from nodal explants **b)** Multiple shoot induction **c)** Multiple shoots with roots **d)** Secondary hardening stage **e)** Hardened healthy plants for planting.

The explants were cultured on MS medium without growth regulators (control) showed less shoot induction with mean number of shoots 2.7 ±0.67 with mean shoot length (2.65 ±0.78). Among the various media combinations, MS medium fortified with combination of BAP of 2.0 mg/L and NAA of 0.5mg/L is responded very effectively for the successful induction and the multiplication of shoots, with the maximum of 15.60 ±3.59 shoots per explants and with mean length of 6.73 ±1.19 (Table 1; Fig. 1a and 1b). In another set of experiment, the MS medium supplemented with BAP of 0.5, 1.0 mg/L alone and in combination with NAA of 0.5mg/L, showed poor induction of shoots per explant. MS medium fortified with BAP of 2.0 mg/L individually and in combination with NAA of 0.5mg/L produced fur like shoots, which are not be suitable for next phase of multiplication. Hence, these combinations were not appropriate towards the production of quality shoots.

The well developed shoots were placed on the medium supplemented with different concentrations of IBA and IAA for root induction (Table 2). Root induction was noticed in all the concentrations, but with varied response with reference to the number and length of the roots per shoot let. Among the various concentrations of IAA, favorable root induction was noticed on MS supplemented with IAA of 1.0mg/L in terms of average number of roots (8.60 ±1.34) with mean root length of 6.75 ±1.06 cm per shoot. In the case of IBA, out of five different concentrations of IBA, the optimal root induction was observed on MS with 1.0 mg/L of IAA. This media showed the maximum average number of roots (6.00 ±1.15) with mean length of 5.30 ±0.88 cm per shoot (Fig. 1c). After 30 days *in vitro* developed shoots were harvested and washed with running tap water and transferred to green house for primary hardening process (Fig. 1c). After 50 days, the primary hardened plants were transferred to polybag for secondary hardening (Fig. 1d and 1e) and 95% of the survival rate was noticed in the end of the secondary hardening phase.

TABLE 2: Effect of growth regulators on root induction

Growth regulators		Number of roots Mean ±SD	Length of roots (cm) Mean ±SD
IBA (mg/L)	IAA (mg/L)		
0.20	0.00	2.00±0.81^{a}	2.75±0.47^{a}
0.40	0.00	3.10±0.73^{a}	3.72±0.47^{b}
0.60	0.00	4.60±0.96^{bc}	4.25±0.79^{bcd}
0.80	0.00	2.60±0.84^{a}	4.14±0.98^{bc}
1.00	0.00	6.00±1.15^{d}	5.30±0.88^{d}
0.00	0.20	2.70±0.94^{a}	5.09±0.86^{cd}
0.00	0.40	4.30±1.05^{b}	5.10±0.69^{cd}
0.00	0.60	5.20±0.91^{bcd}	5.33±0.49^{d}
0.00	0.80	5.70±1.25^{cd}	6.44±1.10^{e}
0.00	1.00	8.60±1.34^{e}	6.75±1.06^{e}

The values represent the Mean ±SD of ten replicates and all experiments were repeated thrice, mean difference of significant is at the 0.05 level. Means with different letter within column are significantly different from each other at $P \leq 0.05$.

DISCUSSION

Plant tissue culture plays an important role in the conservation of medicinal plants through rapid multiplication and rejuvenation of the endangered species (Tiwari Bhrigu Narayan *et al*., 2010). Ajithkumar *et al*., (1998) reported that the nodal segment was the best explants for the initiation of shoots. The reason for the suitability of nodal segment is attributed due to the presence of protected axillary buds, which are not damaged during surface sterilization. In the present study, all media produced shoot but number and the length of shoots varied. The quantity of shoots was comparatively more on the MS medium supplemented with the combination of BAP and NAA than that of in MS medium with BAP alone. Morphogenic responses vary with different concentration and combination of plant growth regulators (Bejoy *et al.,* 2006). *In vitro* propagation of plant species through tissue culture technique has been achieved by using the appropriate concentration and combinations of plant growth hormones (Murashige 1990; Uranbey *et al.,* 2005).

In the present study, the MS medium fortified with BAP-2.0 mg/L + NAA-0.5mg/l was found to suitable one for the successful induction and the multiplication of *P. zeylanica* shoots, with the maximum of 15.60 ±3.59 shoots per explants and with mean length of 6.73 ±1.19 cm. The present study findings varied from the earlier reports (Antony Ceasar *et al.,* 2013; Sivanesan and Jeong 2009). Dohare *et al.* (2012) reported that the maximum shoots with an average shoot length of 7.00 cm was obtained on the MS medium supplemented with 1.0 mg/L of BAP and 1.0 mg/L of NAA. In this study, different concentrations of IBA and IAA were tried among these two auxins IAA showed an efficient root induction. The MS medium fortified with 1.0 mg/L of IAA was found to be the effective one for successful root induction. This result coincides with the earlier reports of Dohare *et al.* (2012); Sivanesan and Jeong (2009); Antony Ceasar *et al.* (2013).

CONCLUSION

In vitro micropropagation is an advanced technique which offers a large number of genetically uniform and disease free plants in limited duration and space and also helps in multiplication of plant species, which have restrictions of conventional breeding. *In vitro* propagation technique would be suitable methodology for direct regeneration of shoot lets as a source of disease free quality planting material, which could be stored and transported easily.

The protocol developed in the present investigation will help in the mass production of *P. zeylanica* in commercial level.

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