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Full Research Article

Simple Protocol for Micropropagation and *in Vitro* Conservation of *Plumbago zeylanica* L: An Important Indigenous Medicinal Plant

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Abstract

A simple protocol was developed for *in vitro* clonal propagation by multiple shoot induction of *Plumbago zeylanica* L., a plant having high medicinal values. Root of *P. zeylanica* is widely used as a traditional medicine. The plant is very popular to different pharmaceutical industry and is mainly harvested from the natural habits. Population growth, urbanization and the unrestricted collection of these plants from the wild population is resulting in an over-exploitation of natural habits. To cope up this alarming situation and to ensure its sustainability, plant tissue culture technique was applied. Micropropagation methodology is a potent alternative approach which has opened extensive areas of research for biodiversity conservation. High frequencies of multiple shoot regeneration (10 to 12 shoots explant^{-1}) were achieved from *P. zeylanica* from nodal explant on to MS medium fortified with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. MS medium along with 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} GA_3 showed the highest shoot elongation. The elongated shoots were subcultured for rooting on MS medium supplemented with 1.0 mg l^{-1} IBA. The survival rate of 95% *in vitro* raised plantlets were acclimatized in poly greenhouse and successfully transplanted to natural condition. *In vitro* conservation was also evaluated by supplementing various osmotic agents. For *in vitro* mid-term conservation, the complete plantlets were maintained healthy condition upto 10 months at 10^{0}C without any subculture by using MS medium in combination with 2% manitol and 2% sorbitol. All regenerated plants are cytologically stable (2n=24), their chromosomal status same as source plant.

1. Introduction

Plumbago zeylanica L., (family: Plumbaginaceae) is an important indigeneous medicinal plant indigenous medicinal plant, distributed throughout most of the tropics and subtropics and throughout India, including West Bengal, Bihar, Maharashtra and various parts of Southern India; growing in deciduous woodland, savannas' and scrublands from sea level up to 2000 m altitude. (Vijver and Lotter 1971; Pant et al., 2012). It is an erect, semi-climbing sub-herb widely used as a complementary and alternative medicine around the world, including India, Bangladesh, Sri Lanka and Australia (Chopra et al., 1956). It is used in the traditional system of Indian medicine against a number of ailments including skin disease, abdomen enlargement, anemia, diabetes, leprosy, dyspepsia, elephantiasis and diarrhea (Kirtikar and Basu, 1993). The root and root bark of this plant are bitter, stomachic carminative, astringent to bowels, anthelmintic, piles bronchitis, itching, disease of lever, consumption, ascetics (Pawar et al., 2010). Leaves are caustic, vesicant aphrodisiac and good for scabies (Handa, 1999). Plant contains number of napthaquinone derivatives viz., plumbagin, 3-chloroplumbagin, 3,3'-biplumbagin, elliptinone, chitranone, zeylione, isozeylione, droserone, plumbagic acid, plumbazeylanone, napthelenone and isishinanolone (Handa, 1999). Fructose, glucose, invertase and protease were isolated from the bark. 3, 3'-biplumbagin, chitranone (binapthaqunione), droserone, elliptinone, isozeylinone, catechol tannin, amino acids, plumbagic acid, vanillic acid were also isolated from the plant (Phondke, 1992). The compound is plumbagin, a yellow crystalline bioactive phytoconstituent present in the roots of this plant (Bothiraja et al., 2011) and it shows as anticancer and antitumor activity (Handa, 1999). The pharmacological studies carried out by several workers indicate that *P. zeylanica* possesses astringent, diuretic, antibacterial, antifungal, anticarcinogenic, antitumor and radio modifying properties (Sing et al., 2011; Gupta et al.,

2011). Due to the medicinal value, these plants are collected and used as raw material for large scale medicinal industry, leading to over exploitation and it becomes an endangered plant species (Dohare et al., 2012). Normal propagation of this plant species through seed is difficult due to their very poor seed setting and germination rate and vegetative propagation through small cuttings are also not adequate for mass plantation. Propagation by seeds does not even maintain homogeneous population, resulting in great variability in its important composition. Under such circumstances plant tissue culture technique has been considered as the sole mean of multiplication for mass cultivation programme. Conventional propagation cannot meet the increasing demand and will ultimately lead to extinction if no attention is given to its conservation and propagation (Amoo et al., 2009). Tissue culture techniques offer a viable tool for mass multiplication and germplasm conservation of elite medicinal plants while at the same time facilitating pharmaceutical and other commercial needs (Sahoo and Chand, 1998; Anis and Faisal, 2005). Plant tissue culture techniques have been successfully used for rapid multiplication and for obtaining useful variants in several medicinal species (Tisserat and Murashige, 1977). Genetic improvement is another approach to augment the drugyielding capacity of the plant (Tejavathi and Shailaja, 1999).

India is one of the twelve mega diversity countries of the world with a rich diversity of biotic resource due to its varied climate, altitudinal variations and ecological habits (Bapat et al., 2008). The use of medicinal plants by people in developing countries is popular because these products are safe, widely available at low cost and easy to access. The World Health Organisation estimates that some 80% of the developing world relies on traditional medicines and that, of these, 85% use plants or their extracts as the active substance. This means that close to 3 billion people rely on plants for medicine (WHO, 2002). Medicinal plants provide meaningful inputs for drugs. Their loss through extinction could lead to considerable loss to the society (Kumar, 2004). The demand for medicinal plant-based raw materials is growing at the rate of 15 to 25% annually, and according to an estimate of WHO, the demand for medicinal plants is likely to increase more than US $5 trillion in 2050. In India, the medicinal plant related trade is estimated to be approximately US $1 billion per year (Joshi et al., 2004). It is expected that India's aim to build a golden triangle between traditional medicine, modern medicine, and modern science will be a boon for developing the traditional herbal medicine and the medicinal plants sector (Ahuja, 2001; Mashelkar, 2005).The objective of the present study based on to develop a simple efficient protocol via node culture for large scale uniform plant production of elite genotype of *P. zeylanica* L. and with the analysis of chromosome constitution of regenerated plants.

2. Materials and Methods

2.1. Plant material

Plumbago zeylanica L. were collected from various locations in West Bengal, India, were grown in the Field Gene Bank at RKMVC College campus under identical environmental conditions of the experimental garden of the RKMVC College, Rahara, Kolkata, W.B., India. In the present study, node explant were used which were collected from six months old disese free healthy plant.

2.2. Surface sterilization of explants

Collected explants from the mother plant were kept under running tap water for 20 minutes and then immersed in 2% bavistin solution (w/v, a systemic fungicide) for 20 minutes and dipped it on to the 5% Tween-20 (v/v, an antisepect liquid soap solution) for 10 minutes and washed with distilled water to make the explants free from detergents. Finally, surface sterilization done with 0.1% (w/v) mercuric chloride solution for 7 minutes and washed thoroughly with sterile distil water before inoculation on to sterilized nutrients agar media pre distributed in culture vessels. Disinfection was performed under aseptic conditions in a laminar airflow cabinet.

2.3. Culture media

Nodal explants were inoculated on to solid MS medium (Murashige and Skoog, 1962) containing 3% sucrose (w/v). The medium solidified with 0.8% agar (Merck, India). The medium enriched for multiplication and shoot proliferation with different concentrations of cytokinin viz., BAP (0.5, 1.0, 2.0, 3.0 and 5.0 mg l^{-1}); along with low concentration of auxin viz., NAA (0.2 mg l^{-1}) and for root initation different concentrations of auxins viz., IAA, IBA (0.5, 1.0, 2.0 mg l^{-1}). The pH of the medium was adjusted to 5.6±0.2 with 1(N) KOH and 1(N) HCl before gelling with 0.7% agar (w/v, Merck, India). Media were steam-sterilized at 121 temperature and 1.1 kg cm^2 pressure for 18 min.

2.4. Culture Conditions

The cultures were incubated at a temperature of 25±2°C, 55-60% relative humidity with a photoperiod of 16 hrs day^{-1} with photon flux density for about 45 μ mol $m^{-2}s^{-1}$ provided by white fluorescent light.

2.5. Multiple shoots induction and plantlet regeneration

Nodal segments (8-12 mm) excised from mother plant were inoculated vertically on to MS medium containing 3% (w/v) sucrose (w/v, Merck, India), 0.8% agar (Merck, India) supplemented with different concentration of BAP along with low concentration of NAA and replicates thrice of each experiment. The multiple shoots were sub-cultured at every

two weeks for 60 days. A control group was maintained (basal medium without hormones) to record the frequency of response.

2.6. Shoot elongation

After 35 days, multiple shoots induced were excised individually and subcultured on MS medium supplemented with BAP (1.0 mg l^{-1}) and varying concentrations of gibberellic acid (GA_3) (0.2, 0.4, 0.6, 0.8, and 1.0 mg l^{-1}) for elongation of shoots. A control group (basal medium without GA_3) was also maintained.

2.7. Induction of rooting

Micro shoots (1-3 cm) were excised from the culture and transferred to half-strength MS medium augmented with different concentrations auxins viz., IAA, IBA (0.5, 1.0 and 2.0 mg l^{-1}, each of three concentrations) and 2% sucrose (w/v, Merck, India) for root initiation. Micro-shoots were cultured in one tube each under the same culture conditions as described above. After 4 weeks the percentage of shoots forming roots, the number of roots per shoot and root length were assessed. One culture set was inoculated in basal MS medium without any plant growth regulator and considered as control.

2.8. In vitro conservation

For *in vitro* conservation, slow growth techniques were used. The effect of osmotic agents and temperature on the survival and re-growth of the *in vitro* cultures of *P. zeylanica*, MS media were supplemented with mannitol (1-3% w/v), sorbitol (1-3% w/v) with 3% sucrose (w/v) and 0.8% (w/v) agar. Shoot tips and nodes were dissected from aseptically grown cultures and inoculated onto the slow growing media in order to increase sub-cultural intervals. Cultures were maintained at 4°C, 10°C and 18°C into growth chambers under a 16 h photoperiod with fluorescent light.

2.9. Re-growth and establishment of plantlets

After conservation period, the plants were sub-culture on normal MS medium for their re-growth. Cultures were monitored during and after storage for survival and subsequently transferring on to the shoot multiplication medium (MS media with PGR) under culture room conditions at 25°C. The number of new buds and shoots induced on multiplication media was counted 30 days after transfer. Collected data reflect the rate of plant conservation from storage buds to proliferating buds, shoots and plantlets. Proliferated shoots were rooted on MS medium containing IBA.

2.10. Acclimatization and field experiments

For hardening, at first the tissue culture derived healthy rooted plantlets were placed at room temperature for 7 to 10 days. Then the plantlets were removed from the agar medium, washed thoroughly under running tap water and transferred to earthen pots containing sterilized soilrite (Keltech Energies Ltd, Bangalore, India). To preserve moisture, the potted plantlets were covered with transparent polythene bags and the pots were placed on a plastic tray containing water under diffused light (16 h, photoperiod) in the poly-house for 25-30 days. After 30 days, these plants were transferred to the earthen tub (8 inch diameter) containing the mixer of garden soil, sand and vermicompost (2:1:1) and placed in the green house. After one month, surviving plants were transferred to the field.

2.11. Cytological study

In connection with chromosomal status, fresh root tips of the source plant as well regenerated plants were excised and subsequently pre-treated in a saturated solution of *p*-dichlorobenzene for 6 hrs. For somatic chromosome counts and karyotypic analysis, fixed root tips were stained with 2.0% aceto-orcein: 1 (N) HCl (9:1, v/v) mixture followed by incubating for 2 h at room temperature. Chromosome plates were observed in Leica DM 750 microscope and photographed with Leica DFC 295 camera. Minimum of 5 metaphase plates from each root tip were analyzed to determine the somatic chromosome number at the metaphase stage.

2.12. Statistical analysis

All the experiments were conducted under controlled conditions with three replications. Means and standard errors were carried out for each experiment and data was analyzed using one way Analysis of Variance (ANOVA) to detect significant differences between means. Data analysis was performed using SPSS v 16.0 software.

3. Results and Discussion

3.1. High frequency shoots proliferation

It was observed that multiple shoot buds originated from nodal explants collected from physiologically healthy source plant (Figure 1A), when MS medium (Murashige and Skoog, 1962) was fortified with different concentrations (0.5, 1.0, 2.0, 3.0

Table 1: Effect of BAP and NAA on shoot proliferation from nodal explant of *Plumbago zeylanica* (Data taken after 28 days of culture)

PGR (mg l^{-1})		Explants response to	No. of shoot
BAP	NAA	shoot induction (%)	per explant
0.5	0.2	85	15.0 ± 0.50^{c}
1.0	0.2	90	18.67 ± 0.44^{b}
2.0	0.2	95	24.0 ± 0.47^{a}
3.0	0.2	60	$5.66\pm0.33f^{gh}$
5.0	0.2	00.00	00.00

(Each value represents the mean±SD of 10 replicates and each experiment was repeated thrice)

and 5.0 mg l^{-1}) of BAP along with the low concentration of auxin viz., NAA (0.2 mg l^{-1}). The nodal explants showed minor swelling prior to the emergence of shoot buds developing from the pre-existing material two weeks after inoculation. Initially two or three axillary shoot buds per explant emerged three weeks after inoculation and gradually the number of shoot buds per explant increased up to 10 to 12 (Table 1) on to MS medium was fortified with 2.0 mg l^{-1} BAP along with combination of 0.2 mg l NAA (Figure 1B). A low number of buds developed in the combination another three concentration BAP (0.5, 1.0, 3.0 mg l^{-1}) along with a combination of 0.2 mg l^{-1} NAA. But in the combination of the concentration of BAP (5.0 mg l^{-1}) along with combination of 0.2 mg l^{-1} NAA, a callus like structure was observed. The nodal explants placed on to MS basal medium lacking of any growth regulators (served as control) did not show any morphogenetic response and failed to produce shoots even after 6 week incubation. The similar results were also recorded in *Plumbago zeylanica* (Gbadamosi and Egunyomi, 2010); *Tylophora indica* (Faisal et al., 2005; Haque and Ghosh, 2013); *Asparagus cooperi* (Ghosh and Sen, 1994); *Mours alba* L. (Anis et al., 2003) by the using of auxin in combination with cytokinin.

3.2. Shoot elongation

Although a maximum of 8.50±0.29 shoots per explants was observed, it was necessary to establish a shoot elongation protocol. Excised shoots that were cultured in medium consisting of BAP (1.0 mg l^{-1}) and in the presence of GA_3 at different concentrations for 2 weeks was evaluated for shoot length (Table 2). The result suggest that small amounts of GA_3 (0.2 mg l^{-1}) in combination with 1.0 mg l^{-1} BAP were effective in stimulating *P. zeylanica* shoot elongation (Figure 1C). Shoots attained a maximum height of 10.27±0.41 cm during GA_3 treatments. Similar result for the effect of GA_3 was also found by Sujatha and Kumari, 2007 in *Artemisia vulgaris*. GA_3 stimulates elongation of shoots by inhibiting the action of action of auxins in meristematic regions (Taiz and Zeiger, 1998). In contrast, it was maintained the primary tissue with the continuous emergence of new shoot initials even after 6 months of sub-culturing.

Table 2: Effect of GA_3 on shoot elongation when cultured on MS medium supplemented with BAP (1 mg l^{-1}) [Data taken after 30 days of culture].

BAP (mg l^{-1})	GA_3 (mg l^{-1})	Shoot elongation response (%)	Mean Shoot (cm)
1.0	0.2	97	9.2 ± 1.5^{a}
1.0	0.4	88	8.9 ± 2.5^{bc}
1.0	0.6	82	7.1 ± 2.0^{c}
1.0	0.8	75	6.0 ± 1.5^{ab}
1.0	1.0	65	4.0 ± 1.9^{gh}

(Each value represents the mean±SD of 10 replicates and each experiment was repeated thrice)

3.3. Rooting of the proliferated shoots

Root development was induced in the *in vitro* proliferated shoots by culturing them on to the MS medium supplemented with 1.0 mg l^{-1} of IAA and 1.0 mg l^{-1} of IBA. Among two types of auxin used in the present experiment, IBA was found to be the most effective at different concentrations tested for producing roots on based of micro-shoots (Figure 1D). Among different concentrations of IBA, 1.0 mg l^{-1} was found to be best concentration auxin for proper rooting of *P. zeylanica* in which 96% shoots rooted within four weeks of the culture (Table 3). The similar results were also reported in *Kaempferia galanga* (Shirin et al., 2000) and *Withania somnifera* (Chatterjee and Ghosh, 2012).

3.4. In vitro conservation

The cultures maintained in good health conditions in controlled medium (MS medium with 3% sucrose, without osmotic agents) with 100% survival rate up to 4 months without subculture, but thereafter survival rate and gradually decrease and finally the control treatment did not help to survive after 6 months of conservation. But when cultures were maintained in manitol and sorbitol survived up to 10 months without subculture.

At low temperature regime (4°C), culture showed poor performance as the shoots degenerated after 45 days and culture did not survive as in case of control at 25°C. In moderate temperature regime (18°C) the cultures grew healthy with reduced growth in comparisons to control. This experiment suggests that culture growth could be reduced at 10°C (Table 4), but storage period could not be increased to maintain healthy cultures. This aspect needs further experimentation to prolong the sub-culture period. It also suggests that low temperature

Table 3: Effect of IAA and IBA on *in vitro* rooting from individual shoot of *Plumbago zeylanica* [Data taken after 28 days of culture].

Plant Growth regulators	Concentration (mg l^{-1})	Induction of roots from shoot (%)	Mean number of roots/ shoot
IAA	0.5	80	4.0 ± 0.56^{bcd}
	1.0	70	3.66 ± 0.30^{cd}
	2.0	60	3.56 ± 0.20^{cd}
IBA	0.5	90	5.66 ± 0.33^{ab}
	1.0	100	7.33 ± 0.35^{a}
	2.0	70	4.30 ± 0.33^{de}

(Each value represents the mean±SD of 10 replicates and each experiment was repeated thrice)

Figure A: Mother plants of *Plumbago zeylanica* L. maintained at the experimental garden of RKMVC College, Rahara

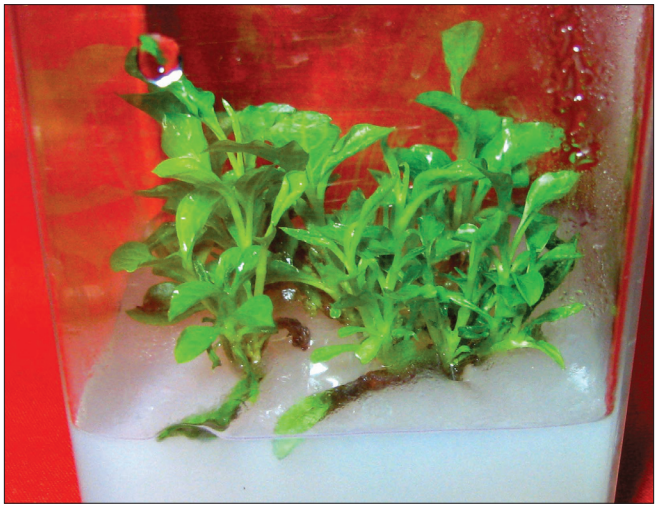

Figure B: *In vitro* multiplication

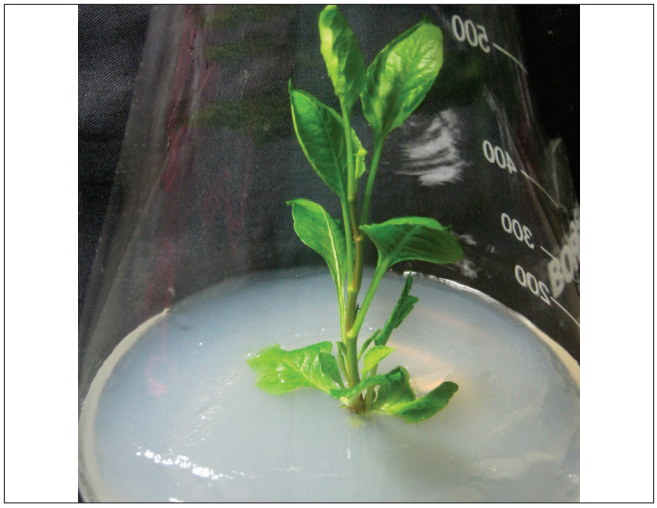

Figure C: *In vitro* elongated shootlet

Figure D: *In vitro* complete plants with roots

Figure E: Hardening of *in vitro* raised plants

Figure F: Hardening of *in vitro* raised plants

Figure G: Tissue culture raised plants

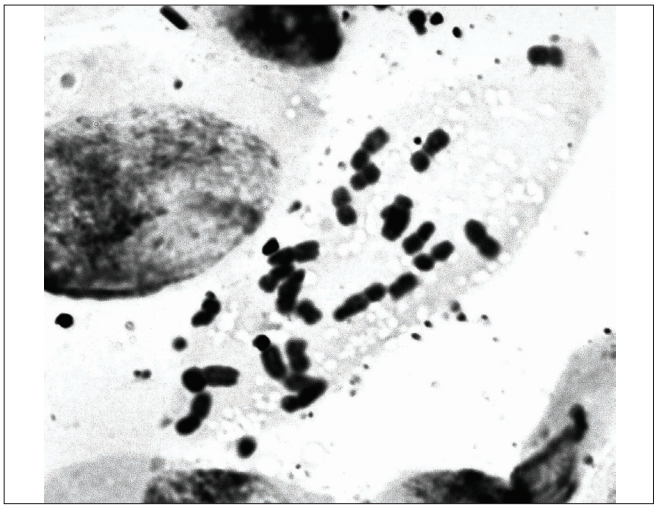

Figure H: Metaphase plate with 2n=24 chromosome number

(4°C) is not suitable for *P. zeylanica in vitro* storage. The growth suppression approach using osmotic agents was attempted in this study and proved to be very useful. The influences of various osmotic agents on this species showed different results. The growth of shoot cultured in medium supplemented with 3% sucrose was controlled for our experiment. The results showed that the addition of sorbitol (w/v) and mannitol (w/v) in MS media, at different concentrations, was more effective for *in vitro* storage of this important medicinal plant than the storage at low temperature (4°C). The addition of osmotic agents 2% sorbitol (w/v) and 2% mannitol (w/v), to each of the media has increased survival rate 90% (Table 5). A declination in survival rate and re-growth occurred when the cultures were stored also at higher concentrations of osmotic agents i.e., with 3% mannitol (w/v) and with 3% sorbitol (w/v) each and with a combination of 2% sorbitol (w/v) and 2% mannitol (w/v). Our results also showed that 18°C and 16 h photoperiod were better than 4°C during slow-growth storage condition. The shoots survived after slow-growth storage had longer shoot height than those not maintained in slow-growth condition. In case of 4°C temperature, the leaves of some plants were curled and withered.

3.5. Re-growth and establishment of plantlets

After 10 months, these shoots were transferred onto fresh MS medium supplemented with different concentration of BAP (0.5 to 5.0 mg l^{-1}), IAA (0.5 to 2.0 mg l^{-1}) and IBA (0.5 to 2.0 mg l^{-1}) for *in vitro* shoot multiplication and *in vitro* rooting and cultured for 6 weeks. Growth suppression had positively reduced the labor during culture maintenance in the tissue culture laboratory and also promoted uniformity of growth among the converted plantlets. No signs of shoot or root growth was noticed during the 10 months of storage. Adding sucrose to the media has prevented dehydration in storage but did not improve shelf-life of germplasm, while frequency of plantlet conservation was higher on shoot tips stored on 2% (w/v) sucrose/agar support with roots breaking during low temperature (4°C) storage condition. All the cultures in storage condition were able to form roots during re-growth and successfully acclimatized in soil rite. After low temperature storage, plantlets improved their survival during acclimatization and more vigorous in field plantings was observed. Similar reports have documented post-storage beneficial effect in apricot by Koubouris and Vasilakakis (2006), Lata et al. (2010), Kanchanapoom and Promsorn (2012), Chatterjee and Ghosh (2012). Our studies provided an effective protocol for storage of medicinal plants under slow growth conditions. Germplasm can be stored effectively for 10 month without subcultures, alleviating maintenance labor in the laboratory.

3.6. Acclimatization

Successful establishment of *in vitro* regenerated plantlets in field conditions requires great care. During this stage plants have to adapt to the new environment of greenhouse or field. The successfully rooted plantlets were transfer to a small earthen pot containing soilrite and covered with transferent polytheen bags for hardening (Figure 1E&F). Plantlets were maintained in the culture room (25±1°C) conditions initially 6-7 weeks and then transferred to normal laboratory in room temperature conditions and maintained for about 4-5 weeks. Finally the plantlets were transferred to Poly House and maintained 3-4 weeks and then it transferred to the experimental field condition (Figure 1G). There was 95% survival rate and the plantlets that were rooted in MS medium containing IBA (1.0 mg l^{-1}) where the plants showed healthy growth. There was no noticeable variation among the acclimatized plants with respect to morphological and growth characteristics compared

Table 4: Effect of temperature on growth and survival percentage of *in vitro* growing plants of *P. zeylanica*. (Intial plant length 2.0 cm)

Storage temperature	Re-growth after 6 months storage (Plant height in cm)	Survival (%)	Re-growth after 10 months storage (Plant height in cm)	Survival (%)
5^0C	1.9±0.5[b]	76	1.5±1.0 [c]	50
10^0C	13.3±1.0[a]	90	18.1±1.5 [a]	90
18^0C	4.3±0.4[c]	70	3.8±1.2 [c]	60

(Each value represents the mean±SD of 10 replicates and each experiment was repeated thrice)

Table 5: Effect of osmotic agents on growth and survival percentage of *in vitro* growing plants maintained at 25C conditions of *P. zeylanica.*[Intial plant length 2.0 cm)

Storage conditions / Osmotic agents	Re-growth after 6 months storage (Plant height in cm)	Survival (%)	Re-growth after 10 months storage (Plant height in cm)	Survival (%)
1% Sorbitol	3.7±0.3[bc]	35	3.0±1.5[c]	60
2% Sorbitol	6.5±2.3[b]	70	6.1±0.4[c]	70
3% Sorbitol	3.4±0.3[c]	37	3.6±1.0[bc]	50
1% Mannitol	2.9±0.5[c]	35	2.5±0.5[b]	45
2% Mannitol	5.1±0.4[bc]	67	5.8±0.3[c]	65
3% Mannitol	3.1±0.5[c]	40	2.3±0.4[b]	40
2% Sorbitol+ 2% Mannitol	7.5±1.2[a]	90	8.7±1.3[a]	90

(Each value represents the mean±SD of 10 replicates and each experiment was repeated thrice)

to conventional grown plant in the experimental garden. All the micropropagated plants were free from external disease.

3.7. Cytological study

Root tips from the source plant showed 2n=24 chromosomes; cytological preparations from the *in vitro* derived plantlets (25 root tips and 50 metaphase/root tip) showed predominantly diploid number (2n=24) of chromosomes. All the regenerates so far analysed do not show any visible cytological changes (Figure 1H). The chromosome number (2n=24) remains the same without any marked structural alternations. Thus the somatic chromosome complements of *in vitro* generated *P. zeylanica* plants remained stable even after passing through three cycles of multiplication. A similar cytogenetically stable plants was also observed in *Asparagus cooperi* (Ghosh and Sen, 1994).

4. Conclusion

The present study developed a simple micropropagation protocol of *P. zeylanica* for mass propagation via node culture within a short time period. In addition, we established a simple method for maintenance of culture in the mode of slow growth that play important role for *in vitro* conservation purpose.

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