RESEARCH ARTICLE

Micropropagation of *Plumbago zeylanica* L. using *in vitro* germinated seedling explants

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ABSTRACT

In vitro seed germination in Plumbago zeylanica L. has been achieved using combined treatment of seed storage, seed soaking, mechanical scarification and MS-basal medium supplemented with GA_3. P. zeylanica seeds stored for six months soaked in distilled water for 30h showed maximum seed germination (95.55%) on ½–Murashige and Skoog basal+GA_3 (3.61 µM). Hardening and survival of seedlings obtained from seed germination was 86%. Maximum multiple shoots/explant (18.02±0.09) proliferated from nodal explants cultured on MS-basal medium supplemented with BAP, Kn and Zea (4.44+4.56+2.32 µM). Maximum shoots rooted on ½–MS basal+IBA (6.15 µM) having 21.91±1.44 roots/shoot and root length 9.36±0.56 cm. Successful hardening of plantlets showed 94% of survival after transferring to garden condition. To the best of our knowledge, this is the first report where in vitro developed seedling has been used as an explant source which will nullify the level of contamination in cultures. We have successfully established an efficient protocol for in vitro seed germination, multiple shoot production through nodal culture and rooting of regenerated shoots to achieve an optimized plant regeneration system along with acclimatization in natural condition. This protocol can be used for large scale commercial micropropagation and conservation purpose.

Keywords - Plumbagin, seed storage, mechanical scarification, seed germination, multiple shoot induction.

INTRODUCTION

Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) exhibits a variety of therapeutic, traditional, ethnobotanical and pharmacological uses (Rana 2011). Because of its diversified multipurpose medicinal importance, high-plumbagin producing plants are of interest. Plumbagin occurs in families like Nepenthaceae (Raj *et al.* 2011), Droseraceae (Jayaram and Prasad 2005), Drosophyllaceae (Crouch *et al*. 1990), Plumbaginaceae (van der Vijver 1972) and Ebenaceae (Robinson 1991).

Plumbago zeylanica L. (Family- Plumbaginaceae) is distributed throughout the tropical and sub-tropical regions of the world (Anonymous 1989, Khare 2008). Plumbagin is mainly produced in the roots of *P. zeylanica* (van der Vijver 1972) and indiscriminate collection from the natural habitat for its roots is done to meet the growing demands of the pharmaceutical companies. *P. zeylanica* grows slowly and it takes several years to produce quality roots that could yield sufficient amount of plumbagin (Kitanov and Pashankov 1994). Though *P. zeylanica* produces large number of seeds, propagation through seed is difficult due to poor seed quality and viability, erratic germination and higher rate of seedling mortality as under natural field conditions (Chaplot *et al.* 2006, Ceasar *et al.* 2013). Seed-coat can interfere with water uptake and gaseous exchange and also prevents leakage of inhibitors, if present (Bradford and Nonogaki 2007) which can hinder seed germination. Various methods have been practiced to facilitate seed germination such as scarification, hot water, dry heat, acid and other chemicals, cold and warm stratification, and light (Bradford and Nonogaki 2007). Certain techniques such as soaking of seeds, mechanical scarification and supplementation of gibberellic acid (GA_3) have been used to improve germination in various plant systems (Menon *et al.* 2001, Kabir *et al.* 2008, Belwal *et al.* 2015). Also, storage of seeds plays an important role in germination (Belwal *et al.* 2015, Kildisheva *et al.* 2011, Martine *et al.* 2009).

Application of modern biotechnological methods are required for the replenishment that has led to the severe depletion of the natural population of *P. zeylanica* and can also suffice the needs of pharmaceutical industries by providing plant resource required for the isolation of drugs without depleting natural plant resources. Biotechnological methods would also reduce the pressure on the species' natural population and thus help conserve these valuable plants. Protocols have been developed for *in vitro* regeneration of *P. zeylanica* using nodal explants (Chaplot *et al.* 2006, Ceasar *et al.* 2013, Sivanesan and Jeong 2009), axillary buds (Selvakumar *et al.* 2001) and leaf (Chaplot *et al.* 2006, Das and Rout 2002, Sivanesan 2007).

In the present study, we aimed to establish an efficient protocol for *in vitro* seed germination of *P. zeylanica*. Further, these well developed *in vitro* grown seedlings were used for the development of a protocol for efficient multiple shoot production through nodal culture and successful rooting of regenerated shoots to achieve an optimized regeneration system along with acclimatization in natural condition.

MATERIALS AND METHODS

Explant source/ collection of seeds – Seeds of *P. zeylanica* (Fig. 1 a, b) were collected from Lonavala, Pune district, Maharashtra (latitude 18.75ºN, longitude 73.39ºE, altitude of 577 m above sea level). The plant was identified and authenticated from Botanical Survey of India, Western Regional Centre, Pune.

Treatment of seeds – Experiment was designed for a combined treatment of seed storage, seed soaking, mechanical scarification and supplementation of GA_3.

Seed storage - Seeds were stored in vials at room temperature and labeled (collection site and collection date) for further experiments. Freshly collected, 3, 6 and 9 months' old seeds were used for the experiment.

Seed soaking - Seeds of *P. zeylanica* were categorized into two sets - Soaked and un-soaked seeds. Seeds were soaked in sterile distilled water for different time intervals (12, 18, 24 and 30 hours) at room temperature, of which the optimum germination results were obtained in 30 h, which was used for the further experiments. The other set of seeds was not soaked and used directly. **Mechanical scarification –** After surface-decontamination, seed explants were mechanical cut at micropylar end before inoculation. **GA_3 treatment** - MS-basal medium (full and ½-strength) was supplemented with different concentrations of GA_3.

Surface sterilization – The 30 h soaked seeds were initially rinsed thoroughly under running tap water for 10 min. followed by washing with 2–3 drops of Tween-20 for 20 min. Seeds were surface sterilized using three disinfectants serially: 70% ethanol for 45 s, 1% (w/v) bavistin for 30 min. on a shaker (120 rpm) and finally treatment of 0.1% (w/v) aqueous mercuric chloride solution for 1–2 min. was given in a laminar airflow chamber with vigorous shaking. These seeds were rinsed 4–5 times with sterile distilled water and further used for inoculation.

Culture medium– Murashige and Skoog's (1962) (MS) medium (PT011, tissue culture grade, Himedia, India) was used throughout the experiment. For seed germination two strengths of MS basal media namely, full and ½-strength were used containing 8 g/l and 4 g/l agar respectively, supplemented with different concentrations of GA_3 ranging from 0.72–4.33 µM. Full–MS basal and ½–MS basal medium served as a control for all treatments.

Culture conditions – The pH of the medium was adjusted to 5.8 prior to autoclaving and 15 ml of molten medium was dispensed into sterile 25 × 200 mm culture tubes. Cultures were incubated at 25±1ºC, relative humidity 55–60% and a 16 h photoperiod. Fifteen replicates were used and the experiment was repeated three times.

Inoculation of seed explant – The sterilized seed explants were mechanical scarified by cutting the micropylar end and were inserted in MS basal and ½–MS (one explant per tube) supplemented with different concentrations of GA_3 (0.72 – 4.33 µM) and control (MS basal and ½–MS basal). The *in vitro* seed germination (SG) response was observed upto 28 d.

Shoot induction – Nodal segments (10±1 mm) from 28 d old *in vitro* seedlings grown on ½–MS medium (180 – 200 mm in length with 7 or 8 nodes) were used as explants. MS basal was supplemented with BAP (6-benzylaminopurine) alone (2.22–6.66 µM), Zea (zeatin) alone (2.28–6.84 µM), Kn (kinetin) alone (1.16–6.97 µM) and BAP, Kn and Zea in combinations and control (MS basal). Twenty replicates were used and the experiment was repeated three times.

Root induction – The obtained multiple shoots were separated and transferred to ½–MS basal medium supplemented with IAA (1.43–8.56 µM), IBA (1.23–7.38 µM) and NAA (1.34–8.06 µM).

Hardening: *In vitro* germinated seedlings and rooted plantlets were transferred to plastic pots (7 cm diameter) containing autoclaved cocopeat : soil (1:1 v/v). High humidity (RH ~75%) was maintained by covering the pots with plastic covers and were supplied with sterile distilled water for 45 d. Established plants were repotted to small clay pots containing autoclaved sand : soil (1:1 v/v) and kept in the shade in the garden.

Statistical analysis – All the experiments were carried out in triplicates and the data were expressed as means ± SE. One way ANOVA analysis followed by the DMRT was used to determine significant ($p \geq 0.05$) differences..

RESULTS AND DISCUSSION

As seen from Table-1 seeds of *P. zeylanica* stored for six months' soaked in sterile distilled water for 30 h and cut at micropylar end showed maximum SG (95.55 %) in ½-MS + GA_3 (3.61 µM) while minimum was observed in fresh unsoaked seeds in ½-MS + GA_3 (0.72 µM). Highest SG % was also observed in seeds stored for 6 months in all treatments of ½-MS + GA_3 as compared to fresh (unsoaked, soaked), 3 and 9 months. Overall, ½-MS + GA_3 showed significant results than full-MS + GA_3 (data not presented).

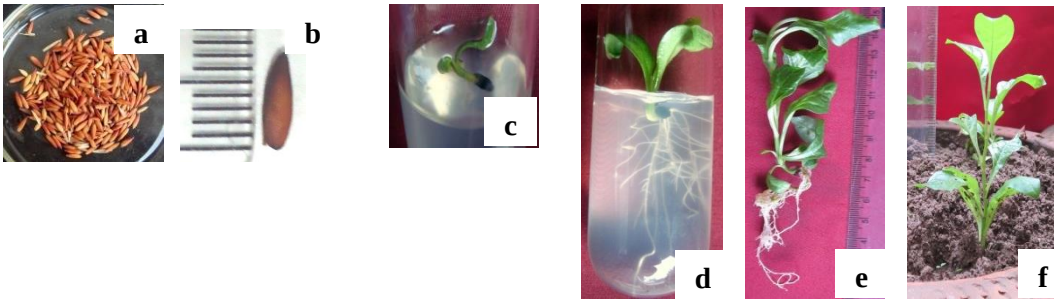

Figure-1 : *P. zeylanica* **- a and b:** seeds, **c:** micropylar end cut seed germinated on ½-MS+GA_3 (3.61 µM) stored for six months, soaked in sterile distilled water for 30 h, **d, e, h:** seedling development.

Table-1: *P. zeylanica* L. – *in vitro* seed germination in ½–MS supplemented with different concentrations of GA_3 after seed soaking and mechanical scarification.

GA_3 (µM)	Unsoaked seeds	Seeds soaked for 30 h			
	Fresh	Fresh	3 months	6 months	9 months
Cont.	00.00 ± 0.00^{c}	15.55 ± 2.22^{e}	24.45 ± 2.22^{g}	31.11 ± 2.22^{e}	22.22 ± 2.22^{g}
0.72	06.67 ± 0.00^{bc}	24.44 ± 2.22^{d}	37.78 ± 2.22^{f}	53.33 ± 3.85^{d}	37.78 ± 2.22^{f}
1.44	11.11 ± 2.22^{ab}	31.11 ± 2.22^{d}	44.45 ± 2.22^{e}	71.11 ± 2.22^{c}	53.33 ± 0.00^{e}
2.17	13.33 ± 3.85^{ab}	42.22 ± 2.22^{c}	55.55 ± 2.22^{d}	82.22 ± 2.22^{b}	68.89 ± 2.22^{d}
2.89	**17.78 ± 2.22^{a}**	51.11 ± 2.22^{ab}	64.45 ± 2.22^{c}	91.11 ± 2.22^{a}	80.00 ± 3.85^{b}
3.61	15.55 ± 2.22^{a}	**57.78 ± 2.22^{a}**	**86.67 ± 0.00^{a}**	**95.55 ± 2.22^{a}**	**93.33 ± 0.00^{a}**
4.33	11.11 ± 2.22^{ab}	48.89 ± 2.22^{bc}	77.78 ± 2.22^{b}	88.89 ± 2.22^{b}	75.78 ± 2.22^{c}

Data represent mean values ± SE of three replicates mean with same letters are not significantly different at 0.05 % probability level according to DMRT

Table-2:– *in vitro* multiple shoot induction response of *P. zeylanica* L.

BAP	Zea	Kin	Shoot proliferation	No. of explant with MS	No. of shoot/explant
	(µM)				
0	0	0	11.33 ± 0.33^{fg}	0.00 ± 0.00^{l}	1.00 ± 0.00^{l}
2.22	0	0	13.67 ± 0.67^{de}	6.48 ± 0.79^{fghi}	4.24 ± 0.23^{h}
3.33	0	0	15.67 ± 0.33^{c}	8.16 ± 0.80^{ef}	6.79 ± 0.21^{f}
4.44	0	0	17.67 ± 0.33^{b}	11.79 ± 1.04^{bc}	**8.26 ± 0.43^{e}**
5.55	0	0	14.67 ± 0.88^{cd}	6.34 ± 1.21^{fghi}	7.25 ± 0.24^{f}
6.66	0	0	12.00 ± 0.58^{f}	4.34 ± 0.91^{ijk}	3.02 ± 0.09^{j}
0	2.28	0	9.00 ± 0.58^{h}	3.22 ± 0.76^{jk}	1.43 ± 0.16^{kl}
0	3.42	0	12.67 ± 0.33^{ef}	5.17 ± 0.71^{hij}	3.31 ± 0.16^{ij}
0	4.56	0	14.67 ± 0.33^{cd}	7.12 ± 0.92^{efgh}	**4.25 ± 0.19^{h}**
0	5.70	0	10.33 ± 0.88^{gh}	3.34 ± 0.31^{jk}	1.90 ± 0.10^{k}
0	6.84	0	7.33 ± 0.88^{i}	2.05 ± 0.45^{kl}	1.49 ± 0.09^{kl}
0	0	1.16	11.33 ± 0.33^{fg}	5.43 ± 0.44^{ghij}	3.75 ± 0.36^{hij}
0	0	2.32	12.67 ± 0.33^{ef}	7.67 ± 0.50^{efg}	**5.12 ± 0.39^{g}**
0	0	3.48	15.33 ± 0.33^{c}	10.66 ± 0.68^{cd}	4.13 ± 0.14^{hi}
0	0	4.65	11.67 ± 0.33^{fg}	5.33 ± 0.58^{ghij}	3.22 ± 0.28^{j}
0	0	6.97	9.00 ± 0.58^{h}	3.66 ± 0.41^{djk}	2.14 ± 0.28^{k}
4.44	0	2.32	18.00 ± 0.00^{ab}	12.67 ± 0.84^{bc}	13.80 ± 0.28^{c}
4.44	0	3.48	18.33 ± 0.33^{ab}	13.66 ± 0.50^{b}	12.02 ± 0.20^{d}
0	4.56	2.32	13.67 ± 0.33^{de}	9.33 ± 0.73^{de}	8.72 ± 0.09^{e}
0	4.56	3.48	15.67 ± 0.33^{c}	11.88 ± 0.87^{bc}	6.63 ± 0.03^{f}
4.44	4.56	2.32	18.67 ± 0.33^{ab}	12.66 ± 1.01^{bc}	**18.02 ± 0.09^{a}**
4.44	4.56	3.48	19.33 ± 0.33^{a}	15.88 ± 0.76^{a}	15.78 ± 0.80^{b}

Data represent mean values ± SE of three replicates mean with same letters are not significantly different at 0.05 % probability level according to DMRT

Table-3: *in vitro* root induction response of *P. zeylanica* L.

IAA	IBA	NAA	Shoots rooted	No. of roots/shoot	Root length (cm)
	(µM)				
0	0	0	0.00 ± 0.00^{i}	0.00 ± 0.00^{i}	0.00 ± 0.00^{k}
1.43	0	0	7.67 ± 0.33^{gh}	3.42 ± 0.47^{hi}	1.09 ± 0.09^{j}
2.85	0	0	8.67 ± 0.33^{fgh}	4.27 ± 0.63^{gh}	1.89 ± 0.39^{ij}
4.28	0	0	9.33 ± 0.88^{fg}	5.80 ± 0.99^{gh}	3.87 ± 0.39^{h}
5.71	0	0	10.33 ± 0.88^{ef}	8.07 ±0.83^{efg}	4.93 ± 0.39^{efg}
7.14	0	0	15.00 ± 0.58^{bc}	11.74 ± 1.46^{cde}	6.96 ± 0.19^{c}
8.56	**0**	**0**	**16.33 ± 0.33^{ab}**	**16.55 ± 2.43^{b}**	**8.04 ± 0.41^{b}**
0	1.23	0	9.33 ± 0.33^{fg}	3.99 ± 1.02^{h}	2.61 ± 0.33^{i}
0	2.46	0	10.33 ± 0.88^{ef}	6.13 ± 1.22^{fgh}	3.96 ± 0.17^{gh}
0	3.69	0	12.00 ± 0.58^{de}	13.02 ± 1.68^{bcd}	4.14 ± 0.31^{fgh}
0	4.92	0	14.67 ± 0.88^{bc}	16.04 ± 1.37^{b}	7.95 ± 0.22^{b}
0	**6.15**	**0**	**17.33 ± 0.88^{a}**	**21.91 ± 1.44^{a}**	**9.36 ± 0.56^{a}**
0	7.38	0	15.33 ± 0.33^{ab}	15.61 ± 2.08^{b}	6.39 ± 0.37^{cd}
0	0	1.34	6.67 ± 0.67^{h}	3.11 ± 0.40^{hi}	1.17 ± 0.16^{j}
0	0	2.69	8.67 ± 0.67^{fgh}	4.99 ± 0.80^{gh}	2.54 ± 0.49^{i}
0	0	4.03	11.67 ± 0.88^{de}	9.82 ± 0.85^{def}	5.82 ± 0.21^{de}
0	**0**	**5.37**	**15.67 ± 0.67^{ab}**	**13.64 ± 1.05^{a}**	**7.00 ± 0.37^{c}**
0	0	6.71	15.33 ± 0.88^{ab}	9.90 ± 0.96^{def}	5.06 ± 0.14^{ef}
0	0	8.06	13.00 ± 1.16^{cd}	6.45 ± 1.16^{fgh}	2.85 ± 0.42^{i}

Data represent mean values ± SE of three replicates mean with same letters are not significantly different at 0.05 % probability level according to DMRT

Unsoaked fresh seeds when inoculated on MS basal did not germinate at all whereas, when inoculated on ½-MS showed significantly least germination as compared to the soaked seeds in all combinations

Effect of dry storage on seed dormancy has been studied earlier (Menon *et al.* 2001, Belwal *et al.* 2015, Kildisheva *et al.* 2011, Karimmojeni *et al.* 2014). To germinate seeds, mechanical scarification by cutting the micropylar end has been reported in *P. zeylanica* (Menon *et al.* 2001) and in other plant systems (Kabir *et al.* 2008, Chanchula *et al.* 2013). Gibberellic acid (GA_3) has shown to break dormancy and increase SG in several genera (Bachelard 1968, Bhatt *et al.* 2000, de Mello *et al.* 2009, Chellopil *et al.* 2012, Brar *et al.* 2013). In the preliminary experiments, seeds were inoculated on MS basal and ½–MS basal medium without any treatment, but seeds failed to germinate.

Germination followed by seedling development was recorded (Fig. 1 c-f). Highest SG (95.55%) achieved in seeds stored for 6 months inoculated on ½-MS + GA_3 (3.61 µM) showed conformity with results of Kildisheva et al. (2011) in *Sphaeralcea munroana* where seeds were mechanically scarified along with storage for 6 months. *In vitro* mechanical scarification of seeds at micropylar end helped in faster imbibition of water efficiently thereby leading to faster germination and might have helped in fast leaching of inhibitors from the seeds. According to Chanchula et al., (2013), partial trimming or complete removal of seed-coat in *Globba* spp. effectively relieved mechanical restraint and/or barriers to gas exchange and stimulated metabolism of the seed to germinate into a seedling. To overcome the problem of unpredictable germination and seedling mortality

under natural field conditions in *P. zeylanica*, in recent years there has been an increased interest in *in vitro* culture techniques which provide a practical tool for mass multiplication in a comparatively short time and in an efficient way. Thus, an attempt was made for *in vitro* seed germination. Half-MS medium supplemented with different concentrations of GA_3 proved the best strength medium for germination as compared to full-MS. Seed swelling was observed from day-3 of inoculation in ½-MS + GA_3 treatment, followed by successful germination from day-6 onwards, further leading to develop into healthy seedlings by day-21. The maximum SG% was achieved at 3.61 µM of GA_3. It can be concluded that optimum GA_3 concentration of 3.61 µM is necessary to stimulate germination in *P. zeylanica* seeds. Seed-coat can be responsible in preventing water uptake and gaseous exchange necessary for seed germination and inhibit the leaching out of inhibitors, if present, that restricts seed germination. Plumbagin, an important naphthoquinone present in *P. zeylanica* has been reported to inhibit germination (Menon *et al.* 2001,

Ferguson *et al.* 2004, Meyer *et al.* 2007). In order to germinate seeds rapidly seed soaking has been reported in various plants (Menon *et al.* 2001, Sabongari S & Aliero 2004, Amusa 2011, Saleem *et al.* 2014) which help in leaching out water-soluble inhibitors present in seeds and facilitate germination. According to Menon et al. (2001) seed storage reduced the plumbagin content (inhibitor level) within the seed. Seed germination percentage increased from fresh to 3- to 6-months stored seeds and declined in 9-months stored seeds. Our results showed concurrence with the findings of Karimmojeni et al. (2014) in *Descurainia sophia*, where it was shown that these seeds might have reached a maximum dormancy release after 150-180 days of dry storage and maximum germination capacity. The 28 day old seedlings were further divided into two groups; half were transferred for hardening and remaining half were used as explant for multiple shoot development. The well-developed plantlets were hardened and the survival rate of plantlets was high (86 %) as compared to earlier report by Mallikadevi et al (2008) which is 80 %.

A higher level of contamination in cultures has been reported in *P. zeylanica* explants collected from wild (Selvakumar *et al.* 2001). The *in vitro* developed seedlings served as sterile explant source. Multiple shoot induction was observed in all concentrations of BAP, Kn and Zea alone and in combination. The combinations of these plant growth regulators (PGRs) was used by selecting the concentrations of growth regulators showing highest shoot proliferation rate, number of explants responding for multiple shoot production and highest multiple shoots per explant. BAP (4.44 µM) showed significant and highest shoot proliferation (17.76 ± 0.33) and 11.79 out of these 17.76 proliferated shoots produced multiple shoots, each having 8.26 ± 0.43 shoots per explant. Similarly, highest shoot proliferation (14.67 ± 0.33) was recorded in Zea (4.56 µM) having 4.25 ± 0.19 shoots per explant; and Kn (2.32 µM) had maximum (5.12 ± 0.39) shoots (Table-2). These individual best concentrations were further combined for assessing their responses with respect to multiple shoot induction. Maximum shoots per explant (18.02) was recorded in MS medium supplemented with BAP, Kn and Zea (4.44 + 4.56 + 2.32 µM) (Table-2). Use of various PGRs for shoot proliferation has been reported in *P. zeylanica* (Chaplot *et al.* 2006, Selvakumar *et al.* 2001, Das and Rout 2002, Sahoo and Debata 1998). However, in present study supplementation of combined PGRs resulted in 49% increase in multiple shoot induction as compared to Chaplot et al. (2006). These multiple shoots were separated and cultured on ½–MS basal medium supplemented with various concentrations of IAA, IBA and NAA. Maximum shoots rooted on ½–MS basal medium supplemented with IBA (6.15 µM) having 21.91 ± 1.44 roots per shoot (Table-3), which is 47 % more as compared to Chaplot et al. (2006). Our results are in accordance with Chaplot et al. (2006) who have reported best results on MS supplemented with IBA as compared to IAA.

CONCLUSION

In the present study, a reliable protocol for seed germination in *P. zeylanica* is established. This established protocol for seed germination of *P. zeylanica* will provide a tool to develop *in vitro* seedlings. These *in vitro* germinated seedlings served as explant source for micropropagation. To the best of our knowledge, this is the first report where *in vitro* developed seedling has been used as an explant source which will nullify the level of contamination in cultures. Acclimatization to natural conditions and survival rate of plantlets obtained from multiple shoots was higher (94 %) than hardened plants from

seedlings obtained after germination (86 %). Also, from a single nodal explant multiple shoots were generated into 18 plantlets as compared to a single seed generating into a single plant which is 18 times more as compared to one single plant. We have successfully developed protocol for a large scale micropropagation of this medicinally important plant and can be used for commercial use and conservation purpose.

Conflicts of interest: The authors stated that no conflicts of interest.

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