Effects of Sargassum Ilicifolium Seaweed Extract on Enhanced in Vitro Seed Germination, Mass Propagation, and Accumulation of Plumbagin in Plumbago zeylanica L.

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Abstract

The present study describes a simple, reproducible and cost-effective regeneration system using seaweed liquid extract (SLE) of *Sargassum ilicifolium* supplemented in Murashige and Skoog (MS) medium for *in vitro* seed germination, multiple shoot and root induction, and plumbagin accumulation in *P. zeylanica*. High rate of seed germination and seedling length was recorded on ½–MS + SLE (2 %) which was 1.11 and 1.28 fold higher as compared to GA_3 (2.89 µM). The best response in terms of explants producing maximum number of shoots was recorded on MS medium supplemented with 4 % SLE that was 2.26 and 1.37 folds higher than BAP (3.33 µM) alone and in combination with IAA (4.28 µM), respectively. High frequency root induction and plumbagin accumulation was recorded on ½–MS + 2.5 % SLE which showed 1.34, 1.49, and 1.44 fold increase for the roots per shoots, average root length, and plumbagin, respectively when compared with IBA (6.15 µM). Besides being an alternative to commercially available PGRs, this is the first report demonstrating the potential of SLE for enhanced growth and plumbagin accumulation in *P. zeylanica*.

Introduction

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) has been reported from several plant families such as Plumbaginaceae, Drosophyllaceae, Ebenaceae, Droseraceae, Nepenthaceae, Iridaceae, Juglandaceae, Dioncophyllaceae, and Anicistrocladaceae (Checker et al. 2018; Badwaik et al. 2019). Plumbagin is reported to possess anti-inflammatory, antioxidant, antifungal, antibacterial, antimicrobial, anti-leishmanial, anti-parasitic, anti-malarial, antitumor, anti-fibrotic, anti-depressant, cyto-protective, neuroprotective, hypolipidemic, and hyperglycemic activities (Mallavadhani et al. 2002; Padhye et al. 2010; Checker et al. 2018) and exhibits anticancer activity against several types such as lung, ovarian, breast, prostrate, colon, cervical, liver, pancreatic, esophageal and gastric cancers; and acute promyelocytic leukemia, lymphoma, melanoma, myeloma, osteosarcoma, tongue and oral squamous cell carcinomas (Liu et al. 2017; Cao et al. 2018; Checker et al. 2018; Badwaik et al. 2019). *Plumbago* species (family Plumbaginaceae) are the most common source of plumbagin (Checker et al. 2018) which makes this family significantly noteworthy and has attracted attention of scientific community to explore its bioactivities. The National Medicinal Plants Board (NMPB), India has listed *P. indica* (syn. *P. rosea*), *P. zeylanica* and *P. auriculata* as high demanded medicinal plants, of which the estimated annual trade of *P. zeylanica* for its roots or whole plant is 500–1000 metric tons (https://www.nmpb.nic.in/medicinal_list). The chemical synthesis of plumbagin is not economically feasible because of its compound structure, specific stereo-chemical requirement of the compound and high cost of chemicals (Ahmad et al. 2015). This problem of chemical synthesis and high annual demand is creating a heavy pressure on the wild population making the plant vulnerable in near future and can soon be threatened. According to Ved and Goraya (2007) this situation may need immediate management attention considering the utilization of wild populations and demands a need of developing cultivation practices to suffice the requirement of raw material for industrial use.

Plant tissue culture is an alternative method of mass propagation and has proved to be valuable to obtain secondary metabolites without hampering plants' natural strands. For *in vitro* culturing of *P. zeylanica* different synthetic growth media and plant growth regulators (PGRs) have been widely used (Selvakumar et al. 2001; Das and Rout, 2002; Verma et al. 2002; Sivanesan and Jeong, 2009; Nayak et al. 2015). However, the higher cost of synthetic growth media and toxic nature of PGRs are a major constrain for mass multiplication in the commercial application (Garabrant and Philbert, 2002; Satish et al. 2015; Soumare et al. 2021). Auxins and cytokinins are the most common PGRs used in plant tissue culture and its aberrant use can stimulate stress in plant cells or tissues which alter physiological responses, consequently leading to phenotypic abnormalities (Krishna et al. 2016) that may result into epigenetic and genetic changes and hyperhydrated shoots and yellowing of stems and leaves (Kahia et al. 2016; Garcia et al. 2019). Though various *in vitro* culture techniques have been practiced for micropropagation and enhancement of plumbagin in *P. zeylanica*, until now no reports on development of cost effective approach is documented.

For centuries seaweeds have been used as a source of diet, in therapeutics and as fertilizers in agriculture. Though the use of seaweeds as fertilizers is ancient, only recently its use as plant growth promoters or biostimulants has been documented (Du Jardin, 2015; Yeganeh and Adel, 2019; Boukhari et al. 2020). Seaweed liquid extracts (SLEs) can be used as an alternative to synthetic growth media and PGRs as it is a source of macro- and micro-nutrients, vitamins, minerals, amino acids, organic acids, and antioxidants, and plant growth regulating substances which are involved in seed germination and mediating growth of shoots and roots (Rency et al. 2016; Khan et al. 2009). Brown algae (Phaeophyta) are the second most abundant group and many genera such as *Ascophyllum* sp., *Fucus* sp., *Laminaria* sp., *Turbinaria* sp., and *Sargassum* sp. are used as biofertilizers or as soil conditioners to enhance plant growth and yield (Khan et al. 2009). *Sargassum ilicifolium* (Turner) C. Agardh is commonly reported from the coastal region of Maharashtra, India and is economically important (Waghmode, 2017) with wide variety of phytochemicals (Murugaiyan, 2020; Nazarudin et al. 2020). Unlike other brown algae, *S. ilicifolium* SLE has not been explored for its biostimulating effect for medicinal plants under *in vitro* conditions.

Thus, in the present investigation we focused to evaluate and compare the effect of *S.ilicifolium* SLE with the PGRs for *in vitro* seed germination, multiple shoot and root induction, and plumbagin accumulation in *P. zeylanica*. It also emphasis on the use of SLEs as an alternative to the PGRs for *in vitro* regeneration protocols which has potential for significant results.

Materials And Methods

Collection and preparation of seaweed liquid extract

S. ilicifolium (Turner) C. Agardh was collected from the coastal area of Malvan, Sindhudurg District of Maharashtra state, India. The collected *S. ilicifolium* was cleaned on collection site with seawater to remove impurities and macroscopic epiphytes. Later, in the laboratory these were washed several times

with double distilled water (DDW) to remove sand particles and other impurities. The cleaned *S. ilicifolium* was shade dried for a week and grounded to coarse powder and the SLE was prepared according to Satish et al. (2015). About 500 g of *S. ilicifolium* powder was added to 1 L of sterile DDW (1:2; w/v) and was autoclaved for 30 min at 121°C. The autoclaved sample was filtered through muslin cloth, allowed to cool at room temperature and was later filtered using Whatman filter paper no. 1. From 1 L of the extract prepared, 850 mL of filtrate was obtained and considered as 100 %. The obtained filtrate (stock solution) was stored at 4°C and working concentrations of SLEs were prepared by diluting it using sterile DDW.

Seed germination in *P. zeylanica*

Seeds of *P. zeylanica* were collected from Bhimashankar, Pune district, Maharashtra (19º4'17.19"N, 73º32'10.99"E). Surface sterilized seeds were inoculated on ½–strength MS basal medium (Murashige and Skoog, 1962; Kakade and Malpathak, 2017) fortified with 0.4 % agar and gibberellic acid (GA_3) (0.72 – 4.33 µM) or SLE (1 – 5 %). Half–strength MS basal medium without GA_3 or SLE served as control. The percentage of seed germination and seedling development (mean length of seedlings, cm) were recorded on day 7 and 28, respectively for all studied media.

Shoot induction and multiplication in *P. zeylanica*

For shoot induction and multiplication, nodal segments from 28 days old *in vitro* seedlings grown on ½–MS medium with GA_3 and SLEs were used as explants. MS media along with 6-benzylaminopurine (BAP) (1.11 – 5.55 µM), Kinetin (Kin) (1.16 – 6.97 µM), and Zeatin (Zea) (1.14 – 4.56 µM) were used alone. To assess high frequency multiple shoot induction, higher responsive media [MS + BAP (3.33 µM)] was employed in combination with indole-3-acetic acid (IAA) (1.43 – 7.14 µM), 1-naphthaleneacetic acid (NAA) (1.34 – 6.70 µM) and indole-3-butyric acid (IBA) (1.23 – 6.15 µM) in combinations. In another set of experiment, MS medium was supplemented with different concentrations of SLEs (1 – 10 %). MS basal (without any PGRs or SLEs) was considered as control. Responses of PGRs and SLEs were analyzed in terms of shoot proliferation (%), number of explants with multiple shoots (%), and mean number of multiple shoots induced per explant after 4 weeks.

Root induction in *P. zeylanica*

The separated multiple shoots were transferred to ½–MS medium supplemented with different concentrations of IBA (3.69 – 7.38 µM), IAA (1.43 – 5.71 µM), and NAA (1.34 – 5.36 µM), and SLEs (0.5 – 5 %). Half–MS medium without any PGRs or SLE served as control. The shoots rooted (%), the mean number of roots per shoot, and length of roots (cm) were determined after 4 weeks of study.

Culture conditions

The pH of the MS medium was adjusted to 5.8 ± 0.2 prior to autoclaving. The medium was sterilized by autoclaving at 121°C for 20 min. The culture tubes were incubated at 25 ± 2°C, 16/8 h photoperiod,

relative humidity 55–60 %, and at an irradiance of 80 µmol photons m^{-2} s^{-1} of cool white fluorescent light. Twenty replicates were used per treatment and each experiment was repeated three times (*n*=60).

Quantification of plumbagin using High Performance Thin Layer Chromatography

Sample preparation

Forty-five day's old *in vitro* generated *P. zeylanica* plantlets having roots were dried, powdered, sieved, and stored in air-tight containers prior to analysis. Extract was prepared by adding 1 g sample powder in 10 mL chloroform and cold macerated at 4ºC for 48 h. Stock solution of standard plumbagin (Himedia, India) was prepared in chloroform at 1mg mL^{-1} (Ariyanathan et al. 2010).

Instrumentation and experimental conditions

Camag (Muttenz, Switzerland) High Performance Thin Layer Chromatography (HPTLC) instrumentation system equipped with Linomat V sample applicator along with Camag TLC Scanner-3 and WinCATS 4 software for data interpretation was used. Chromatographic analyses of the extracts were performed on aluminium plate pre-coated with silica gel $60F_{254}$ TLC plates (20×10 cm; Merck, Darmstadt, Germany). Samples were applied to the plates as sharp bands of bandwidth of 6 mm by means of Camag Linomat V sample applicator. The distance between the tracks was 5 mm. The development chamber was saturated for 1 h with the solvent system n-hexane : ethyl acetate (7 : 3) v/v and the plates were developed in the TLC chamber at a development distance of 90 mm. After development, the plates were derivatized with anisaldehyde – sulphuric acid (Anonymous, 2001) and kept in a pre-heated oven at 110ºC for 15 min. Detection and quantification was performed with a Camag TLC Scanner-3 at 254 nm and 366 nm after derivatization.

Statistical analysis

All the plant tissue culture experiments were carried out in triplicates and the data was expressed as means ± SE. One-way analysis of variance (ANOVA) was used and the significance of variations with means was compared with the Duncan's Multiple Range Test (DMRT) using SPSS 16.0 (SPSS, IBM Statistics).

Results And Discussion

Seed germination in *P. zeylanica*

In the present study, ½-MS + GA_3 showed seed germination and seedling length in the range of 76.67 – 88.33 % and 6.50 – 10.53 cm respectively with higher response at 2.89 µM of GA_3 (Table 1, Figure 1 a – c). Whereas, significantly higher seed germination (86.67 – 98.33 %) and seedling growth (9.27 – 13.57 cm) was observed on different concentrations of ½-MS + SLEs (Table 1). Maximum seed germination (98.33 %) and seedling length (13.57 cm) was recorded for 2 % SLE which showed 1.11 and 1.28 fold

increase for studied parameters as compared to the GA_3 (2.89 µM) (Table 1, Figure 1 d – f). SLEs can be a new low-cost alternative to GA_3 that can aid to overcome the production cost of GA_3 (Camara et al. 2018) and its toxic effects in animals and humans (Boğa et al. 2009; Hosseinchi et al. 2013) which can limit its use for large scale seed germination and *in vitro* applications. The results in this study revealed that seed germination and development of seedling length in *P. zeylanica* is dependent on the concentrations of GA_3 and SLEs, as at higher concentration there was a substantial decrease in response (Table 1). Similar response was reported in *Lycopersicon esculentum* (Vinoth et al. 2012) and *Solanum melongena* (Satish et al. 2015). Researchers have reviewed the chemical constituents of SLEs obtained from green and brown algae and have stated presence of GA_3 (Nabti et al. 2016; Stirk et al. 2020), which could be the reason for higher seed germination and seedling development in *P. zeylanica*. In the present investigation, the *in vitro* seed germination protocol was not only established for rapid regeneration of seedlings, but also served as a source of sterile explants for *in vitro* studies that will eliminate the contamination in cultures.

Effect of PGRs on multiple shoots and root induction

Effect of PGRs on shoot multiplication has been reported in *P. zeylanica* earlier (Chaplot et al. 2006; Kanungo et al. 2012; Ceasar et al. 2013; Roy and Bharadvaja, 2017). In the present investigation two-stage culture system was used, where in the first stage MS media was fortified with cytokinins alone, while in the second stage the responsive media was used in combination with auxins. In first stage, MS media fortified with BAP showed a substantial response as compared to Kin and Zea (Table 2). MS + BAP (3.33 µM) showed 81.67 % shoot proliferation, 75.51 % explants with multiple shoot, 8.67 shoots (Table 2, Figure 2 a). These results are in accordance with the previous reports on *in vitro* regeneration of *P. zeylanica* which stated that BAP exhibited significant response for multiple shoot induction (Sahoo and Debata, 1998; Rout et al. 1999; Sivanesan and Jeong, 2009). Further this responsive media was used in combination with auxins like IAA (1.43 – 7.14 µM), NAA (1.34 – 6.70 µM) and IBA (1.23 – 6.15 µM) to enhance the number of multiple shoots (Table 2). Maximum response of shoot proliferation (96.67 %), shoots per explant (14.33), and cultures with multiple shoots (96.55 %) were observed on BAP + IAA (3.33 + 4.28 µM) after 4 weeks (Figure 2 b). Similar results are reported by Chaplot et al. (2006) where MS medium supplemented with different combinations of BAP and IAA developed multiple shoots. Thus, our results imply that two-stage culture system is superior to obtain high multiple shoot induction in *P. zeylanica*. Present study showed better response as compared to previous studies (Rout et al. 1999; Chaplot et al. 2006; Kanungo et al. 2012; Ceasar et al. 2013; Roy and Bharadvaja, 2017). Further, the multiple shoots grown on MS + BAP (3.33 µM) + IAA (4.28 µM) was excised and cultured on ½–MS medium having IBA, IAA, and NAA alone for rooting (Table 3). Rooting was successfully achieved on all the concentrations of studied PGRs where significantly higher response was recorded on IBA (6.15 µM) for shoots rooted (83.33 %), roots per shoot (26.33), and average root length (9.17 cm) (Table 3, Figure 2 c). This rooting response showed agreement with the previous studies in *P. zeylanica* (Chaplot et al. 2006; Caesar et al. 2013). The percentage of shoots rooted and average root length obtained on IBA (6.15 µM) was approximately 2.5 times higher, while the number of roots per shoot were 7 times higher as

compared to the control (Table 3). Though the MS media supplemented with PGRs is found to be effective for micropropagation of *P. zeylanica*, its production on large scale can be hindered due to high cost of chemicals and longer culture duration which is 4 weeks for every stage of culturing. However, these limitations with micropropagation can be overcome by the employment of cost effective SLEs (Sharma et al. 2015).

Effect of SLEs on multiple shoots and root induction

SLEs contain several growth promoting hormones, nutrients, vitamins, amino acids, brassinosteroids, betains, sterols, polyamines and antibiotics which are responsible for growth development of plants by increasing the nutrient uptake and resistance to biotic and abiotic stresses (Stirk et al. 2014; Battacharyya et al. 2015; Nabti et al. 2016). Several reports bring to light use of SLEs for *in vitro* mass propagation in crop plants (Fakihi Kachkach et al. 2014; Satish et al. 2015; Vinoth et al. 2019). This study describes the effect of SLEs prepared from *S. ilicifolium* on the production of shoots and roots in *P. zeylanica* under *in vitro* condition. After 4 weeks' study, maximum shoot proliferation of 98.33 % was observed in MS + SLE (5 and 6 %), whereas the highest cultures with multiple shoots (100 %) and number of multiple shoots (19.67) was noted on MS + 4 % SLEs (Table 4, Figure 3 a). The response of multiple shoots obtained on 4 % SLEs was 2.26 and 1.37 folds higher than BAP (3.33 µM) alone and in combination with IAA (4.28 µM) respectively (Table 2 and 4) and were used for root induction experiment. For root induction, explants were inoculated on ½–MS medium supplemented with SLEs (0.5 – 5 %) and the maximum rooting response for shoots rooted (88.33 %), roots per shoot (35.33), and root length (13.67) was recorded on 2.5 % SLE (Table 5 and Figure 3 b). The response observed on 2.5 % SLE was further compared with the rooting response of IBA (6.15 µM) which demonstrated 1.34 and 1.49 fold increase for the roots per shoots and average root length, respectively (Table 3 and 5). A similar study in *Solanum melongena* was carried out by Satish et al. (2015) who stated that SLEs played vital role in the *in vitro* multiple shoot induction and rooting response. Similar *in vitro* mass propagation of medicinal plants using SLEs is reported in *Withania somnifera* (Kannan et al. 2014), *Ceropegia thwaitesii* (Muthukrishnan et al. 2015), and *Bacopa monnieri* (Rency et al., 2016). Studies have reported the presence of cytokinins, auxins, and gibberellic acid from seaweed extracts (Khan et al. 2009; Battacharya et al. 2015), while brown algae are reported to be the most important source of PGRs due to the presence of high content of active compounds, continuous, and high availability all year round (Yalcin et al. 2019). Craigie (2011) enlisted the PGRs from a number of brown seaweeds, of which auxins, cytokinins and gibberellins have been reported from genus *Sargassum*. Elemental and hormone analyses of different *Sargassum* sp. revealed the presence of potassium, copper, manganese, zinc, iron, cobalt, magnesium, sodium, calcium, nitrogen, molybdenum, chloride, phosphate, sulphate and nitrate; and cytokinins, auxins, and gibberellins (Vijayanand et al. 2014; Ramya et al. 2015; Bharath et al. 2017; Uthirapandi et al. 2018). The *in vitro* response of *P. zeylanica* seed germination, multiple shoot induction and rooting can be because of the presence of these biostimulants. In the present study, all studied concentrations of SLEs showed exceedingly significant response for multiple shoots and root induction as compared to PGRs. These responses can perhaps be because of the presence of additional macro- and micro-elements, amino acids, and antibiotics in the SLEs (Nabti et al. 2016; Yalcin et al. 2019) that are essential for the growth

and development of plants. We observed that the low concentration of SLEs are effective to attain optimum response which is similar to earlier studies in different plants (Vijayanand et al. 2014; Ramya et al. 2015; Bharath et al. 2017; Uthirapandi et al. 2018).

Effect of PGRs and SLEs on plumbagin accumulation

The 45 d old plantlets developed on ½–MS supplemented with auxins and SLEs were analyzed for the accumulation of plumbagin using HPTLC. For this analysis, various solvent systems were tested, of which n-hexane : ethyl acetate (7:3) showed better resolution and had reproducible peaks at *Rf* 0.60 representing plumbagin in the test samples (Figure 4). Significantly higher plumbagin content was observed on 2.5 % SLE (1588 µg/mg) which was 1.44 and 11 folds higher than that of 6.15 µM IBA (1101.33 µg/mg) and control (½–MS basal), respectively (Table 6, Figure 4 a – d). As compared to the PGRs and control, the plantlets cultured on SLEs showed higher accumulation of plumbagin, this is in concurrence with the previous study in *Picrorhiza kurroa* for the enhancement of picroside-I (Sharma et al. 2015). The increase in plumbagin content might be related to the higher availability of macro- and micro-elements from SLEs, which are used in media optimization and elicitation of cultures to enhance secondary metabolites in different plant systems (Battacharyya et al. 2015; Murthy et al. 2014). Also the SLEs contain different PGRs that modulate the accumulation of secondary metabolites in plant tissue culture (Jamwal et al. 2018). The present study elucidates that SLEs at lower concentrations are effective to accumulate plumbagin which are in concurrence with Ramya et al. (2015) who stated that lower concentrations of SLEs are more effective to alter biochemical responses.

Conclusions

This study describes simple, highly reproducible, and cost-effective *in vitro* seed germination, multiple shoot, and root induction protocol of *P. zeylanica* using *S. ilicifolium* extract. The SLEs significantly enhanced seed germination, shoot proliferation and multiple shoot induction, percentage of shoots rooted, number of shoots per root and root length as compared to the synthetic PGRs like gibberellic acid, cytokinins (BAP, kinetin, and zeatin) and auxins (IBA, IAA, and NAA). This is the first report for an efficient *in vitro* mass propagation protocol of *P. zeylanica* using SLEs. The aim of the investigation was to cut down the use of commercially available PGRs used for *in vitro* mass propagation, and our study indicates that smaller amount of SLEs with MS media can be used as a substitute to the commercially available PGRs and can reduce the expenses. This study also showed that SLEs can enhance plumbagin production in *P. zeylanica* and can be applied for commercial production. Apart from the use as biofertilizers in agriculture and horticulture, SLEs should now be endorsed for large-scale micropropagation of medicinal plants and can be utilized for commercial production of medicinally important secondary metabolites to suffice the needs of pharmaceutical industries. However, further studies are needed for the chemical characterization of *S. ilicifolium* extract which will aid in understanding the mechanisms involved in the growth and development and plumbagin accumulation.

Abbreviations

ANOVA: Analysis of variance

BAP: 6-benzylaminopurine

DDW: Double distilled water

DMRT: Duncan’s Multiple Range Test

GA3: Gibberellic acid

HPTLC: High Performance Thin Layer Chromatography

IAA: indole-3-acetic acid

IBA: indole-3-butyric acid

Kin: Kinetin

MS: Murashige and Skoog

NAA: 1-naphthaleneacetic acid

PGRs: Plant growth regulators

SLEs: Seaweed liquid extracts

Zea: Zeatin

Declarations

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Authors' contributions: Study conception and design was done by PSK and NPM. PSK and SBZ performed the experiments. Analysis and interpretation of data was done by PSK. Drafting of manuscript was done by PSK and SBZ. Critical revision was done by NPM. All authors read and approved the final manuscript.

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Consent for publication: Yes

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Tables

Table 1 Effect of ½-MS basal medium supplemented with GA_3 and *S. ilicifolium* seaweed liquid extracts on *in vitro* seed germination of *P. zeylanica*.

PGRs/ SLEs	Percentage of germination*	Mean length of seedlings (cm)**
GA_3 (µM)		
0	73.33 ± 0.33^{e}	5.97 ± 0.29^{e}
0.72	76.67 ± 0.33^{de}	6.50 ± 0.17^{de}
1.44	81.67 ± 0.33^{bcd}	7.13 ± 0.20^{d}
2.17	83.33 ± 0.33^{abc}	8.30 ± 0.21^{c}
2.89	88.33 ± 0.33^{a}	10.53 ± 0.23^{a}
3.61	86.67 ± 0.33^{ab}	9.47 ± 0.23^{b}
4.33	78.33 ± 0.33^{cde}	6.80 ± 0.17^{d}
SLEs (%)		
0	73.33 ± 0.33^{c}	5.97 ± 0.29^{e}
1.0	88.33 ± 0.33^{b}	9.87 ± 0.33^{cd}
2.0	98.33 ± 0.33^{a}	13.57 ± 0.24^{a}
3.0	96.67 ± 0.33^{a}	12.40 ± 0.26^{b}
4.0	90.00 ± 0.58^{b}	10.47 ± 0.23^{c}
5.0	86.67 ± 0.33^{b}	9.27 ± 0.29^{d}

*Percentage of germination recorded on day 7 and **mean seedling length on day 28. Data represents mean values ± SD of three replicates mean (n=20 number of seeds per replicate) with same letters are not significantly different at 0.05 % probability level according to Duncan's Multiple Range Test (DMRT).

Table 2 Effect of different cytokinins (BAP, Kinetin and Zeatin) alone and in combinations with auxins (IAA, NAA, and IBA) on shoot proliferation (%), cultures with multiple shoot (%), and number of shoots per explant in *P. zeylanica* after 4 weeks study.

PGRs (μM)	Shoot proliferation %	Cultures with multiple shoot %	No. of shoots per explant
0.0	NR	NR	NR
BAP			
1.11	66.67 ± 0.33^{b}	52.50 ± 0.58^{c}	6.00 ± 0.58^{bc}
2.22	76.67 ± 0.33^{a}	65.22 ± 0.58^{b}	7.33 ± 0.33^{ab}
3.33	81.67 ± 0.67^{a}	75.51 ± 0.33^{a}	8.67 ± 0.33^{a}
4.44	68.33 ± 0.33^{b}	68.29 ± 0.33^{b}	6.67 ± 0.33^{b}
5.55	56.67 ± 0.58^{c}	58.82 ± 0.67^{c}	5.00 ± 0.58^{c}
Kin			
1.16	51.67 ± 0.33^{e}	51.61 ± 0.58^{d}	2.67 ± 0.33^{d}
2.32	58.33 ± 0.33^{cd}	57.14 ± 0.33^{c}	3.33 ± 0.67^{cd}
3.48	66.67 ± 0.58^{ab}	60.00 ± 0.58^{b}	4.67 ± 0.33^{ab}
4.65	71.67 ± 0.33^{a}	67.44 ± 0.33^{a}	5.67 ± 0.33^{a}
5.81	63.33 ± 0.67^{bc}	65.79 ± 0.33^{b}	4.33 ± 0.33^{bc}
6.97	56.67 ± 0.33^{de}	50.00 ± 0.58^{cd}	2.33 ± 0.58^{d}
Zea			
1.14	68.33 ± 0.33^{b}	68.29 ± 0.33^{b}	5.67 ± 0.33^{b}
2.28	78.33 ± 0.33^{a}	72.34 ± 0.58^{a}	7.33 ± 0.33^{a}
3.42	73.33 ± 0.67^{ab}	63.64 ± 0.33^{b}	6.00 ± 0.58^{b}
4.56	66.67 ± 0.33^{b}	57.50 ± 0.67^{c}	4.67 ± 0.58^{b}
BAP + IAA			
3.33 + 1.43	71.67 ± 0.33^{d}	86.05 ± 0.33^{c}	9.33 ± 0.33^{d}
3.33 + 2.86	81.67 ± 0.33^{c}	87.76 ± 0.33^{b}	11.67 ± 0.33^{bc}
3.33 + 4.28	96.67 ± 0.33^{a}	96.55 ± 0.33^{a}	14.33 ± 0.33^{a}
3.33 + 5.71	88.33 ± 0.33^{b}	86.79 ± 0.67^{b}	12.33 ± 0.33^{b}
3.33 + 7.14	73.33 ± 0.33^{d}	86.36 ± 0.33^{c}	10.67 ± 0.33^{c}

BAP + NAA			
3.33 + 1.34	75.00 ± 0.58^{c}	91.11 ± 0.33^{c}	9.33 ± 0.33^{c}
3.33 + 2.68	83.33 ± 0.33^{b}	92.00 ± 0.33^{b}	10.33 ± 0.33^{bc}
3.33 + 4.02	91.67 ± 0.33^{a}	94.55 ± 0.33^{a}	12.67 ± 0.33^{a}
3.33 + 5.36	95.00 ± 0.58^{a}	92.98 ± 0.33^{a}	11.33 ± 0.33^{ab}
3.33 + 6.70	88.33 ± 0.33^{ab}	88.68 ± 0.33^{b}	10.33 ± 0.33^{bc}
BAP + IBA			
3.33 + 1.23	78.33 ± 0.33^{c}	85.11 ± 0.33^{c}	8.33 ± 0.33^{c}
3.33 + 2.46	86.67 ± 0.33^{b}	88.46 ± 0.33^{b}	9.00 ± 0.33^{bc}
3.33 + 3.69	93.33 ± 0.33^{a}	89.29 ± 0.33^{ab}	10.33 ± 0.33^{ab}
3.33 + 4.92	88.33 ± 0.33^{ab}	92.45 ± 0.33^{a}	11.00 ± 0.58^{a}
3.33 + 6.15	80.00 ± 0.58^{c}	85.42 ± 0.33^{c}	9.67 ± 0.33^{abc}

NR: No Response. Data represents mean values ± SD of three replicates mean with same letters are not significantly different at 0.05 % probability level according to DMRT.

Table 3 Effect of ½-MS media supplemented with different concentrations of auxins (IBA, IAA, and NAA) on shoots rooted (%), number of roots per shoot, and root length (cm) in *P. zeylanica* after 4 weeks study.

PGRs (μM)	Shoots rooted %	No. of roots per shoot	Root length (cm)
IBA			
0.0	31.67 ± 0.33^{b}	3.67 ± 0.33^{e}	3.70 ± 0.32^{d}
3.69	78.33 ± 0.33^{a}	17.00 ± 0.58^{d}	6.43 ± 0.58^{c}
4.92	78.33 ± 0.67^{a}	21.33 ± 0.33^{c}	7.13 ± 0.54^{bc}
6.15	83.33 ± 0.33^{a}	26.33 ± 0.33^{a}	9.17 ± 0.37^{a}
7.38	80.00 ± 0.58^{a}	23.33 ± 0.88^{b}	8.00 ± 0.29^{ab}
IAA			
0.0	31.67 ± 0.33^{b}	3.67 ± 0.33^{d}	3.70 ± 0.32^{c}
1.43	70.00 ± 0.58^{a}	13.67 ± 0.33^{c}	4.80 ± 0.49^{bc}
2.86	73.33 ± 0.33^{a}	15.67 ± 0.67^{b}	5.73 ± 0.45^{ab}
4.28	78.33 ± 0.67^{a}	19.67 ± 0.33^{a}	7.07 ± 0.55^{a}
5.71	75.00 ± 0.58^{a}	17.00 ± 0.58^{b}	6.27 ± 0.47^{ab}
NAA			
0.0	31.67 ± 0.33^{c}	3.67 ± 0.33^{d}	3.70 ± 0.32^{c}
1.34	70.00 ± 0.58^{b}	16.67 ± 0.33^{c}	5.90 ± 0.38^{b}
2.68	80.00 ± 0.58^{a}	23.67 ± 0.67^{a}	7.83 ± 0.43^{a}
4.02	76.67 ± 0.33^{ab}	20.00 ± 0.58^{b}	7.13 ± 0.52^{ab}
5.36	71.67 ± 0.33^{b}	17.67 ± 0.33^{c}	6.50 ± 0.42^{ab}

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

Table 4 Effect of MS basal medium supplemented with *S. ilicifolium* seaweed liquid extracts on multiple shoot induction in *P. zeylanica* after 4 weeks study.

SLE %	Shoot proliferation %	Cultures with multiple shoot %	No. of shoots per explant
0.0	1 ± 0.00^{f}	NR	1 ± 0.00^{h}
1.0	93.33 ± 0.33^{ab}	96.43 ± 0.58^{bc}	12.33 ± 0.33^{ef}
2.0	93.33 ± 0.33^{ab}	98.21 ± 0.33^{abc}	13.67 ± 0.88^{de}
3.0	95.00 ± 0.00^{ab}	96.49 ± 0.33^{abc}	15.67 ± 0.67^{c}
4.0	96.67 ± 0.33^{ab}	100.0 ± 0.33^{a}	19.67 ± 0.88^{a}
5.0	98.33 ± 0.33^{a}	98.31 ± 0.33^{a}	18.00 ± 1.00^{b}
6.0	98.33 ± 0.33^{a}	96.61 ± 0.33^{ab}	17.33 ± 0.33^{b}
7.0	96.67 ± 0.58^{ab}	96.55 ± 0.33^{ab}	14.33 ± 0.33^{cd}
8.0	91.67 ± 0.33^{bc}	94.55 ± 0.33^{cd}	12.67 ± 0.58^{def}
9.0	88.33 ± 0.33^{cd}	92.45 ± 0.58^{de}	11.33 ± 0.58^{fg}
10.0	86.67 ± 0.58^{d}	92.31 ± 1.00^{e}	9.67 ± 0.33^{g}

NR: No Response; SLE: seaweed liquid extract. Data represents mean values ± SD of three replicates (n=60) mean with same letters are not significantly different at 0.05 % probability level according to Duncan's Multiple Range Test (DMRT)

Table 5 Effect of ½–MS medium supplemented with *S. ilicifolium* seaweed liquid extracts on multiple shoot induction in *P. zeylanica* after 4 weeks study.

SLE %	Shoots rooted %	No. of roots per shoot	Root length (cm)
0.0	31.67 ± 0.33^{d}	3.67 ± 0.33^{i}	3.70 ± 0.32^{h}
0.5	81.67 ± 0.33^{abc}	13.33 ± 0.88^{h}	6.40 ± 0.62^{fg}
1.0	81.67 ± 0.33^{bc}	20.00 ± 0.58^{fg}	7.50 ± 0.53^{ef}
1.5	83.33 ± 0.33^{abc}	26.67 ± 0.88^{d}	9.37 ± 0.41^{bcd}
2.0	85.00 ± 0.58^{abc}	30.67 ± 0.88^{c}	10.67 ± 0.35^{b}
2.5	88.33 ± 0.33^{a}	35.33 ± 0.33^{a}	13.67 ± 0.50^{a}
3.0	86.67 ± 0.33^{ab}	33.33 ± 0.67^{b}	12.73 ± 0.29^{a}
3.5	83.33 ± 0.67^{abc}	27.00 ± 0.58^{d}	10.13 ± 0.47^{bc}
4.0	81.67 ± 0.58^{abc}	23.00 ± 0.58^{e}	8.10 ± 0.58^{de}
4.5	80.00 ± 0.58^{bc}	21.33 ± 0.58^{ef}	7.17 ± 0.41^{efg}
5.0	78.33 ± 0.33^{c}	19.00 ± 0.88^{g}	6.00 ± 0.46^{g}

Data represents mean values ± SD of three replicates (n=60) mean with same letters are not significantly different at 0.05 % probability level according to Duncan's Multiple Range Test (DMRT)

Table 6 Accumulation of plumbagin in plantlets of *P. zeylanica* grown on ½- MS media containing different auxins and SLEs after 45 days.

PGRs (µM)	Plumbagin (µg/mg)	SLEs %	Plumbagin (µg/mg)
0.0	140.67^{d}	0.0	140.67^{d}
IBA		0.5	1071.33^{g}
3.69	862.67^{c}	1.0	1186.67^{f}
4.92	965.00^{b}	1.5	1323.33^{de}
6.15	1101.33^{a}	2.0	1409.33^{c}
7.38	1023.33^{ab}	2.5	1588.00^{a}
IAA		3.0	1500.67^{b}
1.43	682.00^{d}	3.5	1388.33^{cd}
2.86	765.33^{c}	4.0	1278.67^{e}
4.28	960.67^{a}	4.5	1197.67^{f}
5.71	877.67^{b}	5.0	1130.33^{fg}
NAA			
1.34	754.67^{c}		
2.68	997.33^{a}		
4.02	971.33^{a}		
5.36	882.67^{b}		

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

Figures

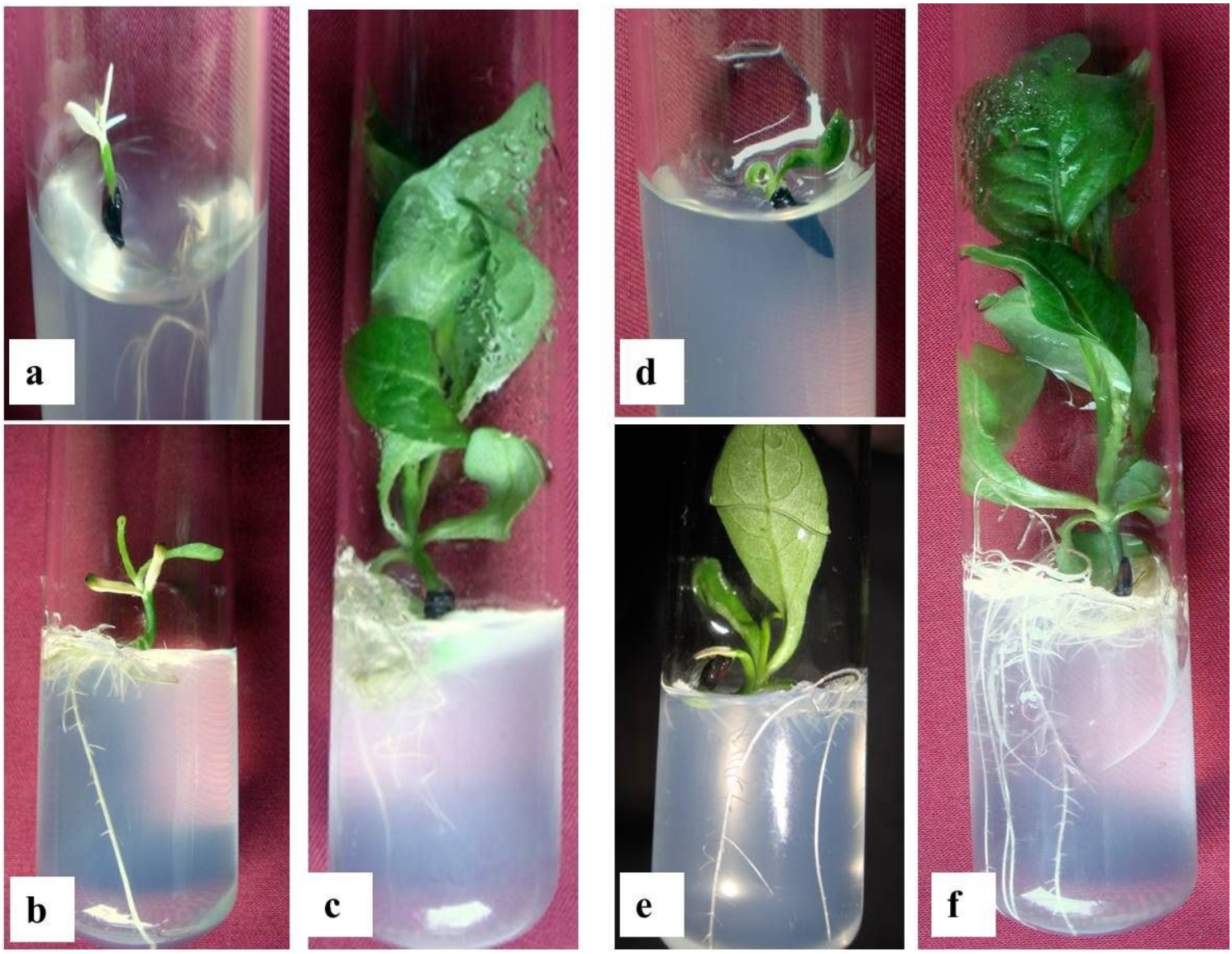

Figure 1

In vitro response of P. zeylanica seeds cultured on ½-MS + GA3 (2.89 µM) a: P. zeylanica seed germination on day 3; b and c: seedling development on day 7 and 21 respectively; ½-MS + SLE (2.0 %) d: P. zeylanica seed germination on day 3; e and f: seedling development on day 7 and 21 respectively.

Figure 2

In vitro response of P. zeylanica for production of multiple shoots on MS medium supplemented with a: BAP (3.33 µM) b: BAP + IAA (3.33 + 4.28 µM), and c: rooting on ½-MS media containing IBA (6.15 µM) after 4 weeks study.

Figure 3

In vitro response of P. zeylanica for production of multiple shoots on MS medium supplemented with a: SLE (4.0 %) b: rooting on ½-MS media containing SLE (2.5 %) after 4 weeks study.

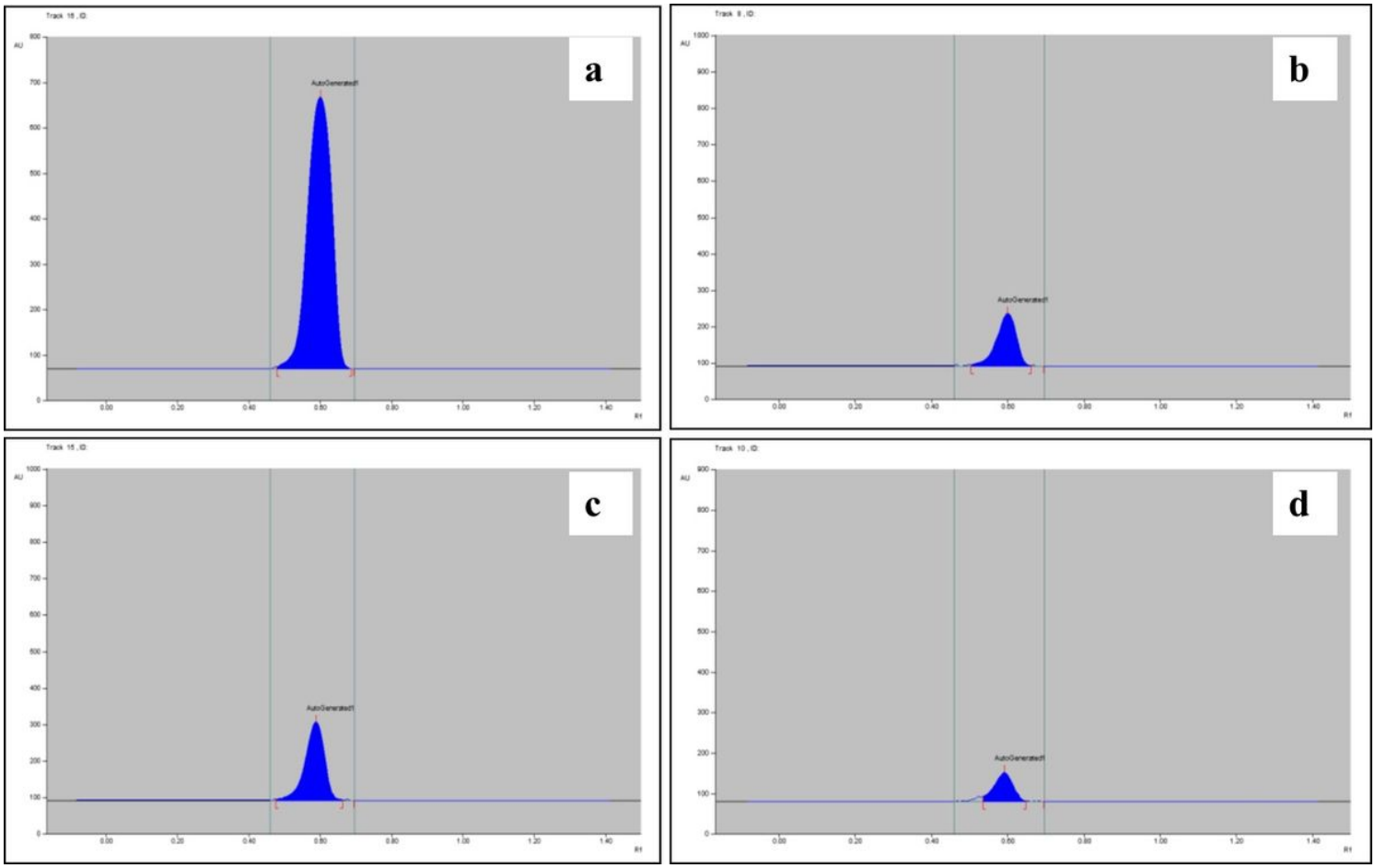

Figure 4

HPTLC chromatograms of a: standard plumbagin, b: plantlet on ½-MSB+6.15 µM IBA, c: plantlet on ½-MSB + 2.5 % SLE, and d: plantlet on ½-MSB (control) after 45 days.