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Swagata Nag
Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India

Suman Kumaria
Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India

Correspondence
Suman Kumaria
Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India

In vitro propagation of medicinally threatened orchid *Vanda coerulea*: An improved method for the production of phytochemicals, antioxidants and phenylalanine ammonia lyase activity

Swagata Nag and Suman Kumaria

Abstract
Vanda coerulea Griff ex. Lindl. is an endangered epiphytic orchid with various medicinal usages in traditional medicines. Different phytochemicals such as flavidin, imbricatin, coelonin, methoxycoelonin, gigantol have been reported to be present in *V. coerulea*. An attempt has been made in the current study to enhance the *in vitro* production of phytochemical constituents by the application of chitosan, a biotic elicitor which may reduce the pressure on its unmindful collection from wild. *In vitro* cultures of *V. coerulea* were raised via asymbiotic seed germination on B_5 medium. Highest number of PLBs which give rise to full grown plantlets was achieved in B_5 medium supplemented with 3µM thidiazuron. The effect of chitosan both on plantlet development and enhancement of phytochemical constituents was studied. The present study revealed that chitosan at 4 mg/l in the medium benefited the accumulation of the secondary metabolites, and greatly affected both the antioxidant as well as PAL enzyme activities. There was an increase of flavonoid and alkaloid contents by 2.3 and 4.17 fold in the methanolic leaf extract as compared to the control. However, the highest total phenolic and tannin contents were found in chloroformic leaf extract with 3.07 and 2.57 fold increases, respectively. The PAL activity of treated sample was found to be 5 fold higher compared to the untreated sample. A significant higher yield of antioxidant activities was observed after exogenous application of chitosan. The present investigation suggests that chitosan may have triggered the metabolic pathway in treated *V. coerulea* plantlets by inducing the PAL enzyme activity through the enhancement of various phytochemicals such as phenolics, alkaloids and flavonoids, which potentially reported to impart the medicinal value to the plant.

Keywords: chitosan; multiple shoot development; secondary metabolites; phenylalanine ammonia lyase

1. Introduction
Vanda coerulea Griff ex. Lindl. Commonly known as "Blue *Vanda* of Asia" and "Autumn lady's tresses" belongs to the family Orchidaceae. It is an epiphytic, monopodial orchid, mainly found in the Northeast India (Meghalaya, Assam and Manipur) and northern parts of Thailand and Burma. The species is mainly used for ornamental and horticultural purposes. Apart from its ornamental values, this species is also important ethno botanically and used to prepare various traditional medicines, owing to the presence of various biochemical compounds such as flavidin, imbricatin, coelonin, methoxycoelonin, gigantol, phytosterol (Simmler *et al*., 2010) [38]. Traditionally, the leaf extract is used to prevent diarrhea, dysentery and dermal disorders, whereas flower decoctions are used to cure glaucoma, cataract and blindness (Medhi and Chakrabarti, 2009) [29]. Habitat destruction, overexploitation, extensive collection, deforestation are the main factors for the decline of this species from its natural habitat. Due to these it has been enlisted as threatened species and included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Hrahsel and Thangjam, 2015) [20]. In order to conserve endangered and threatened species from extinction and to increase its population size, *in vitro* propagation plays a significant role (Wochok, 1981) [42]. *In vitro* seed germination protocols have been introduced for many orchid species such as *Dendrobium longicornu, D. formosum*, *Cymbidium eburnium* (Dohling *et al*., 2010; Gogoi *et al*., 2012) [13, 17]. In general, plant growth hormones have been incorporated into basal media to stimulate the plant growth of orchid (Roy *et al*., 2011) [35]. However, at present various elicitors such as chitosan, salicylic acid and jasmonic acid have also been used instead of plant growth regulators to enhance the plantlet formation and secondary metabolite production (Barka *et al*., 2004) [3]. Further, elicitors help to protect plants from numerous types of abiotic and biotic stresses.

Chitosan is a natural biopolymer derived from chitin. This natural biodegradable polysaccharide is non-toxic in nature and composed of glucosamine and *N*-acetylglucosamine. Chitosan is mainly obtained from the exoskeletons of crustaceans, insects, and fungi some algal cell walls (Sanford and Hutchings, 1987) [37]. In an earlier report, it was found that chitosan enhanced the plant growth as well as the development of various orchid species (Restanto *et al*., 2016) [34]. Besides its role in plant development, chitosan also enhances the accumulation of various enzymes involved in phenylpropanoid pathway, such as PAL. This enzyme helps in synthesizing of secondary metabolites, such as polyphenols, flavonoids, alkaloids, phytoalexins and lignin (Ferri and Tassoni, 2011) [14]. The enhancement of phenolic compounds after chitosan treatment has also been reported in butter lettuce, greek oregano, apricot and sweet basil (Kim *et al*., 2005; Ghasemnezhad and Shiri, 2010; Yin *et al*., 2011; Złotek *et al*., 2014) [24, 16, 44, 46]. Therefore, chitosan treatment has been recognized as an important biotechnological approach to augment the production of secondary metabolites as well as plantlets growth. The enrichment of phytochemicals in plants is not only important for the medical industry; but it is also useful in improving the public health through diet of plant-based foods. The objectives of these research were to study the effect of chitosan on plantlets development and enhancement of phytochemical contents in tissue culture raised plantlets.

2. Materials and Methods

2.1 Asymbiotic seed germination

Plants of *V. coerulea* were collected from forests of Upper Shillong, Meghalaya, India and maintained in the laboratory's green house (Fig.1A, B). Plant sample was authenticated and deposited in NEHU herbarium with the accession number NEHU - 12066. About 7- 8 months old hand pollinated mature capsules of *V. coerulea* were collected and washed thoroughly with liquid detergent under running tap water to remove all dead tissues and then surface-sterilization was performed with 70% ethanol for 1min followed by surface flaming for 2-3 sec inside the laminar air flow cabinet. Then the capsules were dissected longitudinally with a surgical blade, and seeds were scooped out and sown on different types of basal media namely, MS, KC, VW and B_5 (Murashige and Skoog, 1962; Knudson, 1946; Vacin and Went, 1949; Gamborg *et al*., 1968) [30, 25, 41, 15]. All the media were supplemented with 3% sucrose. The pH was adjusted to 5.6 prior to adding solidifying agent agar (0.8%) and then the media were autoclaving at 121 ºC temperature at pressure of 15 psi for 15-20 min. For each basal medium, ten replicates were taken and the experiments were repeated three times. Cultures were incubated at 25 ± 2 ºC for 16 h photoperiod under 50 µmol m^{-2} s^{-1} of light intensity. Periodically, different parameters such as days required for spherule, protocorm development and seed germination percentage were recorded.

Seed germination percentage was calculated as follows:

% seed germination = (Number of seeds forming spherule/Total number of seeds) × 100

2.2 Protocorm multiplication and explants preparation

For protocorm multiplication and subsequent protocorm - like bodies (PLBs) development, cytokinins such as BAP, KI and TDZ were used. Initially protocorms were inoculated in B_5 medium supplemented with BAP, KI at concentrations ranging from 2-10 µM whereas TDZ in 1-5 µM individually. Each treatment constituted of ten replicates and repeated thrice. Various parameters of protocorm development were documented after four weeks of culture initiation. Single PLB with shoot apex was used as explants for further experiments.

2.3 Effect of chitosan on seedling development

For plantlets development, different concentrations of chitosan (1-5 mg/l) were added in B_5 medium prior to autoclaving. Single PLB with shoot apex was inoculated on the medium (Fig. 2C). In order to assess the plantlets development after chitosan treatment following growth parameters were measured: a. response frequency (%), b. number of shoots and roots formation and c. length of shoots and roots. To see the influence of chitosan on biochemical and phytochemical parameters plantlets were used for further experiments.

2.4 Estimation of total chlorophyll

The total chlorophyll contents (Chl_a and Chl_b) was measured by the method of Harmut and Lichtenthaler (1987) [19] with some modifications. Briefly, 500 mg *in vitro* raised fresh leaf samples were cut and soaked in 8 ml acetone and incubated at room temperature for overnight in dark condition. After overnight incubation, the supernatant was collected via centrifugation (10,000 rpm for 5 min). The chlorophyll contents were measured using the following equation (Harmut and Lichtenthaler, 1987) [19]:

Chlorophyll a (µg/gm FW) = $11.24(A_{662}) - 2.04(A_{645})$

Chlorophyll b (µg/gm FW) = $20.13(A_{645}) - 4.19(A_{662})$

Total chlorophyll (µg/gm FW) = $7.05\ (A_{662}) + 18.09(A_{645})$

2.5 Extract preparation for secondary metabolites

Plantlets (leaves and roots) from *in vitro* raised cultures treated with chitosan and control sample (without chitosan) were washed under running tap water, blot dried with tissue towel at room temperature and ground with the help of mortal pestle. The powdered tissue (100 mg) was suspended in 100 ml of different solvents *viz.*, methanol, acetone, chloroform and distilled water. The solutions were kept for overnight in shaking condition (180 rpm). After overnight incubation, the supernatant was collected through centrifugation (10,000 rpm for 20 min).

2.5.1 Quantification of total flavonoid content (TFC)

Total flavonoid content was measured according to the method of Chang *et al*. (2002) [7]. Plant extract (500 µl) was mixed with 1.5 ml of methanol, 100 µl of $AlCl_3$ (10%), 100 µl of sodium acetate (1 M) and 2.8 ml of distilled water (). The reaction mixture was vortexed thoroughly and kept for 30 min at room temperature. The absorbance was measured at 415 nm in UV-vis spectrophotometer. Finally, the results were expressed in mg of quercetin equivalents (QE) per gram of fresh tissue weight.

2.5.2 Quantification of total phenolic content (TPC)

Total phenolic content was estimated by Folin–Ciocalteu method described earlier (Ainsworth and Gillespie, 2007) [1]. Gallic acid was used as standard for this experiment. Equal volume of plant extract and double distilled water (1: 1) were mixed with 0.5 ml of Folin–Ciocalteu reagent (Sigma–aldrich, USA) and solution was incubated at 25°C for 5 min. After incubation, the solution was mixed with 1 ml of 5% Na_2CO_3 and again kept in dark for 1 h. The absorbance was measured at 725 nm. The phenolic content was evaluated in mg of gallic acid equivalents (GAE) per gram of fresh weight.

2.5.3 Quantification of total tannin content (TTC)
Total tannin content was analyzed by the method reported earlier (Bhattacharyya *et al*., 2014) [5]. Initially, 0.5 ml Folin–Dennis reagent (Sigma–Aldrich, USA) was mixed with 250 µl of each sample extract. Then equal volume of 20% sodium carbonate solution and double distilled water were added to the reaction mixture and incubated at 25°C for half an hour. The absorbance of the reaction was measured spectrophotometrically (775 nm). To determine the equivalent tannin content, tannic acid used as a standard.

2.5.4 Quantification of total alkaloid content (TAC)
The quantification of total alkaloid content was performed using the method of Sreevidya and Mehrotra (2003) [40]. The plant extract (250 µl) was mixed with freshly prepared Dragendorff's reagent (500 µl) and incubated at room temperature for 45 min. The optimal density was estimated at 700 nm and the content of the total alkaloid was described by mg of atropine equivalent (AE) per gram of fresh tissue.

2.6 Quantification of antioxidant activity
2.6.1 DPPH radical scavenging assay
The extracts were analyzed for antioxidant activity using the protocol of Jagtap *et al*. (2011) [22]. The plant extract (0.5 ml) was mixed with equal volume of DPPH solution prepared in methanol (100 µM). The mixtures were kept in dark for half an hour. The color change was quantified in terms of optical density at 517 nm using spectrophotometer. The ascorbic acid was used as standard sample. The percentage of radical scavenging activity was calculated by
(1- sample OD/control OD) X100
Where control OD means absorbance value of control and sample OD means absorbance value of various sample.

2.6.2 Metal chelating assay
The chelating assay was quantified using the method described earlier (Dinis *et al*., 1994) [12]. At first, the reaction mixture was prepared by adding plant extract (500 µl), 2mM of $FeCl_2$ (100 µl) and 5mM of ferrozine (40 µl). The solution was mixed and incubated for 10min at room temperature. The absorbance was measured at 562 nm. The percentage of ferrozine – Fe^{2+} complex inhibition was calculated from:
$[(A_0-A_1)/A_0]$ X 100, where A_0 is absorbance value of control and A_1 is absorbance of sample extract.

2.6.3 Hydrogen peroxide (H_2O_2) radical scavenging assay
The hydrogen peroxide radical scavenging assay was measured using the method described earlier (Kumaran and Karunakaran, 2007) [28]. Briefly, 1 ml of sample extract was mixed with 0.6 ml of H_2O_2 (2 mM) which was prepared in phosphate buffer (pH 7.4). The mixed solutions were incubated for 10 min and the absorbance was recorded at 230 nm. The data was calculated as follows:
% inhibition = $[(A_0 – A_1)/A_0]$ X 100, where A_0 stands for absorbance of control sample and A_1 stands for absorbance of plant extract.

2.7 Phenylalanine ammonia lyase enzyme activity
The PAL enzyme activity was determined followed by the method of Kováčik and Klejdus (2012) [27]. Crude enzyme extract was prepared using ice-chilled mortar and pestle. Briefly, 0.5 gm of each sample (leaves and roots) was homogenized in 2 ml of sodium borate buffer (0.1M; pH 8.8) and immediately centrifuged at 10,000 rpm for 15 min at 4ºC. The supernatant was collected for enzyme assay and stored on ice prior to use.
At first, the reaction mixtures (350 µl of homogenate and 500 µl sodium borate buffer) were pre-incubated in water bath (40ºC) for 5 min. After incubation, 300 µl of L-phenylalanine (50 mM) was mixed with the solution to start enzymatic reaction. The reaction mixture was then incubated for 1 h at 40ºC. The reaction was stopped by addition of 50 µl HCl (5N). Parallelly control sample was prepared without adding L-phenylalanine to quantify trans-cinnamic acid (CA) content. The absorbance was recorded at 275 nm. Cinnamic acid was used as a standard sample. The PAL enzyme activity (one unit) was equivalent to the amount of cinnamic acid production (nM) by the deamination of L-phenylalanine.

2.8 Data analysis
All experiments were repeated thrice. Statistical analysis was performed using one-way analysis of variance (ANOVA). The significant differences among the mean values were assayed by Tukey's HSD test. The Pearson correlation coefficient was also performed to develop the relationship between phytochemical constituents and phenylalanine ammonia lyase enzyme activity.

3. Results and discussion
3.1 Asymbiotic seed germination and protocorm formation
Seed germination and protocorm development of orchid species depends on the condition of cultured media which is directly related to the plants growth and morphogenesis. The response of seed germination was recorded by observing the color change and spherule formation (Fig. 1C, D) which got converted to single protocorm (Fig. 1E). Among the four media (MS, B_5, VW and KC) tested, the highest seed germination (71.06%) was achieved on B_5 medium followed by MS (59.34%), KC (22.53%) and VW (43.36%) (Table 1).

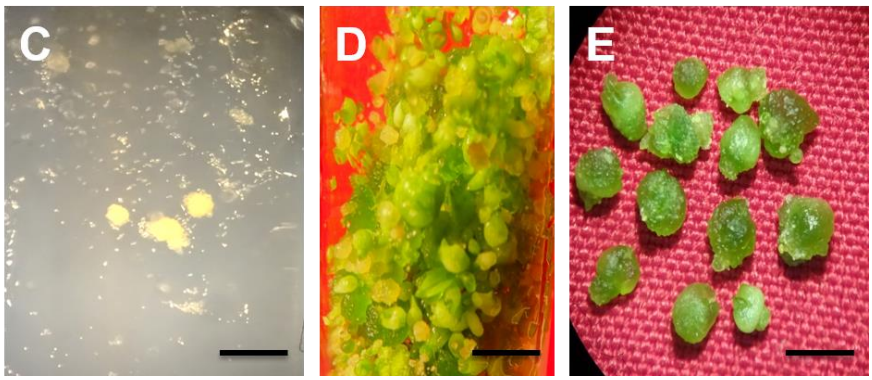


Fig 1: *In vitro* seed germination of *Vanda coerulea*. (A) mature plant in natural habitat, (B) matured flowering plant in green house condition, (C) seeds sown on B_5 medium at day 0, (D) protocorms formation after six weeks of culture initiation, (E) microscopic view of single protocorm used for PLBs formation. Bar = 5 mm

The B_5 medium was found to be most effective in terms of spherule formation (14.5 days) compared to MS (18.34 days), VW (35.58 days) and KC (39.95 days) (Table 1). This suitability of B_5 basal medium on seed germination has been also reported for *Grammatophyllum speciosum* (Samalaa et. al., 2014) [36]. The seed germination of *V. coerulea* occurred in 14.5 days as compared to the earlier report (Roy *et al*., 2011) [35]. The seed germination and protocorms development on different media are dependent on the composition of growth media, supplementation of vitamins, various salts and ions (Chen *et al*., 2015) [10]. Based on the composition of B_5 medium, it has low salts contents as compared to other media which is more suitable for seed germination of *V. coerulea*. The following findings were supported by the report of Zeng *et al*. (2016) [45] which suggested that for some selected orchid species the seeds require very less nutrients for initiating germination.

Table 1: Influence of different basal media on seed germination of *V. coerulea*.

Basal media	Seed germination (%)	Days taken for spherule formation	Days taken for protocorm formation	Total number of protocorms	Protocorm weight (mg)
MS	59.34±0.69^{b}	18.34±0.07^{c}	38.32±0.88^{c}	35.50±0.91^{c}	2.40±0.29^{b}
KC	22.53±0.57^{d}	39.95±0.18^{a}	59.57±1.18^{a}	16.15±0.96^{d}	1.46±0.15^{d}
B5	71.06± 0.99^{a}	14.5±0.22^{d}	34.67±0.19^{d}	63.03±0.80^{a}	3.15±0.19^{a}
VW	43.36±0.74^{c}	35.58±0.38^{b}	55.07±0.77^{b}	43.11±1.39^{b}	1.54±0.13^{c}

Values represent means ± SE of ten replicates and all the experiments were repeated thrice. Means followed by the same letter are not significantly different according to Tukey's HSD test ($P \leq 0.05$). MS: Murashige and Skoog; KC: Knudson C; B5: Gamborg *et al*.; VW: Vacin and Went

3.2 Effect of cytokinins on PLBs and multiple shoot formation

In the present study, additional use of external cytokinins stimulated PLBs formation from single protocorms cultured in B_5 medium. The protocorms cultured in same basal medium devoid of any cytokinins failed to develop PLBs. Depending on the types and concentrations of cytokinins the experimental results varied. Amongst the tested cytokinins (BAP, KN, and TDZ), the highest survival rate (70.25%) was found in B_5 medium supplemented with 3.0 µM TDZ (Table 2; Fig. 2A). Similar culture resulted with highest numbers of PLBs (4.82) per explant along with maximum number of shoot-apex formation (Table 2; Fig. 2B). Apart from BAP and KN, TDZ at lower concentration was found to be best for shoot bud induction from PLBs which is supported by the earlier reports of Bhattacharyya *et al*. (2016) [4]. On the other hand, the present study revealed that TDZ used singly showed more effectiveness over conventional cytokinins (BAP and KN) in terms of PLBs and shoot bud formation. The similar observation was also reported by Cheruvathur *et al*. (2010) [11].

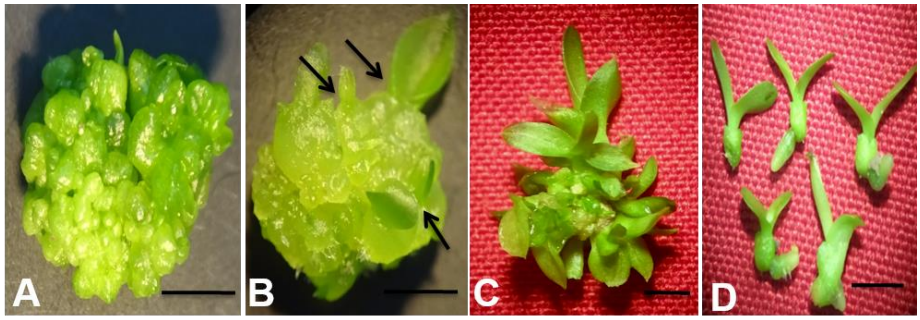


Fig 2: (A) Protocorm like bodies (PLBs) in B_5 medium supplemented with 3.0 µM TDZ, (B) shoot apex protruding from PLB after two weeks in same medium, (C) Multiple shoot proliferation, (D) Small plantlets with shoot and root initiation used as explants for chitosan treatment, (E) complete plantlets in chitosan (4 mg/l) supplemented B_5 medium arranging in time interval (I = 2; II = 3; III = 4; IV = 6 and V = 8 weeks respectively) Bar = 10 mm.

3.3 Chitosan treatment for plantlets development

Extended sub culturing of the small plantlets (Fig. 2C) to the above-mentioned medium did not enhance the plantlets development. Thus, to do that B_5 medium was supplemented with different concentrations of chitosan to assess its role on plantlets formation (Table 3). Highest response (82.80%) in terms of single plantlet formation was obtained in B_5 medium supplemented with 4 mg/l chitosan (Fig. 2D). After eight weeks of culture, the highest number of shoots (10.28±0.77) and roots (5.67±0.47) containing plantlets were reported (Table 3). The development of plantlets in various time intervals in the same chitosan containing medium have been showed in Fig. 2E. The present investigations confirmed the beneficial roles of chitosan multiple shoot induction and seedling developments. It has been reported that chitosan enhances the growth of orchids and flower production by eliciting the expression of the same genes that are regulated by plant growth regulators like cytokinin, auxin as reported in *Dendrobium* species, *Coleus aromaticus* (Chandrkrachang *et al*., 2005; Govindaraju and Arulselvi, 2016) [8, 18]. In the present study, like other plant growth hormones limited concentration of chitosan is more effective in development of multiple shoots. However, the higher concentration of chitosan reduced the growth rate as well as shoot formation. These observations are in accordance to those of Barka *et al*. (2004) [3] in *Vitis vinifera*, and Kim *et al*. (2005) [24] in *Ocimum basilicum*.

Table 2: Effect of cytokinins on PLBs formation and multiple shoot induction.

Phytohormones (µM)			Response (%)	No. of PLBs formation	Shoots		Roots	
BAP	KN	TDZ			Numbers	Length (cm)	Numbers	Length (cm)
2.0			53.34±0.98^{h}	2.95±0.57^{j}	1.23±0.35^{l}	0.62±0.02^{k}	1.00±0.18^{m}	0.24±0.07^{m}
4.0			56.93±0.74^{e}	3.84±1.13^{g}	2.44±0.25^{i}	0.69±0.13^{i}	1.14±0.51^{l}	0.32±0.02^{j}
6.0			60.70±0.69^{d}	4.65±1.08^{c}	3.23±0.37^{e}	0.72±0.42^{h}	1.33±0.11^{k}	0.39±0.12^{h}
8.0			66.19±0.55^{b}	4.72±1.12^{b}	3.75±0.68^{d}	0.79±0.01^{d}	1.67±0.08^{f}	0.45±0.09^{g}
10.0			41.74±0.71^{k}	4.65±0.25^{c}	4.08±1.05^{c}	0.72±0.14^{h}	1.98±0.13^{d}	0.47±0.21^{f}
	2.0		35.51±0.74^{m}	2.25±0.77^{l}	2.34±0.39^{j}	0.62±0.08^{k}	1.06±0.15^{u}	0.12±0.08^{n}
	4.0		42.31±0.66^{k}	2.65±0.56^{k}	2.12±0.48^{k}	0.76±0.07^{e}	1.55±0.04^{h}	0.29±0.42^{k}
	6.0		62.96±0.51^{c}	3.87±0.19^{f}	3.75±0.11^{d}	0.85±0.34^{b}	1.74±0.22^{e}	0.34±0.09^{i}
	8.0		45.79±0.24^{j}	4.46±0.68^{e}	4.25±1.52^{b}	0.74±0.42^{g}	1.42±0.34^{i}	0.54±0.14^{e}
	10.0		37.47±0.54^{l}	3.78±0.35^{h}	2.78±0.34^{h}	0.61±0.27^{l}	1.37±0.02^{j}	0.28±0.03^{l}
		1.0	55.87±0.51^{f}	3.86±0.99^{f}	2.45±0.22^{i}	0.64±0.04^{j}	1.12±0.11^{l}	0.59±0.12^{d}
		2.0	60.45±0.76^{d}	4.63±0.32^{d}	3.11±0.76^{f}	0.75±0.12^{f}	1.62±0.28^{g}	0.68±0.05^{b}
		3.0	70.25±0.62^{a}	4.82±1.01^{a}	4.34±1.03^{a}	0.89±0.04^{a}	2.86±0.05^{a}	1.04±0.66^{a}
		4.0	54.00±0.63^{g}	3.58±0.41^{i}	2.89±0.42^{g}	0.81±0.13^{c}	2.21±0.19^{b}	0.64±0.07^{c}
		5.0	49.45±0.75^{i}	2.07±1.03^{m}	2.12±0.09^{k}	0.62±0.24^{k}	2.05±0.12^{c}	0.32±0.02^{j}

Values represent means ± SE of ten replicates and all the experiments were repeated thrice. Means followed by the same letter are not significantly different according to Tukey's HSD test (P≤0.05)

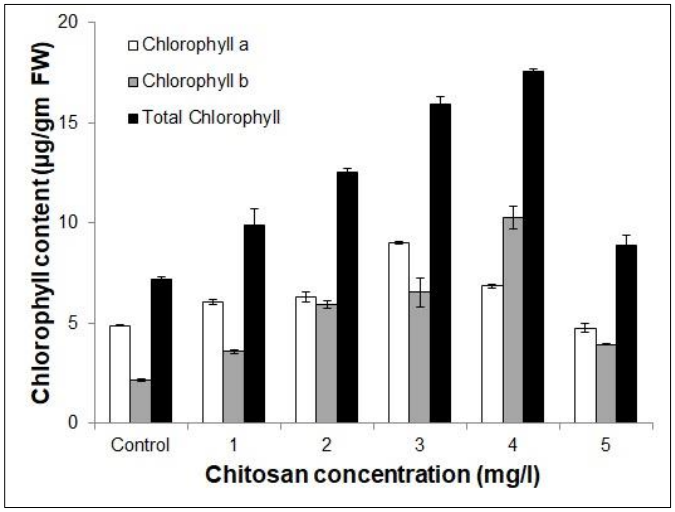


Fig 3: Estimation of total chlorophyll content in chitosan treated samples of *V. coerulea*. Vertical bars indicate ± SE of five replicates.

3.4 Effect of chitosan on chlorophyll

The changes on photosynthetic pigmentation were observed in plantlets treated with different concentrations of chitosan. Chitosan not only helped to increase the plantlets development but it also enhanced the photosynthetic pigmentation. The present experiment showed that effect of chitosan was positively correlated with photosynthetic pigments (Fig. 3). As compared to control samples, the levels of chlorophyll a, chlorophyll b and total chlorophyll were much higher in case of chitosan treated plantlets (Fig. 3). The positive effect of chitosan on enhancing the photosynthetic contents was also reported by another group (Pongprayoon *et al*., 2013) [33].

3.5 Secondary metabolite accumulation

The secondary metabolites were evaluated through total flavonoid, total phenolic, total tannin and total alkaloid content indices. The results of quantitative analysis of phytochemicals are shown in Table 4. The plant derived secondary metabolites have different polarities, thus various solvents (water, acetone, methanol and chloroform) were used to extract the phytochemicals. Earlier study reported that, polyphenolic content from the basil leaves varies with the types of solvents used for the extraction purpose (Złotek *et al*., 2016) [47]. In present study, it is clearly observed that the phytochemical contents yield depends on the solvent nature (Table 4).

Table 3: Effects of chitosan on seedling development of *V. coerulea.*

Chitosan (mg/l)	Response frequency (%)	No. of shoots	Length of shoots (cm)	No. of roots	Length of roots (cm)
Control	68.24±0.19^{d}	4.98±0.06^{f}	4.55±0.21^{e}	2.28±0.16^{f}	2.02±0.07^{f}
1.0	73.9±0.99^{c}	6.29±0.44^{e}	5.50±0.41^{d}	4.80±0.38^{b}	2.54±0.13^{e}
2.0	74.34±1.00^{c}	7.95±1.02^{cd}	6.03±0.42^{cd}	4.34±0.21^{d}	4.32±0.12^{c}
3.0	79.73±0.73^{b}	9.21±0.34^{bc}	7.49±0.21^{b}	4.44±0.22^{c}	4.79±0.09^{b}
4.0	82.80±1.04^{a}	10.28±0.77^{a}	7.73±0.32^{a}	5.67±0.47^{a}	5.85±0.16^{a}
5.0	65.72±0.80^{e}	8.98±0.02^{c}	6.54±0.21^{c}	3.91±0.29^{e}	3.67±0.09^{d}

Values represent means ± SE of ten replicates and all the experiments were repeated thrice. Means followed by the same letter are not significantly different according to Tukey's HSD test ($P \leq 0.05$).

The 4mg/l chitosan treated sample showed maximum phytochemical contents compared to control. The highest TFC was found in the methanolic leaf extract at 4mg/l chitosan treatment, which was increased by 2.38 fold (28.34 ± 0.37 mg QE/ gm FW) and the least was found in the acetone root extract of untreated sample (4.66 ± 0.11 mg QE/ gm FW). Likewise, the chloroformic leaf extract showed 3.07 fold higher phenolic content (27.24 ± 0.21 mg GAE/gm FW) with respect to the control leaf sample of chloroform extract (9.19 ± 0.27mg GAE/gm FW). There was no further enhancement in total flavonoid and phenolic contents was observed at 5 mg/l of chitosan treatment. For extraction of flavonoid content, methanol was found to be the best solvent, similar observation was also reported by Bhattacharyya *et al*. (2016) [4]. Our findings were similar to the earlier reports which suggested that chitosan at low concentrations could induce phenolic compounds in soybean and tomato leaves (Köhle *et al*., 1984; Pearce *et al*., 1998) [26, 31]. The variation in total amounts of alkaloid and tannin contents were also found in extract of leaf and root samples after chitosan treatment. With 4 mg/l chitosan concentration, a higher yield of alkaloids was observed in methanolic leaf extract which is 4.17 fold higher than that of the untreated leaf whereas the amount of TTC increased by 2.57 fold in chloroformic leaf extract. The variation in the secondary metabolite contents is not only depends on the chitosan concentrations but also depend on the nature of solvents used for extraction. Similar.

Table 4: Total flavonoids, phenolics, alkaloids and tannin contents (TFC, TPC, TAC and TTC respectively) in different parts of *V. coerulea* with respect to different solvents (flavonoids: mg QE / gm FW; phenolics: mg GAE / gm FW; alkaloids: mg AE / gm FW; tanins: mg TAE / gm FW) after chitosan treatments.

Chitosan Conc. (mg/l)	Solvent	Leaf				Root			
		TFC	TPC	TAC	TTC	TFC	TPC	TAC	TTC
Control	Methanol	12.32±0.46^{l}	13.21±1.06^{k}	5.95±0.52^{s}	6.67±0.52^{q}	8.33±1.06^{k}	5.18±0.88^{n}	10.17±0.57^{f}	4.52±0.28^{m}
	Chloroform	12.23±1.01^{l}	9.19±0.27^{n}	7.54±0.22^{r}	7.56±0.22^{o}	4.31±0.75^{v}	4.78±0.43^{q}	7.34±0.31^{l}	4.27±1.09^{p}
	Acetone	15.87±0.88^{k}	9.54±0.64^{m}	9.77±0.65^{p}	5.22±0.96^{r}	4.66±0.61^{u}	5.21±0.51^{m}	3.66±0.54^{s}	1.27±0.72^{v}
	Distilled water	9.12±0.82^{m}	7.58±0.11^{q}	8.46±1.02^{q}	4.24±0.29^{s}	5.23±0.22^{s}	3.38±0.21^{t}	2.38±0.21^{w}	3.21±0.43^{t}
1	Methanol	21.38±0.44^{f}	15.89±0.12^{h}	11.36±0.78^{n}	8.44±0.48^{n}	8.52±0.66^{i}	6.69±0.06^{g}	10.36±0.19^{e}	4.76±0.41^{l}
	Chloroform	17.56±0.21^{j}	14.98±0.34^{j}	12.23±0.12^{m}	8.33±0.71^{n}	4.76±0.88t	4.98±0.28^{o}	7.69±0.17^{k}	4.46±0.78^{n}
	Acetone	18.56±0.64^{i}	11.45±0.12^{l}	11.86±0.37^{n}	7.45±1.05^{p}	5.33±0.21^{r}	6.05±0.66^{k}	3.86±0.16^{r}	1.87±0.63^{u}
	Distilled water	8.76±0.12^{n}	7.82±0.21^{p}	10.67±0.4^{o}	8.76±1.14^{l}	6.22±0.05^{q}	4.62±0.18^{r}	2.67±1.03^{v}	3.58±0.18^{r}
2	Methanol	24.5±0.24^{c}	22.38±0.24^{e}	18.69±0.61^{f}	11.34±0.61^{g}	10.21±0.41^{d}	8.34±0.29^{e}	12.32±0.37^{c}	4.85±0.59^{k}
	Chloroform	19.36±0.54^{h}	18.02±0.11^{g}	16.79±0.32^{h}	10.45±0.32^{h}	8.06±0.29^{l}	6.02±0.15^{k}	9.21±0.32^{i}	6.21±0.66^{f}

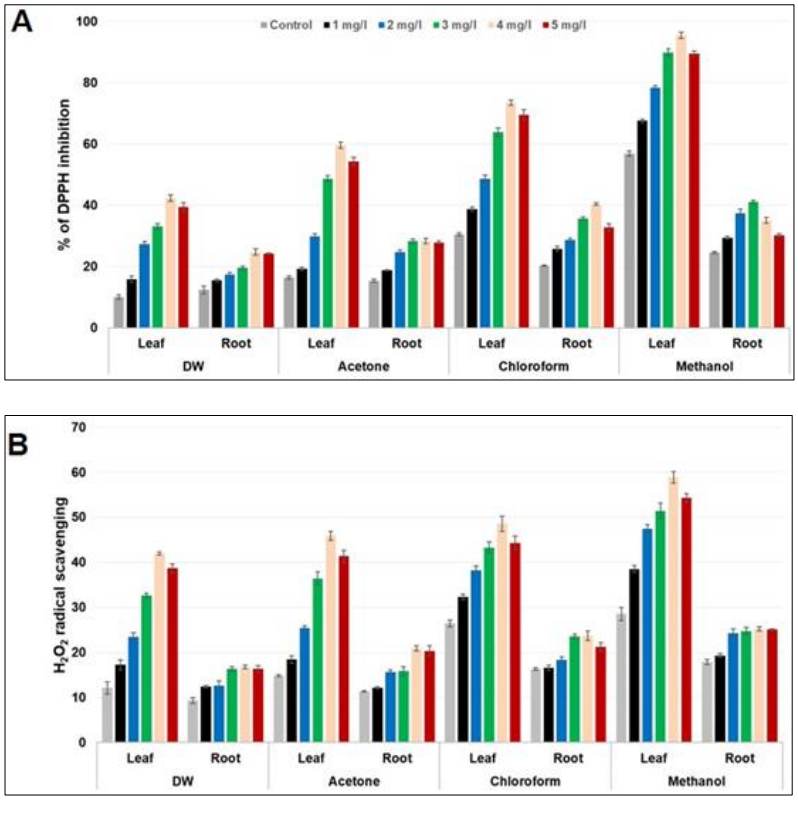
	Acetone	20.42±0.24^{g}	15.27±0.51^{i}	12.85±0.51^{l}	11.56±0.08^{f}	6.21±1.07^{q}	6.27±0.32^{j}	3.92±0.08^{q}	3.35±0.12^{s}
	Distilled water	7.45±1.17^{p}	8.22±0.59^{o}	12.68±0.23^{l}	9.87±0.33^{k}	6.85±0.13^{o}	4.85±0.7^{p}	3.21±0.44^{u}	5.18±0.36^{j}
3	Methanol	26.34±0.86^{b}	25.85±0.38^{b}	22.02±0.22^{c}	14.56±0.22	10.76±0.42^{b}	8.53±0.38^{d}	12.59±0.48^{b}	6.37±0.61^{e}
	Chloroform	24.89±0.11^{c}	25.35±0.27^{b}	20.79±0.66^{d}	17.34±0.27^{c}	8.39±0.33^{j}	8.35±0.31^{e}	9.48±0.62^{h}	6.73±0.27^{c}
	Acetone	22.18±0.34^{e}	19.57±0.33^{f}	15.39±0.61^{j}	16.33±0.37^{e}	8.86±0.18^{h}	6.57±0.27^{h}	4.31±0.51^{o}	3.92±0.11^{q}
	Distilled water	8.02±0.29^{o}	9.34±0.79^{n}	13.06±0.11^{k}	11.95±0.11^{f}	7.19±0.44^{n}	6.34±0.43^{i}	3.57±0.37^{t}	5.49±0.74^{i}
4	Methanol	28.34±0.57^{a}	27.17±0.61^{a}	24.84±0.45^{a}	16.33±0.45^{e}	11.35±0.38^{a}	9.61±0.17^{b}	14.22±0.77^{a}	6.55±0.26^{d}
	Chloroform	26.25±0.36^{b}	27.24±0.21^{a}	22.94±0.19^{b}	19.44±0.19^{a}	9.26±0.41^{f}	10.23±0.22^{a}	9.73±0.58^{g}	7.38±0.19^{a}
	Acetone	26.32±1.07^{b}	23.95±0.9^{d}	16.78±0.21^{h}	18.93±0.21^{b}	10.17±0.37^{e}	7.12±0.35^{f}	4.74±1.02^{m}	5.47±1.05^{i}
	Distilled water	8.95±0.38^{n}	9.58±0.27^{m}	16.58±0.62^{i}	16.58±0.62^{d}	7.45±0.45^{m}	6.58±0.21^{h}	4.58±0.51^{n}	5.77±0.49^{g}
5	Methanol	23.96±0.11^{d}	22.11±0.42^{e}	18.73±0.3^{e}	10.76±0.38^{h}	10.43±0.48^{c}	9.38±0.43^{c}	12.06±0.08^{d}	3.21±0.43^{t}
	Chloroform	18.01±0.42^{i}	25.12±0.61^{c}	18.33±0.27^{g}	8.56±0.27^{m}	7.44±0.24^{m}	4.32±0.39^{s}	8.45±0.24^{j}	7.11±0.44^{b}
	Acetone	20.11±0.33^{g}	15.45±0.24^{h}	10.66±0.09^{o}	10.32±1.09^{i}	9.02±0.94^{g}	5.85±0.22^{l}	4.71±0.72^{m}	4.38±0.96^{o}
	Distilled water	5.67±0.24^{q}	6.34±0.05^{r}	11.56±0.32^{n}	9.98±0.32^{j}	6.33±0.31^{p}	3.34±0.37^{t}	4.18±0.37^{p}	5.65±0.28^{h}

Values represent means ± SE of ten replicates and all the experiments were repeated thrice. Means followed by the same letter are not significantly different according to Tukey's HSD test (P≤ 0.05)

Finding was also observed in *Nardostachys jatamansi* (Bose *et al*., 2016) [6]. Earlier report suggests that various parts of *V. coerulea* is used for traditional folk medicine and cure diseases as they are rich in various phytochemical constituents *viz.*, phenolics, flavonoids, alkaloids and tannins (Medhi and Chakrabarti, 2009) [29]. These phytochemicals have profuse application in treating diseases like inflammation, diabetes and cancer by acting as free radical scavengers (Xanthopoulou *et al*., 2010; Bahadoran *et al*., 2013) [43, 2]. The present findings revealed that compare to untreated samples chitosan treated samples show high yield of phytochemical contents which implies that chitosan might have up regulated the pathway of secondary metabolite synthesis. Similar observations were also found in basil and spinach plants (Singh, 2016; Pirbalouti *et al*., 2017) [39, 32].

3.6 Antioxidant activity after chitosan treatment

The antioxidant activities were measured by three different assays namely, DPPH, H_2O_2 radical scavenging and metal chelating (Fig. 4). There was an increase in the antioxidant activities in all the solvents (water, acetone, methanol and chloroform) with the increase in chitosan concentrations (1-5 mg/l). It was observed that in methanolic extract the best response was recorded for all the three antioxidant activities methods. The highest antioxidant activity was observed by DPPH method in methanolic leaf extract with 4mg/l chitosan treated sample (95.45%) whereas the lowest activity was shown by the untreated leaf sample extracted with distilled water (Fig. 4A). The results obtained from DPPH assay are analogous with H_2O_2 radical scavenging and metal chelating assay. Methanolic leaf extract of chitosan treated sample (4 mg/l) exhibited the strong inhibitory effect against metal ion (87.56%) as well as highest yield of H_2O_2 radical scavenging (Fig. 4B, C). Similarly, highest antioxidant activities have been reported in methanolic extracts of various plants *namely*, *Ceropegia santapaui*, *Nardostachys jatamansi* (Chavan *et al*., 2014; Bose *et al*., 2016) [9, 6]. The occurrence of antioxidant potential could be due to the high yield of phenolic, flavonoid, alkaloid and tannin contents. Correlation coefficient deduced between phytochemicals and antioxidant activities also revealed that the presence of antioxidant activities in micropropagated.



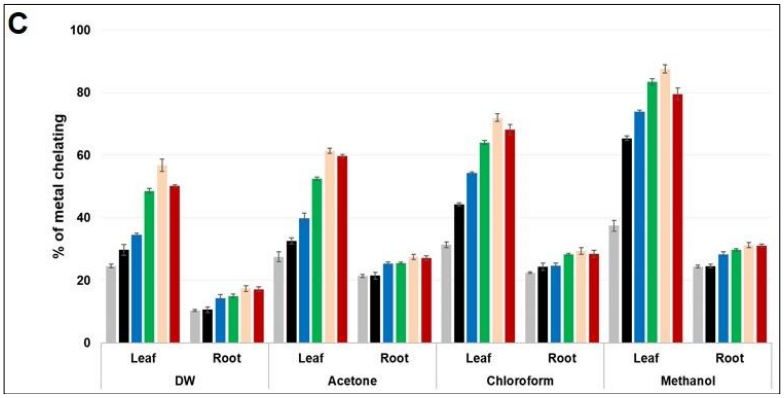


Fig 4: Antioxidant activity of *in vitro* raised chitosan treated plantlets of *V. coerulea* using four different solvents *viz*. distilled water (DW), acetone, chloroform and methanol by (A) DPPH, (B) H_2O_2 radical scavenging and (C) metal chelating assay. Vertical bars indicate ± SE of five replicates.

Table 5: Correlation between antioxidant activity, phytochemical contents and PAL enzyme activity.

Variables	TFC	TPC	TAC	TTC	DPPH	HRS	MC	PAL
TFC	1	0.906	0.958	0.728	0.922	0.938	0.990	0.868
TPC	0.906	1	0.948	0.737	0.994	0.976	0.947	0.965
TAC	0.958	0.948	1	0.813	0.972	0.969	0.959	0.940
TTC	0.728	0.737	0.813	1	0.746	0.695	0.695	0.782
DPPH	0.922	0.994	0.972	0.746	1	0.990	0.954	0.972
HRS	0.938	0.976	0.969	0.695	0.990	1	0.962	0.968
MC	0.990	0.947	0.959	0.695	0.954	0.962	1	0.893
PAL	0.868	0.965	0.940	0.782	0.972	0.968	0.893	1

Pearson correlation matrix; Values in bold are different from 0 with a significance level α = 0.05; DPPH = 1, 1-diphenyl-2-picrylhydrazyl; HRS = hydrogen peroxide radical scavenging; MC = metal chelating; TFC = Total flavonoid content; TPC = total phenolic content; TAC = Total alkaloid content; TTC = Total tannin content; PAL = Phenylalanine ammonia lyase

Plantlets of *V. coerulea* was positively correlated with its phytochemical constituents (Table 5). Similar findings have been reported in other medicinal plant species (Singh, 2016; Bose *et al*., 2016) [39, 6].

3.7 Activity of PAL enzyme

The PAL enzyme is a key regulatory enzyme which produces variety of phytochemicals through phenylpropanoid pathway in plants. The PAL enzyme activity was estimated to correlate the enhancement of secondary metabolites after chitosan treatment. It was observed that PAL activity significantly increased in chitosan treated leaves of micropropagated plantlets (Fig. 5). The highest PAL activity was observed in the plantlets treated with 4 mg/l chitosan in which the activity was 5.12 times greater than that of the control sample. A positive correlation was observed among the enzyme activity and the amount of various phytochemical contents (Table 5), which indicates that chitosan regulated the accumulation of phytochemicals by stimulating the PAL enzyme activity.

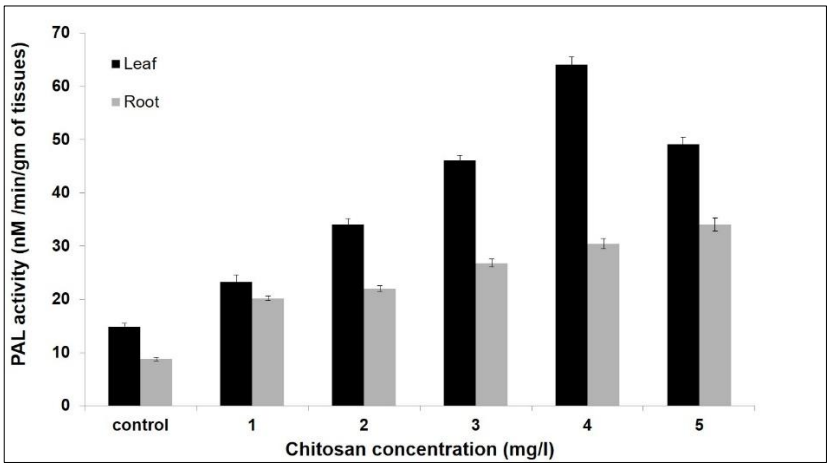


Fig 5: Quantification of phenylalanine ammonia lyase (PAL) enzyme activity of *V. coerulea* after chitosan treatment. Vertical bars indicate ± SE of five replicates.

Similar types of findings have also been documented in soybean and maize leaves (Khan *et al*., 2003; Hura *et al*., 2008) [23, 21]. Our study clearly indicates that chitosan treatment may induce secondary metabolites production via phenylpropanoid pathway in this species of *Vanda* without being subjected to any form of stresses.

4. Conclusion

The present study shows that chitosan has positive effect on shoot multiplication than conventional plant growth regulators within a shorter time span. Besides this, chitosan also helps in enhancing the major secondary metabolites, antioxidant activity along with PAL enzyme activity. An increase in the PAL enzyme activity can be considered as a biochemical indicator for the resistance of the orchid to the biotic stress; given that PAL is the key enzyme for synthesis of secondary metabolites.

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6. Conflict of interest

The authors declare that they do not have any conflict of interest.

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