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# An Efficient *In Vitro* Propagation, Antioxidant and Antimicrobial Activities of *Aphyllorchis Montana* (Reichenb.f.)

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Abstract

An *in vitro* plant regeneration protocol was successfully established in *Aphyllorchis montana*, a saprophytic achlorophyllous orchid by culturing immature seeds. Among the six basal media evaluated for seed germination, BM-TM medium was found to be the best followed by KC medium. After 40 days, all the media turned brown and the growths of the protocorms were arrested. Activated charcoal, 1 g/l in half strength BM-TM was found to be suitable for further development of protocorms. Half strength BM-TM medium was supplemented with different growth regulators either individually or in combinations for multiplication of shoots. Of the five cytokinins tested, TDZ at 6.8 µM was found to be most effective for multiple shoot induction yielding 17.24 ± 0.27 shoots after 10 weeks of culture. Addition of low concentration of NAA (1.3 µM) in MS medium supplemented with the cytokinin TDZ (6.8 µM) favoured shoot multiplication. A mean number of 27.56 ± 0.54 shoots with 3.92 ± 0.11 number of roots were produced per explant. The response of the seed derived protocorm to the different types of organic additives viz., peptone and yeast extract and coconut water was also evaluated. The addition of these organic additives to the medium containing TDZ enhanced the number of shoot regeneration. The plantlets were acclimatized in plastic pots containing sterilized vermiculite. The survival rate was 100 % when maintained in the culture room condition (25 ± 2 °C). Screening of the antibacterial, antioxidant activity and estimation of total phenolics and flavonoid content of methanolic extracts of micropropagated plants were also carried out and compared with that of the wild-grown plants. In all the tests, methanolic extract from wild-grown plants showed higher antioxidant, antimicrobial activity, total phenolics and flavonoid content than *in vitro* propagated plants.

**Keywords:** Antimicrobial activity, Antioxidant activity, *Aphyllorchis montana*, Asymbiotic seed germination, *In vitro* propagation, Plant growth regulators, 2,2-Diphenyl-1- picrylhydrazyl.

**Abbreviations:** IAA (Indol-3-acetic acid), BM-TM (BM-1Terrestrial orchid medium), IBA (Indole-3-butyric acid), NAA (α- Naphthaleneacetic acid), TDZ (Thidiazuron), BA (6-Benzyl adenine), KIN (6-Furfurylaminopurine), 2-iP (2-Isopentenyladenine), $GA_3$ (Gibberellic acid), Zt (Zeatin), DPPH (2, 2-diphenyl-1-picrylhydrazyl), ABTS (2, 2'azinobis (3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt), TPTZ (2, 4, 6-tripyridyl-S-triazine), EDTA (Ethylenediamine tetraacetic acid).

## INTRODUCTION

Orchids belong to the most diverse plant family known to man. They have complex lifecycle, mycorrhizal association (seed germination) and specific pollination syndrome. It is a family of considerable economic importance particularly in horticulture and floristry. Apart from the horticultural value, orchids are used in traditional herbal medicine. Many orchid species are threatened globally by over collection from the natural habitat for horticultural purpose. Plant tissue culture and micropropagation techniquesplay an important role in conservation programs and management of botanical collection.

*Aphyllorchis montana* (family Orchidaceae) is native to the region and rare in natural habitat (Sinu *et al.*, 2012) has drawn much attention in recent years. The extract of it has been in use since olden times to cure cough, cold, anemia and for its vitality strengthening properties (Prajapati *et al.,* 2003). A. montanais used in traditional Indian systems for diabetic activity (Bhavani *et al.,* 2012; Sreenu *et al.*, 2013). The species is also one of the important components of 'Astvarga' and used in the preparation of 'Chyavanprash', a highly popular Ayurvedic tonic. The medicinal properties are possibly due to production of secondary metabolites, including phenolic compounds. The role of antioxidants due to the phenolic compounds in this species cannot be ignored. To fulfill the high demand of pharmaceutical industries, at present raw material is largely being drawn from the wild; this has severely affected its availability in natural habitat and the species has been considered rare in the Western Ghats (Sinu *et al.*, 2012). In spite of this, there has been hardly any effort to commercially cultivate this species.

The vegetative growth of the terrestrial mycohetrrotrophic orchid is absolutely an underground mechanism; it challenges to locate them in vegetative condition, species-specific fungi for seed germination, poor seed viability and low rate of germination in natural condition and poor rooting ability of vegetative cuttings of rhizomes. In spite of this, there has been hardly any effort to commercially cultivate this species. Inevitably, therefore, rapid multiplication of this important drug yielding genotype is imperative. Alternatively, *in vitro* micropropagation would be beneficial in accelerating large scale multiplication and conservation of this important plant species. *In vitro* seed germination has been suggested as a suitable propagation method for conservation of orchids (Kauth *et al.,* 2006; Stewart and Kane, 2006).Therefore, development of rapid protocol for high frequency *in vitro* plant regeneration in this important medicinal herb became necessary in order to reduce the existing pressure on natural populations and continuous supply of plant materials for the pharmaceutical industry. As tissue culture technique has now become a well–established method for large scale plants developed for commercial utilization of several endangered medicinal plants. The major goal of the present investigation was to standardize the best media for seed germination, growth regulators combinations for high frequency plantlet production from protocorm explants of *A. montana* species. In order to enhance the shoot bud multiplication rate, various growth regulators were examined to identify the best growth hormone combinations for maximum number of shoot buds production and to evaluate the antioxidant, antimicrobial activity of both wild-grown and *in vitro* regenerated plants.

## MATERIALS AND METHODS

### Plant material, explant preparation and surface sterilization

Immature seeds of *Aphyllorchis Montana* (Reichenb.f.) were collected from Vellingiri Hills (longitude 60-40' and 70-10' E and latitude 10o-55 and 11o-10' N 1200) at an attitude of 1000-1250 msl. Tamilnadu, India. Freshly collected green capsules were washed thoroughly under running tap water. The pods were then immersed in Teepol3 - 5%(v/v) for 2 - 5 minutes by continuous shaking and then rinsed with sterile distilled water thrice and the pod were pretreated with 0.1% (W/V) of Bavistin, a fungicide for 5 minutes and rinsed in double distilled water. Then the explants were surface sterilized in 0.01% mercuric chloride solution for 5 minutes and rinsed thoroughly with sterile distilled water (5-7 times). The capsules were dipped in 70% ethanol for 30 seconds

and flamed. The surface sterilized pods were cut opened with sterile blade and seeds were extracted using sterileforceps and spread as thin film in test tubes containing 20 ml of culture media. Cultures were maintained at 25 ± 2 ºC under cool white fluorescent tubes at a light intensity of 50 µmol $m^{-2}$ $s^{-1}$ with 16/8-h L/D photoperiod.

**Seed viability test**

Seed viability was tested according to Vellupillai *et al.* (1997). For enumerating seed viability percentage, seeds from the fresh capsules were treated with 1% (w/v) 2, 3, 5-triphenyl tetrazolium chloride (pH 7.0) in the dark overnight. Treated seeds were observed with a light microscope and scored as either viable (red embryo) or nonviable (white embryo).

**Optimization of culture medium for asymbiotic seed culture**

Immature seeds of *A. Montana* were inoculated on Knudson C modified Morel (Morel1965; KCM), Lindemann (Lindemann *et al*., 1970; LM), Mitra medium (Mitra *et al.*, 1976; M), Knudson C medium (Knudson, 1946; KC), Murashige and Skoog medium (Murashige and Skoog, 1962, MS) and BM-1-Terrestrial Orchid media (Van Waes and Debergh,1986 procured from Hi-Media Laboratories Mumbai, India) initially to find out the suitable medium for maximum seed germination. The best medium for seed germination was selected for further studies. All media contained 2% sucrose and were solidified with 0.8% agar (Hi Media Laboratories, India). Activated charcoal (Hi-Media Laboratories Mumbai, India) was added at 0.5, 1.0 or 2.0 g/l to half strength basal BM-TM medium to arrest phenolic exudation and further development of the protocorms. The pH of the media was adjusted to 5.6–5.8 with 1 N NaOH or HCl before autoclaving at 121ºC, 105 kPa for 20 min.

**Multiplication of protocorms**

For the multiplication of protocorms, BM-TM medium was supplemented with cytokinins such as BA (1.10 to 8.80 µM), Kn (1.15 to 9.20 µM), 2-iP (1.01 to 8.12), Zt (1.1 to 9.1 µM) and TDZ (1.1 to 9.0 µM) either individually or in combination with NAA (1.3 µM). All media contained 20 $gl^{-1}$ sucrose and were solidified with 0.8% agar (Hi Media Laboratories, India). The cultures were maintained at 25 ± 2ºC temperature with 75–80% relative humidity and a 16/8 h (light/dark) photoperiod provided with diffuse light (50 µmol $m^{-2}$ $s^{-1}$). Final observation on the number of multiple shoot and the shoot length, root number and root length were recorded after 70 days of culture.

***Ex vitro* plant establishment**

For *ex vitro* establishment, well-developed plantlets were rinsed thoroughly with tap water to remove residual nutrients and agar from the plant body and transplanted to plastic pot containing vermiculite. The plastic pots were covered initially with polyethylene bags and maintained for two months inside the culture room for acclimatization under cool white tubular fluorescent lights (40 W, 220 V, Philips Electronics India Ltd.) at 50 µ$mol^{-1}$ $m^{-2}$ $s^{-1}$ with a 16 h photoperiod at 25±2°C. The transplants were transferred to National Orchidarium, Yearcaud, Tamil Nadu.

**Preparation of methanol extracts**

The biomass from *in vitro* propagated plants (8 week old culture, BM-TM medium supplemented with TDZ (6.8µM) and NAA (1.3µM) shoots were collected) and wild grown plants (whole plants) were washed under tap water and dried in oven at 60ºC for two days. The material was powdered by using electric blender and stored in clean labelled airtight bottles. The powder (100g) was extracted by maceration in 300 ml of methanol (100%) for 3 days with frequent agitation. The mixture was filtered through Whatman No. 1 filter paper and the filtrate was concentrated and dried in petridishes at 60ºC in the oven.

**Estimation of total phenolics (TPC) and flavonoids content (TFC)**

The total phenolics content of the extracts was determined and calculated as gallic acid equivalent (GAE) in mg/g DW from the calibration curve according to method described by Siddhuraju and Becker (2003). The total flavonoids content of sample extract was determined following a colorimetric method and values were expressed as mg/g rutin equivalent (RE) of extract according to the method described by Zhishen *et al*.(1999).

***In vitro* antioxidant activity**

The free radical scavenging activity of the *A. Montana* methanol extracts of wild-grown plants and *in vitro* propagated plants were evaluated by using DPPH$^{\cdot}$ (Blois, 1958), ABTS$^{\cdot+}$ cation radical (Re *et al*., 1999) and ferric reducing antioxidant power (FRAP) activity (Pulido *et al.*, 2000) were measured using standard methods.

**Antimicrobial activity**

**Test bacteria**

The antibacterial activity of isolated compounds 1-6 were evaluated against 6 pathogenic bacteria such as *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), *Streptococcus pnumoniae* (ATCC 33400) *Klebsiella pnumoniae* (ATCC 10031) and *Bacillus subtilis* (ATCC 6633) procured from the Institute of Microbial Technology (IMTECH) Chandigarh, India. All the strains were stored in the appropriate medium before use.

**Disc diffusion method**

Disc diffusion method (Joshi *et al*., 2010) was used for the evaluation of antibacterial activity of *A. Montana* methanol extracts of wild-grown plants and *in vitro* propagated plantsusing 100 µl of suspension containing $10^8$ CFU/ml of bacteria spread on the inoculated agar. A sterile cotton swab was dipped into the inoculums suspension to remove the excess of fluid. Whatmann filter paper discs (6 mm diameter) were prepared at the concentration of 25 µg/disc for wild-grown plants and *in vitro* propagated plants extracts and 10 µg/disc reference antibiotic (Ciprofloxacin). A disc prepared with only the corresponding volume of DMSO was used as negative control. The petriplates were then incubated and antimicrobial activity was evaluated by measuring the diameter of the zones of inhibition around the disc. The experiments were repeated in triplicate and the result was expressed as average value.

**Minimum inhibitory concentration**

The minimum inhibitory concentration (MIC) of wild-grown plants and in -vitro propagated plants of A. montanawas determined using the micro-dilution assay in 96-well microplates (Siddiqi *et al*., 2011). Briefly, 500 µl of each re-suspended sample (1.0 mg/ml) in DMSO (2%). Serial two-fold dilutions were prepared from the stock solution to give concentrations ranging from 500µg to 3.90 µg/ml of the wild-grown plants and *in vitro* propagated plants extracts. The highest concentration of DMSO remaining after dilution (5%, v/v) caused no inhibition to bacterial growth. DMSO served as negative control. Streptomycin and Ciprofloxacin were served as positive controls. An aliquot of 100 µl standardized suspension of the test bacteria (108 CFU/ ml) was transferred to a well of 96 plates. Then, 100 µl diluted samples were also added to each well and incubated at 37°C for 24h. The MIC was defined as the lowest concentration of samples which inhibited the visible growth of tested microorganisms. For further reconfirmation, 20 µl of MTT reagent (1mg/ml) was added as an indicator for microbial growth to each well of the microtitre plates, followed by 20 min incubation at 37°C. The reagent-suspension colour will remain clear or yellowish indicating complete inhibition activity as opposed to dark blue for growth (Eloff, 1998). The MIC was recorded as the most repeatable minimum concentration of triplicate.

**Experimental design and statistical analysis**

The percent of seed germination was recorded after 10, 20 and 30 days of culture. Percent germination was calculated by dividing the number of germinated seeds by the total number of seeds inoculated. Number of multiple shoots, height, root number and root length was recorded after 70 days of culture. The experiments were repeated thrice and each set had five replicates. The significance of differences among means was assessed out using Duncan's multiple range test at P < 0.05 (ANOVA). Data of the antioxidant, total phenolic and flavonoid assays were expressed as the mean ± standard deviation (SD) of three independent measurements. Correlation analysis was performed between phenolics and flavonoids with antioxidant activity using Pearson correlation two-tailed. The results were analyzed statistically using SPSS Version 17 (SPSS Inc., Chicago, USA).

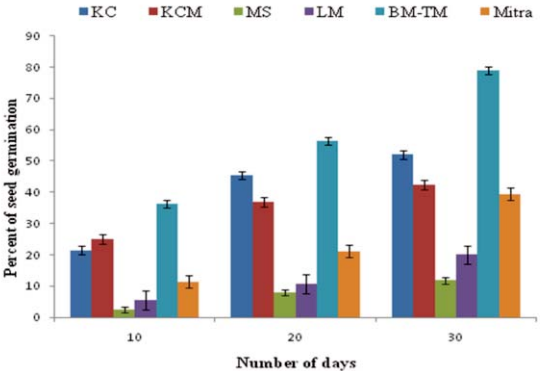


Fig.1. Effect of various culture media on seed germination of *A. montana*
Error bars indicates ± S.E

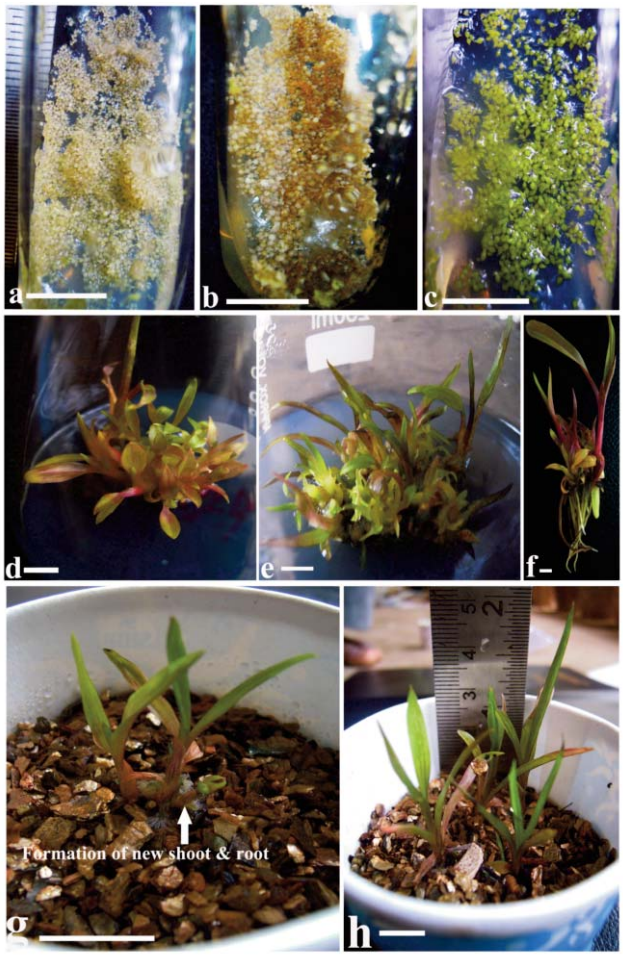


Fig.2. Asymbiotic seed germination and multiple shoot development of *A. montana* (Bar-1cm)
a. Seed derived protocorms developed on BM-TM medium after 30 days of culture
b. Phenolic exudation on BM-TM medium
c. Development of protocorms on half strength BM-TM medium with activated charcoal 1 g/l
d. Development of multiple protocorm on half strength BM-TM medium supplemented with TDZ at 9.8 µM
e. Multiple protocorm development on half strength BM-TM medium fortified with TDZ (6.8µM) and 20% CW
f. Regenerated plantlet
g. Hardened plantlet
h. Hardened plantlet after 2 months under *ex vitro* condition

## RESULTS

### *In vitro* seed germination

Tetrazolium (TZ) viability test indicated a mean embryo viability of 90%. Seeds germinated on all the media tested, however, the percentage of seed germination and protocorm developmentwas not consistent and varied in different media. The embryos enlarged and occupied the entire seed coat within 10 days after sowing. Germination as evidenced by enlargement of the embryo was first observed in BM-TM medium, followed by both KC and KCM medium. The seed germination was low in MS, Mitra and Lindmann orchid medium (Fig. 1).

After 30 days, the embryos by repeated cell divisions emerged and rupturing the testa in BM-TM medium (Fig. 2a). The percentage seed germination was 79% in BM-TM basal medium (Fig. 2a), 52% in KC medium and 42.50 % in KCM medium. After 40 days, all the media turned brown possibly indicating phenolic exudation and further development of protocorms were arrested (Fig. 2b).The above medium was reduced half strength and supplemented with activated charcoal at various concentrations (0.5 to 3 g/l) to pre-

Table 1. Effect of cytokinins on multiple shoot induction from seed derived protocorm of *A. montana.*

| BA (µM/l) | Kn (µM/l) | Z (µM/l) | TDZ (µM/l) | 2ip (µM/l) | No. of hoots/explant | Shoot length (cm) | No. of Root/explant | Length of root (cm) |
|---|---|---|---|---|---|---|---|---|
| 1.10 | - | - | - | - | 1.20±0.71 $^{g}$ | 2.60±0.66 $^{bc}$ | 3.76±0.23 $^{b}$ | 1.53±0.22 $^{d}$ |
| 2.20 | - | - | - | - | 2.56±0.54 $^{f}$ | 2.00±0.12 $^{d}$ | 3.25±0.45 $^{c}$ | 1.09±0.46 $^{de}$ |
| 3.30 | - | - | - | - | 5.12±0.33 $^{d}$ | 2.41±0.34 $^{c}$ | 2.30±0.76 $^{de}$ | 1.22±0.34 $^{de}$ |
| 4.40 | - | - | - | - | 9.51±0.49 $^{c}$ | 2.01±0.52 $^{d}$ | 2.38±0.25 $^{de}$ | 2.19±0.63 $^{c}$ |
| 6.60 | - | - | - | - | 8.51±0.12 $^{c}$ | 1.16±0.22 $^{f}$ | 2.13±0.67 | 2.72±0.62 $^{b}$ |
| 8.80 | - | - | - | - | 5.76±0.21 $^{d}$ | 1.00±0.40 $^{f}$ | 1.84±0.12 $^{f}$ | 3.30±0.20 $^{a}$ |
| - | 1.15 | - | - | - | 1.10±0.73 $^{g}$ | 1.00±0.56 $^{f}$ | 1.90±0.71 $^{f}$ | 3.09±0.90 $^{a}$ |
| - | 2.32 | - | - | - | 1.32±0.26 $^{g}$ | 2.11±0.77 $^{d}$ | 2.18±0.29 $^{e}$ | 3.12±0.17 $^{a}$ |
| - | 3.45 | - | - | - | 2.65±0.12 $^{f}$ | 2.19±0.14 $^{d}$ | 2.64±0.21 $^{d}$ | 2.16±0.24 $^{c}$ |
| - | 4.64 | - | - | - | 3.97±0.75 $^{de}$ | 2.52±0.40 $^{bc}$ | 1.89±0.78 $^{f}$ | 2.06±0.87 $^{c}$ |
| - | 6.90 | - | - | - | 5.14±0.65 $^{d}$ | 2.82±0.49 $^{b}$ | 3.65±0.62 $^{bc}$ | 1.72±0.89 $^{d}$ |
| - | 9.20 | - | - | - | 7.50±0.30 $^{cd}$ | 2.42±0.43 $^{c}$ | 3.12±0.62 $^{cd}$ | 1.16±0.51 $^{de}$ |
| - | - | 1.1 | - | - | 4.60±0.16 $^{de}$ | 2.63±0.87 $^{bc}$ | 1.34±0.52 $^{g}$ | 1.71±0.41 $^{d}$ |
| - | - | 2.2 | - | - | 1.25±0.50 $^{g}$ | 2.61±0.54 $^{bc}$ | 2.00±0.70 $^{e}$ | 1.54±0.26 $^{d}$ |
| - | - | 3.3 | - | - | 2.53±0.11 $^{f}$ | 3.00±0.20 $^{a}$ | 3.12±0.41 $^{cd}$ | 2.73±0.31 $^{b}$ |
| - | - | 4.5 | - | - | 5.54±0.64 $^{d}$ | 2.81±0.28 $^{b}$ | 3.89±0.63 $^{b}$ | 2.39±0.32 $^{bc}$ |
| - | - | 6.8 | - | - | 7.20±0.81 $^{cd}$ | 3.00±0.79 $^{a}$ | 3.60±0.29 $^{bc}$ | 3.66±0.12 $^{a}$ |
| - | - | 9.1 | - | - | 12.65±0.16 $^{b}$ | 2.84±0.65 $^{b}$ | 2.72±0.56 $^{d}$ | 2.53±0.34 $^{bc}$ |
| - | - | - | 1.1 | - | 2.30±0.83 $^{f}$ | 3.16±0.66 $^{a}$ | 2.29±0.12 $^{de}$ | 1.75±0.34 $^{d}$ |
| - | - | - | 2.2 | - | 3.65±0.76 $^{e}$ | 3.00±0.79 $^{a}$ | 2.69±0.34 $^{d}$ | 2.00±0.00 $^{c}$ |
| - | - | - | 3.3 | - | 5.65±0.76 $^{d}$ | 2.71±0.54 $^{b}$ | 2.93±0.18 $^{de}$ | 2.19±0.11 $^{c}$ |
| - | - | - | 4.5 | - | 8.97±0.75 $^{c}$ | 2.01±0.02 $^{d}$ | 2.31±0.51 $^{de}$ | 2.30±0.13 $^{bc}$ |
| - | - | - | 6.8 | - | 17.24±0.27 $^{a}$ | 1.46±0.02 $^{e}$ | 2.12±0.11 $^{e}$ | 2.70±0.71 $^{b}$ |
| - | - | - | 9.0 | - | 12.30±0.65 $^{b}$ | 1.00±0.40 $^{f}$ | 4.94±0.22 $^{a}$ | 2.00±0.92 $^{c}$ |
| - | - | - | - | 1.01 | 2.15±0.58 $^{f}$ | 2.11±0.57 $^{d}$ | 2.12±0.34 $^{e}$ | 1.09±0.92 $^{de}$ |
| - | - | - | - | 2.03 | 2.87±0.98 $^{f}$ | 2.41±0.54 $^{c}$ | 3.81±0.70 $^{b}$ | 1.48±0.44 $^{d}$ |
| - | - | - | - | 3.04 | 3.87±0.70 $^{e}$ | 3.00±0.69 $^{a}$ | 3.61±0.07 $^{bc}$ | 2.00±0.23 $^{c}$ |
| - | - | - | - | 4.06 | 5.82±0.23 $^{d}$ | 3.00±0.10 $^{a}$ | 3.53±0.75 $^{bc}$ | 2.50±0.32 $^{bc}$ |
| - | - | - | - | 6.09 | 9.70±0.65 $^{c}$ | 2.42±0.40 $^{c}$ | 3.50±0.65 $^{bc}$ | 2.43±0.43 $^{c}$ |
| - | - | - | - | 8.12 | 12.65±0.45 $^{b}$ | 2.84±0.92 $^{b}$ | 3.12±0.29 $^{c}$ | 2.87±0.69 $^{b}$ |

Values represent mean ± S.E. of twice repeated experiments each with 5 replications. Means in a column with the different letter (superscript) are significantly different according to DMRT ($P < 0.05$).

vent the phenolic exudation and induce further development of protocorms (Fig. 2c). Among the different concentrations of activated charcoal tested, 1g/l completely arrested phenolic exudation and it was found to bethe optimum concentration for the development of protocorms.

**Effect of cytokininson development of multiple shoots**

The seed derived protocorm explants cultured on half strength BM-TM medium without any growth regulators formed a single shoot. In the presence of cytokinin, the explants responded positively in producing multiple shoots (multiple seedlings). Of the five cyokinins tested, TDZ was found to be more efficient than other cytokinins with respect to initiation and subsequent proliferation of shoots (Table 1). Among the different levels TDZ tested, the maximum number of shoots was observed on the half strength BM-TM medium containing 6.8 µM of TDZ (17.24 ± 0.27) (Table 1, Fig. 2d).The shoot buds first appeared as small white protuberances at basal surface at the protocorm, which eventually developed into multiple shoots within 30-35 days.The number of shoot buds increased with increasing concentration of TDZ up to an optimal level of 6.8 µM. Among the various concentrations of Zt and 2-ip tested, maximum number of the multiple shoots was recorded in half strength BM-TM medium supplemented with 9.1 µM Z and 8.12 µM 2-iP (12.65 shoots/explant). NAA (1.3 µM) in combination with cytokinins also favored shoot formation. However, the response for multiple shoot induction varied (Table 2). Among the different combinations, maximum number of shoots were produced in half strength BM-TM medium supplemented with TDZ (6.8 µM) and NAA (1.3 µM).

**Effect of TDZ and growthadjuvants on multiple shoot induction**

Multiple shoots were also formed when seed derived protocorms were cultured on BM-TM medium supplemented with TDZ at 6.8 µM in combination with growth adjuvants like pep-

Table 2. Effect of cytokinins and auxin on multiple shoot developed from seed derived protocorm of *A. montana.*

| BA (µM) | Kn (µM) | Z (µM) | TDZ (µM) | 2-ip (µM) | NAA (µM) | No. of shoot /explant | Shoot length (cm) | No. of Root/ex-plant | Root length (cm) |
|---|---|---|---|---|---|---|---|---|---|
| 1.10 | - | - | - | - | 1.3 | 1.48±0.61 $^{h}$ | 2.06±0.12 $^{bc}$ | 4.56±0.11 $^{bc}$ | 1.00±0.11 $^{d}$ |
| 2.20 | - | - | - | - | 1.3 | 3.76±0.54 $^{fg}$ | 2.34±0.87 $^{b}$ | 5.12±0.34 $^{bc}$ | 1.22±0.54 $^{d}$ |
| 3.30 | - | - | - | - | 1.3 | 7.62±0.53 $^{e}$ | 2.65±0.66 $^{b}$ | 6.74±0.54 $^{b}$ | 1.76±0.76 $^{d}$ |
| 4.40 | - | - | - | - | 1.3 | 12.47±0.34 $^{d}$ | 2.70±0.33 $^{b}$ | 10.12±0.61 $^{a}$ | 1.00±0.12 $^{d}$ |
| 6.60 | - | - | - | - | 1.3 | 10.51±0.54 $^{d}$ | 3.11±0.44 $^{b}$ | 7.09±0.12 $^{b}$ | 1.22±0.62 $^{d}$ |
| 8.80 | - | - | - | - | 1.3 | 6.76±0.63 $^{e}$ | 1.00±0.31 $^{c}$ | 4.17±0.44 $^{bc}$ | 2.00±0.43 $^{c}$ |
| - | 1.15 | - | - | - | 1.3 | 1.76±0.21 $^{h}$ | 1.22±0.31 $^{c}$ | 1.30±0.41 $^{e}$ | 1.56±0.23 $^{d}$ |
| - | 2.32 | - | - | - | 1.3 | 2.11±0.46 $^{g}$ | 2.23±0.54 $^{bc}$ | 2.32±0.18 $^{d}$ | 1.12±0.21 $^{d}$ |
| - | 3.45 | - | - | - | 1.3 | 3.54±0.14 $^{fg}$ | 2.43±0.54 $^{b}$ | 3.11±0.65 $^{c}$ | 2.16±0.32 $^{c}$ |
| - | 4.64 | - | - | - | 1.3 | 4.32±0.80 $^{f}$ | 2.65±0.10 $^{b}$ | 4.50±0.23 $^{bc}$ | 2.06±0.43 $^{c}$ |
| - | 6.90 | - | - | - | 1.3 | 5.64±0.55 $^{f}$ | 3.13±0.23 $^{a}$ | 4.21±0.11 $^{bc}$ | 1.10±0.54 $^{d}$ |
| - | 9.20 | - | - | - | 1.3 | 7.50±0.37 $^{e}$ | 3.61±0.55 $^{a}$ | 4.82±0.62 $^{bc}$ | 1.21±0.11 $^{d}$ |
| - | - | 1.1 | - | - | 1.3 | 1.60±0.56 $^{h}$ | 2.97±0.16 $^{ab}$ | 1.02±0.11 $^{e}$ | 1.65±0.20 $^{d}$ |
| - | - | 2.2 | - | - | 1.3 | 2.62±0.84 $^{g}$ | 3.20±0.19 $^{a}$ | 2.34±0.45 $^{d}$ | 1.23±0.43 $^{d}$ |
| - | - | 3.3 | - | - | 1.3 | 2.70±0.65 $^{g}$ | 3.61±0.89 $^{a}$ | 2.23±0.41 $^{d}$ | 2.73±0.31 $^{b}$ |
| - | - | 4.5 | - | - | 1.3 | 4.47±0.07 $^{f}$ | 3.44±0.29 $^{a}$ | 3.89±0.63 $^{c}$ | 2.39±0.32 $^{c}$ |
| - | - | 6.8 | - | - | 1.3 | 5.32±0.66 $^{f}$ | 3.87±0.64 $^{a}$ | 3.60±0.29 $^{c}$ | 3.66±0.12 $^{a}$ |
| - | - | 9.1 | - | - | 1.3 | 7.33±0.87 $^{e}$ | 3.98±0.16 $^{a}$ | 2.72±0.56 $^{d}$ | 2.53±0.34 $^{b}$ |
| - | - | - | 1.1 | - | 1.3 | 4.81±0.33 $^{f}$ | 3.16±0.62 $^{a}$ | 2.29±0.12 $^{d}$ | 1.75±0.34 $^{d}$ |
| - | - | - | 2.2 | - | 1.3 | 7.12±0.87 $^{e}$ | 3.45±0.69 $^{a}$ | 2.69±0.34 $^{d}$ | 2.00±0.00 $^{c}$ |
| - | - | - | 3.3 | - | 1.3 | 12.57±0.61 $^{d}$ | 2.67±0.21 $^{b}$ | 2.93±0.18 $^{cd}$ | 2.19±0.11 $^{c}$ |
| - | - | - | 4.5 | - | 1.3 | 19.78±0.81 $^{bc}$ | 2.35±0.98 $^{b}$ | 3.31±0.51 $^{c}$ | 2.30±0.13 $^{c}$ |
| - | - | - | 6.8 | - | 1.3 | 27.56±0.54 $^{a}$ | 1.56±0.33 $^{c}$ | 3.92±0.11 $^{bc}$ | 2.70±0.71 $^{b}$ |
| - | - | - | 9.0 | - | 1.3 | 22.65±0.11 $^{b}$ | 1.34±0.54 $^{c}$ | 4.23±0.32 $^{bc}$ | 3.41±0.12 $^{a}$ |
| - | - | - | - | 1.01 | 1.3 | 2.98±0.12 $^{g}$ | 2.43±0.67 $^{b}$ | 2.12±0.34 $^{d}$ | 1.32±0.14 $^{d}$ |
| - | - | - | - | 2.03 | 1.3 | 1.41±0.17 $^{h}$ | 2.78±0.88 $^{b}$ | 3.78±0.32 $^{c}$ | 1.98±0.35 $^{cd}$ |
| - | - | - | - | 3.04 | 1.3 | 3.25±0.24 $^{fg}$ | 3.22±0.23 $^{a}$ | 3.24±0.34 $^{c}$ | 2.23±0.34 $^{c}$ |
| - | - | - | - | 4.06 | 1.3 | 5.98±0.35 $^{f}$ | 3.54±0.82 $^{a}$ | 3.76±0.87 $^{c}$ | 2.17±0.41 $^{c}$ |
| - | - | - | - | 6.09 | 1.3 | 10.70±0.89 $^{d}$ | 2.17±0.10 $^{bc}$ | 3.87±0.76 $^{c}$ | 2.21±0.91 $^{c}$ |
| - | - | - | - | 8.12 | 1.3 | 16.43±0.54 $^{c}$ | 1.65±0.92 $^{c}$ | 3.15±0.33 $^{c}$ | 2.11±0.43 $^{c}$ |

Values represent mean ± S.E. of twice repeated experiments each with 5 replications. Means in a column with the different letter (superscript) are significantly different according to DMRT ($P < 0.05$).

Table 3. Effect of growth adjuvants on multiple shoot induction from seed derived protocorm of *A. montana.*

| TDZ (µM) | Peptone (g/l) | Coconut water (%) | Yeast extract (g/l) | No. of shoots | Shoot length (cm) | Root number | Root length (cm) |
|---|---|---|---|---|---|---|---|
| 6.8 | Control | | | 17.24±0.27 $^{e}$ | 1.46±0.02 $^{c}$ | 2.12±0.11 $^{d}$ | 2.70±0.71 $^{a}$ |
| 6.8 | | | | 18.66±0.11 $^{e}$ | 1.11±0.31 $^{e}$ | 2.19±0.32 $^{d}$ | 1.92±0.22 $^{b}$ |
| 6.8 | 0.5 | - | - | 20.21±0.26 $^{de}$ | 1.16±0.82 $^{e}$ | 1.98±0.18 $^{e}$ | 1.70±0.43 $^{b}$ |
| 6.8 | 1.0 | - | - | 23.82±0.32 $^{c}$ | 0.97±0.12 $^{f}$ | 1.50±0.12 $^{f}$ | 1.40±0.65 $^{c}$ |
| 6.8 | 2.0 | - | - | 20.89±0.44 $^{de}$ | 0.46±0.14 $^{g}$ | 1.00±0.43 $^{g}$ | 0.98±0.36 $^{e}$ |
| 6.8 | 3.0 | - | - | 21.71±0.54 $^{d}$ | 1.76±0.23 $^{b}$ | 2.12±0.22 $^{d}$ | 1.10±0.12 $^{d}$ |
| 6.8 | - | 5 | | 23.61±0.11 $^{c}$ | 1.49±0.62 $^{c}$ | 3.92±0.31 $^{c}$ | 1.87±0.11 $^{b}$ |
| 6.8 | - | 10 | | 27.24±0.76 $^{b}$ | 1.17±0.39 $^{e}$ | 5.54±0.52 $^{a}$ | 1.72±0.18 $^{b}$ |
| 6.8 | - | 15 | | 28.33±0.27 $^{a}$ | 1.98±0.42 $^{a}$ | 7.21±0.29 $^{a}$ | 1.45±0.13 $^{c}$ |
| 6.8 | - | 20 | | 17.89±0.61 $^{e}$ | 1.21±0.26 $^{d}$ | 1.00±0.32 $^{g}$ | 0.50±0.17 $^{f}$ |
| 6.8 | - | - | 0.5 | 14.32±0.79 $^{f}$ | 1.11±0.13 $^{e}$ | 1.00±0.11 $^{g}$ | 0.40±0.11 $^{f}$ |
| 6.8 | - | - | 1.0 | 12.18±0.43 $^{g}$ | 1.00±0.43 $^{f}$ | 0.91±0.11 $^{g}$ | 0.10±0.03 $^{g}$ |
| | - | - | 2.0 | 9.29±0.99 $^{h}$ | 1.00±0.54 $^{f}$ | 0.00±0.00 $^{h}$ | 0.00±0.00 $^{h}$ |
| | | | 3.0 | | | | |

Values represent mean ± S.E. of twice repeated experiments each with 5 replications.
Means in a column with the different letter(superscript) are significantly different according to DMRT ($P < 0.05$).

tone, yeast extract and coconut water (0.5 g- 3 g/l or 5-20%). The mean number of multiple shoots varied among the treatments (Table 3). A significantly higher number of multiple shoots (28.33 ± 0.27)were obtained on medium supplemented with TDZ (6.8 µM) and 20% CW (Fig. 2e). Other organic additives were also effective in inducing multiple shoots, however their number remained low when compared to CW (20%). The mean length of the shoot per explant was in the range of 0.46 ± 0.14 - 1.76 ± 0.23 cm which is lower when compared to the medium supplemented with TDZ (6.8 µM) individually (Table 1).

Table 4. Comparison of total phenol, flavonoids content and antioxidant activity of in vitro propagated plant and wild grown plant of *A. montana*

| Sample | Total phenolics content (TPC) (mg/ga) | Total flavonoids content (TFC) (mg/gb) | DPPH $IC_{50}$ (µg/ml) | FRAP (mmol Fe(II)/g extractc) | ABTS (µmoltrolox/g extractd) |
|---|---|---|---|---|---|
| Wild grown plant extract | 121.34 ±1.23 | 93.62 ±1.17 | 26.87 [a] | 2987.10±19.33 [a] | 38912.19± 456.12 [a] |
| *In vitro* propagated plants | 61.89 ±3.19 | 34.59 ±1.14 | 41.45 [c] | 1034.23±67.44 [c] | 20246.53± 291.24 [c] |
| BHA | | | 34.59 [b] | 1213.89±80.86 [b] | 21682.08± 359.47 [b] |

Values are mean of three replicate determinations standard deviation. Mean values followed by different superscript in a column are significantly different (P< 0.05).
[a] gallic acid equivalents (GAE)
[b] rutin equivalent (RE)
[c] mmol of ferrous equivalents / g extract;
[d] µmol of trolox equivalents/ g extract;
BHA- Butylated hydroxyl anisole

Table 5. Correlation between phenolics, flavonoids and different antioxidant parameters of *in vitro* regenerated and wild grown plant methanol extract of *A. Montana*.

| Parameters | Phenolics | | Flavonoids | |
|---|---|---|---|---|
| | Wild grown plant extract | *In vitro* propagated plants | Wild grown plant extract | *In vitro* propagated plants |
| DPPH | -0.987** | 0.478 | -0.971** | 0.669 |
| ABTS | 0.980** | 0.898 | 0.973** | 0.732 |
| FRAP | 0.992** | -0.672 | 0.966* | 0.980** |

### *Ex vitro* establishment of plantlets

The plantlets (Fig. 2f) were transferred to the potting medium containing vermiculite and covered with polythene bag. After 2 months, the cover was gradually loosened, thus dropping the humidity (65–70%). This procedure subsequently resulted in *in vitro* hardening of the plants. The survival rate was 100% when maintained in culture room condition (25±2 °C). New shoot and root were formed after two months of *in vitro* hardening (Fig. 2g & h).The hardened plantlets are maintained in the National Orchidarium, Tamil Nadu, India with 80% field establishment rate.

### Total phenolics and flavonoid content of the samples

The results of the phenolics and flavonoid content of both methanol extract of the wild plant and *in vitro* regenerated plants are shown in Table 4. The results revealed that the phenolics content of the wild grown plant of *A. montana* (121.34 ±1.23 mg/1g) was significantly ($P < 0.05$) higher than that of the *in vitro* regenerated plant of *A. montana* (61.89 ±3.19 mg/1g). Also, the flavonoid content of the methanol extracts as revealed in Table 4 indicated that wild grown plant *A. montana* (93.62 ± 1.17 mg/1g) had significantly ($p< 0.05$) higher flavonoid content than *in vitro* regenerated plants (34.59 ±1.14 mg/1g).

### Antioxidant activity

In the DPPH assay, the wild-grown plants of A. montanashowed higher scavenging activity ($IC_{50}$ = 26.87mg/ml) compared to the positive standard (BHT) ($IC_{50}$ = 34.59mg/ml) and *in vitro* propagated plant ($IC_{50}$ = 41.45mg/ml) (Table 4). To determine the relationship between the levels of the total phenolics and the antioxidant capacity of the extracts, correlation and regeneration analysis was performed. Total phenolic content of wild grown plant methanolic extract correlated with radical scavenging activity against DPPH ($r^2$ = -0.987, $P<0.01$) and flavonoid ($r^2$ = -0.971, $P<0.01$) are presented in Table 5 whereas, in *in vitro* regenerated plant methanolic extract, the significant correlation was not observed.

Table 6. Evaluation of antimicrobial activity of methanolic extract of *A. montana* by agar disc diffusion method and microdilution method.

| Micro-organ | Inhibition zone diameters of the test compounds (mm) | | | Minimum inhibitory concentration (MIC, µg/ml) | | | |
|---|---|---|---|---|---|---|---|
| | Wild grown plant extract 25µg/disc | *In vitro* propagated plants 25µg/disc | Ciprofloxacin 10 µg/disc | Wild grown plant extract | *In vitro* propa-gated plants | Ciprofl oxacin | Streptomycin |
| *E. coli* (ATCC 25922) | 8.11±0.91 | 4.61±0.23 | 21.77±0.11 | 31.25 | 125 | 3.90 | 1.90 |
| *P. aeruginosa* (ATCC 27853) | 7.11±0.34 | 5.54±0.21 | 20.47±0.17 | 62.5 | 125 | 1.90 | 3.90 |
| *S. aureus* (ATCC 29213) | 11.87±0.13 | 6.42±0.98 | 20.77±0.11 | 15.62 | 31.25 | 7.81 | 1.90 |
| *S. pnumoniae* ( ATCC 33400) | 6.55±0.62 | 4.54±0.31 | 21.07±0.05 | 62.5 | 125 | 3.90 | 3.90 |
| *K. pnumoniae* (ATCC 10031) | 10.21±0.91 | 8.12±0.67 | 19.17±0.11 | 7.81 | 15.62 | 1.90 | 3.90 |
| *B. subtilis* (ATCC 6633) | 10.17±0.23 | 9.97±0.11 | 22.67±0.11 | 15.62 | 31.25 | 3.90 | 7.81 |

The Trolox equivalent antioxidant capacity (TEAC) was measured using the improved ABTSradical cation decolorization assay. The decolorization of $ABTS^{\bullet+}$ cation radical is an unambiguous way to measure the total equivalent antioxidant capacity of test compounds or plant samples. Since, TEAC is a measurement of the effective antioxidant activity of the extract. A higher TEAC value would imply greater antioxidant activity of the sample. Similarly to DPPH assay, the wild grown plant showed the highest amount of $ABTS^{\bullet+}$ radical quenching ability and then BHA and in-vitro plant material (Table 4). From the correlation analysis, it is conceived that phenolics ($r^2 = 0.980$, $P<0.01$) and flavonoids (0.973, $P<0.01$) of *A. montana* wild grown plantmethanol extract wasthe main contributors for their reducing activity (Table 5).

Antioxidative activity has been proposed to be related to reducing power. Therefore, the antioxidant potential of *A. montana* wild grown plant and *in vitro* propagated methanolic extract was estimated for their ability to reduce TPTZ–Fe (III) complex to TPTZ–Fe (II) (Table 4). The FRAP activities of wild-grown plant of *A. montana* (2987.10 ± 19.33 mmol Fe (II)/g extract) and *in vitro* propagated plant (1034.23 ± 67.44 mmol Fe (II)/g extract) are presented in Table 4. Similar to DPPH and ABTS radical scavenging activity, the reducing power of wild-grown plant was significantly higher than *in vitro* samples. From the correlation analysis, it is conceived that phenolics ($r^2 =0.992$, $P<0.01$) and flavonoids ($r^2 =0.966$, $P<0.05$) of wild grown plant methanolic extract of *A. montana* were the main contributors for their reducing activity (Table 5).

**Antimicrobial activity**

Antibacterial activity was compared between *in vitro* propagated and wild-grown plants. The results of antibacterial assay are presented in Table 6. The result from the disc diffusion method measured in inhibition zone (IZ in mm) of *in vitro* propagated and wild-grown plants methanol extracts against bacterial strains ranged from 4.54 ± 0.31 to 22.67 ± 0.11mm (Table 6). Methanol extract of wild-grown plants of S. corymbosa showed high antimicrobial activity against *S. aureus, B. subtilis* and *K. pnumoniae* at 25 µg/ml. The antimicrobial activity of *in vitro* propagated and wild-grown plants methanol extracts of *A. montana* was quantitatively assessed by MIC against the six bacterial strains at various concentration range from 500 to 3.90 µg/ml. The wild-grown plants methanol extract exhibited potent growth inhibitory activity against *K. pnumoniae, B. subtilis* and *S. aureus* with MIC value of 7.81 and 15.62 µg/ml respectively (Table 6).

## DISCUSSION

Tetrazolium staining indicated that *A. montana* seed viability was higher than that of observed during germination experiments. However, many studies on the Orchidaceae indicate that the viability testing often is not a good indicator of germinability (Johnson *et al*., 2007; Mahendran and Narmatha Bai, 2009; Mahendran *et al*., 2013). Because of this, the viability estimates should be confirmed by germination tests. Mass propagation of orchids through asymbiotic seed germination is a tool for conservation of the declining orchid propagation in nature (Kauth *et al*., 2006; Stewart and Kane, 2006; Mahendran and Narmatha Bai, 2009; Roy *et al*., 2011; Mahendran *et al*., 2013). The success of asymbiotic seed germination is dependent upon identification of suitable medium and abiotic conditions. The nutritional requirements of most orchids vary due to their enormous diversity and complex mycorrhizal interactions (Arditti *et al*., 1990). Burgeff (1959) has reported that under culture condition the seedling of saprophytic species do not develop beyond the first leaf primordia and the first root. However in the present study fully developed seedlings were regenerated on BM-TM medium supplemented with growth regulators. In *A. montana*, among the six basal media evaluated for asymbiotic seed germination, BM-TM medium was found to be the best for seed germination followed by KC medium. Though all the media favoured seed germination, the growth of the protocorm was arrested beyond protocorm stage due to browning. Browning, a problem in *in vitro* culture of orchids, results from the accumulation of phenolic compounds and causes the loss of growth capacity and tissue death during culture (Rittirat *et al*., 2012), but can be induced by different factors. In *Grammatophyllum speciosum*, as much as 70% PLB browning occurred after 8% glucose was added as the carbon source to MS medium (Pimsen and Kanchanapoom, 2011) while in *Phalaenopsis cornu-cervi*, New Dogashima medium induced up to 30% PLB browning (Rittirat *et al*., 2012). MS medium containing 0.5 mg/l NAA was the optimal medium for *Cymbidium faberi*, 74% of PLBs turning green and 26% of PLBs undergoing browning (Tao *et al*., 2011). The application of TDZ (3 mg/l) in half-strength MS medium with 60-day subculture interval stimulated browning in as many as 45% of *Phalaenopsis* somatic embryos (Gow *et al*., 2009a). In a separate study, Gow *et al*., (2009b) found that incubation of *Phalaenopsis* leaf explants in the dark for 15 days followed by 45 days under light induced browning in as many as 90% of embryos after 60 days. In *Eria bambusifolia*, MS medium reduced PLB browning more effectively than Knudson C medium (52% and 64%, respectively) with subsequently higher percentage germination (48% and 36%, respectively) (Basker and Narmatha Bai, 2010). However, in this study, protocorms browning was presumably caused by the high salt concentration of full-strength medium. Since terrestrial orchids usually require the medium with lower salt concentrations for seed germination (Rasmussen, 1995), the diluted macro-elements of MS medium (1/2, 1/4, or 1/8 MS) was preferable in several orchid species (Lee, 1998). In the present study, half strength BM-TM medium supplemented with activated charcoal was necessary for further growth of the protocorm in *A. montana*. This promotive effect has been attributed to the ability of activated charcoal to absorb phenolic compounds released by the plantlet into the media (Pan and van Staden, 1998).

The varied responses in different media might be due to the composition of the media. All the media used in the present study have different mineral composition. BM-TM medium is enriched with organic nitrogen sources such as casin hydrolysate, amino acids and vitamins, thus conditions are suspected to be responsible for enhancing seed germination which is in agreement with many workers (Kauth *et al*., 2006; Stewart and Kane, 2006; Roy *et al*., 2011; Mahendran *et al*., 2013). These findings indicate the existence of species–medium specificity. Such species – specific medium for seed germination have also been reported by many workers (Arditti and Ernst, 1993; Roy *et al*., 2011; Mahendran *et al*., 2013).

Orchids needauxin or cytokinin for the formation of new protocorms and plantlets development. The type and concentrations of growth regulators play an important role during *in vitro* multiplication of many orchid species (Arditti and Ernst,1993). In *A. montana*, the protocorm developed into multiple shoots directly on half strength BM-TM medium fortified with cytokinins or cytokinin in combination with NAA. Among the cytokinins, TDZ was more effective for inducing maximum

number of multiple shoots (17.24 ± 0.27). TDZ also found to be suitable for protocorm proliferation for *Dendrobium* hybrids, *Dendrobium candidum, Satyrium nepalense* and *Phalaenopsis gigantean* (Martin and Madassery, 2006; Zhao *et al*., 2007; Mahendran and Narmatha Bai, 2009; Niknejad *et al*., 2011). In *A. montana* TDZ in combination with NAA enhanced the number of secondary protocorms/multiple shoots. Combined effects of cytokinin (BA or TDZ) and auxin (NAA) proved to be useful in induction of protocorm and seedling in many orchid species like *Phalaenopsis* and *Doritaenopsis* (Park *et al*., 2002; 2003) and *Dendrobium candidum* (Zhao *et al*., 2008). The ratio of auxin and cytokinin for the initiation of shoot buds or PLB formation varies from species to species (Teng *et al*., 1997). Cytokinin and auxin in the ratio of 2:1produced shoot buds in *Rhynchostylis gigantea* (Van Le *et al*., 1999), *Vanda spathulata* (Decruse *et al*., 2003), *Phalaenopsis* and *Renanthera imschootiana* and *Vanda coerulea* (Seeni and Latha, 2000). An auxin in combination with cytokinin has been well documented for multiplication in various species (Javed *et al*., 2013) as observed in our study. However, the concentration of auxin required was found to be very low which indicate a high endogenous level of auxin in the explants. This may be due to reinforced by high requirement of exogenous cytokinin as the auxin has been shown to inhibit the transcription of cytokinin biosynthesis geneas suggested by Tanaka *et al*.(2006) and Javed *et al*.(2013).

The organic supplements have been shown to stimulate seed germination and seedling growth of many orchids (Arditti and Ernst, 1993; Zeng *et al*., 2012; Zhang *et al*., 2013). However, their effects are complex and may vary depending on species, the types of explants or the developmental stages. In the present study the combined effect of organic additives with TDZ (6.8 µM), various organic additives (peptone, yeast extract and coconut water) at different concentrations (0 .5, 1.0, 2.0, 3.0 g/l or 5, 10, 15 and 20%) were used together with the optimal TDZ concentration (Table 3). This study was needed so as to familiarize their ability to affect the shoot induction and multiplication rate and to optimize the medium composition for maximum plantlet regeneration. Addition of organic additives (peptone, yeast extract and coconut water) at various concentrations (0 .5, 1.0, 2.0, 3.0 g/l or 5, 10, 15and 20%) in combination with the optimum concentrations of cytokinins was found to be superior for shoot initiation and multiplication. Among the different levels of organic additives tested, TDZ (6.8 µM) in combination with coconut water (20%) was found to be the best for multiplication of shoots (28.33±0.27) with a mean number of 1.98 ± 0.42 shoot length per explant (Table 3). This increase in the number of multiple shoots could be attributed to the biochemical compounds of the tender coconut milk such as amino acids, organic acids, inorganic ions, vitamins, sugars, lipids, nitrogenous compounds and hormones. Similarly, the effectiveness of coconut water for promoting shoots differentiation has also been reported in *Phalaenopsis gigantean* (Murdad *et al*., 2006), *Gastrochilus calceolaris* (Pathak *et al*., 2011), *Vanda* Kasem's Delight (Gnasekara *et al*., 2012) and *Dendrobium* AlyaPink (Nambiar *et al*., 2012).

In the present study, wild–grown plant gave higher phenolic compounds and total flavonoid content probably due to a higher stress associated to their growth conditions. Otherwise, *in vitro* cultured seem to be under less stress producing lower amounts of phenolics and total flavonoids compounds (secondary metabolites). In fact, *in vitro* cultured plant grown with controlled light, temperature and nutrients. Similarly early researchers found that lower amounts of phenolic compounds *in vitro* cultured samples in *Poliomintha glabrescens* (Garcia-Perez *et al*., 2012), *Hypericum* spp (Danova *et al*., 2012), *Cichorium pumilum* (Khateeb *et al*., 2012), *Hypericum undulatum* (Rainha *et al*., 2013), *Melissa officinalis* (Barros *et al*., 2013) and *Swertia corymbosa* (Mahendran and Narmatha Bai, 2014).

Free-radical scavenging activity (FRSA) was determined to evaluate the antioxidant potential of *in vitro* propagated plants and these were compared with levels in wild-grown plants. Considering the fact that the mechanisms of antioxidant processes are complex, therefore, an approach with multiple assays in screening work is highly advisable (Matkowski, 2008; Amoo *et al*., 2012; Mahendran and Narmatha Bai, 2014). In the present study, various methods of *in vitro* assays were performed to determine the antioxidant activity of *in vitro* propagated plants and wild grown plants of *A. montana*.

The DPPH free radical scavenging assay has been widely used to evaluate antioxidant capac-

ities. Antioxidants react with DPPH, reducing a number of DPPH molecules equal to the number of available hydroxyl groups (Matthaus, 2002). The degree of discoloration indicates that the samples to scavenge DPPH radical due to its ability to donate hydrogen proton. Similar to DPPH, the decolorization of $ABTS^{\bullet+}$ radical reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species. The $ABTS^{\bullet+}$ radical cation is generated from the reaction of ABTS with potassium persulfate overnight in water (Re *et al*., 1999). In the present study, the methanolic extract of plants growing wild seemed to exhibit a higher antioxidant potential compared to plants growing *in vitro* under controlled conditions. The differences between the responses of methanolic extracts prepared from wild and *in vitro* growing explants could be attributed to the differences in the chemical constituents of the plant cells that are likely to develop in response to the surrounding environmental conditions and different components are accumulated during different growth phases or due to mutations (Parsaeimehr *et al*., 2010; Mohanty *et al*., 2011). The radical scavenging activity of extracts can be credited to the presence of its major phenolic compounds (Guimaraes *et al*., 2010). The antioxidant activity of phenolic compounds is related to the hydroxyl groups linked to the aromatic ring, which are capable of donating hydrogen atoms with electrons and stabilizing free radicals (Dorman *et al*., 2003; Yanishlieva *et al*., 2006). In *P. glabrescens*, *C. pumilum*, *M. officinalis* and *S. corymbosa* higher antioxidant activity correlated with higher total phenolic contents (Garcia-Perez *et al*., 2012; Khateeb *et al*., 2012; Barros *et al*., 2013; Mahendran and Narmatha Bai, 2014).

## CONCLUSION

In conclusion, the present study is the first to report seed derived protocorm explants and the successful plant regeneration in *A. montana*. The protocol described here could be applied in a propagation program for genetic resource conservation and commercial purposes. The total phenolics, flavonoid content and antioxidant activity of *in vitro* propagated plants methanol extract were lower than that of wild-grown plants' methanol extract. In addition, we provide evidence that anti-bacterial activity of *A. montana* extracts revealed that wild-grown plants' methanol extract at 25 µg/ml exhibited strong antibacterial activity against *S. aureus, B. subtilis* and *K. pnumoniae* with MIC values of 7.81 and 15.62 µg/ml respectably. Further work on the type of phytoconstituents and purification of individual groups of bioactive components can reveal the exact potential of the plant to inhibit pathogenic microbes and induce the antioxidation. Tissue culturing and the *in vitro* propagation of *Aphyllorchis* explants could be a possible method for the large scale commercial production of biologically active components.

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