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High Frequency Plantlet Regeneration via Direct Organogenesis in *Andrographis paniculata*

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ABSTRACT

An efficient protocol was standardized for high frequency plant regeneration via organogenesis from stem base explants of *Andrographis paniculata.* Seeds of *A. paniculata* were germinated on MS basal media. Shoot multiplication occurred on MS media supplemented with various concentrations of cytokinins viz. benzyladenine (BA), kinetin (Kn), thiodiazuron (TDZ) and zeatin (Zn). However, high frequency direct organogenesis generated maximum number of adventitious shoots (62.0 ± 4.2/explant) on MS media supplemented with 2.0 mg/l Zn. Histological observations revealed the organogenic path of regeneration. Shoot elongation from proliferated shoots occurred on MS media supplemented with BA (2.0 mg/l). Shoots rooted after they were dipped in indole-3-butyric acid (1000 mg/l) and upon its further transfer to MS basal media.

Keywords: andographolides, *in vitro* culture, multiple shoots, plant growth regulators, stem base explants, zeatin
Abbreviations: 2ip, 2-isopentyl adenine; **BA**, benzyladenine; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **TDZ**, thiodiazuron; **Zn**, zeatin

INTRODUCTION

Plants have been used as an exclusive source of life-saving drugs since time immemorial. Several pharmaceuticals have been discovered from plants; however, they often have no useful synthetic substitutes yet are found to possess the same efficacy of pharmacological specificity as plants (Oksman–Caldentey and Inzé 2004). In recent years, the traditional system of medicine has become a topic of global importance and people are paying much more attention towards the use of plant medicaments. On the other hand the natural habitats of medicinal plants are disappearing fast and it is becoming difficult to acquire plant-derived compounds (Mulabagal and Tsay 2004). Plant cell cultures have been used not only as a continuous and reliable potential source of natural products alternative but also as an apt way for conservation of valuable plant genetic resources (Mulabagal and Tsay 2004; Debnath *et al*. 2006). There is an urgent need for the conservation of plant genetic resources with particular reference to medicinal plants (Faisal *et al*. 2007). A recently reported finding on *in vitro* production of huperzine A, a promising drug candidate for Alzheimer's disease is a step in this direction (Ma and Gang 2008).

Andrographis paniculata (Family: Acanthaceae) commonly known as king of bitters or kalmegh grows in Southeastern Asia, India, Pakistan, and Indonesia but is cultivated in China and Thailand. Amongst the several medicinal uses of *A. paniculata*, neoandrographolide and dexandrographolide were found to possess antimalarial activity against *Plasmodium* species (Mishra *et al.* 1992). Extracts of *A. paniculata* are used as a remedy for fever, pain reduction, liver protection, immune system enhancer and improvement of intestinal tract disorders (Rastogi and Malhotra 1993). The immune-stimulating and regulatory actions of *A. paniculata* are responsible for the prevention and treatment of many diseases (Ram 2001). Andrographolides in particular also showed action against human lymphocytes (Pannosian *et al.* 2002). Further, the extracts of *A. paniculata* have great promise for interfering with the viability of the HIV-1 virus (Calabrese *et al.* 2000). Andrographolide is a potentially anti-inflammatory diterpenoid lactone, which has been effectively used for the treatment of infection, inflammation, cold, fever and diarrhea in China for centuries (Ko *et al*. 2006). Andrographolide significantly decreased the number of surviving hepatoma-derived Hep3B cells in an MTT assay and induced cell apoptosis (Ji *et al.* 2007).

Clonal propagation is an alternative for rapid mass-scale propagation of plants and a continuous source of plant material. Conventional vegetative propagation of *A. paniculata* is very slow and difficult to meet the increasing demand. Moreover, variability among seed derived progeny coupled with scanty and delayed rooting of seedlings curbs its propagation via seeds (Martin 2004). Experience with *Andrographis* indicated that growing region and season play an important role for concentration of these important diterpene lactones. Recently, it has been found that the variation exists in growth and diterpene latones among field-cultivated *A. paniculata* (Prathanturarug *et al.* 2007). Therefore, the production of andrographolides using *in vitro*-propagated plants will be of immense interest besides its role for conservation of this valuable plant. Recently, an adventitious root culture system was used for production of andographolides (Praveen *et al.* 2009). Different plant growth regulators influence morphogenesis. *In vitro* regeneration reports in this plant are scanty (Prathanturary *et al*. 1996; Martin 2004; Purkayastha *et al.* 2008). The present study reports high frequency organogenesis in *A. paniculata* using stem base explants.

MATERIALS AND METHODS

Seeds of *A. paniculata* Wall. were procured from the Regional Centre of the Central Institute of Medicinal and Aromatic Plants (CIMAP), Hyderabad, India. Seeds were treated with a mild detergent (Tween 20) followed by washing under running tap water for 15-20 min. Seeds were surface sterilized with 0.1% (w/v) mercuric chloride solution for 4-5 min followed by a wash with sterile distilled water 8 to 10 times. Surface-sterilized seeds were inoculated aseptically on MS (Murashige and Skoog 1962) media for germination and establishment of aseptic cultures. All cultures were

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Research Note

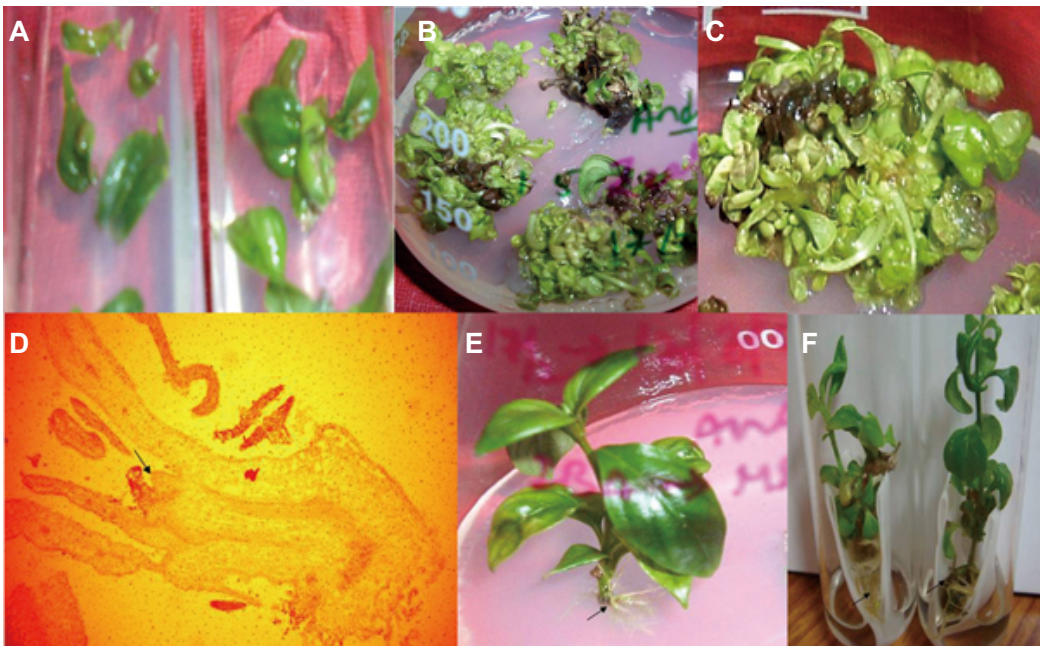

Fig. 1 High frequency direct organogenesis and rooting of *in vitro* propagated shoots of *Andrographis paniculata*. (**A**) Stem base explants in culture, (**B**) High frequency organogenesis response, (**C**) Magnified view of profuse organogenesis from stem base explants, (**D**) Histological section showing two shoot bud initiation (arrows), (**E**) Rooting response of *in vitro* propagated shoots in semisolid agar medium (arrow), (**F**) Rooting response in filter boat culture in liquid medium (arrow).

maintained under controlled environmental conditions at 25 ± 2°C, 16-h photoperiod with irradiance of 50 µmol/m^2/s^1. All the experiments were repeated three times with 20 explants/treatment.

Explants were excised from one-month-old plantlets maintained *in vitro*, transferred to MS media supplemented with cytokinins (benzyladenine, kinetin, thiodiazuron, zeatin) at different concentrations (0.25, 0.5, 1.0, 2.0, 3.0, 4.0 mg/l). Explants were cultured in 250 ml Erlenmeyer flasks containing 75 ml media an observations recorded four weeks after inoculation.

Proliferating adventitious shoots were dissected and transferred to MS media (full strength and half-strength) supplemented with auxins (indole-3-acetic acid, indole-3-butyric acid and α-naphthalene acetic acid) at different concentrations (0.1, 0.25, 0.5, 1.0 and 2.0 mg/l) for rooting. In a parallel experiment *in vitro* propagated shoot were cut at the base, and dipped in a concentrated filter sterilized solution (1000 mg/l) of IBA for 5 min and transferred to MS basal media as per the procedure followed for root initiation in *Aconitum heterophyllum* (Giri *et al.* 1993). Alternatively, these shoots were cut at the base, and base of the shoot was dipped in a concentrated filter sterilized solution (1000 mg/l) of IBA for 5 min on a Petri dish and inoculated onto a filter paper bridge and placed on MS liquid media in test tube.

Histological studies were carried out by fixing the tissue at initial stage of organogenesis in FAA (formalin: glacial acetic acid: absolute alcohol) at a 5: 5: 90 ratio for 24 hrs. Fixed tissue was dehydrated through an ethanol-xylene series, and passed through different grades of ethanol and xylene. Infiltration was carried out by adding paraffin wax in the specimen tube and uniform sections of 6.0-10 µm thickness were cut using a Leitz Rotary Microtome (Model 1512). Sections were obtained from freshly prepared wax blocks with tissue specimen by adjusting the microtome and stained with safranin (Johansen 1940; Giri and Giri 2007).

Statistical analysis were done based on the observations and scoring of data on culture responses at different stages of the experiments conducted. Response has been expressed in terms of number of shoots per explant and average length of shoots. Each treatment with 20 replicates and all experiments were repeated three times. Statistical analysis such as mean, standard deviation was done using Matlab version 5.3, and SPSS version 10.0 Math Works Inc., USA, statistical packages.

RESULTS AND DISCUSSION

Germination of *A. paniculata* seeds was observed after a week of inoculation. The germination frequency was low (20%) on MS basal media. The stem base explants gave rise to a maximum number of adventitious shoots on MS media supplemented with Zn (**Fig. 1A, 1B**). Amongst different concentrations of cytokinins, 2.0 mg/l Zn was found to be most effective for direct adventitious shoot regeneration. A maximum of 62 ± 4.2 shoots were obtained in this concentration (**Fig. 1B**). The cultures maintained their growth ability through subculture on the same medium (**Fig. 1C**). However, a subculture passage of more than 28 days leads to decrease in multiple shoot proliferation. Direct organogenesis from stem base explants was confirmed by histological studies (**Fig. 1D**). Number of multiple shoots varied in different cytokinins at different concentrations when the shoot tip explants were used. However, the stem base explants gave rise to a large number of adventitious shoots with varied responses with media containing different cytokinins.

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators provided in the medium and those produced endogenously by an explanted tissue. While most growth regulators exert a direct effect on cellular mechanisms, many synthetic regulators modify the level of endogenous growth substances (Radhika *et al.* 2006). High frequency organogenesis in *A. paniculata* on Zn-supplemented medium indicates its positive role in probable modification of the endogenous level of plant growth regulators.

The frequency of shoot multiplication reported in this study is very high. In earlier studies somatic embryogenesis has been reported in *A. paniculata* by Martin (2004); however, indirect somatic embryogenesis via a callus phase is not a reliable alternative for production of true to type plants. Martin 2004 reported production of more than 1800 plantlets from 1 g callus within 200 days. However, our study reports a successful attempt of achieving plantlet regeneration through adventitious shoot formation only on MS medium supplemented with Zn and it's rooting within a short duration.

The frequency of shoot regeneration in this study is very high. Zeatin used in this present investigation proved to be superior compared to all other cytokinins as the frequency of shoot induction is enormously high (**Fig. 2**). Zeatin which is natural cytokinin holds promise for high

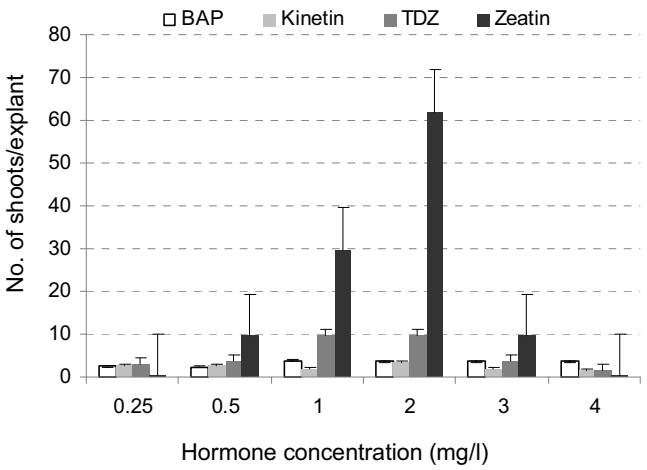

Fig. 2 Graphical representation of organogenesis using different plant growth regulator concentrations in *Andrographis paniculata*.

frequency regeneration of *A. paniculata.* This is one of the first reports of successful attempt on direct shoot regeneration from stem base explants using zeatin as the suitable hormone. In this study medium supplemented with other cytokinins BAP, Kn, and TDZ also induced multiple shoot formation, however, the number of multiple shoots was less. Recently, there was a report on the *in vitro* multiplication of *A. paniculata.* However, the multiple shoot formation was obtained from nodal explants of *in vitro* grown plants (Purkayastha *et al.* 2008). In their study it was evident that amongst different cytokininins such as BA, Kn, TDZ, and 2ip, BA was found to be suitable to generate maximum number of shoots. In another study, TDZ-induced high frequency plant regeneration through multiple shoot formation in *Cichorium intybus* L. (Sujatha and Ranjitha Kumari 2007; Yucesan *et al.* 2007). Recently, TDZ induced high frequency direct shoot regeneration (organogenesis) in *Cannabis sativa* has been reported (Lata *et al.* 2009). However, in our study TDZ did not promote higher shoot organogenesis compared to other cytokinins with particular reference to zeatin. The statistical analysis (ANOVA, *post hoc* tests, multiple comparison test) revealed the effect of hormone zeatin for multiple shoot induction as significant compared to other plant growth regulators (data not shown).

Root induction from shoots regenerated on MS medium supplemented with different cytokinins could not be achieved on MS medium with or without exogenous auxin supplements. However, rooting could be effectively induced when the base of adventitious shoots were dipped in autoclaved concentrated solution of IBA (1 mg/ml) for five minutes followed by transfer to semisolid MS basal media. High frequency of rooting (100%) was observed after 12–15 days of transfer to MS basal medium (**Fig. 1E**). Alternatively, rooting was also observed when adventitious shoots were dipped in autoclaved concentrated solution of IBA (1 mg/ml) for 5 min followed by transfer to liquid MS basal media by placing shoots on filter boat (**Fig. 1F)**. Adventitious shoots regenerated on Zeatin containing medium when transferred to MS media fortified with 2 mg/l of BAP showed flowering of shoots after 2-3 subculture passages. Fruit formation in shoots, grown *in vitro* was also observed in subsequent culture passages.

CONCLUSION

The present communication reports an efficient method for high frequency adventitious shoot regeneration and further rooting of the plantlets from stem base explants of *A. paniculata*. Presently described regeneration system has potential for genetic transformation studies thereby enhancing the andrographolide content of this medicinal plant using biotechnological approaches.

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