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Full Length Research Paper

Influence of plant growth regulators on indirect shoot organogenesis and secondary metabolite production in *Aconitum violaceum* Jacq.

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Influence of plant growth regulators on indirect regeneration and secondary metabolite production in *Aconitum violaceum* Jacq. was evaluated. Among the different plant growth regulators studied, 2.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 µM kinetin (Kn) promoted the highest frequency of callus production for indirect regeneration. 6-Benzyl aninopurine (BAP) was more effective in improving shoot regeneration and secondary metabolite production compared to thidiazuron (TDZ). The highest frequency of regeneration (61.8%) was obtained when calli were transferred to Murashige and Skoog medium supplemented with 1 µM BAP and 0.5 µM α-naphthalene acetic acid (NAA) and was more than two-times higher when compared to the treatments with cytokinin only. Supplementation with low NAA concentrations resulted reduction in *in vitro* secondary metabolite production in most cases, when compared to treatments with cytokinin only. Moreover, differences in cytokinin concentrations significantly affected secondary metabolite production in some cases. The current findings highlighted the differential effects of auxin-cytokinin interactions on indirect shoot regeneration and the production of secondary metabolites in *A. violaceum*.

Key words: Cytokinins, auxins, plant tissue culture, indirect regeneration, *aconitum violaceum,* secondary metabolites.

INTRODUCTION

Aconitum violaceum Jacq. member of family Ranunculaceae is an important medicinal plant found in subalpine and alpine areas of Indian Himalayan Region at 3500-4000 m elevations, and shares its position with threatened plant species of IHR (Chaudhary and Rao, 1998; CAMP, 2003). Roots of this plant are the natural source of alkaloid aconitine, a neurotoxin which attributes to the medicinal properties of the plant (Anonymous, 1988). The crude extract of underground parts possess antipyretic and analgesic properties and traditionally been used in renal pain, rheumatism, high fever and for the treatment of snake and scorpion bites, contagious infections and inflammation of the intestines (Kirtikar and Basu, 1984; Ameri, 1998; Chauhan, 1999).

Abbreviations: MS medium, Murashige and Skoog medium; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **TDZ,** thidiazuron; **BAP,** 6-benzylamino purine; **NAA,** α-naphthalene acetic acid; **Kn,** kinetin; **IAA,** indole-3- acetic acid; **PGRs,** plant growth regulators; **HPLC,** high performance liquid chromatography.

Due to increased demand from pharmaceutical industries, uncontrolled collection and lack of organized cultivation, plant is going depleted in natural habitat. Therefore, to protect the natural germplasm and to collaborate with rising demand of the plant material there is an immense need of biotechnology based interventions to ascertain secure conservation of elite germplasm. *In vitro* propagation techniques have contributed signify-cantly to the growth of pharmaceutical industry over the past several decades in a variety of ways including varietal improvement. The use of *in vitro* techniques for rapid and mass propagation offers possibilities for 'recovery' of endangered species, thus reducing the risk of extinction (Nadeem et al., 2001).

Regeneration from secondary meristems is useful for both these approaches because it facilitates the production of nonchimeric plants. The indirect regeneration of adventitious shoots is an alternative method to somatic embryogenesis in obtaining whole plant regeneration of explants. Shoot organogenesis and somatic embryogenesis from various explants have been reported earlier (De Wit et al., 1990; Kintzios et al., 1999; Vergne et al., 2010). One of the major factors affecting the success of *in vitro* plant propagation is the choice of plant growth regulators (PGRs). As a result, PGRs (especially cytokinins and auxins) are often added to culture media for the purpose of controlling different physiological responses *in vitro*, leading to the production of tissues (such as callus), organs (such as shoots and roots) or whole plants.

In spite of extensive hype in worldwide research interests in medicinally important plants and pharmaceutically active compounds there from, no conventional scientific attempts have so far been reported to conserve *A. violaceum*. The reports on *in vitro* multiplication and conservation of genetic diversity of genus *Aconitum* provides a realistic insight of its medicinal importance (Cervelli, 1987; Shiping et al., 1988; Giri et al., 1993; Watad et al., 1995; Giri et al., 1997; Mitka et al., 2007; Hatwal et al 2011). Thus, due to uncontrolled exploitation, the risk of loss of natural genetic resource of *A. violaceum* and biodiversity conservation aspects has drawn the attention in current research. Keeping above points in mind, the present study was aimed to investigate the role of plant growth regulators in improving/optimizing indirect shoot regeneration in *A. violaceum*. The influence of an auxin-cytokinin interaction on secondary metabolite production *in vitro* was also studied.

MATERIALS AND METHODS

Plant material and culture conditions

Plants of *A. violaceum* Jacq. were collected from Hemkund (30° 41'55" N to 79° 47' E 4100 m amsl, District Chamoli, Uttarakhand) of Garhwal Himalaya during the month of September. Plants were brought to the laboratory and used immediately for *in vitro* propagation following the protocol of Mishra-Rawat et al. (2013). Shoot tips and nodal segments of the plants were used as explants for the establishment of *in vitro* cultures. Surface sterilization of the explants was done with 0.5% Bavistin (30 min) and 0.1% mercuric chloride solution (2 min) followed by four times subsequent washing with sterile water. The sterile explants were cultured on MS (Murashige and Skoog, 1962) basal medium containing 0.8% (w/v) agar and sucrose (3% w/v) without any plant growth promoters (Figure 3). The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were maintained at 25 ± 2°C in 16/8 h light/dark cycle on racks fitted with cool fluorescent tubes (Philips 40 W; 42.0 and 60.0 µmol/m^2/s irradiance inside and outside the culture flasks, respectively). Sub-culturing was carried out at three to four weeks interval for optimal growth.

Callus induction, maintenance and plant regeneration

Three types of explants (leaf, shoot tip and nodal segment, taken from the same mother plant) were inoculated on the MS medium supplemented with 0.5 to 10.0 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 to 2.0 µM kinetin (Kn), 3% (w/v) sucrose and 0.8% agar for callus induction. Callus cultures were maintained through subculturing after every 30 days. Consequently, calli subcultured within three generations were used to induce adventitious shoots. Adventitious shoot induction medium was MS medium supplemented with various concentration of 6-benzyl aninopurine (BAP), thidiazuron (TDZ) and α-naphthalene acetic acid (NAA) (0.5 to 5.0 µM BAP, 0.5 to 5.0 µM TDZ and 0.25 to 2.0 µM NAA: single or in combination) and ultimately plants were regenerated. Regenerated shoots were cultured up to eight weeks and further used for secondary metabolite analysis. Culture conditions were same as described above for shoot induction.

Experimental design and statistical analysis

Each experiment was repeated three times and each repeat had seven replicates. Data were analyzed in a factorial based on completely randomized design (CRD). Data were analyzed using statistical programs MSTAT-C and SPSS. Statistically significant averages were compared using Duncan's Multiple Range tests. Graphs were plotted with the Excel program. Differences were regarded as significant at $P \leq 0.05$.

Active ingredient analysis

Ten gram (FW) of plant material of each treatment was used for analysis. Plants were washed to remove media particle and dried at room temperature (25°C) for 20 days. The air dried material were powdered and made into a composite mixture before chemical analysis. Extraction of active ingredients was done following the method of Hikino et al. (1983). The powdered samples (1.0 g) were extracted (25 ml x 3; 30 min each) with ammoniacal ether (ether containing 5% v/v, ammonia solution); the residue was then extracted with methanol (25 ml) for 16 h followed by two more extractions for 3 h each.

Column chromatography

Samples were further purified on neutral alumina (Sisco Research Laboratories Pvt Ltd., Mumbai) columns (8 x 2 cm; length and diameter) eluted with 50 ml of ethyle acetate and methanol (7.3, v/v). The eluates were dried *in vacuo* (30°C) in a rotary film evaporator, dissolved in high performance liquid chromatography (HPLC) grade methanol (1.0 ml) for further analysis by HPLC.

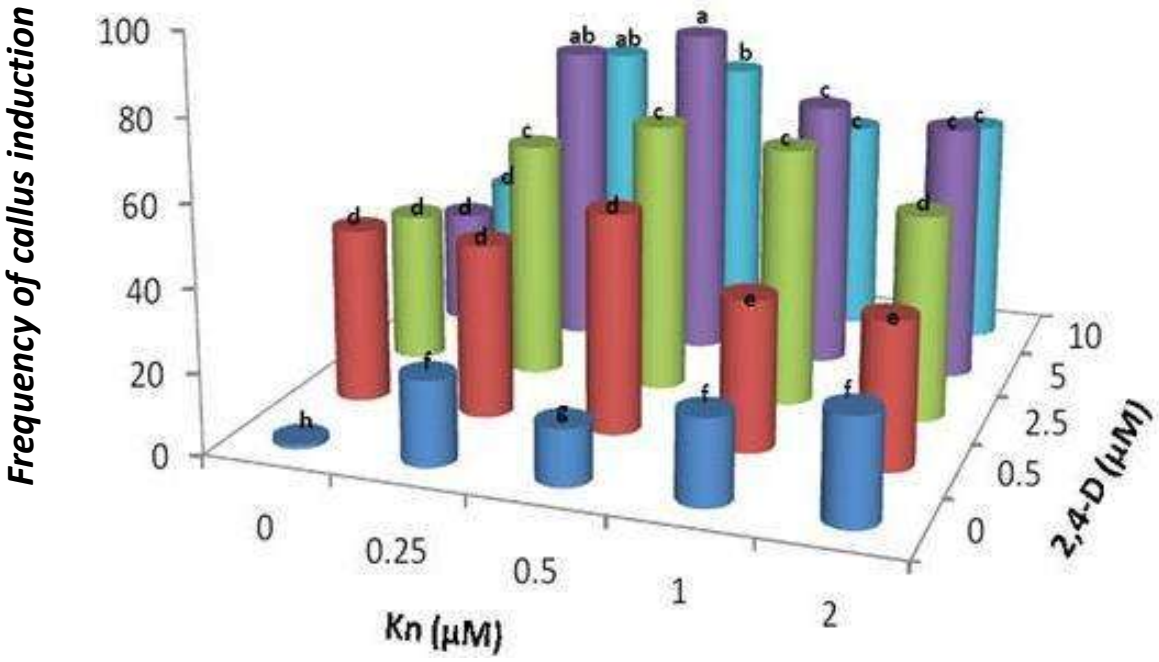


Figure 1. Effects of different concentration of 2,4-D and Kn on Indirect regeneration (somatic embryogenesis and organogenesis) of *Aconitum violaceum*. Means with different letters are significantly different according to Duncan's Multiple Range Test (P≤0.05).

High performance liquid chromatography (HPLC)

The quantification of aconitine was carried out using a HPLC system (Shimadzu corporation, Japan; Model LC-10 ATVP) in RP-1 Spherisorb column (250 x 4.6 mm id, 5 µm; Merck Darmstadt, Germany), eluted in an isocratic mode with methanol and water (60:40, v/v) containing 0.1% of acetic acid. The column elutes were monitored using an online UV detector set at 263 nm. The peaks were identified on the basis of retention time and quantification was carried out on peak area basis using a dose- response curve prepared with authentic compounds. Three analyses were done per sample. The lower limit of detection was approximately 100 ng. Aconitine was obtained from Sigma chemicals Co. St. Louis, USA.

RESULTS

Callus induction and maintenance

Callus induction started with enlargement of the exposed surface and cut regions of the explants (leaves, shoot tips and nodal segments), 21-28 days after the initial culture. All types of explants produced callus on all callus induction media. A greater proportion of explants on medium with 5.0 µM 2,4-D and 0.5 µM Kn initiated callus (leaves, 83.1%, shoot tips, 24.1%, nodal segments, 39.0%) than other media (data not shown). As the leaf explants showed maximum callus formation, further experiment regarding the effect of types and concentration of plant growth regulators on indirect regeneration was carried out with callus induced from leaf explants only (Figure 1). Media containing 5.0 µM 2,4-D and 0.5 µM Kn produced embryogenic globular and light green callus (Figure 2a), whereas media containing 10.0 µM 2,4-D produced soft watery callus creamy or light brown in color (Figure 2b).

Effect of cytokinins on regeneration of adventitious shoots from callus

The calli generated from different callus induction media were transferred to regeneration medium. The highest frequency of regeneration (61.8%) was obtained from media containing 1 µM BAP and 0.5 µM NAA (Figures 2c and 3a). The media containing TDZ showed lesser number of shoots compared to the media containing BAP (Figure 3a). Figure 3b shows the effects of different types and concentrations of cytokinins alone or in combination with NAA on shoot production after 8 weeks of culture. Increased shoot production was observed in the medium supplemented with cytokinin and auxin when compared to the PGR-free treatment in all the cases (Figure 3b). Maximum shoot production was observed with medium supplemented with 1 µM BAP and 0.5 µM NAA (10.3 ± 0.8) which was more than three times higher than the control (2.9 ± 0.3), although this was not significantly different from the result of the treatment with 2.5 µM TDZ and 0.5 µM NAA (8.1 ± 0.3). The highest shoot fresh weight was recorded in the treatment with 2.5 µM BAP

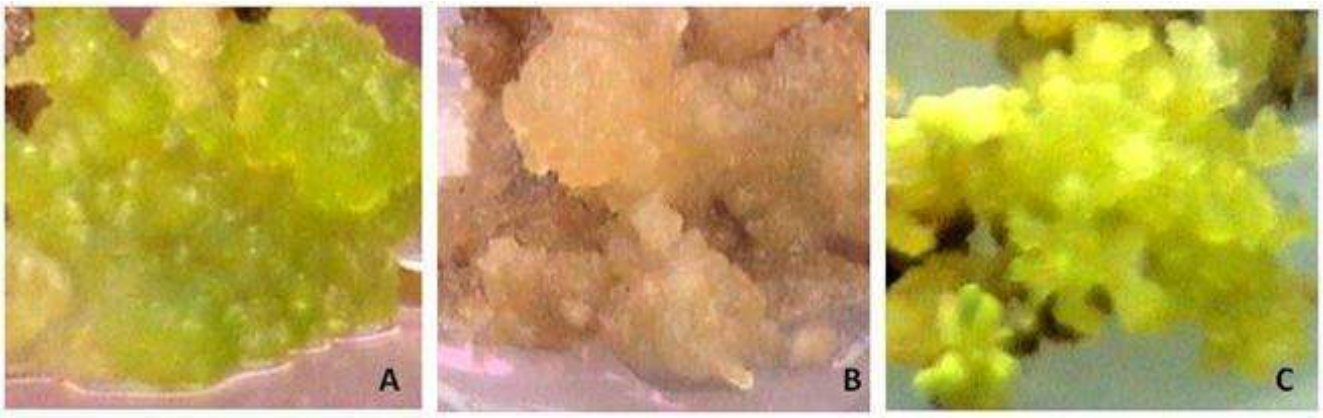


Figure 2. Indirect regeneration in *A. violaceum.* A, Formation of embryogenic callus on MS medium supplemented with 5.0 µM 2,4-D and 0.5 µM Kn; B, white brown callus formation on MS medium supplemented with 10.0 µM 2,4-D only; C, organogenesis in MS medium supplemented with 1 µM BAP and 0.5 µM NAA.

and 0.25 µM NAA (Figure 3c).

Active ingredient analysis

The active ingredient content of regenerated shoots from different cytokinin treatments singly or in combination with NAA concentrations are presented in Figure 3d. The aconitine content recorded in treatments containing cytokinin alone or in combination was significantly higher compared to that of the PGR-free medium in most of the cases. An increase in NAA concentration gave increased aconitine content in some cases. The supplementation of medium with cytokinins and low NAA concentrations (0.25 µM) resulted in significantly reduced aconitine content in most cases (Figure 3d). Maximum secondary metabolite production (0.87% of dry weight) was recorded in the medium supplemented with BAP and NAA (2.5 µM BAP +1.0 µM NAA). These results suppo-sedly suggest a possible antagonistic interaction of low NAA concentrations with cytokinin on aconitine produc-tion *in vitro*, in this plant species.

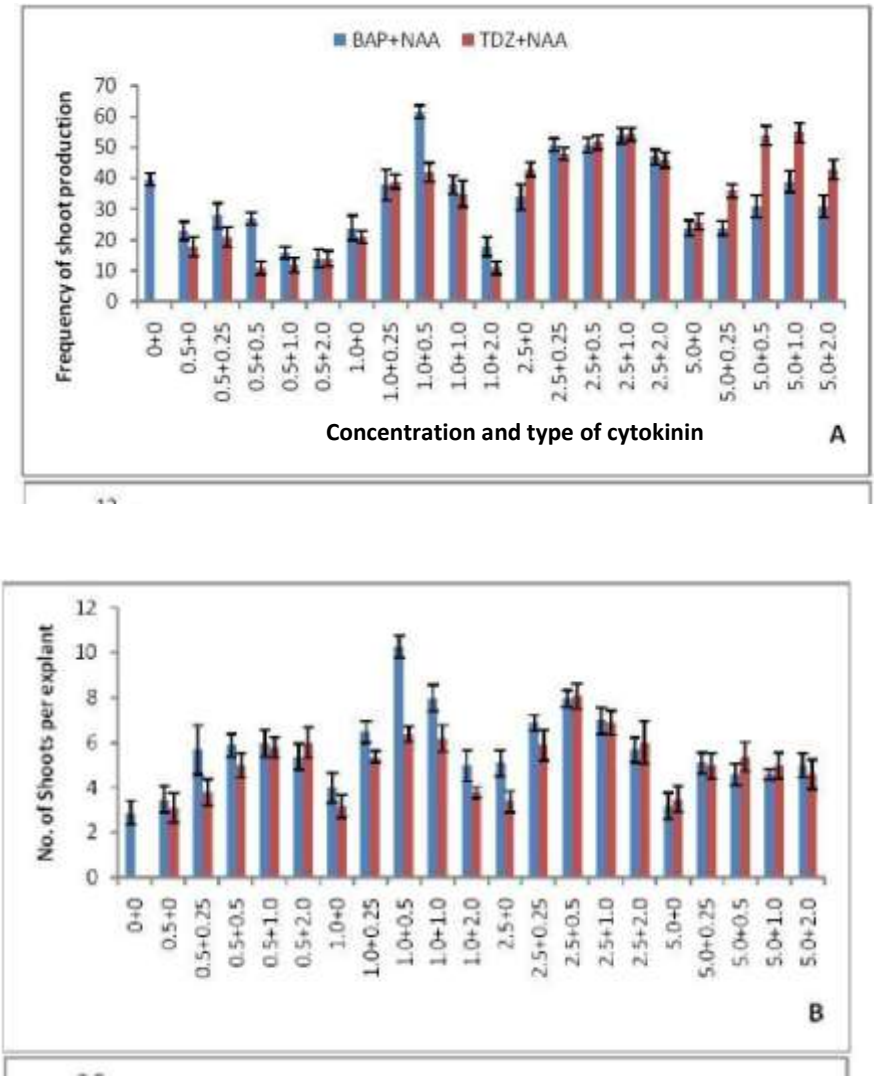
DISCUSSION

The indirect regeneration of adventitious shoots is an alternative method to somatic embryogenesis in obtaining whole plant regeneration of explants. In the present study, all the three types of explants produced callus with 5.0 µM 2,4-D and 0.5 µM Kn. Initiated callus were embryogenic and light green in colour. The auxin 2,4-dichlorophenoxyacetic acid (2,4-D) has been employed (single or in combination) in induction of somatic embryo-genesis in various plant species (Rout et al., 1991; Dohm et al., 2001; Li et al., 2002; Estabrooks et al., 2007). Increasing 2,4-D concentration from 0.5 to 10 µM resulted in a decrease in frequency of callus induction, which is an agreement with Li et al., (2002) who stated that increasing 2,4-D from 11.3 to 181 µM decreased callus induction in R. hybrida cv. Carefree Beauty.

MS medium containing 1 µM BAP and 0.5 µM NAA showed maximum frequency of regeneration, whereas media containing TDZ showed lesser number of shoot compared to the media containing BAP. Different plant growth regulators play significant roles in the regene-ration process of plants in the *in vitro* conditions. TDZ and BAP are the most frequently used cytokinins to induce regeneration, but their effectiveness depends on genotype and other factors (Magyar-Ta´bori et al., 2010). Although, maximum shoot production was observed with medium supplemented with 1 µM BAP and 0.5 µM NAA, this was not significantly different from the result of the treatment with 2.5 µM TDZ and 0.5 µM NAA. In most of the cases, the addition of NAA significantly increased shoot production, it suggest a synergistic/ additive effect of NAA on shoot proliferation. The highest shoot fresh weight was also recorded in the treatment with BAP and NAA. Other reports also showed the synergistic effect of auxins with cytokinins on shoot regeneration and proliferation in medicinal plants (Sudha et al., 1998; Sreekumar et al., 2000; Martin 2002; Beena et al., 2003). Although the number of shoots produced per explant in most treatments was higher (significantly in some cases) than that of the control (Figure 3b), there was no significant difference in the shoot fresh weight of the control when compared to other treatments (1 µM BAP and 0.5 µM NAA, Figure 3c). Shoot fresh weight could have been affected more by the growth of an individual plant rather than the proliferation rate (Bairu et al., 2007; Amoo et al., 2009).

Although the bioactive alkaloid, that is, aconitine, responsible for therapies, are mainly accumulated in the roots of aconitum plant, alkaloid content were also found in the vegetative parts for example, leaves and so on and constantly changes throughout the growth period

Figure 3. Effects of plant growth regulators on shoot proliferation of *A. violaceum* after 8 weeks of culture. A, Frequency (%) of shoot production; B, mean number of shoots regenerated per stem explants; C, mean shoot fresh weight (mg); D, aconitine content in % dry weight.



(Maknickiene 2008; Sinam and Devi, 2011). In the present study, the secondary metabolite analysis has been done in the vegetative part to know the effect of different types and concentration of PGRs. Sinam and Devi (2011) reported that maximum stock of alkaloids in leaves are accumulated before flowering. It is important to mention here that aconitum alkaloid contents can vary with the species, place of origin, time of harvest and most importantly the method and adequacy of processing (Chan et al., 1994).

According to Coenen and Lomax (1997), auxins are known to exhibit synergistic, antagonistic and additive interactions with cytokinins at multiple levels (depending on the plant species and tissue type) in regulating physiological responses. While the level of cytokinins in plants can be regulated by auxins and vice versa, Nordstrom et al. (2004) in their study using transgenic Arabidopsis plants, observed that auxin is a rapid and potent regulator of cytokinin biosynthesis compared to the reverse (where cytokinin regulates auxin synthesis). In addition to controlling fundamental growth and developmental processes in plants, PGRs are also known to regulate the production of plant secondary metabolites in plant tissue culture (Dornenburg and Knorr, 1995). The stimulatory role of cytokinin on the production of secondary metabolite in this plant species might be due to the repression of certain macronutrient transporters, leading to the expression or up-regulation of

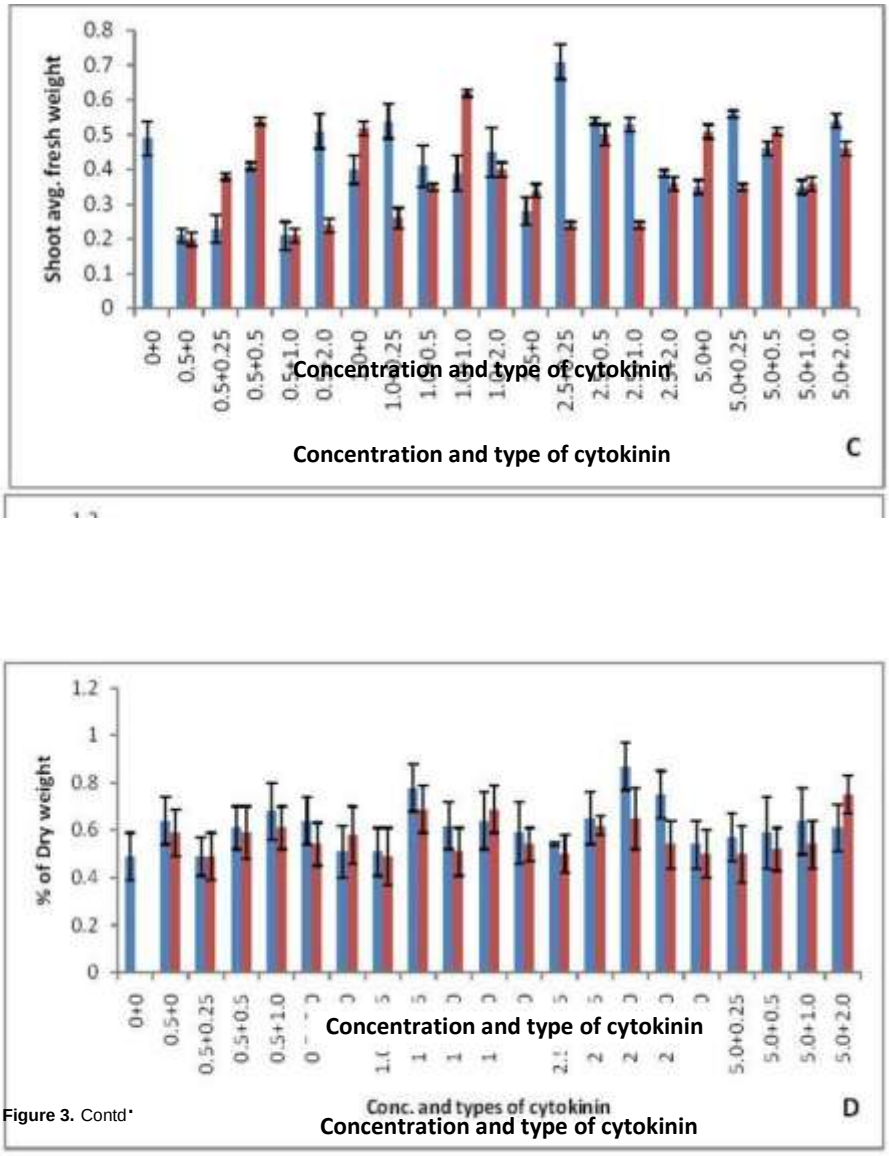


Figure 3. Contd.

genes involved in the biosynthetic pathway of secondary metabolites (Sakakibara et al., 2006). According to Sakakibara et al. (2006), cytokinins significantly repress some transporters of macronutrients such as nitrate, ammonium, sulphate and phosphate on one hand, while nitrate on the other hand regulates the expression of genes involved in secondary metabolite pathways. The influence of ytokinins such as BAP, Kn and TDZ as well as auxins such as NAA, 2,4-D and IAA on *in vitro* secondary metabolite production either alone or in combination have been reported by several workers (Meyer and Van Staden 1995; Miura et al., 1998; Luczkiewicz and Cisowski 2001; Coste et al., 2011).

The current study shows the effectiveness of BAP in increasing *in vitro* shoot proliferation and secondary metabolite production in *A. violaceum*, when compared to TDZ. Furthermore, the choice of cytokinin concentration makes a difference in the production level of secondary metabolites. Exogenously applied NAA interacted with cytokinin in a synergistic/additive manner on shoot proliferation, thus increasing the production of regenerated shoots per explant more than three times. On the other hand, low NAA concentrations reduced *in vitro* secondary metabolite production in most cases when compared to treatments with cytokinin only. The current findings highlighted the differential effects of auxin-cytokinin interaction on shoot proliferation and the production of secondary metabolites.

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