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# ANTIOXIDANT POTENTIAL IN REGENERATED TISSUES OF MEDICINALLY IMPORTANT *ATROPA ACUMINATA*

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**Abstract**

Due to random exploitation from natural resources, an efficient regeneration system of medicinally important but rare plant species, *Atropa acuminata* for conservation was inevitable. Leaf explants were incubated on MS medium with different level of various plant growth regulators (PGRs) alone and in combination for callus induction and induced organogenesis. After 4 weeks of culture, callus induction was recorded with the highest frequency with 1.0 mg/l thidiazuron (TDZ) supplement. After 5 weeks of subsequent sub-culturing, optimum shoot induction frequency of 89% was achieved with 1.0 mg/l TDZ and 1.0 mg/l α-naphthaleneacetic acid (NAA) supplement. Highest number of shoots/explant (8.2) were recorded on MS medium with 2.0 mg/l 6-benzyladenine (BA)+1.0 mg/l NAA supplement. Shoots in elongation medium was recorded 5.8 cm long in two medium i.e., 1.0 mg/l TDZ supplement and 1.0 mg/l TDZ+1.0 mg/l NAA supplement. Successful *In vitro* rooting was induced on MS medium with all applied level of indole butyric acid (IBA). The regenerated shoots with well developed roots were successfully acclimatized in sterilized soil and transferred to greenhouse conditions. Furthermore higher activity for detoxifying DPPH free radical was shown by regenerated shoots in this medicinally important plant species.

**Key words:** *Atropa*, Regenerated, Thidiazuron, Benzyladenine, DPPH.

## Introduction

*Atropa acuminata* belongs to the genus *Atropa*, family Solanaceae and is an important source of medicinally important tropane alkaloids; atropine, hyoscyamine and scopolamine which are commonly used as sedative, analgesic, mydriatic, antiasthmatic, anodyne and antispasmodic (Anon., 1948; Singh & Chand, 1995; Nasir & Ali, 1982).

Due to indiscriminate exploitation of wild plants from natural resources without any focus on its cultivation practices, this species is considered endangered (Shinwari & Qaisar, 2011; Rauf *et al.,* 2007; Ahuja *et al*., 2002; Banerjee *et al.,* 2008). Owing to the threat of extinction there is a need for development of necessary conservation strategies and efficient propagation protocols (Shinwari *et al*., 2012). A major constraint is the low seed germination and seedling survival rate of *Atropa acuminata* in conventional propagation methods (Wani *et al.,* 2007). The alternative method of propagation has been taken over by *In vitro* regeneration techniques, which include useful tools for conservation and large scale production of many threatened plant species (George & Sherrington, 1984; Abbasi *et al*., 2007; Hussain *et al*., 2013).

*In vitro* regeneration techniques have been employed for many threatened and medicinally important plant species (Liu *et al*., 2004; Murch *et al*., 2004; Khan *et al*., 2014). Of the plants species, many species of genus *Atropa* have also been used in *In vitro* regeneration techniques (Bajaj & Simola, 1991; Zarate *et al.,* 1997). The techniques of *In vitro* regeneration may be helpful in the mass-multiplication of this endangered species for large scale cultivation in the natural populations (Abbassi *et al*., 2011). To the best of our knowledge, successful *In vitro* regeneration of *Atropa acuminata* from leaf explants exploited from *In vitro* seed derived plants has not been reported so far. However, reports on literature shows that limited success has been achieved by employing shoot apices and nodal explants for *In vitro* propagation of *Atropa acuminata* (Ahuja *et al.,* 2002; Hussain *et al*., 2011).

Normal metabolism in human's body by the use of active oxygen results in reactive oxygen species (ROS), which in high levels create oxidative damage, causing a variety of biochemical and physiological disturbances in metabolic impairment and ultimately causes cell death. Synthetic antioxidants have been suspected of possessing certain levels of toxicity and being responsible for liver damage and carcinogenesis. Therefore, it is vital to develop and isolate natural antioxidants from plant sources (Blemekki and Bendimarad). Furthermore, plant *In vitro* regeneration is accomplished by different biochemical mechanisms of complex nature which involves specific enzymatic regulation and activation at specific times for accomplishment of organogenesis (Abbasi *et al.,* 2007; Meratan *et al.,* 2009). The increased and rapid generation of ROS is the consequence of plant responses to stress conditions (Yang *et al.,* 2010). In growth and development of plants, free radicals are known for their vital role; however, oxidative stress is the consequence of their uncontrolled production (Abbasi *et al.,* 2011). To prevent oxidative damage or eliminate the ROS plants develop different protection mechanisms (Yang *et al.,* 2010).

The current study aimed for developing an efficient and reproducible protocol for *Atropa acuminata In vitro* mass-multiplication and to evaluate the antioxidant potential of different *In vitro* derived tissues.

## Materials and Methods

Seeds of *Atropa acuminata* were collected from wild grown plants in Khyber Pakhtunkhwa province of Pakistan. Taxonomic identity was confirmed by Herbarium of Plant Sciences, Quaid-i-Azam University Islamabad by comparison with the reference standards. Surface sterilization of seeds were performed by the method of Abbasi *et al.* (2011) by immersion in 70% ethanol for three minutes, and then by dipping in 0.1% mercuric chloride for one minute, followed by washing three times with sterile distilled water. These seeds were

germinated on half strength MS (Murashige & Skoog 1962) solid medium for 45 days in growth chamber with a 16/8-hour light/dark photoperiod under cool-white light (40-50 $\mu$mol/m$^2$/sec).

Leaves were collected from *In vitro* germinated plantlets (~5 cm height) and placed onto MS solid medium with had different level of PGR i.e. TDZ, Kn, BA alone and in combination with NAA. Additionally, NAA alone was also tested. The frequency of callus induced shoot organogenesis and number of shoots per explants were recorded after 5 weeks of culture. The regenerated healthy elongated shoots of 4-6 cm in length were removed and incubated on MS medium with having varying levels of IBA, NAA at 0.5, 1.0, 2.0, 5.0, and 10.0 mg/l for induction of roots. All media contained 0.8% agar and 30 g/l sucrose. The pH of all the media were adjusted with standard development of the species which was 5.7 and certainly autoclaved at 121ºC for 20 minutes.

The *In vitro* regenerated plantlets isolates were first washed with water for removal of any attached medium and then shifted to potting soil mixture that were placed in green house. To ensure high humidity (~ 90%) these plantlets were covered with polyethylene bags. The polyethylene bags were removed gradually after 25 days and exposed under green house conditions.

The regenerated plantlets and tissues including calluses, shoots and seed derived plantlets of *Atropa acuminata* were evaluated for antioxidant activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Antioxidant activity was determined by some modification in a protocol described by Amarowicz *et al.* (2004). Briefly, dried plant tissue of 10.0 mg was dissolved in methanol 4 ml which was further then dissolved in methanolic solution of DPPHº (1 mM, 0.5 ml). The mixture was vortexed for 15 sec and then kept at room temperature for 30 minutes. The resultant solution was read by spectrophotometrically which indicated absorbance of 517 nm. For background correction, the non-purple color solution which indicated methanolic solution of DPPHº being decayed was due to the addition of butylated hydroxyanisole (BHA). The radical scavenging activity was calculated as percentage of DPPHº discoloration with the following equation:

$$\% \text{ Scavenging DPPH}^{o} \text{ free radical} = 100 \times (1 - A_E/A_D)$$

$A_E$ = Absorbance of solution, when extract has been added at a particular level
$A_D$ = Absorbance of the DPPHº solution with nothing added
The data was collected of triplicate treatments each and further analyzed statistically. Analysis of variance and Duncan's multiple range tests were used for comparative analysis of different treatments means.

## Results and Discussion

Light green calluses emerged from cut margins of leaf explants which after 28 days became compact green. The highest callogenic response (92%) was recorded for 1.0 mg/l TDZ alone among all the PGRs tested (Fig. 1). Previously, similar observation was reported by Osman *et al.* (2010) for callus induction in *Lycopersicon esculentum*. According to the findings of Chalupa (1988) high concentrations of TDZ induces formation of large calluses in *Quercus robur* L. The present study shows that addition of 1.0 mg/l NAA to other PGRs were inhibitory for callus induction in *Atropa acuminata*. Similar results were previously observed by Abbasi *et al.* (2010a, b) for callus induction in some other plant species.

Fig. 1. Plant regeneration in *Atropa acuminata*. **A**. Callus Induction **B.** Shoot Regeneration **C.** Shoot Multiplication **D.** Shoot Elongation **E.** Rooting **F.** Acclimatization **G.** Regenerated Plantlet.

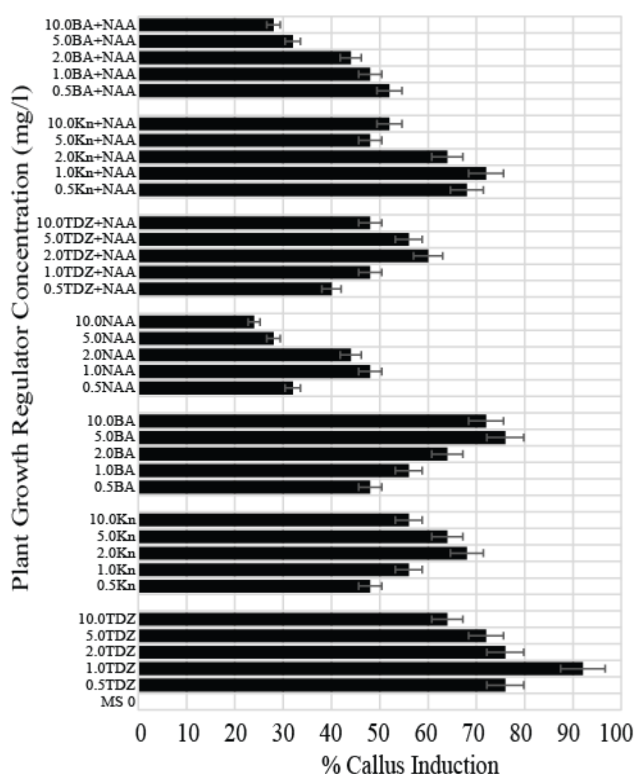


Fig. 2. Effects of various concentrations of thiodiazuron (TDZ), 6-benzyladenine (BA), α-naphthaleneacetic acid (NAA), kinetin (Kn) with or without 1 mg/l α-naphthaleneacetic acid (NAA) on the percent callus induction frequency of *Atropa acuminata.* The data were collected after 4 weeks of culture. The values are the means of five replicates.

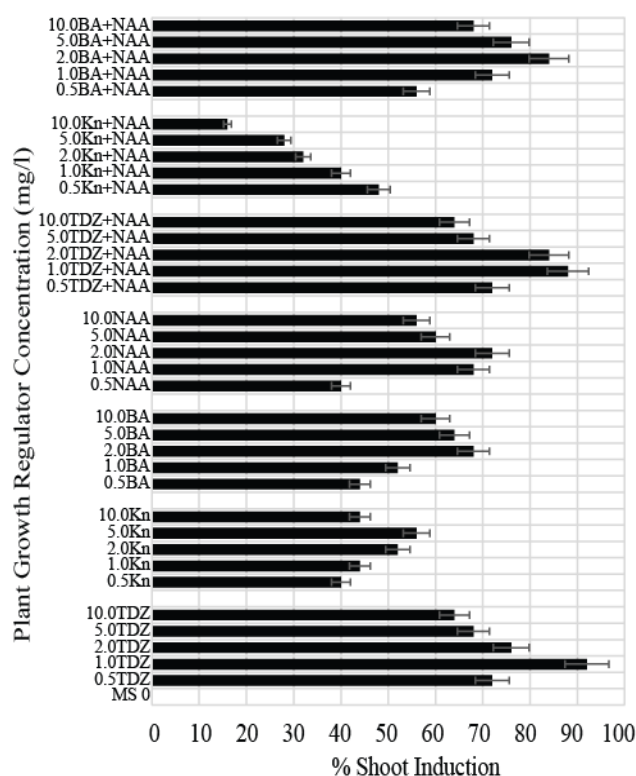


Fig. 3. Effects of various concentrations of TDZ, NAA, BA and Kn with or without 1 mg/l NAA on the percent shooting in *Atropa acuminata*. The data were collected after 5 weeks of subculture to MS media with similar PGRs. The values are means of five replicates.

Data on different parameters of shoot regeneration was determined after 35 days of subculture and the highest shoot induction response of 89% was recorded for explants cultured on medium containing TDZ (1.0 mg/l) with NAA (1.0 mg/l) in combination and 88% at 1.0 mg/l TDZ alone (Fig. 2). Modest concentration of TDZ alone as well as with NAA combination enhanced shoot organogenesis while higher concentration showed inhibitory action. Similar results for TDZ were previously also reported by Osman *et al.* (2010) for *Lycopersicon esculentum*. However, combination of TDZ with NAA failed to induce shoot induction in *Lycopersicon esculentum*. This is in contrast to our results where maximum response of shoot induction was recorded for TDZ in combination with NAA. Shoot induction frequency was markedly enhanced by the addition of NAA in MS medium already containing BA and TDZ. These results were comparable to the findings of Abbasi *et al*. (2010a,b) and in contrary to the results of Ahmad *et al.* (2010) for other plant species.

TDZ alone and BA in combination with NAA were more efficient to induce maximum no. of shoots/explant as compared to all other PGRs used (Figs. 3 & 4). Highest numbers of shoot/explant (8.2) were obtained on 2.0 mg/l BA along with 1.0 mg/l NAA, which was followed by shoots/explant (8.1) to which 10.0 mg/l TDZ alone was supplied. The addition of NAA in medium augmented with either TDZ or BA significantly enhanced the number of shoots/explant. Our results support the findings of Ahuja *et al*. that synergistic effects of auxin with other plant growth regulators enhanced shoot regeneration in *Atropa acuminata.* Similar results were obtained for TDZ alone on some other plant species of family Solanaceae which are in agreement with our results (Uranbey, 2005; Osman *et al.,* 2010).

Data on mean shoot length shows that maximum mean shoot length (5.8 cm) was observed on TDZ (1.0 mg/l) alone as well as its combination with NAA (1.0 mg/l) among all the PGRs (Fig. 5). The present study reveals that an optimized concentration of TDZ with NAA was more inductive towards mean shoot length as compared to others PGRs combinations. Recently, Fatima & Anis, (2011) observed similar results for *Withania somnifera* L. for mean shoot length which justify our results.

The best rooting medium for root organogenesis was MS medium supplemented with IBA (0.5 mg/l), where 90% of regenerated plants induced rooting (Table 1). Moderate concentrations of IBA were more beneficial for rooting while further increase in concentrations inhibited percent rooting response. The addition of NAA to MS medium failed to induce rooting in *Atropa acuminata.* Similar results were previously reported by Ahuja *et al.* (2002) for *Atropa acuminata* on full strength RT medium containing IBA. Our results also support the findings of Amiri *et al.* (2011) for root induction in *Datura stramonium* L. by IBA. In another study on *Atropa baetica* species, rooting was reported on medium containing NAA which is in contrast to our results (Zarate *et al.,* 1997).

The *In vitro* regenerated plants when transferred to soil in green house, a survival rate of 80% was recorded.

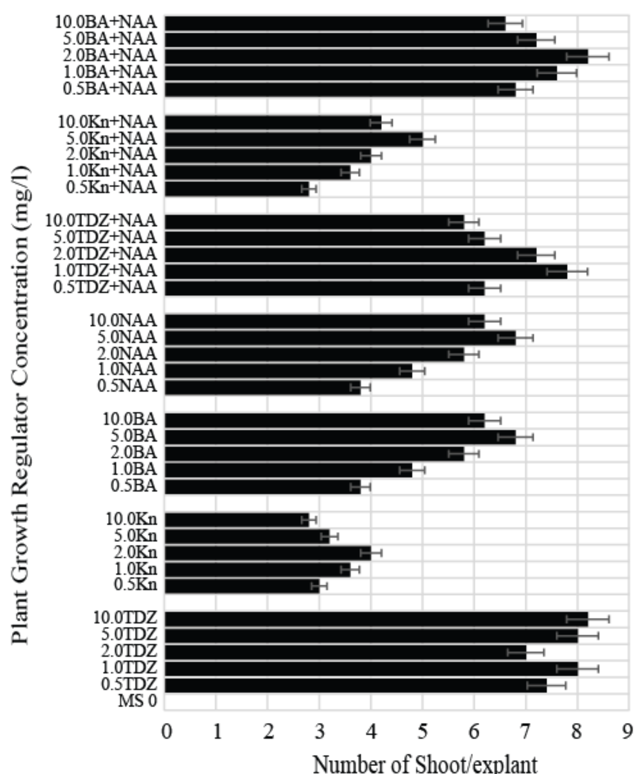


Fig. 4. Effects of various concentrations of TDZ, NAA, BA and Kn with or without 1 mg/l NAA on no of shoots per explant in *Atropa acuminata.* The data were collected after 5 weeks of subculture to MS media with similar composition of PGRs. The values are means of five replicates.

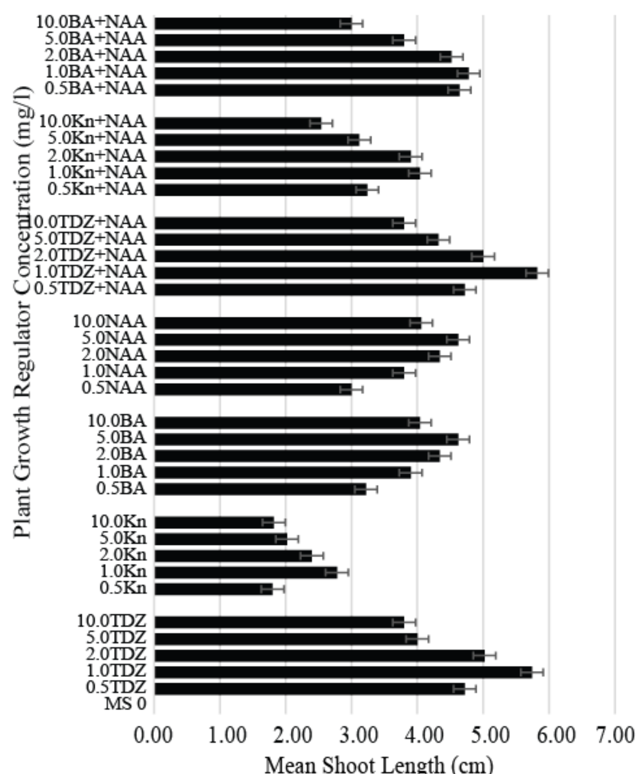


Fig. 5. Effects of various concentrations of TDZ, NAA, BA and Kn with or without 1 mg/l NAA on the mean shoot length in *Atropa acuminata.* The data were collected after 5 weeks of subculture to MS media with similar compositions of PGRs. The values are means of five replicates.

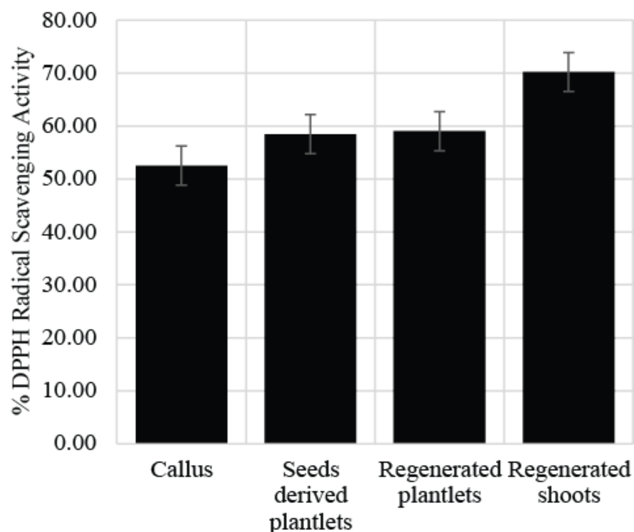


Fig. 6. Antioxidant activity in seed derived plantlets, callus and regenerated tissues of *Atropa acuminata.* Values are mean of three replicates.

**Table 1. Effects of different concentrations of indole butyric acid on percentage rooting of *Atropa acuminata.***

| IBA Concentration (mg/l) | Rooting (%) |
|---|---|
| 0.5 | 90 |
| 1.0 | 77 |
| 2.0 | 50 |
| 5.0 | 40 |
| 10.0 | 25 |

Regenerated shoots showed higher activity (71%) to detoxify free radicals followed by regenerated plantlets and seed derived plantlets (Fig. 6). However, callus showed less antioxidant activity in present study. The higher antioxidant potential in regenerated tissues could be ascribed to the plant's response against the increased production of ROS as a result of combinatorial effect of PGRs and growth room conditions on *In vitro* incubation of *Atropa acuminata*. Our results are in accordance with Abbasi *et al.* (2011) who reported higher antioxidant activity in regenerated tissues of *Sinapis alba* against DPPH free radical. Furthermore, Ahmad *et al.* (2010) had similar results for black pepper. On the contrary, Abbasi *et al.* (2010) determined high antioxidant activity in callus cultures of *Silybum*. This difference in data suggests that plant system accumulate different components during different phases of *In vitro* growth conditions. Regenerated plantlets may have the same profiles of secondary metabolites like that present in the mother plant (Shilpa *et al.,* 2010). In the current study, the regeneration protocol established showed feasible amounts of active antioxidants that from regenerated shoots, redirecting towards scaling up bioreactor level for production of chemically consistent *Atropa acuminata* plantlets.

The current study entails establishment of an efficient and reproducible system for the *In vitro* regeneration and conservation of medicinally important

and endangered species of *Atropa acuminata*. This protocol can be further exploited for potential large scale production, as these species do not propagate suitably under natural conditions and callus cultures can be helpful to provide valuable materials for the long term storage and preservation of this important species.

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