IN VITRO PROPAGATION OF *AMSONIA ORIENTALIS* FROM NODAL SEGMENTS OF ~~FIELD-GROWN~~ ADULT PLANTS

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Authors: medium — singular
media — plural

Abstract

Amsonia orientalis Decne. (syn. *Rhazya orientalis* (Decne.)) is an ornamental and medicinal plant which has very restricted distribution only in the northwest of Turkey and the northeast of Greece. In the present study, *in vitro* propagation of *A. orientalis* using nodal segments taken from field-grown adult plants was performed by testing the effects of plant growth regulators (PGRs) used at different concentrations. Nodal explants taken from adult plant were cultured in Murashige and Skoog (MS) media containing 0.5 to 2.0 mg l^{-1} 6-benzylaminopurine (BAP) after surface sterilization. Media containing 1.0 mg l^{-1} BAP gave the best result for the shoot multiplication. Obtained nodes were cultured in MS media supplemented with combinations of various plant growth regulators. Lengths of shoots proliferated from axillary buds were measured. Maximum mean shoot length was measured from the media supplemented with 0.5 mg l^{-1} indole-3-acetic acid (IAA) + 1.5 mg l^{-1} BAP and 0.5 mg l^{-1} IAA + 0.5 mg l^{-1} Kinetin (Kn) individually. Rooting experiments were also conducted in full and half strength MS media supplemented with IAA or indole-3-butyric acid (IBA). The best condition for rooting was found to be the full strength MS media supplemented with 0.5 mg l^{-1} IAA. Plantlets went through a hardening phase in a controlled plant growth chamber prior to *ex vitro* transfer. Through this study, an efficient and reliable *in vitro* propagation protocol for *A. orientalis* was developed for the first time by using nodal explants taken from field-grown adult plants.

Key words: *Amsonia orientalis*, Apocynaceae, *in vitro* propagation, nodal segment, bud breaking

Running title: *In vitro* propagation of *Amsonia orientalis*

The abstract requires a complete re-write. The authors should include only the most important results !

Introduction

The plants belonging to Apocynaceae (dogbane) family are widely known by their great pharmacological and ornamental properties. *In vitro* propagation studies were conducted on various species of this family in order to increase the number of individuals available for several purposes (Kumar et al., 2005; Nishita et al., 2006; Ozden-Tokatli et al., 2008). As a member of Apocynaceae family, the genus *Amsonia* Walter contains 22 species, most of which have horticultural merit or potential. The majority occur in a wide range of habitats throughout the central, the southern and the eastern North America (Davis, 1978). A few others are native to the southern Europe, Turkey, Japan, Korea and China. *Amsonia* species are commonly known as "blue stars" since they have pale blue colored and star shaped flowers. *Amsonia orientalis* is an ornamental and medicinally important plant which has very restricted natural distribution only in the northwest of Turkey and the northeast of Greece (Tutin et al., 1972). The plant grows best in well-drained soil and also needs full sun or partial shade. It can be found along margins of lakes and streams. Field studies have indicated that plant is now very rare and near extinction (Özen, 2006). In the "Red Data Book of Turkish Plants" *A. orientalis* is listed in the category of "critically endangered" (CR) (Ekim et al., 2000). Moreover, in the Bern Convention, The European Council placed the plant in the list of the plant species that must be conserved on European scale. In addition to its extinction risk, certain researchers have declared that the plant has a strong antimicrobial activity against several yeasts and bacteria (Akyalçın et al., 2006). Furthermore, six new flavonoid glycosides and some indole alkaloids known by their anticancer and anti-tumour activities were isolated from the plant over the last two decades (Rahman et al., 1989; Itoh et al., 2002). Propagation of *A. orientalis* was carried out through *in vitro* germination of seeds and callus formation achieved by Öz et al. (2008). Although its medicinal importance and threat of extinction were emphasized above, a limited number of papers about the conservation of the plant are found

in the literature. Out of this reason, in order to preserve the plant and supply plant material for ornamental purposes and potential medicinal studies, description of an optimized *in vitro* propagation protocol is urgently required.

In vitro propagation of rare and threatened plants is generally undertaken to enhance the biomass and conserve the germplasm, especially when population numbers are low in the wild (Sarasan et al., 2006). The application of plant tissue culture in *ex situ* conservation of rare and endangered plant taxa including medicinal and economically important plants has long been recognised (Latha and Seeni, 1994; Balaraju et al., 2009; Ishii et al., 2011). The present paper describes an efficient and reliable *in vitro* propagation protocol of *A. orientalis* using nodal explants taken from field-grown adult individuals, followed by successful *ex vitro* establishment of regenerated plants.

Materials and Methods

Plant material and disinfection

Shoots, 20–25 cm in length, were collected from field-grown individuals of *Amsonia orientalis* before flowering in May 2011. For the sake of easier manageability the shoots were cut into shorter pieces about 10 cm long and washed under tap water for 15 minutes. All leaves were cut off before washing step. These shoots were cut into 1-2 cm long pieces which have a single node at least and they were disinfected by dipping in 70% ethyl alcohol (EtOH) for 2 minutes and 1% sodium hypochlorite (NaOCl) for 10-12 minutes respectively. Traces of NaOCl were removed by two or three rinses with distilled sterile water.

Shoot multiplication and nodal culture

Disinfected single node explants were inoculated vertically in culture boxes containing 40 ml of Murashige and Skoog's (1962) basal medium (MS) with vitamins. MS media were

enriched with BAP at 4 different concentrations at a range of 0 (control) to 2.0 mg l^{-1} in order to multiply shoots and test the effects of different concentrations of 6-benzylaminopurine (BAP) on shoot multiplication. Following the inoculations at the end of the culture period of 4 weeks, numbers and lengths of shoots were scored.

Multiplied shoots were cut into small segments about 1 cm length which have a single node at least. Shoot multiplication was continued on the same fresh media by subculturing shoot segments with 2-3 nodes every 4 week in the same conditions. For investigating the effects of media on shoot initiation and elongation, single nodes obtained from multiplication phase were cultured on MS media supplemented with three different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), BAP, indole-3-acetic acid (IAA) and kinetin (Kn) at various concentrations.

Rooting and acclimatization

Proliferated shoots, not less than 3 cm but approximately at same length, were employed for rooting experiments. A period of 4 weeks was determined for rooting phase. For the rooting of regenerated shoots, various treatments were tested by transferring the shoots onto the solid half- or full-strength MS basal media supplemented with 0.5-5 mg l^{-1} IAA or 0.5-5 mg l^{-1} IBA. After 4 weeks, lids of culture boxes were half opened and kept on incubation 3 days more as the first step of acclimatization. Roots washed under tap water and rests of media were removed. Rooted plantlets, not less than 3 cm length, were transferred into pots containing a mixture of peat and compost (1:1). The pots were kept covered with transparent plastic bags for 3-4 days. After this period, small holes were opened on plastic bags for air circulation.

Culture media and conditions

All media were supplemented with 30 g l^{-1} sucrose (Duchefa) and 7 g l^{-1} of Plant agar (Duchefa). The pH of all media was adjusted to 5.7 with 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg cm^{-2}, 121°C for 20 min. Disinfection treatments and inoculations were carried out aseptically in a laminar air flow cabinet.

Cultures were maintained at 23±1°C ~~temperature~~ and a relative humidity of 65% (only at the first step of acclimatization) in a plant growth chamber with a 16/8 h light/dark photoperiod under an illumination of 80 µmol m^{-2} s^{-1} photosynthetic photon flux intensity provided by cool-white fluorescent lights.

Data collection and statistical analysis

Each treatment for both shoot multiplication and investigating the effects of media on shoot initiation was conducted with 15 nodes and repeated thrice. Rooting experiments also were carried out with 15 shoots for each treatment and repeated thrice. All measurements were done at the end of the incubation periods. Means were compared using Duncan's multiple range test at $P \leq 0.05$ significance level. The software IBM SPSS Statistics was used for statistical analysis.

Results and Discussions

Establishment of in vitro cultures and shoot multiplication

After 4 weeks of ~~inoculation~~ culture, emerged multiple shoots directly from axillary ~~nodes~~ buds of the cultured explants ~~were recorded~~ in all media. Axillary buds started breaking after 5-7 days ~~of~~ post inoculation. The highest percentage of bud breaking (98%) was observed in medium enriched with 0.5 mg l^{-1} BAP (Figure 1A). Amongst the five different media tested (basal or supplemented with increasing levels of BAP), media supplemented with 1.0 mg l^{-1} BAP ~~were~~ was

2 What is it Control with BAP?

the most effective for *in vitro* shoot multiplication of nodal explants while the basal MS media did not support the proliferation of multiple shoots (Table 1). Almost 91% of cultures showed an average shoot number of 9.20 ± 0.2 within 30 days of culture (Figure 1B). Recently, Adel et al. (2012) have declared that MS media fortified with 2.0 mg l^{-1} BAP plus 1.0 mg l^{-1} Kn responded better than the other tested combinations and reached 15 shoots per nodal explant in the propagation of *Rhazya stricta*. Therefore, the usage of different cytokinins together in shoot multiplication may be a greater option and can be tested in future propagation studies on *A. orientalis*.

Effects of PGRs on axillary bud breaking and shoot formation

Variation in the percentages of axillary bud breakings and also shoot lengths revealed that type and concentration of PGRs are decisive for *in vitro* shoot regeneration of this plant while axillary shoot initiations were observed in all media. Axillary buds started breaking in 7-10 days of inoculation. Of the three combinations tested, IAA plus Kn was found to be superior for bud breaking and also more effective for shoot elongation. In the present study, combined IAA (2.0 mg l^{-1}) plus Kn (1.0 mg l^{-1}) and 2,4-D (0.5 mg l^{-1}) plus BAP (1.5 mg l^{-1}) in the culture medium promoted the bud breaking frequency (95.6%) whereas the combination of 2,4-D (2.0 mg l^{-1}) plus BAP (1.5 or 2.0 mg l^{-1}) induced it at lower percentages (40 - 44.4%) (Table 2). According to the hypothesis, apically derived auxin is transported basipetally and plays a role in the inhibition of axillary buds. The inhibitory effect of auxin on the lateral bud breaking has long been recognized (Thimann, 1937, Prusinkiewicz et al., 2009). Tamas et al. (1989) investigated the effect of plant growth substances on axillary bud growth of common bean and reached the conclusion that basipetal auxin transport from apex is essential for inhibition of bud growth. Subsequent to this finding, Chatfield et al. (2000) working with isolated nodal segments of *Arabidopsis thaliana*, declared that basally supplied auxins failed to inhibit lateral bud outgrowth whereas apical application did not cause the same effect.

Similar to other micropropagation studies, in our study, auxins applied to nodal segments basally in combination with cytokinins. Bud breaking was not significantly reduced ~~in case of~~ auxin/cytokinin ratio was increased fourfold in any combination. This result is parallel to the previous studies mentioned above. In the presence of 2,4-D plus BAP or IAA plus BAP at respectively balanced levels, bud breaking was decreased, nevertheless, IAA and Kn showed positive synergistic effect on bud growth when applied in combination. Therefore, there is a complexity in the interaction of these PGRs.

Abnormalities such as falciform and crinkly leaves were observed in media containing 2,4-D at 2.0 mg l^{-1}. ~~These abnormal situations were not common and observed only in few medium.~~ Shoot lengths were negatively influenced by increased 2,4-D levels. Internodes of regenerated shoots from axillary buds of nodal explants cultured on the media ~~enriched~~ with 2,4-D plus BAP ~~concentrations~~ were relatively short, but more nodes were obtained. This appears to be an advantage for generating *in vitro* explant resources that can be used for multiplying shoots in future subcultures. In contrast, shoots induced by IAA plus Kn ~~concentrations~~ had ~~less~~ nodes ~~due to having~~ longer internodes. Maximum shoot length of 4.4 ± 0.7 cm was observed from media fortified with the combination of ~~the balanced levels of~~ IAA plus Kn (both at 0.5 mg l^{-1}) and the combination of IAA plus BAP at the concentration of 0.5 mg l^{-1} and 1.5 mg l^{-1} respectively (Figure 1C). We measured the maximum shoot lengths from media enriched higher cytokinin plus lower auxin concentrations, however the media supplemented with lower Kn and BAP levels combined with reduced auxin levels stimulated shoot lengths well whereas Öz et al. (2008) reported that the combination of IBA (1.0 mg l^{-1}) plus Kn (0.5 mg l^{-1}) induced direct shoot regeneration from shoot explants in 4 weeks and after 8 weeks of inoculation slightly root formation was observed. As predicted, all concentrations of 2,4-D in combination with BAP ~~successfully~~ induced ~~callus initiation~~ at basal cut ends of explants. Callus formation was found to be weaker in the media enriched with other combinations,

Although effects of external PGR levels on shoot lengths, numbers and callus formations can be predicted, internal hormone levels could be decisive for both.

Effects of PGRs on root induction

Effects of half- and full-strength MS media supplemented with various IBA and IAA concentrations were tested to constitute an effective rooting protocol. In our study, full-strength media with lower auxin levels and half-strength media even if without auxins stimulated root induction well (Table 3). Öz et al. (2008) cultured shoot explants of *A. orientalis*, derived from aseptically grown seedlings, in the MS medium fortified with 1.0 mg l^{-1} IBA plus 0.5 mg l^{-1} Kn for direct regeneration and declared that same medium can initiate rooting weakly within 8 weeks. When the rooting performance and culture period are taken into account, our results can offer a more effective protocol for rooting phase. Root inductions in all IAA-containing media were observed earlier than all media supplemented with IBA. The earlier response given to IAA can be relevant to its rapid metabolism within plant tissues. Nevertheless, not only physical effects such as temperature and light but also oxidative degradation of IAA can lead to lose its effects in time (Machakova et al., 2008). According to our findings, the effect of IAA on the number of roots was relatively weaker than the effect of IBA. This situation may be attributed to degradation of IAA in time. Although in terms of root initiation plantlets responded to IBA late, it was observed that higher levels of IBA showed a strong positive effect on the number of roots (Figure 1D). Among tested concentrations of auxins, IBA at 5.0 mg l^{-1} induced the highest number of roots (17.7 ± 0.4) compared to IAA. In accordance with our study, in the micropropagation of *Chonemorpha grandiflora*, another plant from Apocynaceae family, IBA showed better performance than IAA and α-naphtaleneacetic acid (NAA) (Nishitha et al., 2006). Similar results dealing with the superiority of IBA on *in vitro* rooting of plants, either belong to dogbane family or not, can be found in several reports (Nissen and Sutter, 1990; Ahmad et al., 2010). On the other

hand, in our study, ~~increasing levels of~~ IAA and IBA promoted average number of roots while root lengths were negatively affected by them. Increasing levels of both IAA and IBA caused a reduction on root lengths. Maximum mean root length of 2.8 ± 0.2 cm was ~~measured from~~ the full strength media supplemented with 0.5 mg l^{-1} IAA. Shoots cultured on both half- or full-strength MS media fortified with lower levels of auxin ~~showed~~ increased length of roots in time (Figure 1E). Nordström et al. (1991) attributed the greater ability of IBA to promote adventitious root formation compared with IAA to the higher stability of IBA versus IAA both in solution and plant tissue. Although all media with or without auxins induced the callus formation in two weeks, only the media without any auxins and the media fortified with 0.5 mg l^{-1} IAA caused minimal callus formation at ends of basal parts. During rhizogenesis shoot elongations were observed. Lower IBA levels successfully supported these shoot elongations. Similar results in the presence of IBA alone in MS medium were also observed in *Swietenia macrophylla* and various *Vitis vinifera* cultivars (Roubelakis-Angelakis and Zivanovitc, 1991; Nakamura and Soda, 2002). It can be considered that higher IBA concentrations showed an inhibitory effect on shoot elongation due to an increase in ethylene production.

Acclimatization of plantlets

~~Relative humidity (RH) plays an important role on acclimatization of *in vitro* propagated plants.~~ In culture boxes, RH can be found at high ratios. For acclimating ~~them~~ to outdoor conditions, a stepped protocol was conducted. In the first step, RH, inside growth chamber, was adjusted to 65% and lids of boxes were half opened, thus plants were acclimated to 65% RH and all of them survived (Figure 1F). Normally, some bacterial contaminations were observed during the first step. In this situation, Sherwood (1994) stated that the contamination of media ~~don't~~ cause any problem unless the culture boxes are kept open more than one week. In the second step, plantlets were transferred to pots and covered with transparent plastic bags. Keeping pots covered with transparent plastic bags more than 3-4 days (without holes)

caused fungal contamination due to excessive increase of inside RH. At the end of the period, more than 80% of transferred plants survived and successfully acclimated (Figure 1G).

Consequently, the seeds of *A. orientalis* are known by their slow and uneven germination. Germination frequency of seeds was found to be 15% by Öz et al. (2008). Culturing mature explants could be a more effective way for micropropagation of this plant than trying to germinate its recalcitrant seeds for such purposes. Through this study, a regeneration protocol for *A. orientalis* was developed for the first time by using nodal explants taken from field-grown plants. The outlined procedure is the first report that offers an effective and reliable potential system for conservation and *in vitro* propagation of *Amsonia orientalis*.

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342 **Table headings and figure legends**

343 **Table 1.** Influence of BAP on nodal explants from field-grown *Amsonia orientalis* plants on

344 MS medium

345 **Table 2.** Influence of plant growth regulators on *in vitro* regenerated nodal explants of

346 *Amsonia orientalis* on MS medium

347 **Table 3.** Effects of medium strength and auxin concentration on root formation from

348 regenerated shoots

349 **Figure 1.** *In vitro* propagation through adult nodal explants excised from field-grown

350 *Amsonia orientalis* plants. A, Shoot induction from nodal segments on MS medium

351 containing 0.5 mg l^{-1} BAP. B, Shoot multiplication on MS medium with 1.0 mg l^{-1} BAP at the

352 end of initial culture. C, Shoots developed from nodal microcuttings of *in vitro* regenerants on

353 MS medium supplemented with 0.5 mg l^{-1} IAA and 1.5 mg l^{-1} BAP in combination. D,

354 Rooting of *in vitro* derived shoots in full strength MS medium with 3.0 mg l^{-1} IBA at the end

355 of 4th week of culture. E, Elongated roots in full strength MS medium with 0.5 mg l^{-1} IAA at

356 the end of 6th week of culture. F, A rooted plant is ready to transfer into pots after the first step

357 of acclimatization. G, Successfully acclimated plant.

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Tables

Table 1.

Concentrations of BAP (mg l^{-1})	Shoot proliferation (%)	No. of shoots / explant	Mean shoot length (cm)
Control	82	$1.53^{d} \pm 0.1$	$1.92^{e} \pm 0.2$
0.5	98	$4.24^{c} \pm 0.1$	$4.28^{b} \pm 0.2$
1.0	91	$9.20^{a} \pm 0.2$	$4.87^{a} \pm 0.1$
2.0	87	$7.07^{b} \pm 0.2$	$3.85^{c} \pm 0.1$
4.0	80	$4.64^{c} \pm 0.2$	$2.34^{d} \pm 0.2$

Data represented ~~average~~ means ± SE of three replicates, each with 15 explants. Means having the same letter in a column were not significantly different by Duncan's multiple-range test ($P \leq 0.05$).

Table 2.

Concentrations (mg l^{-1})	Growth regulator					
	2,4-D + BAP		IAA + Kn		IAA + BAP	
	Shoot initiation (%)	Average shoot length (cm)	Shoot initiation (%)	Average shoot length (cm)	Shoot initiation (%)	Average shoot length (cm)
0.5 + 0.5	82	2.5 ± 0.5^{abcdef}	89	4.4 ± 0.7^{a}	76	3.0 ± 0.6^{b}
1.0 + 0.5	51	3.9 ± 1.1^{ab}	80	3.4 ± 0.8^{ab}	69	2.1 ± 0.5^{bcd}
1.5 + 0.5	69	3.0 ± 0.9^{abcd}	78	3.0 ± 0.5^{abc}	80	1.6 ± 0.4^{cd}
2.0 + 0.5	73	2.1 ± 0.7^{bcdef}	91	2.8 ± 0.4^{abc}	89	1.2 ± 0.3^{d}
0.5 + 1.0	78	3.4 ± 0.8^{abc}	82	3.1 ± 0.8^{abc}	60	1.2 ± 0.5^{d}
1.0 + 1.0	58	1.9 ± 0.6^{bcdef}	82	4.3 ± 0.7^{a}	56	$2.1b \pm 0.3^{cd}$
1.5 + 1.0	64	1.6 ± 0.4^{cdef}	89	3.7 ± 0.7^{a}	78	1.8 ± 0.3^{bcd}
2.0 + 1.0	64	1.2 ± 0.3^{def}	96	3.6 ± 0.5^{a}	73	2.1 ± 0.3^{bcd}
0.5 + 1.5	96	4.3 ± 0.7^{a}	87	2.6 ± 0.5^{bc}	71	4.4 ± 0.7^{a}
1.0 + 1.5	84	2.8 ± 0.6^{abcde}	78	1.8 ± 0.4^{bcd}	49	1.4 ± 0.4^{d}
1.5 + 1.5	78	2.1 ± 0.6^{bcdef}	84	1.4 ± 0.3^{cd}	60	1.3 ± 0.4^{d}
2.0 + 1.5	40	0.7 ± 0.3^{f}	84	1.6 ± 0.4^{bcd}	60	1.2 ± 0.3^{d}
0.5 + 2.0	76	2.0 ± 0.4^{bcdef}	93	1.7 ± 0.3^{bcd}	78	2.8 ± 0.3^{cd}
1.0 + 2.0	51	1.0 ± 0.3^{def}	89	1.6 ± 0.4^{cd}	62	2.0 ± 0.3^{bcd}
1.5 + 2.0	67	1.1 ± 0.3^{def}	82	1.5 ± 0.4^{cd}	67	1.6 ± 0.2^{cd}
2.0 + 2.0	44	0.9 ± 0.3^{ef}	87	0.8 ± 0.2^{d}	53	1.6 ± 0.2^{cd}

Data represented ~~average~~ means ± SE of three replicates, each with 15 explants. Means having the same letter in a column were not significantly different by Duncan's multiple-range test ($P \leq 0.05$).

372 **Table 3.**

Growth regulator and media	Concentrations (mg l^{-1})	Rooting (%)	Average root length (cm)	Average No. of roots
IAA - MS	0	100	2.1 ± 0.2^{ab}	4.5 ± 0.1^{i}
	0.5	100	2.8 ± 0.2^{a}	7.9 ± 0.3^{f}
	1.0	100	2.7 ± 0.3^{a}	7.7 ± 0.2^{f}
	2.0	100	1.7 ± 0.1^{bc}	9.8 ± 0.2^{d}
	3.0	73	1.4 ± 0.2^{bc}	6.7 ± 0.3^{g}
	5.0	87	1.2 ± 0.2^{c}	8.9 ± 0.2^{e}
IAA - ½MS	0	90	2.6 ± 0.2^{a}	4.2 ± 0.2^{i}
	1.0	90	2.1 ± 0.3^{ab}	6.0 ± 0.2^{h}
	3.0	70	1.5 ± 0.2^{bc}	4.4 ± 0.3^{i}
	5.0	90	1.5 ± 0.1^{bc}	5.5 ± 0.2^{h}
IBA - MS	0	100	2.1 ± 0.2^{abc}	4.5 ± 0.1^{i}
	0.5	100	2.3 ± 0.2^{ab}	8.0 ± 0.2^{f}
	1.0	100	1.8 ± 0.1^{bc}	9.5 ± 0.3^{d}
	2.0	98	1.5 ± 0.3^{cd}	13.4 ± 0.5^{c}
	3.0	100	0.7 ± 0.1^{e}	14.5 ± 0.5^{b}
	5.0	100	0.9 ± 0.0^{e}	17.7 ± 0.4^{a}
IBA - ½MS	0	90	2.6 ± 0.2^{a}	4.2 ± 0.2^{i}
	1.0	85	1.6 ± 0.1^{cd}	5.6 ± 0.3^{h}
	3.0	80	1.1 ± 0.1^{de}	8.2 ± 0.4^{f}
	5.0	58	0.5 ± 0.0^{e}	4.7 ± 0.3^{i}

373 Data represented average ± SE of three replicates, each with 15 explants. Means having the same letter in a
374 column were not significantly different by Duncan's multiple-range test ($P \leq 0.05$).

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