In vitro Propagation of the Endangered Medicinal Tree *Arbutus andrachne* L.

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Abstract—

The Grecian Strawberry tree, *Arbutus andrachne* L., Arabic name "Qayqab", is a medicinal small tree with evergreen foliage grows in Palestine on rocky hills with high pH. In its natural habitats, very limited regeneration of new plants is observed because of difficult seed germination and slow plant growth. As many other wild plants in Palestine, populations of the Grecian Strawberry tree are deteriorating and recently the plant is listed among the endangered species. Plant tissue culture offers a good alternative to propagate difficult and endangered plant species. In the present work, we put an effective micropropagation protocol for conserving *A. andrachne* plant. Micropropagation protocol was initiated from seeds. Seeds were cold stratified then soaked in GA_3 then culture on Lloyd & McCown (WP) plant growth media. High germination percentage was observed (84%) after this treatment. Culturing microshoots on WP media supplemented with 6.0 mg/l zeatin gave the highest proliferation results (27.1 shoots/explant). For *in vitro* rooting, WP media supplemented with 15 g/l sucrose and 1.5 mg/l IBA gave 100% rooting response with 21.1±5.5. Rooted plantlets gave the highest survival percentage (83%) when acclimatized and transferred to *ex vitro* conditions.

***Keywords: Arbutus andrachne*, micropropagation, medicinal plats, species conservation.**

I. INTRODUCTION

Palestine is a land with rich floral diversity (1). There are around 600 species from total of 2700 species are used for food and medicinal purposes. Many of plant populations with edible and medicinal uses in Palestine are subjected to genetic erosion and listed as endangered. One of these species is *Arbutus andrachne* L. (Ericaceae), the Grecian Strawberry tree, Arabic name: Qayqab. It is an evergreen small tree, widely distributed from the East Mediterranean to Northern Black Sea area (3). In Palestine, the plant occurs on rocky hills with high clay content and low aeration. The reddish stems and evergreen foliage make the tree very attractive with high value in landscape. Additionally, fruits become sweet with good taste when ripe and can be eaten as fresh or dried or making jam (4,5). Furthermore, *A. andrachne* is used traditionally as astringent and urinary antiseptic and for the treatment of urinary system, and also used as a blood tonic, against joints ache and for treating wounds. These uses caused increase demand for this species and consequently it became threatened (2, 8).

A. andrachne was found to be the highest among 51 other medicinal plant species in Jordan that have antioxidant content (9). According to the available literature, triterpenoides, sterols, and lipids were isolated from bark, leaves, and fruits, Arbutin, monotropeins, unedoside, and catechin were also isolated from bark and leaves (9).

In the past few decades, populations of *A. andrachne* as many other wild plants in Palestine are facing severe habitat fragmentation, over-exploitation, destruction by extensive agricultural and human activities, overgrazing, and premature harvest by local people. All these activities lead to rarity of many wild plants and accelerated their extinction in many areas (6). Very limited recovery of *A. andrachne* trees in natural habitats is observed possibly due to difficult seed germination in natural conditions and slow plant growth (7). Because of difficult germination conditions of *A. andrachne*, there is a strong challenge to conserve this species by applying modern techniques in biotechnology used for multiplying endangered plant species.

In vitro propagation offers a good alternative to multiply novel and endangered plant species. Moreover, it is also used to provide sufficient number of plantlets for planting from few stock plants, which does not produce seeds or does not respond well to vegetative propagation (10). *In vitro* propagation in many cases is more rapid than traditional methods and can offer virus and disease- free plants all year round.

This study was conducted to optimize a micropropagation protocol of *A. andrachne.* The protocol also addressed breaking seed dormancy in *A. andrachne*, then optimizing a suitable medium and optimal concentration of growth regulators for aseptic culture initiation, shooting, and rooting, and finally acclimatizing of *in vitro* growing *A. andrachne* plantlets.

I. Materials and Methods

1) Collection of plant material

Seeds of *A. andrachne* L. were collected in November 2007 from wild grown plants near Taffouh Town located 8.0 km to the west of Hebron. Seeds were surface sterilized by washing thoroughly under running tap water for 2.0 min, then by soaking in sterilized distilled water (SDW) with few drops of detergent for 30 min. Antiseptic solution of 20% (v⁄v) sodium hypochlorite (Clorox®) was prepared. Under the Laminar air-flow cabinet, seeds were immersed in the antiseptic solution for 5.0 min with continuous shaking. Seeds were then rinsed with SDW for three times (2 min. each). Finally, 70% ethanol solution was added to the seeds for 30 seconds then washed with SDW three times (2 min. each). In all experiments sterilized seeds were cultured aseptically on different basal growth media; Murashige and Skoog MS (11), Gamborg *et al*. B5 (12), or woody plant WP (13) media to overcome seed dormancy and obtain a sterile seedlings.

2) Breaking seed dormancy and seed germination

Four *in vitro* experiments have been conducted to adjust the effects of pretreatment with GA_3 and cold stratification on breaking seed dormancy and germination, respectively. 1) Control treatment where seeds were untreated and planted directly on the media, 2) Cold pretreatment at 4°C for 24 h, 3) Seeds soaked in 5.0 mg⁄l GA_3 solution for 24 h at room temperature,4) Seeds treated by stratification then immersed in GA_3 for 24 hours.

Treated seeds were surface sterilized and cultured on four types of basal growth media; control (water agar), MS, B5, and WP. Media were prepared without using sucrose. Treatments were arranged in a Completely Randomized Design CRD with fifty replicates (test tubes) per treatment and one seed/replicate. Seeds were kept in the growth room under cold fluorescent light for 16-h: 8h dark at photoperiod photosynthetic photon flux density (PPFD) = 40-45 µmoles/m2/sec. Temperature was kept at 24 ± 1°C. Data was reported after six weeks for the percentage of germination in each treatment.

3) Micropropagation

3.1) *In vitro* shoot proliferation:

After six weeks of inoculating, microshoots from germinated seedlings were cultured in 250 ml Erlenmeyer flasks filled with 50.0ml of solid media. WP medium supplemented with zeatin or 2i-P at 0, 2.0, 4.0, 6.0, or 8.0 mg/l. Data were recorded after 6 weeks for the percentage of shoot proliferation, shoot height, number of proliferated leaves/shoots, and plant performance was also monitored. Treatments were arranged in a CRD with ten replicates (flask) per treatment and one microshoot /replicate.

3.2) *In vitro* rooting

In vitro rooting experiment was conducted using microshoots of 1.0cm long formed on seedlings in plant growth regulator PGR-free WP medium. Each microshoot was cultured in test tube filled with 8.0 ml of WP medium supplemented with 15 g/l sucrose and 0, 0.5, 1.0, or 1.5 mg/l IAA or IBA. Data were recorded after 6 weeks for the percentage of rooting, root number and length, shoot height, number of leaves, and also plant performance was monitored. Treatments were arranged in a CRD with ten replicates (test tube) per treatment and one microshoot/replicate.

3.3) Acclimatization to *ex vitro* conditions

Ex vitro acclimatization was performed as cited in (14). *In vitro* young rooted plantlets were taken out of the test tubes, washed thoroughly with distilled water to remove any remaining of medium. Plantlets were transferred to plastic pots containing peatmoss and perlite at 1:1 (v/v), potted plantlets were covered with transparent polythene bags to ensure high humidity and watered when needed for 2 weeks under 16 h light (PPFD = 40-45 µmoles/m2/sec) /8h dark at (24 ± 1°C). Polythene bags were removed after 2 weeks in order to acclimatize plants to field conditions. Success of hardening protocol was determined by calculating survival percentage.

4) Statistical analysis

Each experiment was set up at Completely Randomized Design (CRD). Data in each experiment was analyzed with the analysis of variance (ANOVA) using SigmaPlot version 11.0 (Inc. SigmaPlot for Windows). Means were separated according to Fisher's least significant difference (LSD) test at the p=0.01 level of probability. Sample numbers for each measurement are provided in the captions of related illustrations.

5) Result

5.1) Breaking seed dormancy and germination:

Successful seed germination of *A. andrachne* was achieved in this study (Table.1). Our control treatment indicated that *A. andrachne* seeds could not germinate. Stratified and GA_3 treated seeds showed the highest germination percentage 84% compared to the control treatment 0.0%. The results confirmed that both stratification at 4ºC for 24 h and treatment with 5.0 mg/l GA_3 are directly involved together to breaking seed dormancy. Germination was not also observed in all treatments when seeds inoculated on MS and B5 media. Both WP and water- agar media showed germination at different percentages according to the treatment. Stratified seeds gave 4% in water- agar and 16% on WP media. Seeds pretreated with 5.0 mg/l GA_3 gave higher germination percentage 8% and 30% in water- agar and WP media respectively. The highest germination percentage 84% (Fig.1a) was obtained when both treatments (stratification and GA_3 pretreatment) were tested with WP media.

Table.1 Influence of different treatments and basal growth media on seed germination percentage.

Treatment	Basal growth media			
	Water-agar	WP	B5	MS
a) Control (untreated)	0.0[b]	0.0[d]	0.0	0.0
b) Stratified at 4° C for 24h	0.04[b]	0.16[c]	0.0	0.0
c) Soaked in 5mg/l GA3	0.08[b]	0.30[b]	0.0	0.0
d) Both(b+c)	0.44[a]	0.84[a]	0.0	0.0
P value	0.0	0.0	-	-
LSD	0.119	0.138	-	-

Fifty replicates (tubes) per treatment and one seed /replicate. Means were separated by Fisher's LSD at *p*=0.01.

5.2) Micropropagation

5.2.1) *In vitro* shoot proliferation

Resulted or proliferated microshoots that were cultured on WP medium with zeatin are shown in (Table.2). Culture of microshoots on 2i-P supplemented media resulted with no shoot proliferation (data not shown). Exhibited shoot proliferation varied depending on concentration of zeatin. Percentage of shooting on WP medium was 50% and 90% at (0.0, 2.0 mg/l) of zeatin respectively and increased to 100% at (4.0, 6.0, and 8.0 mg/l) of zeatin. Zeatin increased the number of shoots (3.7-27.1) and leaves (13-31.2) compared with the control and resulted in the greatest shoot height (4.4 cm) at 6.0 mg/l (Table.2, Fig.1b). Zeatin at 8.0 mg/l decreased all growth parameters, likely due to its inhibitory effect at this high concentration.

Table.2. Influence of zeatin levels on the percentage of shooting, shoot height, number of shoots, and number of *in vitro* leaves microshoots of *A. andrachne*. Percentage of 100% were set to 1.0 for simplicity.

Concentration (mg/l)	Shooting (%)	No. of shoots	No. of leaves	Shoot height (cm)	Callusing
Zeatin					
0.0	0.5[b]	3.7[d]	13.0[b]	3.0[c]	-
2.0	0.9[a]	6.8[c]	20.0[b]	3.85[b]	-
4.0	1.0[a]	15.7[b]	20.4[b]	3.38[c]	-
6.0	1.0[a]	27.1[a]	31.2[a]	4.4[a]	-
8.0	1.0[a]	3.8[d]	18.2[b]	3.08[c]	-
P value	0.0005	0.0	0.0	0.008	
LSD	0.248	1.635	5.806	0.431	

Ten replicates (flask) per treatment and one microshoot /replicate. Different letters within columns showed significant difference at *P*= 0.01 as determined by Fisher's LSD.

5.2.2) *In vitro* rooting

Rooting rate and characteristics of the roots and explants were influenced by the type and concentration of auxin. Microshoots were successfully gave roots by adding IBA at 1.5 mg/l. There were high significant variations among and within rooting parameters of experiment (Table.3). No rooting was observed on PGR-free medium. Rooting rate was increased when the concentration of auxin was increased especially when IBA was used at 1.5 mg/l. IBA induced 100% of rooting at 1.5 mg/l whereas it dropped to 50% at 1.5 mg/l IAA, the highest number of root/explants were 21.1, and root length was 5.2 cm (Table 3., Fig.1c).

Table.3. Influence of IBA levels on number of root, root length, shoot height, and number of leaves and shoots of *in vitro* microshoots *A. andrachne*.

Concentration (mg/l)	Rooting (%)	No. root/ explant	Root length (cm)	Shoot height (cm)	No. of leaves/explant
IBA					
0	0.2[b]	0.3[bc]	0.6[bc]	2.1[b]	1.6
0.5	0.9[a]	8.3[a]	2.2[a]	2.24[a]	2.1
1.0	0.8[a]	5[a]	1.42[a]	2.35[a]	3.7
1.5	1.0[a]	21.1[a]	5.2[a]	3.4[a]	2.3
P value	NS	0	0	0.0008	NS
LSD	0.306	5.511	1.384	0.645	-
IAA					
0	0.2	0.3b	0.6	2.1	1.6
0.5	0.4	4.1a	1.1	2.8	1.0
1.0	0.2	0.7b	0.25	2.25	1.4
1.5	0.5	1.4ab	0.5	2.3	1.7
P value	NS	0.034	NS	NS	NS
LSD	-	2.736	-	-	-

Ten replicates (test tube) per treatment and one microshoot /replicate. Different letters within columns showed significant differences at *P* = 0.01 as determined by Fisher's LSD.

5.2.3) Acclimatization to *ex vitro* conditions

A. andrachne plantlets were transferred to plastic pots containing peatmoss and perlite mixture 1:1. Agar was washed away from rooted plantlets. *Ex vitro* acclimatization was successfully done (Fig.1d) for the *in vitro* rooted plantlets of *A. andrachne*. Rooted plantlets showed different survival percentages according to the PGR used in the rooting experiment (Table.4).

Table.4. Total survival and survival percentages, after 8 weeks of transferring rooted plantlet to *ex vitro* acclimatizing conditions according to the type of auxin used in rooting.

PGR	Survival Number	Survival Percentage
IBA	33/40	83%
IAA	26/40	65%

Figure.1. (a) *Arbutus andrachne* plant germinated seeds on WP Woody Plant medium after stratification, and treatment with GA_3, (b) shoot proliferation on WP at 6.0 mg/l zeatin (c) rooted plantlet on WP at 1.5 mg/l IBA (d) acclimatized rooted plant. A, b, c and d after six weeks of the beginning of each experiment.

6) Discussion

Seed treatment with GA_3 and stratification were successful for germination and breaking seed dormancy. The stratification process appears to enhance the production of some types of growth-promoting substances such as GA_3 (15). According to (7), various methods tested to break dormancy of *A. andrachne* seeds and found that nontreated seeds were not able to germinate. The same reference also reported that either stratification of seeds at 4ºC for three to four months or treatment of seeds with 250 ppm GA_3 gave 86% of seed germination. Compared to this study, the germination percentage was 84% that obtained by reducing the stratification time to 24 h and doubled the concentration of GA_3 to 500 ppm. WP media gave the highest germination result compared with B5 and MS media, the possible explanation of our result is that the major nutrient composition in WP medium corresponds to the mineral composition of the *A. andrachne* seeds, however, detailed mineral analysis for the seeds should be done before confirming this conclusion. It is well documented in literature that WP medium is being commonly used as a basic medium for propagation of woody plant species (16).

Success of shoot proliferation on WP medium with zeatin (Table.1) indicates that zeatin increases the rate of shoot proliferation and results in production of a better quality shoot. (17) were obtained only an average of 3.8–4.8 shoots/explants and 1.3–1.9 cm shoot height using zeatin-containing WP medium, which is much less than 27.1 shoots/explants and 4.4 cm shoot height that was obtained at 6.0 mg/l zeatin-containing WP medium in the present study. According to (18), they found that the proliferation was successful on MS medium supplemented with zeatin as a cytokinin type. Zeatin has increased shoot proliferation to 90–100% and gave average number of shoots 13.4–22.8 which is lower than 27.1 in this study. Zeatin also resulted in the highest shoot height 3.0 cm at 6.0 mg/l, while resulted in 4.4 cm when using zeatin with WP medium at 6.0 mg/l. Accordingly, it is apparent that zeatin was the most effective cytokinin for shoot proliferation of *A. andrachne* in both MS and WP media.

The result of rooting experiment showed that IBA and presence of sucrose were critical factors to initiate root formation. Absence of rooting on PGR-free medium indicates the significance of auxin in enhancing rooting *in vitro*. Rooting was inhibited with IAA in the current study, which is not in agreement with findings of (20) who reported that *A. unedo* microshoots were rooted successfully on WP medium supplemented with 2.0 mg/l IAA or IBA. The maximum number of roots in the present study was observed in media with 1.5 mg/l IBA, this result was in accordance with the findings of (17). They were reported that rooting rate was 90% for *A. andrachne* on WP medium supplemented with 1.0 mg/l IBA.

Plantlets rooted with IBA exhibited 83% survival, while with IAA 65%. Similar results were reported in (20) who found *A. unedo* rooted plantlets at IAA showed low survival rate during acclimatization. Survival rate obtained in the current study was relatively high 83% and similar to that reported for *A. andrachne* by (17, 18).

Conclusion

Establishment of culture medium by adjusting their PGRs concentration and maintenance of aseptic conditions are the keys to success for *in vitro* multiplication of *A. andrachne*. Seeds are considered a good starting material for *in vitro* establishment of *A. andrachne* but seeds successfully germinate especially if they are pretreated with 5.0 mg/l GA_3 at 4°C for 24h, then cultured on WP medium containing 1.0 mg/l GA_3. Culturing microshoots on WP medium supplemented with 6.0 mg/l zeatin is recommended to achieve 100% shoot proliferation and the greatest shoot height, shoot, and leaf number. Rooting was most successful on WP medium supplemented with 1.5 mg/l IBA. Sucrose work as an inhibitor of shoot proliferation, but inducer of rooting at low percentage

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