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The Effect of Plant Growth Regulators on Callus Induction and Regeneration of *Amygdalus communis*

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Abstract

The Almond (*Amygdalus communis*) is one of the most important and oldest commercial nut crops, belonging to the *Rosaceae* family. Almond has been used as base material in pharmaceutical, cosmetic, hygienically and food industry. Propagation by tissue culture technique is the most important one in woody plants. In the current research, *in vitro* optimization of tissue culture and mass production of almond was investigated. In this idea, explants of actively growing shoots were collected and sterilized, then transferred to MS medium with different concentrations and combinations of plant growth regulators. The experiment was done in completely randomized blocks design, with 7 treatment and 30 replications. After 4 weeks, calli induction, proliferation, shoot length and number of shoot per explants were measured. Results showed that the best medium for shoot initiation and proliferation was MS + 0.5 mg/l IAA (Indol-3-Acetic Acid) + 1 mg/l BA (Benzyl Adenine). Autumn was the best season for collecting explants. The shoots were transferred to root induction medium with different concentrations of plant growth regulators. The best root induction medium was MS + 0.5 mg/l IBA (Indol Butyric Acid).

Keywords: *in vitro*, *Amygdalus communis*, micropropagation, tissue culture, shoot proliferation

Introduction

The almond (*Amygdalus communis*) is one of the major tree crops of the world (Kester *et al.*, 1996).

From Middle and West Asia, it has diffused to other regions and continents which include Middle East, China, Mediterranean region and America (Ladizinsky, 1999). Conventional breeding of woody fruit species is a slow and difficult process due to high levels of heterozygosity and long generation cycles (Sriskandarajah *et al.*, 1994).

Because almond is highly heterozygous, and most common commercial cultivars are self-incompatible, almond trees are virtually as variable as wild populations. Vegetation propagation via layerge or cutting is inefficient in order to minimize the problem of the enormous genetic variation and to obtain genetically identical populations, due to the great problems of this fruit species regarding rooting *in vivo* (Henry *et al.*, 1992).

Also, to maintain clonal purity, seed-derived material is not generally used for propagation. Thus, plant tissue culture techniques are more valuable for the clonal propagation of almond trees. Therefore, the aim of this study was to determine the conditions needed to optimize micropropagation methods for almond, from buds explants by *in vitro* culture. Yet, there have been no useful protocol on the optimization of propagation of almonds through tissue culture.

Material and methods

Plant materials

Actively growing shoots of almonds were collected in four seasons from suitable trees growing at the Agricultural and Natural Resource Research Center of Razavi Khorasan in Iran. After removing the leaves, the shoots were cutted into segments of 1-2 cm in length, and then washed with running water for 1 hour. After that, the scions were surfaced sterilized in 0.02% (w/v) mercuric chloride for 3 min, and then rinsed with 70% ethanol for 2 min. Later, the segments were sterilized by immersion in 30% (w/v) calcium hypochlorite solution, containing 0.05% (v/v) Tween 20, for 15 min; finally they were rinsed four times with sterile distilled water.

Micropropagation

Shoot tips of 0.5 to 1 cm, containing a single bud, were established in tubes containing 20 mL of Murashig and Skoog (1962) medium (MS), supplemented with 30 gl^{-1} sucrose, 7 gl^{-1} agar (Agar-Agar, Sigma) and different concentrations and types of plant growth regulators (PGRs) (Tab. 3). The pH was adjusted to 5.8 prior to autoclaving at 120°C for 20 min. The cultures were maintained at 25±2°C with 16 h photoperiod (35 µmol $m^{-2}s^{-1}$), provided by white fluorescent lamps. Proliferating axillary shoots were subcultured once every 3 weeks. In the second stage, proliferating shoots were separated and transferred to a

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medium containing the same composition. The MS medium without PGRs was used as control. Each treatment was replicated for 30 times. Shoot induction, number of shoot per explants, length of shoot per explants, callusing and rate of infection were reported.

Rooting treatment

Four weeks old micropropagated shoots were transferred to 20 mL tubes containing a basal medium of MS with different types and concentrations of plant growth regulators (Tab. 1). The MS medium without PGRs served as control. Each treatment was replicated for 10 times. Rate of root induction, number of shoots and length of roots were records after 4 weeks.

Hardening off

Plantlets with roots' length between 0.5 and 1 cm were selected for establishing in green house. Explants were removed from culture and the roots were gently washed in distilled water to remove any residual medium. Then, shoots were potted in 1:2:1 mixture of perlite, sand and soil, respectively. Plantlets were covered with clear borosilicate beaker to maintain a 90 ± 5% relative humidity, for 4 weeks, before transferring into the growth room. Relative humidity was slowly decreased by gradually removing beakers. Plantlets were acclimatized after 3 weeks in a green house at 25 ± 2°C under natural daylight conditions.

Tab. 1. Composition of PGRs used during rooting experiment

Treatment Codes	Medium	Composition
E	MS	(1 mg/l) IBA
A	MS	(0/5 mg/l) NAA
N	MS	(0/5 mg/l) IAA + (1 mg/l) BA
B	MS	(1 mg/l) NAA
L	MS	(0/5 mg/l) NAA + (0/5 mg/l) IBA
D	MS	(0/5 mg/l) IBA
T	MS	(0/5 mg/l) IAA
Control	MS	---

Experimental design and statistical analysis

A completely randomized blocks design was used for the study, with data from each experiment being analyzed separately. In micropropagation the treatments were replicated 30 times, and in rooting induction, treatments were replicated 10 times, with each replicate comprising one explants.

The Duncan's test was adjusted at p = 0.01 probability level to separate mean differences when significant treatment effects were detected.

Results and discussion

The best multiple shoot initiation was obtained on the MS medium supplemented with BA at 1 mgL^{-1} and IAA at 0.5 mgL^{-1} (Tab. 1 and 3) with a shoot number of 3.15 per explants, on the 4th week of culture (Tab. 3). The higher length of shoots, with a shoot length of 1.21 cm and the most callus initiation, with 9.5, were observed on MS medium supplemented with the same PGPs as the above (Tab. 3). Conclusions show that BA treatments caused better shoot proliferation and better shoot multiplication (Fig. 1, 2, 3).

The results indicated that between the different seasons, autumn was the best time for collecting explants from trees on the field (Tab. 4). There were significant differences between the rate of contaminations and the dates of collecting the explants (Tab. 2).

Tab. 2. Summary of treatment interaction as determined analysis of variation for shoot proliferation

Source of variation	df	Rate of contamination	Callus initiation	Shoot length (cm)	Shoot number	Shoot induction
Date of collecting explants	3	107753/96**	0/86**	1/44**	8/95**	1.14**
PGRs treatment	6	554/56**	1/10**	1/78**	12/89**	1.05**
Date of collecting explants × PGRs treatment	18	149/80 ns	0/07 ns	0/14 ns	0/97 ns	0/06 ns
Error	812	2032/84	0/14	0/11	0/78	0/11

*p≤0.05; **p≤0.01; ns no significant

Tab. 3. Effect of different PGRs treatment on shoot proliferation

Treatment codes	Composition of PGRs treatment	Mean rate contamination	Mean callusing	Mean shoot length (cm)	Mean shoot number	Mean Shoot induction
N	IAA(0/5mg/l)+BA(1mg/l)	42/91 a	9/5 a	1/21 a	3/15 a	9 a
H	BA(2mg/l)+IBA(0/01mg/l)	42/91 a	7 ab	0/77 cb	2/57 b	6/25 b
J	TDZ(4mg/l)	39/58 a	5/75 b	0/84 b	1/93 c	6 bc
K	IBA(0/01mg/l)+BAP(1/27mg/l)	37/50 a	5/5 b	0/6 cd	1/52 cd	4/5 bc
F	IBA(0/01mg/l)+BAP(0/68mg/l)	37/91 a	5/75 b	0/73 cb	1/57 cd	4/5 bc
C	IAA(0/1mg/l)+BA(1/5mg/l)	40/41 a	4/75 b	0/75 cb	1/67 cd	3/8 c
Control	---	40/41 a	0.0 d	0.0 d	0.0 d	0.0d

Values with the same letters in the same column are not significantly different (p≤0.01) according to Duncan's test

Tab. 4. Effect of different date of collecting explants on shoot proliferation

Date of collecting explants	Mean rate contamination	Mean callusing	Mean shoot length (cm)	Mean shoot number	Mean shoot induction
May	36/19 b	7 ab	0/81 b	2/43 b	5/83 b
August	39/76 b	5/6 cb	0/72 c	1/95 c	4 bc
November	15 c	9/3 a	0/98 a	3/57 a	8/5 a
February	70 a	3/8 c	0/67 c	1/90 c	3/75 c

Values with the same letters in the same column are not significantly different (p=0.01) according to Duncan's test

Tab. 5. Summary of treatment determined analysis of variance for shoot proliferation

Source of variation	df	Root length (cm)	Root number	Root induction
Treatment	8	2/103**	3/07**	0/9**
Error	81	0/239	0/362	0/128

*p≤0.05; **p≤0.01

Tab. 6. Effect of different PGRs treatment on rooting stage

Treatment	Control	T	N	L	B	A	E	D
Mean root induction	0c	0c	1cb	2b	3b	3b	6a	7a
Mean root number	0c	0c	1/2b	1/2b	1/37ab	1/3b	1/92a	2/11a
Mean root length(cm)	0c	0c	2/8a	0/85b	0/79b	0/93b	1/2a	1/7a

Values with the same letters in the same column are not significantly different (p=0.01) according to Duncan's test

The best root formations were observed in MS medium supplemented with 0.5 mgL^{-1} IBA (Tab. 6). The maximum number of root induction was 7, the mean value for roots per seedling was 1.92 and for root length was 2.8 cm, data obtained on MS medium supplemented with 0.5 mgL^{-1} and 1.0 mgL^{-1} IBA, for the firts two (treatments D and E, respectively) and with MS medium + 0.5 mgL^{-1} IAA+1.0 mgL^{-1} IBA for the last expriment (Tab. 3). No root development was observed in control groups.

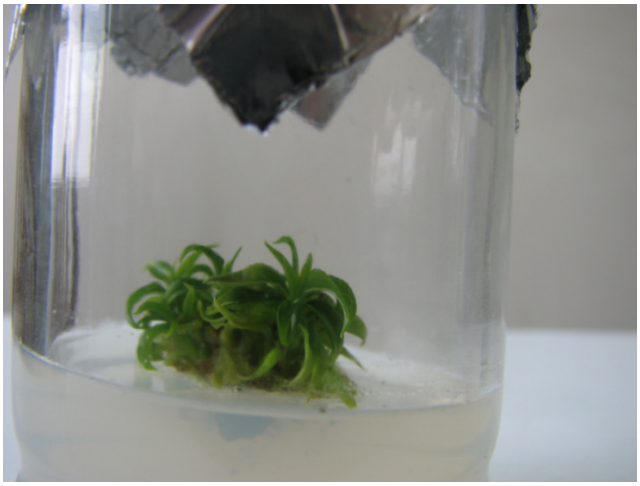

Fig. 1. Multiple shoot proliferation on a MS medium with IAA (0.5 mgL^{-1}) and BA (1 mgL^{-1})

The present results are in agreement with Tabachnic and Kester (1977) observations. They reported that the use of BA in shoot multiplication was absolutely necessary.

Hisajima (1982) reported that the best result for proliferation of the almond was obtained from MS medium supplemented with 0.2 mgL^{-1} BA + 0.005 mgL^{-1} IBA.

Isikalan *et al.* (2008) determined that the best multiple shoot initiation for almond was obtained on the MS medium supplemented with BA 2 mgL^{-1}, with a shoot number of 5.7 ± 1.04 mgL^{-1} per explant.

Increasing BA concentration from 1 mgL^{-1} to 3 mgL^{-1} may significantly reduce the length of shoots, as observed by Shekafandeh and Khush-khui (2008) in guava (Psidi-

Fig. 2. Developed shoot on a MS medium with IAA (0.5 mgL^{-1}) and BA (1 mgL^{-1})

Fig. 3. Seedlings on MS medium with IBA (0.5 mgL^{-1})

um guajava, *Myrtaceae*). Ahmad *et al.* (2004) reported that IBA is the preferable auxin for *in vitro* rooting of peach rootstock GF677 and the maximum number of roots and root length were obtained when applying 0.4 mgL^{-1} IBA.

Besides plant grow regulation factors, the year's seasons have been observed to influence in different degrees the micropropagation of temperate cultures. Shahrzad and Emam (2000), for instance, reported that summer and early autumn were the best times for collecting *Populous euphratica* explants in order to shoot proliferation. Nasiri (2000) show that mid autumn is the best time for taking explants from Olea europaea trees.

The present experiments demonstrated that shoot multiplication could be achieved on MS medium supplemented with 1 mgL^{-1} BA and 0.5 mgL^{-1} IAA. BA treatments resulted in a better elongation of almond scion. However, MS medium with 0.5 mgL^{-1} IBA was also effective for root induction and root elongation.

After 3 weeks, rooted shoots of almond were acclimatized and then were successfully transferred to natural condition. Channuntapipat *et al.* (2003) showed that the maximum rooting of shoots for some almond hybrid rootstocks occurred on half strength MS medium with 2.4 mgL^{-1} IBA. Ainsley *et al.* (2001) determined that IBA and NAA are the most suitable auxin for rooting seedlings of 'Nec plus ultra' and 'Nonpariel' almond cultivars, *in vitro* conditions.

The type and concentration of auxin during rooting period strongly influenced the quality of the root system during rooting period. The application of NAA resulted in poor rooting of the almond shoots. This might be explained by the NAA resistence to degradation by the auxin-oxidase enzyme (Smulders *et al.*, 1990). Nissen and Sutter (1996) have shown that, in tissue culture, media IAA is rapidly photo-oxidized (50% in 24 h), while the IBA oxidized slowly (10%) and NAA is very stable.

Conclusions

The results obtained in the present research can be used as guidelines for improving propagation of almond as a commercial fruit tree. In addition, the results demonstrated which is the optimized stage for root induction. Since in micro propagation rooting of micro cutting is often a challenging step, losses at this stage have vast economic consequences. In conventional propagation via cuttings many woody plants are also recalcitrant to root. Thus, a research on root formation is highly important from the practical point of view. It can be conclude that proliferation and multiplication of almond by tissue culture is a fast, economic and valuable method.

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