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Adventitious shoot development from leaf and stem explants of *Amygdalus communis* L. cv. Yaltinski

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Abstract

In the present research effect of plant growth regulators (PGRs) on callus formation and adventitious shoot from different explants of *Amygdalus communis* L.cv. Yaltinski was investigated. Callus was initiated from leaf and stem explants on MS medium supplemented with four different auxin (IAA, NAA, IBA and 2, 4-D) combined with BAP (1.0 mg l^{-1}) under dark and light conditions. Among all the concentrations of auxin the best result from leaf explants was observed with the 1.0:1.0 and 2.0:1.0 NAA plus BAP treatments (90%, 88% respectively) under light conditions. Only non-embryogenic callus on leaf explants obtained in the concentrations (1.0, 2.0, 4.0 mg l^{-1}) of auxin. The obtained calli from leaf explants cultured on MS media containing different concentrations of BAP (1.0, 2.0, 4.0, 6.0, 8.0 mg l^{-1}) for adventitious shoot development. Calli showed some proliferation after it was transferred to the media, but did not show any embryogenic response. In our research, the highest callus induction (80%) was obtained on medium containing 1.0:1.0 2,4-D plus BAP, under the dark (7 days) conditions for stem explants. Moreover, at the same medium and under the same conditions embryogenic calli was obtained as well. Calli showed adventitious shoot development after it was transferred to medium containing 4.0 mg l^{-1} BAP, but other concentrations of BAP (2.0, 6.0, 8.0 mg l^{-1}) did not show any embryogenic response. The medium supplemented with 4.0:1.0 IAA plus BAP provided root development directly from the yaltinski stem explants under the dark conditions (7 days).

Key words: *Amygdalus communis* L.cv. Yaltinski, (PGRs) Plant Growth Regulators, Callus, Adventitious shoot

Abbreviations: IAA-Indole- 3-acetic acid; IBA- Indole butyric acid; NAA- α-Naphthalene acetic acid; 2,4-D-2,4-Dichlorophenoxyacetic acid; BAP- Benzylaminopurine; PGRs- Plant Growth Regulators

Introduction

The almond (*Prunus dulcis*, syn. *Prunus amygdalus*, or *Amygdalus communis*) is a small decidious tree belonging to the subfamily, Prunoideae of the family, Rosaceae. Almonds are one of the oldest commercial nut crops of the world. However, it is highly heterozygous and most common commercial cultivars are self-incompatible. To maintain clonal purity, seed-derived material is generally not used for propagation. Thus, It is important to develop a efficiently protocols for regenerate plants from adult tissue (Ainsley et al., 2000). It is traditionally propagated by seedling, budding or grafting on to seedling which is laborious and slow. Non uniform germination, prolonged seedling emergence, and disease susceptibility to mycoplazma are other problems related to traditional propagation (Hammerschlag, 1986; Hartmann *et al.*, 1990). The application of tissue culture techniques as alternative propagation methods has been reported as early as the 1960s. Tissue culture has numerous potential applications for temperate fruit and nut tree species, including propagation of rootstocks, own-rooted scion cultivars, virus-free stock plants, and elite genotypes (Hutchinson, 1987; Gella and Errea, 1998). These techniques also offer unprecedented opportunities for the evaluation of horticultural traits in breeding program. There are a number of reports of regeneration of adventitious shoots from various explants of *Prunus* species. Regeneration using leaf tissues and apical shoots has been reported for almond (*P.dulcis* Mill.) (Miguel et al., 1996, Ainsley et al., 2000, Akbaş et al., 2009), sour cherry (*P.cerasus* L.) (Song and Sink, 2005), black cherry (*P.serotina*) (Xiaomei and Paula, 2008). Regeneration of adventitious shoots also has been achieved from immature cotyledons of peach (*P. persica*) (Wu et al., 2005), sour cherry (Tang et al., 2000). Micropropagation and rooting of microshoots of several prunus species has been achieved, including *P. dulcis* (Ainsley et al., 2001a, Channuntapipat et al., 2003). Breeding fruit tree species via conventional procedures is a difficult and expensive process due to heterozygosity, polyploidy, long breeding cycles, and lengthy field trial procedures**.** (Canli and Tian, 2008).

To our knowledge there has been only one report related to micropropagation from mature apical shoot explants of the *A.communis* L.cv Yaltinski (Akbaş et al. 2009**).** But indirect plant regeneration and transformation protocol of yaltinski has not yet been reported. Development of an effective genetic transformation system for its depends largely on the availability of an efficient and reliable regeneration system. The objective of the present study was to determine required conditions to facilitate regenaration of plantlets from leaf and stem explants derived from *in vitro* cultures of the almond paper-shell cultivar, yaltinski.

Table 1. Effect of cytokinin and auxins on callus induction from leaf explants of *Amygdalus communis* L.cv. Yaltinski

Cytokinin (mgl^{-1})	*Auxins (mgl^{-1})*				*% Response*		*Types of callus*
BAP	*IAA*	*NAA*	*IBA*	*2,4-D*	*Light*	*Dark*	
1	-	-	-	-	0	10	Callus not formed
1	1	-	-	-	75	71	Slightly loose & yellowish green
1	2	-	-	-	12	0	Friable & white callus
1	4	-	-	-	0	13	Callus not formed
1	-	1	-	-	90	82	Hard, compact & dark green callus
1	-	2	-	-	88	46	Hard & green calli
1	-	4	-	-	25	40	Friable (fragile) & light yellow callus
1	-	-	1	-	25	15	Friable (fragile) & yellowish green callus
1	-	-	2	-	39	8	Loose & light green callus
1	-	-	4	-	53	28	Light yellow callus
1	-	-	-	1	75	0	Friable & yellowish green callus
1	-	-	-	2	50	0	Friable & white callus
1	-	-	-	4	0	0	Callus not formed

Materials and methods

Establishment of in vitro shoot cultures

Young offshoots were collected from *Amygdalus communis* L. cv. yaltinski, growing at the botanical garden of the university of Harran, in Şanlıurfa, a province located in the Southeastern part of Turkey. All the explants used in the subsequent experiments were proliferated from almond cultivar, yaltinski trees.

For establishment of *in vitro* shoot cultures, apical shoot tips (10–15 cm long) were dipped in 70% ethanol for 30 seconds, surface sterilized in 10% (w/v) commercial bleach solution (NaOCI) for 10 min, and rinsed five times with sterile distilled water. Apical shoot tips were trimmed into 1 cm-long pieces, and they were cultured on full strength MS (Murashige and Skoog 1962) medium supplemented with 1.0 mg l^{-1} benzylaminopurine (BAP), 3% (w/v) sucrose and 0.55% agar (w/v). A detailed method for the optimized surface sterilization and culture initiation from mature almond trees was reported by Akbaş et al. (2009). The regenerated shoots (new shoots were subcultured on the fresh medium every 4 weeks) were maintained and proliferated on the initiation medium for more than one year. In this experiment, all the media were adjusted to pH 5.8 prior to autoclaving (120°C for 20 min), and the cultures were maintained at 25 ± 2°C with 16 h photo period (40 µmol m^{-2} s^{-1}) provided with mercury fluorescent lamps.

Callus induction for adventitious shoot from leaf and stem explants

In the present research, the effect of initial explants (stem and leaf) on callus induction and adventitious shoot development was investigated. The explants were cultured separately on media supplemented with different concentrations of auxins combined with one concentration of BAP.

In the first stage of our study, the leaves with a portion of the petiole were excised and cultured on medium with their petioles and abaxial sides in contact with the medium. The media supplemented with different concentrations (1.0, 2.0 and 4.0 mg l^{-1}) of indole-3-acetic acid (IAA), Indole butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) combined with 1.0 mg l^{-1} BAP. Also stem explants (0.5 cm) isolated from *in vitro* shoots were cultured on the same media separately. In addition, the leaf and stem explants were cultured on the same media and incubated in the dark for 7 days (Table 1, 2), and then transferred to the light for 3 weeks.

In the second stage of our study, calli was transferred to MS media containing different concentrations of BAP (2.0, 4.0, 6.0, 8.0 mg l^{-1}) for embryogenic callus and adventitious shoot development. The calli was subcultured on a fresh medium every 4–5 weeks.

Results and discussion

Almond (*Amygdalus communis)* is an important crop, with a growing number of applications for its kernel and oil. Unfortunately, the information about the induction of organogenesis from leaf and genetic transformation of almond paper-shell cultivar, yaltinski is very limited. Therefore, the basic procedures was investigated for the establishment of callus culture and adventitious shoot regeneration from leaf and stem explants. The PGR combinations that were used in the experiments are presented in Table 1 and 2. For establishment of *in vitro* shoot cultures, apical shoot tips were cultured on MS medium supplemented with 1.0 mg l^{-1} BAP (Fig1A) and proliferated (Fig 1B). In our study, the leaves and stem explants isolated from *in vitro* shoots were cultured separately (Fig 1C and Fig 1D) . In our research, the media containing different PGR concentrations and combinations have highly significant impact on growth percentages of callus and adventitious shoot development. Our results are supported by Khan et al. (2009), who reported that *in vitro* regeneration of an explant is always affected by internal or external factors. Therefore, researchers examined regeneration response of the leaf citrus explants in various culture conditions. It is shown that culture medium has a critical factor for induction of organogenesis in plant tissue culture.

In the first stage of cultures, the leaf explants enlarged and developed globular structures on the margins as well as on the mid-rib region of the leaves within 3 weeks of culture on MS medium supplemented with NAA (1.0, 2.0 and 4.0 mg l^{-1}) plus BAP (1.0 mg l^{-1})**.** After 5 weeks of callus induction, it turned green, hard and compact in the surface of all leaves at lower concentrations of NAA (1.0 and 2.0 mg l^{-1}). All the treatments (from 1.0 to 4.0 mg l^{-1}) provided callus induction (Table 1). However, the rate of callus formation decreased with increasing concentration of NAA on medium, under

Table 2. Effect of cytokinin and auxins on callus induction from stem explants of *Amygdalus communis* L.cv. Yaltinski

Cytokinin (mgl^{-1})	*Auxins (mgl^{-1})*				*% Response*		*Types of callus*
BAP	*IAA*	*NAA*	*IBA*	*2,4-D*	*Light*	*Dark*	
1	-	-	-	-	0	0	Callus not formed
1	1	-	-	-	0	0	Callus not formed
1	2	-	-	-	0	0	Callus not formed
1	4	-	-	-	50	0	Fragile & ligth green callus
1	-	1	-	-	70	0	Loose & light green callus
1	-	2	-	-	60	0	Loose & light green callus
1	-	4	-	-	0	0	Callus not formed
1	-	-	1	-	0	0	Callus not formed
1	-	-	2	-	0	0	Callus not formed
1	-	-	4	-	0	0	Callus not formed
1	-	-	-	1	0	80	İntensive & hard callus
1	-	-	-	2	0	0	Callus not formed
1	-	-	-	4	0	0	Callus not formed

Table 3. Effect of different concentrations of BAP on callus proliferation and adventitious shoot development

BAP (mgl^{-1})	*Types of callus*	*Shoot growth*
2.0	Loose- light green	Shoot not formed
4.0	Intensive-dark green -hard	Shoot formed
6.0	callus not occured	Shoot not formed
8.0	callus not occured	Shoot not formed

dark and light conditions. The best callus formation from leaf explants was observed with the 1.0:1.0 NAA plus BAP treatments (90%) (Fig 1E). As shown in Table 2, for stem explants NAA plus BAP treatments did not promote callus initiation, under dark conditions. However, the callus (loose texture, light green) on 1.0:1.0 and 2.0:1.0 NAA plus BAP treatments (70%, 60% respectively) was obtained, under light conditions.

In our study, the media containing various concentrations of IBA plus BAP showed callus production on leaf explants, but the growth rate was very slow. Callus formation increased with increasing concentration of IBA (Table 1). Callus induced only in the petiol region of leaf explants. But browning observed in the 3 weeks of cultures (Fig 1F,G). As presented in Table 2, no callus from stem explants occurred on IBA plus BAP treatments, under dark and light conditions.

The medium containing 4.0 mg l^{-1} of IAA did not promote callus initiation on leaf explants. The other concentrations (1.0, 2.0 mg l^{-1}) of IAA showed callus production (slightly loose, yellowish green) after 3.weeks of culture. The best callus induction rate (75%) occurred on medium with 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BAP (Table 1). Both light/dark conditions and low concentrations of IAA did not promote callus induction from stem explants. Callus induction (50%) was obtained with the only 4.0:1.0 IAA plus BAP and under light conditions (Table 2). On the contrary, under dark period and on high concentration of IAA (4.0 mg l^{-1}) root was obtained from stem explants directly (Fig 1H). Table 1 and 2 show the effects of various concentrations of 2,4-D on callus induction from stem and leaf explants. 2,4-D plus BAP (4.0:1.0) treatments did not promote callus initiation from leaf explants. However, the other concentrations (1.0 and 2.0) showed callus production (75%, 50%). The callus obtained on low concentration has hard texture, granular-formed, and green-colored. But, no callus from stem explant occurred on 2.0:1.0 and 4.0:1.0 2,4-D plus BAP treatments under light/dark conditions. Only one concentration (1.0:1.0 2,4-D+BAP) showed embryogenic calli (80%) from stem explants under the dark conditions (Table 2) (Fig 1I). Initially the calli was growing slowly, friable and yellowish green in color. After 4 weeks of callus induction, it turned dark green, hard and compact on stem explants.

Auxin 2,4-D, by itself or in combination with cytokinins, has been widely used to enhance embryogenic callus induction and maintenance (Ziauddin and Kasha 1990). In our research, we found a similar response where 2,4-D was more effective on embryogenic callus formation from stem or leaf explants of yaltinski than all other treatments. The regular light conditions have much more impacts than dark conditions on the callus induction from leaf explants. However, lighting conditions were not effective on embryogenic callus formation.

In the our research, IBA, IAA were less effective than NAA for non-embryogenic calli induction from leaf explants. The best callus production was successful in the presence of 1.0:1.0, 2.0:1.0 NAA plus BAP (90%, 88% respectively) for leaf explants. On the contrary, It was reported that 4.0 mg l^{-1} BAP was more effective on callus formation from shoot tips of mature embryos (*in vitro*-derived) (Yapar et al., 2006). The effectiveness of dark incubation in promoting callus formation and/or shoot regeneration from cotyledon explants was reported in *P. dulcis* (Ainsley et al., 2001a) and P. armeniaca (Nedelcheva and Tsoneva, 1998). It was shown that dark incubation is critical for regeneration of shoots in some *Prunus* species. Also our results are in agreement with the previous reports on regeneration of shoots in *Prunus* species. After 2 times subcultures, the compact, friable callus was selected and transferred to the media supplemented with concentrations of BAP for callus proliferation or adventitious shoot development (Fig 1J). After calli transferred to media supplemented with high concentrations of BAP (6.0, 8.0 mg l^{-1}), browning of the stems explants was observed in the 2 weeks of culture (Fig 1K). When the hard compact middle calli were subcultured on different concentrations of BAP, nodulation and growth in calli resumed in hard compact callus started from stem explants within 4 weeks of incubation (Table 3).The medium containing 4.0 mg l^{-1} of BAP was found to be the most effective than all other concentrations for adventitious shoot development (Fig 1L).

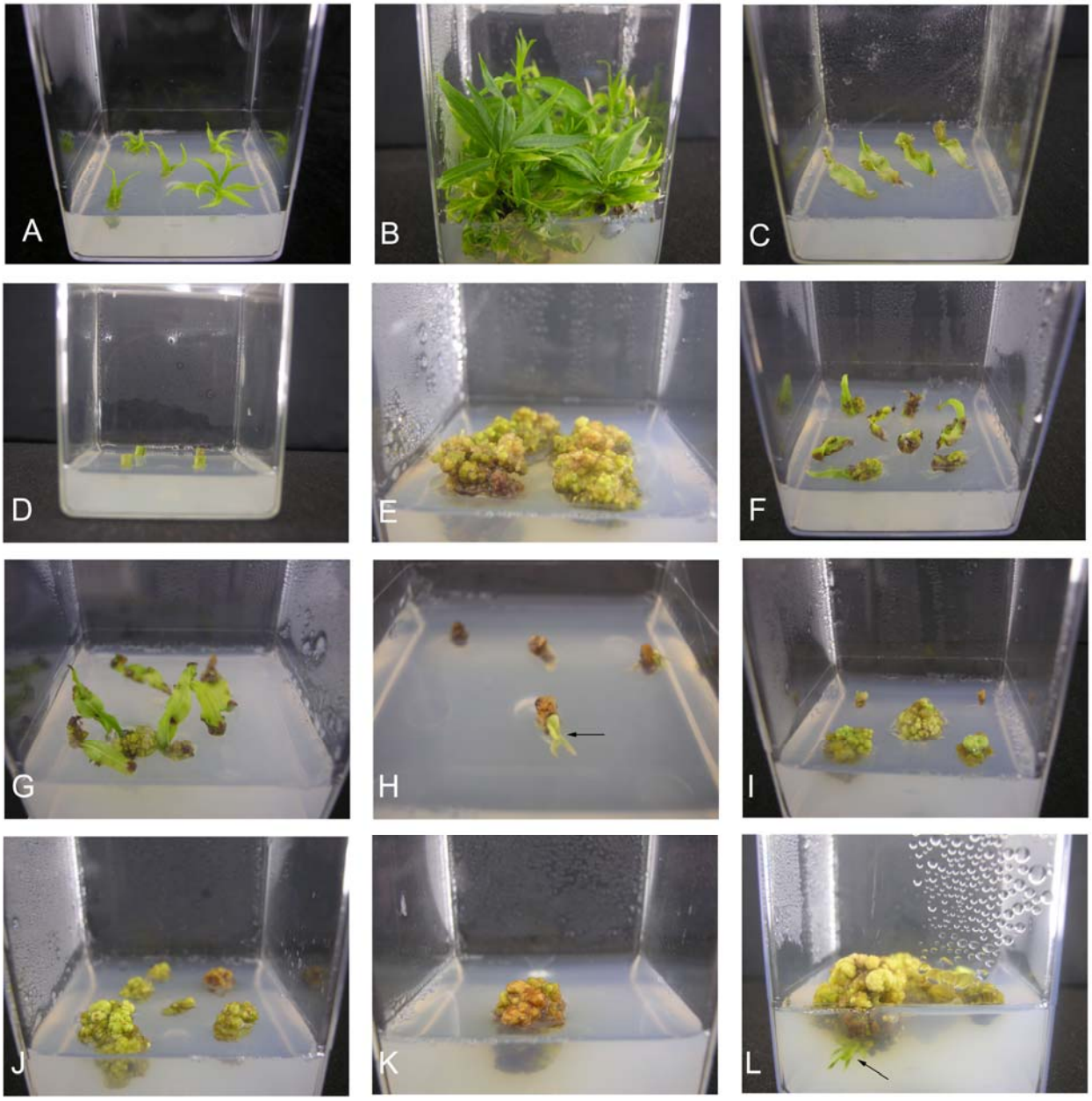

Fig 1. Callus induction and adventitious shoot development from leaf and stem explants of *Amygdalus communis* L. cv. Yaltinski. **(A)** Aspect of apical shoot tips cultured on basal MS medium. **(B)** Multiple shoots grown on MS medium supplemented with 1.0 mgl^{-1} BAP. **(C)** Aspect of leaf explants cultured on MS medium. **(D)** Aspect of stem explants cultured on MS medium. **(E)** Calli formation from leaf explants in the presence of 1.0:1.0 NAA plus BAP. **(F, G)** Low calli formation from leaf explants on MS medium supplemented with IBA plus BAP. **(H)** Root formation from stem explants in the presence of 4.0:1.0 IAA plus BAP. **(I)** Calli formation from stem explants on medium supplemented with 1.0:1.0 2,4-D plus BAP under the dark conditions. **(J)** Aspect of calli was transferred to MS medium containing various concentrations of BAP. **(K)** Aspect of browning calli on medium supplemented with high concentrations of BAP. **(L)** Adventitious shoot formation from stem explants on MS medium containing 4.0 mg l^{-1} BAP.

Embryogenic calli are also a good source of protoplasts, allowing manipulations at the single-cell level, such as gene transfer, somatic hybridization (Lührs and Lörz 1987). Therefore, the callus cultures are rather important in the plant tissue cultures studies. To our knowledge this research is the first study on embryogenic callus induction and regeneration of adventitious shoots from mature stem or leaf explants of yaltinski. The findings of this research will be beneficial for callus culture and transformation protocol of the almond paper-shell cultivar, yaltinski.

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