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Application of plant tissue culture technique to micropropagation of the most important almond cultivars in Syria

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Abstract

Plant tissue culture has become an important technique in plant production. This research was carried out in the Laboratory of Biotechnology for Medicinal Plants of the National Commission for Biotechnology/Damascus, during the period between 2018-2019, to study the effect of some growth regulators on multiplication and rooting of four almond cultivars (Shami Furk, Dafadii, Ferragnes and Fournat), and determine which combination of growth regulators lead to the highest rate of multiplication (in terms of number of shoots and length), and the best rooting (in terms of rooting percentage, number of roots and length). The highest survival and uncontaminated explants percentages were 75%, 62.5% and 62.5% obtained with 0.5% NaOCl for 10 min and 0.1% $HgCl_2$ for 30 sec in cultivars Dafadii, Shami Furk and Fournat, respectively, while the Ferragnes cultivar showed highest response to surface sterilization when explants were disinfected with 0.1% $HgCl_2$ for 1 min, with an average of 50% of survival and uncontaminated explants percentage. The results indicated that Murashige and Skoog (MS) medium supplemented with 1.0 mg/l BA , 0.1 mg/l IBA and 0.2 mg/l GA_3, achieved the highest shoot multiplication with an average of 5.31 and 3.67 shoots per explant and an average of 6.23cm and 4.98cm shoot length in cultivars Shami Furk and Dafadii, respectively, and MS medium supplemented with 1.0 mg/l BA and 0.2 mg/l GA_3, achieved the highest shoot multiplication with an average of 3.84 shoots per explant and an average of 4.67 cm shoot length in cultivar Ferragnes, while the cultivar Fournat did not respond to growth in the multiplication phase. The greatest rooting percentages were 75%, 40% and 35% and the largest average number of roots were 5.17, 3.65 and 3.87 obtained when using IBA auxin at a concentration of 1.0 mg/l for studied cultivars Shami Furk, Dafadii and Ferragnes, respectively with an average of 3.03 cm root length. The acclimatization percentages ranged between 50% and 85% in both cultivars Ferragnes and Shami Furk, respectively.

Keywords: Almond, cultivars, Micropropagation, Cytokinin, Auxin, Rooting.

Introduction

The cultivated almond (*Prunus dulcis* Mill.) belongs to the sub-genus Amygdalus from the Rosaceae family and the Prunoideae sub-family and is adapted to dry and semi dry areas (Al-Ghzawi et al., 2009). Almonds have great nutritional value, high fat content, and are added to many nutritional products (Ahrens et al.,2005). Martins et al. (2004) reported that almond trees are widely spread throughout the Mediterranean region. They are considered one of the most important and ancient nut trees. They also represent the largest nut tree crop produced around the world (Sorkheh et al., 2009). In addition to the industrial uses of the almond tree, it is also used as an ornamental tree (Isıkalan et al., 2008).

Syria is rich in genetic diversity among wild species of almond, such as P. orientalis, P. arabica, P. communis, P. korschinskii and P. spartioids (Ladizinsky, 1999), which are used for stabilizing water sheds and controlling soil corrosion (Mortazavi, 1986).

In addition, they are a rich source of important characteristics used by breeding programs to improve cultivars (Gradziel et al., 2001, Rahemi et al., 2010). Numerous difficulties are encountered in almond cutting during the process of propagation due to the tree's poor rooting ability (Hartmann et al., 1997). The traditional method of almond propagation is through T-budding either in the late spring or in the fall (Hartmann et al., 1997), which is an exhaustive and time-consuming method. Hence, the need for new and rapid methods for propagation is growing, and that is possible only through micropropagation (Jain and Häggman, 2007). Micropropagation is a convenient and rapid procedure to obtain a large number of genetically identical plants (Antonopoulou et al., 2005). Recent reports demonstrate the feasibility of producing plantlets in almond via shoot-tips (Gürel and Gülşen, 1998), immature seed cotyledons (Ainsley et al., 2001), and leaf explants (Ainsley et al., 2000). Akbas et al.(2009) mentioned that the explants of Amygdalus communis L. which were cultured on MS medium containing various concentration of BA, Kn, for shoot multiplication were best achieved from explants on MS medium containing 30 g/l sucrose, 7g/l agar and 2.0 mg/l BA. This amount of BA (2.0 mg/l) gave the best multiple shoot formation response with average of 16.10 shoots per explant. Abou Rayya et al. (2010) found that the most effective cytokinine for enhancing *in vitro* growth was BA followed by kinitine and zeatin respectively. Lower concentration of BA and kinitine at (0.5 and 1.0 mg/l) gave healthier plants than 2.0 or 4.0 mg/l. Gürel and Gülşen (1998) obtained the best rate of shoot multiplication for almond by using the combination of 1.0 mg/l BA and 0.1 mg/l IBA. However, on the contrary, Isıkalan et al. (2008) obtained the highest rate of shoot multiplication for almond cultivar 'Nonpareil' in an MS medium supplemented with 1.0 mg/l BA.

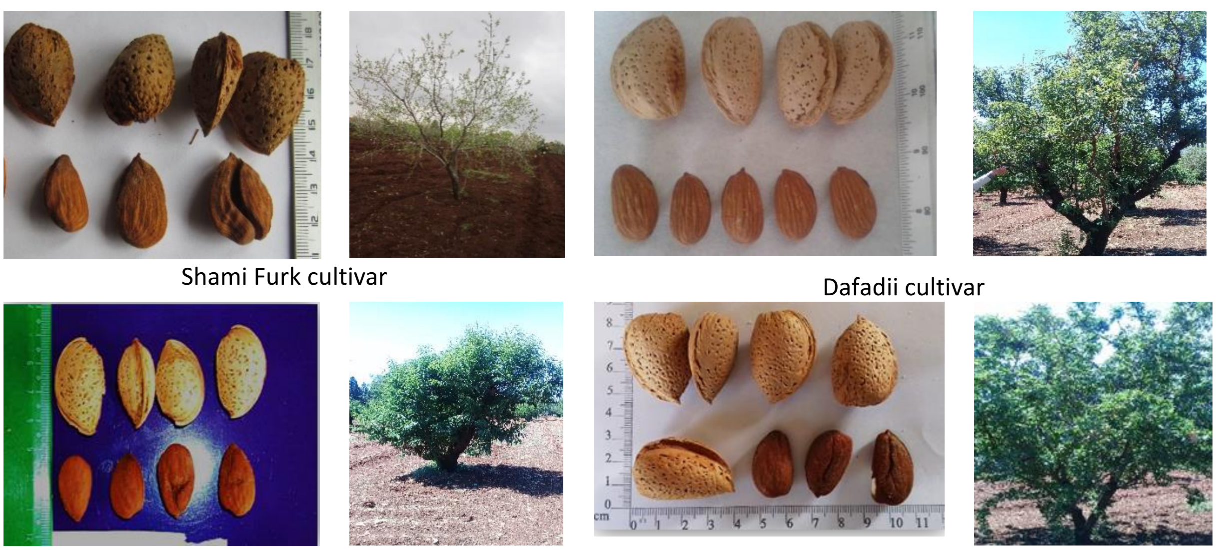
Indole butyric acid (IBA) is the most widely used auxin to stimulate the rooting process in cuttings because of: 1) its high ability to promote root initiation (Weisman et. al., 1988) and 2) its weak toxicity and great stability in comparison to naphthalene acetic acid and indole- 3-acetic acid (Hartmann et. al., 1997). Ainsley et al. (2001) determined that IBA and NAA are the most suitable auxin for rooting seedlings of Ne Plus Ultr and Nonpariel almond species *in vitro* conditions.

Wolella et al. (2017) reported that the best results for rooting of *Prunus domestica* cv. Stanley was obtained from half strength MS medium supplemented with 1.0 mg/l IBA, with an average number of 4.25 roots per shoot and 3.6 cm average root length.

Therefore, the aim of this research was to develop an effective sterilization protocol for *in vitro* propagation of four commercially important almond cultivars (Shami Furk, Dafadii, Fournat and Ferragnes), determine the best combination of growth regulators to use in order to achieve the highest rate of multiplication and find a reproducible method for the successful rooting of studied almond cultivars.

Materials and Methods

Plant Materials: This research was carried out in the Laboratory of Biotechnology for Medicinal Plants of the National Commission for Biotechnology/ Damascus, during the period between 2018–2020. The source of plant materials was the nodal segments of two local almond varieties, *Prunus dulcis* cvs. 'Shami Furk' and 'Dafadii' and two entrance almond varieties, *Prunus dulcis* cvs. 'Fournat' and 'Ferragnes' that are grown at the Homs Research Centre of the General Commission for Scientific Agricultural Research. The studied cultivars have a major economical prominence in Syria.



Shami Furk cultivar Dafadii cultivar

Fournat cultivar Ferragnes cultivar

Figure 1: studied almond cultivars

Surface disinfection (Surface sterilization of explants): New shoots of 15-20 cm in length were collected in mid-May from ten-year-old trees of almond cultivars (Shami Furk, Dafadii, Fournat and Ferragnes) and brought to the laboratory. After removing the leaves, shoots were cut into nodal cuttings, which contained 1-2 lateral buds and with lengths that ranged from 0.5 to 1 cm. For the purpose of disinfection, the explants were washed with running water for one hour. Next, they were surface sterilized by dipping in 70% (v/v) ethanol for 30 seconds, then in different sterilants for varying time duration.

1. In the first experiment, the explants were surface sterilized with 30% sodium hypochlorite (NaOCl) solution containing 2 drops of Tween for (10, 20) minutes.
2. In the second case, the explants were surface sterilized with 0.1% mercuric chloride (HgCl2) solution for (1, 2) minutes.
3. In the third experiment, the explants were surface sterilized first with 10% sodium hypochlorite (NaOCl) solution containing 2 drops of Tween for 10 minutes followed by treatment of 0.1% mercuric chloride for 30 seconds.

Finally, In all the above cases, the explants after surface sterilization process were rinsed three times (for five minutes each time) in distilled water. Surface sterilization was performed in sterilized conditions.

Shoot initiation: After sterilization, each explant was cultured in an MS medium (Table 1). All the cultured tubes were placed and observed for four weeks in a growth chamber at 22±1 °C, with 16 h of photoperiod, and at a light intensity of 30 µmol $^{m-2\ s-1}$ provided by cool daylight fluorescent lamps (Figure 3).

Table 1: Composition of MS Medium (Murashige and Skoog, 1962)

Ingredients	Amounts (mg/ litre)
Macronutrients	
NH4NO3	1650
KNO3	1900
CaCL2. H2O	0440
MgSO4.7H2O	0370
KH2PO4	0170
Micronutrients	
KI	0.830
H3BO3	06.20
MnSO4. H2O	15.60
ZnSO4.7H20	08.60
Na2MoO4.2H2O	0.250
CuSO4.5H2O	0.250
CoSo4.6H2O	0.025
FeSO4.7H2O	27.80
Na2EDTA	37.30
Vitamins	
Thiamine HCl	0.100
Pyridoxine HCl	0.500
Nicotinic acid	0.500
Myo- inositol	100
Others	
Glycine	2**mg**/l
Sucrose	30g/l
Agar	**7** g/l
pH	5.7

Shoot multiplication: For shoot multiplication, the newly-formed microshoots were sub-cultured in MS medium including various types of growth regulators in order to achieve shoot multiplication:

- MS1: MS + 0.5 mg/l BA + 0.2 mg/l GA_3.
- MS2: MS + 1.0 mg/l BA + 0.2 mg/l GA_3.
- MS3: MS + 2.0 mg/l BA + 0.2 mg/l GA_3 .
- MS4: MS + 1.0 mg/l BA + 0.2 mg/l GA_3 + 0.1 mg/l IBA.

All of the cultivated tubes were placed in a growth chamber at 22±1 °C, with 16 h of photoperiod, and a light intensity of 30 µmol m-2 s-1 provided by cool daylight fluorescent lamps. Number of shoots per explant and Shoot length per explant were recorded after 30 days of sub-culturing.

In vitro regenerated shoots were micropropagated and sub-cultured every three weeks.

Rooting of shoots: Uniform proliferated shoots (2- 3 cm) were transferred to half MS medium supplemented with various concentrations of IBA:

- R1: ½MS without plant growth regulators served as a control.
- R2: ½MS+ 0.5 mg/l IBA
- R3: ½MS+ 1.0 mg/l IBA
- R4: ½MS+ 2.0 mg/l IBA

All the treatments were maintained in the dark for one week and then were transferred to photoperiod of 16/8 h light/dark for three weeks. The rooting parameters: Rooting percentage (%), average number of roots and average length of roots (cm) were recorded after after 4 weeks of *in vitro* culture.

Acclimatization: *In vitro* rooted shoots were removed from cultured tubes and the roots were gently washed in distilled water to remove any residual medium. Subsequently, they were transplanted into individual commercial plastic pots filled with an autoclaved mix of perlite and peat [1:2 (v/v)]. 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 ºC under natural daylight conditions.

Experimental Design and Statistical Analysis:_All experiments were carried out according to completely randomized design. The multiplication experiment contained four almond cultivars, Five Surface sterilization treatment, four proliferation treatments (medium), three rooting treatments, three replicates per each treatment, and 20 explants per replicate. The results were analysed using the analysis of variance (ANOVA) method to determine the significant differences between the means of all treatments. Duncan's multiple range test was used at 1% level of significance to assess the significance of difference among means using the Genstat 12 statistical program. Means followed by the same letter are not significantly different.

Results and Discussion

1. Surface sterilization of explants:

Several factors can affect success of sterilization such as season of year, position of culture, location of explant on mother plant, method of sterilization, both type and concentrations of sterilization chemical materials and finally exposure period to sterilization materials. As shown in Table (2) and Figure. (2) cleared the effect of sodium hypochlorite and/or mercuric chloride on surface sterilization of studied almond cultivars explants for micro propagation. The responses of explants to various types and concentrations of sterilization agents were different.

The highest percentages of uncontaminated and survival explants (75% and 62.5%) were recorded when explants of almond cultivars (Dafadii, Shami Furk and Fournat) were disinfected with 0.5% NaOCl for 10 min and 0.1% $HgCl_2$ for 30 sec, while Surface sterilization of explants with 0.1% $HgCl_2$ for 1 min achieved the highest percentage of uncontaminated and survival explants (50%) for Ferragnes cultivar. Meanwhile, the lowest concentration of NaOCl (1.5%) at all exposure periods possessed the lowest percentage of uncontaminated survival explants. These results clearly revealed that the varaiable impact of sterilizing agents and success of sterilization is dependent on genotype used. The local cultivar Dafadii showed the highest response to surface sterilization treatments (Figure. 3), with an average of 46% for all treatments. The results obtained in this study revealed that the sodium hypochlorite alone was the least effective in the sterilization process, while the use of mercury chloride $HgCl_2$ with sodium hypochlorite were the most effective, although $HgCl_2$ is extremely toxic (Alizadeh-Arimi et al., 2020).

Table 2. Effects of sterilizing agents used in a different concentration with varying time of sterilizing nodal cuttings of the studied almond cultivars.

Treatments	Uncontaminated and survival explants percentage				Mean
	Ferragnes	Fournat	Shami Furk	Dafadii	
1.5% NaOCl for 10 min	12.5 m	0 p	7.5 o	10 n	7.5 E
1.5% NaOCl for 20 min	22.5 j	15 l	12.5 m	40 g	22.5 D
0.5% NaOCl for 10 min and 0.1% $HgCl_2$ for 30 sec	37.5 h	62.5 b	62.5 b	75 a	59.38 A
0.1% $HgCl_2$ for 1 min	50 e	50 e	57.5 d	60 c	54.38 B
0.1% $HgCl_2$ for 2 min	25 i	17.5 k	25 i	45 f	28.12 C
Mean	29.5 C	29 C	33 B	46 A	……
L.S.D 0.01	Cultivars	0.70			
	Treatments	0.78			
	Interaction	1.57			

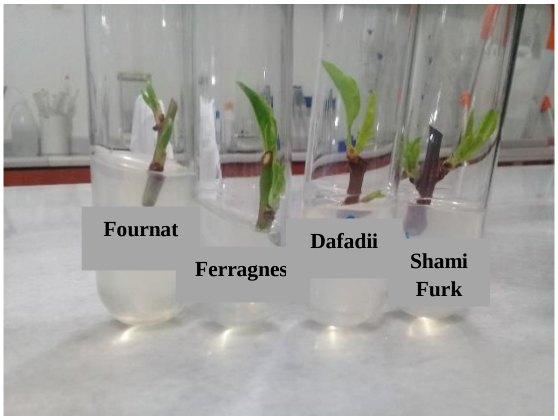


Figure 2. New shoot of studied cultivars at the second week of initiation stage.

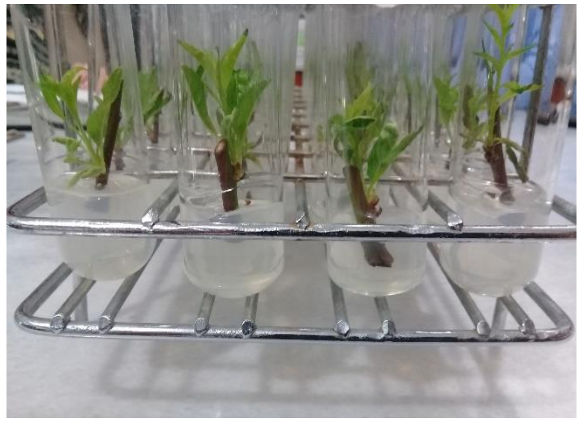


Figure 3. New shoot of Dafadii cultivar at the end of initiation stage.

2. **The Effect of Different Plant Growth Regulator Combinations on the Shoot Multiplication of Almond Cultivars:**

The data in Table 3 show the effect of different concentrations of BA in combination with GA_3 at 0.1 mg/l alone or with GA_3 at 0.1 mg/l and IBA at 0.1 mg/l on the average number of shoots produced per explant as well as the mean length of the shoots. According to the data, the MS medium culture supplemented with 1.0 mg/l BA plus 0.2 GA_3 plus 0.1 IBA, resulting in a mean number of 2.70 shoots with an average shoot length of 3.13 cm, is the most suitable treatment. As for the interactions between studied cultivars and growth regulator combinations, the results clarified that the highest significant average number of shoots/explant (5.31 and 3.67) and the longest shoot (6.23 and 4.98 cm) for both Shami Furk and Dafadii cultivars, respectively, were obtained in a medium supplemented with BA at 1 mg/l plus GA_3 at 0.2 mg/l plus IBA at 0.1 mg/l (Figure 4), while the highest significant average number of shoots/explant (3.84) and the longest shoot (4.67 cm) for Ferragnes cultivar were obtained in a medium supplemented with BA at 1.0 mg/l plus GA_3 at 0.2 mg/l, while the Fournat cultivar did not respond to growth in the multiplication phase and elongation. Prior research indicates that each plant species propagated *in vitro* needs different requirements and concentrations of plant growth regulators. Most of them are based on BAP and auxins IBA, IAA and NAA (Channuntapipat, 2002). Brison et al. (1995) found that the simultaneous presence of cytokinin, giberellin and auxin in the medium was more effective for *Prunus* rootstocks *in vitro*. It was indicated that the cytokinin, such as BA, encourages cell division by activating DNA synthesis, inducing growth of lateral buds, and promoting shoot formation (Dobranszki and Silva, 2010). The selection of BA as a cytokinin was due to its effect *in vitro* with several woody plants (Bennett and Davies, 1986). In this regard, it is important to observe that the multiplication medium should be supplied with more cytokinin in relation to auxin (Murashige, 1974). The effect of cytokinins on tissue or organ cultures differs based on the culture type, the cultivar used, and explant age (George et al., 2007). The auxins control cytokinin levels through repressing its synthesis ratio and its gathering size (Nordstrom et al., 2004). Kodad et al. (2020) showed that the regulators'best concentration and type depends on the genotype to get a successful multiplication rate of various almond explants. The results obtained from Isıkalan et al. (2011) proved that the medium supplied with only BAP were more beneficial than the medium containing IBA and BAP for shoot development of almond cultivars (Nonpareil).

Table 3. Micropropagation of almond cultivars explants in MS medium supplemented with different plant growth regulators combinations

Media	Avg No of shoots/ explants				Mean	Avg length of shoots (cm)				Mean
	Shami Furk	Dafadii	Ferragnes	Fournat		Shami Furk	Dafadii	Ferragnes	Fournat	
MS1	1.34 i	1.12 j	1.29 i	0 k	o. 93 D	2.53 c	2.40 cd	1 e	0 f	1.48 D
MS2	3.22 d	3.15 e	3.84 b	0 k	2.55 B	2.62 c	2.43 cd	4.67 b	0 f	2.43 B
MS3	2.34 f	1.07 j	1.54 h	0 k	1.24 C	2.46 cd	2.02 d	2.54 c	0 f	1.75 C
MS4	5.31 a	3.67 c	1.8 g	0 k	2. 70 A	6.23 a	4.98 b	1.3 e	0 f	3.13 A
Mean	3.05 A	2.25 B	2.11 C	0 D	.....	3.46 A	2.96 B	2.37 C	0 D	.....
l.S.D (0.01)	**Cultivars**	0.03				0.14				
	Media	0.03				0.14				
	Interaction	0.06				0.28				

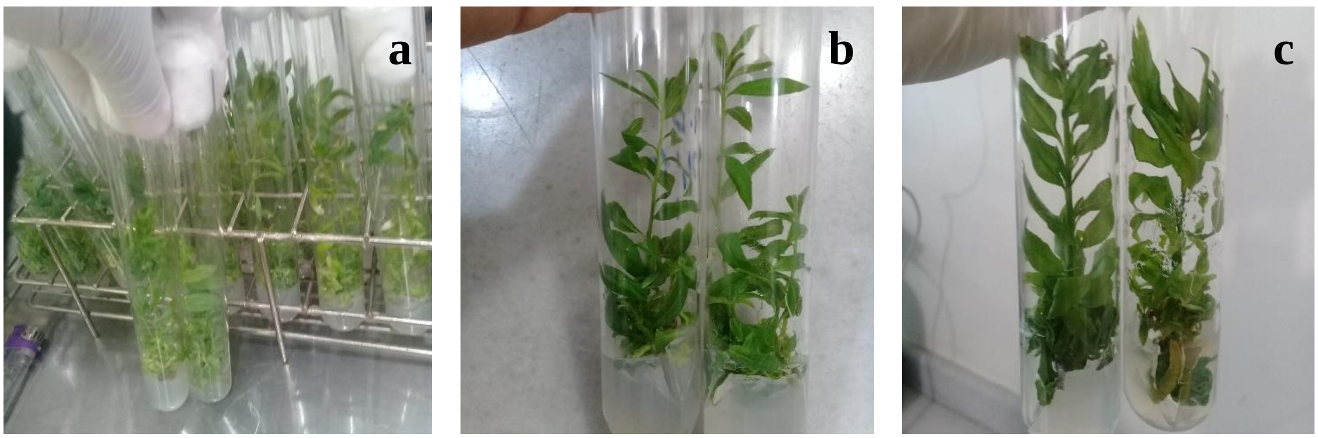


Figure 4. Shoot culture at the end of multiplication stage for Shami Furk cv. (a) and Dafadii cv. (b) in MS medium supplemented with BA at 1 mg/l plus GA_3 at 0.2 mg/l plus IBA at 0.1 mg/l (b), and for Ferragnes cv. (c) in MS medium supplemented with BA at 1 mg/l plus GA_3 at 0.2 mg/l

3. The Effect of Different concentrations of auxin on rooting of Almond Cultivars:

The data of percentage of rooting, roots/explants, and average length of roots (cm) as affected by the concentration of auxin were presented in table 4 and figure 5. Based on the results obtained, the effect of IBA concentrations was significant on the rooting percentage (%), root number and root length (Table 4).

The highest rooting percentage (50%), maximum number of roots/shoot (4.23), and highest root length (3.03 cm) were obtained in half strength MS medium supplemented with 1.0 mg/l IBA. However, no rooting was noticed with IBA at 0.50 mg/l for Ferragnes or by IBA at 2.0 mg/l for all used cultivars and in the control treatment.

In this study, the use of the IBA with concentration of 2.0 mg/l induced the callus formation for Shami Furk cultivar (Figure 5).

The interactions between treatments and cultivars, showed the highest rooting percentages (30 %, 16.25%, 8.75%) and the maximum number of roots (5.17, 3.65, 3.87) were obtained in half strength MS medium supplemented with 1.0 mg/l IBA for all studied cultivars (Shami Furk, Dafadii and Ferragnes, respectively).

For the average length of roots, results indicated that IBA at 1.0 mg/l produced the highest root length (3.25 cm, 3.14 cm) for Dafadii and Ferragnes cultivars, respectively, while IBA at 0.5 mg/l produced the highest root length (2.92 cm) for Shami Furk cultivar. Indole-3-butyric acid (IBA) is commonly used to promote root initiation both *in vitro* and with cuttings (Pan and Zhao, 1994). IBA can enhance rooting via increased internal free IBA or may synergistically modify the action of endogenous synthesis of IAA (Krieken et al., 1993). Thus, keeping cultures in the dark for a short period prior to transfer them into light condition can enhance *in vitro* rooting ability because photoreceptor activation in dark is one of the factors which are involved in plant growth processes (Tian et al., 2007; Lamrioui et al., 2011; Housman, 2003). Furthermore, IBA is more stable and less sensitive to auxin degrading enzymes (Riov, 1993). Sabatini et al. (1999) reported that differentiation of phloem ray parenchyma cells into root primordia depends upon the type and concentration of auxin. In the literature, the concentration of 1.0 mg/1 of IBA is usually the one mostly used (Drew et al., 1993; Kalinina and Brown, 2007). Baker and Wetzstein (2004) have reported that higher concentrations of auxin induce the higher level of degradative metabolites in tissues, thus blocking the regeneration process. Moreover, Sugiyama (1999) has reported that the effect of an auxin on rooting is promontory at low concentrations and inhibitory at supra-optimal concentrations.

Table 4. Effect of various levels of IBA on rooting of almond cultivars shoots on half strength MS medium.

IBA treatment (mg/l)	Rooting percentage (%)			Mean	Avg No of roots/shoot			Mean	Avg length of root (cm)			Mean
	Shami Furk	Dafadii	Ferragnes		Shami Furk	Dafadii	Ferragnes		Shami Furk	Dafadii	Ferragnes	
0.00	0 f	0 f	0 f	0 C	0 f	0 f	0 f	0 C	0 f	0 f	0 f	0 C
0.50	45 b	25 e	0 f	23.33 B	4.22 b	2.93 e	0 f	2.38 B	2.92 c	2.54 e	0 f	1.82 B
1.00	75 a	40 c	35 d	50 A	5.17 a	3.65 d	3.87 c	4.23 A	2.70 d	3.25 a	3.14 b	3.03 A
2.00	0 f	0 f	0 f	0 C	0 f	0 f	0 f	0 C	0 f	0 f	0 f	0 C
Mean	30 A	16.25 B	8.75 C	......	2.34 A	1.64 B	0.97 C	0 C	1.4 B	1.45 A	0.8 C	....
l.S.D (0.01)	**Cultivars**		0.73		0.008				0.008			
	Media		0.85		0.007				0.007			
	Interaction		1.47		0.014				0.014			

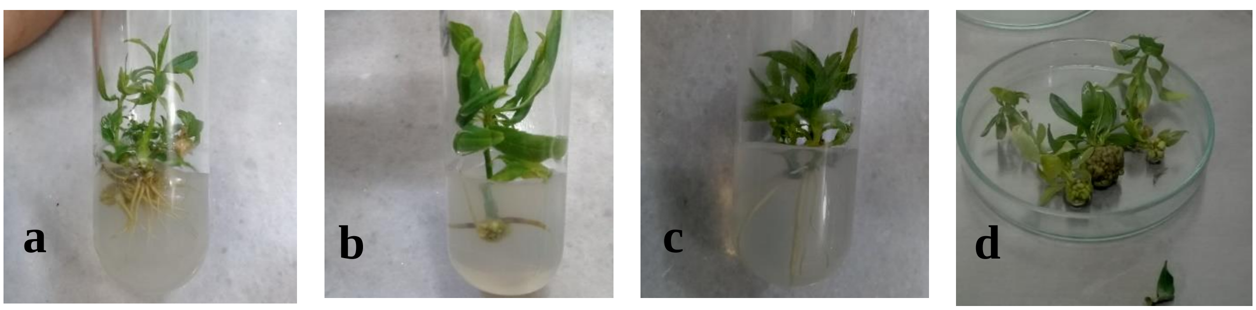


Figure 5. Rooting of almond cultivars on half strength MS medium containing 1.0 mg/l IBA (a. Shami Furk cv., b. Dafadii cv., c. Ferragnes cv.);
d: callus formation in treatment containing 2.0 mg/l IBA for Shami Furk cv.

Acclimatization: The data in Table 5 show the survival percentage ranged between 50% and 85%. The cultivar Shami Furk showed the highest survival rate (85%). It has now been observed that the process of acclimatization depends on a number of crucial factors, including genotype, which not only influences the response of the explant to different culture media, but also the organogenesis and ability of the regenerated plants to withstand the ex vitro growing conditions (Hazarika et al., 2006). Figure 6 shows the survival percentage at 90 days after transfer to an autoclaved mix of perlite and peat.

Table 5. Percentage Survival Rate of almond Plantlets at 90 Days after Transfer to an autoclaved mix of perlite and peat.

Cultivar	Percentage Survival Rate (%)
Ferragnes	50 c
Dafadii	66 b
Shami Furk	85 a
L.S.D 0.01	3.03

Figure 6. Acclimatized of almond cultivars plantlets after 2 months

Conclusions

- In this study, a protocol for surface sterilization of four almond cultivars was developed using nodal segments.
- MS medium containing 1.0 mg/l BA, 0.1 mg/l IBA, and 0.2 mg/l GA_3 was chosen as the optimum medium for multiplication and development of Shami Furk and Dafadii shoots.
- MS medium containing 1.0 mg/l BA and 0.2 mg/l GA_3 was chosen as the optimum medium for multiplication and development of Ferragnes shoots.
- half strength MS medium containing 1.0 mg/l IBA was chosen as the optimum medium for rooting of Shami Furk, Dafadii and Ferragnes shoots.

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