African Journal of Biotechnology Vol. 7 (12), pp. 1875-1880, 17 June, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

In vitro micropropagation of almond *(Amygdalus communis* L. cv. Nonpareil)

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Accepted 9 May, 2008

An efficient *in vitro* propagation method was developed for almond (*Amygdalus communis* L. cv. Nonpareil). The effect of BA and kinetin (0.0, 0.5, 1.0, 2.0, 4.0 mgl^{-1}) on the culture initiation of zygotic embryos isolated from mature seeds was investigated. A Murashige and Skoog (1962) (MS) medium containing 30 gl^{-1} sucrose, 0.5 and 1.0 mgl^{-1} N^6-benzylaminopurine (BA) and 7 gl^{-1} agar resulted in a multiple shoot initiation at the rate of 11.0 ± 1.32 and 14.7 ± 2.12 shoot per explant, respectively, in 28 days of culture. The effects of a low concentration of BA (0.1, 0.5, 1.0 and 2.0 mgl^{-1}) and different combinations of auxin + cytokinin were investigated for shoot proliferation. The best results for new shoot production were obtained from a MS culture medium which was supplemented with 1.0 mgl^{-1} BA. The rooting was achieved in a ½ MS medium supplemented with 8.0 mgl^{-1} indole acetic acid (IAA). The *in vitro* raised plants were acclimatized in a growth room and successfully transplanted to the field. This method here in described will be useful for the rapid multiplication of almond (*A. communis* L. cv. Nonpareil) in commercial exploitation.

Key words: *Amygdalus communis* L.cv. Nonpareil, micropropagation, zygotic embryos, *in vitro.*

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; from the Middle and West Asia, it has diffused to other regions and continents which include the Middle East, China, the Mediterranean region and America (Ladizinsky, 1999). Besides its commercial use as a nut crop, the almond can be used for ornamental planting because it has beautiful flowers which are white or pale pink.

The improvement of almond cultivars has traditionally been carried out by controlled crosses among selected clones, seed isolation and germination, followed by selection and vegetative propagation for evaluation of superior stocks (Kester and Asay, 1975). Due to cross-pollination, commercial almond trees are virtually as variable as wild populations. The breeding strategy was to exploit this genetic variation using seed orchards and controlled crossing (Kester et al., 1977). Since it is a cross-pollinated species, a continuous genetic variation has occurred in the almond through the years, which has led to the formation of very different types in terms of crop development, fruit yield and quality, and tolerance to environmental stresses. To minimize the problem of the enormous genetic variation and to obtain genetically identical populations, vegetative propagation via layerage or cutting is inefficient due to the great problems of this fruit species in rooting *in vivo* (Henry et al., 1992; Gjuleva and Atanassov, 1994). This long and difficult process can be shortened by newly developed biotechniques that allow for the introduction of specific genes, through direct or mediated gene transfer methods, and the recovery of improved genotypes of elite cultivars. This, therefore, makes the plant tissue culture techniques more valuable for the clonal propagation of almond trees.

In vitro micropropagation methods have been intensively used for the clonal propagation of many tree species, of which fruit and timber trees are of great eco-

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Abbreviations: BA, Benzylaminopurine (N^6-benzyladenine); kin, kinetin; MS, Murashige and Skoog medium; IAA, indole acetic acid; NAA, naphtalen acetic acid; PGRs, plant growth regulator(s).

nomic importance (Gomez and Segura, 1995). However, many tree species are not suitable for vegetative multiplication and in those which are suitable, to a certain extent, the process is rather slow. There have been few studies on the propagation of almonds through tissue culture. In the almond, plantlet regeneration through adventitious induction has been achieved from juvenile explants or endosperm (Mehra and Mehra, 1974; Seirlis et al., 1979; Rugini and Verma, 1983; Bimal and Jha, 1985).

In this study, an efficient *in vitro* micropropagation method for *A. communis* cv. Nonpareil was developed. At the same time, the present study has shown that, axenic shoots which have been developed rapidly from the mature seed of the almond "*A. communis* cv. Nonpareil" can be used as healthy rootstocks for micrografting studies. The method which was used and is described here will be a useful protocol for further studies similar to micrografting of the almond.

MATERIALS AND METHODS

In this study, mature seeds from the *A. communis* cv. Nonpareil were used (Figure 1). These were provided by the Şanlıurfa-Koruklu Agricultural Research Station in the Southeast of Turkey. *In vitro* laboratory techniques, which were suggested by Gautheret (1959) were used.

Zygotic embryos which had been isolated from the mature seeds were used for culture initiation. The mature seeds, from which the outer pericarp and shells had been removed, were surface-sterilized by immersion in a 10% (w/v) commercial bleach solution (NaOCl) for 30 min after pre-sterilisation processes, which included washing with tap water for 3-5 min and then, rinsing with 70% (w/v) ethanol for 30 s. Later, the mature seeds were rinsed with sterilized water 5 times for 5 min each to remove the NaOCl. After that, the zygotic embryos were isolated from the mature seeds in a sterile cabine before being inoculated onto the MS (Murashige and Skoog, 1962) basal medium (Figure 2). The medium was supplemented with 3% sucrose (w/v) and solidified with agar (0,7%, w/v, Agar-Agar, sigma). The media were adjusted to pH 5.7 prior to autoclaving (120°C for 20 min). Plant growth regulators (PGRs) were added to the medium prior to the adjustment of pH and sterilization. The cultures were maintained at 25 ± 2°C with 16 h photo period (40 µmol m^{-2} s^{-1}) provided mercury fluorescent lamps. All the shoot tips used in the subsequent experiments were proliferated from zygotic embriyos of the mature seeds.

For *in vitro* culture inititation, a modified MS medium supplemented with 30 gl^{-1} sucrose, 7 gl^{-1} agar and a different concentration of BA and kin (0.5, 1.0, 2.0 and 4.0 mgl^{-1}) was used together with a control group which did not contain any PGRs. Ten days after culture, the mature seeds of almond germinated, and after four weeks of culture developing seedlings produced actively growing apical shoots. In the first stage, for *in vitro* proliferation of axenic germinated explants which have at least an apical shoot, the explants were aseptically cut and cultured in Magenta GA7, but the media were supplemented with different concentrations of BA (0.1, 0.5, 1.0, 2.0 mgl^{-1}) (Figure 3).

In the second stage of our study, the shoots which had been obtained from early culturing were used for an investigation of the effect of the BA + IAA or BA + NAA combination on shoot proliferation. According to the results of our previous experiments, the media were supplemented with BA used as a control (1.0 mgl^{-1}) together with IAA or NAA (0.1, 0.2, 0.5 mgl^{-1}) for shoot proliferation. The newly formed shoots were excised and used for the induction of *in vitro* rooting. For root initiation, the explants were incubated in Magenta GA-7 vessels, each containing a 50 ml half strength MS medium with IAA at 8.0, 10.0, 12.0 and 0.3% sucrose. In addition, the effects of light and darkness on different concentrations of (0.5, 1.0, 2.0 mgl^{-1}) IAA and NAA for *in vitro* rooting of Nonpareil were investigated. The modified half strength MS medium without plant growth regulators served as a control. The rooting response was scored after 40 days of culture.

In vitro rooted shoots were washed in running water before being potted in an 1:1:1 mixture of perlite, sand and soil. Plantlets, were covered with a pyrex beaker to maintain a 90 ± 5% relative humidity for 3-4 weeks before transfer into the growth room. The growth room was illuminated by mercury fluorescent lamps (400 W). The plants were irrigated every 2-3 days with water and, after 21 days, were succesfully adapted to the *in vivo* condition. A four to five week period of acclimatization by progressive reduction of humidity (90 ± 5% to 60 ± 5) using an 1:1:1 mixture of perlite, sand and soil for higher plantlet survival rates.

The data on the number of shoots obtained through zygotic embryos proliferation, shoot length and nod number per explants were analysed using standard ANOVA procedures. The student's *t*-test was adjusted at p=0.05 probability level to separate mean differences when significant treatment effects were detected. A chi-square (χ^2) test was used to test differences between the percentages of surviving plantlets.

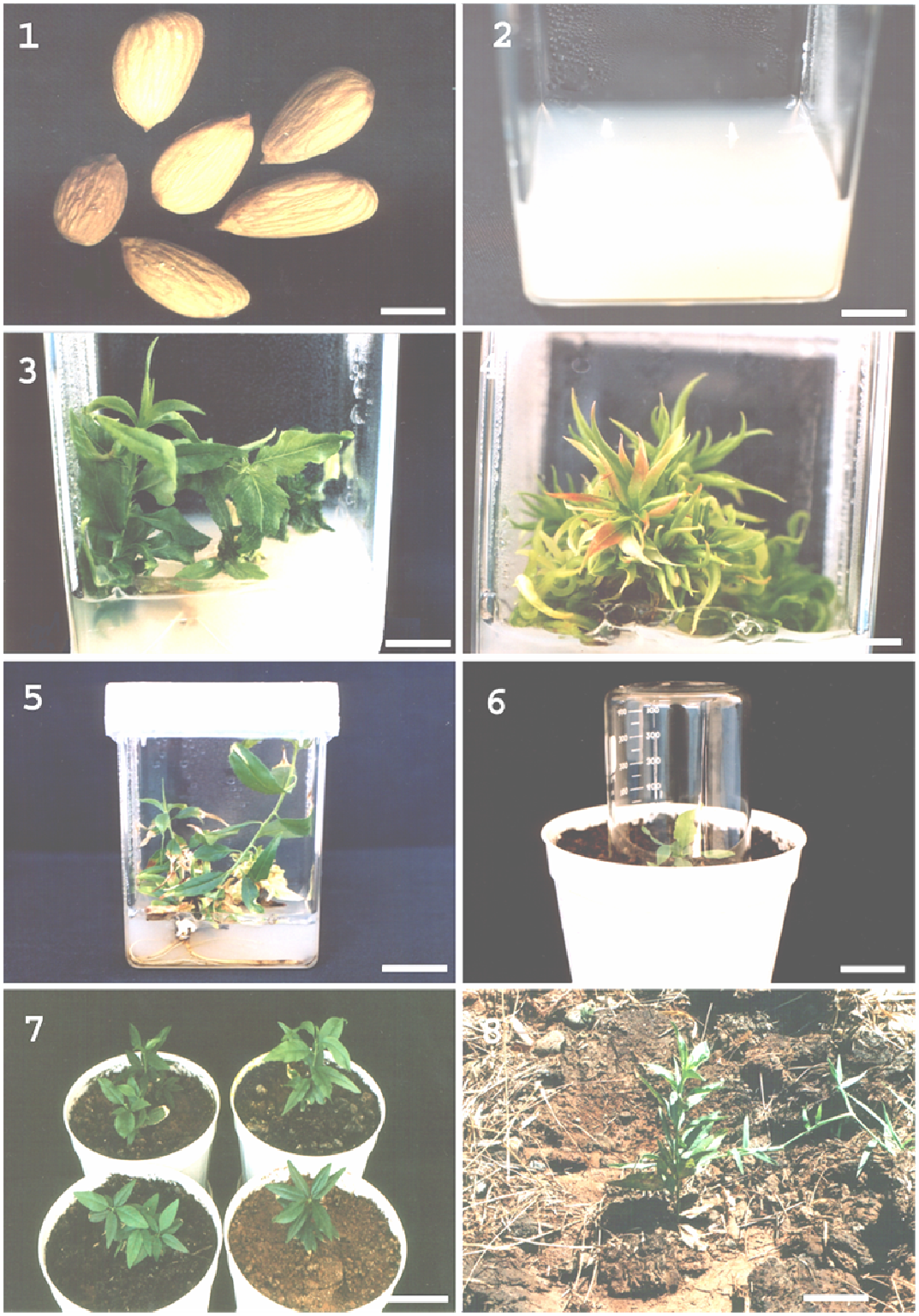
RESULTS AND DISCUSSION

Micropropagation of woody and semi-woody trees is reported as problematic (Babaoğlu et al., 2002) and to obtain a material without viruses is a long-term, complex process. To obtain stocks without viruses, it is necessary to use seeds without viruses as rootstocks. For this purpose, many *in vitro* experiments have been conducted for these economically important fruit trees (Kester and Asay, 1975; Wang and Hu, 1980; Hisajima, 1982; Barghchi and Alderson, 1983; Maynard et al., 1991; Gürel and Gülşen, 1998; Onay et al., 2003; Adıyaman et al., 2004; Tilkat et al., 2005). This study used homozigot diploid Nonpariel, which is an economical important woody tree, and the effects of different PGRs on *in vitro* micropropagation were investigated.

Culture initiation

The effect of cytokinins on the culture initiation of the zygotic embryos isolated from the mature seeds of almond (*A. communis* L.cv. Nonpareil) was investigated. PGRs did not significantly affect the frequencies of seed germination. It was determined that the low concentrations of BA (0.5 and 1.0 mgl^{-1}) used on the zygotic embryos helped to create a lot of lateral shoots, but also led to inhibition of apical development.

From the results presented (Table 1), the number of shoots formed were 1.3 ± 0.15 in the control group, and 11.0 ± 1.33 and 14.7 ± 2.12 in 0.5 and 1.0 mgl^{-1} BA treatments, respectively. However, a decline in terms of new shoot formation in high BA concentrations (2.0 and 4.0 mgl^{-1}) was also observed. Highly significant differen-



Figures 1-8. *In vitro* Micropropagation of *Amygdalus communis* L. Cv. Nonpareil. **1**) Mature seeds of Nonpareil (*Amygdalus communis* L. Cv. Nonpareil), bar = 15 mm. **2**) Zygotic embryos isolated from mature seeds bar = 14 mm. **3**) Four weeks after culturing aseptically seedling of Nonpareil on a MS medium supplemented with BA (1 mgl^{-1}), bar = 14 mm. **4**) After 28 day in cultures, multiple shoot proliferation on a MS medium supplemented with BA (1 mgl^{-1}), bar = 11 mm. **5**) *In vitro* rooted plantlets on a MS medium with IAA (8 mgl^{-1}), bar: 22 mm. **6**) Plantlets undergoing acclimatization in a soil, sand and perlite mixture (1:1:1) in a growth room, bar: 37 mm. **7**) Plantlets regenerated from cloned shoots four weeks after transplanting, bar = 55.5 mm. **8**) Almond seedlings transfered into the field bar = 36 mm.

Table 1. Effects of different BA concentrations on shoot growth and the number of cultured explants.

Treatment	Germinated seeds (%)	Mean shoot length (cm)	Mean shoot number	Mean nod number
Control	100	4.1 ± 0.31a	1.3 ± 0.15c	5.3 ± 0.39a
0.5 mgl^{-1} BA	100	4.1 ± 0.23a	11.0 ± 1.32a	5.7 ± 0.53a
1.0 mgl^{-1} BA	100	4.3 ± 0.39a	14.7 ± 2.12a	4.3 ± 0.29b
2.0 mgl^{-1} BA	100	3.1 ± 0.10b	6.2 ± 0.63b	3.9 ± 0.14c
4.0 mgl^{-1} BA	60	3.3 ± 0.33ab	6.8 ± 0.71b	3.1 ± 0.23d

Data was recorded on the 28th day and representing an average of 10 replicates per treatment with two repetitions of the experiment.
Values followed by the same letter are not significantly different (p = 0.05) according to student's *t*-test.

Table 2. Effects of different kin concentrations on shoot growth and the number of cultured explants.

Treatment	Germinated seeds (%)	Mean shoot length (cm)	Mean shoot number	Mean nod number
Control	100	4.1 ± 0.31a	1.3 ± 0.15a	5.3 ± 0.39a
0.5 mgl^{-1} kin	100	4.2 ± 0.41a	1.7 ± 0.49a	5.7 ± 0.71a
1.0 mgl^{-1} kin	100	3.5 ± 0.30a	1.7 ± 0.39a	4.7 ± 0.65a
2.0 mgl^{-1} kin	100	3.7 ± 0.53a	1.8 ± 0.41a	5.6 ± 0.70a
4.0 mgl^{-1} kin	100	4.2 ± 0.31a	2.0 ± 0.29a	6.1 ± 0.67a

Data was recorded on the 28th day and representing an average of 10 replicates per treatment with two repetitions of the experiment.
Values followed by the same letter are not significantly different (p = 0.05) according to student's *t*-test.

Table 3. Effects of different BA concentrations on shoot proliferation.

Treatment	Mean shoot length (cm)	Mean shoot number	Mean nod number	Mean leaf number
0.1 mgl^{-1} BA	2.9 ± 0.24 b	2.2 ± 0.35c	3.0 ± 0.52bc	13.2
0.5 mgl^{-1} BA	3.0 ± 0.25b	3.2 ± 0.48b	3.7 ± 0.63b	18.7
1.0 mgl^{-1} BA	3.9 ± 0.27a	5.1 ± 0.62a	6.4 ± 0.81a	30.0
2.0 mgl^{-1} BA	3.6 ± 0.27a	5.7 ± 1.04a	4.4 ± 0.60b	21.7

Data was recorded on the 28th day and representing an average of 10 replicates per treatment with two repetitions of the experiment.
Values followed by the same letter are not significantly different (p = 0.05) according to student's *t*-test.

ces were observed between tested concentrations using the student's t-test ($P<0.05$). Consequently, the results suggest that different BA concentrations have an effect on new shoot formation.

Kinetin also induced multiple shoots, but not as effectively as the BA treatments. According to the data presented in Table 2, kinetin concentrations have no effect on new shoot formation. But application of the kinetin indicates that it promotes apical development. There were no statistical differences between all tested concentrations of kinetin and a control group regarding shoot and nod numbers and shoot length ($p>0.05$).

Shoot multiplication

Since the BA treatments gave better shoot proliferation, it was used for shoot multiplication. Multiple shoots were observed in all of the BA treatments tested after 28 days in culture. Both shoot multiplication and elongation of all the explants used in culture differed significantly with different BA concentrations (0.1, 0.5, 1.0 and 2.0 mgl^{-1}). It appears that shoot production decreased as BA concentrations decreases. The best multiple shoot initiation was obtained on the MS medium supplemented with BA at 1 or 2 mgl^{-1}, with a shoot number of 5.1 ± 0.62 and 5.7 ± 1.04 per explant, respectively, on the 28th day of culture (Table 3). Although higher shoot number (5.7 ± 1.04) was obtained from the MS medium supplemented with 2 mgl^{-1} BA, there were no significant differences between compared to 1.0 mgl^{-1} BA, (Figure 4) and the use of the lower concentrations of BA was more economical.

After shoot proliferation, the surviving explants were subcultured regularly every four weeks. None of the treatments produced callusing tissues during the four weeks of culture.

Table 4. Effects of BA (1.0 mgl^{-1}) used in combination with different concentration of two auxin types (IAA and NAA) on the multiplication of shoots from *Nonpareil* explants.

Treatment	Mean shoot length (cm)	Mean shoot number	Mean nod number
0.1 mgl^{-1}NAA + 1.0 mgl^{-1}BA	0.8 ± 0.12b	1.7 ± 0.36b	1.3 ± 0.37c
0.2 mgl^{-1}NAA + 1.0 mgl^{-1}BA	0.9 ± 0.13b	1.2 ± 0.36c	1.3 ± 0.32c
0.5 mgl^{-1}NAA + 1.0 mgl^{-1}BA	1.2 ± 0.22a	2.3 ± 0.46a	1.8 ± 0.35b
0.1 mgl^{-1}IAA + 1.0 mgl^{-1}BA	1.4 ± 0.33a	1.8 ± 0.26b	2.5 ± 0.37a
0.2 mgl^{-1}IAA + 1.0 mgl^{-1}BA	1.3 ± 0.18a	1.4 ± 0.24c	1.8 ± 0.37b
0.5 mgl^{-1}IAA + 1.0 mgl^{-1}BA	1.5 ± 0.25a	2.8 ± 0.51a	2.0 ± 0.37a

Data was recorded on the 28th day and representing an average of 10 replicates per treatment with two repetitions of the experiment.
Values followed by the same letter are not significantly different (p = 0.05) according to student's *t*-test.

To optimize an efficient proliferation method, one concentration of BA (1.0 mgl^{-1}) was used in combination with two auxin types (IAA and NAA) at different concentrations (0.1, 0.2 and 0.5 mgl^{-1}). As can be seen from Table 4, the best response for shoot proliferation was obtained from the explants cultured on the MS medium supplemented with 0.5 mgl^{-1} IAA. At the same time, it was determined that there were no significant differences between 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} IAA.

Hisajima (1982) reported that the best results for proliferation of the almond was obtained from MS medium supplemented with 0.2 mgl^{-1} BA + 0.005 mgl^{-1} IBA. In contrast, in our study determined that the shoot number and the length are considerably reduced when the auxin was added to the nutritient media, and using 1.0 mgl^{-1} BA alone was more economical and effective (Table 4).

Our results are in agreement with those obtained by Gürel and Gülşen (1998) who found that the best shoot growth and proliferation for the commercial cultivars of almond (Nonpareil and Texas) was a MS medium containing 1.0 mgl^{-1} BA. Similar results were reported by Tabachnik and Kester (1977) where reducing BA concentration inhibited new axillary shoot formation. The results showed that using BA in the shoot multiplication was an absolutely necessity.

Rooting and establishment in the field

Nonpareil shoots obtained from *in vitro* conditions were used as material for rooting studies. For *in vitro* rooting of shoots, different concentrations of IAA (8.0, 10.0 and 12.0 mgl^{-1}) were used. At the end of 40 days of culture, half of all explants (50%) cultured on half strength MS medium supplemented with 8.0 mgl^{-1} IAA were observed to have long primer and secondary roots (Figure 5). Root formations were observed only in an MS medium supplemented with 8.0 mgl^{-1} IAA, and the maximum number of roots and root length obtained was 1.75 ± 0.47 and 4.22 ± 0.74 cm, respectively. However, no root development was observed in any of the other treatments of IAA and NAA (0.5, 1.0 and 2.0 mg l^{-1}) and control groups.

Our results are in agreement with Caboni and Damiano's (1994) observations. They studied the effect of darkness and auxin (IAA and IBA) for *in vitro* rooting of two different almond genotypes (Caboni and Damiano 1994). They found a variation in accordance with almond genotype caused by the influence of IAA and IBA on the rooting of scions. Hartmann et al. (1990) reported that the rooting of the almond shoots are difficult, and the established success was quite limited. However, the same researchers indicated that satisfactory rooting results were obtained from only a few cultivars. Ainsley et al. (2001) determined that IBA and NAA are the most suitable auxin for rooting seedlings of Ne Plus Ultra and Nonpariel almond species *in vitro* conditions. They reported that the best results for both species were obtained using 1.0 µM IBA in a 0.6% liquid agar solution for 12 h and then keeping them in a 100.0 µM phloroglucinol (PG) auxin-free medium for 2 weeks. In addition, they remarked that for Nonpareil species, the best nutrition medium is 1/1 strength AP (Almehdi and Parfitt , 1986).

In our study, although the effects of light and darkness on different concentrations of (0.5, 1.0 and 2.0 mgl^{-1}) IAA and NAA for of Nonpareil were analyzed, no positive results were obtained. Besides, a half strength of MS medium supplemented with different concentration of IAA (8.0, 10.0 and 12.0 mgl^{-1}) was also used for rooting. Consequently, the results indicated that shoots cultured on a half strength MS medium supplemented with 8.0 mgl^{-1} IAA showed both primary and secondary root formation at the rate of 50%.

The overall frequency of developed plantlets was 100% in the sand, soil and perlite. In this compost, plantlets tended to resume shoot growth very quickly, and there were at least two pairs of new leaves on each plant. The acclimatized plantlets established, themselves well upon transfer to a growth room (Figure 6). Shoot growth reached 3-4 centimeters in the mixture of soil, sand and perlite in 4 weeks; there were at least two to three pairs of new leaves on each plantlet (Figure 7). Subsequent transfer of rooted plants into the field was also successful (Figure 8). The regenerated plantlets resumed their

growth after one year in the field. These findings had not previously been reported for *A. communıs* L. Cv. Nonpareil.

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