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ORIGINAL ARTICLE / ORIGINALBEITRAG

Developing a Promising Micropropagation Method for Several Drought Tolerant and Hard-to-Root Wild and Domesticated Almond Genotypes by Shoot Tips Culture

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Abstract

The possibility of micropropagation of five selected superior almond genotypes for rootstock purposes were investigated. Shoot tips were subjected to surface disinfection and cultured on Murashige and Skoog (MS) medium containing benzyl adenine (BA) and gibberellic acid (GA_3) (2, 3 and 4 mgl^{-1}), indole-3-butyric acid (IBA) 0.1 mgl^{-1} and thidiazuron (TDZ) 1 mgl^{-1} for establishment and shoot induction. In the proliferation stage, five separate experiments (EX1–EX5) having various concentrations of BA (0, 1.5, 2, 3 and 4 mgl^{-1}), TDZ (0, 0.2 and 2 mgl^{-1}), GA_3 (0, 0.2 and 1 mgl^{-1}) and IBA (0.05, 0.1 and 1 mgl^{-1}) were conducted. In the rooting stage ½ MS medium supplemented with IBA and 1-naphthalene acetic acid (NAA) (0, 0.5, 1 mMg/l) was used. The results showed that increased concentrations of BA and GA_3 lead to enhanced growth characteristics of the explants in the establishment and shoot induction stage and the best combination for the establishment was MS containing 3 and 4 mgl^{-1} BA and GA_3 in combination with IBA and TDZ. The best culture medium for proliferation of *Prunus elaeagnifolia, P. scoparia* × *P. elaeagnifolia, P. eburnea, P. scoparia*, and 'Garnem' was MS medium in EX4, EX2, EX1, EX3 and EX5, respectively. The highest percentage of root formation (38.88%) and root number (5) were obtained in 'Garnem', while the highest root length (14.36 cm), root fresh (6144 mg) and dry (801 mg) weight were recorded in *P. eburnea* using ½ MS with 1 mgl^{-1} IBA.

Keywords Establishment · MS media · Proliferation · Shoot induction · Wild genotypes

Introduction

The almond, *P. dulcis (Mill.) D. A. Webb.* [syn. *P. amygdalus Batsch*], is an economically important temperate fruit tree that is widely grown in Iran. Iran ranks fifth in the world with 76,392 hectares of cultivated areas. Also, the annual production of almonds in Iran is 164,348 t, which ranks fourth in the world (FAO 2020). Almonds are very important in human nutrition because they contain essential and mineral elements, fatty acids, phenolics and high protein (Banjanin et al. 2021; Özcan and Lemiasheuski 2020). Iran is also extremely rich in wild almond genetic resources (Gharaghani et al. 2017). Due to cross-pollination, wild species provide an enlarged pool of available germplasm which possesses desired characteristics such as crop development, nut yield and quality, as well as tolerance to environmental and biotic stresses, which can be utilized in scion breeding programs (Gharaghani et al. 2017; Vahdati et al. 2019). On the other hand, some of the wild almonds can be applied as almond rootstocks, usually in non-irrigated conditions (Khadivi-Khub and Anjam 2016), because they can survive irrigation deficiencies due to some anatomical features such as loss of leaves in hot seasons and having roots with more absorption and storage of soil moisture (Madam et al. 2011; Zokaee-Khosroshahi et al. 2014).

Prunus scoparia, P. elaeagnifolia, and *P. eburnean* are among the widely distributed almond species in some regions of Iran. *P. scoparia* is used as a rootstock for almonds. This genotype has characteristics such as strength, having very small leaves, long and slender shoots (Khadivi-Khub and Anjam 2014). *P. elaeagnifolia* is a shrub or small tree that has mostly been used as the rootstock of plum cultivars

Data Availability All necessary data included to the manuscript and there is no associated data.

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in Iran, especially in a region where access to water is scarce (Gharaghani et al. 2017). *P. eburnea* is known as the gray almond and has conservation, nutritional and native importance (Zangiabadi et al. 2021). These almond species have been used in arid and semi-arid regions (Rahimi et al. 2021; Sorkheh et al. 2009) to control soil erosion and watersheds (Khadivi-Khub and Anjam 2016) and even as rootstocks for top working the almond and plum cultivars (Gharaghani and Eshghi 2014). Also, G × N15 or 'Garnem', originated from the crossing of *P. amygdalus* (Garfi) × *P. Persica* (Nemared) (Series GxN) and used as almond rootstock, both in irrigated and rainfed orchards (Felipe 2009).

Micropropagation is a suitable and fast method for obtaining a large number of genetically identical plants (Bhatia et al. 2015). In addition, it is possible to manipulate plant growth in culture media by adding plant growth regulators at certain stages of growth or maturity. At least two cultivation steps including one growth induction/development treatment for each of shoot and root organs are required to obtain a complete plant (Phillips and Garda 2019). Murashige and Skoog (MS) medium is one of the most common culture media used as a medium for many plant species (Oseni et al. 2018). Various researches showed that the use of IBA, BA and TDZ are suitable for rooting and propagation of almonds rootstocks, respectively (Abbasi et al. 2019; Gerdakaneh et al. 2020; Kodad et al. 2021). Organogenesis in almonds strongly depends on the genotype, type of explant, compounds of plant growth regulators and culture conditions (Choudhary et al. 2015).

However, the use of this method is associated with some problems such as the appropriate culture medium, selecting the appropriate method for disinfection of specimens, optimal growth, and propagation conditions, the appropriate concentration of plant growth regulators, as well as high costs of technologies and tissue culture equipment (Papafotiou and Martini 2009; Massa et al. 2008). Therefore, it is necessary to consider all these conditions in order to obtain a suitable method of plant micropropagation. Micropropagation of almond species and hybrids rootstocks have been reported by multiple researchers (Ainsley et al. 2000; Ezazi et al. 2018; Choudhary et al. 2015; Isikalan et al. 2008; Yıldırım et al. 2010; Aghaye and Yadollahi 2012; Abbasi et al. 2019; Şan et al. 2018; Premier 2021). Researchers suggested MS medium supplemented with 2 mgl^{-1} TDZ and half-strength (½) MS and 0.5 mgl^{-1} IBA for proliferation and rooting of *P. scoparia*, respectively (Abbasi et al. 2019). MS culture medium containing different combinations of auxin and cytokinin used for almond native genotypes. TDZ at a concentration of 1 mgl^{-1} with 1 mgl^{-1} and 1 mgl^{-1} IBA combined with ½ MS medium showed the best proliferation and root formation, respectively (Kodad et al. 2021). The highest rate of rooting and the number of roots were obtained on MS containing 0.1 mgl^{-1} IBA and 0.5 mgl^{-1} BA for GF677, respectively (Gerdakaneh et al. 2020). Reports describing micropropagation, in particular, rooting of wild almond explants are limited.

The use of seed propagation will lead to genetic diversity and loss of genetic uniformity. Therefore, to create a clone and produce similar plants, vegetative methods should be used for almond propagation. On the other hand, vegetative propagation of almonds is difficult, because almonds have difficulty in rooting, and vegetative propagation may cause the transmission of the disease-causing virus. Consequently, an alternative method should be used that produces a large number of virus-free and rooted plants in the shortest possible time. Although Iran is within the center of diversity of almond and also is one of the main producers of almond, the majority of Iranian almond orchards relies on only one seedling rootstock called bitter almond. However, to a far lesser extent peach × almond hybrid clonal rootstock of GF677 and GN ('Garnem') has recently also been considered. In order to utilize valuable wild almond genetic resources and to diversify almond and stone fruits rootstocks, a comprehensive rootstock breeding program started recently at Shiraz University, Fars, Iran. This program aimed to select proper genotypes with desired agronomic traits from seedling populations developed from seeds collected from natural wild almond habitats (Rahimi et al. 2021). As many of selected superior genotypes are difficult to root by common vegetative methods, this study aims to develop an effective in vitro protocol for mass clonal propagation of various superior almond genotypes including three genotype of *P. scoparia, P. elaeagnifolia* and *P. eburnean*, a natural hybrid of (*P. scoparia* × *P. elaeagnifolia*), and newly introduced GN rootstocks to Iran's almond industry by examining the different combinations as well as concentrations of cytokinin, gibberellin and auxin on shoot induction, proliferation and rooting.

Materials and Methods

Plant Material

Young and fresh shoots were collected during May from 7-year-old trees of *P. elaeagnifolia, P. scoparia, P. eburnean* selected genotypes, *P. scoparia* × *P. elaeagnifolia* (natural hybrid) and GN rootstocks grown in the almond collection of Shiraz University (Fig. 1a). Young shoots were used for disinfection after being separated from the tree. The leaves of young shoots were removed and cut to a length of 10–15 cm. These explants were washed with tap water for 30 min. It was then disinfected in 5% fungicide for 90 min. Then, explants in the laminar hood were immersed in 70% ethanol for 60 s, sterilized in 1% Sodium hypochlorite (w/v) for 10 min, and washed three times with sterile distilled wa-

Fig. 1 Young shoots used as explants (**a**). Disinfection of explants in a laminar hood (**b**)

ter. The temperature of the water to wash the young shoots was 36–38 °C (Fig. 1b).

Establishment and Shoot Induction

The surface-sterilized apical branches of various almond genotypes (length 1.5–2.0 cm) were cultured on MS (Murashige and Skoog 1962) medium containing different concentrations of plant growth regulators (PGRs) (BA, GA_3, TDZ, IBA). The medium was supplemented with 3% sucrose (w/v) and solidified with agar (0.7% w/v, agar-agar, Sigma, Ronkokoma, NY, USA). In this study, all media were adjusted to pH 5.5–5.7 (prior to autoclaving at 121 °C and 15 psi for 20 min), and cultures were placed in a growth chamber at 25 ± 2 °C with 16 h photo period (40 µmol m-2 s-1) provided with mercury fluorescent lamps. The various combination of BA, GA_3 and TDZ were used for shoot proliferation (Table 1).

The explants were placed on the culture medium containing the mentioned concentrations for 4 weeks. Due to the low growth rate of the plant samples, they were again transferred to a new medium with the same concentrations as the previous medium, and let them grew in this medium for 4 more weeks. After this period, the desired traits including number of shoots per explants, shoot length (cm), establishment rate (%), leaf number, node number and internode length were recorded.

Table 2 The various combinations of BA, GA_3, IBA and TDZ in shoot proliferation medium of several almond genotypes

PGRs	EX1	EX2	EX_3	EX4	EX5
BA (mgl^{-1})	0	2	3	4	1.5
TDZ (mgl^{-1})	0	0.2	0	2	0
GA_3 (mgl^{-1})	1	0.2	0	0	0
IBA (mgl^{-1})	1	0.05	0.1	0.1	0.1

Shoot Proliferation

The elongated shoots were transferred onto a proliferation medium as new explants. This stage was performed as five independent experiments and, in each experiment, the culture medium contained different concentrations of growth regulators. All genotypes were included to all experiments (Table 2). Other components of the medium and growth chamber conditions were similar to that of the first stage. After 4 weeks, traits including callus induction (%), shoot number, shoot length, survival rate (%) and number of leaves were measured.

Rooting

The proliferated culture were transferred to ½ MS medium supplemented with 40 gl^{-1} sucrose and 6 gl^{-1} agar, IBA and NAA (0, 0.5, 1 mgl^{-1}) and adjusted to pH 5.6. Other compo-

Table 1 The various combinations of BA, GA_3, IBA and TDZ for establishment and shoot induction medium of several almond genotypes

	BA (mgl^{-1})	GA_3 (mgl^{-1})	TDZ (mgl^{-1})	IBA (mgl^{-1})
Treatment 1 (T1)	0	0	0	0
Treatment 2 (T2)	2	0	1	0.1
Treatment 3 (T3)	3	0	1	0.1
Treatment 4 (T4)	4	0	1	0.1
Treatment 5 (T5)	0	2	1	0.1
Treatment 6 (T6)	0	3	1	0.1
Treatment 7 (T7)	0	4	1	0.1

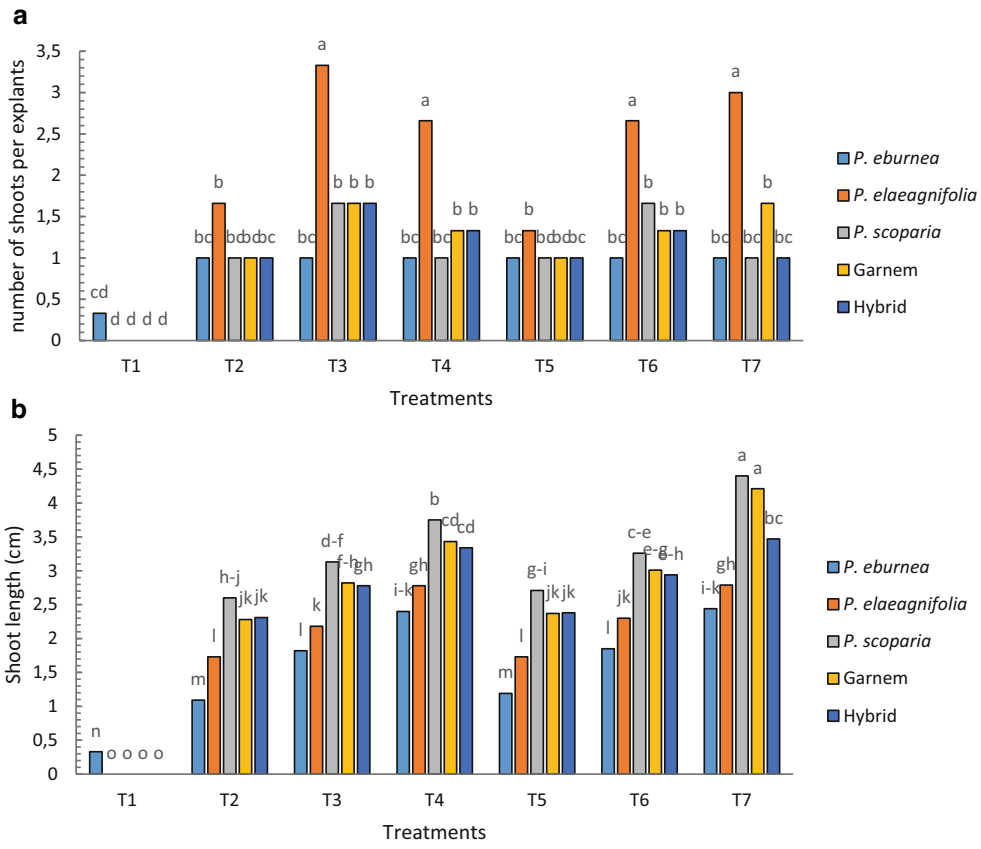

Fig. 2 The interaction effect of plant growth regulator concentrations and plant genotypes on the number (**a**) and length (**b**) of shoots. Means with the same letter are not significantly different from each other ($P = 0.01$)

nents of the medium and growth chamber conditions were same as that of during multiplication. The jars were placed in the dark for 1 week and then in the light for 4 weeks. Root percentage (%), number of roots induced per rooted shoot, root length per rooted shoot (cm), fresh weight (FW) (mg) and dry weight (mg) were recorded.

Statistical Analysis

The shoot induction stage was conducted as a factorial experiment based on a completely randomized design with seven treatments (T1–T7) and four replications. The proliferation stage performed as five independent experiments based on a completely randomized design with four replications. Rooting stage was done as a factorial experiment based on a completely randomized design with three factors (hormone, genotypes and concentration) and three replications. Data analyzed based on analysis of variance (ANOVA) using SAS software (9.1, SAS Institute, Inc, Cary, NC, USA). Then, Duncan's multiple range test (DMRT) was used with a probability of 95% to show the differences in the average data values among different genotypes and different hormone concentrations.

Results

Shoot Induction and Establishment

Shoot Number and Length

The effect of PGR concentrations, plant genotype and the interaction effect of PGR concentrations and plant genotypes on the number and length of shoots were significant at the 1% level. The highest and lowest shoot number was recorded in *P. elaeagnifolia* in almost all of treatments, in particular T3 (3.33), and T1 in all genotypes, respectively (Fig. 2a). *P. scoparia* in T7 had the highest shoot length (4.4 cm), while the lowest shoot length was related to T1 in all genotypes (Fig. 2b).

Establishment Rate (%)

The effect of PGR concentrations on establishment rate was significant at the 1% level, while the effect of plant genotype and the interaction effect of PGR concentrations and plant genotypes were not significant for this trait. T7 and T1 showed the highest (82%) and lowest (4%) explant's establishment rate, respectively (Fig. 3a). Among the plant genotypes, *P. eburnea* and hybrid had the highest (70.23%)

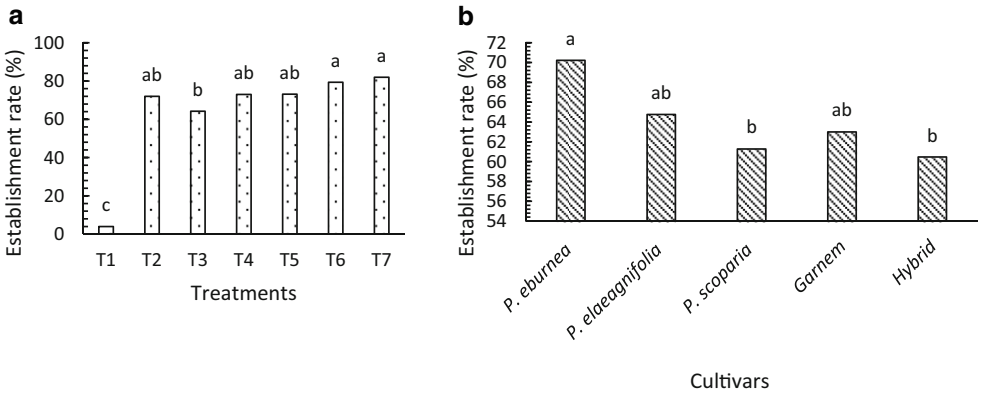

Fig. 3 The effect of various plant growth regulator treatments (**a**) and plant genotypes (**b**) on the establishment rate of the explants. Means with the same letter are not significantly different from each other ($P = 0.01$)

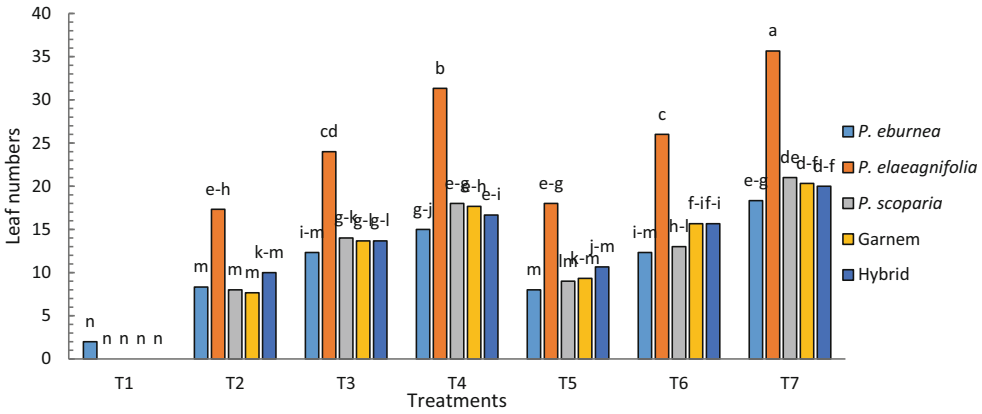

Fig. 4 The interaction effect of plant growth regulator concentrations and plant genotypes on leaf number of almond genotypes. Means with the same letter are not significantly different from each other ($P = 0.01$)

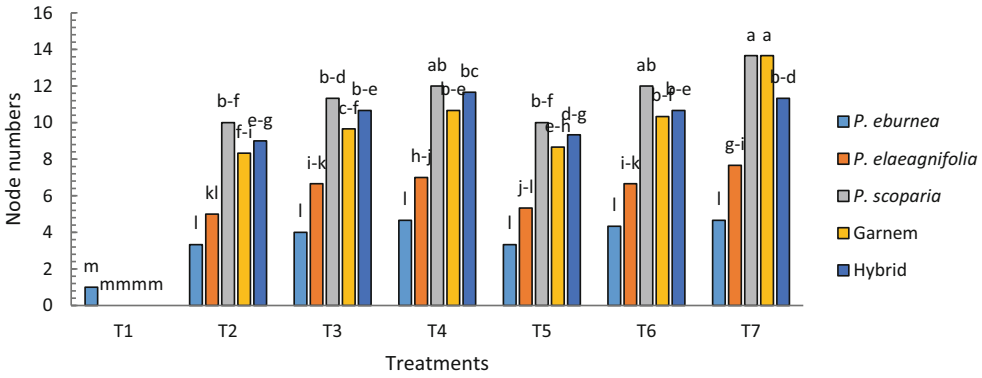

Fig. 5 The interaction effect of plant growth regulator concentrations and plant genotypes on nodes number and internode length of almond genotypes. Means with the same letter are not significantly different from each other ($P = 0.01$)

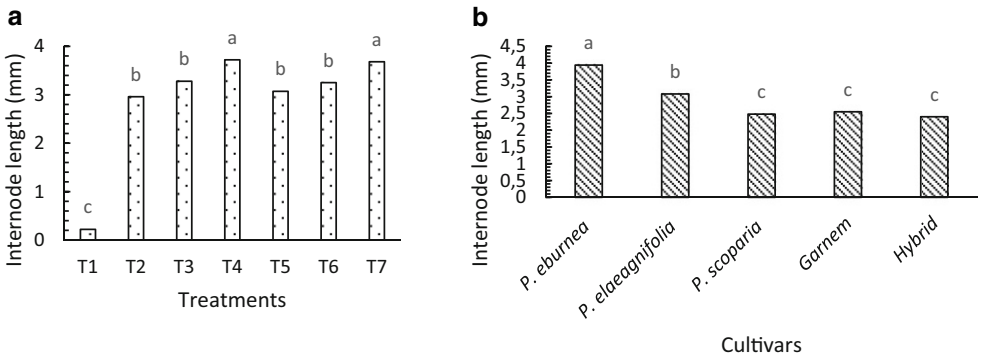

Fig. 6 The effect of various plant growth regulator treatments (**a**) and plant genotypes (**b**) on internode length. Means with the same letter are not significantly different from each other ($P = 0.01$)

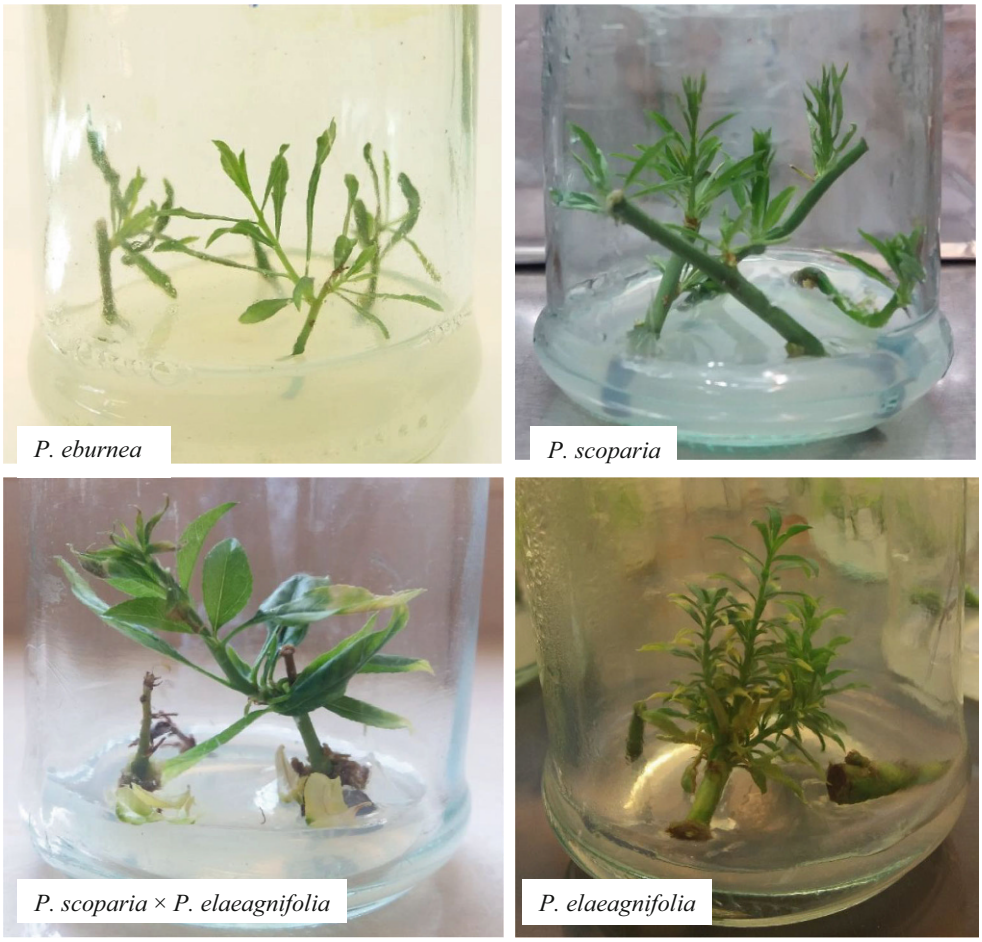

Fig. 7 Growth of different almond genotypes in the establishment and shoot induction stages

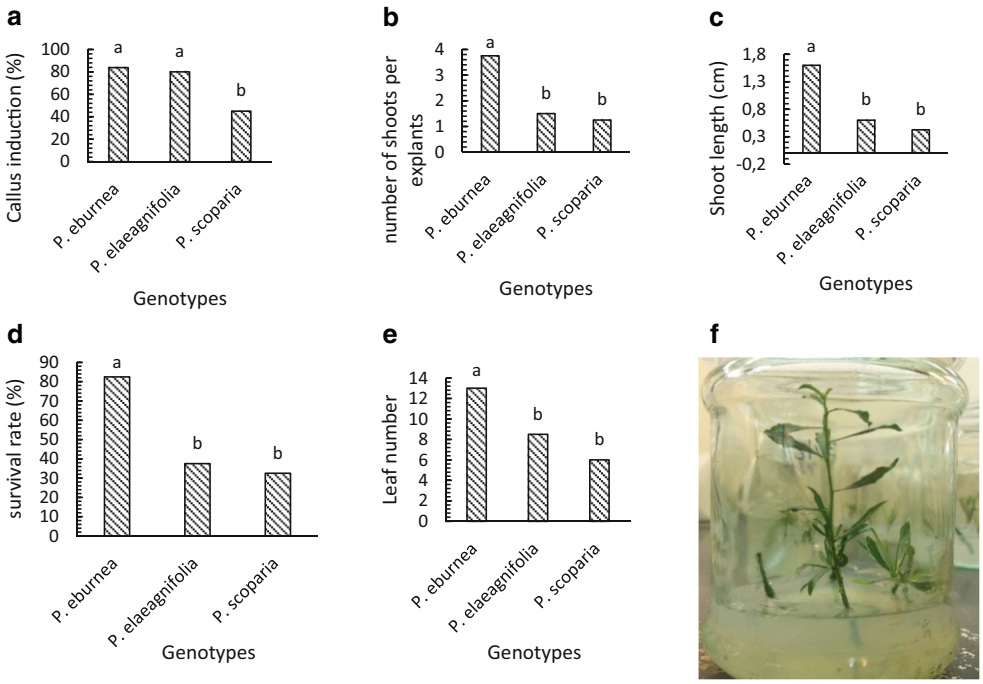

Fig. 8 The effect of plant growth regulator combination of EX1 (1 mgl^{-1} GA_3 and 1 IBA) on callus induction percentage (**a**), shoot numbers (**b**), shoot length (**c**), survival rate (**d**) and leaf numbers (**e**) of the genotypes studied. Growth performance of the *P. eburnea* in EX1 medium (**f**). Means with the same letter are not significantly different from each other ($P = 0.01$)

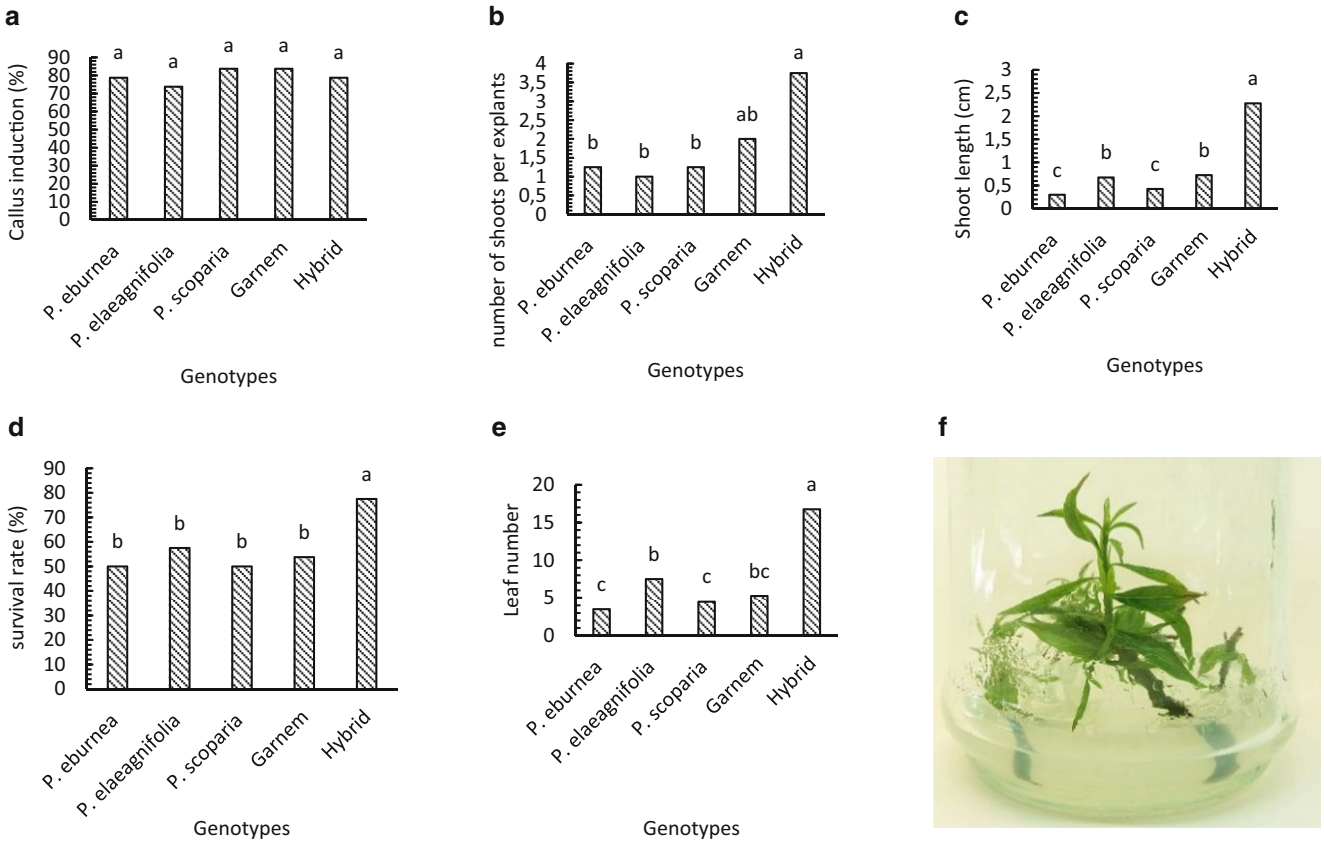

Fig. 9 The effect of plant growth regulator combination of EX2 (BA, TDZ, GA3 and IBA = 2, 0.2, 0.2 and 0.05 mgl^{-1}, respectively) on callus induction percentage (**a**), shoot numbers (**b**), shoot length (**c**), survival rate (**d**) and leaf numbers (**e**) of the genotypes studied. Growth of the *P. scoparia* × *P. elaeagnifolia* in EX2 medium (**f**). Means with the same letter are not significantly different from each other ($P = 0.01$)

and lowest (60.4%) establishment percentages, respectively (Fig. 3b).

Leaf Number

The effect of PGR concentrations, plant genotype and the interaction effect of PGR concentrations and plant genotypes on the number of leaves were significant at the 1% level. The highest leaf number (35.66) belonged to *P. elaeagnifolia* in T7 and the lowest value was recorded in T1 in all genotypes (Fig. 4).

Node Number and Internode Length

The effect of PGR concentrations and plant genotype and interaction effect on the node number and internode length were significant at the 1% level. *P. scoparia* and 'Garnem' had highest number of nodes in T7 treatment (13.66). The lowest number of nodes was observed under T1 hormonal combination in all genotypes (Fig. 5). The highest (3.72 mm) and lowest (0.22 mm) internode length was measured in T4 and T1, respectively (Fig. 6a). Considering the genotypes, *P. eburnea* and hybrid showed the highest (3.94 mm) and lowest (2.4 mm) internode length, respectively (Fig. 6b). Figure 7 represents the growth of different genotypes in the stage of establishment and shoot induction.

Shoot Proliferation

Experiment 1

That the effect of different treatments in all measured traits was significant at the 1% level. The GN and hybrid did not grow on the medium and hormonal combination used in this experiment. *P. eburnea* had the highest number (3.75) and length of shoots (1.6 cm), the percentage of survival (82.5%) and the number of leaves (13) (Fig. 8a, b, c, d, e). In general, *P. eburnea* had better growth on this medium and hormonal mixture compared to other genotypes (Fig. 8f).

Experiment 2

The effect of the treatments on the percentage of callus induction was not significant, while it was significant for shoot length, survival rate and number of leaves at the 1% level and for the number of shoots at the 5% level.

Results showed that genotypes did not differ significantly in callus induction percentage (Fig. 9a), while the hybrid genotype had the highest number (3.75) and length of shoots (2.27 cm), survival rate (77.5%) and number of leaves (16.75) compared to the others (Fig. 9b, c, d, e). Figure 9 shows the growth of *P. scoparia* × *P. elaeagnifolia*.

Experiment 3

The effect of treatments in all measured traits was significant at the 1% level. The highest (87.5%) and lowest (40%) percentage of callus induction were observed in *P. scoparia* and hybrid genotypes, respectively (Fig. 10a). 'Garnem' showed the highest number of shoots (3.5) in this medium (Fig. 10b). The highest and lowest shoot lengths were obtained in *P. eburnea* and hybrid, respectively (Fig. 10c). The highest survival rate (87.5%) was recorded in *P. scoparia*, while other genotypes had no significant difference to each other for this trait (Fig. 10d). In addition, *P. scoparia* and *P. eburnea* produced higher number of leaves (Fig. 10e). *P. scoparia* and *P. eburnea* performed better than other genotypes on this medium. Figure 10f shows the performance of *P. scoparia*.

Experiment 4

The effect of the treatments on the percentage of callus induction was not significant, while it was significant for shoot length, survival rate and number of leaves at the 1% level and was significant at 5% for shoot number. No significant difference was observed between genotypes in the percentage of callus induction (Fig. 11a). *P. elaeagnifolia* produced the highest shoot number (3) and length (3.92 cm), survival rate (72.5%) and leaf number (20.5) (Fig. 11b, c, d, e). Therefore, in this culture medium, the best growth response was related to *P. elaeagnifolia* (Fig. 11f).

Experiment 5

Treatment was significant on all traits at the 1% level except shoot number. The percentage of callus induction in *P. eburnea, P. elaeagnifolia* and 'Garnem' was not signifi-

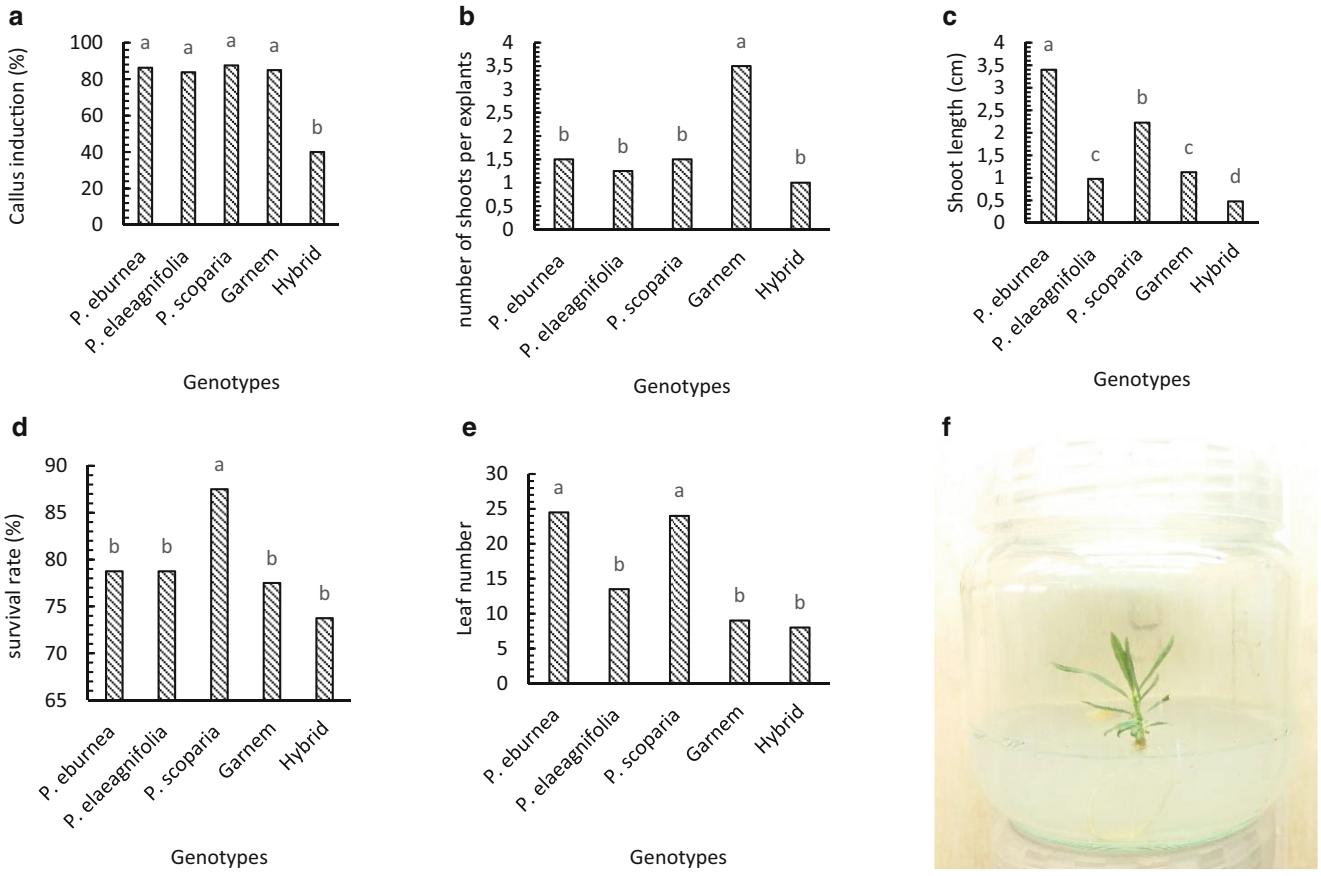

Fig. 10 The effect of plant growth regulator combination of EX3 (BA and IBA = 3 and 0.1 mgl^{-1}, respectively) on callus induction percentage (**a**), shoot numbers (**b**), shoot length (**c**), survival percentage (**d**) and leaf numbers (**e**) of the genotypes studied. Growth performance of *P. scoparia* in EX3 medium (**f**). Means with the same letter are not significantly different from each other ($P = 0.01$)

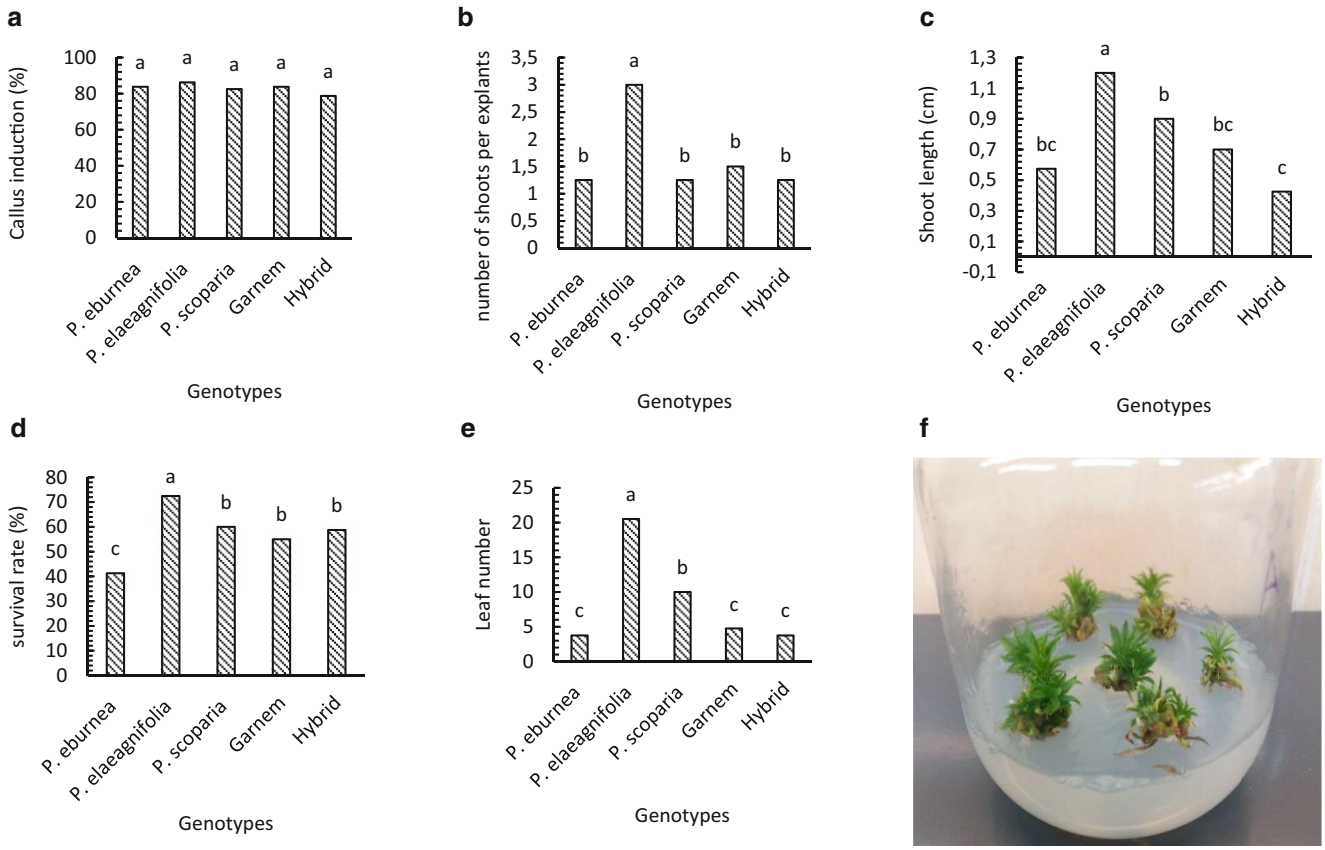

Fig. 11 The effect of plant growth regulator combination of EX4 (BA, TDZ and IBA = 4, 2 and 0.1 mgl^{-1}, respectively) on callus induction percentage (**a**), shoot numbers (**b**), shoot length (**c**), survival percentage (**d**) and leaf numbers (**e**) of the genotypes studied. Growth performance of *P. elaeagnifolia* in EX4 medium (**f**). Means with the same letter are not significantly different from each other ($P = 0.01$)

cantly different and was higher than those of *P. scoparia* and hybrid genotypes (Fig. 12a). Also, no significant difference was observed in the number of shoots (Fig. 12b). Survival rate did not show a significant difference in *P. eburnea, P. elaeagnifolia, P. scoparia* and 'Garnem' (Fig. 12c), while, the highest number of leaves (12) and shoot length (2.55 cm) were recorded in 'Garnem' (Fig. 12d, e). In general, it seems 'Garnem' had better growth performance on this medium than other genotypes (Fig. 12f).

Rooting

In the rooting stage, only three genotypes (*P. eburnea*, 'Garnem' and hybrid) generated root, thus the results are presented just for these three genotypes. Also, as no root formation was obtained in the medium supplemented with NAA (in all concentrations), these results were excluded. The plant genotypes and concentration of IBA had significant effects on measured root attributes (at 1% level). The highest rooting percentage (38.88%) and root number (5) were obtained in 'Garnem', while the highest root length (14.36 cm), root FW (6144 mg) and root DW (801 mg) belonged to *P. eburnea*. The best rooting performance was observed on the medium containing 1 mgl^{-1} IBA (Fig. 13). Figure 14 shows rooting performance of the *P. eburnea*, 'Garnem' and hybrid.

Discussion

Establishment and Induction

The results showed that different hormonal combinations had different effects on their measured traits. The use of BA 3 mgl^{-1} in combination with the 0.1 mgl^{-1} IBA + 1 mgl^{-1} TDZ produced the highest number of shoots in each explant, while in the media that were free of BA, very few shoots generated in almost all genotypes, except for *P. elaeagnifolia*, which produced a large number of shoots in MS medium containing 3 and 4 mgl^{-1} GA_3 (very similar to medium containing 3 mgl^{-1} BA). It has been shown that the best result for shoot growth and development was obtained from the combination of 0.1 mg l^{-1} IBA and 1.0 mg l^{-1} BAP (Gurel and Gulsen 1998). Choudhary et al. (2015) reported that no growth was observed when almond explants were placed on a culture medium without cytokinin and

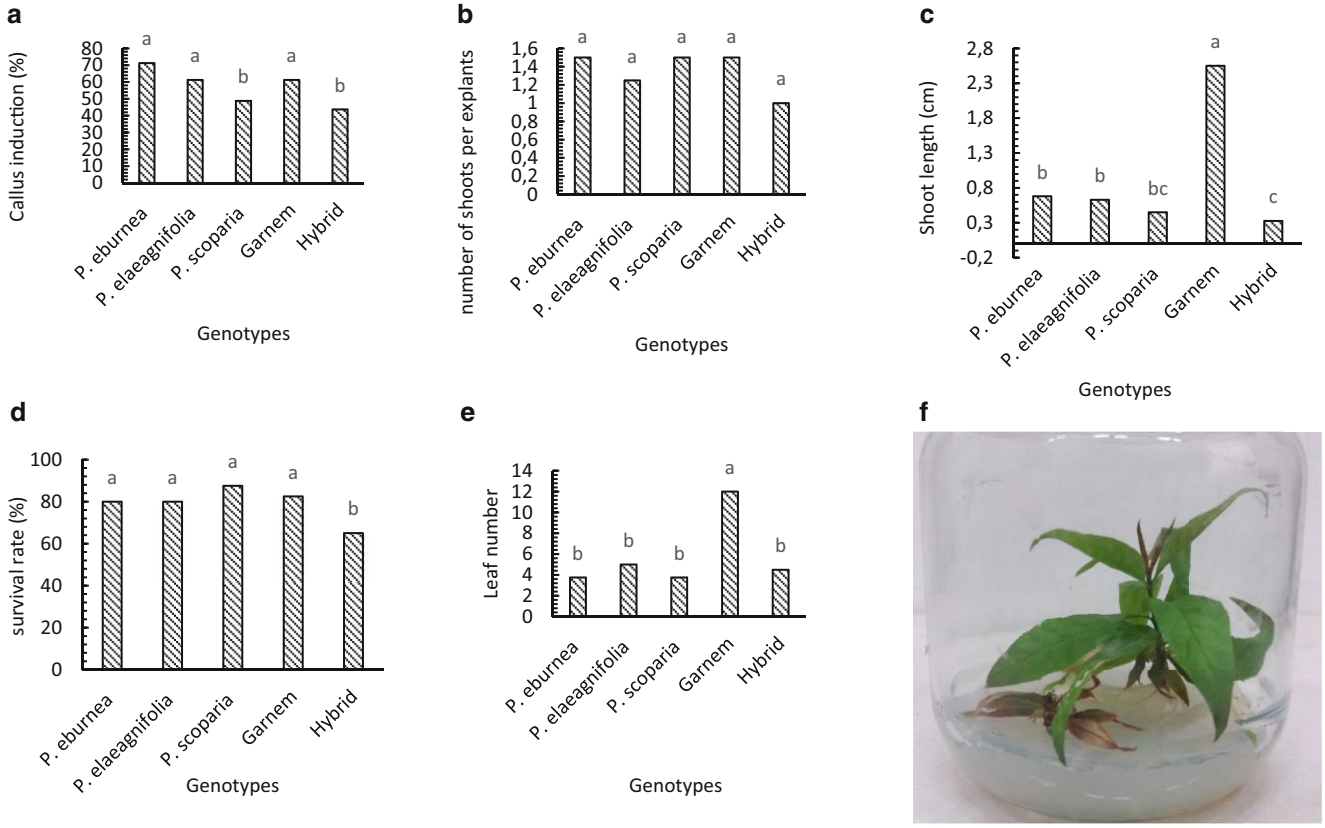

Fig. 12 The effect of plant growth regulator combination of EX5 (BA and IBA = 1.5 and 0.1 mgl^{-1}, respectively) on callus induction percentage (**a**), shoot numbers (**b**), shoot length (**c**), survival percentage (**d**) and leaf numbers (**e**). Growth of the 'Garnem' in EX5 medium (**f**). Means with the same letter are not significantly different from each other ($P = 0.01$)

auxin. Therefore, according to the obtained results, as well as those of previous researches, it can be concluded that BA is a very suitable source of cytokinin for the regeneration phase of almonds under *in vitro* culture. Also, it can be concluded that the combined use of BA and IBA is a suitable mixture for the higher number of new shoots production. These findings are consistent with the results of previous research on *Prunus* species, which stated that the combination of a cytokinin source (BAP) with an auxin (IBA) was the most effective treatment for shoot growth (Yıldırım et al. 2010; Gurel and Gulsen 1998; Kodad et al. 2021).

TDZ promotes shoot proliferation and has a positive effect on increasing the number of regenerated shoots in woody plants (Ainsley et al. 2001b; Şan et al. 2018; Abbasi et al. 2019; Bhagwat and David Lane 2004; Arab and Shekafandeh 2016; Kodad et al. 2021). This is why we used a fixed concentration of 1 mgl^{-1} TDZ in all medium compositions for promoting shoot proliferation. Recently, it was also reported that the combined use of TDZ and IBA played a key role in stimulating callus induction and unwanted shoot growth in fig micropropagation (Abdolinejad et al. 2020). Like other PGRs, gibberellins play an important role in plant growth and development. In order to stimulate the growth of explants of woody plants, GA_3 is added to the culture medium before the rooting stage to increase the length of the shoot through stimulating and elongating the cells (Kumar et al. 2008). The present results proved that increasing GA_3 concentration enhanced shoot length in various almond genotypes.

According to the results, it was found that elevated concentration of BA caused an increased number of leaves in almond genotypes studied, which is consistent with the results of Alizadeh Arimi et al. (2020) on almond, who showed that there is a positive relationship between increasing the BA concentration and establishment rate, and the more establishment rate is associated with increasing of leaf number. The highest number of leaves and establishment rate were obtained from the hormonal combination of 1 mgl^{-1} BA plus 0.05 mgl^{-1} IBA, while the lowest leaf number was obtained from the explants that were on the control media (Alizadeh Arimi et al. 2020).

Also, results indicated that the number of nodes increased with the elevated concentration of BA, which is in contrast to the results reported by Isikalan et al. (2008) on *Amygdalus communis* L. cv. Nonpareil, who stated that the treatment of 1 mgl^{-1} of BA produced more nodes than the treatment of 2 mgl^{-1}. Also, these researchers showed that there was no statistical difference between all the tested

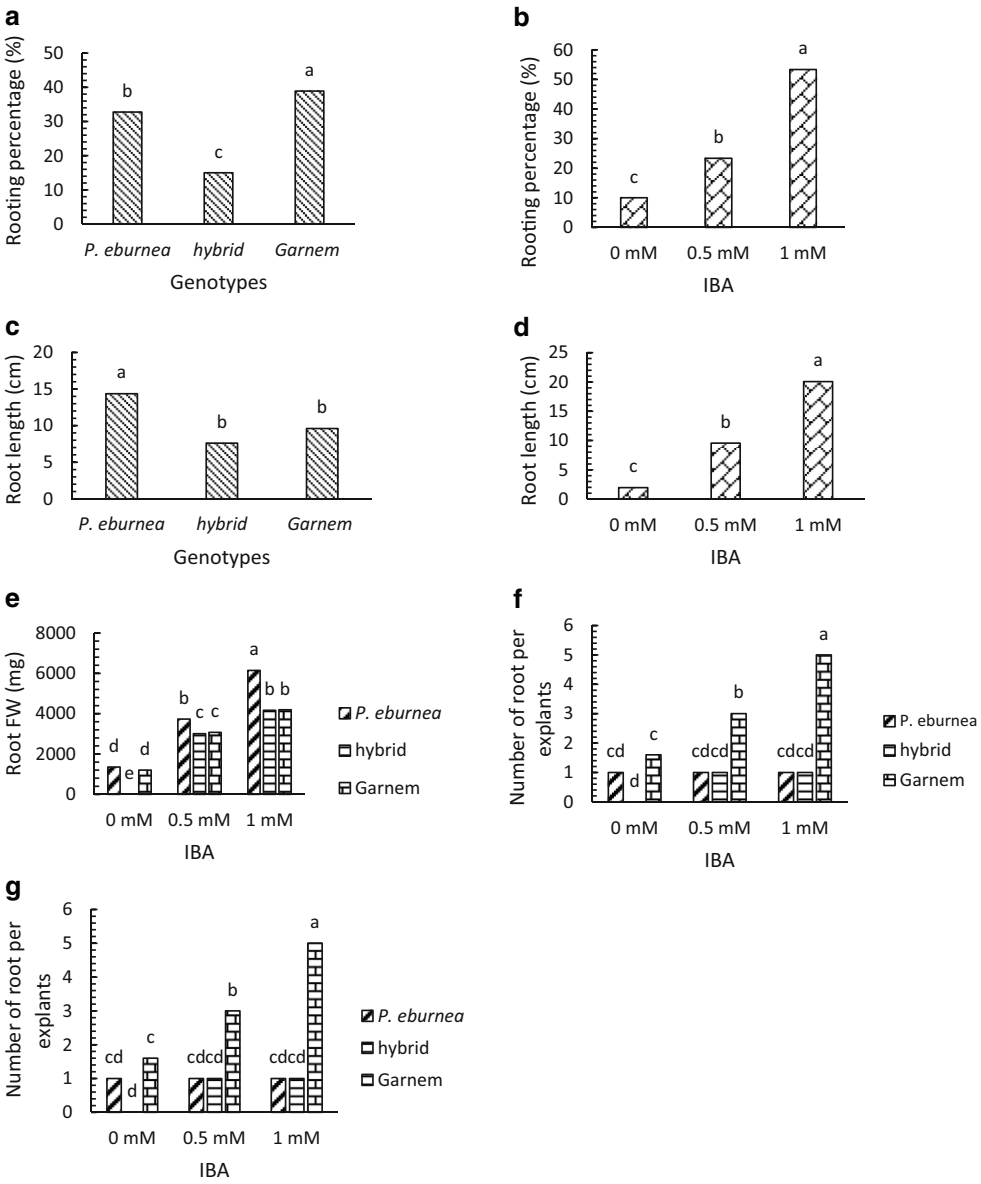

Fig. 13 Effect of Genotypeon rooting percentage and root length (**a**, **c**), indole-3-butyric acid (*IBA*) concentration on rooting rate (**b**) and root length (**d**), and interaction effect of genotype and IBA concentrations on root fresh weight (**e**) and number of roots per explant (**f**). Means with the same letter are not significantly different from each other ($P = 0.01$)

concentrations of kinetin and the control group in the number of nodes (Isikalan et al. 2008). MS medium supplemented with 1 or 2 mgl^{-1} of BA resulted in the highest node number in GF677 rootstock (Matani Borkheyli et al. 2021). Results of current study showed that GA_3 can also have a positive effect on increased number of nodes in various almond genotypes. Adding gibberellin to the culture medium increases the cell growth and even cell division, as a result, the number of nodes will be increased (Polat and Eskimez 2022). Results also confirmed an enhancement in the internode length by combined increasing of the BA and GA_3 concentrations. In contrast to findings of this study, Sulusoglu and Cavusoglu (2013) showed that higher concentrations of BAP (0.6 or 0.8 mgl^{-1}), leads to short internodes in *Prunus laurocerasus* L. (cherry laurel) (Sulusoglu and Cavusoglu 2013).

Proliferation

Benzyl adenine (BA) is a type of cytokinin hormone that use in tissue culture to induce callus formation in plants (Zhang et al. 2005). In this study, increased BA concentration enhanced callus formation, and the highest percentage of callus induction was related to the mediums supplemented with the highest concentration of BA (3 and 4 mgl^{-1}) without GA_3. In other research that was done on *Amygdalus communis* L. cv. Yaltinski, the MS medium containing 2,4-D + BAP (1.0:1.0) showed the highest callus induction (80%) for stem explants in dark conditions (Isikalan et al. 2010). MS medium containing a high concentration of GA_3 (1 mg l^{-1}) had the higher rate of callus induction compared to others. In general, in this research, it was found that the use of BA and GA_3 is necessary for callus induction.

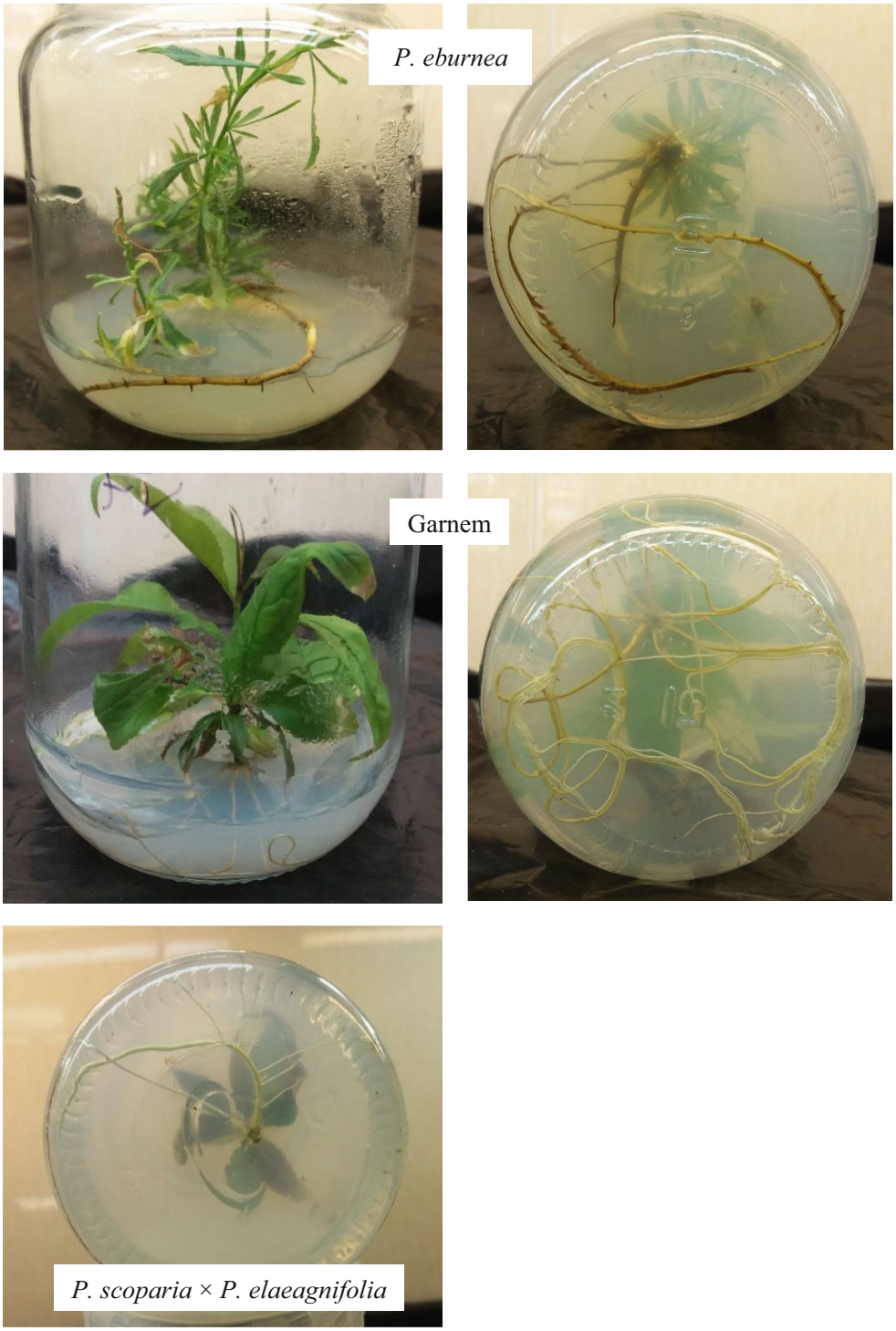

Fig. 14 Rooting in the *P. eburnea*, 'Garnem' and hybrid in the MS medium containing 1 mgl^{-1} IBA

EX1 medium (free of BA + 1 mg l^{-1} GA_3) callus induction observed. Also, callus induction was observed in EX3 and EX4 that are free of GA_3 + BA 3 and 4 mg l^{-1}.

It has been reported by many researchers that a cytokinin source is essential for shoot development of *Amygdalus communis* L. cv. 'Nonpareil' (Tabachnik and Kester 1977; Isikalan et al. 2008; Akbas et al. 2009). Previous research showed that kinetin alone has no effect on shoot production of almonds, while BA at a concentration of 1 mg l^{-1} in combination with IBA at 0.5 mg l^{-1} resulted in the highest total number of shoots produced per explant (Akbas et al. 2009). Recently, Kodad et al. (2021) showed that the use of 1 mg l^{-1} BAP improved the average number of shoots induced per explant and the average shoot length in al-

mond genotypes (Kodad et al. 2021). Also, in another study the highest number and length of shoots were obtained on MS medium containing 1 mg l^{-1} BAP, 0.01 mg l^{-1} IBA and 0.5 mg l^{-1} GA_3 for almond cultivars (Ebrahimi et al. 2022). Results of all mentioned studies are in general agreement with the results of current study. The lack of growth of two genotypes ('Garnem' and hybrid) on EX1 (free of BA and TDZ) medium also confirms the necessity of a cytokinins source for plant growth in tissue culture conditions.

According to the results, various almond genotypes showed different growth responses to the same hormonal concentrations or combinations. It has been reported that same species or cultivar which treated with the same growth regulator may give different responses, which could be due to the possible tissue specificity of phytohormone receptors or the interaction of endogenous plant hormones in tissues and growth regulators that are exogenously supplied in tissue culture (Phillips and Garda 2019). Based on the obtained results, the suitable medium for proliferation of *P. elaeagnifolia* was MS containing GA and IBA 1 mg/l, as well as the medium supplemented with BA 3 mg l^{-1} and IBA 0.1 mg l^{-1}, while the best culture medium for *P. scoparia* × *P. elaeagnifolia* was MS medium containing BA 2 mg l^{-1}, TDZ 0.2 mg l^{-1}, GA_3 0.2 mg l^{-1} and IBA 0.05 mg l^{-1}. *P. eburnea* showed the best growth in the culture MS medium containing BA 4 mg l^{-1}, TDZ 2 mg l^{-1} and IBA 0.1 mg l^{-1}. *P. scoparia* had the best growth in MS medium supplemented with BA 3 mg l^{-1} and IBA 0.1 mg l^{-1}. 'Garnem' showed the highest growth in the MS culture medium containing BA 1.5 mg l^{-1} and IBA 0.1 mg l^{-1}.

Rooting

Almond rooting response can be different depending on the genotype (Kodad et al. 2021), concentration and type of auxin (Isikalan et al. 2008) and cultivation conditions (Abbasi et al. 2019), and the optimal in vitro rooting method can guarantee the successful adaptation of almonds (Kodad et al. 2021). According to the results, dark condition and the use of auxin hormone had a positive effect on the rooting of almond genotypes. The highest rooting percentage, root length, root number, root FW and root DW in the studied almond genotypes were observed in the treatment of 1 mg l^{-1} IBA. In research conducted by Şan et al. (2018) on different genotypes of almonds, rooting occurred on ½ MS medium containing 1 mg l^{-1} IBA and 120 mg l^{-1} sequestrene (iron source), while other researchers showed that MS medium containing 0.50 mg l^{-1} IBA showed the highest rooting percentage, root number and root length in *P. scoparia* (Abbasi et al. 2019) and *P. empyrean* (Sadeghi et al. 2015).

Generally, most almond-related species had a low rooting percentage (Ainsley et al. 2001b). In the present research, the percentage of rooting was moderate, which is consistent with results reported by others authorities in almonds (Abbasi et al. 2019; Choudhary et al. 2015a). 'Beldi' almond ecotypes showed the highest rooting rate (60.41% ± 0.81) of the propagated shoots and the number of roots per shoot (7.3 ± 1.36) on 1/2 MS culture medium with IBA 1 mg l^{-1} (Kodad et al. 2021). Also, 30, 60 and 60% of root formation were reported in a ½ MS medium by immersing the shoots in 1.0 g l^{-1} IBA solution in 'Nonpareil' almond (Namli et al. 2011) and bitter almond (Faustino et al. 2022) and 1.0 mM IBA in 'Nonpareil' and 'Ne Plus Ultra' almond cultivars (Ainsley et al. 2001a), respectively. In research conducted on 'Garnem', rooting was achieved at the rate of 42.8% in ½ MS culture medium containing 2 mg l^{-1} IBA (Ak et al. 2021), while in the present research, the best rooting of 'Garnem' was obtained in the culture medium containing 1 mg l^{-1} IBA.

Conclusion

It can be emphasized that the different almond genotypes that were tested in this study showed different responses to PGR combinations in micropropagation. Several suitable mediums were introduced for each stage of the tissue culture to use proper cultivation medium that has the best results for the micropropagation of each genotype of almonds in order to mass and quickly produce drought-resistant rootstocks almonds. For the establishment and induction of shoots, the use of MS containing concentrations of 3 and 4 mg^{-1} BA and GA_3 in combination with IBA and TDZ had the best results. In the proliferation stage, the best results for proliferation of *P. elaeagnifolia, P. scoparia* × *P. elaeagnifolia, P. eburnea, P. scoparia* and 'Garnem' were obtained by MS containing different concentrations of PGRs used in EX4, EX2, EX1, EX3 and EX5, respectively. In the rooting stage, 1/2 MS medium containing 1 mg l^{-1} of IBA is the best medium. Therefore, it can be stated that the results of this research are a suitable guide for the tissue culture of these valuable almond genotypes, which can be used as a fast and efficient method for the commercial micropropagation of these drought-resistant rootstocks.

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Author Contribution Amir Rezaei conducted all the experiments, performed statistical analysis and wrote the manuscript. Ali Gharaghani designed all experiments, provided materials and research facilities, supervised the practical issues of the experiments, data collection, and data analysis, and finally revised the manuscript. Akhtar Shekafandeh and Saeid Eshghi gave advice regarding the designation and conduction of the experiments as well as writing the manuscript.

Conflict of interest A. Rezaei, A. Gharaghani, A. Shekafandeh and S. Eshghi declare that they have no competing interests.

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