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The Effect of Plant Growth Regulators on Shoot Regeneration of Two Wild Almond Species *Amygdalus scoparia* L. and *Amygdalus lycioides* Spach.

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ABSTRACT

Two species of *Amygdalus scoparia* L. and *A. lycioides* Spach. from the Rosaceae family can be found in suitable environmental conditions due to their high resistance to drought and heat and resistance to calcareous soils and attacking nematodes and diseases. The propagation of fruit tree rootstocks through vegetative means can be enhanced through the optimization of micropropagation techniques. This research was done in plant tissue culture laboratory of the Agricultural and Natural Resources Research Center of Kermanshah in 2018. In this research, the improved micropropagation protocol of these plants, explants of disinfected nodes in MS medium in combination with BA (6-Benzyladenine) hormones, at 6 levels (0, 0.25, 0.5, 1, 2, and 3 mg L^{-1}) in four levels of Indole Butyric Acid (0, 0.1, 0.25 and 0.5 mg L^{-1}). The experiment was carried out with four replications in a completely randomized factorial design. The results indicated that the highest percentage of explant regeneration in the *A. lyciodes* genotype was achieved with a combination of 0.5 mg L^{-1} BA and 0.25 mg L^{-1} IBA, while in the A. scoparia genotype, the highest regeneration percentage was observed with a combination of 2 mg L^{-1} BA and 0 mg L^{-1} IBA. The *A. lyciodes* genotype exhibited the highest number of shoots when treated with 0.5 mg L^{-1} BA and 0.25 mg L^{-1} IBA, while the *A. scoparia* genotype showed the maximum shoot number at a BA concentration of 2 mg L^{-1} and IBA concentration of 0 mg L^{-1}.

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1. Introduction

Iran is the origin of most wild almond species in the world and about 21 species have been reported in different regions of the country (Rahemi *et al*., 2011). It represents a wide range of morphological characteristics that have grown naturally in different regions of the country. Due to their adaptability to extreme environmental conditions (drought), these can be used as rootstocks in almond or peach (Rahemi *et al*., 2011; Ansari *et al*., 2019; Imani *et al*., 2021). *Amygdalus lycioides* is a native species of Iran. It is a small deciduous shrub with tough and thorny branches that height is about 1 to 1.5 meters (Rahemi and Gradziel, 2024). *Amygdalus scoparia* is another native species of Iran that exists in many areas of the country. It is a large deciduous shrub with a height of about 2 to 2.5 meters, which can sometimes reach up to 6 meters (Fig. 1). It produces a large number of long, straight, narrow, smooth green shoots (Rouhi *et al*., 2007).

Drought stress is one of the most important limitations in the world that restricts the yield of agricultural products (Alaei, 2019). In order to prevent the reduction of the yield of agricultural products in drought conditions, it is necessary to choose plants that tolerate unfavorable environmental conditions (Ashrafi *et al*., 2018). So, one of the ways to reduce drought stress is the selection of drought-tolerant rootstocks. Resistance to drought stress in plants is caused by the

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interaction of biochemical, physiological and morphological characteristics (Lotfi *et al*., 2019).

The species *A. scoparia* and *A. lycioides* are the most important species that are used as almond rootstocks in non-irrigated conditions. Development of drought-tolerant almond orchards using *A.* scoparia rootstocks will result in more sustainable production in arid regions (Khadivi-Khub and Anjam, 2016). Phenotypic diversity is the essential characteristic of plants to respond to environmental changes for their long-term survival (Imani *et al*., 2021). Some reports showed that leaves of *A. scoparia* species fall under stress conditions, but some leaves of *A. lycoides* species survive and are able to carry out photosynthesis (Rouhi *et al*., 2007). Also, these species grow in shallow and dry limestone soils (Ali *et al*., 2015).

Climate change is one of the most important agricultural challenges in the world, especially in dry and semi-arid climates. To deal with this problem, it will be necessary to introduce new varieties and rootstocks (Gharaghani *et al*., 2017). Achieving dwarf rootstocks resistant to pests, diseases and environmental stress is one of the goals of many rootstocks breeding programs (Panis *et al*., 2020; Volk *et al*., 2023). It has been reported that grafting almond cultivars on *A. scoparia* causes a decrease in plant height and an increase in almond fruit yield under drought stress conditions and calcareous soils. Almond cultivars are propagated sexually and asexually (Rahemi *et al*., 2011). On the other hand, rooting in almonds is difficult and propagation through seeds leads to genetic variation and will not be suitable for use as a clone. Therefore, the propagation method through tissue culture is an efficient alternative system (Farsi *et al*., 2016; Eshghi Khas *et al*., 2020).

In order to use a specific species as a vegetative rootstock and propagate it through tissue culture, it is necessary to develop effective regeneration and propagation. So this research reports a micropropagation protocol using segment nodal explants of *A. lycioides* and *A. scoparia* species in Iran. For commercial propagation of these species in the nursery, an optimal protocol is required for micropropagation of this species. We did not find any reports on shoot micropropagation of *A. lycoides*. Therefore this is the first study reporting the *in vitro* regeneration of *A. lycoides*. Therefore, in this research, we investigated the micropropagation of *A. lycoides* and *A. scoparia* species using different concentrations of cytokinin and auxin.

2. Materials and methods

This research was done in the Plant Tissue Culture Laboratory of the Agricultural and Natural Resources Research Center of Kermanshah in 2018. The growing branches were collected in May from the young parts of two wild almond species of *A. scoparia* and *A. lycioides* in the Bilawar region of Kermanshah province in Iran (Fig. 1).

The branches were kept in an ice box during transport to the laboratory. The shoot samples were washed under tap water for 30 minutes to remove surface contamination. Surface sterilization was performed inside the laminar air flow cabinet by immersing the explants in 70% (v/v) ethanol for 30 seconds and by immersing them in 1% (v/v) sodium hypochlorite solution for 15 minutes. After washing three times in sterile double distilled water, sterilized uniform nodal branch segments (length 3-4 cm) were cultured on MS culture medium in combination with BA (benzyladenine) hormones, at 6 levels (0, 0.25, 0.5, 1, 2, and 3 mg L^{-1}) in four levels of Indole Buteric Acid (0, 0.1, 0.25 and 0.5 mg L^{-1}). The pH of the medium was adjusted to 5.8 using 0.1N of NaOH or HCl and before autoclaving, the medium was solidified by adding 0.8% agar.

For regeneration of shoot culture, individual sterile nodal segments were placed in 40 ML polycarbonate tubes containing medium. The cultures were incubated in a growth chamber under 16/8 h light/dark cycle under 40 mol m^{-2} s^{-1} illuminations in cool, white fluorescent light at 25 ± 1°C. After 6 weeks, regeneration percentage, shoot number, shoot length, leaf number, leaf length, and internodal distance were measured.

The experiment was carried out as factorial in a completely randomized design with four replications. Factorial analysis of variance was conducted using statistical analysis system (SAS) and all data were analyzed according to Duncan's Multiple Range Test ($p \leq 0.05$).

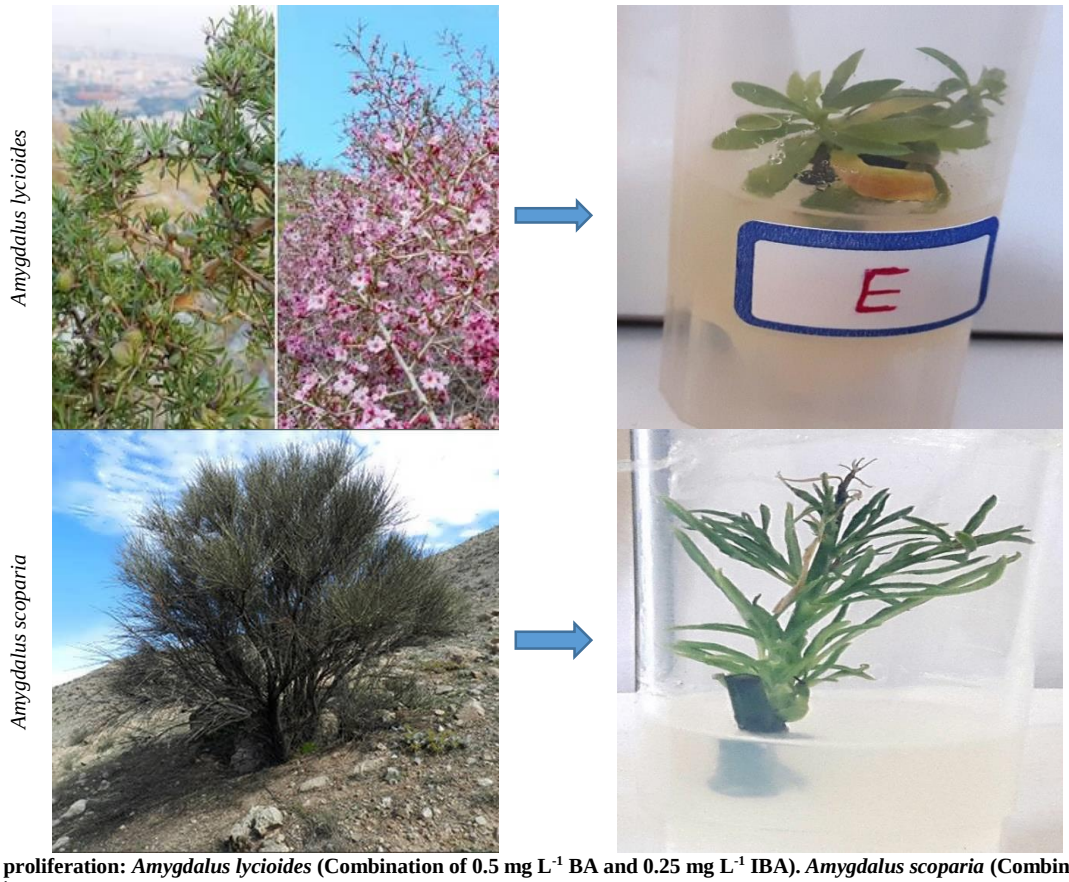

Figure 1. Shoot proliferation: *Amygdalus lycioides* (Combination of 0.5 mg L^{-1} BA and 0.25 mg L^{-1} IBA). *Amygdalus scoparia* (Combination of 2 mg L^{-1} BA and 0 mg L^{-1} IBA).

3. Results and discussion

For regeneration of wild almond species including *A. scoparia* and *A. lycioides*, individual sterile nodal segments were cultured on MS medium supplemented with BA at the concentration of 0, 0.25, 0.5, 1, 2, and 3 mg L^{-1}, or BA combined with IBA at the concentration of 0, 0.1, 0.25 and 0.5 mg L^{-1}. Analysis of variance showed that the effect of different levels of genotype, growth regulators of cytokinin (BA) and auxin (IBA) and interaction effects between these factors on the regeneration percentage, shoot number, shoot length, leaves number, leaf length, and internodal distance traits were significant at ($p \leq 0.01$) level.

3.1. Regeneration percentage

The average comparison results showed that the lowest regeneration frequency was obtained on a hormone-free MS medium for both species. The BA concentrations less than 0.05 mg L^{-1} did not show shoot induction and proliferation in both genotypes. The highest percentage of regeneration of explants in *A. lycioides* was obtained in the combination of 0.5 mg L^{-1} BA and 0.25 mg L^{-1} IBA. In the *A. scoparia* plant, the highest percentage of explant regeneration was obtained in the combination of 2 mg L^{-1} BA and 0 mg L^{-1} IBA. In fact, the highest regeneration frequencies (98.25% and 93.00%) were obtained on MS medium supplemented with BAP and IBA for *A. scoparia* and *A. lycioides* genotypes, respectively (Table 1).

3.2. Shoot number

Among BA concentrations, only 0.5, 1, 2, and 3 mg L^{-1} treatments generated shoot, while there were no shoots in the media containing 0 and 0.25 mg L^{-1} BA concentrations. Results revealed that *in vitro* shoot proliferation response increased by combining 0.5 mg L^{-1} of BA with 0.25 mg L^{-1} of IBA in *A. lycioides* genotype. However, the highest number of shoots per explant of *A. scoparia* genotype was recorded only with cultivated in 1 mg L^{-1} BA medium without the use of IBA. On this medium, *A. lycioides* and *A. scoparia* explants produced 2.12 and 4.25 average number of shoots per explant, respectively (Table 1).

3.3. Shoot length

The results showed that the maximum length of shoots in *A. lycioides* plant was obtained in the combination of 1 mg L^{-1} BA and 0.1 mg L^{-1} IBA. In *A.*

scoparia plant, the maximum shoot length was observed in the combination of 2 mg L^{-1} BA and 0 mg L^{-1} IBA. Culturing the explants on various media showed different results in terms of shoot length. Culturing on this medium, *A. lycioides* and *A. scoparia* explants generated 4.37 and 23.75 cm as average length of the tallest shoot per explant, respectively (Table 1).

Table 1. Mean comparison of the effect of different BA and IBA concentrations on on different traits of two wild almond genotypes.

plant growth regulator (mg L^{-1})		Regeneration (%)		Shoot number		Shoot length (cm)	
		wild almond genotypes		wild almond genotypes		wild almond genotypes	
BA	IBA	*A. lycioides*	*A. scoparia*	*A. lycioides*	*A. scoparia*	*A. lycioides*	*A. scoparia*
0	0	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
	0.1	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
	0.25	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
	0.5	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
0.25	0	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
	0.1	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
	0.25	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
	0.5	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
0.5	0	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
	0.1	31.00^{h}	53.50^{e}	0.48^{h}	0.48^{h}	3.20^{jk}	3.25^{j}
	0.25	93.00^{b}	77.25^{c}	2.12^{c}	0.71^{g}	2.75^{kl}	7.43^{c}
	0.5	26.25^{i}	73.25^{d}	1.00^{f}	0.63^{g}	2.37^{l}	3.25^{j}
1	0	0.00^{k}	98.25^{a}	0.00^{j}	4.25^{a}	0.00^{m}	10.25^{b}
	0.1	48.75^{f}	73.75^{d}	1.37^{he}	2.11^{c}	4.37^{g}	4.81^{f}
	0.25	0.00^{k}	48.25^{f}	0.00^{j}	1.00^{f}	0.00^{m}	6.25^{de}
	0.5	0.00^{k}	0.00^{k}	0.00^{j}	0.00^{j}	0.00^{m}	0.00^{m}
2	0	0.00^{k}	22.00^{j}	0.00^{j}	0.44^{h}	0.00^{m}	23.75^{a}
	0.1	43.75^{g}	72.00^{d}	1.43^{e}	1.94^{d}	4.17^{gh}	4.25^{g}
	0.25	0.00^{k}	96.25^{a}	0.00^{j}	2.42^{b}	0.00^{m}	3.75^{hi}
	0.5	0.00^{k}	45.25^{g}	0.00^{j}	0.53^{h}	0.00^{m}	3.37^{ij}
3	0	0.00^{k}	25.00^{ij}	0.00^{j}	0.47^{h}	0.00^{m}	2.90^{jk}
	0.1	0.00^{k}	50.00^{f}	0.00^{j}	0.72^{g}	0.00^{m}	4.92^{f}
	0.25	0.00^{k}	73.75^{d}	0.00^{j}	1.46^{e}	0.00^{m}	6.50^{d}
	0.5	0.00^{k}	23.00^{j}	0.00^{j}	0.23^{i}	0.00^{m}	5.89^{e}

The means with the same letters in each trait are not significantly different at the 5% level using Duncan's multiple range tests.

3.4. Internode length

The results of comparing the averages showed that the maximum internode distance of *A. lycioides* plant was obtained in MS culture medium with 0.5 mg L^{-1} BA and 0.1 mg L^{-1} IBA. In *A. scoparia* plant, the highest internode distance in the environment was obtained with the combination of 1 mg L^{-1} BA and 0.25 mg L^{-1} IBA (Table 2).

3.5. Leaf number

As shown in Table 2, the number of leaves on shoots produced on media containing only 0.5, 1, 2, and 3 mg L^{-1} while there were no shoots in the media containing other BA concentrations. The highest number of leaves of *A. lycioides* plant was obtained in MS culture medium with 2 mg L^{-1} of BA and 0.1 mg L^{-1} of IBA. In *A. scoparia* plant, the highest number of leaves was obtained in the environment with the combination of 2 mg L^{-1} BA and 0 mg L^{-1} IBA (Table 2).

3.6. Leaf length

The results of comparing the averages showed that the maximum leaf length of *A. lycioides* plant was recorded in MS culture medium with 0.5 mg L^{-1} BA and 0.5 mg L^{-1} IBA. In the *A. scoparia* plant, the maximum leaf length was obtained in the MS culture medium with 0.5 mg L^{-1} BA and 0 mg L^{-1} IBA (Table 2).

Table 2. Mean comparison of the effect of different BA and IBA concentrations on on different traits of two wild almond genotypes.

plant growth regulator (mg L^{-1})		Leaf number		Leaf length		Internode length	
		wild almond genotypes		wild almond genotypes		wild almond genotypes	
BA	IBA	*A. lycioides*	*A. scoparia*	*A. lycioides*	*A. scoparia*	*A. lycioides*	*A. scoparia*
0	0	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
	0.1	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
	0.25	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
	0.5	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
0.25	0	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
	0.1	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
	0.25	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
	0.5	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
0.5	0	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
	0.1	7.18^{e}	7.75^{e}	2.97^{gh}	3.55^{ef}	0.44^{i}	0.42^{ij}
	0.25	13.50^{b}	14.00^{b}	3.00^{gh}	3.56^{fe}	0.21^{l}	0.53^{gh}
	0.5	11.25^{c}	6.50^{f}	3.81^{e}	2.93^{ghi}	0.21^{l}	0.51^{h}
1	0	0.00^{i}	13.88^{b}	0.00^{l}	8.81^{a}	0.00^{m}	0.73^{c}
	0.1	11.50^{c}	7.15^{e}	3.25^{fg}	4.32^{d}	0.38^{j}	0.68^{d}
	0.25	0.00^{i}	4.87^{g}	0.00^{l}	3.85^{e}	0.00^{m}	1.27^{a}
	0.5	0.00^{i}	0.00^{i}	0.00^{l}	0.00^{l}	0.00^{m}	0.00^{m}
2	0	0.00^{i}	39.50^{a}	0.00^{l}	2.87^{hi}	0.00^{m}	0.60^{ef}
	0.1	14.13^{b}	7.50^{e}	2.62^{lj}	5.62^{b}	0.29^{k}	0.56^{fg}
	0.25	0.00^{i}	6.50^{f}	0.00^{l}	3.87^{e}	0.00^{m}	0.62^{e}
	0.5	0.00^{i}	6.15^{f}	0.00^{l}	2.40^{jk}	0.00^{m}	0.55^{gh}
3	0	0.00^{i}	4.25^{h}	0.00^{l}	2.25^{k}	0.00^{m}	0.68^{d}
	0.1	0.00^{i}	5.25^{g}	0.00^{l}	3.25^{fg}	0.00^{m}	0.94^{b}
	0.25	0.00^{i}	10.50^{d}	0.00^{l}	4.75^{c}	0.00^{m}	0.62^{e}
	0.5	0.00^{i}	6.25^{f}	0.00^{l}	3.45^{f}	0.00^{m}	0.94^{b}

The means with the same letters in each trait are not significantly different at the 5% level using Duncan's multiple range tests.

As a general conclusion, it can be emphasized that different wild almond genotypes show different reactions to the type and concentration of plant growth regulators used in culture media. Therefore, it is

recommended that if a wild almond genotype is selected as a rootstock for almonds and if there is a need for micropropagation, it is recommended to use a special culture medium which is the best result for its multiplication.

The data obtained in this study indicated that the response of regeneration percentage of explants was greatly dependent on the concentration of BA combination with different concentrations of IBA. BA combination with the low concentration of IBA improves regeneration percentage of explants. The BA concentrations less than 0.05 mg L^{-1} did not show shoot induction and proliferation in both genotypes. The lowest value of regeneration was for all concentrations of BA combination with the high concentration of IBA combination (0.5 mg L^{-1}). Callus induction was observed within three weeks after inoculation of nodal segments on MS medium containing different concentrations of BA combination with the high concentration of IBA (Table 1). These results showed that the use of BA is necessary for the proliferation of explants. The proliferation of shoots in tissue culture conditions is influenced by many factors such as the type and concentration of plant growth regulators, explant type and genotypes which was consistent with the results of other researchers (Kodad *et al*., 2021). Both auxin and cytokinin hormones are important in explant organogenesis. Mozafari et al. (2016) reported that a low concentration of benzyl adenine in medium, decreased the number of shoots, but with an increase in BA concentration, the number of shoots increased. Various studies have shown that plant growth regulators are necessary for the regeneration of explants (Lizarraga *et al*., 2017).

MS media supplemented with BA, had a significant effect on shoot number. The results showed that a hormone-free MS nutrient medium does not shoot proliferation for both genotypes. A genotype effect was observed between the different tested genotypes since *A. lycioides* and *A. scoparia* show different proliferation rates in the same media. Regarding the interaction between genotype and plant growth regulators, 0.5 and 1 mg L^{-1} BA allowed for better results in both genotypes since proliferation rate was much more enhanced in MS medium than in 2 and 3 mg L^{-1} BA medium. *A. lycioides* exhibited significantly greater shoot numbers compared to most other treatments when 0.25 mg L^{-1} of IBA was combined with 0.5 mg L^{-1} of BA. In contrast, the highest number of shoots in the A. scoparia genotype was achieved only with 1 mg L^{-1} of BA. BAP growth regulator is commonly used as shoot proliferation hormone in plant tissue culture. Renu and Singh (2018) also reported that the highest number of branches was observed in Fuji red apple on MS culture medium with 4.44 M BAP and 0.05 NAA. It is reported that in MS culture medium with 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} GA3, causes 70% regeneration of Gisela-5 cherry rootstock explants (Thakur *et al*., 2016). In a study, it was found that culturing the shoot tips of the peach rootstock Hansen 536 on WPM medium + 1 mg L^{-1} BA and 0.1 mg L^{-1} NAA obtained the highest shoot regeneration (Sabbadini *et al*., 2019). The use of BA for shoot regeneration has also been reported in wild almond (Abbasi *et al*., 2019; Ebrahimi *et al*., 2022). Other researchers also reported that BA for shoot regeneration is more effective than other cytokinins (Nas *et al*., 2010).

Plant growth regulators had a great influence on shoot length. The results of this test showed that shoot length of genotype was affected by BA and IBA combinations. The two genotypes significantly were different in shoot length as affected by the treatments. The *A. scoparia* genotype produced the longest shoot length, while the shortest value was recorded with the *A. lycioides*. Our experimental results are in agreement with those observed for the Peach Rootstocks (Abou Elyazid Doaa *et al*., 2021) and *A. scoparia* (Abbasi *et al*., 2019). It has been reported that the most effective medium for increasing shoot length is MS medium containing 0.5 mg L^{-1} BA (Ak *et al*., 2021). This study showed that by increasing BA concentration to 2 mg L^{-1}, the number of leaves increased. In the almond genotype 2-22, the hormonal combination of 1 mg L^{-1} BA + 0.05 mg L^{-1} IBA resulted in the highest number of leaves and shoot length (Alizadeh-Arimi *et al*., 2020). Furthermore, according to Abbasi et al. (2019) for micropropagation of *A. scoparia* only 2 mg L^{-1} BA was the optimum concentration.

4. Conclusion

Shoots regeneration in this study was influenced by the type and concentration of plant growth regulators and genotypes. The data obtained in this study indicated that the response of regeneration percentage of explants were greatly dependent on the

concentration of BA combination with different concentrations of IBA. BA combination with the low concentration of IBA improves regeneration percentage of explants.

Conflict of interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No humans or animals were used in the present research.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

All authors had an equal role in study design, work, statistical analysis and manuscript writing.

Informed consent

The authors declare not to use any patients in this research.

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