Original Article

Effects of culture medium and plant hormones in organogenesis in olive (CV. Kroneiki)

Bahman Fazeli-Nasab[1]*, Ahmad Farid Rahmani[2], Hamideh KHajeh[3]

[1] *Research Department of Agronomy and Plant Breeding, Agricultural Research Institute, University of Zabol, Zabol, Iran*
[2] *Department of Horticulture, Faculty of Agriculture, Herat University, Herat, Afghanistan*
[3] *Agricultural Biotechnology Research Institute, University of Zabol, Zabol, Iran*

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ABSTRACT

A long time has passed since the first experience of *In Vitro* propagation of olive, but the presence of very strong end dominance in the neoplasm stage, which cannot be controlled by various cytokinin treatments, has limited the possibility of propagation in vitro. Therefore, the aim of the present study was to evaluate the micro-propagation feasibility inside olive glasses of olive cultivar Kroneiki. For branching and rooting of lateral buds, the experiment was carried out as a factorial experiment based on completely randomized design with three replications. Branching factors include five types of culture media (MS, MS1/2, MSM, MSM1/2 and OM), two types of hormones (BAP and Zeatin), and three types of hormone concentrations (control, 0.5 and 1 mg/L) and there were three types of time periods. Rooting factors include five types of culture media (MS, MS1/2, MSM, MSM1/2 and OM), two types of hormone combinations (IBA and NAA), four types of hormone concentrations (control, combination of 0.2 with 0.5, combination 0.5 with 1 and a combination of 0.75 with 1.5 mg/L) and three types of time intervals. Analysis of variance was performed by Statistix10 software and the comparison of mean traits was performed using the least significant difference test. The effects of culture medium, different hormones and also different concentrations of hormones used in the period, on the number of green leaves, number of yellow leaves, fresh weight and dry weight of seedlings as well as rooting were examined , which were found different ($P < 0.01$). The highest length of branch (1.8 cm) was obtained in MSM culture medium with BAP hormone in the third week. The highest fresh weight (0.06 g), the highest dry weight (0.003 g) and the highest amount of green leaves (55.39%) were obtained from the treatment of MSM medium with 1 ppm of BAP hormone. The highest rooting rate (95.17%) was obtained from the treatment of MS1/2 medium with 1.5 ppm of BAP hormone and 0.75 ppm of NAA hormone in the third week. The most effective culture medium and hormone on branching was MSM culture medium with 1 ppm BAP hormone and on rooting, MS1 culture medium with 1.5 ppm BAP hormone and 0.75 ppm NAA hormone.

***Corresponding Author:** Bahman Fazeli-Nasab (bfazeli@uoz.ac.ir)*

1. Introduction

The olive *Olea europaea* L. is a small, evergreen tree species of the olive family *Oleaceae*. This plant is native to the coastal areas of the eastern Mediterranean, including Lebanon and the coastal parts of Asia Minor [1]. The Iranian plateau is one of the known origins of olives [2] which is found in northern Iran and south of the Caspian Sea and can be cultivated in different parts of the world due to its long life and adaptation to different climatic conditions [1]. Considering the average rooting power of the majority of olive cultivars under natural conditions and the reduction of their propagation through cuttings on the one hand and the economic importance of olives in canning and olive oil on the other hand, using tissue culture methods for more efficient olive propagation is necessary [2].

In different olive cultivars, obtaining body embryos by cultivating different parts of immature embryos, mature embryos, petioles, etc. has been successful. In addition to the age of the embryo, other factors such as light and quality of growth regulators are involved in this matter [1]. Physical embryogenesis is the process by which body cells undergo the same developmental process as egg embryos. This is an important technique in plant micro-propagation and is an essential tool for basic research on embryonic development and other aspects of plant physiology [1]. The first report of bodily embryogenesis in olives is presented from the root[3].

Calluses from adult embryo fragments have been reported to have the ability to take root, and only micro-samples of immature embryo cotyledons (Picclin, Frangiento, Frantio cultivars) harvested between 60 and 90 days after flowering were suitable to stimulate embryonic callus formation. The difference in embryogenesis rate was dependent on the origin of the micro-samples (near, middle, and far from the egg embryo) and the last survival time of the micro-samples to induce callus in the culture medium. Various studies have shown that single body embryos are more easily transformed into complete and healthy plants than integrated embryos[1].

The composition and concentration of growth regulators have been studied to regulate the environment at different stages of body embryogenesis (from callus stimulation to embryonic growth and regeneration). Absolute dark conditions and light period of 16 light hours/ 8 hours of darkness were both effective in stimulating callus formation and body embryogenesis. It has been reported that liquid culture medium is useful for inducing callus production and solid culture medium is useful for callus development and seedling regeneration from vegetative embryogenesis. Obtaining body embryos from tissues is still difficult, although this has been done with two cultivars, Canino and Morailo, using a process called the dual regeneration system. In the first stage, regenerated stems were obtained from the culture of buds located in the place of petiole in the glass, and then the leaflets were cultured in a suitable culture medium until progeny masses appeared. In early embryos, the body embryonic cycle is easy to obtain from the epidermis or under the epidermis, and when the embryo is acquired, the body embryonic cycle can be maintained indefinitely [2].

Although it has been a long time since scientists first attempted to propagate inside olive jars, it is still difficult to reproduce through this method. The rate of germination in explants obtained from tissue culture is limited to a few cultivars. Very strong terminal dominance is the main feature of the olive germination stage, which is not eliminated by various cytokinin treatments. Therefore, the need to use different methods to modulate the final dominance is quite obvious [2].

Due to the slow growth of olives under natural conditions, this study is dedicated to improving the growth of this plant in vitro. Owing to the fact that plant growth regulators control the growth and development of plants and play an effective role in micro-propagation in vitro conditions, some of these substances were used in different concentrations. The results of various studies have shown that

different types of plant growth regulators and their different concentrations have played a significant role in increasing the micro-propagation of ornamental plants [4].

Cytokinins are commonly used in branch culture media for branch propagation. Many findings showed that increasing auxins to culture media is effective to increase the number and length of roots [4]. Rooting is a critical stage for micro-propagation success. Without an efficient root system, plant adaptation is impaired and the amount of propagation is affected. Some studies have shown a positive effect of cytokinins on rooting [5]. However, some other studies have reported that cytokinins play a negative role in shoot rooting [6]. In a study on in vitro culture of orchids, auxin stimulated root growth [7]. At low concentrations, auxins are used more for rooting than for continuous growth. Auxin has a positive effect on rooting and shoot length of plants. In addition, many other studies have positively evaluated the role of auxins in the rooting of shoots produced in vitro, and from a physiological point of view, the relationship between the role of auxins and cytokinins in branching and rooting is consistent with the known role of these plant growth regulators[8].

Micro-propagation, during tissue culture, allows a large number of plants to regenerate from a small piece of the parent plant (micro-sample) in a short period of time without seasonal restrictions. In the field of tree plants, tissue culture has led to the high production of superior cultivars, which in turn leads to the commercialization of uniform and healthy plant materials. This method also provides an opportunity for plant breeding. Micro-propagation of virus-free plants in vitro is the best way to ensure the production of healthy plants. There are many factors involved in plant micro-propagation, the most important of which is the type and concentration of plant growth regulators.

Micro-propagation uses embryonic axis explants, seeds, and apical and lateral buds compared with other explants due to greater genetic stability, less somaclonal diversity, and no need for high concentrations of cytokinins for growth. Lateral buds are simpler and more reliable [5]. Therefore, the use of micro-propagation method is one of the ways to achieve a large number of olive cultivar Kroneiki seedlings with the same genetic structure and reduce production costs and the possibility of continuous and rapid production.

2. Materials and Methods

2.1. Preparing an Explant

Olive plant of olive cultivar Kroneiki was prepared in 2020 from Sistan Agricultural and Natural Resources Research and Training Center (Zahak) (Figure 1). To obtain sterile seedlings, lateral buds were placed under running water for half an hour, and then washed with Twin X20 and washed three times with sterile distilled water. After washing, the sprouts were placed in 7% mercury chloride. They were rinsed again with sterile distilled water for three minutes. The buds were transferred again to 10% sodium hypochlorite for ten minutes. After this step, they were washed three times with sterile distilled water and then the explants were placed in MS medium [9] with 0.5 mg of BA hormone and prepared for cultivation in the main media for branching.

Figure 1. Appearance characteristics of olive cultivar Kroneiki (Photo; E, Elahi-Moghadam)

2.2. Branching

For branching lateral buds, experimental factorial experiments were performed in a completely randomized manner with three replications in the tissue culture laboratory of Zabol University Agricultural Biotechnology Research Institute. The main factors included culture medium, plant hormone, different concentrations of hormone and duration of treatment. Sub-factors included five types of culture medium (MS [9], MS1/2, MSM [10], MSM1/2 and OM [11]), two types of hormones (BAP and Zeatin), three types of hormone concentration (control (zero mg) (0.5 and 1 mg/l) and three types of time periods (first week, second week and third week after treatment).

2.3. Rooting

For rooting, first the best branches were produced, which belonged to the third week, MSM hormone treatment and BAP hormone at a concentration of one milligram per liter, and before the experiment, the produced branches were placed in the dark for five days and they were then immersed in ABA hormone for 20 seconds and finally entered the rooting test. Experimental body was performed factorially in a completely randomized manner with three replications. The main factors included culture medium, plant hormone, different concentrations of hormone and duration of treatment. Sub-factors included five types of culture medium (MS [9], MS1/2, MSM [10], MSM1/2 and OM [11]), two types of hormone composition (IBA * NAA and IAA * NAA), four types of hormone concentration (Control (zero mg), combination of 0.2 with 0.5, combination of 0.5 with 1 and combination of 0.75 with 1.5 mg/L, respectively, for combining NAA hormones with IBA and IAA with NAA) and three types. There were time periods (first week, second week and third week after treatment).

2.4. Data Analysis

Data were analyzed by Statistix10 software. Comparison of mean traits was evaluated using the least significant difference test.

3. Results

3.1. Branching

The effect of culture medium, different hormones and also different concentrations of

hormones used in the time period on the number of green leaves, number of yellow leaves, fresh weight and dry weight of seedlings were different (P <0.01) (Figure 2) but only the effect. The culture medium was different in different time periods on the longitudinal growth of seedlings (P <0.01). The interaction of culture medium and hormone as well as the interaction of culture medium and different concentrations of hormones on longitudinal growth, number of green leaves, number of yellow leaves, fresh weight and dry weight were different, but the interaction of culture medium and different concentrations of hormones on longitudinal growth was not effective. (P<0.01). In addition, the interaction of culture medium and hormones in a period of time except dry weight was not effective on other traits. The type of culture medium had no effect on the measured traits over time. Different concentrations of hormones only affected fresh weight, dry weight and number of green leaves and had no effect on longitudinal growth. Hormone type and different concentrations of hormones over time did not affect the measurement traits of the branches formed. The interaction of hormone, culture medium and different concentrations of hormone on fresh weight, dry weight and number of leaves was effective (P <0.01) but did not affect the number of yellow leaves and longitudinal growth. The interaction of culture medium, hormones and different concentrations of hormones over time, except for dry weight, was not effective on other traits.

LSD post hoc test showed that the highest branch length (1.8 cm) was obtained in MSM culture medium with BAP hormone in the third week (Figure 3). The highest fresh weight (0.06 g) and the highest dry weight (0.003 g) (Figure 4) and the highest amount of green leaves (55.39%) (Figure 5) from MSM culture medium treatment with 1 ppm of BAP hormone were obtained.

Table 1. Analysis of branching traits variance based on the LSD test

S.O.V	df	MS				
		Longitudinal growth	Number of green leaves	Number of yellow leaves	Wet weight	Dry weight
Medium(M)	4	11.1875**	3970.78**	16021.3**	0.00129**	5.0E-06*
Hormone(H)	1	11.0100ns	8113**	986.8**	0.01422**	3.2E-05**
Concentration(C)	1	2.6907ns	4195.67**	5517.1**	0.03254**	9.4E-05**
Weak (W)	2	9.8397*	674.98**	205.9**	0.01005**	3.6E-05**
M * H	4	8.5929*	3242.1**	1270.6**	0.00607**	2.1E-05**
M * C	4	0.2568ns	1016.7**	2370.4**	0.00383**	1.6E-05**
M * W	8	2.6969ns	25.08ns	40 ns	0.00025ns	1.7E-06ns
H *C	1	0.3453ns	1617.14**	4.9 ns	0.00262**	9.9E-06*
H* W	2	2.2441ns	8.01ns	0.5 ns	0.00067ns	4.2E-06ns
C * W	2	2.8160ns	44.48 ns	11.1 ns	0.00057ns	2.4E-06ns
M * H * C	4	0.5543ns	786.03**	47.3 ns	0.00215**	9.0E-06**
M * H * W	8	2.4235ns	12.5ns	3.8 ns	0.00031**	3.0E-06ns
H * C * W	2	4.3511ns	5.08 ns	3.5 ns	0.00045**	5.2E-07ns
M *H * C * W	16	3.4305ns	3.42 ns	0.8 ns	0.00017**	1.7E-06ns
Error	300	3.1278	46.05	35.8	0.00026	1.8E-06
Total	359					

*, **; significant at 5 and 1 level, respectively, and ns; none significant

Figure 2. Shoots produced in MSM medium with BAP hormone in olive cultivar Kroneiki

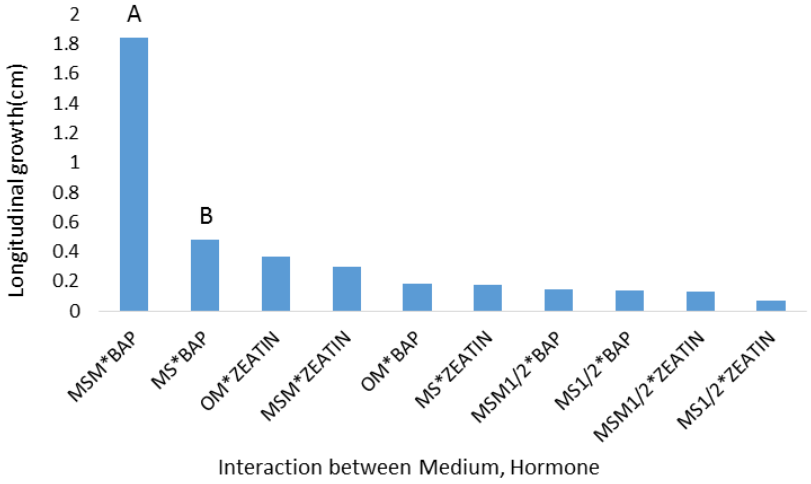

Figure 3. The effect of culture medium and hormone on the longitudinal growth of branches produced from the micronutrient of the lateral bud of the olive cultivar Kroneiki

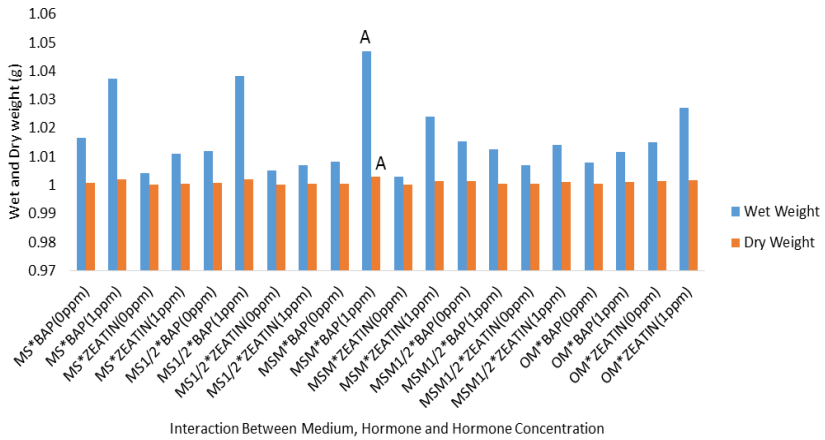

Figure 4. Effect of culture medium and hormones on fresh weight and dry weight of shoots produced from lateral bud micronutrients of olive cultivar Kroneiki due to the distance between the wet weight and dry weight numbers, all data plus two are doubled and then the logarithm based on two is taken from them

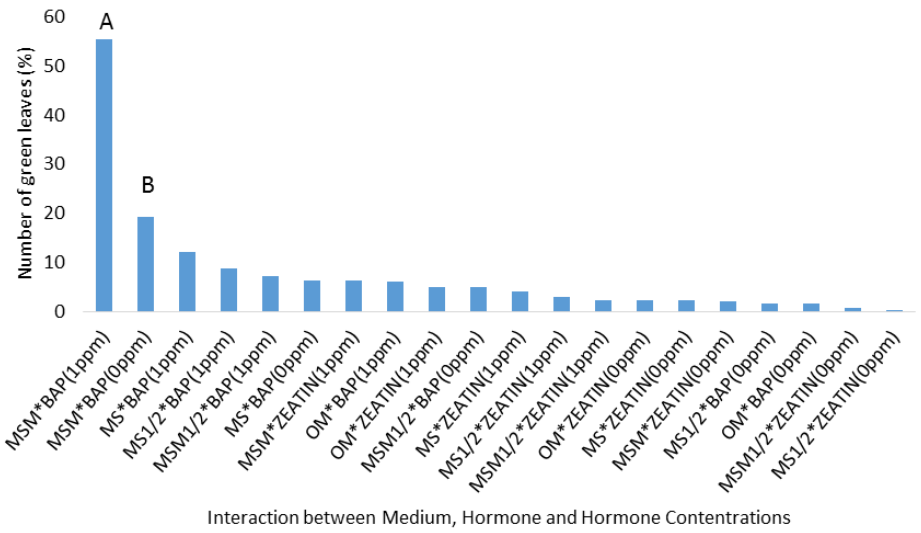

Figure 5. The effect of culture medium and hormones on the number of green leaves of branches produced from the micro-sample of lateral buds of olive cultivar Kroneiki

3.2. Rooting

The type of culture medium, the type of hormone and different concentrations of hormones over time have been effective in rooting. Also, the interaction between the type of culture medium and the type of hormone and different concentrations of hormones over time have been effective on rooting (P <0.01). LSD post hoc test showed that the highest rate of rooting (95.17%) was obtained from the treatment of MS1/2 medium with 1.5 ppm of BAP hormone and 0.75 ppm of NAA hormone in the third week (Fig. 6).

Table 2. Analysis of rooting variance based on the LSD test

Source	DF	SS	MS	F
Medium(M)	4	41601	10400.4	1101.93**
Hormone(H)	1	3276	3276.1	347.11**
Concentration(C)	3	22764	7587.9	803.95**
Weak(W)	2	1408	703.8	74.56**
M * H	4	12991	3247.9	344.11**
M * C	12	21674	1806.2	191.37**
M * W	8	705	88.1	9.33**
H *C	3	2790	930.1	98.54**
H* W	2	101	50.6	5.36**
C * W	6	552	91.9	9.74**
M * H * C	12	9902	825.2	87.43**
M * H * W	8	474	59.3	6.28**
H * C * W	6	158	26.4	2.8*
M *H * C * W	48	1051	21.9	2.32**
Error	240	2265	9.4	
Total	359	121713		

*, **; significant at 5 and 1 level, respectively, and ns; none significant

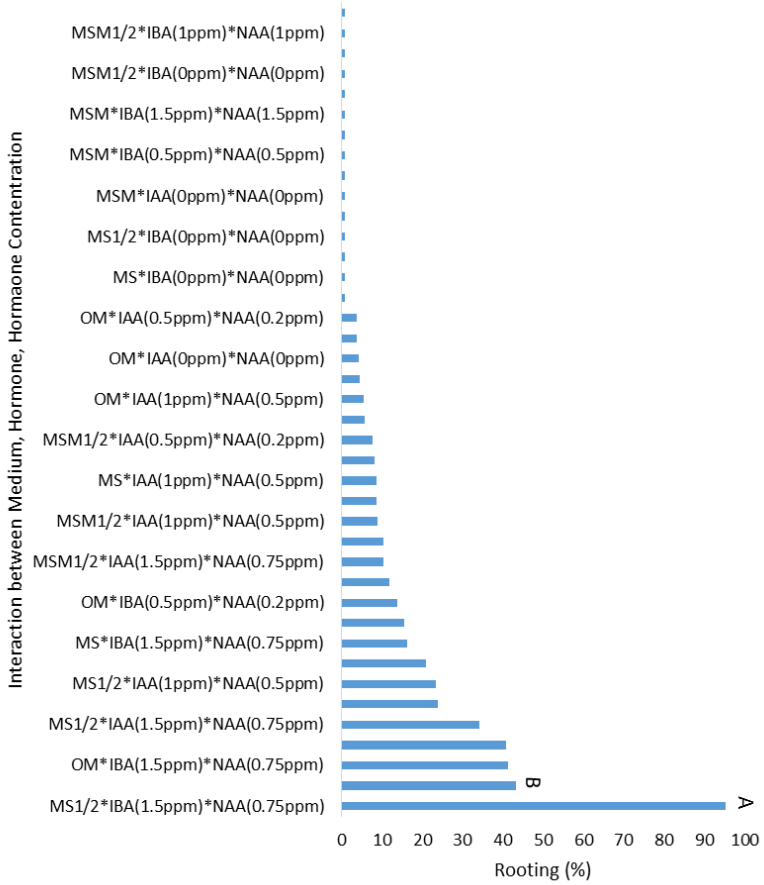

Figure 6. Effect of culture medium and different concentrations of hormone on microbial branching of lateral buds of olive cultivar Kroneiki

4. Discussion

The results of the present study showed that the most effective culture medium and hormone on branching were MSM culture medium with 1 ppm of BAP hormone in the third week, respectively. The highest rooting rate was obtained from the treatment of MS1/2 medium with 1.5 ppm of BAP hormone and 0.75 ppm of NAA hormone in the third week.

According to the results of this study, the type of culture medium and hormones had a significant difference on branching and rooting. Studies have reported that the highest callus production was due to the interaction of two growth regulators, auxin and cytokinin [12, 13], and the results [13] reported that no callus was formed in the bean leaf explant and the highest effect of BAP growth regulators (In 4 mg/l) for induction of bean callus was about 11%, but in combination with NAA growth regulator was able to increase callus production up to 42%. In the present study, the interaction of two growth regulators had a greater effect on branching and rooting. Considering that it has been reported that the salts in the culture medium have a negative effect on growth and callus formation to some extent [14], on the other hand, in the present study, two yellow and olive yellow cultivars were evaluated for branching and rooting. It was very weak and even in some cases did not respond to branching and rooting at all, so it was not mentioned in the research. Therefore, it can be mentioned that one of the reasons for the weak and low branching and rooting of yellow olive cultivar can be because this plant was not resistant to salinity [15] and could not provide a positive response, but the olive cultivar Kroneiki was resistant to salinity and was able to provide better results in the presence of growth regulators.

As a result, the best calli were obtained in MS medium containing growth regulator BA with a concentration of 1 mg/L and NA with a concentration of 0.1 mg/L [12]. It is noteworthy that NAA and Kin growth regulators are weaker growth regulators and have been more appropriate in most studies of 2, 4-D and BAP[16].

Regarding the importance of growth regulators in callus production, it has been shown that the presence of growth regulators is necessary for callus production and has not been produced in culture medium without growth regulator and without callus auxin [17]. Similar results have been recorded by other scientists in several plant species [18-20]. Some studies have shown that callus is produced in culture medium with auxin and without cytokinin, but callus is not produced without the presence of auxin [21]. The need for auxin regulators to initiate callus formation in other herbaceous species has also been observed[22, 23] because auxins have a direct effect on cell growth [24]. It has been reported that callus formation in explants is directly related to increasing auxin content and increasing auxin to cytokinin ratio [25, 26].

It has also been reported in walnut husks [27] that no callus is produced without auxin in the culture medium; but in general, different plant species have different needs for auxins for callus formation [28]. Therefore, callus production depends on the combination of growth regulators used in the culture medium and the balance between auxin and cytokinin growth regulators is a determining and important morphogenetic factor [29, 30]. In this regard, it has been reported [31] that the auxin/cytokinin ratio is close to 10 times, suitable for the rapid growth of undifferentiated calluses, the ratio close to 100 is suitable for root development and the ratio close to 4 is suitable for new shoot growth. It was effective for branching of 100% cytokinin and for rooting of Sadr in 100% auxin. In another study, the best callus was produced in the presence of cytokinin (0.25 mg) and auxin (4 mg), with the best callus formation percentage being obtained in the same ratio of 1: 10 [30].

Callus formation and regeneration in free forest species (*Zelkova carpinifolia*) under in vitro culture conditions was addressed and it was concluded that the percentage of callus formation in leaves (82.2%) was higher than stem explants (72.9%). MS medium containing 2 mg/L BAP and 1 mg/L NAA had the highest callus production rate (94.3%) and was considered as the best culture medium. The highest percentage of induction of callus germination (7.97%) was observed on MS medium with 0.05 mg/L GA3. MS medium enriched with 2 mg/l IBA and 2 mg/l NAA with 70% rooting were the best rooting media [32]. In the present study, it was found that the highest rooting rate (95.17%) was obtained from the treatment of MS1/2 medium with 1.5 ppm of BAP hormone and 0.75 ppm of NAA hormone in the third week, which is similar to the presented research.

The effect of growth regulators on regeneration of lateral bud explants of four species (Ziziphus spp.) in vitro culture showed that the highest branching in culture medium containing 6 mg/L BA and ip2 with 0.03 mg was obtained in IAA liters. Comparison of the interaction of growth regulators and lateral type showed that in the culture medium containing 6 mg/L BA with 0.03 mg/L IAA, the highest number of shoots with 4 seeds was obtained next to seedless Bengal. In the rooting stage, the culture medium containing 10 mg/L IBA showed the best rooting treatment for seedless Bengal [5]. In the present study, BAP and NAA also played an effective role in rooting.

Proper rooting leads to proper survival of seedlings resulting from the growth of explants grown in vitro and adapted plants [33]. Looking at the results obtained in this study, it can be seen that explants grown in auxin-free media had the least rooting. However, the use of high concentrations of these hormones in the culture medium is not recommended for rooting. Most rooting is obtained in media containing both IBA and NAA hormones.

In the present study, optimal rooting was the result of a combination of the hormones auxin and cytokinin. However, in other studies, similar

and even different results have been presented, which show the effect of plant species, hormone type and experimental conditions on rooting [17, 28, 34, 35]. For example, the findings on oak showed that the use of 0.3 mg/L IBA alone or in combination with 0.1 mg/L NAA was the most effective treatment on the longitudinal growth of primary and secondary roots [36] [36]. In many studies, only auxin hormones without combination with cytokinin hormones have been cited as more suitable stimuli for rooting [37]. Other results show that in the presence of cytokinins, rooting is reduced [38] [38]. One of the effects of cytokinins is complete inhibition or reduction of rooting. The most important reason for this difference should be sought in species differences and differences in the amount of endogenous plant growth regulators, especially auxins and cytokinins in different plant species [33]. For example, a juvenile micro-propagation study showed that 5 mg/L IBA was the best treatment for rooting [39]. Optimal rooting of eucalyptus was obtained in culture medium containing 2 and 1 mg/L IBA [40]. In *Jatropha curcas*, yew, almond, eucalyptus and hawthorn (*Crataegus* sp.), the highest rate of rooting was observed in the medium enriched with 1 mg/L IBA [33]. Also, the highest rate of rooting was observed in culture medium enriched with 0.5 or 2 mg/L IBA [38]. In various other studies, the superior role of IBA over other auxins in stimulating root formation was identified [37].

Micro-propagation of Caspian boxwood (Buxus hyrcana Pojark.), an endangered ornamental species, was found and the highest number of shoots was obtained in explants treated with 1 mg/L BAP with 0.5 mg/l IBA. Also, the highest number of roots was measured in explants treated with 1 mg/L BAP along with 1.5 mg/l IBA. The highest shoot height was obtained in explants treated with 1 mg/l BAP along with 1.5 mg/L IBA. The highest root length was related to seedlings treated with 0.5 mg/l BAP with 1.5 mg/L IBA [41]. In the present study, the highest branching was obtained from the treatment of MSM medium with 1 ppm of BAP hormone and the highest rooting rate was obtained from the treatment of MS1/2 medium with 1.5 ppm of BAP hormone and 0.75 ppm of NAA hormone.

Optimal conditions for micro-propagation of hawthorn (*Cratagus aronia*) under in vitro culture conditions showed that the best branching rate was 3 mg/L BAP and 1 mg/L IBA and the highest rooting rate was MS1/2 ms in the treatment was 3 mg/L IBA [33]. In the present study, the most effective hormones for rooting BAP and NAA were in MS1/2 medium.

Selection of an explant plays a key role in the success of tissue culture in vitro. The morphological complexity of an explant along with the selection of appropriate plant growth regulators has a significant effect on callus induction [42], although the age of the plant and how it is located on the culture medium is also important in some plants[43]. Also, due to the fact that callus induction starts from the incised areas [44], therefore, it is suggested to use young and herbaceous specimens. The surfaces of the explants also should be injured before inoculation.

5. Conclusion

The use of new technologies, especially tissue culture techniques in crop production, will be able to promote the process of mass and controlled production of this plant as an alternative method. In single node culture, the bud or part of the stem is separated for stem formation and development. This is the most natural way to propagate plants in the laboratory. Vegetative propagation is difficult in the usual way, but single-node cultivation is preferred to accelerate large-scale reproduction in a short time and help maintain plant survival [33]. Micro-propagation is a very important method for growing plants in in vitro conditions, especially plants that are difficult to propagate in natural conditions. The results of the present study showed that the most effective culture medium and hormone on branching, respectively MSM culture medium with 1 ppm of BAP hormone and the most effective culture medium and hormone on rooting were the treatment of MS1/2 medium with 1.5 ppm BAP hormone and 0.75 ppm NAA hormone.

Authors' contributions

In this study the first author presented the design, supervised, carried out the formal and statistical analysis and wrote the manuscript; the second author carried out the experiment, and the third author helped to write the original draft.

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Ethics approval and consent to participate

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Orcid:

Bahman Fazeli-Nasab
https://orcid.org/0000-0002-3268-8351
Ahmad Farid Rahmani
https://orcid.org/0000-0001-5473-7065
Hamide Khajeh
https://orcid.org/0000-0002-0521-7861

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