



Optimization of the Sterilization and Establishment Steps for Almonds 2-22 Genotype

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ABSTRACT

The almond is one of the most important nut crops in many countries, including Iran. On the other hand, due to the difficult rooting in conventional propagation methods such as cuttings, its propagation is faced challenges. Therefore, this study was aimed at optimizing in vitro culture conditions for the promising 2-22 genotype as a high-yield and late-blooming cultivar. To this end, after collecting apical and lateral buds of 2-22 genotype, surface sterilization and establishment treatments were applied to explants. The second-order equation is selected for the central compound design (CCD) with two variables (HgCl₂ and NaClO) to obtain a good fit in the sterilization treatment. The results showed that 1.8 % NaClO for 12 min + 0.1 % HgCl₂ for 3 min and 1.5 % NaClO for 8 min with 81.25%, and 10% of healthy seedlings were the best treatment in contamination control and explant Viability respectively. The experimental establishment was conducted as a factorial experiment using completely randomized design (CRD) with four replications. The establishment treatments indicated that the WPM medium was more effective than the MS medium and Knop medium. The hormone composition of 1 mgL⁻¹ BA + 0.05 mgL⁻¹ IBA had the best results in the percentage of establishment (72.25%), number of foliage (7.24), and shoot length (13.77 mm).

Introduction

Almond (*Prunus amygdalus* L.), belongs to the Rosaceae family, is one of the essential and profitable nut crops in the world with a very high commercial, nutritional, and therapeutic value (Ansari & Gharaghani, 2019). In 2017, world production of almonds was 2.2 million tons and the leading producers include the United States, Australia, Spain, Iran, and Italy (FAO, 2018). Fortunately, almond production continues to grow year to year. During the 2018-2019 season, the almond world crop was increased by 2% from the prior season and 20% from the previous 10-year average, amounting to over 1.25 million metric tons (kernel basis). Moreover, world almond exports in 2017 reached the highest

level in the last decade: over 943,600 metric tons (kernel equivalent, shelled + in-shell converted to kernel basis) (INC., 2018-2019). Given the content and high economic value of almond, it is evident that with proper planning, the use of appropriate technology, horticultural knowledge, improved cultivars, and advanced horticultural methods, we can see a very high currency exchange rate soon through the production of this nut crop.

One of the valuable genotypes of almonds being introduced is the 2-22 genotype, which is distinguished by its very high performance compared to other cultivars. This genotype is self-incompatible, with semi-spread growth

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habit, large tree size, late-blooming, very high yield and mixed fruiting habit. Furthermore, with the medium-size kernel and semi-hard shell, fruits are medium to late-ripening (This promising genotype is being studied physiologically, morphologically, biochemically in the Horticultural Department of Seed and Plant Improvement Institute (SPII), Karaj, Iran and according to the results, will be soon introduced as a cultivar with unique characteristics such as late-blooming and very high yield).

Similar to other nut crops, almond cultivars multiplication is accomplished sexually and asexually. Methods of sexual reproduction lead to genetic variability. Therefore, genetic uniformity will be lost, and generated plants will not be proper to use as a clone (Dixon & Gonzales, 1996). On the other hand, since the almond has difficulty in rooting and there are centers of viral infection in almond trees and the ability to transmit the virus causing the disease by propagating materials (graft, cuttings, and seeds), as well as for mass reproduction in the shortest time and its multiplied roots, the method of asexual reproduction is not an excellent method. Therefore, in vitro propagation methods present an efficient alternative system (producing many disease-free and true-to-type plants) for the commercial mass propagation of rootstocks and cultivars (Kose & Canli, 2015; Farsi et al., 2016; Eshghi Khas et al., 2020). Tissue culture technique has been used to regenerate or reproduce many plant species. The multiplication of shoots in vitro is affected by many factors (Ahmad et al., 2007) such as the type of explant, obtaining aseptic explant, explanting time, the composition of the basal medium, plant growth regulators, growth additives and carbon source (Matt & Jehle, 2005; Hassankhah et al., 2014; Pourkhaloe & Khoshkhui, 2015).

One of the most essential steps in tissue culture is obtaining an aseptic explant. Microbial contamination (including fungus, bacteria, viruses, and yeasts) is a severe problem of tissue culture causing a significant number of plant losses in micropropagation (Da Silva & Kulus, 2014; Taghizadeh & Solgi, 2014). Thus, sterilization is considered as the most important steps in vitro tissue cultures including

sterilization of tools, equipments, and nutrient medium as well as surface sterilization of the explants. In in vivo culture sterilization of other factors are essential, including, soil, greenhouses, gardens, are essential (Babaoglu et al., 2001). Generally, contamination causes slow growth of explants, tissue necrosis, reduced regeneration rate of different tissues which results in culture disruptions and finally explant death (Mihaljevic et al., 2013). An appropriate surface sterilization method should be determined which is varying according to the plants and the species. Factors like concentrations of chemical agents and the duration of application of chemical sterilizer influence the success of the surface sterilization even within the same varieties of a single species. Different chemicals including, sodium hypochlorite (NaClO), calcium hypochlorite (Ca(ClO)₂) and ethanol (C₂H₆O) are densely used for sterilization of the explants in vitro culture (Grouh et al., 2011; Mihaljevic et al., 2018; Ugur et al., 2020). In addition, the chemical agents such as mercury chloride (HgCl₂), hydrogen peroxide (H₂O₂) and silver nitrate (AgNO₃) are used as disinfectants (Arab et al., 2014). Ugur (2020) optimized sterilization protocol for DO-1 (*Prunus domestica*) rootstock of in vitro. In recent years, micropropagation of the genus *Prunus* have been studied in various dimensions such as the effect of different culture media and chemical elements on the growth of explants (Arab et al., 2018; Fotopoulos & Sotiropoulos, 2005), the effect of different concentrations of growth regulators (Abbasi et al., 2018; Erfani et al., 2017; Antonopoulou, 2007), the effect of vitamins and proline (Antonopoulou, 2007), iron in the medium (Modgil, 2010), and various percentages of sucrose (Lloyd & McCown, 1980). According to culture medium, explant age and variety cytokines may impose differential effects on the initiation of lateral bud activity, cell stimulation and division, lateral bud formation, and proliferation. According to the results of George (1996), the use of high levels of adenosine cytokines compared to natural cytokines was considered necessary. Given such a result (Ruzic & Vujovic, 2008; Ansar et al., 2009) also observed the effect of natural cytokinins on the growth of

woody plants in pear, apricot (*Prunus armenica*) and cherry (*Prunus avium*) and olive varieties ‘Moraiolo’. Arab et al, (2014) noticed that the MS medium with BAP were efficient on the proliferation rate of G×N15 rootstock. However, the presence of high amounts of NH₄NO₃ and cytokinin in the culture medium triggered an inhibitory effect on shoot growth. Moreover, they reported that if high concentrations of cytokinin were used in the proliferation phase, the tissue of regenerated seedlings would be more turgid usual. The leaves would become abnormal in some cultivars.

This study was aimed at evaluating the effect of surface sterilization, basal medium, and plant growth regulators on micropropagation of 2-22 genotype to achieve plants with optimal quality and quantity in in-vitro conditions.

Material and Methods

This study was conducted at the Plant Tissue Culture Laboratory of the Faculty of Agriculture, Tarbiat Modares University, Iran on June and August 2019. Explants of almond 2-22 genotype were taken from plants grown in Horticultural Department of Seed and Plant Improvement Institute, Karaj, Iran, in June 2019. One-year-old shoots were cut into 1–1.5 cm long pieces, each including one bud. The factors such as dust and waste of insects that existing on the shoots and negatively affects sterilization process were cleaned out with running water for 30 minutes. Then, the explants were kept in 70% ethanol for 30 seconds as a

pretreatment procedure before the application of the chemical agents. Later, they were rinsed with distilled water three times to avoid the caustic effect of the alcohol. Then, explants were kept in sterilization solutions prepared at different concentrations, as shown in Table 1, for different periods in such a way that immersion in 2%, 1.8%, and 1.5% (w/v) NaClO respectively for 15, 12, and 8 min depending on the status of explant tissues and rinsed three times with sterile distilled water. Then, explants were dipped in 0.1% HgCl₂ for 5, 3, and 0 minutes. Finally, they were washed four times with sterile distilled water. The second-order equation presented for the central compound design with four replications and four explants in each replicate is an equation as following:

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_{i-1} + \sum_{i < j} \beta_{ij} x_i x_j + \sum_{i=1}^k \beta_{ii} x_i^2 + \epsilon$$

y: Model output (healthy and dead explant percentage, bacterial and fungal contamination), k: Number of input variables (concentration of sterilization agents and immersion time) β₀: Constant coefficient, β_i: linear coefficient on variable, β_{ij} : The coefficient of interaction effect on the variables, β_{ii}: The coefficient of the second-order on the variable, and ε: Includes the residual value of the model. In addition, Analysis of tests was performed by Minitab version 19 software.

Table 1. Chemicals used in surface sterilization and their application durations

Experiment	Chemical agents	Application Combinations	
		Concentrations (wv ⁻¹ %)	Durations (minute)
T1	NaClO	2	15
	HgCl ₂	0.1	5
T2	NaClO	1.8	12
	HgCl ₂	0.1	3
T3	NaClO	1.5	8
	HgCl ₂	-	-

In the culture establishment stage, culture media MS (Murashige & Skoog, 1962), WPM (Lloyd and McCown,

1980) and Knop (Knop W, 1865), and containing different concentrations of BA (0. 0.5 and 1 mgL⁻¹) and (0, 0.05 and

0.1 mgL⁻¹ IBA) were evaluated on the percentage of active buds and bud growing quality index. The pH of the culture medium was 5.6, and the culture medium was autoclaved at 121°C for 20 minutes. Growth chamber conditions were 16 hours of light at 25°C and 8 hours of dark at 23°C. Leaf number, shoot length, establishment percentage were measured after six weeks.

This study was conducted as a factorial experiment using a CRD. Each treatment had four replicates and four explants in each replicate in a 125 ml culture glass jar containing 35 ml of medium. Statistical analysis of the data was carried out through using (IBM SPSS Statistics Version 22) software, and difference among treatment means were compared by Duncan's Multiple Range Test (DMRT) at 1% and 5% levels.

Results

According to the obtained results, T2 and T3 resulted in 81.25% and 100% of healthy seedlings, therefore, both treatments were the best in production of healthy and viable plants without contamination, respectively. Alternatively, T1 resulted in 25% of healthy seedlings and 75% of dead explants were the least effective in the sterilization process

(Fig. 1, 2). Results of analysis of variance revealed that the effect of concentration and immersion duration of sodium hypochlorite on explants viability both were significant at 0.05 probability level (Table 2). The use of a higher concentration of sodium hypochlorite and the longer immersion time has been associated with an increase in the percentage of burn or dead explants. In the following, mercury chloride treatment had a significant effect on the viability of explants and was significant at 0.05 probability level. Furthermore, the immersion duration of mercury chloride effect on the survival of the explants was significant at the level of 0.01. Application of higher concentrations and longer immersion time in mercury chloride solution led to an increase in the percentage of dead explants, (Table 3). Moreover, all three treatments were very effective in controlling bacterial and fungal infections. Almost no infection was observed in the explants of the three treatments. From this table of analysis of variances No. 2 and 3, the difference between the treatments in terms of infection control was not significant. These results indicate the high efficiency of the applied treatments in terms of contamination control.



Fig. 1. Images of the results of establishment experiments T1, T2, and T3

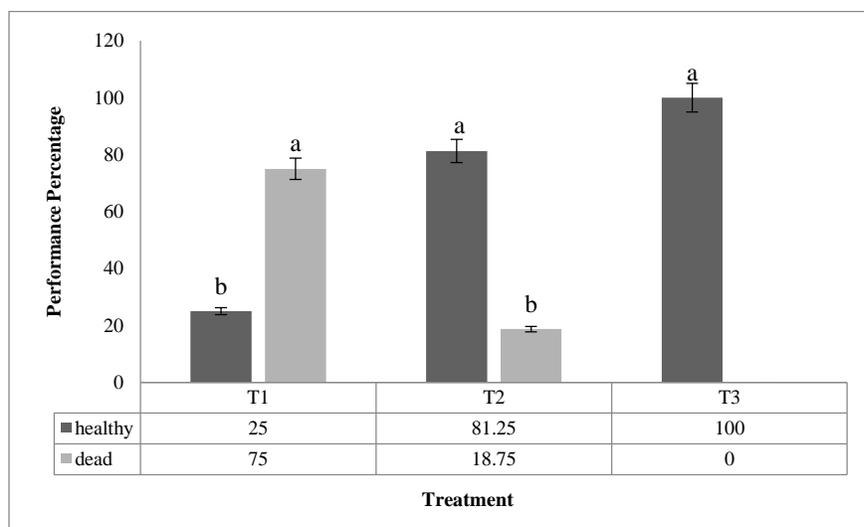


Fig. 2. Percentage of healthy and dead explants in sterilization treatment

Table 2. Analysis of variance for the effect of concentration and immersion duration of sodium hypochlorite on explants viability

Sources of changes	DF	Healthy explants	Dead explants	Bacterial contamination	Fungal contamination
sodium hypochlorite treatment	1	1539*	1539*	0 ^{ns}	52.72 ^{ns}
time	1	1813*	1813*	4.11 ^{ns}	37.01 ^{ns}
Error	9	2969	2969	3437.50	1718.75

*and ns, respectively, indicating significant at 5% and is non-significant

Table 3. Analysis of variance for the effect of concentration and also immersion duration of mercury chloride on explants viability

Sources of changes	df	Healthy explants	Dead explants	Bacterial contamination	Fungal contamination
Mercury chloride treatment	1	1833*	1813*	4.11 ^{ns}	37.01 ^{ns}
time	1	6328**	6328**	703.12 ^{ns}	78.13 ^{ns}
error	9	2969	2969	3437.50	1718.75

*, ** and ns, respectively, indicating significant at 1%, 5% and is non-significant

Results of analysis of variance showed that different media and different concentrations of BA, IBA, as well as the interaction of medium with BA and IBA growth regulators on leaf number, shoot length, and establishment percentage at 1% level were significant (Table 4, Figs. 3, 4).

Table 4. Analysis of variance for the effect of different media and different concentrations of BA and IBA on percentage of establishment, shoot length and leaf number

Sources of changes	DF	Average of squares		
		Percentage of establishment	Shoot length	Leaf number
Media (A)	2	8654.134**	431.714**	47.181**
BA + IBA (B)	8	5097.290**	97.573**	44.731**
A × B	16	172.642**	3.769**	1.205**
Error	81	6.698	0.300	0.090

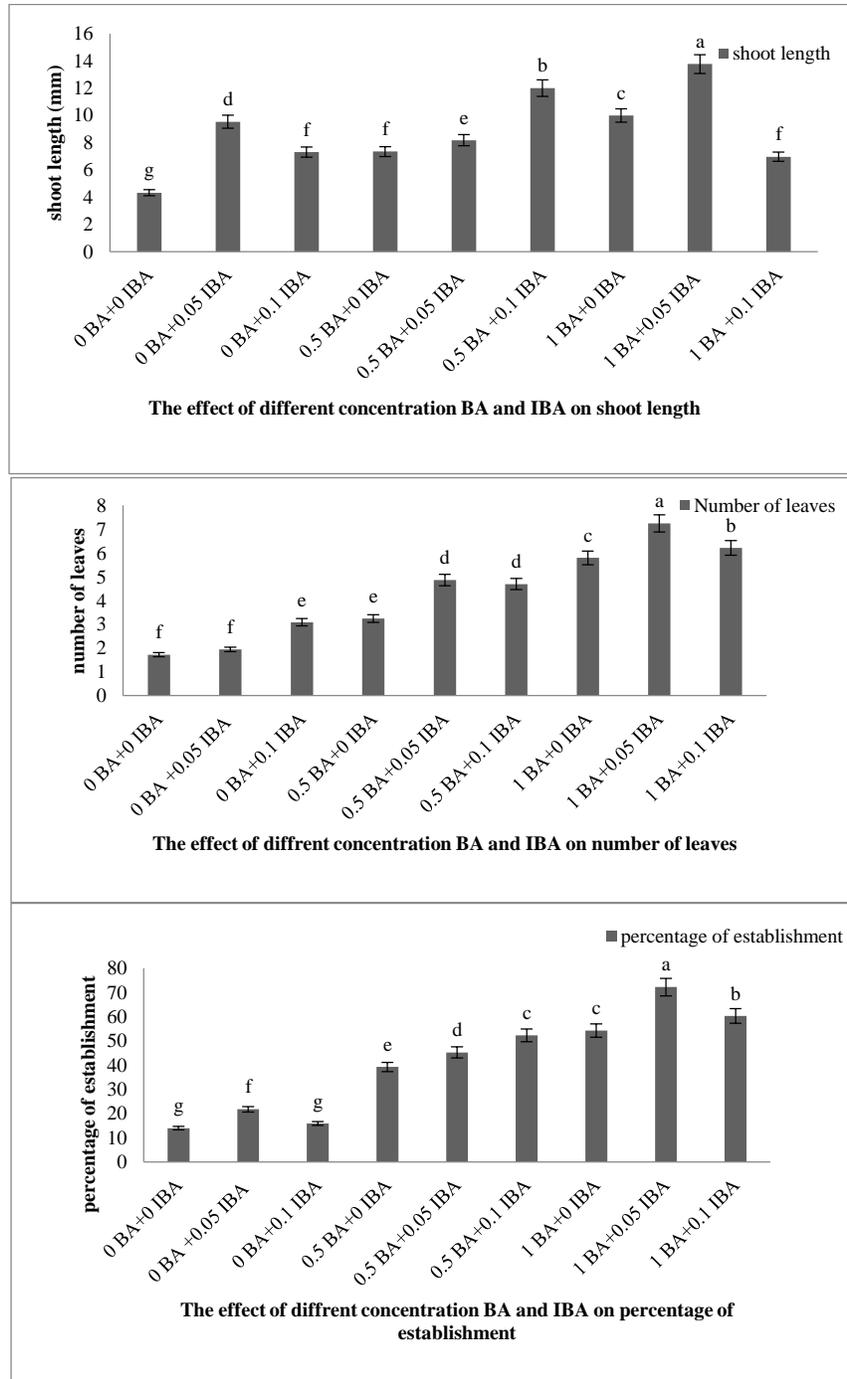


Fig. 3. The effect of growth regulators BA (0, 0/5 and 1) and IBA (0, 0.05 and 0.1) in the establishment stage on indicator of leaf number, shoot length and percentage; data collection of the mentioned indicators was done after four weeks.

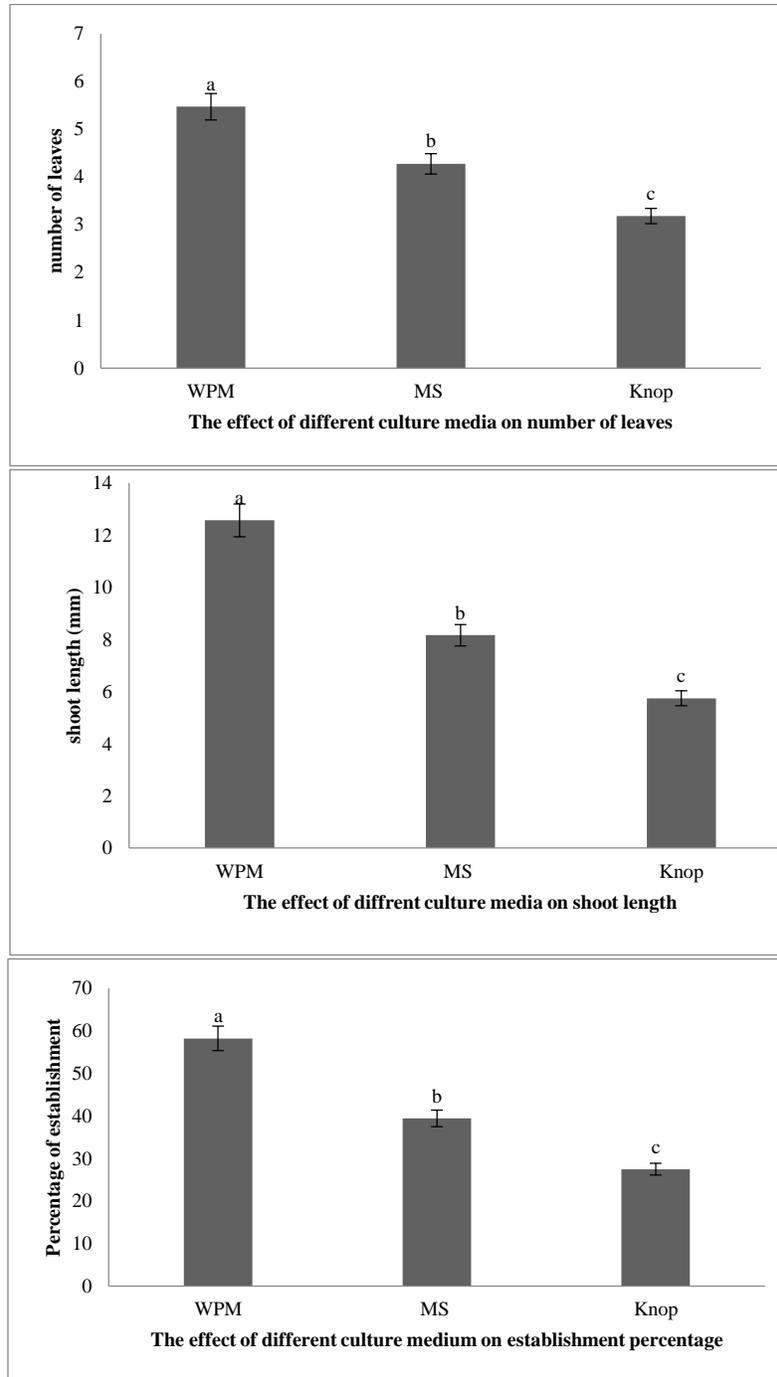


Fig. 4. Different culture media, including MS, WPM, and Knop contains different concentrations of IBA (0, 0.05, and 0.1) BA (0, 0.5 and 1, sucrose (30 g/l) with agar (7 g/l) and pH = 5.8. They were placed in an autoclave at 121 °C for 20 minutes for sterilization. After autoclaving, 30 ml of media were distributed in jars. The explants were then placed in the environments and placed in a growth chamber at 25 ± 2 °C under 16/8 h light/dark cycles for four weeks. After four weeks, indicators such as leaf number, shoot length, and establishment percentage were measured.

Based on the results of the mean comparison, the highest leaf number (7.24), shoot length (13.77 mm), and establishment percentage (72.25%) in the WPM medium with 1 mgL^{-1} BA and 0.05 mgL^{-1} of IBA and lowest leaf number (1.71), shoot length (4.32 mm), and establishment percentage (13.97%) were obtained in hormone-free Knop's medium. WPM medium had the highest number of leaves (5.46), shoot length (12.56 mm), and establishment percentage (58.19%). Knop's medium had the lowest leaf number (3.18), shoot length (5.73 mm) and establishment percentage (27.43%) and MS medium with leaf numbers (4.27), shoot length (8.16 mm), and establishment percentage (39.38%) were placed between WPM and Knop's medium [Figs. 3, 4]. This study showed that with increasing BA concentration to a certain extent, the number of leaves and establishment rate increased, indicating a positive relationship between the increase in concentration and establishment rate that the percentage of establishment peaked was at a concentration of 1 mgL^{-1} BA. The establishment treatments indicated that the WPM medium was more effective than the MS medium and Knop's medium. Besides, the hormonal composition of 1 mgL^{-1} BA plus 0.05 mgL^{-1} IBA had the best results in the percentage of establishment and number of foliage.

Discussion

The success of micropropagation and setting up a plant regeneration protocol is highly dependent on the efficiency of the sterilization stage (Da Silva *et al.*, 2016). This efficiency can be achieved through the optimized concentration of the sterilizing materials as well as the period of exposure (Da Silva *et al.*, 2016; Hesami *et al.*, 2018). Our results showed that the effect of concentration and duration of sodium hypochlorite and mercury chloride treatments was active in three experiments designed for explant viability but did not control the bacterial and fungal contamination. Moreover, T2 and T3 showed the best results in explants' survival, while T1 was not an excellent treatment for sterilization due to the

high immersion time of mercury chloride. Of course, none of the three treatments affected the control of bacterial and fungal infections. These results are in line with (Hesami *et al.*, 2019) that showed that the explant viability could be reduced by increasing the concentration of NaClO and immersion time. Furthermore, their results revealed that 1.5% NaOCl at 15 min immersion time resulted in 100% explant viability. Ugur (2020) showed that the highest aseptic explant rate (91%) was achieved with 2% NaClO application in 15 minutes, the lowest aseptic explant rate (23.33%) was achieved with 0.5% HgCl_2 application in ten days. Furthermore, Hesami *et al.* (2018) demonstrated that an increase in the concentration of NaOCl and immersion time had a negative effect on the explant viability of *Chenopodium quinoa*. Besides, NaClO is highly reactive with amides, nucleic acids, amines, and amino acids due to its strong oxidizing properties (Mihaljevic *et al.*, 2013). These reactions can produce CO_2 , respective aldehyde, and NH_4Cl (Da Silva *et al.*, 2016). Ugur (2020) also reported that the lowest contamination rate was observed in NaClO applications (18.34%) and the highest contamination rate was observed in HgCl_2 applications among the sterilization agents and HgCl_2 at a high level, causing a decrease in explant viability. In the current study, HgCl_2 that has an extremely toxic effect, led to negative results in surface sterilization of plant explants in in-vitro studies. Generally, the use of HgCl_2 in the sterilization stage is not recommended due to Hg neurotoxic and immunotoxic properties, which are environmental pollutants (Da Silva *et al.*, 2016).

This study showed that with increasing BA concentration to a certain extent, the number of leaves and establishment rate increased, and this indicates a positive relationship between the increase in concentration and establishment rate that the percentage of establishment peaked was at a concentration of 1 mgL^{-1} BA. The results are in line with the Aghaei and Yadollahi (2012) findings that the highest number and length of the stem for almond GF rootstock micropropagation were obtained in MS medium with 1 mgL^{-1} . They also stated that increasing BA concentration decreased

the number of branches. Tatari and Musavi (2013) produced the highest number and length of Tetra, Nemaguard, and GF677 seedling in the combination of 0.6 mgL^{-1} BA and 0.01 mgL^{-1} NAA. Moreover, they reported that increasing the concentration of growth regulators by 0.8 and 1 mgL^{-1} of BA and 0.1 and 0.5 mgL^{-1} NAA resulted in vitrification and thalli callus that can be related to the difference in cytokinin influence based on the kind of culture, the variety of plants, and the age of explants. It is inconsistent with some researches that suggested concentrations above 1 mgL^{-1} increased the number of branches, which can be attributed to the fact that shoot branching depends on the initiation and activity of axillary meristems, which are hormonally controlled mainly by cytokinin (Dobranszki & Teixeira, 2010). Rezaei and Hosseipour (2015) reported that the highest number of shoots was obtained with 2 mgL^{-1} BA. Accordingly, Kose and Canli (2015) proposed that the highest numbers of shoots per explant were obtained at higher concentrations of BA. Furthermore, according to Abbasi et al. (2018), for micropropagation of *Prunus scoparia* only 2 and 4 mgL^{-1} BA and 2 mgL^{-1} TDZ, was the optimum concentration. Of course, increasing the concentration of BA in the medium produce more, but shorter shoots due to the limited capacity of biomass synthesis of the plantlet in in-vitro condition. As a result, it is evident that the amount of nutrients substances absorbed by each shoots reduced by increasing the number of proliferated shoots.

Besides, the results showed that the dormant buds of 2-22 genotype in the WPM culture medium had the highest establishment rate, leaf number, and shoot length relative to the two others mediums. The composition of culture medium plays an important role in the growth of in vitro plants. In in vitro culture, the culture medium has been very active in plant growth caused by the combination of salts in the medium. Various types of growth abnormalities, such as leaf chlorosis, dead-end tissue Leaves, dehydration, and browning of tissues, are related to the type of culture medium. The Knop's culture is weak, and the concentration of some

elements in it is several times lower than in other media. The yield of culture medium depends on the genotype. In the almond, Nan Paril cultivar, AP medium was a suitable culture medium, while for Nius plus Ultra, the MS medium had favorable cultivation (Battistini & Paoli, 2002). The effects of two media MS and LP on GF677 in vitro regeneration of leaves were studied and reported that LP media had a more significant effect on the number of buds produced in regenerated samples. The researchers found that the better response to the LP medium was due to the lower amount of chloride presented in the medium and to the $\text{Ca}(\text{NO}_3)_2$ form of Ca, whereas the Ca in the MS medium was CaCl_2 (Hasan et al., 2010). Erfani et al. (2017) have optimized a successful system for the Garnem rootstock (almond \times peach) tissue culture. The results showed that the best media for proliferation in terms of shoot and node number was DKW containing BA.

Conclusions

The results of sterilization treatment showed that T2 and T3 with 81.25% and 100% of healthy seedlings were selected as the best treatment in term of contamination frequency and explant viability, respectively and the application of concentrations and immersion time of explants in sodium hypochlorite and mercury chloride were highly effective in the explants viability. The establishment treatments indicated that the WPM medium was more effective than the MS medium and Knop's medium. Furthermore, the hormonal composition of 1 mgL^{-1} BA plus 0.05 mgL^{-1} IBA had the best results in the percentage of establishment, number of foliage and, shoot length and these amounts have been 72.25%, 7.24 and 13.77mm, respectively. Furthermore, we can safely say that a certain amount of BA is required to obtain the best effect. Thus, in this experiment, the effect of different concentrations of the hormone BA was very significant, and the results reveal that for dormant buds, the BA hormone is essential, while the IBA hormone did not have significant effect.

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