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In vitro microtuberization of Black Zira (*Bunium persicum* Boiss.)

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Bunium persicum or **Black Zira is one of the endangered species in the land of Persia. The main purpose of this study was to investigate microtuberization of *B persicum*in in order to use in germplasm storage and commercial production. Seeds of *B. persicum* were used as explant. Different culture media (MS, ½MS and B_5) along with different concentrations of jasmonic acid (JA) (0, 2 and 5) were used individually as basal media and also in combination with two different temperatures (15 and 20°C) to develop appropriate media for microtuberization. Moreover, propagated microtubers were then vernalized and acclimatized in order to transfer to greenhouse. The results revealed that by increasing in concentration of JA, weight and length of microtubers increased significantly. MS medium seemed to be the most effective basal medium for this plant. In contrary, this study indicated that MS medium and 5 mM JA were the most suitable combination for *in vitro* culture establishment and short-term maintenance of tested *B. persicum.* Also, 15°C showed significant effect on increasing the weight of microtubers.**

Key words: Microtuberization, *Bunium persicum*, jasmonic acid, temperature, medium.

INTRODUCTION

Bunium persicum (Boiss.) or Black Zira is a species from the Apiaceae family, especially grown in the northeast areas of Iran. *B. persicum* seeds are called ‘‘zireh kuhi’’, meaning ‘‘wild cumin’’, and are used as a culinary spice (Mortazavi et al., 2010). Apiaceae family has a short growth period (75 days) (Omidbigi, 2012). It is found wildly growing in Iran, Pakistan, India and central parts of Asia (Omidbigi, 2012). *B. Persicum* seeds have been widely used for medicinal purposes especially as anti-fungus (Sharififar et al., 2010). Several remedial impacts

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including digestive disorders, urinary tract disorders, and diuretic, gynaecologic, anticonvulsion, anti-helmetic have been defined for *B. persicum* seeds (Sekine et al., 2007). It has also been reported that this species has strong anti-inflammatory activities and reduces the pain (Hajhashemi et al., 2012). The demand for this crop is rapidly increasing while its habitat has been shrinking across the country because of overharvesting. Despite all properties of this valuable plant, there are lots of unknown and unstudied cases about this amazing species. Appropriate germination and crop establishment is most critical stage of growth period of this plant (Sharifi and Pouresmael, 2003). It is well documented that the main reason of low germination percentage of Apiaceae species are because of its minute embryo in this family and oxygen deficit in germination process (Carol et al., 1992). Seeds of *B. persicum* need cold temperature for stratification process and cold demands of this plant eliminate across December to January in northeast of Iran. In nature, seeds germinate in 3 to 4 month after passing through the winter and consequently produce only a few leaves and a small tuber (Garval and Rani, 1999). Plant growth rate in first year of cultivation is significantly slow and maximum diameter of *B. persicum* tuber is approximately 4 mm (Omidbigi, 2012). Reproductive phase (seed production) of *B. persicum* starts after 4 years and may maintain up to 12 years as tuber continues growing (Garval and Rani, 1999; Omidbigi, 2012). Generally, economic production of this plant set up after almost 4 years which is the main reason for low tendency of local farmers to cultivation of this valuable plant. Khosravi (1994) reported that the production of *B. persicum* is limited because of seed dormancy and several biotic stresses of which wilt diseases are the most serious. Also, potential genetic variability for conventional production is limited in *B persicum* (Hunault et al., 1989). Chizzola et al. (2014) reported the genetic variability of fruits from different Iranian wild populations.

The use of *in vitro* tuberization has some advantage such as higher control of the different environmental factors in microtuberization and can boost the crop production. Rapid production of microtubers could be useful for the production of pathogen-free seed tubers (Omokolo et al., 2003). Moreover, it has been proved that tissue culture is one of the main methods for gene conservation (Turner et al., 2001). Also, microtubers are important in fundamental researches in plant science and plant germplasm storage. Components of the culture medium vary according to the type of plant and the propagation stage. These components include inorganic salts, organic compound, natural ingredients and inert support. There are few reports about micro-propagation and somatic embryo development of *B. persicum*. Wakhlu et al. (1990) and Valizadeh et al. (2007) obtained the callus of *B. persicum* Boiss from mericarps. In this regard, the present study was built on determination of more suitable medium for enhancing of *B. persicum* tuber size in order to promote its commercial production in future and save this plant from extension.

Numerous investigators engaged in other systems have shown that some growth regulators, such as Jasmonic acid, can act as growth induced stimulator in tuber development processes (Zel et al., 1997; Cenzanoa et al., 2007; Pelacho et al., 1999). It has shown that microtuberization is generally induced directly on the explants culture and is influenced by factors including growth regulators such as Jasmonic acid (Jasik and Mantell, 2000). Hence, the main purpose of this study was to find out the appropriate condition for microtuber development of *B. persicum* under different controlled conditions such as different medium and temperature, and their interactions with Jasmonic acid as a growth regulator. To achieve this, an *in vitro* experiment under different temperatures, mediums and growth regulators was established.

MATERIALS AND METHODS

Plant material

This study was conducted at Ferdowsi University of Mashhad and Khorasan Research Institute for Food Science and Technology in Iran from 2010 to 2012. *B. persicum* seeds were collected from the experimental field of Ferdowsi University of Mashhad. Seeds were washed with tap water for 10 min and surface sterilized with 70% ethanol for 1 min and 1.5% (w/v) sodium hypochlorite solution for 15 min. Seed were then washed with distilled water 3 times. A simple seed medium containing agar (Sigma) (5 g/l) and sucrose (Sigma) (5 g/l) were used in each micro-tube. Each seed were sown individually in 1.5 ml microtubes containing 1 ml prepared seed medium to prevent contamination. All microtubes were kept in 4°C for stratification. Following a period of time (8 weeks), seeds started to germinate. Those seeds which had developed 4 mm of root were used for tuber induction stage. Figure 1 shows the germinated seeds after developing a full radicle.

Preparation of the plant growth regulators and media

For tuber induction, different basal media MS (Murashige and Skoog, 1962) (3% sucrose), B_5 (Gamborg et al., 1968) and MS basal media of half strength of macro- and micro-salts (1/2 MS) with 825 mg/l NH_4NO_3 and 950 mg/l KNO_3, 185 mg/l $MgSO_4.7H_2O$, 85 mg/l KH_2PO_4, 13.9 mg/l $FeSO_4 \cdot 7H_2O$, 110 mg/l $CaCl_2.2H_2O$, 3.1 mg/l H_3BO_3, 0.12 mg/l $NaMoO_4.2H_2O$, 0.012 mg/l $CoCl_2.5H_2O$, 4.3 mg/l $ZnSO_4.4H_2O$, 11.1 mg/l $MnSO_4.4H_2O$, 0.41 mg/l KI, 18.6 mg/l Na_2 EDTA.$2H_2O$ supplemented with 3% sucrose were used. Vitamins were added to all media in same concentration. The pH of the media was adjusted to 5.7 and autoclaved at 121°C for 15 min. Stock solution of Jasmonic acid (JA) was prepared by dissolving in a small amount of 70% ethanol and then adding distilled water to volume for the next steps of experiment (Gao et al., 2003). Also, to assess the effect of JA on morphological characteristics of microtubers, JA was used in concentration of 0 (control), 2 and 5 mg/l. Each germinated seed was planted in a glass tube containing 5 ml of each medium. Temperature treatment was applied after seeds had been germinated at 4°C under darkness conditions. *B. persicum* germinated seeds were transferred to growth chambers controlled to temperature of 15 and 20°C (18 h light/6 h dark

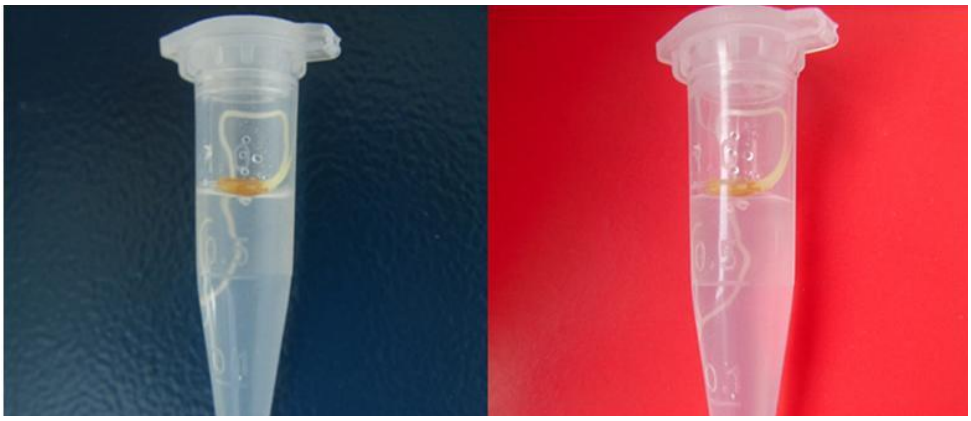

Figure 1. Germinated seeds of *B. persicum* after chilling period at 4°C.

photoperiod) conditions. Light was supplied by cool white fluorescent lamps (40 mMol/m/s).

Data collection

Following a period of growth (usually after 8 weeks), fresh weight, length, width of each single microtuber from each culture tube were recorded. The average final dry weight for the replicates of each treatment was recorded after 8 weeks.

Microtuber vernalization

Some visual morphological changes in the plantlets and micro tubers after vernalization stage *in vitro* and in greenhouse conditions were observed and recorded. To access this purpose, 20 well developed healthy microtubers obtained from all treatments were acclimatized and subcultured in the same medium which had been applied during the incubation stage. At this time, all the microtubers had been passed 8 weeks after the seed culture. Furthermore, to induce the vernalization, microtubers were transplanted to the refrigerator (4°C). After detecting the first true leaves on microtubers, all germinated microtubers were transferred to the greenhouse controlled to temperatures of 25±3°C. MS medium was removed, and washed from the microtubers using distilled water to prevent subsequent infection. All the microtubers were planted in medium composed of peat (20%) and coco peat (20%) and soil (60%). Plants were then maintained in the greenhouse (25°C, 85% humidity). Vernalisation steps of microtubers are provided in Figure 2.

Data analysis

Statistical analysis was performed using the SAS software program (Version 9.0). Data were subjected to the analysis of variance (ANOVA) to detect the significant differences between level of Jasmonic acid, level of temperature and level of medium. The general linear model (GLM) was constructed to generate three-way ANOVA. The results of three-way ANOVA for dry weight and length of microtuber are summarized in Table 1. Mean separation was conducted using Least Significant Difference (LSD) test at 0.01 probability level. All samples, with the exception of occasional contaminated tubes, were computed for tuberization. Among all calculated factor (length, width, weight, weight ratio) impacts of medium and temperature and jasmonic acid had shown a significant influence on length and weight of *B. persicum* microtubers. The effective results were obtained as shown in the present report.

RESULTS AND DISCUSSION

Effect of jasmonic acid

Jasmonic acid significantly increased the growth of *B. persicum* microtubers (2 and 5 mM) on the weight and length of microtubers in comparison with the control ($p \leq 0.01$) (Table 1). This effect of JA on tuber induction and tuber growth ratio has been previously reported. Pelacho and Mingo-Castel (1999) showed that final JA promoted tuberization percentage (100%) after 30 days in culture and it induced potato tuber fresh weight 6.4 times the kinetin induced tuber weight. Similar to those effects, in *B. persicum,* JA might act as a chemical signal to trigger senescence related processes such as tuber induction, which took place after a sufficient vegetative development (8 weeks).

Effect of jasmonic acid and temperature

There was a significant effect in both concentration of Jasmonic acid (2 mM and 5 mM) on the weight and length of microtubers in comparison with the control ($p \leq 0.01$) (Figures 3 and 4). Regardless of culture medium effect, JA promoted the weight of *B. persicum* microtubers under both 15 and 20°C temperature when used at concentrations of 2 and 5 mM (Figure 3). This effect of JA was prominent in microtubers length (Figure 4). A more complete effect of JA occurred when samples were exposed to 5 mM and JA. The response of microtubers to 2 mM JA and 20°C were significantly lower than the other two levels of JA ($p \leq 0.01$). This might be due to pleiotropic effects associated with the result of external addition of Jasmonates, with induction or inhibition of physiological and biochemical processes in specific

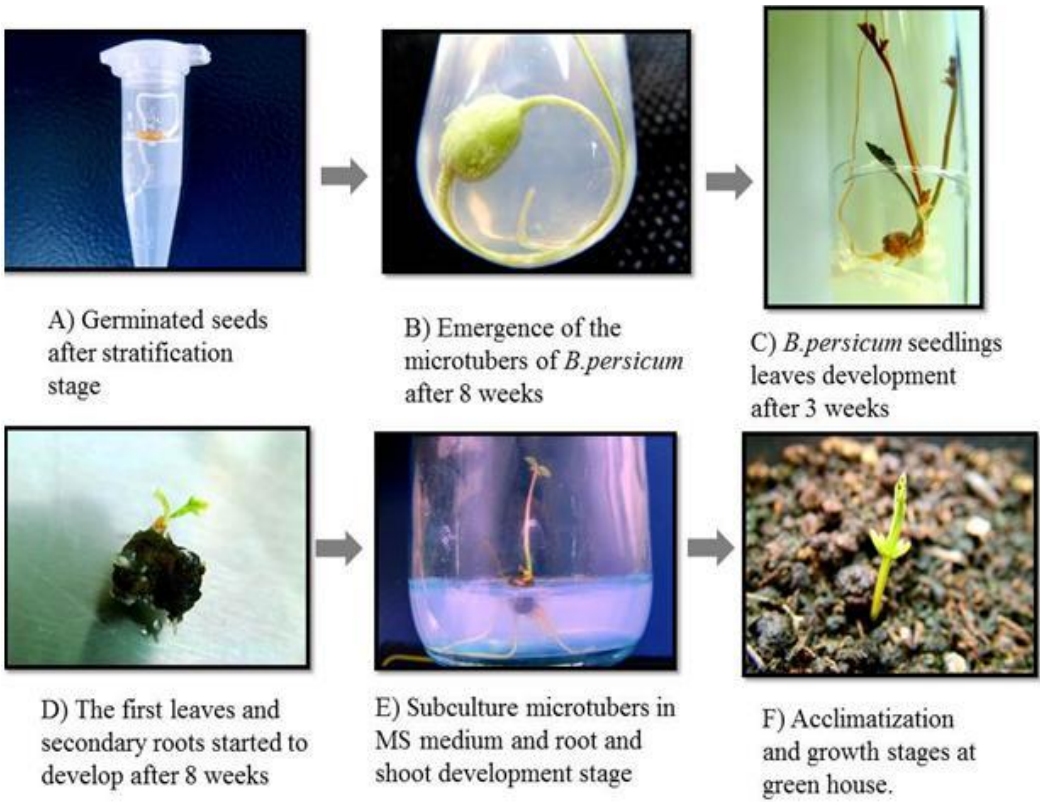

Figure 2. *In vitro* microtuberization, vernalization and acclimatization stages of *B. persicum* (A-f).

Table 1. Summary of the three-way analysis of variance for dry weight and length of *B. persicum* microtubers under three levels of Jasmonic acid (JA), three levels of medium (Med) and two levels of temperature (Temp).

Variable source	Degree of freedom (DF)	Dry weight of microtuber (mg)		Length of microtuber (mm)	
		Means of square	*p*-level	Means of square	*p*-level
Replicate	2	1986.71	<0.0001**	45.45	<0.0001**
JA	2	32368.12	<0.0001**	117.85	<0.0001**
Med	2	23618.74	<0.0001**	34.04	<0.0001**
Temp	1	7302.94	<0.0001**	19.03	<0.0001**
JA*Med	4	2119.99	<0.0001**	1.44	0.0013**
JA*Temp	2	880.19	<0.0001**	0.68	0.0828^{ns}
Med*Temp	2	266.81	<0.0001**	0.47	0.1725^{ns}
JA*Med*Temp	4	215.15	<0.0001**	1.12	0.0056**
Model	19	7099.19	<0.0001**	22.44	
Error	34	10.41		0.25	
Corrected total	53				
Coefficient variance (CV)			1.84		5.67
R^2			0.99		0.98

**- significant at 0.01 level of probability, ^{ns}- non-significant.

organs of a plant or in the whole plant. Previous studies demonstrated that JA induced tuber formation (Zel et al., 1997; Pelacho et al., 1999; Jasik and Mantell, 2000; Cenzanoa et al., 2007). Result of this study shows that JA supported microtubers development in all medium combination tests. However, JA could not influence the microtubers weight ratio as much as weight (Data not shown). It is also believed that this particular manner of

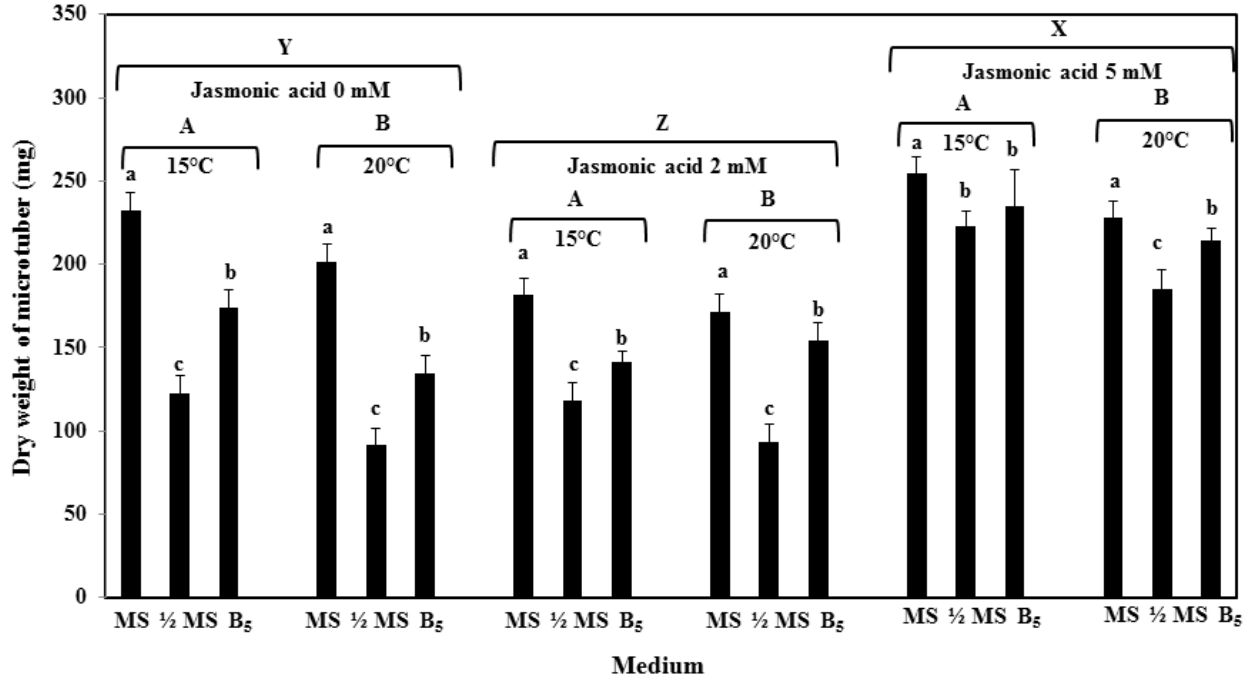

Figure 3. The effect of different mediums (MS, ½MS and B_5), different temperatures (15°C and 20°C) and Jasmonic acid on *B. persicum* microtuber dry weight.. Error bars indicate standard deviation. Small letters (a, b and c) represent significant differences among different mediums. Capital letters (A, B and C) represent significant differences between different temperatures. Capital letters (X, Y and Z) represents significant differences among different concentrations of Jasmonic acid.

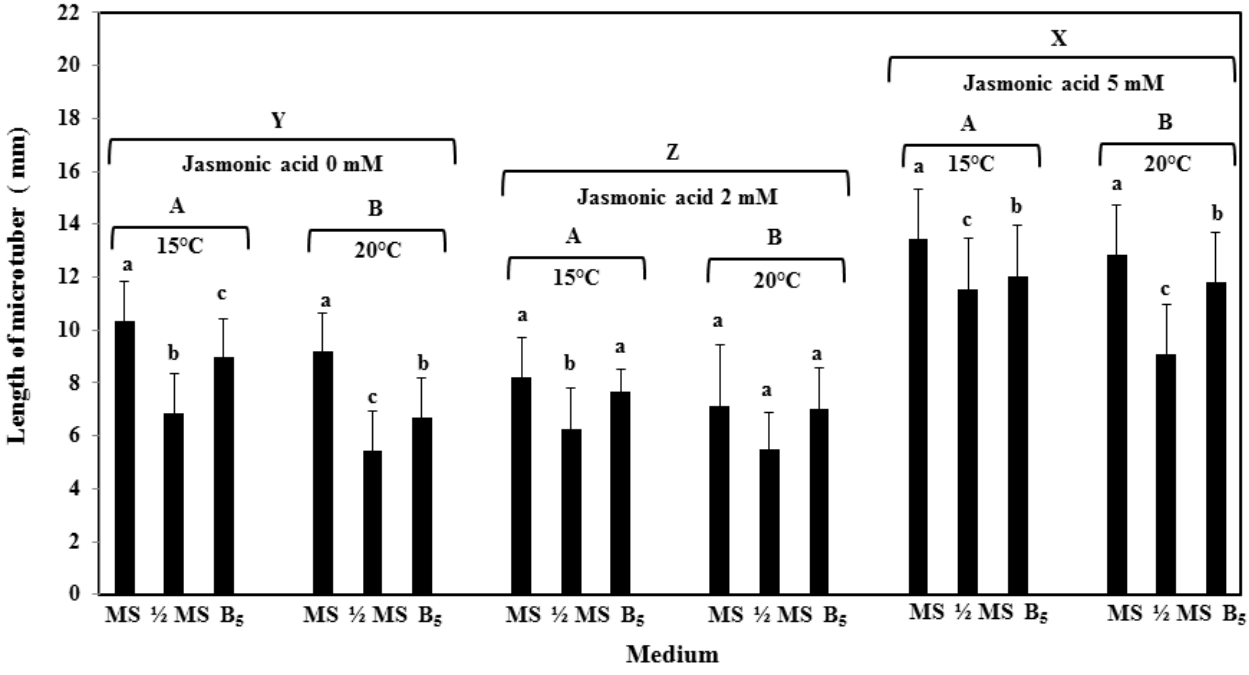

Figure 4. The effect of different mediums (MS, ½MS and B_5), different temperatures (15°C and 20°C) and Jasmonic acid on *B. persicum* microtuber length. Error bars indicate standard deviation. Small letters (a, b and c) represent significant differences among different mediums. Capital letters (A, B and C) represent significant differences between different temperatures. Capital letters (X, Y and Z) represents significant differences among different concentrations of Jasmonic acid.

the JA was greater when *B. persicum* microtubers were grown in 15°C. JA and related compounds play an important role in many morphogenetic events in plants, such as tuberization, flowering, bulb and tuberous root formation and others. All these areas have been extensively reviewed (Castro et al., 1999; Jasik and Mantell, 2000). Moreover, some other reports have suggested that exogenously applied JA affected the formation of storage organs like bulbs in garlic (Ravnikar et al., 1993). In some cases, Jasmonic acid and its related compounds act in an inhibitory manner (Sembdner and Parthier, 1993). Methyl Jasmonates inhibited tubrization when added as vapour, but stimulated this process if added to the medium (Jasik and Mantell, 2000). It seems that JA could stimulate microtuber induction in *B. persicum* specifically when added in lower concentration. It might be because JA increases the young cell expansion in potato buds. Increasing the microtuber weight can be the result of cell expansion in *B. persicum* microtubers. JA increases the tuberonic acid and its glucoside (TAG) in plant which are signals for tuberization (Castro et al., 1999).

Effect of medium and temperature

Basically, it is necessary to optimize the best combination of medium and temperature to develop a beneficial protocol for a particular species. In the present experiment, MS medium could significantly increase the length and weight of microtubers in comparison to the other mediums (½MS and B_5) ($p \leq 0.01$) (Figures 3 and 4). In contrast, ½MS implied the lowest effect on microtubers final dry weight and length of *B. persicum*. Moreover, combination of MS medium and lower temperature seemed to be more effective on growth of microtubers. However, it was found under the conditions employed that MS medium and 15°C is suitable for *in vitro* tuberization and maintenance of *B. persicum*. Several reports show that MS medium can be a good option for microtuberization. Valizadeh et al. (2007) revealed that MS medium was suitable for callus induction of *B. persicum.* Karam and Al-Majathoub (2000) found that MS medium was a satisfactorybasic medium for *invitro* tuber cultures of *Cyclamen persicum*. MS medium contains more nutrients and salts in comparison with the ½MS and possibly it can influence the weight ratio and weight of microtubers. Yamaner and Erdag (2008) also showed that combination of MS and ½MS with 24°C is the best condition for tuber development of *Cyclamen persicum*. It has been reported that the optimum temperature for developing the microtubers of *B. persicum* in the natural habitat is 15°C (Omidbaigi, 2012). Temperature can influence rooting by interfering with nutrient uptake, metabolism and its control, mainly in temperate climates. This environmental factor can influence the plant life cycle in a particular year. Regarding the result of the present study, it looks that higher nutrients (MS) and lower temperatures stimulate (15°C) the development of *B. persicum* microtubers. The effect of the lower temperature might be due to the different origin of the seeds. Apparently, MS medium contains main nutrients necessary for predevelopment of the microtubers of *B. persicum*. The performance of MS medium and 15°C showed the best result on microtubers of *B. persicum*.

Effect of medium, temperature and jasmonic acid

The ½MS induced the lower weight and length in combination with 2 mM JA. This effect of the ½MS basal medium was significant when the microtubers were kept on 20°C (Figures 3 and 4). MS medium combined with 5 mM JA and 15°C demonstrated the greatest effect on stimulating the weight increase on the microtubers. This result is in agreement with the result of Jasik and Mantell (2000) who showed that JA and its compound were able to improve microtuber weight but when *Dioscorea alata* were planted in the medium supplemented with low concentrations of salt and sucrose. This result might be due to the role of JA in uptake of sucrose and nutrients in the medium (Jasik and Mantell, 2000). However, the main storage compounds of the *B. persicum* tubers are unknown. Moreover, this manner of the JA can be greater when added in the lower temperature. In order to improve microtuberization in *B. persicum* in different solid medium, the effect of temperature and JA were tested. According to the present result, JA, temperature and medium combination increased the weight and length of microtubers significantly ($p \leq 0.05$) (Figures 3 and 4). Results show that when microtubers were grown to MS solid medium containing 5 mM JA and kept in 15°C the average weight of microtubers increased up to 250 mg. As the Figure 3 depicts, in general MS medium influenced microtubers weights more than other medium (½MS and B_5) when used in combination with other treatments (JA and temperature). Having analyzed the data, it was found that combination of MS medium, 15°C and 5 mM JA seems to be beneficial for the *B. persicum* microtuber development. In addition, the lowest length of microtubers was obtained with B_5, 20°C and in the absence of JA (Figure 2).

Microtuber acclimatization and vernalization

Microtubers started to grow and the first true leaf emerged 8 weeks after chilling periods. At first, a small green leaf started to emerge followed by very small with secondary roots. In this stage, germinated microtubers were transported to MS basal medium. Results show that 16 (80%) transported microtubers germinated 8 weeks after chilling periods. Based on the result of this study, it seemed that a minimum period of 8 weeks (1344 h) was necessary for vernalization of *B. persicum* microtubers.

Vernalization is a substantial stage for many plant species including many species in Apiaceae family (Alessandro and Galmarini, 2007). However, there is no report about the cold period necessary for verbalization of *B. persicum* tubers. The stage of growth when seedlings are not responsive to low-temperature vernalization is known as juvenility (Alessandro and Galmarini, 2007). In the case of carrot, this condition usually ended when carrot plants had initiated 8 to 12 leaves, and storage roots were larger than 4 to 8 mm in diameter (Atherton et al., 1990). The juvenility stage of *B. persicum* has not been well known and needs further investigation. Acclimatization is an important stage for plant establishment of plant *in vitro* propagation. The development of optimization process in one or more stages of plant micro propagation is of fundamental importance to increase the competitiveness of micro propagated plantlets produced in commercial laboratories (Cardosoa et al., 2013). In this study, it was found that regardless of the effect of the different medium on microtubrisation of *B. persicum* during laboratory stages, 20% of microtubers adapted to the greenhouse condition and started to develop new leaves after 1 week.

To sum it up, *in vitro* microtuber production is very beneficial to propagate and store valuable plants stock and may be adaptable for automated commercial propagation and large scale mechanized field planting. However, there is no particular report on optimization, a medium for development of *B. persicum* microtubers. In the present study three different culture media MS, ½MS and B5 along with different concentrations of JA were used individually as basal media and also in combination with two different temperatures (15 and 20°C) to develop appropriate media for microtubrization of *B. persicum*. The use of JA in a concentration of 5 mM could enhance the *B. persicum* microtubers development. MS medium and JA level of 5 mM seem to be satisfactory for *in vitro* culture establishment and short-term maintenance of tested *B. persicum.* However, more researches are needed to develop a protocol suitable for commercial micro propagation, specifically in plant establishment and acclimatization of *B. persicum.*

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Conflict of interests

The author(s) did not declare any conflict of interest.

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