Indian J. Genet. Plant Breed., (2023); 83(2): 265-269
https://doi.org/10.31742/ISGPB.83.2.13
ISSN: 0975-6906
www.isgpb.org

RESEARCH ARTICLE

In-vitro micro-tuberization in *Bunium persicum* with different media and sucrose concentrations

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Abstract

Bunium persicum (Boiss.) B. Fedtsch. called Blackzira, is one of the economically important members of the Apiaceae family with a tuberous root. Its production is restricted by seed dormancy and long juvenile time. The present study was conducted to determine the best media among MS, WPM, B5, DCR and the optimum concentration of sucrose for *in vitro* micro-tuberization of Black Zira. The results showed the highest mean of the root length (4.5196 cm), tuber length (1.175 cm), tuber width (0.3575 cm), tuber diameter (4.1 mm), and tuber weight (0.1472 g) which was achieved in the WPM medium. The 6% sucrose concentration showed the best micro-tuber diameter (7.675 mm), and weight (0.2687 g). Therefore, the WPM was recognized as the best medium for Balck Zira's micro-tuberization. The newly developed protocol might be an efficient *in vitro* propagation method for tuber induction in order to accelerate Black Zira's mass propagation. It can also be the first step of artificial seeds production in the breeding program of *B. persicum*, a long-life cycle medicinal plant that is hard to propagate.

Keywords: Black zira, micro-tuberization, sucrose concentration, tissue culture, WPM medium

Introduction

Black Zira (*Bunium persicum* (Boiss.) B. Fedtsch.) is a valuable aromatic medicinal herb that belongs to the family of Apiaceae (Bansal et al. 2023). *B. persicum* is an endemic plant to eastern Iran that grows in the North of Khorasan and Kerman provinces, East of the Zagros, and South of the Alborz mountains range in Iran (Valizadeh and Kazemi 2009).This plant is known by its different names as black caraway, Persian cumin, great pignut, Shah zira, kala zeera, Carum carvi, Zire Kuhi, Jira, wild caraway and wild cumin throughout the world (Gani et al. 2020) including Indian states of Jammu and Kashmir and hills of Utharakhand and Uttar Pradesh. In nature, seeds germinate in 3 to 4 months after passing across the winter and accordingly create just a few numbers of leaves and a small tuber. The growth rate of this plant during the first year of cultivation is extremely slow as the tuber diameter is about 4 mm. The reproductive phase of *B. persicum* starts after 4 years and continues up 12 years as the tuber continues its growth. Such a long-life cycle seriously impedes the commercial cultivation of this species. This plant is critically endangered of extinction in natural habitats due to genetic erosion, pasture degradation, and early harvest. Two key challenges facing the economic plantation of *B. persicum* are seed dormancy and long juvenile time. There are different approaches for the germination of dormant seeds (Emamipoor and Maziah 2014). In Black Zira and some other species of the Apiaceae family the cold stratification requirement for dormancy elimination has already been beheld (Singh and Kumar 2021). Generally, crop plants are propagated through seeds and vegetative parts. In several crops the associations of fungal contamination with seeds are responsible for lower germination rate. There are several physiological factors which also hinder seed germination and vegetative propagation. Various unconventional cultivation practices, heavy biotic pressure, overexploitation, deforestation,

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How to cite this article: Ouzhand B., Pourseyedi S., Mohayeji M. and Abdolshahi R. 2023. In-vitro micro-tuberization in Bunium persicum with different media and sucrose concentrations. Indian J. Genet. Plant Breed., **83**(2): 265-269.

Source of support: Nil

Conflict of interest: None.

Received: Sept. 2022 **Revised:** March 2023 **Accepted:** April 2023

climate change, global warming and trade have resulted in the depletion of different populations from forested (Singh et al. 2022) as well as nonforested areas, which has pushed the various genera and species to the endangered level and are on the verge of extinction (Jitsopakul et al. 2023).

Therefore, the tissue culture could be considered as a suitable rapid mass-production tool to preserve its genetic resources (Singh and Kumar 2021). Several reports existed for genes involved in seed dormancy in plants (Graeber et al. 2012). So far, there is no report for the genetic background of prolonged dormancy in Black Zira. There are different approaches for the germination of dormant seeds such as chilling, KNO3, GA3, etc. In Black Zira and some other species of the Apiaceae family, the cold stratification requirement for dormancy elimination has already been carried out earlier. After seeds germination of Black Zira, just a few numbers of leaves and a small tuber are formed followed by a long juvenile time. This plant's long juvenile time depends on the slow growth rate of tubers (Mardani et al. 2015). The size and shape of the micro-tuber are affected by various factors such as sucrose level, growth regulators, light duration, etc. Sucrose has been reported to play the main role in the induction of tuberous root formation (Nazir et al. 2022).

The objective of the present study was to survey the effect of different cold stratification periods on the deep dormancy breaking and assessment of different media effects on micro-tuberization and related traits in *B. persicum*. A study on evaluation of the effects of sucrose different concentrations and on the growth of the *B. persicum* micro-tubers was also carried out.

Materials and methods

Seed sterilization method and breaking dormancy

Seeds of *B. persicum* were collected from the mountains of Kerman Province, Iran. Afterward, seeds were sterilized with 10 times pre-washed using tap water and three times washed with sterile distilled water, respectively. Then, they were surface sterilized by 1.5% (w/v) sodium hypochlorite solution for 20 minutes in laminar airflow cabinet. For removing hypochlorite residue, three times shaking was done in sterile distilled water at intervals of 10 min. They were soaked in 70% (v/v) ethanol for 1.5 minutes and then washed with distilled water 3 times and were cultured in petri dishes containing agar (8 g/L) and sucrose (5 g/L). All petri dishes were kept at 4°C in the dark for dormancy breaking. After seeds germination, the 4 mm of root-length seeds were selected for further studies.

Preparation of different media

Different basal media with 3% sucrose were used for micro-tuber induction, including Murashige and Skoog (MS), Gamborgi's B5 (B5), Woody Plant Medium (WPM) and Gupta and Durzan's DCR (DCR) plantlet formation and micro-tuberization. To evaluate micro-tubers' growth rate, the best media was used with sucrose at varying concentrations (3, 6, 9, 10 and 12%) in dark and light duration. Vitamins were added to all media in different concentrations. pH was adjusted to 5.8 ± 0.2. Autoclaving was done at 121°C for 15 minutes and germinated seeds were planted in a glass containing 50 mL of each medium. After 2 weeks of incubation at 25°C in the growth chamber, and the light intensity of 42 (µmoles/m^2/s) and a photo phase of 16 hours of light per day, plantlets were achieved.

Data collection and analysis

After 6 weeks, root length and shoot length of plantlet and length, width, diameter, and weight of every single micro-tuber were recorded. The experiment was conducted in a randomized complete block design with four treatments and 8 replications per treatment. For sucrose different concentrations (3, 6, 9, and 12%), the split-plot based on an RCBD design was performed with 8 replications as well. Analysis of Variance (ANOVA) was performed by SPSS software. Means comparisons were conducted with Duncan's Multiple Range test and Excel software was applied to draw all the charts.

Results and discussion

In the present investigation, seed germination under cold dark conditions (4°C) was started after 4 weeks on 0.5% sucrose and 0.8% agar medium (Fig. 1). Seed germination percentage increased with increment of the stratification time and reached to 100% in agar + sucrose medium at 8 weeks and (Fig. 2). Banyal and Sharma (2020) stated that seed germination started after 75 days of continuous chilling

Fig. 1. Germination percentage of *B. persicum* seeds over eight weeks

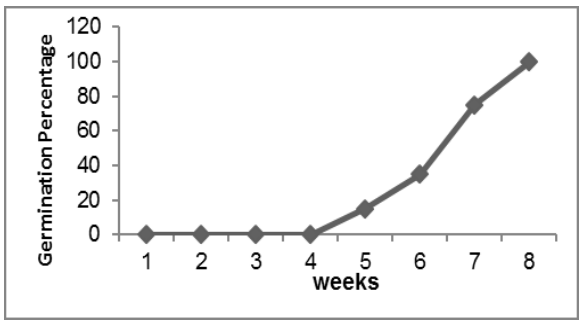

Fig. 2. Percentage germination of seeds *B. persicum* in simple culture medium

Table 1. ANOVA table of growth traits of *B .persicum* plantlet

Source of variation	df	Mean Square					
		Shoot length	root length	Tuber length	Tuber width	Tuber diameter	Tuber weight
Replication	7	0.348^{ns}	0.350^{ns}	0.026^{ns}	0.003^{ns}	0.033^{ns}	0.000397^{ns}
treatments	3	2.411^{ns}	2.158*	0.542**	0.043**	5.232**	0.016666**
Error	21	1.596	0.521	0.013	0.002	0.054	0.000427

*Significant at P = 0.05; **Significant at P=0.01; ns: Non significant

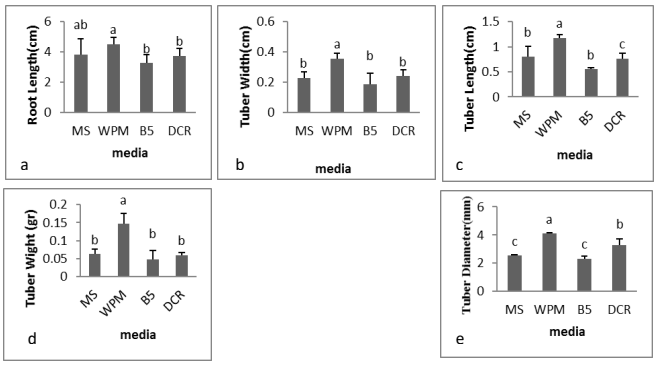

Fig. 3. Mean comparison of WPM, DCR, MS, and B5 media for studied characteristics

treatment at 4 ºC and increased over the time. It seems that the increment in time of cold led to the increment in the germination percentage.

After transferring the germinated seeds to different culture media (MS, B5, WPM and, DCR), cotyledon leaves were formed in most samples within one week. During next two weeks the initial differences were palpable between the studied media. The present findings support previous studies conducted by Bagheri et al. (2014) on *B. persicum* germinated seeds.

Data analysis for the traits in different culture media (MS, B5, WPM and, DCR) indicated that there was a significant difference between all treatments except shoot length (Table 1). Ab Rahman et al. (2018) stated that the semi-solid WPM medium was most effective in shoot initiation and shoot proliferation for micropropagation of *Lepisanthes Fruticosa* as well. Statistical analysis showed that the effect of culture medium on root length was significant at level 5% (Table 1). the comparison of the means showed that the highest mean was observed in the WPM medium (4.52 cm) while the lowest mean was obtained from B5 medium (3.26 cm) (Fig. 3a).

The comparisons of mean for micro-tuber width divided the treatments into two groups. The first group included WPM and DCR media and the second group included DCR, MS and, B5 media. The highest and lowest micro-tuber width average was achieved from the WPM media (0.36 cm) and B5 medium (0.19 cm), respectively (Fig. 3b). Mean comparisons for micro-tuber length also divided the treatments into two groups in which WPM medium has the highest amount (1.18cm) and was significantly separated from the other media. Therefore, it had the greatest effect on the length and width of the micro-tubers (Fig. 3c).

The most effective media for the weight of micro-tuber was WPM media (0.15 g), while B5 media (0.05 g) had a minimum effect on the weight of micro-tuber (Fig. 3d). The results of micro-tubers diameter measurement showed that the WPM culture medium had the highest micro-tubers diameter (4.10 mm) while, B5 medium had the lowest (2.32 mm) amount (Fig. 3e). The results of Purohit et al. (2020) investigation showed that the WPM medium was the best growth medium for the root and shoot initiation for in vitro propagation. The positive effect of the WPM culture medium can be as a result of the different nutrients in this culture medium compared to the other media in this work (Fig. 4). The germination, effect of media, different concentration of sucrose and tuber induction processes are summarized in Fig. 5.

The nitrate to ammonium ratio in B5 and DCR media was about 18.66 and 3.24 times higher than WPM and MS. Moreover, the composition of ammonium in WPM, MS and, DCR media is NH_4NO_3, while its composition in B5 medium is $(NH_4)_2SO_4$. In WPM medium compared to B5, DCR and, MS media, potassium sulfate is replaced with potassium nitrate. The amount of nitrate in the WPM medium is lower than other media (Table 2). It was found that WPM was a favorable medium for rooting which may be the result of lower concentrations of minerals in WPM, especially nitrogen (Tian et al. 2008).

Calcium may directly impact cell and organ growth, cell elongation and division, plant cells signaling, and tuber

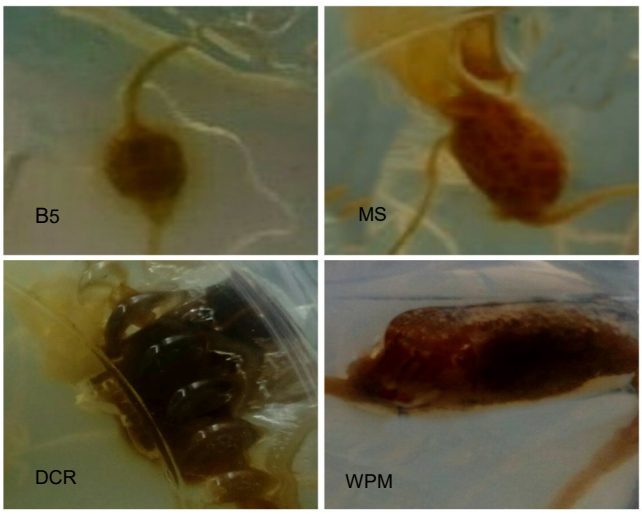

Fig. 4. Comparison of tuber growth in B5, MS, DCR, and WPM

Table 2. The amount of macroelements, microelements, vitamins and, sucrose (mgL-1) in the studied culture media

Composition	MS	WPM	B5	DCR
NH4NO3	1650	400	-	400
KNO3	1900	-	2500	340
Ca(NO3)2.4H20	-	556	-	556
CaCl2.2H2O	440	96	150	85
K2SO4	-	990	-	-
MgSO4.7H20	370	370	250	370
(NH4)2SO4	-	-	134	-
NaH2PO4.H2O	-	-	150	-
KH2PO4	170	170	-	170
MnSO4.4H20	22.3	22.3	-	22.3
MnSO4.H20	22.3	-	-	22.3
Na2MoO4.2H20	0.25	0.25	0.25	0.25
ZnSO4.7H2O	8.6	8.6	2	8.6
Zn(NO3).6H2O	-	-	-	-
KI	0.83	-	0.75	0.83
H3BO3	6.2	6.2	3	6.2
CuSO4.5H20	0.025	0.25	0.025	0.25
CoCl.6H20	0.025	-	0.025	0.03
NiCl2.6H2O	-	-	-	0.03
FeSO4.7H2O	27.8	27.8	27.8	27.8
Na2EDTA.2H20	37.3	37.3	37.3	37.3
Myo-inositol	100	100	100	100
Thiamin-HCL	0.1	1	10	1.6
Nicotinic acid	0.5	0.5	1	0.5
Pyridoxine-HCL	0.5	0.5	1	0.5
Glycine	2	2	-	2
Sucrose	30000	30000	30000	30000

formation (Hirschi 2004). The amount of calcium in WPM is higher than in other media. Therefore, rapid formation and larger micro-tuberization may cause a higher amount of calcium in WPM compared with other media. According to Balamani et al. (1986) calcium could recover the tuberization response in Potato. WPM also has the higher amount of sulfate than in other media. Increasing the amount of sulfate improves the morphological features and result in the yield increment. results of Davara Monali et al. (2019) on coriander indicated that the application of zinc sulfate significantly increased number of branches per plant, number of umbels per plant, number of umbellate per umbel, number of seed per umbellate, and 1000-seed test weight.

Zn, Mn, P, Mg, Na, B and, Cu micronutrients are similar in WPM, DCR, and MS media but lower in the B5 medium. These micronutrients are necessary for growth and play a major role in the plants biomass production. while the deficiency of the minerals may reduce rooting (Haider et al. 2018).

Data analysis for different concentrations of sucrose and

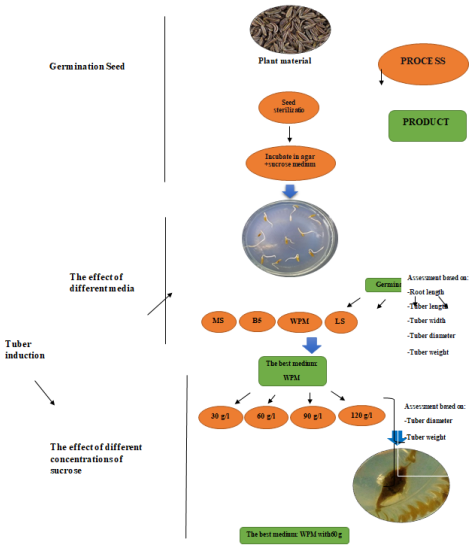

Fig. 5. Schematic diagram showing the germination, effect of media, different concentrations of sucrose and tuber induction processes

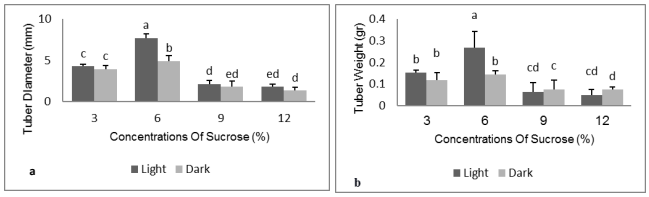

Fig. 6. Effects of different sucrose concentrations on diameter (a) and weight (b) of micro-tubers in light and dark condition

photoperiod on diameter and weight of micro-tuber after five weeks indicated that there was a significant difference between all treatments at 1% level (Table 3). The means comparisons indicated that the maximum diameter of micro-tuber (7.68 mm) was produced in a concentration 6% of sucrose in 16 hrs light condition. However, the minimum diameter (1.78 mm) of micro-tubers was achieved from plants treated with concentrations 9 and 12% of sucrose in dark conditions (Fig. 6a). The highest weight of micro-tubers obtained in 6% concentration of sucrose (0.26875 gr) in 16 h light condition, while 12% concentration of sucrose in dark conditions had lowest (0.04875gr) amount (Fig 6b). The average diameter and weight of micro-tuber were increased at concentrations 3% and 6% of sucrose, however, a decrease happened in 9% and 12% concentrations. The reduction in micro-tuber formation at 9% and 12% of sucrose might be because of the effect of undesirable osmotic condition for water observation that have deleterious effects on the micro-tuber formation of seedlings. The present results were in agreement with several researches (Aslam et al. 2011; Imani et al. 2010). Fufa and Diro (2013) Stated that sucrose lower concentrations might has a positive effect on the micro-tuberization, while at higher concentrations negative effect on it.

MS generally is a practical and common medium for plants propagation, but the results of this study showed

Table 3. ANOVA table of sucrose concentrations on micro-tuber of *B. persicum* plantlet

Source of variation	df	Mean square	
		Tuber diameter	Tuber weight
Replications	7	0.496535*	0.002781*
Light	1	14.69764**	0.031207**
Light ×replication	7	0.102782ns	0.00073ns
Ea	7	0.216746	0.00073
treatment	3	77.15157**	0.091328**
treat × Light	3	5.721931**	0.013582**
Eb	42	0.216746	0.001546

*Significant at a = 0.05, **Significant=0.01, ns not significant

that the WPM and then DCR media were the best culture media for *B. persicum* micro-tuberization. Therefore, this newly developed protocol might be an efficient in vitro propagation method for tuber induction to accelerate Black Zira's mass propagation. It can also be the first step of artificial seeds production in the breeding program of *B. persicum*, a long-life cycle medicinal plant that is hard to propagate.

Authors' contribution

Conceptualization of research (BO, ShP); Designing of the experiments (BO, MM); Contribution of experimental materials (ShP, RA); Execution of field/lab experiments and data collection (BO, MM); Analysis of data and interpretation (MM, RA); Preparation of the manuscript (BO, MM).

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