

Dormancy breaking, indirect somatic embryogenesis, and encapsulation of somatic embryos in Black Zira

Behnaz Ouzhand

Shahid Bahonar University of Kerman

Mehdi Mohayeji (✉ mohayeji@uk.ac.ir)

Shahid Bahonar University of Kerman

Shahram Pourseyedi

Shahid Bahonar University of Kerman

Roohollah Abdolshahi

Shahid Bahonar University of Kerman

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Abstract

Black Zira (*Bunium persicum*) is an important endemic medicinal plant in Iran. This research was conducted out to assay breaking seed dormancy, develop new protocols for callus induction, somatic embryogenesis, and artificial seed. Seeds were treated with ultrasonic waves in different periods (0, 1, 3, 5, 7, and 9 min). Then, two experiments with IBA (0, 1, 2, 3, and 4 mg L⁻¹) and a combination of different levels of 2,4-D (0, 1, 2, and 4 mg/l) and BAP (0, 0.5, 1, and 2 mg/l) were designed. Different concentrations of sodium alginate (2, 3, and 4%) and CaCl₂ (50, 75, and 100 mM) were prepared for artificial seed production. According to the results, the 7 min ultrasonic waves had the maximum effect on germination (81.87%), while the highest negative effect on germination (2.5%) was reported from 9 min exposure time. In the IBA experiment, the highest percentages of callogenesis, callus weight, and adventitious root were obtained from MS medium containing 4 mg/l IBA. Moreover, 1 mg/l IBA resulted in the highest embryogenesis percentage. In the second experiment, the highest percentage of callus formation and callus weight were achieved in 1 mg/l BAP + 4 mg/l 2,4-D, 0.5 mg/l BAP + 4 mg/l 2,4-D, and 2 mg/l 2,4-D media. The maximum embryogenesis percentage was seen in 0.5 mg/l BAP + 1 mg/l 2,4-D, 0.5 mg/l BAP + 2mg/l 2,4-D, and 1 mg/l 2,4-D media. The highest germination rate (77.77%) was obtained from encapsulated embryos with 3% sodium alginate and 100 mM CaCl₂.

Key Message

A protocol with improved results has been developed for indirect somatic embryogenesis and, artificial seed production in *B.persicum* using hypocotyl explants.

Introduction

Bunium persicum (Boiss.) B. Fedtsch, Black Zira (Zireh Kuhi, Zireh-e-Irani) which is originated from Europe and Western Asia, is a perennial aromatic plant. This plant is a valuable endangered herb that is of great economic importance. *B. persicum* is also known as "Persian Cumin", "Shah Zira", "Jira", "Zire kuhi", "Carum carvi", "Black caraway", "Great pignut", "Kala Zeera", "wild cumin" and "Wild caraway" throughout the world (Bansal et al. 2021). Black Zira is an important medicinal plant in the food and pharmacology industries because of various chemical compounds such as phenolic acids, flavonoids, and aldehydes, as well as a high content of mono-terpenes and sesquiterpenes existed in essential oil (Hassanzad Azar et al. 2018). *B. persicum* has the properties of restorative, diuretic, stimulants, fracture treatment, gastric ulcer treatment, fat reduction, anti-allergy, and carminatives; and proved very useful in treating diarrhea, dyspepsia, ureteric disorders, convulsion, and asthma (Shah et al. 2019).

B. persicum propagates naturally through seeds, where seed germination usually takes 4–6 months. Three to four-year juvenile period is required to develop into Black Zira tuber and yield. The long life cycle of this plant prevents commercial cultivation of this plant (Banyal and Sharma 2020). Thus, the plant is critically endangered in natural habitats due to genetic erosion, pasture degradation, and early harvest. One of the limitations of Black Zira cultivation is seed dormancy and exposure to various stresses, which

wilting disease is the most common (Majidi et al. 2020). Therefore, it is necessary to create a proper regeneration system for the mass production of Black Zira. There are different methods for breaking dormancy and seeds germination in plants. Among the treatments of cytokinin, gibberellic acid, light, and potassium nitrate, only cold temperature treatment was effective for breaking Black Zira seeds dormancy (Sharifi and Pouresmael 2006). In addition, ultrasonic waves as a new physical method is suggested for breaking the seed dormancy that involves the application of sound frequencies in the range of 20 to 100 kHz for interaction with materials. This method seems to be nondestructive, inexpensive, safe, and easy compared to other methods. The use of high-energy waves (more than 100 kHz) may have destructive effects such as the demolition of cell membranes and important molecules including enzymes and DNA, while low-intensity waves (20–100 kHz) may have useful effects on increasing and stimulating seed germination (Babaei-Ghaghelestany et al. 2020). Yaldagard et al. (2008) reported effective results of ultrasonic treatment on accelerating and enhancing the germination of barley seed.

Tissue culture is an excellent conservation method, especially in endangered medicinal plants. Also, the vital purpose of tissue culture is shortening breeding programs period as well as producing disease-free plants (Tripathi et al. 2021). Regeneration through somatic embryogenesis is an important way to overcome the restriction of seed availability and shortening long sexual cycle. The somatic embryogenesis protocols were developed based on the use of basic media supplemented with auxins and cytokinins (Hazubska-Przybył et al. 2020). For the first time, callus culture was reported on *Daucus carota* L. (carrot) in the Apiaceae (Hunault 1984). This phenomenon has been observed to be similar to other Apiaceae members such as fennel (*Foeniculum vulgare* Mill.) and celery (*Apium graveolens* L.) (Saranga and Janick 1991). Numerous studies have been done on plant regeneration through indirect embryogenesis on many plant species especially *B. persicum* (Sobańska et al. 2023; Wakhlu et al. 1990). According to Khajuria et al. (2021), MS medium with 0.15 mg/l 2,4-D + 0.05 mg/l Kin had the highest number of somatic embryo in *Viola canescens* Wall.

Nowadays, somatic embryos are mainly used to produce artificial seeds (synseeds). For the first time, Murashige (1977) defined artificial seeds as encapsulated single somatic embryos. It was later described by Gray et al. (1991) as a somatic embryo engineered for practical use in the commercial plants production. Artificial seeds production has been studied in *Daucus carota*, *Artemisia vulgaris* L, *Pinus patula*, *Quercus robur*, *Vitis vinifera*, *Swertia chirayita*, and many other plants (Gantait et al. 2015). Artificial seed production is also a laboratory technique that can be used to protect the germplasm of endangered species. In 1986, Redenbaugh et al. (1986) discovered that a gelatinous material called sodium alginate was appropriate for producing artificial seeds containing somatic embryos. The use of sodium alginate for the production of artificial seeds has advantages including non-toxicity and independence from heat requirement for gelation process. During artificial seeds production with sodium alginate, for the hardening process, somatic embryos are mixed with the calcium chloride solution (50 to 300 mM). Hatzilazarou et al. (2021) stated that explant coating in 2.5% sodium alginate in combination with 100 mM of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ led to the production of firm beads of uniform globular shape, proper for

handling, while hardening in 50 mM of calcium chloride formed soft beads. Also, adding MS culture medium to sodium alginate increases the subsequent germination rate of beads.

The present study aimed to investigate the effect of ultrasonic waves on the dormancy breaking of *B. persicum* seeds and develop a protocol for somatic indirect embryogenesis. Furthermore, in the present study, we optimized the artificial seed production in Black Zira.

Material And Methods

This experiment was carried out in the tissue culture laboratory of the Agriculture Faculty, Shahid Bahonar University of Kerman, in 2022. Mature seeds of *B. persicum* were collected from Kerman Mountains and was used as explants for somatic embryogenesis. This research was conducted in three steps.

In Vitro Seeds Germination Using Ultrasonic Waves

In the first step, the seeds are thoroughly washed and transferred to the laminar airflow cabinet for further disinfection. Sodium hypochlorite solution (1.5% (w/v)) containing a few drops of Tween was used to sterilize the seeds for 20 min, then rinsed three times with sterile distilled water to remove hypochlorite residue. Subsequently, the seeds have soaked in 70% (v/v) ethanol for 1 minute and washed with distilled water three times. To break the seed dormancy, the 40 kHz ultrasonic waves produced by Digital Ultrasonic vclean1-L4 were applied to the seeds at constant temperature (24°C) and different periods (0, 1, 3, 5, 7, and 9 min). The seeds were soaked in a plastic tube containing distillate water and incubated in the ultrasonic apparatus. Afterward, the seeds were placed on filter paper for drying, and then transferred to 1/2 MS medium. They were kept at 4°C in the dark. Finally, germinated seeds were cultured on basal MS medium with full strength of macro and microelements, vitamins, and sucrose (30 g) for one week at $25 \pm 2^\circ\text{C}$ in the dark to generate two-leaf seedlings. After five weeks the percentage of seed germination of every plate was recorded.

Callogenesis And Somatic Embryogenesis Induction

In the second step of this experiment, two-leaf seedlings were used as explants to induce callogenesis, and somatic embryogenesis. Root, hypocotyl, and cotyledon leaf segments (5 mm in size) were used as explants for further experiments. To investigate the explants response to callus induction and somatic embryogenesis, two experiments were conducted in MS medium. In the first experiment, five different concentrations of IBA (0, 1, 2, 3, and 4 mg L⁻¹) were evaluated with five replications. In the second experiment, the combination of different levels of 2,4-D (0,1, 2, and 4 mg/l) and BAP (0, 0.5, 1, and 2 mg/l) were assessed in a factorial analysis with five replications. The explants were placed in each sterilized glass dish containing 30 ml of the media combined with the required dose of plant growth regulators. All media were cultured with 60 g/L sucrose, and solidified with 8 g/L agar. Media pH was adjusted to $5.8 \pm$

0.2. The media were autoclaved at 121°C for 15 min. To callus formation, the glass dishes were wrapped with a parafilm and they were incubated in a phytotron at $25 \pm 2^\circ\text{C}$ in a dark condition. To increase the number of embryogenic calli, the callus proliferation was conducted via four times of subculturing in MS medium (40 g sucrose) by 16 hours of light per day with the light intensity of $42\mu\text{moles/m}^2/\text{s}$. The explants are subcultured every 21 days. Subsequently, the globular embryos were transferred to the glass dishes containing plant growth regulations (PGRs) free MS medium (60 g sucrose) for 40 days in order to develop somatic embryos.

The percentage of explants producing callus, adventitious root and the weight of every callus after four weeks, and the percentage of somatic embryogenesis from each culture glass at the different treatments were recorded after the calli subculture.

Artificial Seed Production:

Synthetic seeds were formed using different percentage of sodium alginate (2, 3, and 4%) in MS salt solution as the gel matrix and different concentrations of calcium chloride (50, 75, and 100 mM) for complexation. Both the sodium alginate gel and CaCl_2 solution were sterilized by autoclaving at 121°C and a pressure of 1.1 kg/cm^2 for 20 minutes. After preparing the solutions, the somatic embryos were separated using a scalpel in the cotyledon stage under laminar hood airflow. The explants were then placed in 20 ml of different concentrations of sodium alginate solution using a sterile 2 mm stainless steel spoon and swirled gently into the calcium chloride solution. After that, coated embryos were kept on an orbital shaker for proper shape formation and polymerization. After 30 min, the alginate beads were collected, and rinsed with sterile distilled water to remove calcium chloride residue, and cultured on MS medium containing 1 mg/L IBA + 2 mg/L Kin medium for two weeks. Synthetic seeds were incubated at $25 \pm 2^\circ\text{C}$ with a 16-h photoperiod for germination. All the procedures were done under the laminar air flow chamber to avoid microbial contamination. Finally, to acclimatize, the grown seedlings were transferred to a pot containing a sterile peat-based substrate, surrounded by a plastic cover. This cover was gradually removed after two weeks. Seedlings were irrigated with 1/2 MS during the experiment.

The experiment was performed based on a completely randomized design with three replications using a factorial experiment. Analysis of variance (ANOVA) was performed by SPSS (IBM SPSS Statistics 26) software. The means comparisons were conducted by Duncan's Multiple Range test and Excel software was applied to prepare the charts.

Result

Effect of ultrasonic waves on germination of *B. persicum*

The results of statistical analysis of *B. persicum* seeds germination showed that there was a highly significant difference between ultrasonic waves treatments (0, 1, 3, 5, 7, and 9 min) at probability level of 1% (prob 0.000). The greatest effect on germination percentage (81.87%) was belong to the 7 min

ultrasonic. The increment of exposure time to 9 min had a highly negative effect on germination percentage (2.5%) even compared to the control (17.5%) (Fig. 1). The results indicated that ultrasonic treatments could have a positive impact on dormancy breaking in Black Zira.

Initiation, Stimulation, And Production Of Embryonic Callus

Among different explants (root, hypocotyl, and cotyledon leaf) which were taken from the two-leaf seedlings (Fig. 2), hypocotyl explants expressed the best results and were subsequently cultured at different concentrations of the PGRs to determine which medium is suitable for callus production and somatic embryogenesis. In the first experiment, IBA treatments induced callus formation after ten days. Then, the calli increased in size until the end of the fourth week. The formation of callus initiated from the wound edges of the hypocotyl explants and progressively grew to completely cover the explants. After four weeks, two types of non-embryogenic and embryogenic calli were observed according to their color and texture. The non-embryogenic type included yellowish soft calli and the embryogenic type was greenish hard ones with lower growth rate (Fig. 3). During callus formation, adventitious roots were observed as well (Fig. 4). However, no adventitious shoot was observed in this media after repetitive subcultures. The results of variance analysis for different IBA treatments showed that the callogenesis percentage, adventitious root and callus weight traits were significantly affected by IBA concentration (Table 1). The means comparison of the treatments indicated that the highest percentages of callogenesis, callus weight, and rooting traits were observed in the 4 mg/l IBA treatment while the 2 mg/l IBA treatment showed the lowest value (Fig. 5 and Fig. 6).

Table 1
ANOVA results of IBA treatments on different traits in explants of *B.persicum*

S.O.V	df	Mean Square			
		Percentage of callugenesis	Weight of callus	Adventitious root	Percentage of embryogenesis
Treatments	3	1562.500**	13.550*	2104.167**	1742.134*
Error	16	89.844	5.543	125.000	251.731
*Significant at a = 0.05, **Significant = 0.01, ns not significant					

Globular embryos were seen for the first time in the second subculture (Fig. 7.a). The globular embryo numbers increased with subcultures number increment. Afterward, the globular embryos were placed in a PGRs-free MS medium containing 60g sucrose and were differentiated into heart-shaped, torpedo and cotyledon embryos in the first and second subcultures (Fig. 8). The results of the ANOVA on the IBA treatments for the percentage of somatic embryogenesis trait showed that there are significant differences at probability level of 5% (Table 1). The treatment of 1 mg/l IBA had the highest percentage of embryogenesis. However, 3 mg/l IBA had the lowest percentage of embryogenesis.

In the second experiment with different BAP + 2,4-D treatments, callus formation started from the second week and showed a slower growth rate than the first experiment. However, no callus was seen in the 0 mg/l BAP and 0 mg/l 2,4-D treatment combination. The increment in size of calli was slowed down after four weeks. Then, calli were subcultured in the same media for embryogenesis induction. In most cases, no adventitious root was observed in any sub-cultured calli. Occasionally, adventitious shoots were generated from calli after some subcultures with low frequency and thus no significant difference was observed in the analysis of variance for this trait. There was a significant difference in the percentage of callus formation and callus weight (Table 2). The maximum percentage of callus formation and callus weight was measured in the 1 mg/l BAP + 4 mg/l 2,4-D, 0.5 mg/l BAP + 4 mg/l 2,4-D, and 2 mg/l 2,4-D media, while the lowest percentage of callus formation was seen in 0.5 mg/l BAP treatment (Fig. 9 and Fig. 10). As a result, the high frequency of callogenesis was achieved in a high auxin media. After 28 days the globular embryos were observed in the same media. The number of globular embryos increased in media containing low auxin supplemented with cytokinin (Fig. 7. b). Differentiation of globular embryos was completed in the PGRs-free MS medium (60 g sucrose) after 30 days. The ANOVA results of the BAP + 2,4-D treatments for the percentage of somatic embryogenesis trait showed that there are significant differences at level 5% (Table 2). The mean comparison of treatments BAP + 2,4-D indicated that the highest percentage of embryogenesis was observed in treatment 0.5 mg/l BAP + 1 mg/l 2,4-D, 0.5 mg/l BAP + 2 mg/l 2,4-D, and 1 mg/l 2,4-D. While the lowest percentage of embryogenesis was detected in treatment 0.5 mg/l BAP + 4 mg/l 2,4-D, 1 mg/l BAP + 4 mg/l 2,4-D, and 4 mg/l 2,4-D (Fig. 9).

Table 2

ANOVA results of BAP + 2,4-D treatments on different traits in hypocotyl explants of *B.persicum*

S.O.V	df	Mean Square			
		Percentage of callogenesis	Weight of callus	Adventitious Shoot	Percentage of embryogenesis
BAP	3	636.252*	1.258**	18.341 ^{ns}	2619.589*
2,4-D	3	4751.808**	3.530**	31.808 ^{ns}	5403.552**
BAP×2,4-D	9	2016.651**	1.050**	49.012 ^{ns}	1619.029*
Error	64	117.966	0.117	67.797	418.490
*Significant at a = 0.05, **Significant = 0.01, ns not significant					

Artificial Seed Production

The first important step in encapsulating the somatic embryos was the determination of the best concentrations of sodium alginate and calcium chloride. The seeds prepared with 2% sodium alginate were difficult to make, fragile for handling, and have no right shape which resulted in reducing the frequency of germination. Coating embryos with 4% sodium alginate made uniform and circular beads

with considerably hard shapes resulting in a delay in germination. Subsequently encapsulated somatic embryos were incubated on MS medium containing 1 mg/l IBA + 2 mg/l Kin at $25 \pm 2^\circ\text{C}$ for two weeks. Statistical analysis showed that the effect of sodium alginate and calcium chloride solution on the germination percentage of artificial seed was significant at the probability level of 5% (prob .018). According to this, the beads prepared with 3% sodium alginate solution and 100 mM CaCl_2 showed significantly higher germination percentage (77.77%) while 4% sodium alginate and 75 mM calcium chloride showed the lowest germination percentage (11.11%) in light conditions (Fig. 11 and Fig. 12).

Discussion

Seed germination is a critical period for plant establishment. Breaking dormancy and stimulating seed germination are very important for propagation of endangered plants. Recently, the application of ultrasonic waves as an easy and safe method for breaking seed dormancy has attracted the consideration of many researchers. The reasons and mechanism of how to happen remain unclear yet. Though, many researchers believe that the use of ultrasonic waves has a significant effect on seed coat permeability and water absorption would leading accelerate seed germination (Miano et al. 2016). Many articles have been published about the positive effect of ultrasound waves on seed germination. The results of a research (Nazari et al. 2014) on seed dormancy breaking by ultrasonic waves illustrated that the treatment with ultrasonic waves had a positive impact on the seed dormancy breaking. But there was no linear correlation between germination speed and time of exposure which was agree with the present experiment. The results of our experiment showed that the seed germination was significantly reduced at the highest ultrasonic exposure time. A similar study by Sharififar et al. (2015) mentioned that 5 and 7 min of ultrasonic exposure had the highest seed germination percentage, while 9 min of exposure negatively affected on germination compared to other treatments.

The results of this experiment led to a protocol for the induction of *B. persicum* embryogenesis callus and the development of somatic embryos. Bedir et al. (2022) reported that successful induction of somatic embryogenesis depends on the selection of the explant, explant tissue, explant growth stage, genotype, and appropriate combination of PGRs levels. In terms of the explant and its type, according to the results of this study, hypocotyl explants had the best results for somatic embryogenesis. Callus Induction using hypocotyl explants has been reported in many plants (Karakas 2020; Li et al. 2021). Plant propagation through somatic embryogenesis is important for obtaining a large number of plants (Mukherjee and Datta 2007). The first stage in embryogenesis is callus induction. Auxins play important role in embryogenesis by predisposing cells to enter the differentiation stage. The concentration of auxin depends on the type of explants (Mathesius 2008). Regarding the effects of PGRs, in the first experiment, the best results for callogenesis were obtained with 4 mg/l IBA. This indicated that the presence of auxin is essential for callus induction. Ling et al. (2013) stated that the highest callogenic response from leaves and stem explants was obtained in the media supplemented with IBA. Also, the results of our first experiment indicated that some adventitious roots were observed during callus formation as well. IBA hormone is used commercially for the rooting of various plant species worldwide (Hartmann et al. 1997).

There are several possibilities to explain the better performance of IBA versus IAA including higher stability, difference in metabolism, and difference in transport. Moreover, IBA is a slow-release source of IAA. In this study, the highest weight of callus was achieved from callus cultured on MS medium supplemented with 4 mg /l IBA. Also, the results of the first experiment indicated that IBA was just useful for callus induction and somatic embryogenesis, which was consistent with the results of previous reports (Cheruvathur et al. 2013; Nakhooda and Mandiri 2016). Shirali et al. (2022) reported that 1 mg/l IBA without BAP had the highest rate of organogenesis.

In the second experiment, the high frequency of callogenesis was achieved in a high auxin media which agreed with the findings of the first experiment. The Bano et al. (2022) showed that the combined 2,4-D and BAP indicated the highest callus weight in 1 mg/l 2,4-D + 1 mg/l BAP medium.. Besides, the result of the second experiment indicated that sometimes calli generated adventitious shoots as well. Several studies showed that the adventitious shoots have been seen in different concentrations of BAP + 2,4-D (Andaryani et al. 2019; Kantia and Kothari 2002). In the present study, after 28 days of culture, the globular structures emerged on the callus surface which later formed heart and torpedo stages. Iva Doycheva et al. (2022) showed that MS medium supplemented with 4.53 μ M 2,4-D + 2.27 μ M TDZ + 2.22 μ M BAP was the most suitable medium for embryogenesis of *Papaver degenii*. The results of our experiment showed that when auxin reduced or completely removed, the number of globular embryos were rised. Therefore, the low concentration of auxin and cytokinin in the media led to an increment of globular embryos. After embryogenesis induction, the role of auxin would change and embryos start to synthesize their own auxin (Choudhary et al. 2009; Pérez-Pérez et al. 2019). When the somatic embryos are produced under controlled in vitro conditions, they can be directly transformed into seedlings. However, using artificial seeds increase the compatibility of embryos like true seeds (Rihan et al. 2017).

In this research, artificial seeds of *B. persicum* were produced in 3% sodium alginate prepared in liquid MS nutrient medium and hardened in 100 mM calcium chloride which was eventually turned into seedling. In accordance with our research observations, several studies stated that the best combination for somatic embryogenesis encapsulation was obtained in 3.0% sodium alginate with 100 mM calcium chloride (Mishra et al. 2022; Muda and Awal 2019). The MS compounds in synthetic coating (carbon source) improve seedlings germination as well. Although, Redenbaugh et al. (1987) reported that it is necessary to remove Ca^{2+} from the MS medium to form calcium alginate capsules and carry out the ion exchange process. In this research, even calcium chloride didn't remove from the MS culture medium before hardening process, and the process of replacing sodium with calcium ions was done well. The lower concentration of sodium alginate not only increased the polymerization time, but also led to the production of soft beads that were difficult to work with, during transfer to the culture medium. Singh et al. (2010) reported that low concentrations of sodium alginate led to a decrease in gelatinization ability. High concentrations of sodium alginate and calcium chloride produced almost the same diameter but hard seeds, which had a delay in germination. These results were consistent with the findings of Danso and Ford-Lloyd (2003) and Singh et al. (2009).

Conclusions

In this study, we optimized the seed germination of *Bunium persicum* using different exposure times of ultrasonic waves. The most effective treatment was obtained from treatment of 7 min ultrasonic exposure. Furthermore, a novel protocol was established to clarify the effect of auxin (IBA) and auxin-cytokine (2,4-D + BAP) composition on the hypocotyl explant of *B. persicum* to induce indirect somatic embryogenesis. In the first experiment, somatic embryogenesis was promoted at the concentrations of 1 mg/l IBA, while, in the second experiment it was promoted at 0.5 mg/l BAP + 1 mg/l 2,4-D, 0.5 mg/l BAP + 2 mg/l 2,4-D, and 1 mg/l 2,4-D media. The germination percentage of encapsulated somatic embryos was affected by the exposure time of CaCl_2 solution and the type of encapsulation matrix. The 3% sodium alginate and 100 mM CaCl_2 for 30 min produced optimal beads. Therefore, artificial seed production using this protocol facilitated the propagation of this valuable medicinal plant.

Abbreviations

IBA

Indole-3-butyric acetic acid

BAP

6-Benzylaminopurine

2,4-D

2,4-Dichlorophenoxyacetic acid

NAA

1-Naphthaleneacetic acid

Kin

Kinetin

PGRs

plant growth regulators

Declarations

Author contributions BO and ShP conceived and conceptualized the manuscript; BO and MM wrote and prepared the original draft. BO and MM conducted the experiments. MM and RA analyzed data. BO and MM contributed to reviewing and critically revising the manuscript. RA and ShP visualized and carefully supervised the work and the manuscript. All authors have read and agreed to the published version of the manuscript

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Conflict of interest the authors declare that there is no conflict of interest for conducting this study and did not receive any external funds for the study. We are free to write, interpretation, publication, and share our results.

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Figures

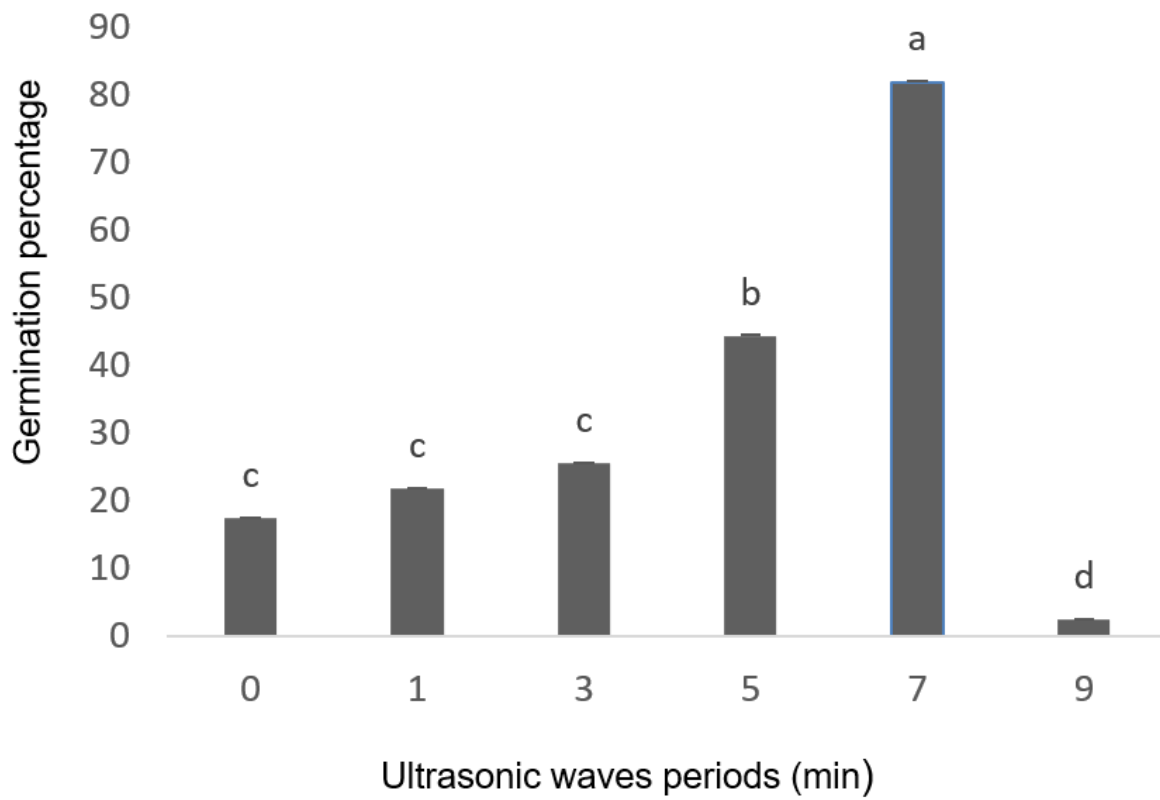


Figure 1

Germination percentage at different ultrasonic waves periods of *B. persicum*



Figure 2

Two-leaf seedlings after one week of germination

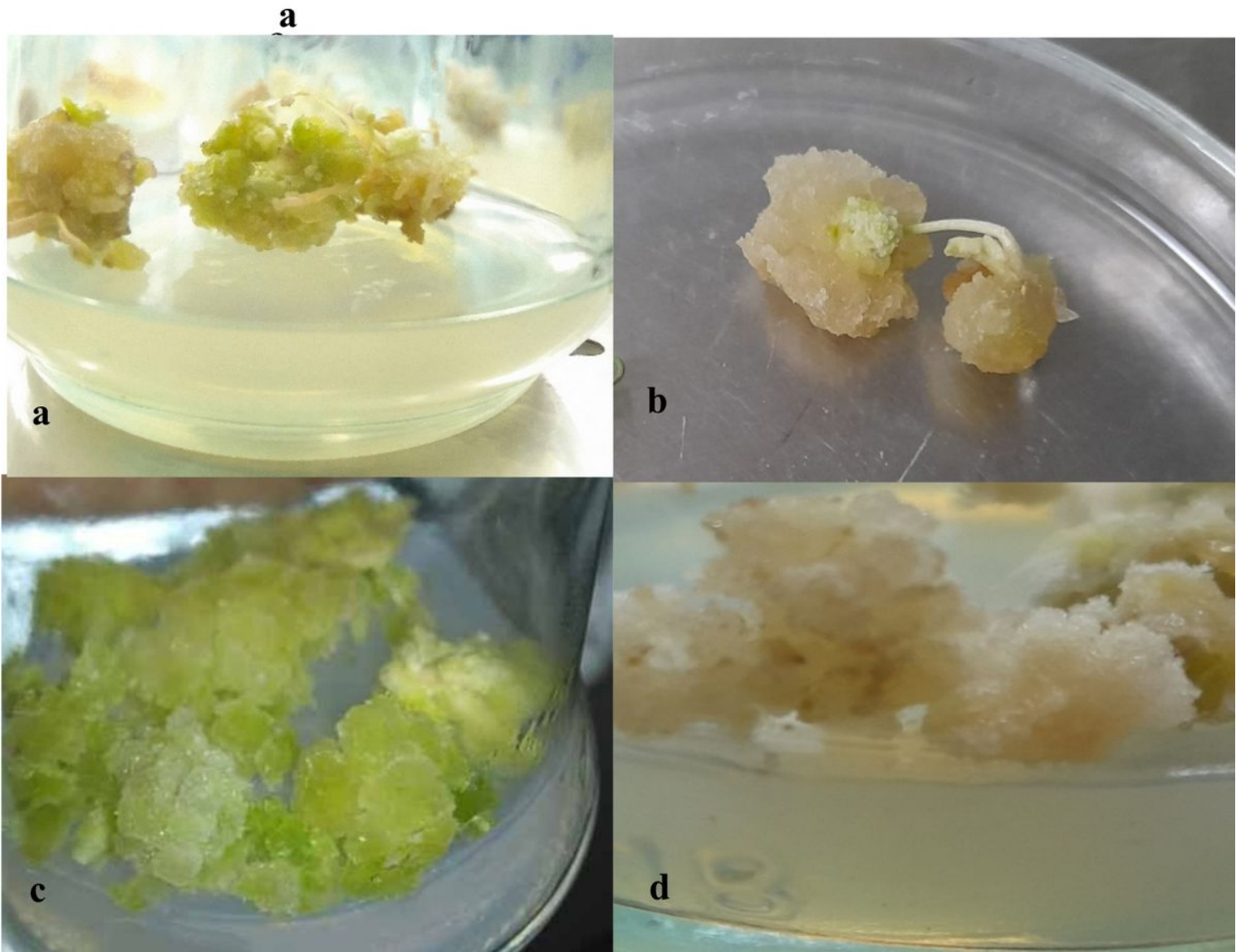


Figure 3

Morphology of embryogenic and non-embryogenic calli in two experiment **a)** embryogenic calli in first experiment **b)** non-embryogenic calli in first experiment **c)** embryogenic calli in second experiment **d)** non-embryogenic calli in second experiment



Figure 4

Adventitious root induction from callus in MS medium supplemented with 4 mg L⁻¹ IBA

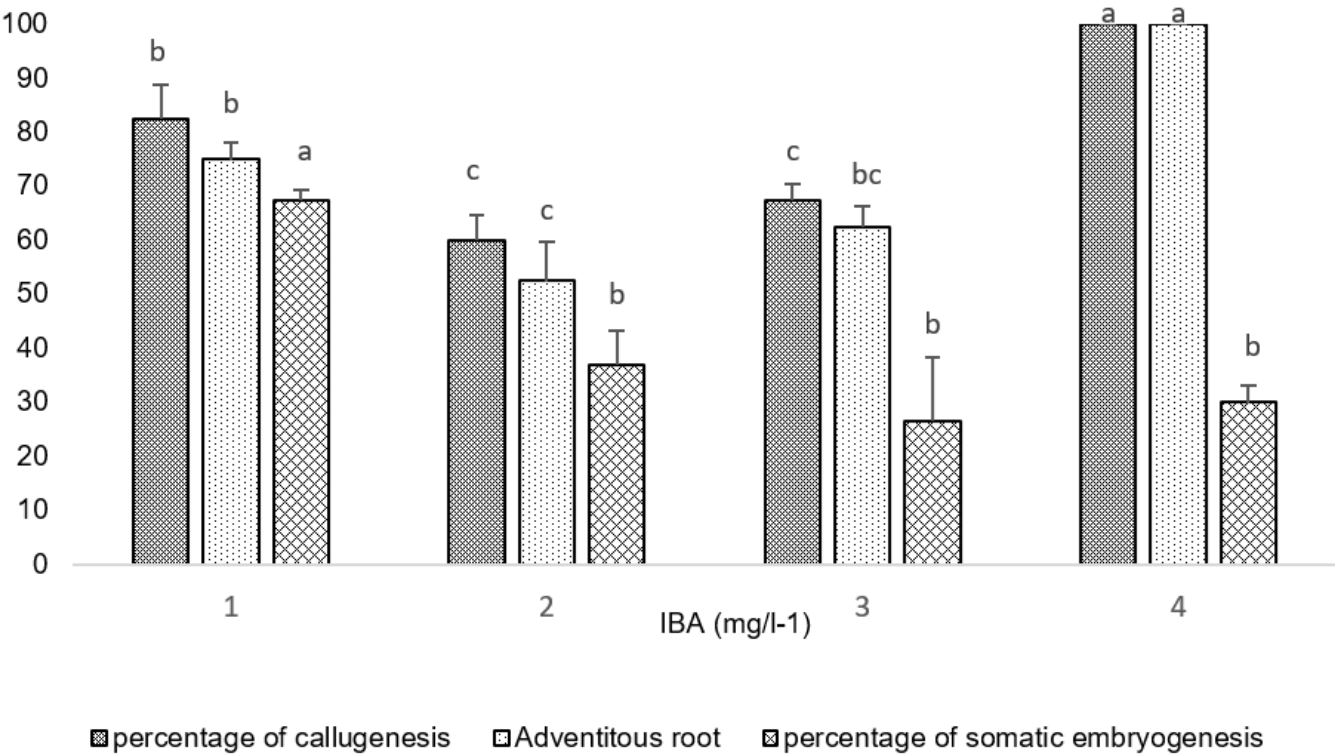


Figure 5

The effect of different concentrations of IBA on percentage of callogenesis, adventitious root and percentage of somatic embryogenesis

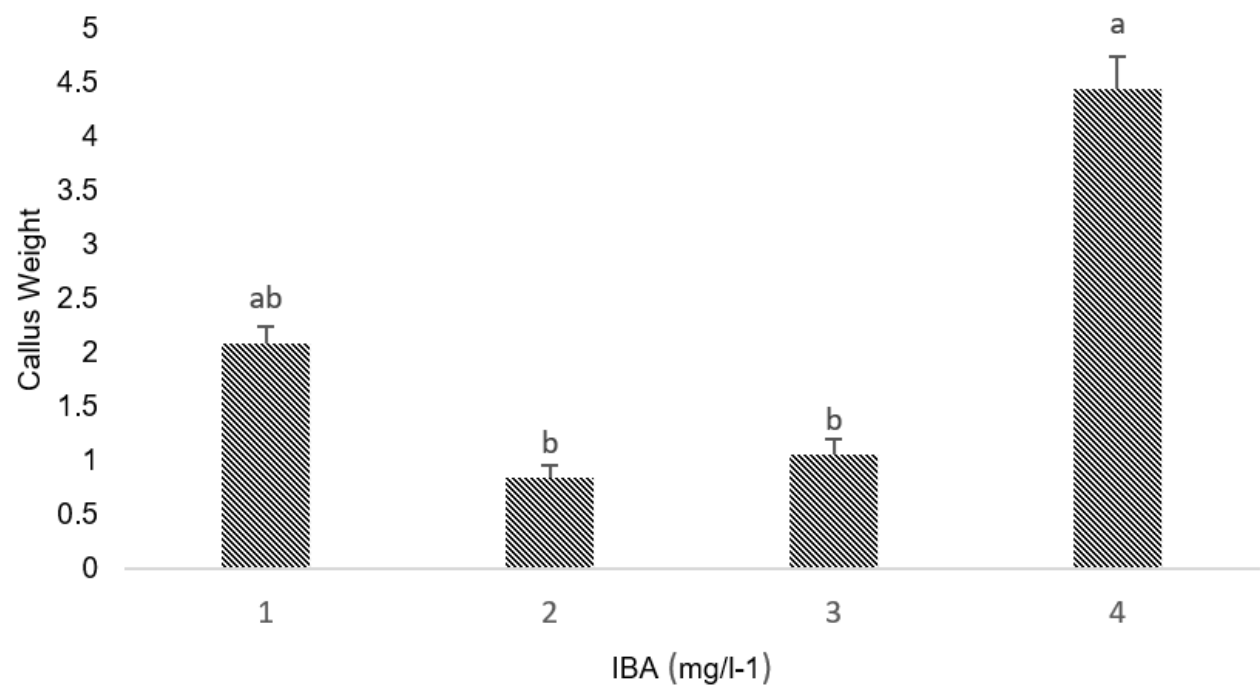


Figure 6

The effect of different concentrations of IBA on callus weight

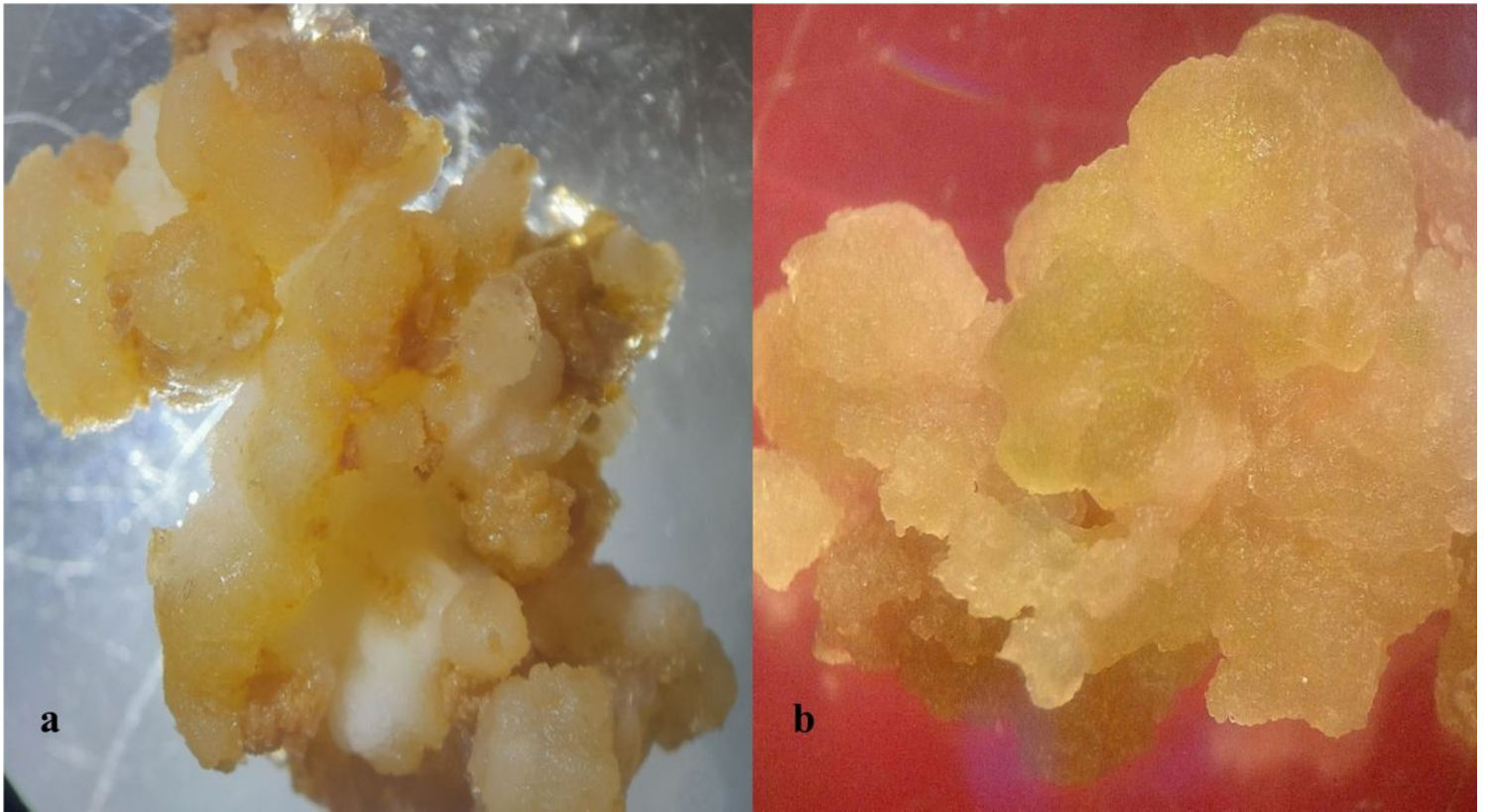


Figure 7

Numerous globular embryos in **a)** first experiment **b)** second experiment



Figure 8

Somatic embryogenesis from callus cultures of *B.persicum* **a)** globular-shaped somatic embryos derived from embryogenic callus **b)** heart-shaped somatic embryo **c)** torpedo-shaped somatic embryo **d)** cotyledonary somatic embryo

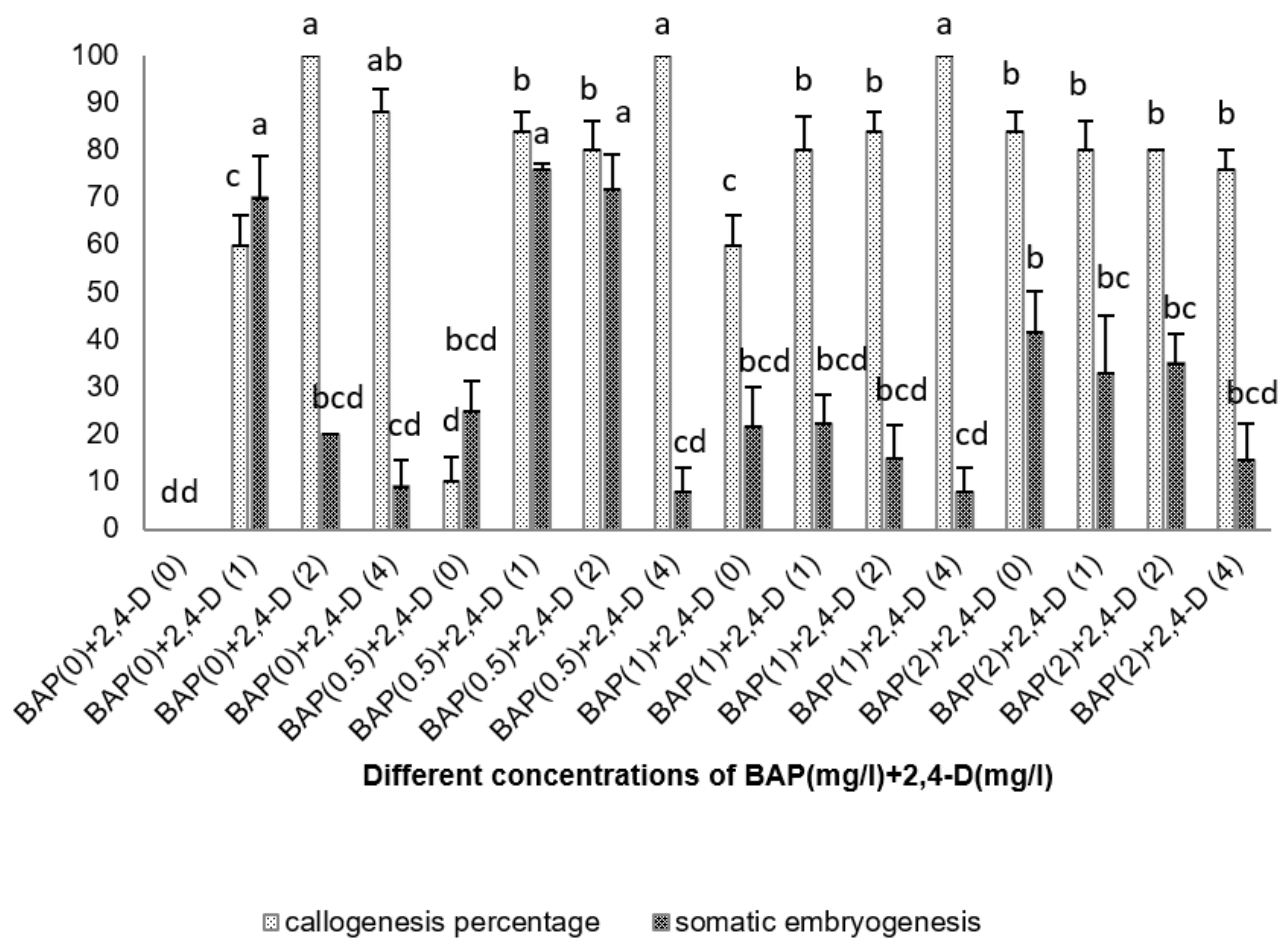


Figure 9

The effect of different concentrations of BAP+2,4-D on percentage of callus, and percentage of somatic embryogenesis

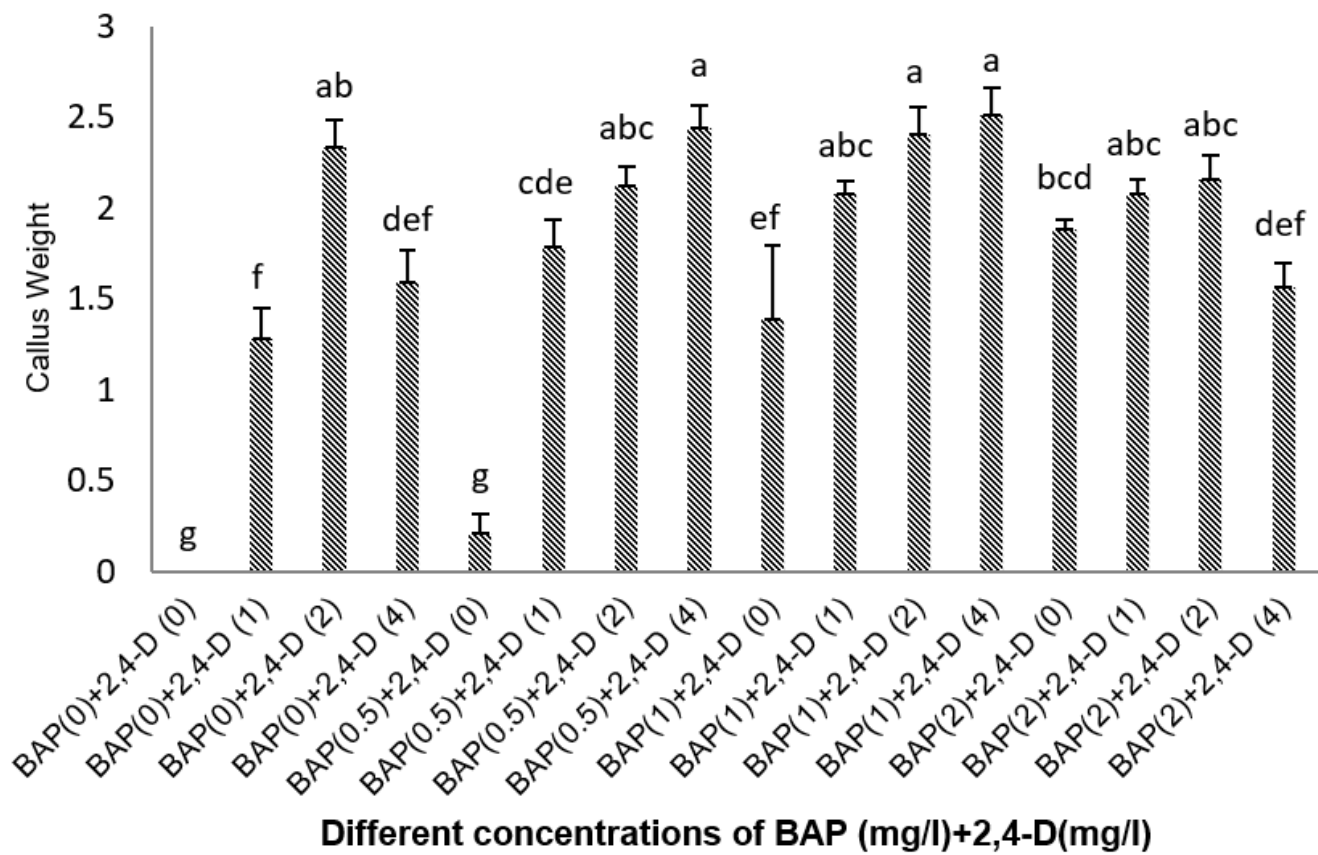


Figure 10

The effect of different concentrations of BAP (mg/l) +2,4-D (mg/l) on callus weight



Figure 11

B. persicum synthetic seed production, germination and transferring to pot containing peat based substrate **a)** synthetic seeds production using cotyledon shaped somatic embryos **b)** germination of a synthetic seed on MS medium after 14 days of culture **c)** germinated seedling from synthetic seed in peat based substrate **d)** acclimated plant after transferring to pot containing peat based substrate

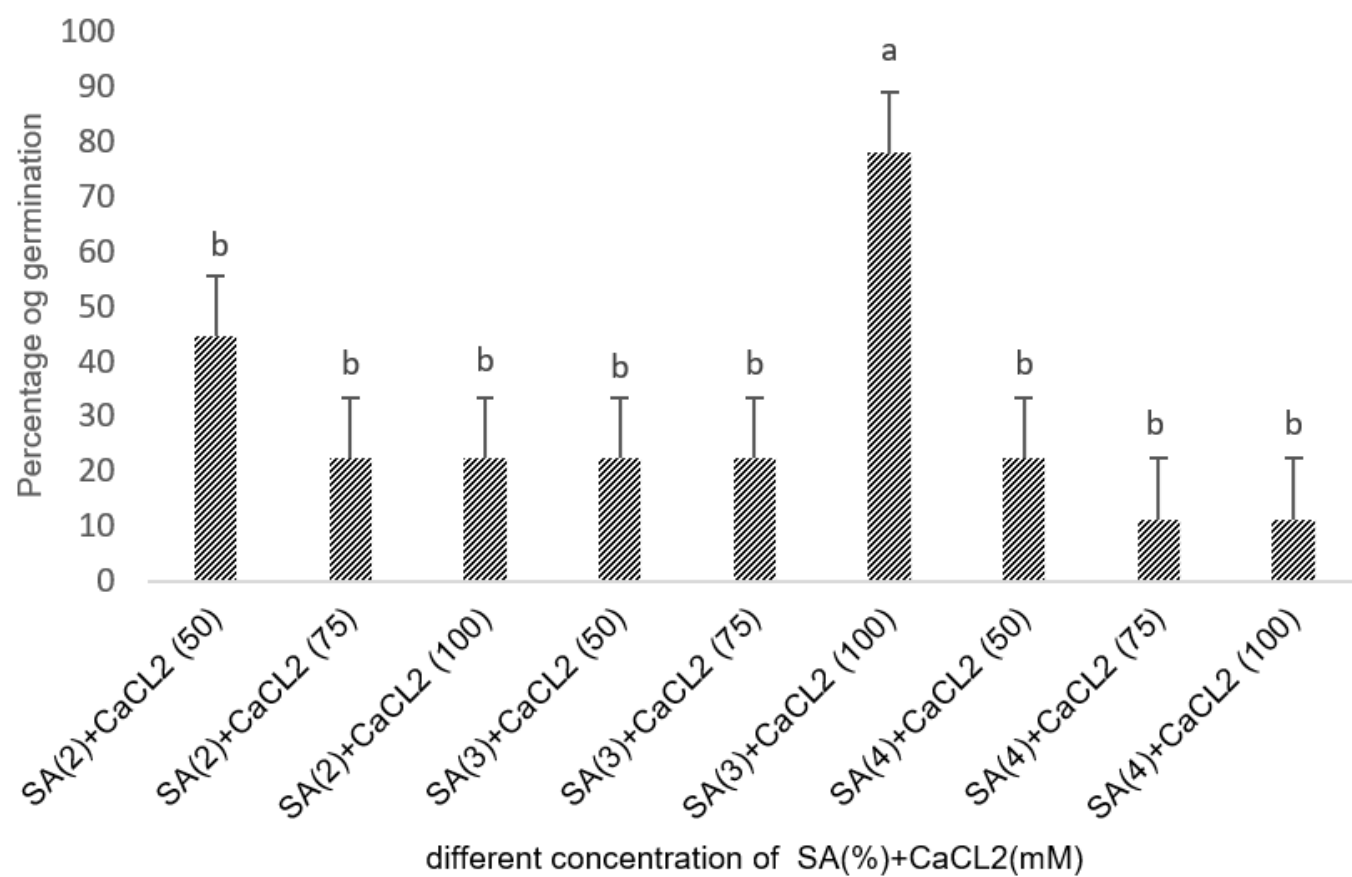


Figure 12

The effect of different concentration of sodium alginate (SA) and calcium chloride (CaCl₂) on germination percentage of artificial seed