INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 12, No. 5, p. 311-323, 2018
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Role of biotechnology in protection of endangered medicinal plant, *Bunium persicum*; the most important herb of Iran

Yousef Emamipoor*

Agricultural Research, Education & Extension Organization (AREEO), Kerman Branch, Iran

Article published on May 30, 2018

Key words: *Bunium persicum*, Somatic embryogenesis, Plant growth regulators, Corm derived callus

Abstract

Bunium persicum (Boiss) Fedtsch is a valuable medicinal plant that is facing extinction. A study was conducted to adopt various strategies and techniques to conserve and protect the biodiversity of *B. persicum*. Application of corm explant from six-month-old seedling on MS medium supplemented with various auxins showed that corm derived callus on MS medium supplemented with 1.0mg/L 2,4-dichlorophenoxyacetic acid (2, 4-D) by 74.9% induced somatic embryogenesis callus. The somatic embryos transferred to medium supplemented with different concentrations of benzylaminopurine (BAP), kinetin, spermidine, forchlorfenuron (CPPU), chlormequat chloride (CCC), paclobutrazol (PBZ), casein hydrolysate (CH), poly ethylene glycol (PEG) and banana powder, led to maximum plantlet regeneration, which was 65.8 ± 2.6 obtained in ½ MS medium supplemented with 20g/l banana powder. Consequently, induction of somatic embryogenesis under 1.0mg/L 2, 4-D was found to be more suitable than other auxins and capacity of banana powder for plantlet development by having indol acetic acid (IAA), cytokinins and gibberellins (GAs) was more than other additives and PGRs. Also, the effect of different concentrations of sucrose, BAP, PBZ and GA_3 on size of B. persicum corms was investigated. Results showed that 90 g/L sucrose with 164.9 ± 2.8 mg corm fresh weight (FW) was the most suitable sucrose concentration for growth of corm and shoot numbers. The corms coated with sodium alginate and calcium nitrate to create artificial seeds. The seeds transferred to soil and produced seeds in the first year.

***Corresponding Author:** Yousef Emamipoor ✉ u.emami@areeo.ac.ir

Introduction

Bunium persicum is a medicinal plant belonging to the Umbelliferae family. The seeds are used in traditional medicine for treatment of disorders related to digestive and blood system. In addition, the seeds are added to food as carminatives and spice. This shrub is found in Central Asia, India, Pakistan, Afghanistan and Iran. In the natural habitats, seeds will germinate in late winter and seedlings will grow in the following spring. The discovery of *B. persicum* seeds inside the pottery dishes of the first civilization shows the demand for this medicinal plant from five thousand years ago (Gazerani, 2012). With increase in the human population the demand for this medicinal plant has been increased (Pezhmanmehr *et al.*, 2009). Non-regulated harvesting, including extensive and or early seed collection was the reason for the destruction of the plant habitat, depletion of this species and increasing the probability of extinction (Abraham, 2010).

In countries such as Seri Lanka and Morocco, the ethnobotanical studies for domestication of medicinal plant have been started and economical evaluation shows high income from plantation of medicinal plants (El-Hilaly, 2003; Russell-Smith *et al.*, 2006). However, a reliable protocol for economic plantation of *B. persicum* has not been recorded yet. Ecological studies showed that cold winter or chilling temperature (4-5°C) is needed for breaking of seed dormancy (Sharma *et al.*, 2006). The seedlings grow and a small underground corm will appear at the end of first growth season. The corm is responsible for the vegetative growth and after 4-7 years reproductive stage will start with formation of inflorescence and seed (Khosravi, 2005). In fact, long juvenile time, which is more than four years and seed dormancy have restricted the economic plantation of *B. persicum*.

Moreover, appetency of 70% of the people for traditional medicines for primary health care (Khan *et al.*, 2009) and dependency of most pharmaceutical industries on medicinal plants have increased the fear of extinction of medicinal plants such as *B. persicum* (Thatoi & Patra, 2011). Subsequently, different strategies and techniques are needed to protect the biodiversity of this plant. Callus formation and callus maintenance are often the first steps in tissue culture experiments (Carter & Gunawardena, 2011). Plant tissue culture is a useful system for basic studies such as biochemistry, physiology and also molecular biology of cells. In addition, by spreading the viral diseases in the farmland, such as happened in passion fruit farms, application of tissue culture technique for massive production of virus free plants could be mandatory (Prammanee *et al.*, 2011). Nowadays, tissue culture methods have also been applied for the production of desirable plants by genetic transformation (Kothari *et al.*, 2010). In fact, tissue culture of rare or endangered medicinal species is necessary in order to maintain the sustainability of plants (Singh & Tiwari, 2010). Regeneration of *B. persicum* for massive plant production was surveyed. Ecological studies revealed that vegetative plantation of *B. persicum* via corm gave 604.1g seed's yield by plantation of 200 corms per square meter (Faravani, 2004). Thus, this study could be useful for future studies on the biochemistry, physiology and also molecular biology of corm to improve vegetative plantation of *B. persicum*. Also, by optimization of proper plantlet regeneration method, the results could be applied in the genetic transformation and production of desirable plants.

However, a broad field of natural resources and the absence of codified roles are reasons for the significant decrease in the distribution area of *B. persicum*. Such human activity and also the recent occurrences of drought have raised fears about the possible extinction of this wild plant. It is therefore hoped that domestication of this plant will not only lead to economic plantation-type production but will also ensure the survival of this valuable natural resource. The main objectives of this research were as follows:

- To develop a methodology for initiating somatic embryogenesis callus.
- To optimize a method for increasing the corm size to reduce the juvenile period of *B. persicum*.

- To study the optimum gelling agent type for artificial seed production.
- To find the proper concentration of calcium nitrate in artificial seed production.
- To produce deliverable artificial seeds to farmers.

Materials and methods

Seeds were harvested from wild *B. persicum* shrubs of Bolboloie Mountains in late June. The area located 26 kilometers on the way from Kerman to Sirch in South-East of Iran. The area is one of the well-known distributed areas of *B. persicum* and identification of species was done by research Institute of Forest and Rangeland (Ghoncheie & Emamipoor, 2007).

Germination is the phenomenon of testa rupture and radicle emerging from plant seeds. Some seeds are able to germinate in a specific period under suitable physical condition, called non-dormant seeds (Baskin & Baskin, 2004). Some seeds don't have this capacity and called dormant seeds. Sharma *et al*., (2006) revealed that *B. persicum* seeds are not shown germination under normal physical conditions (temperature, moisture and light) and so are dormant seeds.

The three-month-old seeds were washed under tap water for six hours followed by submerging in 70% ethanol for 60 seconds. To reduce the rate of contamination, seeds were double sterilized with 100ml of 20% commercial bleaching solution, CLOROX, with 2 drops of Tween 20 for 15min. The sodium hypochlorite was removed by three times rinsing in sterile distilled water at 10-minute intervals.

Plant materials for callus induction

Seedlings developed on ½ MS medium after treated by TDZ at low and GA_3 at high concentrations (Emamipoor & Maziah, 2012). Corm segments from sterile seedlings were used for callus induction.

Initiation of somatic embryogenesis callus

Corm derived calluses were transferred to MS medium supplemented with plant growth regulators to induce somatic embryos. Observation of callus under stereo microscope to see globular, heart shape, torpedo and dicotyledony stages done every 3 days. Percentage of somatic embryogenesis capacity, the time of somatic embryogenesis browsing and quantity of derived embryos compared with each other.

Development of plantlets

Plantlets transferred to ½ MS medium with different concentrations of sucrose to increase development of plants with proper size of corm. The corm initiation, the number of corms, the size and number of roots as well as root length measured for selection the suitable medium.

Optimum concentration of sodium alginate and calcium nitrate for corm coating

Sodium alginate is the common gelling agent for artificial seed coating. The regenerated corms with more than 3.0g fresh weight selected and transferred to 0.5, 1.0, 2.0 and 3.0% of sodium alginate. The encapsulated corms were transferred to 100mM calcium nitrate for 5, 10 or 20min. Then capsules rinsed in water and stored in air tight container. The statistically designed method was randomized complete with five repeat by encapsulation of 60 corms and results were analysed by MSTATC version 9.

Results

Callus induction

Corms are underground storage organs and have been used for propagation of *Colocasia esculenta* and *Alocasia amazonica* (Deo *et al*., 2009; Deo, *et al*, 2010). This study was focused on callus formation from corm slices of six-month-old plant on MSB medium (MS salts and B5 vitamins) in the presence of auxins. Analysis of variance of data shows that auxins significantly influence the percentage of callus formation (Appendix 1) and callus initiation time (Appendix 2) from corm slices of *B. persicum*. Results show that 2,4-D, NAA, dicamba and picloram with 83.33%, 50.00%, 46.67% and 43.33% respectively influence callus formation from corm slices (Table & Fig. 1). Also 2, 4-D, picloram, NAA and dicamba with 20.3 ± 0.6, 21.6 ± 0.5, 28.5 ± 0.4 and 37.4 ± 0.8 days of callus initiation time respectively influence callus formation from com slices (Table 1).

Table 1. Callus induction from corm explants of six-month-old of *B. persicum* on MS medium containing 1.0mg/L auxins.

1.0mg/L auxins	Day to callus formation	Callus score**	Percentage of callus formation	Callus morphology
Free-hormone	0*	-	0	0
2,4-D	20.3^{c}	+++	83.33^{a}	Green, compact
Dicamba	37.4^{a}	++++	46.67^{b}	Pale green, friable
NAA	28.3^{b}	++++	50.00^{b}	Pale yellow, friable
Picloram	21.6^{c}	++	43.33^{b}	Greenish, friable

*= no callus formation; **Callus initiation were scored from + to ++++; += callus only formed at the edge; ++= callus formed more at the edge; +++=callus covered the surface; ++++= callus covered the surface massively.Data represent the mean ± SD. Data within a column with the same letter (a-c) are not significantly different (p = 0.05). n=3.

Fig. 1. *B. persicum* seed collection from distributed area in South-East of Iran (from Google earth, 08.08.2017 at 7557 ft). Arrow shows collection area.

The results revealed that in the presence of 1.0mg/L 2, 4-D, corm segments showed maximum callus formation, which was 83.33% and minimum requirement time which was 20.3 ± 0.64 days (Table). Similar results were also reported by Zhang *et al.*, (2011), who showed that corm segments are suitable explants for callus induction in the presence of 2, 4-D on medium.

In tissue culture condition, the exogenous level of auxins influences the endogenous level of natural auxins such as IAA. In fact, 2,4-D specifically influence corm callus induction because this PGR increases the level of endogenous IAA more than the other auxins (Deo *et al.*, 2009). Also, duration of explant exposure to auxins is an important factor in callus formation. Corm explants need less exposure time for callus induction if 2,4-D has been used as callus inducer. As a matter of fact, a threshold of endogenous auxin was needed for callus induction from corm explants.

Calluses were separated from explants and subculture on the same callus induction media. It was observed that the callus was in a steady state of growth after one month of culturing. The callus was subculture twice on the similar medium to obtain homogenous callus. The results showed that auxins with 1.0 mg/L significantly affect callus proliferation (p = 0.05). Callus fresh weight and dry weight were recorded after one month of culture. The results showed that picloram gave the highest callus fresh weight for all explants while 2, 4-D significantly influenced callus dry weight.

In fact, increase in callus dry weight is related to biosynthesis of mass cellular products and more synthesis while increase in callus fresh weight shows more watery of cells. Thus callus dry weight shows real growth of callus. The results showed that corm-derived callus had maximum dry weight which was 0.126 ± 0.003 mg/l callus culture on MSB media with 2, 4-D (Table 2).

Table 2. The influence of various auxins on fresh and dry weight of callus from corm explants of *B. persicum* after one month culture on MS medium supplemented with 1.0mg/ l auxins.

Medium	Explant	Auxin (1.0 mg/L)	Fresh weight of callus (g)	Dry weight of callus (mg)
MS salts With B5 vitamins	Corm	0	0	0
		2,4-D	4.06 ± 0.03[a]	0.126 ± 0.003[a]
		Dicamba	4.50 ±0.06[a]	0.075 ± 0.008[c]
		NAA	3.27 ± 0.02[b]	0.105 ± 0.004[b]
		Picloram	4.16 ± 0.04[a]	0.104 ± 0.004[b]

Data represent the mean ± SD. Data within a column with the same letter (a-d) are not significantly different (p = 0.05). n=3.

Potential Embryogenic Callus of B. persicum

According to Wakhlu *et al*., (1990) presence of 2, 4-D in tissue culture medium prevents regeneration potential of *B. persicum* callus. In this study, one-month-old corm-derived callus from proliferated calli was implanted on Perti dishes with 20 ml MS free hormone medium. Observations of calli after 45 days revealed that 2, 4-D and NAA affected the frequency of somatic embryogenesis (Table).

Results showed corm-derived callus with 1.0mg/L 2,4-D induced 74.9% embryogenic callus (Table 3). Fig. shows the different stages of somatic embryogenesis from one-month-old corm-derived callus on MS free media after two months. Analysis of data on the effect of 2,4-D on formation of embryogenic callus showed that corm-derived callus significantly influenced induction of embryogenic callus. Also, observation showed that embryogenic callus was not detected in the presence of kinetin or BAP with 0.5, 1.0 and 1.5mg/L.

Regeneration of plantlets from corm-derived somatic embryos

The amounts of 500 mg embryogenic callus in cotyledon stage were transferred on media supplemented with cytokinins, polyphenylureas, polyamines, growth retardants and osmotic modifier (PEG). Results of implantation of corm-derived somatic embryos on ½ MSB medium containing BAP, kinetin and forchlorfenuron (CPPU) are shown in**Error! Reference source not found.**4. The study indicated that kinetin at 0.5mg/L is the suitable cytokinin in regeneration of B. persicum from corm-derived somatic embryos. The specific role of kinetin on somatic embryos could be due to the effect of kinetin on cytokinin-binding protein (CBP) and accumulation of cytokinins in cell divisions (Barciszewski, Massino, & Clark, 2007). Evidence implicated that kinetin gave specific results in regeneration of plants via somatic embryogenesis (Silveira, Floh, Handro, & Guerra, 2004).

Table 3. Effect of auxins on induction of somatic embryogenic callus from one-month-old corm derived callus of *B. persicum* on MS medium.

Medium	Explant	Auxin (1.0 mg/L)[1]	Callus morphology on free-hormone medium[2]	% of callus forming EM
	Corm	2,4-D	Nodular, yellow	74.9 ± 4.5[a]
		Dicamba	Friable, yellowish	0[b]
		NAA	Friable, yellowish	0[b]
		Picloram	Friable, yellowish	0[b]

EM= Embryogenic callus, 1= One-month-old callus, 2=after two months implantation of 100 mg callus. Data represent the mean ± SD. Data within a column with the same letter (a-c) is not significantly different (p = 0.05).

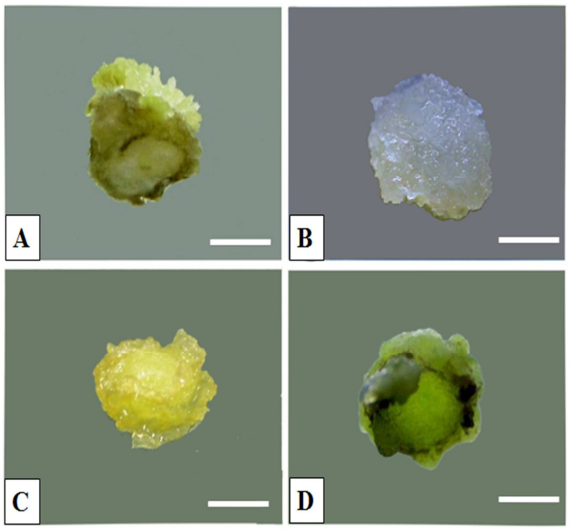

Fig. 2. Callus formation from corm segments of *B. persicum* on MSB media supplemented with 1.0mg/L auxins. (A) Picloram at day 40. (B) Dicamba at day 50. (C)NAA at day 40(D) 2,4 –D at day 30. Scale bar= 5mm.

Results revealed that kinetin at 0.5 mg/L with higher shoots (30.6 ± 1.1mm) and higher rate of regeneration (38.4 ± 2.9 plantlet/ culture) was the ideal phytohormone (Table 4 & **Error! Reference source not found.** In addition, kinetin at 0.5 mg/L formed two corms per culture with the size of 1.9 ± 0.3mm. Results also showed that the other cytokinins, which was BAP (maximum at 0.5mg/L with 13.00±1.6 plant/culture and 11.2 ± 0.5mm length) and phenylurea compound, which was CPPU (maximum at 1.0mg/L with 31.60 ± 2.3 plant/culture and 18 ± 1.0mm length) affected the regeneration of *B. persicum* from corm-derived embryos, but the number of plantlets and shoot length was less than control. Also, corm formation didn't happen in the presence of BAP and CPPU.

Polyamines and growth retardances are positive factors of tuberization. Spermidine is a useful polyamine and chlorocholine chloride (CCC) and paclobutrazol (PBZ) are common growth retardances, which use for tuberization in tissue culture methods (Hussain *et al.*, 2006). In this experiment effect of these two groups of tuber inducers were studied.

Results demonstrated in the result revealed that polyamines such as spermidine and growth retardances including chlorocholine chloride (CCC) and paclobutrazol (PBZ) influenced plantlet regeneration from mature corm-derived embryos on ½ MSB medium. CCC at 0.5mg/L with regeneration rate of 60.6 ± 2.5 plantlet/culture, shoot length of 53.6 ± 2.3mm and higher number of corm, which was four with diameter of 4.28 ± 0.4 mm, is an ideal factor for regeneration of plant from corm-derived embryos (Table 5 & Fig. 5).

Table 4. Effect of different level of PGRs on development of somatic embryos (derived from mature corm explants) on ½ MS medium**.**

PGRs	Concentration (mg/L)	No. of regenerated plantlets / culture[2]	Shoot length (mm)[2]	Corms/culture[2]	
				Size (mm)	Number
BAP	0	33.20 ± 1.9^{b}	24.2 ± 0.8^{b}	2.13 ± 0.35^{a}	1 ± 0^{b}
	0.2 mg/L	6.00 ± 0.7^{e}	4.2 ± 0.5^{e}	0.0^{d}	0^{c}
	0.5 mg/L	13.00 ± 1.6^{d}	11.2 ± 0.5^{d}	0.0^{d}	0^{c}
	1.0 mg/L	2.00 ± 0.0^{f}	3.1 ± 0.1^{e}	0.0^{d}	0^{c}
Kin	0	33.20 ± 1.9^{b}	24.2 ± 0.8^{b}	2.13 ± 0.35^{a}	1 ± 0^{b}
	0.2 mg/L	8.40 ± 0.5^{e}	6.6 ± 0.6^{e}	0.0^{d}	0^{c}
	0.5 mg/L	38.4 ± 2.9^{a}	30.6 ± 1.1^{a}	1.9 ± 0.3^{b}	2 ± 0^{a}
	1.0 mg/L	19.40 ± 1.1^{c}	16.2 ± 0.8^{c}	0.0^{d}	0^{c}
CPPU	0	33.20 ± 1.9^{b}	24.2 ± 0.8^{b}	1.75 ± 0.46^{c}	1 ± 0^{b}
	0.5 mg/L	15.20 ± 1.4^{d}	11.6 ± 0.9^{d}	0.0^{d}	0^{c}
	1.0 mg/L	31.60 ± 2.3^{b}	18 ± 1.0^{c}	0.0^{d}	0^{c}
	2.0 mg/L	12.40 ± 1.1^{d}	10.2 ± 0.8^{d}	0.0^{d}	0^{c}

2=Data have been recorded after two months, BAP= 6-benzylaminopurine, CPPU= Forchlorfenuron. Data shown by mean ± SD. Mean values ± SD within a column with the same letter (a-e) are not significantly different (p = 0.05). n=3.

Table 5. Effect of different level of polyamines (spermidine) and plant growth retardants on development of somatic embryos from mature corm-derived callus of *B. persicum*, cultured on ½ MSB medium.

Polyamines, growth retardants	Concentration (mg /L)	No. of plantlets / culture[2]	Shoot length (mm)[2]	Corms/ culture[2]	
				Size (mm)	Number
SPE	0	33.20±1.9^{c}	24.2±0.8^{c}	2.13± 0.35^{c}	1 ± 0^{c}
	0.5	9.80±0.8^{e}	7.6±0.6^{f}	0^{d}	0^{d}
	1.0	42.60 ±1.1^{b}	33±2.2^{b}	2.12 ± 0.64^{c}	1 ± 0^{c}
	1.5	17.0 ±1.0^{d}	11.6 ± 0.6^{o}	0^{d}	0^{d}
CCC	0	33.20±1.9^{c}	24.2±0.8^{c}	2.13± 0.35^{c}	1 ± 0^{c}
	0.1	43.40±1.1^{b}	37.4±1.1^{b}	3.0±0.53^{b}	3 ± 0^{b}
	0.5	60.60±2.5^{a}	53.6±2.3^{a}	4.28± 0.4^{a}	4 ± 0^{a}
	1.0	16.60±1.3^{d}	12.0±0.5^{d}	0^{d}	0^{d}
PBZ	0	33.20±1.9^{c}	14.8±0.8^{d}	2.13± 0.35^{c}	1 ± 0^{c}
	0.1	0.00^{f}	0^{g}	0^{d}	0^{d}
	0.5	0.00^{f}	0^{g}	0^{d}	0^{d}
	1.0	5.60±0.6^{e}	3.6±0.6^{f}	0^{d}	0^{d}

2=Data have been recorded after two months, SPE = Spermidine, CCC= Chlorocholine Chloride, PBZ= Paclobutrazol. Data represent the mean ± SD. Data within a column with the same letter (a-g) are not significantly different (p = 0.05). n=3.

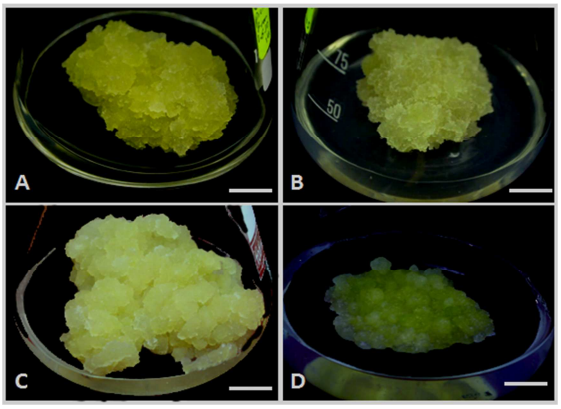

Fig. 3. Corm-derived callus from six-month-old *B. persicum* on MSB medium supplemented with 1.0mg/L auxins. Proliferated corm-derived-callus cultured on medium supplemented with 1.0mg/L (A) Picloram (B) Dicamba (C) NAA and (D) 2, 4-D after one month. Scale bar= 10mm.

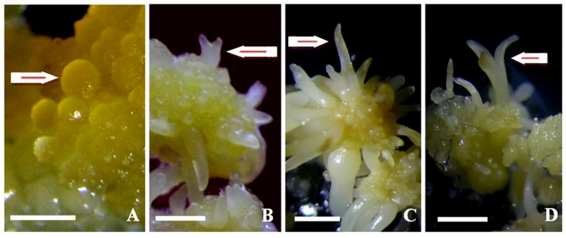

Fig. 4. Different stages of somatic embryogenesis from corm-derived callus on MSB free hormone medium. Arrows show (A) Globular stage after 30 days, (B) Heart shape after 45 days, (C) Torpedo stage after 50 days and (D cotyledon stage after 60 days of implantation. Bar= 2mm.

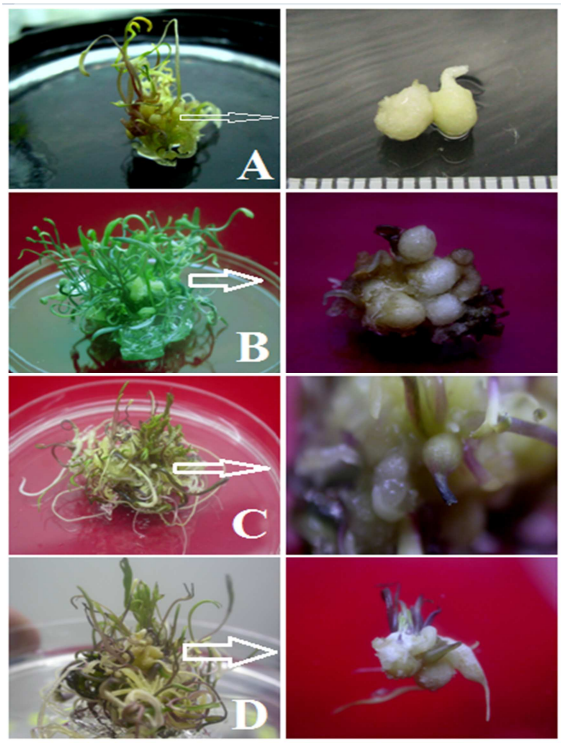

Fig. 5. Corm formation from mature corm-derived embryos cultured on 1/2 MS medium containing (A) 0.5mg/L kinetin, (B) 0.5mg/L CCC, (C) 1.0g/L casein hydrolysate and (D) 0.1mg/L CCC. Scale= 5mm.

Also, CCC at 0.1mg/L with regeneration rate of 43.4 ± 1.1 plantlet/culture and shoot length of 37.4 ± 1.1mm and high number of corm, which was three with 3.0 ± 0.53mm diameter influenced regeneration of *B. persicum* from mature corm-derived embryos. Table & **Error! Reference source not found.**).

Moreover, the results implicated that spermidine at 1.0mg/L with 42.6 plantlet/culture regeneration rate and 33.0 ± 2.2mm shoot length influenced regeneration of somatic embryos. Also, spermidine affected corm formation with one corm per culture with size of 2.12 ± 0.64mm. In addition, influence of additives such as casein hydrolysate and banana powder, and also influence of PEG, an osmotic potential modifier on plantlet regenerations, was studied. Data represented in showed that organic additives (casein hydrolysate and banana powder) as well as polyethylene glycol (PEG) influence plantlet regeneration via somatic embryogenesis from mature corm-derived embryos.

Results implicated that casein hydrolysate at 1.0 g/l with regeneration rate of 43.20 ± 1.9 plantlet/culture, shoot length of 28.4 ± 0.6 and one corm with 2.4 ±0.3 mm diameters influenced the regeneration of corm-derived embryos. Also, results showed that banana powder at 20g/L with regeneration rate of 35.6 ± 2.3 plantlet/culture and shoot length 25.8 ± 2.3mm significantly influenced regeneration of embryos. The corm size for plantlets was 3.3 ± 0.4mm. moreover, PEG at 15g/L affected regeneration rate (43.20 ± 1.9 plantlets/culture) and plantlet height (33.6 ± 1.5mm) of somatic embryos.

Table 6. Effect of different level of additives and PEG on development of somatic embryos from mature corm-derived callus cultured on ½ MSB medium.

Additives or, osmotic potential modifier	Concentration (g /L)	No. of plantlets / culture[2]	Shoot length (mm)[2]	Corms/culture[2]	
				Size (mm)	Number
Casein hydrolysate	0	33.20±1.9^{ab}	24.2±0.8^{c}	1.9 ± 0.3^{a}	1 ± 0^{a}
	0.2	7.60±0.6^{d}	6.2±0.5^{f}	0^{b}	0^{b}
	0.5	13.20±0.8^{c}	10.4±0.6^{de}	0^{b}	0^{b}
	1.0	43.20±1.9^{a}	28.4±0.6^{b}	1.9 ± 0.3^{a}	1 ± 0^{a}
Banana powder	0	33.20±1.92^{ab}	16.4±1.1^{d}	1.9 ± 0.3^{a}	1 ± 0^{a}
	5.0	30.40±2.3^{b}	21.2±1.9^{c}	0^{b}	0^{b}
	10.0	31.40±1.1^{b}	23.8±1.6^{c}	0^{b}	0^{b}
	20.0	35.60±2.3^{ab}	25.8±2.3^{c}	0^{b}	0^{b}
PEG	0	33.20±1.9^{ab}	24.2±0.8^{c}	1.9 ± 0.3^{a}	1 ± 0^{a}
	10.0	5.20±0.8^{d}	2.2±0.5^{g}	0^{b}	0^{b}
	15.0	43.20±1.9^{a}	33.6±1.5^{a}	0^{b}	0^{b}
	20.0	6.60±0.6^{d}	3.2±0.5^{g}	0^{b}	0^{b}

2=Data have been recorded after two months, PEG = Polyethylene glycol. Data shown by mean ± SD. Mean values ± SD within a column with the same letter (a-g) are not significantly different (p = 0.05). n=3

Table 7. Effect of media on development of *B. persicum* plantlets from corm-derived callus regeneration medium (MSB medium with 0.6mg/L kinetin).

Medium type	Medium strength	No. of Swollen roots	Shoot length(mm)[1]
DKW	full	0^{b}	8.8 ± 0.4 ^{d}
MS salts with MS vitamins (MS)	full	0^{b}	22.4 ± 1.5 ^{b}
MS salts with B5 vitamins (MSB)	full	1^{a}	13.2 ± 0.9 ^{c}
DKW	half	0^{b}	14.6± 1.2 ^{c}
MS salts with MS vitamins (MS)	half	1^{a}	23.8± 1.8 ^{b}
MS salts with B5 vitamins (MSB)	half	1^{a}	27.9 ± 2.0 ^{a}

1 =regenerated explants, with five mm size, were implanted on media (N=10, triplicates). The results were recorded after 30 days. Data are shown by mean ± SD. Mean values ± SD within a column with the same letter (a-d) is not significantly different (p = 0.05).

Maturation of B. persicum plantlets

The previous results showed that regenerated plantlets on MSB medium contain 0.6 mg/L kinetin produced maximum length of shoot and root compare to other cytokinins.

Microscopic observation showed that corms were initiated from swollen roots after 25 days. Medina *et al.*, (2009) showed that under *in vitro* condition, the swollen roots sprouted root tubers and reported that for terrestrial plants the storage organs such as tubers or corms are necessary for survival.

The previous experiment showed that a large number of healthy plantlets regenerated on MS medium supplemented with 0.6 mg/L kinetin. These plantlets are small and fragile in comparison with plants from corm sprouting. The micropropagation techniques can exploit the potential of swollen roots to have mature plants with storage organs (corms) for successful domestication of *B. persicum*.

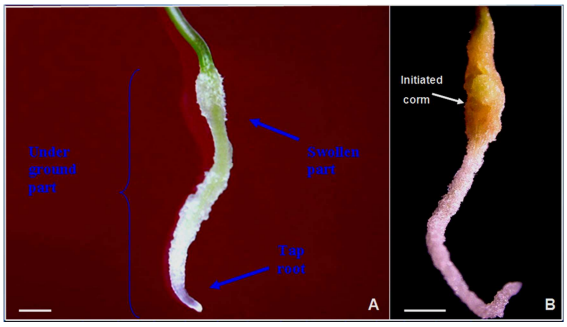

Fig. 6. Initiation of swollen root on indirect shoot regeneration pathway of *B. persicum*. (A) Underground parts of regenerated plant, from callus in ½ MSB media at day 15, shows two separate parts, swollen and tap root parts. (B) Corm initiation from swollen underground part of regenerated plant at day 25. Scale bar=1mm.

Effect of basal medium on maturation of B. persicum plantlets

In this section, the effect of basal media with half and full strength in utilization of swollen root formation were studied. The experiment designed according to Moon & Stomp (1997) for maturation of plantlets on half and full MS, MSB and DKW media. Swollen root formation and plant heights were recorded after 30 days (Fig.).

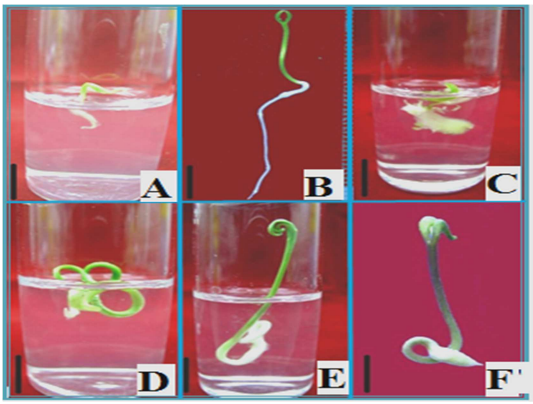

Fig. 7. Effect of medium on development of plantlets. (A) DKW, (B) MS, (C) MSB, (D) ½ DKW, (E) ½ MS and (F) ½ MSB media. Regenerated shoots originated from MSB medium supplemented with 0.6 mg/L kinetin.

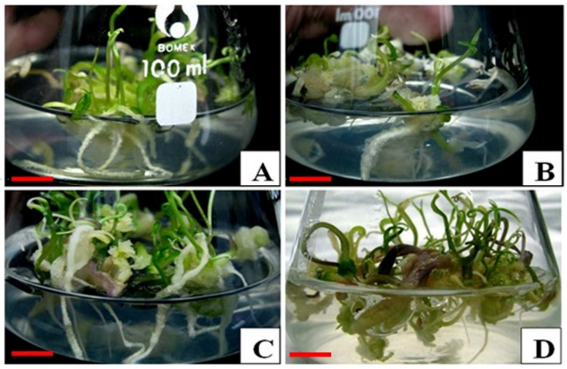

Fig. 8. Influence of IBA on rooting of *B. persicum* plantlets. The numbers of 20 plantlets from regenerated medium (MSB+0.6mg/L kinetin) implanted on ½ MSB medium supplemented with (A) 0.0mg/L IBA, as control, (B) 0.1mg/L IBA, (C) 0.2mg/L IBA and (D) 0.5mg/L IBA. Bar= 10mm.

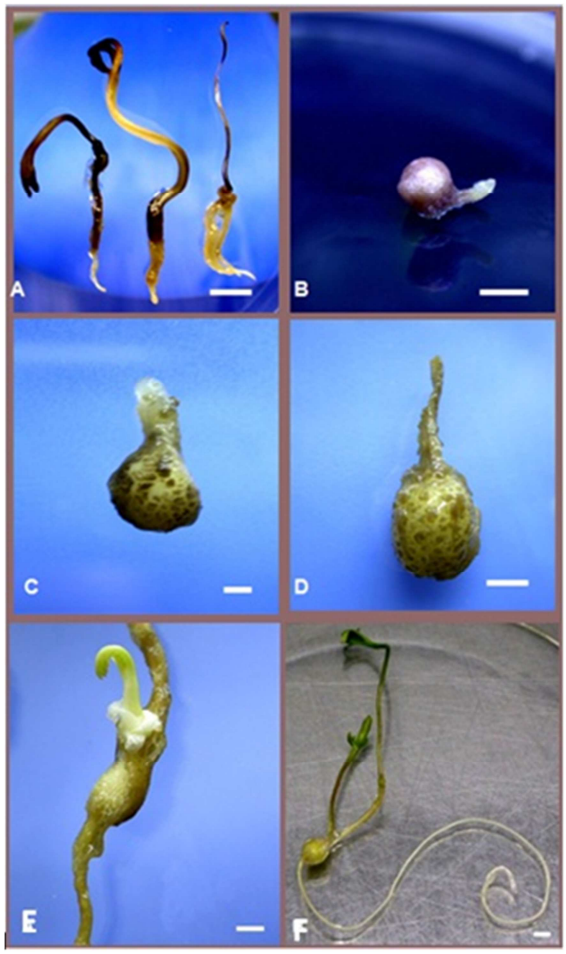

Fig. 9. Effect of sucrose concentrations on corm development in regenerated plants after 60 days in ½ MS medium. (A) Without sucrose, (B) With 30g/L, (C) 60g/L, (D) and 90g/L sucrose. (E) Initiation (at 20th day) and (F) Development (at 45th day) of the second leaf in ½ MSB medium with 90g/L sucrose. Bar = 5mm.

Analysis of data showed that type of media influenced regenerated plant height and swollen root formation. Data as shown in revealed that half strength of MSB medium with higher plant length, which was 27.9 ± 2.0mm and swollen root formation influenced maturation of regenerated plantlets. In addition, plantlets which cultured on full MS and full MSB media showed 23.8 ± 1.8mm and 13.2 ± 0.9mm long respectively with swollen root formed. Also, results showed that DKW medium with full and half strength have no effect on swollen root formation. It is suggested that swollen root formation is a phenomenon related to presence of pyridoxine and high amounts of thiamine. Furthermore, the ratio of ammonium to nitrate is a determining factor in swollen root formation and the media strength is the last factor. Subsequently, low concentration of pyridoxine, more ratio of ammonium to nitrate, high concentration of thiamine and half strength of salts respectively effect on swollen root formation.

Table 8. Effect of different concentrations of IBA on *in vitro* rooting of *B. persicum* cultured in ½ MSB media with different levels of IBA.

IBA (mg/L)	% of rooting	No. of roots per shoot	Root length (mm)
0	53.3 ± 2.9^{c}	1	13.6 ± 1.2^{c}
0.1	68.3 ± 2.9^{b}	1	15.3 ± 1.8^{c}
0.2	83.3 ±2.8^{a}	1	22.57 ± 1.7^{a}
0.5	60.0 ± 5.0^{b}	1	17.14 ± 1.2^{b}
1.0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}

NO= root not observed, Data recorded after 30 days of culturing of regenerated shoots on media. The number of regenerated shoots was 20 and experiment was repeated three times. Data are shown by mean ± SD. Mean values ± SD within a column with the same letter (a-d) is not significantly different (p = 0.05).

Table 9. Effect of different concentrations of sucrose, PBZ, BAP and GA_3 on corm formation of plantlets culture on1/2 MSB media.

Corm inducers	Concentration	No of corms	Corm FW (mg)	No of shoots
Sucrose	00.0 g/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}
	30.0 g/L	1.0 ± 0.0^{a}	89.6 ± 5.3^{c}	1.0 ± 0.0^{b}
	60.0 g/L	1.0 ± 0.0^{a}	113.7 ± 5.0^{b}	1.0 ± 0.0^{b}
	90.0 g/L	1.0 ± 0.0^{a}	164.9 ± 2.8^{a}	2.0 ± 0.0^{a}
	120 g/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}
PBZ	0.5 mg/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}
	1.0 mg/L	0.0 ± 0.0^{b}	-0.0 ± 0.0^{d}	0.0 ± 0.0^{c}
	1.5 mg/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}
BAP	2.0 mg/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}
	5.0 mg/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}
	10.0 mg/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}
GA_3	0.5 mg/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	1.0 ± 0.0^{b}
	1.0 mg/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	1.0 ± 0.0^{b}
	1.5 mg/L	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	1.0 ± 0.0^{b}

Data were recorded after 60 days of culturing and are shown by mean ± SD. Mean values ± SD within a column with the same letter (a-d) are not significantly different (p = 0.05). Experiment was repeated three times with five replicates.

Effect of IBA on rooting of regenerated plantlets

Analysis of data has shown that the addition of IBA to micro propagation medium, which was ½ MS, significantly influenced root length and rooting frequencies of regenerated shoots from root explants. In this section, the amounts of 100mg leaf-derived regenerated callus from regeneration medium (MS supplemented with 0.6mg/L kinetin) including about 20 plantlets implanted on ½ MSB medium with different concentrations of IBA. The results revealed that IBA at 0.2mg/L with 83.3% rooting and 22.57 ± 1.7mm shoot length, was more effective.

Effect of sucrose, paclobutrazol (PBZ), BAP and GA_3 on corm formation and size of corm

Ascough *et al.*, (2011) showed that in *Watsonia* vanderspuyiae, sucrose with 60g/L, GA_3 and paclobutrazol with 1.0mg/L, induce corm formation 50%, 70.4% and 26.3% respectively and BAP prevents corm formation. In this experiment, the effects of different concentrations of PBZ, BAP and GA_3 on corm formation from regenerated shoots of *B. persicum* were investigated. Results in show that PBZ, BAP and GA_3 had no effect on corm formation in regenerated shoots. Results also indicated that sucrose at 90 g/L with higher corm fresh weight and more number of shoots influenced corm formation and number of shoots.

Hussain *et al.*, (2006) reported an increase in the number of tubers by increasing the sucrose concentration in *Solanum tuberosum*. In this experiment the corm FW increased with increasing the sucrose concentration. It is suggested that by increasing the sucrose concentration caused increasing of osmolarity of medium. Thus, the increase in the number of corm is related to osmatic stress and increasing the size of corm is related to ability of plant to increase the reserve materials in storage organ (corm). By the increasing of the storage materials, plant behavior change to maturity with increasing the number of leaves.

Optimization of sodium alginate and calcium nitrate for artificial seed production

The results indicated that corm-derived callus have somatic embryogenesis capacity. Although somatic embryos could coated for artificial seed production, but even artificial seeds produced from this procedure will not decrease juvenile time and for commercial production is not proper. Corms are more suitable for economical production via artificial seed (corm). Results showed that 1% sodium alginate is the proper gelling agent for artificial seed coating. Also results indicated that transferring encapsulated corms to 100 mM calcium nitrate for 20 min gave seeds with flowering ability in the first growth season.

References

Abraham J, Cheruvathur MK, Mani B, Thomas TD. 2010. A rapid in vitro multiplication system for commercial propagation of pharmaceutically important *Cyclea peltata* (Lam) Hook & Thoms. based on enhanced axillary branching. Industrial Crops and Products **31,** 92-98.

Ascough GD, Erwin JE, Staden JV. 2008. Reduced temperature, elevated sucrose, continuous light and gibberellic acid promote corm formation in *Watsonia vanderspuyiae*. Plant Cell, Tissue and Organ Culture **95,** 275-283.

Barciszewski J, Massino F, Clark BFC. 2007. Kinetin a multiactive molecule. International Journal of Biological Macromolecules **40,** 182-192.

Baskin JM, Baskin CC. 2004. A classification system for seed dormancy. Seed Science Research **14(1-16).**

Carter J, Gunawardena AHL. 2011. Regeneration of the aquatic monocot *Aponogeton madagascariensis* (lace plant) through callus induction. Aquatic Botany **94,** 143-149.

Deo PC, Harding RM, Taylor M, Tyagi AP, Becker DK. 2009. Somatic embryogenesis, organogenesis and plant regeneration in taro (*Colocasia esculenta* var. *esculenta*). Plant Cell, Tissue and Organ Culture **99,** 61-71.

Deo PC, Taylor M, Harding RM, Tyagi AP, Becker DK. 2010. Initiation of embryogenic cell suspensions of taro (*Colocasia esculenta* var. *esculenta*) and plant regeneration. Plant Cell, Tissue and Organ Culture **100,** 283-291.

El-Hilaly J, Hmammouchi M, Lyoussi B. 2003. Ethnobotanical studies and economic evaluation of medicinal plants in Taounate province. Journal of Ethnopharmacology **86,** 149-158.

Emamipoor Y, Maziah M. 2014. An efficient method in breaking of dormancy from *Bunium persicum* (Boiss) Fedtsch seeds: a valuable herb of Middle East and Central Asia. Asian Pac J Trop Biomed **4(8),** 642- 649.

Faravani M. 2004. Survey on treatment of seed amount, transplanting time of black zeera bulbs from nursery to field with best cultivation pattern. Iranian Journal of Pharmaceutical Research **3(2),** 65-65.

Ghoncheie M, Emamipoor Y. 2007. Collection, identification, evaluation and protection of medicinal plant's seeds for gene bank Final reports of research studies. Tehran, Iran: Research Institute of Forests and Rangelands.

Hussain I, Chaudhry Z, Muhammad A, Asghar R, Naqvi SMS, Rashid H. 2006. Effect of chlorocholine chloride, sucrose and BAP on in vitro tuberization in potato (*Solanum tuberosum* L. cv. *cardinal*). Pakistan Journal of Botany **38(2),** 275-282.

Khan MY, Aliabbas S, Kumar V, Rajkumar S. 2009. Recent advances in medicinal plant biotechnology. Indian Journal of Biotechnology **8(9-22).**

Khosravi M. 2005. Intercropping Black Zira (*Bunium persicum*) with Saffron and annual crops: Agroecological and economic perspetives. Doctor Of Phylosophy PhD thesis, University of Ferdosi, Mashhad, Iran.

Kothari SL, Joshi A, Kachhwaha S, Ochoa-Alejo N. 2010. Chilli peppers - A review on tissue culture and transgenesis. Biotechnology Advances **28,** 35-48.

Medina RD, Flachsland EA, Gonzalez AM, Terada G, Faloci MM, Mroginski LA. 2009. In vitro tuberization and plant regeneration from multinodal segment culture of *Habenaria bractescens* Lindl., an Argentinean wetland orchid. Plant Cell, Tissue and Organ Culture **97,** 91-101.

Moon HK, Stomp M. 1997. Effects of medium components and light on callus induction, growth, and frond regeneration in *Lemna gibba* (Duckweed). In Vitro Cellular and Developmental Biology–Plant **33,** 20-25.

Pezhmanmehr M, Hassani ME, Jahansooz F, Najafi AA, Sefidkon F, Mardi M, Pirseiedi M. 2009. Assessment of genetic diversity in some Iranian populations of *Bunium persicum* using RAPD and AFLP markers. Iranian Journal of Biotechnology **7(2),** 93-100.

Prammanee S, Thumjamras S, Chiemsombat P, Pipattanawong N. 2011. Efficient shoot regeneration from direct apical meristem tissue to produce virus-free purple passion fruit plants. Crop Protection **30,** 1425-1429.

Russell-Smith J, Karunaratne NS, Mahindapala R. 2006. Rapid inventory of wild medicinal plant populations in Sri Lanka. Biological Conservation **132,** 22-32.

Sharma RK, Sharma S, Sharma SS. 2006. Seed germination behaviour of some medicinal plants of Lahaul and Spiti cold desert (Himachal Pradesh): implications for conservation and cultivation. [Research Communications]. Current Science **90(8),** 1113-1118.

Silveira V, Floh EIS, Handro W, Guerra MP. 2004. Effect of plant growth regulators on the cellular growth and levels of intracellular protein, starch and polyamines in embryogenic suspension cultures of *Pinus taeda*. Plant Cell, Tissue and Organ Culture **76,** 53-60.

Singh J, Tiwari KN. 2010. High-frequency in vitro multiplication system for commercial propagation of pharmaceutically important *Clitoria ternatea* L.—A valuable medicinal plant. Industrial Crops and Products **32,** 534-538.

Thatoi H, Patra JK. 2011. Biotechnology and Pharmacological Evaluation of Medicinal Plants: An Overview, Journal of Herbs, Spices & Medicinal Plants **17(3),** 214-248.

Wakhlu AK, Nagari S, Barna KS. 1990. Somatic embryogenesis and plant regeneration from callus cultures of *Bunium persicum* Boiss. Plant Cell Reports **9,** 137-138.

Zhang S, Liu N, Sheng A, Ma G, Wu G. 2011. Direct and callus-mediated regeneration of *Curcuma soloensis* Valeton (Zingiberaceae) and ex vitro performance of regenerated plants Scientia Horticulturae **130,** 899-905.

Appendix 1

Analysis of variance of the effects of different levels of Auxins on percentage of callus formation from corm segments of *B. persicum*.

Source	DF	MS	F Value
A	4	1311.67	34.98 **
C	1	13653.33	364.09 **
A*C	4	1311.67	34.98 **
Error	20	37.50	
CV (%)		44.13	

A=Auxins, C= Concentration, CV= Coefficient of Variation, DF = degrees of freedom, MS = mean square, F value = frequency of each value, **= highly significant, Least Significant Differences (LSD) = 10.43.

Appendix 2

Analysis of variance of the effects of different levels of Auxins at time of callus initiation from corm segments of *B. persicum*.

Source	DF	MS	F Value
A	4	953.44	117.93 **
C	1	10851.98	1342.28 **
A*C	4	956.72	118.34 **
Error	90	8.08	
CV (%)		44.13	

A=Auxins, C= Concentration, CV= Coefficient of Variation, DF = degrees of freedom, MS = mean square, F value = frequency of each value, **= highly significant, Least Significant Differences (LSD) = 13.53