JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov. 2015. Vol. **9**(Spl. Edn. 2), p. 21-29

Efficient Microspore Embryogenesis Induction in Tomato (*Lycopersicon esculentum* Mill.) using Shed Microspore Culture

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(Received: 13 March 2015; accepted: 15 April 2015)

The effects of cold treatment (4°C for 0, 2, and 5 days), heat shock (30°C for 0, 2, and 5 days), and two induction media (B5 and NLN) supplemented with 2% and 9% sucrose were assessed on microspore embryogenesis induction in tomato using shed microspore culture technique. Significantly higher callusing was obtained when anthers were cultured on the B5 medium with 2.0 mg l^{-1} 2, 4-D and 2.0 mg l^{-1} BAP. In addition, IAA at 0.5 mg l^{-1} in combination with 0.5 mg l^{-1} Kin also substantially improved callogenesis. Chitosan treatment proved to be beneficial to callusing and shoot regeneration so that, high callogenesis (85%) and shoot regeneration (3%) was obtained from anthers treated with 50 mg l^{-1} chitosan. Microspore-derived structures with more than 9 nuclei were observed in both media containing 2% sucrose when treated at 4°C for 2 days and 30°C for 2 and 5 days. Microspore-derived embryos (MDEs) were only obtained in the double-layer B5 medium supplemented with 2% sucrose which exposed to 4°C for 2 days and then 30°C for 2 and 5 days. Microspore embryogenesis in tomato could be efficiently induced when appropriate duration of cold treatment, heat shock, and induction medium were selected.

Key words: *Lycopersicon esculentum* Mill.; Microspore embryogenesis; Shed microspore culture

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Tomato (*Lycopersicon esculentum* Mill.), belonging to the family of *Solanaceae*, is one of the most important vegetable crops that has achieved tremendous popularity over the last century[1]. Modern plant breeding and genetic technologies are used nowadays for breeding tomato varieties, including F1-hybrids. Tomato has tremendous potential of heterosis for earliness, total yield, resistance attributes and uniformity, thus, its improvement, in parallel to contemporary demands of the climate, growing practices and the markets, is mainly carried out via the hybrid breeding using conventional techniques or doubled haploid (DH) technology through the anther and isolated microspore culture (IMC) systems[2].

The anther culture and IMC are likely to remain prominent in plant breeding programs as they offer an opportunity to the plant breeders to develop DH plants on a larger scale which enables them to speed up the breeding process by fixing homozygosity in one generation after a cross has been made. Thus, cultivar development period can be dramatically reduced with the aid of IMC or anther culture in crop species responsive to this method[3]. In addition, this technique is very useful in gene transformation, mutation and breeding, and biochemical and physiological studies[4,5]. Commercial varieties developed through the DH protocols, have been reported for many crops, such as wheat (*Triticum aestivum* L.), barley (*Hordeum*

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vulgare L.), triticale (× *Triticosecale* Wittm.), rice (*Oryza sative* L.), *Brassica* spp., eggplant (*Solanum melongema* L.), pepper (*Capsicum annum* L.), asparagus (*Asparagus afficinalis* L.), and tobacco (*Nicotiana tabacum* L.)[5,6]. Despite the numerous economic importance of tomato, DH technology through the anther culture and IMC are still far from being routinely applied in tomato breeding programs, mostly due the lack of knowledge about microspore embryogenesis induction in this recalcitrant species[7,8].

Microspore embryogenesis consists of inducible reprogramming of the microspores, diverting them from their original i.e. gametophytic pathway towards embryogenesis[9,10]. To deviate microspores, a number of factors must occur at a same time and place[10]. Depending on the species, microspores often inducted by cold and heat shock[9,11], starvation[12], colchicine[13], and antioxidants[14].

Cold stress is commonly used treatment to induce microspore embryogenesis. Cold treatment is usually carried out at 4-10 ºC for a few days up to several weeks[15]. It has been indicated that microspores in the cold pre-treated anthers detach from the tapetum resulting in starvation, causing them to switch from gametophytic pathway to embryogenic development[9] In addition, it has been indicated that in cold pre-treated anthers the total content of free amino acids is increased, which is suggestive of metabolic re-programming that a microspore needs to undergo, in preparation for embryogenesis induction [9,15]. Following cold treatment, small heat shock protein genes e.g. *tom66* and *tom111*, have also been reported to be expressed in tomato and has been argued that this is possibly to protect cells against chilling injury and also results in decreased pollen-specific protein synthesis[15,16,17]. Heat shock is also frequently used to trigger microspore embryogenesis induction[9,11,15]. Heat shock influences microtubule distribution, blocks further gametophytic development, during which, acentric nucleus migrates to more central position and mitosis ultimately results in a symmetrical division with two daughter cells, similar in size and organelle distribution[18,19].

Nearly all attempts to androgenesis induction in tomato have focused on the *in vitro* anther culture[20]. Although, a large number of culture media types, stresses, vitamins, growth regulators i.e. indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), kinetin (Kin), 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D), have been assessed (reviewed by Bal and Abak[21]), only few entire plant regeneration with a clear haploid origin has been reported[7,20,22]. Further investigations are needed to enhance the efficiency of the process.

In this study, the effects of cold treatment (4°C for 0, 2, and 5 days), heat shock (30°C for 0, 2, and 5 days), and two induction media (B5 and NLN) supplemented with sucrose (2% and 9%) on efficiency of symmetrical nucleus divisions of microspores and embryo formation on shed microspore culture and also the effects of IAA (0, 0.5, 1.0, and 2.0 mg l^{-1}) in combination with Kin (0, 0.5, and 1.0 mg l^{-1}), 2,4-D (0, 1, 2, and 3 mg l^{-1}) in combination with BAP (0, 1, and 2 mg l^{-1}), and also various levels of chitosan (0, 10, 20, 50, and 100 mg l^{-1}) treatment in anther culture were assessed in *L. esculentum* Mill. cv. 'Berlina'.

MATERIALS AND METHODS

Donor plants and growth conditions

L. esculrntum cv. 'Berlina' was used as the test plant. Donor plants were grown in a greenhouse at a day/night temperature of 23-28/15-20°C under natural light conditions. Plants were irrigated three times a week.

Shed microspore culture

Flower buds (5-7 mm in length) containing a mixed population of mid to late-uni-nucleate microspores, which was determined using 4',6-diamidino-2-phenylindole (DAPI) nucleic acid staining, were harvested from the main and lateral branches of donor plants that had reached anthesis after about 90-110 days. These buds were immersed in 2% sodium hypochlorite (Golrang, Tehran, Iran) with gentle shaking for 10 min followed by three 5-min washes with cold (4°C) sterile distilled water. Sterilized buds were placed in a sterile Petri dish and then incubated at 4 °C (for 0, 2, 5, 10 days) in the dark. Anthers were then dissected from the buds aseptically and transferred onto the two-layer culture media. The below medium was B5 medium[23] supplemented with 2% sucrose, pH 5.7 and 0.7% agar (Duchefa Biochemie, Haarlem, The Netherlands). The above liquid medium was

comprised of filter-sterilized NLN (2% and 9% sucrose, [24]) or B5 medium (2% and 9% sucrose). The pH of all liquid media was adjusted at 6.0 with 1 N NaOH and 1 N HCl. The cultures were incubated at 30°C (for 0, 2, and 5 days) and then transferred to 25°C in the dark.

Anther culture

Anthers were dissected aseptically from pre-sterilized buds and then transferred onto the MS[25] medium containing various levels of filter-sterilized IAA (0, 0.5, 1.0, and 2.0 mg l^{-1}) in combination with Kin (0, 0.5, and 1.0 mg l^{-1}), 2, 4-D (0, 1, 2, and 3 mg l^{-1}) in combination with BAP (0, 1, and 2 mg l^{-1}), and also various levels of chitosan (0, 10, 20, 50, and 100 mg l^{-1}). Cultures were incubated at 25°C for 14 days in the dark for callogenesis induction (Fig. 1A). Four weeks after initial culture, regenerated calli measuring 1-2 mm in diameter (Fig. 1B, C) were transferred onto the MS medium supplemented with 0.25 mg l^{-1} zeatin and 0.025 mg l^{-1} IAA for shoot regeneration (Fig. 1D).

DAPI staining

Nucleus divisions of microspores were detected two weeks following anther culture using blue-fluorescent DAPI nucleic acid staining, which preferentially stains doubled-stranded DNA[26]. Shed microspore suspension (1 ml) was transferred to 1.5 ml vials and centrifuged at 150 × *g* for 4 min. The supernatant was decanted and microspores were fixed with Carnoy reagent (ethanol: glacial acetic acid, 3:1, v/v) for 15 min. Then, the suspension was centrifuged at 150 × *g* for 4 min, the supernatant was decanted and 300 µl of fresh ethanol: water (1:1, v/v) was added to the pellet using gentle shaking. Finally, the suspension was centrifuged and microspores were stained with DAPI: glycerol (3:1, v/v) solution for an hour. Samples were observed using an inverted microscope (Nikon Eclipse TE 2000-S) with fluorescent illumination.

Experimental design and statistical analysis

The experiments were conducted as factorial experiments based on a completely randomized design (CRD) with four replications (Petri dishes). The entire experiments were repeated two rimes. Data analyses were performed using SPSS software version 17 and the means were compared using Duncan's multiple range test (DMRT) at *á* = 0.05 following analysis of variance.

RESULTS

Shed microspore culture

Anthers from 5-7 mm long buds (Fig. 2A) containing a mixed population of mid to late-uni-nucleate microspores (Fig. 2B, B') were transferred onto the two layered culture medium for embryogenesis induction. Cold treatment in combination with heat shock could efficiently induce symmetrical nucleus divisions in both culture media and sucrose levels tested (Table 1 and 2). Except for control (without cold and heat treatment), 3-5 nuclei microspores (Fig. 2C, C') were detected in all treated cultures, few of which developed further into the 6-8 nuclei microspores (Fig. 2D, D'). Microspore-derived structures with more than 9 nuclei (Fig. 2E, E') were observed in both media containing 2% sucrose when exposed to 4°C for 2 days and 30°C for 2 or 5 days. Our results indicated the superiority of B5 medium and detrimental effect of high sucrose level (9%) to sporophytical divisions of tomato microspores so that, globular MDEs (Fig. 2F, F', G) were only obtained in the B5 medium supplemented with 2% sucrose which treated at 4°C for 2 days and then 30°C for 2 and 5 days. To date, it is the first report of producing MDEs form shed microspores of tomato.

Anther culture

Callogenesis was significantly affected by applied IAA and Kin levels (Table 3). The highest callusing was obtained in the cultures treated with 0.5 mg l^{-1} IAA along with 0.5 mg l^{-1} Kin. High level of IAA treatment was detrimental to callogenesis so that, the lowest callus formation was observed in the cultures treated with 2.0 mg l^{-1} IAA at all Kin levels tested. Callusing was also substantially increased in the B5 medium supplemented with 2.0 mg l^{-1} 2, 4-D and 2.0 mg l^{-1} BAP (Table 4). Regenerated calli (1-2 mm in diameter) were transferred onto the MS medium containing 0.25 mg l^{-1} zeatin and 0.025 mg l^{-1} IAA for shoot regeneration (SR). No regeneration was observed in calli produced form all examined phytohormones. On the other hand, chitosan treatment proved to be beneficial to both callusing and shoot regeneration so that, high callusing (85%) and also SR (3%) was obtained from anthers treated with 50 mg l^{-1} chitosan (Table 5). Higher level was not favorable so that, callogenesis

significantly decreased in the cultures treated with 100 mg l^{-1} chitosan.

DISCUSSION

Application of specific stress treatment(s) is considered to be crucial to divert microspores form their original gametophytic developmental pathway towards embryogenesis[9] Our results indicated that cold treatment in combination with heat shock efficiently induced symmetrical nucleus divisions in both culture media and sucrose levels tested. Microspore-derived structures with more than 9 nuclei were obtained in both media containing 2% sucrose and 2 days treatment at 4°C and 2 and 5 days at 30°C. According to Seguí-Simarro and Nuez[7] cold treatment at 7°C for 6 days could efficiently induce initial microspore symmetrical nucleus divisions and also haploid and DH plant regeneration from *in vitro* cultured anthers of tomato. In another study, Motallebi-Azar and Panahandeh[27] also indicated that

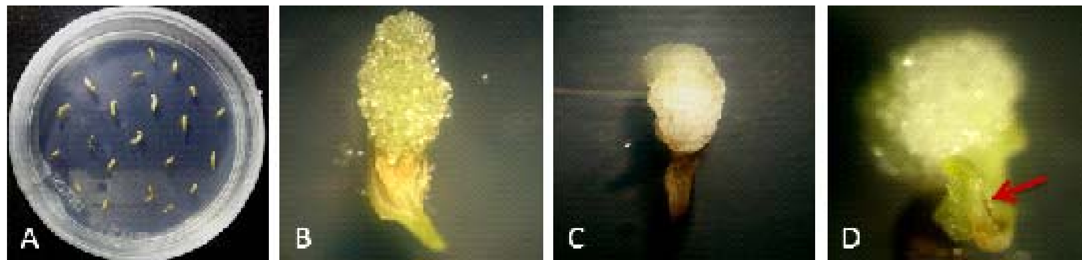

Fig.1. Anther culture in L esculentum cv. Berlina. A: cultured anthers after 14 days; B, C: callus formation 4 weeks after initial culture; D: shoot initiation 2 weeks after transferring calli onto the shoot regeneration medium (showed by arrow)

Table 1. Effect of different levels of sucrose in B5 medium, cold treatment and heat shock on symmetrical nucleus division and microspore-derived embryo (MDE) formation in shed microspore culture of *Lycopersicon esculentum* Mill. cv. 'Berlina'.

Sucrose (%)	Temperature treatment		Nucleus division (%)				
2%	4 °C	30 °C	Non-divided	3-5	6-8	9 e"	MDE
		0	100 a*	-	-	-	-
2%	0	2	68 ± 9.6 cd	27 ± 5.2 ab	5 ± 2.3 a	-	-
		5	62 ± 8.8 de	38 ± 8.8 a	-	-	-
		0	100 a	-	-	-	-
2%	2	2	46 ± 11.6 e	21 ± 8.4 bc	14 ± 6.0 a	11 ± 4.2 a	8 ± 2.8 a
		5	66 ± 12.7 d	15 ± 9.5 bc	10 ± 6.8 a	7 ± 3.3 a	2 ± 0.7 b
		0	100 a	-	-	-	-
2%	5	2	72 ± 8.3 cd	21 ± 6.9 bc	7 ± 4.3 a	-	-
		5	86 ± 7.7 abc	14 ± 7.7 bc	-	-	-
		0	100 a	-	-	-	-
9%	0	2	79 ± 11.1 bc	21 ± 11.1 bc	-	-	-
		5	86 ± 7.0 abc	14 ± 7.0 bc	-	-	-
		0	100 a	-	-	-	-
9%	2	2	68 ± 12.6 cd	23 ± 8.2 abc	9 ± 4.7 a	-	-
		5	78 ± 9.3 bcd	22 ± 9.3 bc	-	-	-
		0	100 a	-	-	-	-
9%	5	2	80 ± 11.9 abc	20 ± 11.9 bc	-	-	-
		5	92 ± 3.0 ab	8 ± 3.0 c	-	-	-

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT (α = 0.05).

Table 2. Effect of different levels of sucrose in NLN medium, cold treatment and heat shock on symmetrical nucleus division and microspore-derived embryo (MDE) formation in shed microspore culture of *Lycopersicon esculentum* Mill. cv. 'Berlina'.

Sucrose (%)	Temperature treatment		Nucleus division (%)				
2%	4 °C	30 °C	Non-divided	3-5	6-8	9 ?	MDE
		0	100 a*	-	-	-	-
2%	0	2	86 ± 7.3 bc	14 ± 7.3 bcd	-	-	-
		5	93 ± 3.8 ab	7 ± 3.8 d	-	-	-
		0	100 a	-	-	-	-
2%	2	2	50 ± 13.4 f	30 ± 9.5 a	11 ± 6.0 a	9 ± 2.9 a	-
		5	70 ± 10.2 e	23 ± 8.3 ab	6 ± 3.4 a	3 ± 1.8 b	-
		0	100 a	-	-	-	-
2%	5	2	72 ± 11.6 de	19 ± 9.7 bc	9 ± 4.4 a	-	-
		5	96 ± 2.2 ba	4 ± 2.2 d	-	-	-
		0	100 a	-	-	-	-
9%	0	2	92 ± 3.6 ab	8 ± 3.6 cd	-	-	-
		5	100 a	-	-	-	-
		0	100 a	-	-	-	-
9%	2	2	68 ± 12.9 e	22 ± 9.3 ab	10 ± 5.5 a	-	-
		5	84 ± 6.4 cd	16 ± 6.4 bc	-	-	-
		0	100 a	-	-	-	-
9%	5	2	91 ± 3.9 ab	9 ± 3.9 cd	-	-	-
		5	100 a	-	-	-	-

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT (α = 0.05).

Table 3. Effect of various levels of IAA and Kin on callogenesis induction and shoot regeneration (SR) from *in vitro* cultured anthers of *Lycopersicon esculentum* Mill. cv. 'Berlina'

IAA level (mg l^{-1})	Kin level (mg l^{-1})	Callogenesis (%)	SR (%)
	0	36 ± 5.6 b*	-
0	0.5	30 ± 3.9 cb	-
	1.0	25 ± 4.2 c	-
	0	31 ± 4.8 bc	-
0.5	0.5	47 ± 6.3 a	-
	1.0	23 ± 5.0 cd	-
	0	18 ± 2.6 cd	-
1.0	0.5	26 ± 3.7 c	-
	1.0	35 ± 5.5 b	-
	0	9 ± 2.1 e	-
2.0	0.5	15 ± 3.8 de	-
	1.0	12± 2.6 e	-

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT (α = 0.05)

androgenesis responses were substantially affected by cold treatment and its duration of exposure so that, the highest number of DH regenerated plantlets was achieved using 4°C for 36 and 72 hours. The stimulatory effect of cold treatment has also been reported in other crop species[28,29]. Working on *Brassica* spp. microspore culture, Gu et al[28] noted that cold pretreatment (1-4 days at 4°C) significantly enhanced microspore embryogenesis by 1-7 fold compared to non-pretreated cultures in *Brassica napus*. Yuan et al[29] reported that cold pretreatment (4°C for 1, 2 and 3 days) induced microspore embryogenesis in *B. oleracea* but the frequency of embryos was low, however, microspore embryogenesis significantly enhanced by 63-72 fold when cold pretreatment combined with heat shock (32.5°C for 1 days) treatment.

Our results also indicated the superiority of B5 medium to NLN medium and also 2% sucrose over 9% with regard to their efficiency in the MDE formation so that, MDEs were only produced in the cultures containing B5 medium supplemented

with 2% sucrose (treated at 4°C for 2 days and then exposed to 30°C for 2 or 5 days). The superiority of B5 medium to other induction media e.g. W14, NLN, and MS has been reported in *Solanaceae* species[30]. Comparing the total N content -which has proved to have profound influences on the efficiency of microspore embryogenesis[31]- of these induction media, Lantos et al[30] found that the B5 medium had the highest N content (20-35 mmol) while others exhibited drastically lower amounts. They also underlined the importance of media components and the ratios of components, especially those of the N sources on microspore embryogenesis induction[30]. Sucrose also is another crucial component of an induction medium. Sucrose not only provides energy, but also as an osmotic agent, regulates the osmotic pressure and movement of nutrients/elements form the cell[32].

Our results also revealed that callogenesis from the *in vitro* cultured anthers was substantially affected by applied IAA and Kin levels. The highest callusing was obtained in the cultures treated with 0.5 mg l^{-1} IAA along with 0.5 mg l^{-1} Kin. Working on anther culture in aromatic rice hybrids, Sakila et al. [33] noted that 1.0 mg l^{-1} of Kin is the optimal level for callus induction. However, relatively higher level (5.0 mg l^{-1}) of Kin has been reported to be more beneficial to callogenesis induction in *Solanum sessiliflorum* Dunal[34]. IAA has also been proved to be beneficial to androgenesis induction in wide variety of crop species[34,35]. According to Abate et al[35] IAA at 1.0

Table 4. Effect of various levels of 2, 4-D and BAP on callogenesis induction and shoot regeneration (SR) from *in vitro* cultured anthers of *Lycopersicon esculentum* Mill. cv. 'Berlina'

2, 4-D level (mg l^{-1})	BAP level (mg l^{-1})	Callogenesis (%)	SR (%)
	0	40 ± 7.3 d	-
0	1.0	33 ± 4.5 de	-
	2.0	24 ± 3.0 ef	-
	0	36 ± 4.9 de	-
1.0	1.0	58 ± 6.6 b	-
	2.0	49 ± 5.3 c	-
	0	27 ± 3.6 ef	-
2.0	1.0	51 ± 6.7 bc	-
	2.0	69 ± 7.8 a	-
	0	6 ± 1.3 g	-
3.0	1.0	21 ± 3.7 f	-
	2.0	26 ± 4.0 ef	-

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT (α = 0.05).

Table 5. Effect of various levels of chitosan treatment on callogenesis induction and shoot regeneration (SR) from *in vitro* cultured anthers of *Lycopersicon esculentum* Mill. cv. 'Berlina'

Chitosan level (mg l^{-1})	Callogenesis (%)	SR (%)
0	36 ± 5.7 c	-
10	47 ± 7.3 c	-
20	63 ± 8.1 b	-
50	85 ± 10.6 a	3 ± 0.5 a
100	42 ± 6.4 c	-

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT (α = 0.05).

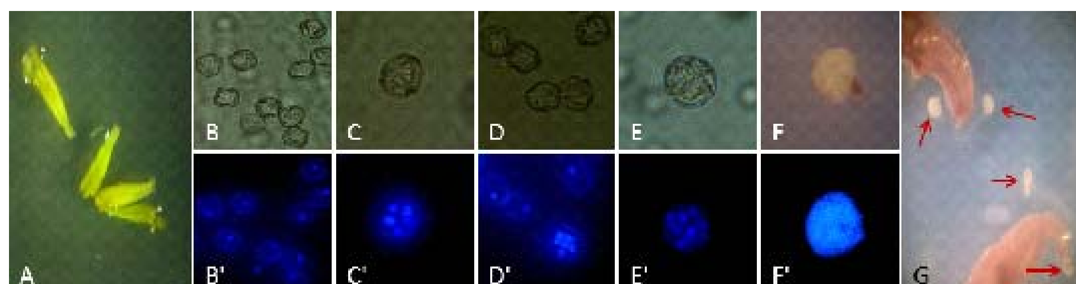

Fig. 2. Shed microspore culture in L.esulentum cv. Berlina. A: anthers at the initial culture; B, B: mid to late-uni-nucleate microspores; c, C': microspore-derived structures with 3-5 nuclei; D, D': microspore-derived structures with 6-8 nuclei; E, E'; microspore-derived structures with more than 9 nuclei; F, F'; microspore-derived embryo; G: globular and torpedo MDEs (Showed by arrows) after two weeks

mg l^{-1} profoundly increased callus formation form *in vitro* cultured anthers of tomato. Our results also indicated that, higher calli were produced when the induction medium was supplemented with 2.0 mg l^{-1} 2, 4-D and 2.0 mg l^{-1} BAP. Callus formation from cultured anthers of cucumber was also efficiently induced in the presence of 0.5 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} BAP[36]. Plant growth regulators, especially auxins and cytokinins, are widely used to androgenesis induction; however, their optimum concentration varies considerably from species to species[37].

According to our results, chitosan treatment significantly affected callogenesis induction and shoot initiation. The highest callusing and SR was observed from anthers treated with 50 mg l^{-1} chitosan. Chitosan, a polymeric deacetylated derivative of chitin, has been reported to act as a plant growth stimulator in some plant species[38,39]. Conversion of orchid's meristematic explants into the protocorm-like bodies was accelerated up to 15 times in the presence of shrimp and fungal chitosan in the liquid medium[38]. The stimulatory effect of chitosan has also been reported in *Vitis vinifera* L.[40], soybean sprouts[41], and *Ocimum basilicum* L.[42]. It has been reported that chitosan plays an important role in the enhancing growth and development by auxin biosynthesis pathway via tryptophan-independent pathway[43]. However, our results revealed that chitosan is not favorable to callogenesis induction and shoot regeneration when applied at high levels so that, calli formation significantly decreased in the cultures treated with 100 mg l^{-1} chitosan.

CONCLUSION

Anther and microspore culture provide an excellent platform to speed up breeding programs. However, these techniques are not available in tomato due to lack of knowledge in this recalcitrant species. We could efficiently induce microspore embryogenesis using shed microspore culture technique for the first time. In this technique, microspores shed from anthers into the liquid medium gradually instead of mechanically isolating. Therefore, microspores remain intact and not adversely affected by inductive stresses during the induction phase. According to our results, MDEs were only obtained in the double-layer B5 medium supplemented with 2% sucrose which exposed to 4°C for 2 days and then 30°C for 2 and 5 days. In addition, chitosan treatment (50 mg l^{-1}) significantly induced callogenesis and shoot regeneration form *in vitro* cultured anthers.

ACKNOWLEDGMENTS

This research was supported by grants from Agricultural Biotechnology Research Institute of Iran (ABRII) Project No. 12-05-05-9451-940001.

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