# Induction of symmetrical nucleus division and multi-nuclear structures in isolated microspores of sugarcane (*Saccharum officinarum* L.)

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## Abstract

In this study, the effects of floret sterilization with sodium hypochlorite, cold stress, heat shock, 2,4-dichlorophenoxyacetic acid and colchicine treatment on microspore viability and induction of symmetrical nuclei divisions were assessed in six genotypes of sugarcane. The highest microspore viability was observed when florets were sterilized with 3.0% and 3.5% sodium hypochlorite in all genotypes tested. More viable microspores were obtained in the cultures exposed to 4 °C. A sharp decrease was observed in viability at higher temperature pretreatments in all genotypes tested. Microspores with 6-10 nuclei were achieved in cultivars 'L62-96' and 'CP57-614' (5% and 18%) when cultures were pretreated at 4 °C. The nuclei division was strongly inhibited in the cultures exposed to 33 °C and 37 °C. High frequency of 3-5 nuclei microspores were obtained when 25 and 50 mg $l^{-1}$ 2,4-D were applied in the induction medium. Multinuclear microspores were only observed in cultivars 'L62-96' and 'CP57-614' (4% and 16%) and in the presence of 25 mg $l^{-1}$ colchicine, however, its higher level (100 $mgl^{-1}$) strongly inhibited nuclei division of cultured microspores. Symmetrical nucleus division could be induced in microspores of sugarcane when appropriate genotypes, temperature pretreatment and optimum level of 2,4-D and colchicine were used.

***Key words:*** Colchicine, Heat shock, 2,4-D, Microspore viability, *Saccharum officinarum* L.

## Abbreviations

2,4-D: 2, 4-Dichlorophenoxyacetic acid, DAPI: 4', 6-Diamidino-2-phenylindole, DH: Doubled haploid, FDA: Fluorescein diacetate, *FUS3: FUSCA3, GmAGL18: AGAMOUS-Like18,* HSPs: Heat-shock proteins, IMC: Isolated microspore culture, MCS: Multi-cellular structure, RAP-PCR: RNA arbitrarily primed PCR.

## INTRODUCTION

Sugarcane (*Saccharum officinarum* L.), tall perennial true grass belonging to the family of *Poaceae*, accounts for providing around 80% of the world's sugar. Sugarcane, an important industrial crop, is also used for ethanol and biomass production as an alternative source of energy (Dahlia *et al.,* 2010). There is an increasing pressure worldwide to enhance the productivity of sugarcane cultivation in order to sustain profitable sugar industries (Hanlon *et al.,* 2000). The conventional breeding programs are used

successfully to develop new hybrid varieties with high yielding potential and sugar contents. However, being a perennial crop, conventional breeding programs often require very long period of time to develop and release elite sugarcane varieties (Lakshmanan *et al.,* 2005). Haploid breeding technology through the anther culture or IMC has been developed successfully among wide varieties of crop species and has become quite popular in breeding programs, as it considerably reduces the time period required in developing homozygous lines (Agarwal *et al.,* 2006). In comparison with anther culture, IMC is simpleer and more affordable and therefore is the method of choice for plant genetic research and programs. Also, IMC is very useful for biochemical and physiological studies of embryogenesis, gene transformation and mutation and selection at early stages (Liu *et al.,* 2005; Brew-Appiah *et al.,* 2013). In addition, IMC gives a homogenous population of haploid or doubled haploid (DH) plants resulting from developing microspores, while removing anther wall tissue.

Under specific stress treatments, isolated microspores can be induced to deviate from their gametic developmental pathway and switch towards embryogenesis, forming haploid or DH embryos (Touraev *et al.,* 1996; Shariatpanahi *et al.,* 2006). Depending on the species, microspores are usually inducted by cold pretreatment (Ayed *et al.,* 2010), heat shock (Prem *et al.,* 2005) and its duration (Ahmadi *et al.,* 2012a), osmotic stress (Ayed *et al.,* 2010), carbon starvation (Kasha *et al.,* 2002), antimitotic (Soriano *et al.,* 2007) and mutagenic agents (Ahmadi *et al.,* 2012b), antioxidants (Hoseini *et al.,* 2014), stress hormones (Ahmadi *et al.,* 2014a), polyamines or antibiotics (Ahmadi *et al.,* 2014b).

Cold (4-10 °C) and heat shock (30-35 °C) are among the most commonly used pretreatments for the induction of microspore embryogenesis, although the type and duration of their application vary with the species or even the variety. Cold pretreatment is widely used in *Triticum turgidum* L. (Ayed *et al.,* 2010), *T. aestivum* L. (Khound *et al.,* 2013), *Hordeum vulgare* L. (Jacquard *et al.,* 2009) but very seldom in *Brassica* microspore culture (Gu *et al.,* 2004). Working on anther culture in 10 clones of *S. spontaneum* L., Fitch and Moore (1983) found that cold pretreatment of harvested panicles (10 °C for 21 d) efficiently switched the developmental pathway of nearly mature microspores towards sporophytic pathway but young microspores were killed following the inductive stressor. Transcriptomic analysis of cold pretreated (4 °C for 3-96 h in the dark) microspores of *H. vulgare* revealed that the genes encoding enzymes involved in oxidative stress, synthesis of jasmonic acid and the phenyl propanoid pathway as well as pathogenesis-related proteins were strongly up-regulated upon stress treatment (Jacquard *et al.,* 2009), all of which are involved in the somatic and microspore embryogenesis regulation (Maillot *et al.,* 2009; Zur *et al.,* 2009; Ahmadi *et al.,* 2014a). In addition to cold pretreatment, a short period of heat shock treatment is usually given to the cultured microspores to enhance the androgenic response in many plant species (Babbar *et al.,* 2004). Working on microspore culture in *B. napus* L., Ahmadi *et al.* (2012a) noted that elevated temperature (30°C) not only inducted microspore embryogenesis but also accelerated the process of embryogenesis. 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 (Fan *et al.,* 1988; Shariatpanahi *et al.,* 2006).

2,4-D, a synthetic auxinic herbicide, is a recently appreciated chemical inducer of microspore embryogenesis (Ardebili *et al.,* 2011). According to Ardebili *et al.* (2011), 2,4-D at 15-45 mg $l^{-1}$ could efficiently induce embryogenesis in *B. napus* microspores. In addition, 2,4-D has been widely used in anther culture (Rodriguez *et al.,* 2004; Robostova *et al.,* 2013) and somatic embryogenesis systems (Flower *et al.,*, 1998; Zheng and Perry 2014). Using RNA arbitrarily primed PCR (RAP-PCR), Flower *et al.* (1998) noted that 2,4-D stimulated cell division and enhanced somatic embryogenesis in cultured leaf explants of *Medicago falcate* L. by inducing calnexin-like protein synthesis, a 67 kDa integral protein and analogous to the heat-shock proteins (HSPs), which is implicated in developmental reprogramming of somatic and microspore embryogenesis induction (Dudits *et al.,* 1995; Touraev *et al.,* 1996).

Colchicine, an anti-microtubular alkaloid which binds to α- and β-tubulin heterodimers and causes de-polymerization of the microtubules (Dorléans *et al.,* 2009), also effectively induced microspore embryogenesis in *Brassicas* (Zhou *et al.,* 2002; Abraha *et al.,* 2008), coffee (Herrera *et al.,* 2002), *Zea mays* (Obert and Barnabás 2004) and *T. aestivum* (Soriano *et al.,* 2007) when exogenously applied in the induction medium. Since treatment of microspores with colchicine triggers microspore embryogenesis by displacing the microspore nucleus towards the center of the cell, it has been proposed that cytoskeleton rearrangements

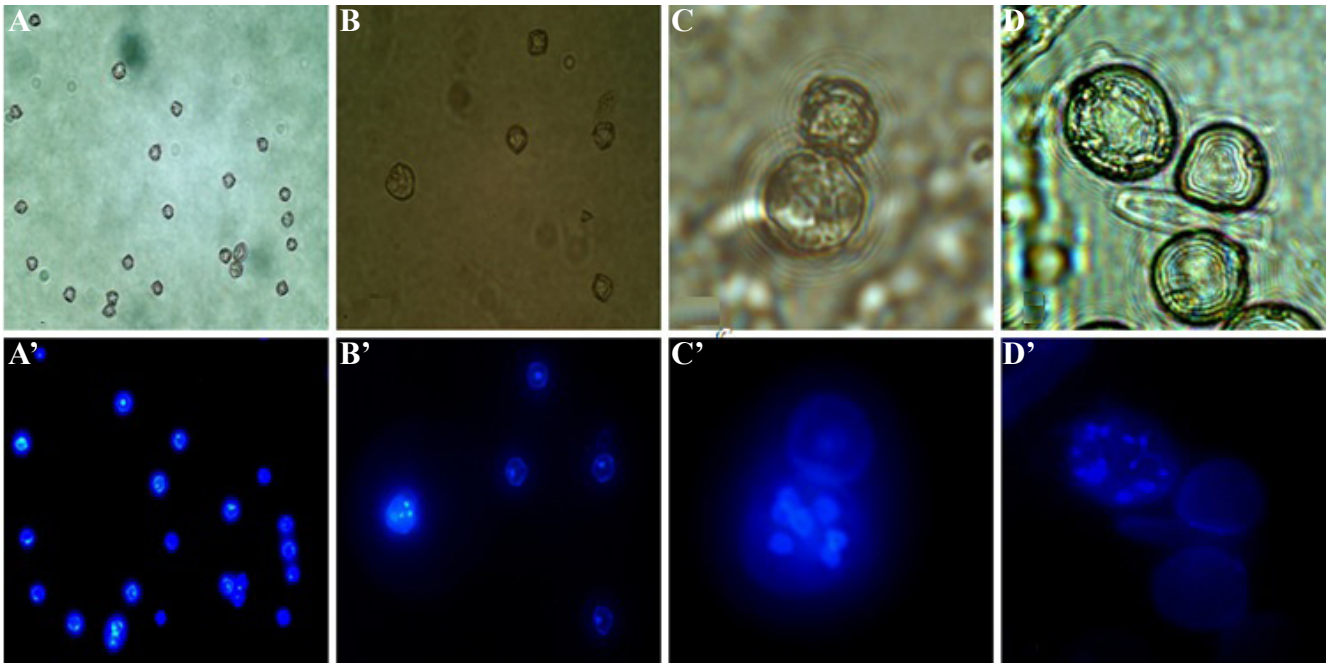


**Figure 1.** Induction of symmetrical nuclei divisions in isolated microspores of *S. officinarum* L. **A,A':** mixed population of mid to late-uni-nucleate microspores; **B, B':** 3-5 nuclei microspore in the cultures subjected to 30 °C; **C, C':** 6-10 nuclei microspore in the cultures incubated at 4 °C; **D, D':** Multinucleate microspores in the presence of colchicine (25 mg $l^{-1}$).

are involved in the microspore embryogenesis induction (Obert and Barnabás 2004; Maraschin *et al.,* 2005). However, the exact mechanism(s) underlying microspore embryogenesis inducted by colchicine treatment is not well explored.

To our knowledge, the culture of isolated microspores of sugarcane in liquid media has not been reported yet. In this study, the effects of floret sterilization, cold, heat shock, 2,4-D and colchicine treatment on microspore viability and induction of nuclei division were assessed in six genotypes of sugarcane.

## MATERIALS AND METHODS

### Donor plants and growth conditions

*Saccharum officinarum* L. Cultivars "CP45-3", "CP65-315", "L62-96", "CP59-73", "CP57-614" and "C113-1" were the test plants. Donor plants were grown in a green house with a day/night temperature of 33-35/23-25 °C and relative humidity of 60-80% under natural light condition in the Sugarcane Research and Training Institute of Khuzestan.

### Floret sterilization

Florets containing a mixed population of mid to late-uni-nucleate microspores, which was determined using 4', 6-diamidino-2-phenylindole (DAPI) nucleic acid stain (Figures 1A, 1A'), were surface sterilized with sodium hypochlorite (3.0, 3.5 and 4.0%) using gentle shaking for 10 min followed by two 5-min washes with cold (4 °C) sterile distilled water. These florets were placed in a glass tube and gently macerated into 10 ml of liquid microspore isolation medium (NLN-13, B medium or mannitol 0.3 M) using a sterile magnet bar. The crude suspension was filtered through a 40 µm metal mesh (Damavand Tes Sieve Ltd. Tehran, Iran), collected into two 15 ml centrifuge tubes and the volume was adjusted with fresh isolation medium to 10 ml. The filtrate was centrifuged at 100×*g* for 4 min. The supernatant was decanted and the pellet was rinsed in fresh isolation medium. This procedure was repeated twice. Finally, the plating density was adjusted at $2\times10^4$ microspores $ml^{-1}$ using a hemocytometer (Precicolor, Germany) by adding liquid NLN-13 medium (Lichter 1982, as embryogenesis induction medium) and the suspension was dispensed (5 ml) into 6 cm sterile plastic Petri dishes (Farazbin, Tehran, Iran).

### Cold, heat, 2,4-D and colchicine treatment

After determining plating density and dispensing suspension into the Petri dishes, cultures were pretreated at 4, 30, 33 or 37 °C for 5 days for cold and heat pretreatment and then transferred to 25 °C in the dark. 2,4-D (Duchefa Biochemie) was dissolved in ethanol (99%) and colchicine (Sigma Aldrich, St. Louis, MO) was dissolved in double-distilled water and with gentle

**Table 1.** Results of analysis of variance of interaction effect of genotype and different applied concentration of sodium hypochlorite on microspore viability of *Saccharum officinarum* L.

| Source of variation | df | Mean squares |
|---|---|---|
| Genotype | 5 | 2348.56** |
| Sodium hypochlorite concentration | 2 | 637.90** |
| Genotype×sodium hypochlorite concentration | 10 | 36.86* |
| Error | 36 | 14.42 |
| Coefficient of variation (%) | 7.25 | |

**,*: Significant at 1% and 5% probability level, respectively; ns: not significant.

shaking at room temperature (25 °C) in the dark. The pH was adjusted to 6.0 with 1 N NaOH and 1 N HCL and maintained in the dark at 4 °C until needed. After determining plating density ($2\times10^4$ microspores $ml^{-1}$) and dispensing microspore suspension into Petri dishes, filter-sterilized (0.22 µm filter) 2,4-D or colchicine (0, 25, 50 and 100 mg $l^{-1}$) was added to the Petri dishes and incubated at 25 °C for 30 min (for 2,4-D treatment) or 2 days (for colchicine treatment) in the dark. Residuals were removed by centrifugation at 100×*g* for 5 min. Plating density was adjusted to $2\times10^4$ microspores $ml^{-1}$ by adding liquid NLN-13 medium and the suspension was dispensed into the same Petri dishes and incubated at 25 °C.

**FDA and DAPI staining**

Fluorescein diacetate (FDA, Sigma Aldrich, St. Louis, MO) hydrolysis assay was used to determine the initial microspore viability immediately following floret sterilizing and microspore isolation according to Heslop-Harrison and Heslop-Harrison (1970). The same method was also used to assess the microspores viability 5 days after being exposed to cold, heat, 2,4-D and colchicine treatment. For this purpose, 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 stained with one drop of the FDA then, samples were observed using an inverted microscope (Nikon Eclipse TE 2000-S) with fluorescent illumination. In each sample, the ratio of shining microspores to total counted microspores was considered as microspore viability (Heslop-Harrison and Heslop-Harrison, 1970).

Nuclei divisions were also detected two weeks following microspore culture using blue-fluorescent DAPI nucleic acid staining, which preferentially stains doubled-stranded DNA. 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 at room temperature. 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 at room temperature. Samples were observed using an inverted microscope (Nikon Eclipse TE 2000-S) with fluorescent illumination.

**Experimental design and statistical analysis**

The experiments were conducted in a factorial experiment based on a completely randomized design (CRD) to evaluate the effect of different factors. Entire experiments were repeated twice. Each treatment had three replications (Petri dishes). Data analyses were performed using SPSS software version 17 and the means were compared using Duncan's multiple range (DMRT) test at $\alpha=0.05$ following analysis of variance.

## RESULTS

**Effects of floret sterilization on viability of microspores**

The results of analysis of variance showed a significant effect of floret sterilization by sodium hypochlorite on viability of microspores at 1% probability level (Table 1). Based on the results of analysis of variance, the interaction effect of genotype×concentrations of sodium hypochlorite on viability of microspores was significant at 5% probability level (Table 1). The results of means comparison analysis showed that the highest mean of microspore viability was observed when the florets were surface sterilized with 3.0% and 3.5% sodium hypochlorite in all tested genotypes (Table 2). However, microspore viability sharply decreased as sodium hypochlorite level was increased (Table 2).

**Effects of cold and heat treatments on viability of microspores**

The results of analysis of variance showed that the microspore viability was significantly affected by the temperature pretreatments (Table 3). Based

**Table 2.** Viability of microspores (%) following floret sterilization with sodium hypochlorite in six cultivars of *Saccharum officinarum* L.

| Hypochlorite level (%) | Cultivars (microspore viability %) | | | | | |
|---|---|---|---|---|---|---|
| | CP45-3 | CP65-315 | L62-96 | CP59-73 | CP57-614 | C113-1 |
| 3.0 | 86±12.8[a]* | 83±11.6[a] | 80±7.9[a] | 84±8.8[a] | 78±8.5[a] | 83±12.6[a] |
| 3.5 | 79±9.7[b] | 75±9.3[b] | 71±12.4[b] | 76±10.7[b] | 81±7.4[a] | 77±11.0[a] |
| 4.0 | 64±8.2[c] | 55±10.5[c] | 63±9.2[c] | 61±7.6[c] | 66±6.1[b] | 53±8.3[b] |

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

**Table 3.** Results of analysis of variance of interaction effect of genotype and different applied temperature pretreatments on microspore viability of *Saccharum officinarum* L.

| Source of variation | df | Mean squares |
|---|---|---|
| Genotype | 5 | 792.19** |
| Heat stress | 3 | 4264.19** |
| Genotype×Heat stress | 15 | 160.57** |
| Error | 48 | 14.30 |
| Coefficient of variation (%) | 9.38 | |

**: Significant at 1% probability level.

**Table 4.** Viability of microspores (%) five days after temperature pretreatment in six cultivars of *Saccharum officinarum* L.

| Temperature treatment (°C) | Cultivars (microspore viability %) | | | | | |
|---|---|---|---|---|---|---|
| | CP45-3 | CP65-315 | L62-96 | CP59-73 | CP57-614 | C113-1 |
| 4 | 73±10.6[a]* | 66±7.2[a] | 71±11.4[a] | 68±9.1[a] | 59±6.7[a] | 69±13.8[a] |
| 30 | 52±11.9[b] | 44±14.1[b] | 38±10.7[c] | 45±8.9[b] | 37±9.5[b] | 37±12.2[b] |
| 33 | 49±13.1[b] | 41±11.0[bc] | 45±13.4[b] | 47±10.6[b] | 23±11.7[c] | 42±9.1[b] |
| 37 | 27±7.8[c] | 25±10.2[c] | 19±9.3[d] | 21±10.5[c] | 16±7.7[d] | 30±12.1[c] |

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

on the results of ANOVA, the interaction effect of genotype×heat treatment on the microspore viability of sugarcane was significant at 1% probability level (Table 3). The results of means comparison analysis showed that the highest mean of microspore viability was obtained in the cultures exposed to 4 °C in all investigated genotypes and sharp decrease in viability was observed at higher temperature pretreatments (Table 4). However, there was no significant difference between viability of microspores pretreated at 30 and 33 °C in all genotypes tested, except for L62-96, and CP57-614 genotypes (Table 4).

The microscopic observations of symmetrical nuclei divisions in isolated microspores showed that the highest frequency (31%) of microspores with 3-5 nuclei (Figures 1B, 1B') were observed in cv. 'CP57-614'in the cultures subjected to 30 °C (Table 5), but the highest frequency of 6-10 nuclei microspores (Figures 1C, 1C') was observed in the cultures incubated at 4 °C for 5 days in cv. 'CP57-614' (Table 5). The results of means comparison analysis showed that higher temperature pretreatments were not advantageous so that, nuclei division was completely inhibited in the microspores exposed to 37 °C in all investigated genotypes (Table 5).

**Effect of 2,4-D and colchicine treatments on viability of microspores**

The results of analysis of variance showed that microspore viability was significantly affected by 2,4-D and colchicine treatment, respectively (Tables 6 and 7). The results of means comparison analysis revealed that the microspore viability of all investigated genotypes of sugarcane decreased as 2,4-D level was increased and the highest and lowest means of viability were observed from interaction effect of CP113-1×25 mg $l^{-1}$

**Table 5.** Frequency (%) of microspores with symmetrically divided nuclei two weeks after temperature pretreatment in six cultivars of *Saccharum officinarum* L.

| Temperature treatment (°C) | Cultivar | Non-divided microspores (%) | Nuclei division (%) | |
|---|---|---|---|---|
| | | 1-2 | 3-5 | 6-10 |
| 4 | CP45-3 | 83±9.8$^{c}$* | 17±9.8$^{bc}$ | - |
| | CP65-315 | 91±7.4$^{b}$ | 9±7.4$^{d}$ | - |
| | L62-96 | 73±11.9$^{de}$ | 22±6.1$^{b}$ | 5±2.8$^{b}$ |
| | CP59-73 | 84±8.8$^{c}$ | 16±8.8$^{bcd}$ | - |
| | CP57-614 | 68±13.1$^{e}$ | 14±6.3$^{cd}$ | 18±4.5$^{a}$ |
| | C113-1 | 95±4.8$^{ab}$ | 5±4.8$^{de}$ | - |
| 30 | CP45-3 | 87±8.2$^{bc}$ | 13±8.2$^{cd}$ | - |
| | CP65-315 | 80±12.4$^{d}$ | 20±12.4$^{b}$ | - |
| | L62-96 | 84±9.3$^{c}$ | 16±9.3$^{bcd}$ | - |
| | CP59-73 | 93±6.6$^{b}$ | 7±6.6$^{de}$ | - |
| | CP57-614 | 69±12.9$^{e}$ | 31±12.9$^{a}$ | - |
| | C113-1 | 86±8.7$^{c}$ | 14±8.7$^{cd}$ | - |
| 33 | CP45-3 | 100$^{a}$ | - | - |
| | CP65-315 | 100$^{a}$ | - | - |
| | L62-96 | 100$^{a}$ | - | - |
| | CP59-73 | 100$^{a}$ | - | - |
| | CP57-614 | 93±5.4$^{ab}$ | 7±5.4$^{de}$ | - |
| | C113-1 | 100$^{a}$ | - | - |
| 37 | CP45-3 | 100$^{a}$ | - | - |
| | CP65-315 | 100$^{a}$ | - | - |
| | L62-96 | 100$^{a}$ | - | - |
| | CP59-73 | 100$^{a}$ | - | - |
| | CP57-614 | 100$^{a}$ | - | - |
| | C113-1 | 100$^{a}$ | - | - |

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

**Table 6.** Results of analysis of variance of interaction effect of genotype and different applied concentration of 2,4-D on microspore viability of *Saccharum officinarum* L.

| Source of variation | df | Mean squares |
|---|---|---|
| Genotype | 5 | 67.33$^{**}$ |
| 2,4-D concentration | 3 | 8.44$^{**}$ |
| Genotype×2,4-D concentration | 15 | 2.24$^{*}$ |
| Error | 48 | 1.19 |
| Coefficient of variation (%) | 23.66 | |

**,*: Significant at 1% and 5% probability level, respectively; ns: not significant.

2,4-D, and CP57-614×100 mg $l^{-1}$ 2,4-D, respectively (Table 8). The results of means comparison analysis showed that colchicine at 50 and 100 mg $l^{-1}$ resulted in a significant reduction in the viability of cultured microspores in all investigated genotypes of sugarcane (Table 9). In this experiment, the highest and lowest means of viability were observed from interaction effect of L62-96×25 mg $l^{-1}$ colchicine, and CP59-73× 100 mg $l^{-1}$ colchicine, respectively (Table 9).

The results of means comparison analysis of interaction effect of genotype×2,4-D level on frequency of microspores with symmetrically divided nuclei showed that the highest frequency of microspores with 3-5 nuclei was achieved when 25 and/or 50 mg $l^{-1}$ of 2,4-D were applied in the induction medium (Table 10). However, all microspores failed to proceed further, so that no microspore with 6-10 or more nuclei was observed following 2,4-D treatment in all tested

**Table 7.** Results of analysis of variance of interaction effect of genotype and different applied concentration of colchicine on microspore viability of *Saccharum officinarum* L.

| Source of variation | df | Mean squares |
|---|---|---|
| Genotype | 5 | 771.16** |
| Colchicine concentration | 3 | 346.40** |
| Genotype×Colchicine concentration | 15 | 67.12** |
| Error | 48 | 2.83 |
| Coefficient of variation (%) | 10.41 | |

**: Significant at 1% probability level.

**Table 8.** Viability of microspores (%) five days after 2,4-D treatment in six cultivars of *Saccharum officinarum* L.

| 2,4-D treatment (mg $l^{-1}$) | Cultivars (microspore viability %) | | | | | |
|---|---|---|---|---|---|---|
| | CP45-3 | CP65-315 | L62-96 | CP59-73 | CP57-614 | C113-1 |
| 0 | 47±9.6$^{a}$* | 43±12.6$^{ab}$ | 41±10.5$^{a}$ | 59±11.0$^{a}$ | 39±13.3$^{a}$ | 52±10.9$^{a}$ |
| 25 | 41±12.8$^{ab}$ | 49±9.3$^{a}$ | 39±11.8$^{ab}$ | 32±10.1$^{b}$ | 28±7.6$^{b}$ | 49±11.2$^{a}$ |
| 50 | 33±8.9$^{b}$ | 35±10.5$^{b}$ | 28±7.7$^{b}$ | 23±6.4$^{c}$ | 21±9.2$^{c}$ | 34±7.4$^{b}$ |
| 100 | 12±5.1$^{c}$ | 15±6.7$^{c}$ | 17±4.9$^{c}$ | 12±5.5$^{d}$ | 7±4.1$^{d}$ | 19±6.0$^{c}$ |

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

**Table 9.** Viability of microspores (%) five days after colchicine treatment in six cultivars of *Saccharum officinarum* L.

| Colchicine treatment (mg $l^{-1}$) | Cultivars (microspore viability %) | | | | | |
|---|---|---|---|---|---|---|
| | CP45-3 | CP65-315 | L62-96 | CP59-73 | CP57-614 | C113-1 |
| 0 | 53±11.9$^{a}$* | 47±9.3$^{a}$ | 46±11.7$^{b}$ | 52±9.0$^{a}$ | 43±10.4$^{ab}$ | 48±9.9$^{a}$ |
| 25 | 46±8.1$^{b}$ | 44±6.9$^{a}$ | 53±9.5$^{a}$ | 49±6.7$^{a}$ | 47±9.8$^{a}$ | 44±10.0$^{a}$ |
| 50 | 31±5.4$^{bc}$ | 27±6.6$^{b}$ | 34±4.3$^{c}$ | 30±7.1$^{b}$ | 36±5.3$^{b}$ | 27±7.6$^{b}$ |
| 100 | 22±6.2$^{c}$ | 17±5.7$^{c}$ | 24±6.9$^{d}$ | 15±5.0$^{c}$ | 20±7.7$^{c}$ | 17±5.8$^{c}$ |

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

genotypes (Table 10).

The microscopic observations of symmetrical nuclei divisions in the isolated microspores of investigated genotypes of *S. officinarum* revealed that multinucleate microspores were only obtained in cultivars 'L62-96' and 'CP57-614' (4% and 16%, respectively) and in the presence of 25 mg $l^{-1}$ colchicine (Figures 1D, 1D'), therefore, the best interaction effects of genotype×colchicine levels for frequency of multinucleate microspores were CP57-614×25 mg $l^{-1}$, and L62-96×25 mg $l^{-1}$ colchicine, respectively (Table 11). However, the higher level of colchicine (100 mg $l^{-1}$) strongly inhibited nuclei division of the cultured microspores (Table 11).

## DISCUSSION

The first step for microspore embryogenesis is the isolation of viable microspores. Optimal sterilization regime, on the one hand, has to provide reliable defense for all types of infections, and on the other hand, keep treated tissues intact and viable. According to our results, the highest microspore viability was observed when the florets were sterilized with 3.0 and 3.5% sodium hypochlorite in all tested genotypes. Working on microspore embryogenesis induction in two tetraploid *Rosa hybrida* L. genotypes, Dehestani Ardakani *et al.* (2016) noted that hypochlorite at 3.5% for 10 min was the optimum level for bud sterilization and higher levels significantly decreased the viability of isolated microspores.

Cold and heat shock are critically important pretreatments required for blocking gametophytic developmental pathway and inducing embryogenesis in competent cultured microspores. However, responses to such triggering stresses are species- and

**Table 10.** Frequency (%) of microspores with symmetrically divided nuclei two weeks after 2,4-D treatment in six cultivars of *Saccharum officinarum* L.

| 2,4-D level (mg $l^{-1}$) | Cultivar | Non-divided microspores (%) | Nuclei division (%) | |
|---|---|---|---|---|
| | | 1-2 | 3-5 | 6-10 |
| 0 | CP45-3 | 82±6.3$^{cd}$* | 18±6.3$^{de}$ | - |
| | CP65-315 | 88±7.5$^{c}$ | 12±7.5$^{f}$ | - |
| | L62-96 | 81±5.2$^{d}$ | 19±5.2$^{de}$ | - |
| | CP59-73 | 88±4.0$^{cd}$ | 12±4.0$^{f}$ | - |
| | CP57-614 | 79±8.6$^{de}$ | 21±8.6$^{d}$ | - |
| | C113-1 | 91±4.4$^{bc}$ | 9±4.4$^{g}$ | - |
| 25 | CP45-3 | 61±11.9$^{f}$ | 39±11.9$^{b}$ | - |
| | CP65-315 | 73±8.8$^{e}$ | 27±8.8$^{cd}$ | - |
| | L62-96 | 55±12.3$^{g}$ | 45±12.3$^{a}$ | - |
| | CP59-73 | 79±6.5$^{de}$ | 21±6.5$^{de}$ | - |
| | CP57-614 | 70±7.2$^{e}$ | 30±7.2$^{cd}$ | - |
| | C113-1 | 85±5.3$^{cd}$ | 15±5.3$^{e}$ | - |
| 50 | CP45-3 | 88±4.7$^{c}$ | 12±7.7$^{f}$ | - |
| | CP65-315 | 81±8.7$^{d}$ | 19±8.7$^{de}$ | - |
| | L62-96 | 73±10.2$^{e}$ | 27±10.2$^{cd}$ | - |
| | CP59-73 | 92±3.7$^{bc}$ | 8±3.7$^{gh}$ | - |
| | CP57-614 | 66±9.6$^{fgh}$ | 34±9.6$^{c}$ | - |
| | C113-1 | 85±8.1$^{cd}$ | 15±8.1$^{e}$ | - |
| 100 | CP45-3 | 100$^{a}$ | - | - |
| | CP65-315 | 100$^{a}$ | - | - |
| | L62-96 | 96±3.6$^{b}$ | 4±3.6$^{h}$ | - |
| | CP59-73 | 100$^{a}$ | - | - |
| | CP57-614 | 82±4.0$^{cd}$ | 18±4.0$^{de}$ | - |
| | C113-1 | 100$^{a}$ | - | - |

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

even genotype-dependent. In this study, the highest microspore viability was achieved in the cultures pretreated at 4 °C and viability strongly decreased as temperature was increased. Working on triticale (×*Triticosecale* Wittm.) microspore culture, Zur *et al.* (2008) observed more viable microspores in the cultures exposed to cold treatment (4 °C) and the yield of isolated viable microspores dropped by cold pretreatment. In the present study, cold and heat pretreatment not only affected microspore viability but also induced nuclei division of cultured microspores so that, the highest nuclei division (6-10 nuclei) was achieved in the cultivars 'L62-96' and 'CP65-315' when microspores were pretreated at 4 °C. Working on anther culture in *S. spontaneum* L., Fitch and Moore (1983) noted that pre-mitotic microspores died upon cold pretreatment (10 °C for 3-4 weeks) but uni- to bi-nucleate microspores often developed into 4 to 6-celled structures during the cold pretreatment. In addition, tri-nucleate microspores at starch-filled stage did not visibly change when pretreated with cold but died when transferred to the culture medium (Fitch and Moore 1983). According to Khound *et al.* (2013), more multi-cellular structures (MCS) were produced after incubating the excised anthers at 4 °C for 5 days compared with untreated anthers in spring cultivars of *T. aestivum*. However, cytological analysis of 30-day-old cultured anthers in soybean (*Glycine max* L.) showed that MCS could be formed following both cold (4 °C) and heat (33 °C) pretreatments and the results of these treatments were not significantly different (Moraes *et al.,* 2004). According to our results, heat shock was less effective than cold treatment and nuclei division was strongly inhibited by 33 °C and 37 °C treatments.

2,4-D is a recently appreciated chemical inducer of microspore embryogenesis and information about its regulatory role(s) in the induction of embryogenesis is rather limited. In this study, viability of cultured microspores were significantly affected by 2,4-D

**Table 11.** Frequency (%) of microspores with symmetrically divided nuclei two weeks after colchicine treatment in six cultivars of *Saccharum officinarum* L.

| Colchicine level (mg $l^{-1}$) | Cultivar | Non-divided microspores (%) | Nuclei division (%) | | |
|---|---|---|---|---|---|
| | | 1-2 | 3-5 | 6-10 | Multinucleate |
| 0 | CP45-3 | 76±8.6$^{e}$* | 24±8.6$^{cd}$ | - | - |
| | CP65-315 | 93±5.1$^{b}$ | 7±5.1$^{f}$ | - | - |
| | L62-96 | 72±9.5$^{ef}$ | 28±9.5$^{cd}$ | - | - |
| | CP59-73 | 85±6.9$^{cd}$ | 15±6.9$^{e}$ | - | - |
| | CP57-614 | 68±8.0$^{ef}$ | 32±8.0$^{c}$ | - | - |
| | C113-1 | 88±7.8$^{cd}$ | 12±7.8$^{e}$ | - | - |
| 25 | CP45-3 | 57±11.2$^{g}$ | 43±11.2$^{b}$ | - | - |
| | CP65-315 | 52±9.2$^{gh}$ | 39±5.6$^{bc}$ | 9±3.9$^{d}$ | - |
| | L62-96 | 36±6.3$^{j}$ | 42±5.4$^{a}$ | 18±3.2$^{b}$ | 4±2.6$^{b}$ |
| | CP59-73 | 65±7.6$^{f}$ | 22±6.5$^{cd}$ | 13±4.1$^{c}$ | |
| | CP57-614 | 44±7.3$^{h}$ | 25±5.9$^{cd}$ | 15±5.1$^{bc}$ | 16±4.2$^{a}$ |
| | C113-1 | 72±10.6$^{ef}$ | 28±10.6$^{cd}$ | - | - |
| 50 | CP45-3 | 68±8.5$^{f}$ | 32±8.5$^{c}$ | - | - |
| | CP65-315 | 64±8.8$^{f}$ | 30±5.7$^{bc}$ | 6±4.4 d$^{e}$ | - |
| | L62-96 | 42±6.0$^{i}$ | 47±7.9$^{a}$ | 11±3.5$^{cd}$ | - |
| | CP59-73 | 76±9.1$^{e}$ | 22±5.7$^{d}$ | 2±1.3$^{e}$ | - |
| | CP57-614 | 52±6.6$^{gh}$ | 25±4.1$^{cd}$ | 23±5.7$^{a}$ | - |
| | C113-1 | 85±5.5$^{cd}$ | 15±5.5$^{e}$ | - | - |
| 100 | CP45-3 | 100±a | - | - | - |
| | CP65-315 | 100±a | - | - | - |
| | L62-96 | 83±6.3$^{cd}$ | 17±6.3$^{de}$ | - | - |
| | CP59-73 | 100±a | - | - | - |
| | CP57-614 | 88±4.7$^{c}$ | 12±4.7$^{e}$ | - | - |
| | C113-1 | 100±a | - | - | - |

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

application. The highest microspore viability was observed in the untreated cultures and also in the presence of 25 mg $l^{-1}$ 2,4-D in all genotypes tested and viability significantly decreased as 2,4-D level was increased. Working on *B. napus* microspore culture, Ardebili *et al.* (2011) noted that 2,4-D treatment (15-45 mg $l^{-1}$) decreased microspore viability relative to untreated cultures at all durations tested. Nuclei division of cultured microspores was also affected by 2,4-D treatment. According to our results, high frequency of microspores with 3-5 nuclei was observed in the cultures subjected to 25 mg $l^{-1}$ 2,4-D, however, all failed to proceed further. Working on somatic embryogenesis in *G. max*, Zheng and Perry (2014) observed that some genes i.e. *AGAMOUS-Like18* (*GmAGL18*) and *FUSCA3* (*FUS3*), which promoted somatic embryogenesis in *Arabidopsis thaliana* and *G. max* when ectopically expressed (Zheng *et al.,* 2013), were up-regulated 3 days after placement immature cotyledon explants in the induction medium containing 40 mg $l^{-1}$2,4-D. 2,4-D causes plethora alterations in the gene expression pattern during somatic embryogenesis induction phase which provides convincing evidence for its implication in this process (Feher *et al.,* 2003). However, according to our results, inhibitory effects of 2,4-D at high levels were observed so that, nuclei division was strongly inhibited when 100 mg $l^{-1}$ was used in the induction medium.

Colchicine treatment at 50 and 100 mg $l^{-1}$ significantly decreased viability of microspores in all genotypes tested. Working on microspore embryogenesis in tomato (*Lycopersicon esculentum* Mill.), Seguí-Simarro and Nuez (2007) found that microspore viability dramatically decreased to about half when 100 mg $l^{-1}$ colchicine was applied in the pretreatment medium. Colchicine is a cytoskeleton-disrupting drug widely used in DH technology to stimulate the first embryogenic divisions and to induce genome doubling in haploid embryos (Shariatpanahi *et al.,* 2006), but its concentration-dependent effects

on cell cycle and microtubule arrangements, a major component of cell function, greatly affects cell progression and viability (Caperta *et al.,* 2006). However, according to our results, colchicine was not detrimental to microspore viability when applied at low concentration i.e. 25 mg $l^{-1}$. Multinucleate microspores were also obtained in cultivars 'L62-96' and 'CP57-614' following colchicine treatment (25 mg $l^{-1}$). The exact biological mechanism(s) underlying microspore embryogenesis induced by colchicine is poorly studied. Re-organization of the microtubule or actin filament cytoskeleton during colchicine treatment is a critical event during microspore embryogenesis induction (Zhao *et al.,* 2003). However, our results revealed that 50 mg $l^{-1}$ colchicine was less effective (in comparison with 25 mg $l^{-1}$) and 100 mg $l^{-1}$ was detrimental to nuclei division in all tested genotypes.

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