African Journal of Biotechnology Vol. 10(59), pp. 12535-12541, 3 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1287
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Microspore derived embryo formation and doubled haploid plant production in broccoli (*Brassica oleracea* L. var *italica*) according to nutritional and environmental conditions

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Accepted 17 June, 2011

In cell culture, the maintenance of proper growing conditions is a key approach for improving the formation of embryos, and is useful in the production of doubled haploid (DH) plants. Optimal nutritional and environmental conditions for the microspore culture of *Brassica oleracea* L. *var italica* were determined in order to reduce time and effort in breeding. The optimal conditions for microspore embryo formation differed depending on genotype. Microspore-derived embryos (MDE) formation was influenced by the strength of the NLN medium, the microelement and sugar concentration, and the heat shock temperature and period. The 0.5XNLN liquid medium was the most favorable for MDE formation. The most efficient formation of MDE was observed in the 0.5X NLN liquid medium, without the addition of microelements. When 13 or 15% sucrose was added to the 0.5X NLN liquid medium, the amount of normal MDE formation increased. The optimum heat shock temperature and period for MDE formation was 32.5°C and 24 h, respectively. A polyploidy test indicated that 30% of the microspore derived plants were diploid throughout the embryogenesis process.

Key words: Embryogenesis, heat shock, microelements, NLN medium, polyploidy test.

INTRODUCTION

Broccoli is considered as a major vegetable, having high nutritional value with various functional materials such as selenium, sulforaphane, indol-3-carbinol and folic acid. It is also a well-known antioxidant food. The microspore culture of *Brassica* plants is a very valuable tool for genetic manipulation via haploid breeding; however, the production of homozygous lines through bud pollination is time consuming and labor intensive. Microspore culture is an efficient technology for the production of homozygous lines when producing F_1 hybrids of modern cultivars, leading to an increase in selection efficiency for desirable genetic recombinants (Dias, 1999). Microspore derived plants provide a rapid means of obtaining homozygous and homogeneous lines of agriculturally important plants (Dias, 2001).

Microspore culture has been used to produce haploid and doubled haploid plants in the genus *Brassica* (Keller and Armstrong, 1979; Lichter, 1989). These plants can be utilized in varietal development, mutant selection, and biochemical and genetic engineering studies (Swanson and Erickson, 1989; Swanson et al., 1988; Taylor et al., 1993). The doubled haploid parental lines can enhance and accelerate plant breeding programs by saving labor and time. These lines have already been developed using anther culture (Farnham, 1998), and they have been introduced into breeding schemes. Successful microspore culture in different broccoli genotypes has been described by Duijs et al. (1992) and Takahata and Keller (1991).

One problem with the practical application of microspore culture, reported by different authors (Dias, 1999; Duijs et al., 1992), is the very low embryo yield in many

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Figure 1. Flower bud removed sepals of *Brassica oleracea* L.*var italica* for microspore derived embryo culture. The stigma is longer than the length of the floral leaf.

broccoli genotypes. There has always been an attempt to adapt and improve the current microspore culture protocols to make this technique available for haploid breeding. There are a few published reports on microspore embryogenesis in broccoli, but improvement in the microspore culture protocols is required. Several factors influencing microspore embryogenesis are donor plant conditions, genotype, developmental stage, media constituents and culture conditions. The objective of this paper was to study nutritional, chemical and physical factors affecting microspore derived embryo (MDE) formation in broccoli, and to also verify the polyploidy of microspore-derived plantlets.

MATERIALS AND METHODS

Gene source K005262 provided by the National Agrobiodiversity Center, located at Suwon Korea was used for donor plants. The donor plants were grown using plastic pots (50 x 29 cm) in a greenhouse under a 16 h photoperiod with 400 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Later, they were vernalized in a cold room maintained at 4±1°C under a 16 h photoperiod with 400 µmol m^{-2} s^{-1} PPFD for eight weeks. After floral differentiation and the start of generative development, plants were transferred to a greenhouse at 25°C under a 16 h photoperiod with 400 µmol m^{-2} s^{-1} PPFD.

Microspore isolation

Flower buds having a shorter floral leaf length as compared to the length of the stigma were chosen. The buds of this stage contained anthers at the late uninucleate stage of microspore development, and their size was 2 to 4 mm (Figure 1). The buds were wrapped in gauze and surface sterilized in 1% sodium hypochlorite for 15 min on the shaker at 70 rpm, and then rinsed three times for 3 minutes each, using sterile water. The buds were gently macerated with 2 ml of B5-13 medium (Gamborg et al., 1968), and ground using a mortar. They were filtered through a 45 µm metal mesh screen, and collected in a 50 ml centrifuge tube. The microspore suspension was washed three times with 10 ml of B5-13 medium by centrifuging at 1,000 rpm for 3 min. Then, the supernatant was removed and pelleted microspores were re-suspended at a density of 40,000 microspores to 1 ml of NLN liquid medium (Lichter, 1982). The number of microspores was estimated using a hemacytometer. The last microspore suspension was re-suspended in NLN liquid medium with 13% sucrose. We dispensed 2.5 ml of the microspore suspension into a 60 x 15 mm sterile Petri-dish that was subsequently sealed with parafilm. All culture media was adjusted to pH 5.8 using NaOH or HCl and filter-sterilized using a 25 µm low protein binding membrane filter (Corning, USA). After a 24h heat shock treatment and 14day incubation in darkness, all microspores were placed on a shaker at 60 rpm and 25°C under a 16 h photoperiod with 50 µmol m^{-2} s^{-1} PPFD (cool, white fluorescent lamps) for 2 weeks.

Microspore treatments

Microspores were incubated in the dark at 32.5°C during the 24 h heat shock treatment, and then transferred to 25°C in the dark. After 15 days, the Petri-dishes were placed on a shaker and agitated at 60 rpm with a 16 h photoperiod at 25°C. The embryo number was scored four weeks after microspore isolation (Figure 2A). Microspores were cultured with various NLN liquid medium strengths (0.25X, 0.5X, 1.0X, 2.0X and 4.0X) to investigate the effect of NLN on MDE induction. To investigate the effects of sugar concentration on embryonic induction, microspores were cultured in 0.5X NLN liquid medium containing 0, 3, 5, 10, 13, 15 and 20% sucrose. Microspores were cultured at four different heat shock temperatures of 25, 32.5, 37 and 42°C for 24h in order to determine the optimal temperature for MDE formation. The heat shock period was also varied at 0, 24, 48 and 72 h at 32.5°C. After 30 days of culture, the number of embryos in each Petri-dish was counted. The experiment was conducted with ten replications. Embryo yields were calculated as the average of ten Petri-dishes.

Germination of microspore derived embryos

For the conversion of microspore embryos into plantlets, the fully developed dicotyledonous embryos and torpedo embryos were picked up and transferred directly to MS medium containing 3% sucrose and 8% agar (Figure 2A and B). All microspore embryos were incubated at 25 ± 1°C under a 16 h photoperiod with 50 mol m^{-2} s^{-1} PPFD (cool, white fluorescent lamps) for 4 weeks. These were transferred *ex vitro* (Figure 2C).

Ploidy analysis using flow cytometry

The nuclear DNA content of the leaves of microspore-derived plantletswasmeasured with a flow cytometer (Cytoflow PA,Partec GmbH, Germany) using the protocol described by Mishiba et al. (2000). Seedling leaves of K005262 (2n = 2x = 18) were used as a standard. Young leaves (0.3-0.5 cm2) from microspore-derived plantlets and seedlings were analyzed for their nuclear DNA content. Fresh tissues were individually chopped with a sharp razor blade to less than 1 mm in a 6 cm glass petri-dish containing

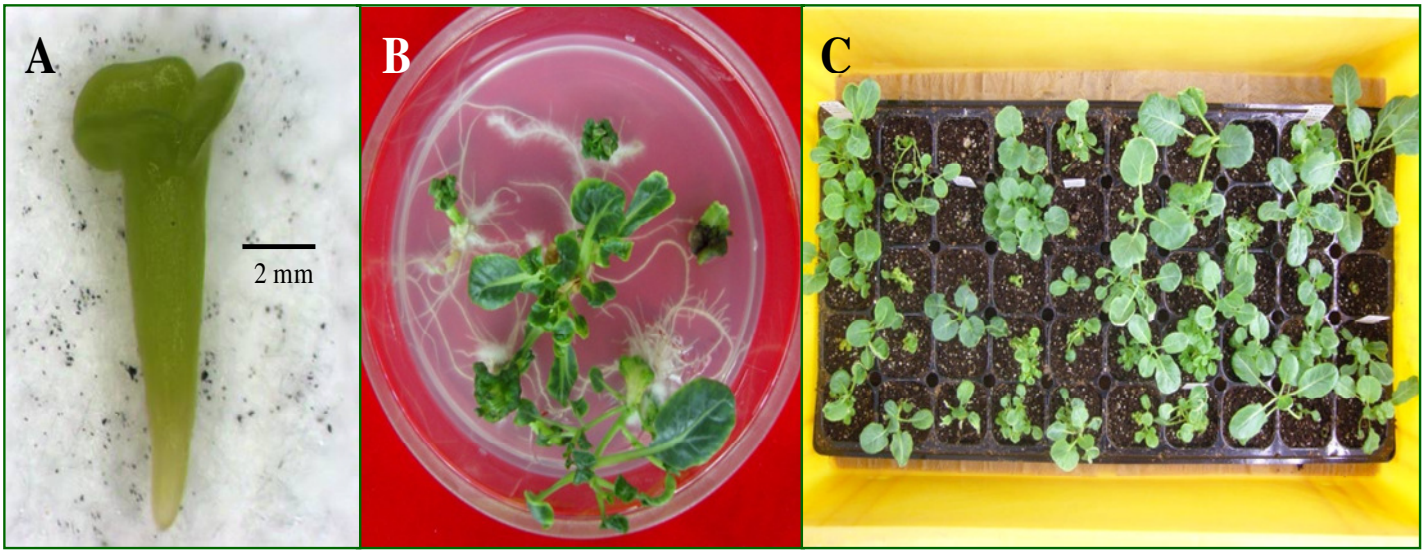

Figure 2. Microspore derived embryo of *Brassica oleracea* L. *var italica* . A, Cotyledonary microspore embryo formation after 4 weeks in culture on 0.5 X NLN medium containing 150 g L^{-1} sucrose; B, microspore derived plantlet formation after 4 weeks on conversion medium (0.5X MS medium containing 30 g L^{-1} sucrose, 0.8% agar); C, acclimatized microspore derived plants in the greenhouse 4 weeks after transfer from *in vitro* culture.

400 µl of extracting buffer (Solution A inthe CyStain UV Precise P Kit, Partec, Germany). After chopping, 1,600ml of the 4, 6-diamidino-2-phenylindol (DAPI) staining buffer (Solution B of the kit) wasadded. The suspension was filtered through a 30 µm nylon mesh (CellTricsTM, Partec, Germany). For each sample, 2,500-5,000 nuclei were analyzed using a flow cytometer equipped with a HBO-100 mercury lamp.

Statistical analysis

Statistical analysis was done to evaluate significant differences among microspore-derived embryos formation and various nutritional and environmental conditions. One way ANOVA was used to assess differences of microspore-derived embryos formation in NLN liquid medium strength, microelement strength of NLN medium, sucrose concentration, heat shock temperature and heat shock temperature period. ANOVA were carried out using statistical analysis systems software SAS 9.2 (SAS Institute., Cary, NC, USA). Means were separated using Duncan's multiple range tests at the 0.05 significance level.

RESULTS AND DISCUSSION

The MDE formation was 6.2 and 6.8 in the 0.25X and 1.0X NLN liquid medium, respectively; however, the difference was not significant. The 0.5X NLN liquid medium had the highest embryo formation, with 8.4. The MDE formation in the 2.0X and 4.0X NLN liquid medium was low (Figure 3). The high concentrations of macro and micro nutrient were not effective for MDE formation. Therefore, reducing the concentration of major salt to one and half in the NLN liquid medium seems to increase embryogenesis frequency in broccoli microspore culture. The nutritional requirements for induction and production of embryos vary widely from species to species. One of the most important media components influencing embryogenesis is basal salt. For MDE formation in broccoli, most experiments use the standard NLN-13 media. In this study, 0.5X NLN liquid medium proved to be significantly better than the other media strengths. Sato et al. (1989) obtained similar results in *Brassica campestris* ssp. Pekinensis, and the same result was reported in somatic embryo formation of *Pimpinellbrachycarpa* (Na and Chun, 2009). A reduction in the concentrations of some of the macronutrients in NLN-13, mainly NO_3, may be useful for promoting embryogenesis. Higher concentrations of macronutrients may be inhibitory to the induction of embryogenesis, as well as to embryo growth (Na and Chun, 2009). The addition of various amounts of micronutrients to the 0.5X NLN liquid medium was less effective than adding no micronutrients at all. The media to which micronutrients were not added had the highest formation rate in MDE (Figure 4) and also in rooted MDE (data not shown). This finding differed from results for Chinese cabbage, which showed an increase in MDE formation after the addition of micronutrients to 0.5X NLN medium (data not shown).

One of the most important medium components influencing the induction of embryogenesis is sucrose. The MDE formation was 72 and 69 in the 13 and 15% sucrose concentrations, respectively. The difference in MDE formation between the two concentrations was not significant, but the MDE formation in the 15% sucrose concentration was the highest. A sucrose concentration less than 10% decreased the embryo formation rate

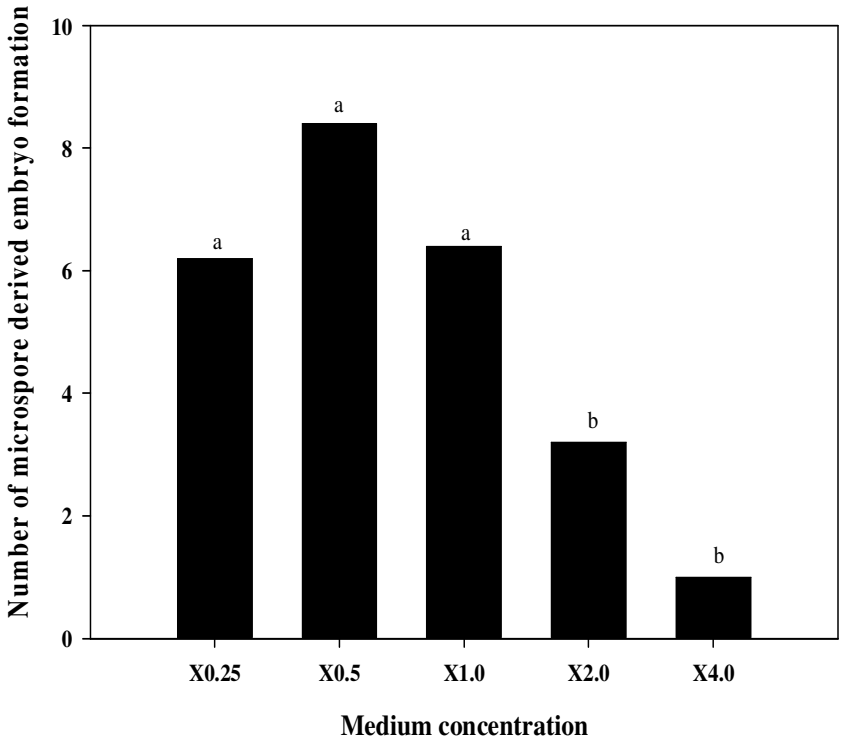

Figure 3. Microspore derived embryo yields (number of embryos/petri-dish) of *Brassica oleracea* L.*var italica* of microspore

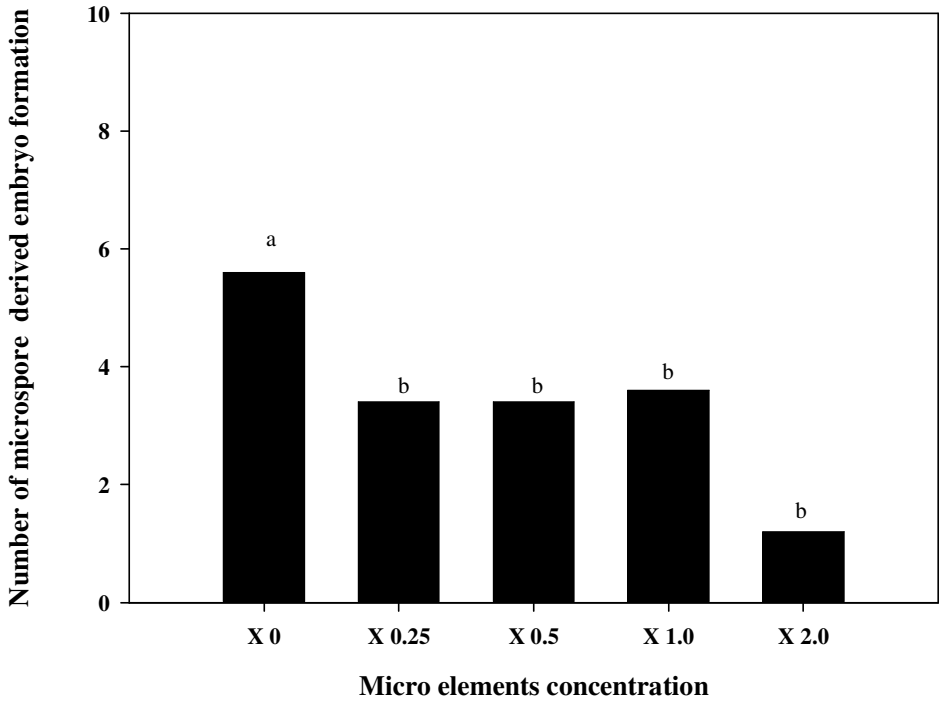

Figure 4. Microspore derived embryo yields (number of embryos/Petri-dish) of *Brassica oleracea* L.*var italica* of microspore culture medium (0.5X NLN) with various microelement strength of NLN liquid medium. Data was collected 30 days after culture. Each value is the average obtained from ten replications. Columns with the same letters are not significantly different by Duncan's multiple range tests at $P < 0.05$.

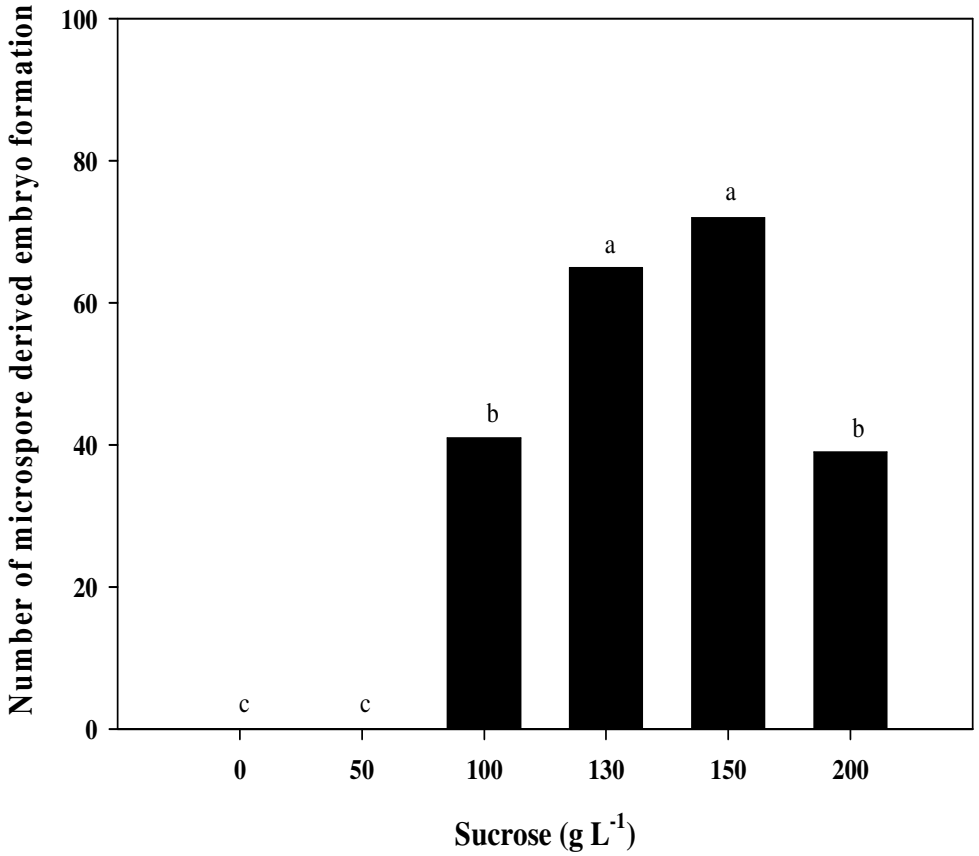

Figure 5. Microspore derived embryo yields (number of embryos/Petri-dish) of *Brassica oleracea* L.*var italica* of microspore cultures treated with various concentration of sucrose. Data was collected 30 days after culture. Each value is the average obtained from ten replications. Columns with the same letter are not significantly different by Duncan's multiple range tests at $P < 0.05$.

remarkably, and there was no microspore formation in the 5% sucrose concentration (Figures 5 and 6). Ferrie et al. (1999) found that 13% sucrose had a higher embryo yield as compared to 10%. However, previous studies showed that a high level of sucrose is required for initial microspore survival and division, but a lower level is important for the continuation of microspore division (Dunwell and Thurling, 1985). Additional research on the different effects of applied sucrose concentration according to MDE formation phase is required.

Microspore embryogenesis is induced by the heat shock stress treatment. In *B. napus*, the most efficient induction is obtained by increasing the culture temperature to 32°C for a minimum of 8 h (Custers et al., 1994; Pechan et al., 1991). Binarova et al. (1997) reported that DNA synthesis was initiated in both generative and vegetative nuclei by the application of heat stress treatment. MDE formation at the heat shock temperatures of 25 and 32.5°C in broccoli was 4.5 and 7.5, respectively; however, it was merely 0.5 at 37°C, and none at 42.5°C (Figure 7). The optimum heat shock temperature for MDE formation was 32.5°C. The MDE formation at a heat shock temperature of 32.5°C was counted at heat shock times of 0, 24, 48 and 72 h. At 0, 48 and 72 h, MDE formation was 1.6, 1.7 and 0.9, respectively. The highest MDE formation was 8.9 at the heat shock time of 24 h (Figure 8). Duijs et al. (1992) established a standard protocol for microspore culture using a pre-treatment (48 h at 30°C). MDE formation was significantly increased in many broccoli genotypes after incubating at the heat shock temperature of 32.5°C for 1 day, as compared to the standard incubation (Duijs et al., 1992).

The results of the polyploidy test for microspore-derived plantlets produced from the earlier experiments showed that the mean percentages of haploid, diploid, tetraploid, haploid + diploid, and diploid + tetraploid nuclei were 52, 30, 2, 8 and 8%, respectively, indicating the existence of endopolyploid cells in the microspore-derived plantlet, which are considered to be mixoploid. These results were consistent with the research of Chen et al. (2009), who obtained various mixoploidy plants from the protocorm-like body of *Phalaenopsis*.

This study described a methodology for achieving a high frequency of microspore embryo formation by controlling nutritional factors. Moreover, the efficient microspore culture protocols developed in this study could be useful in the production of a homozygous line used to produce F_1 hybrids.

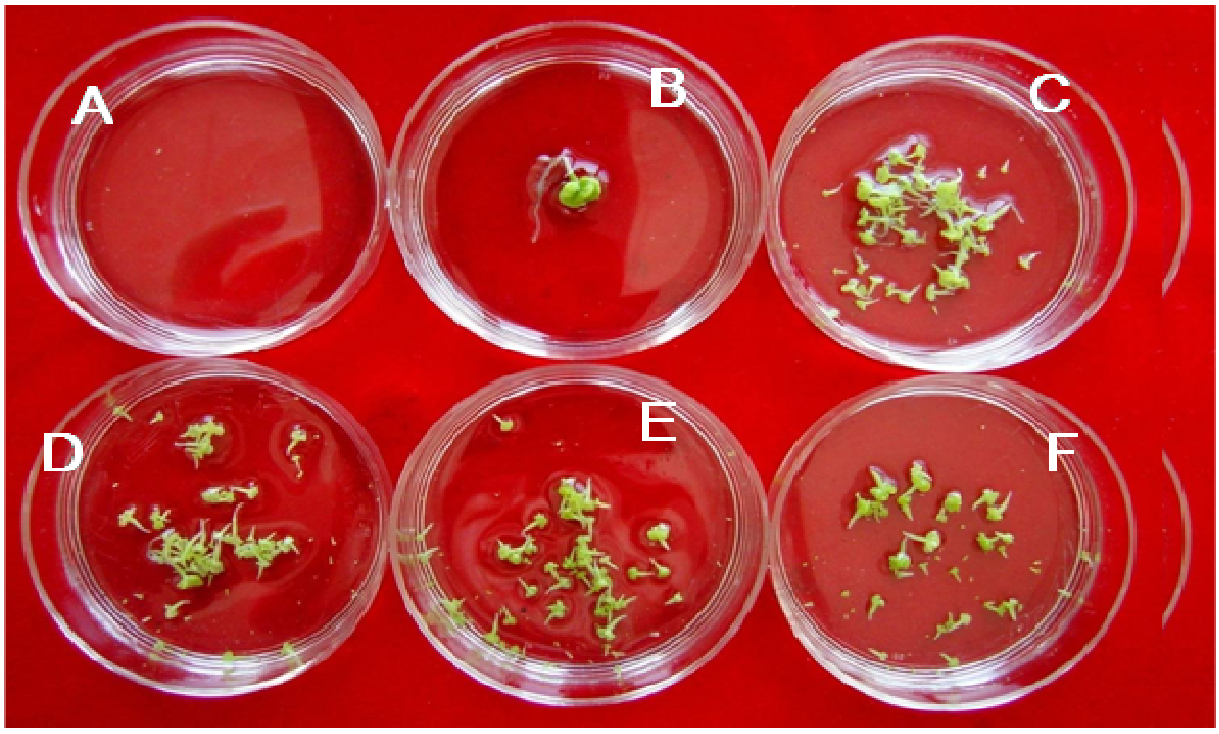

Figure 6. Morphology of microspore derived embryo formed from microspores cultured in an NLN liquid media with various concentrations of sucrose. A, 0; B, 50; C, 100; D, 130; E, 150; F, 200 g L-1.

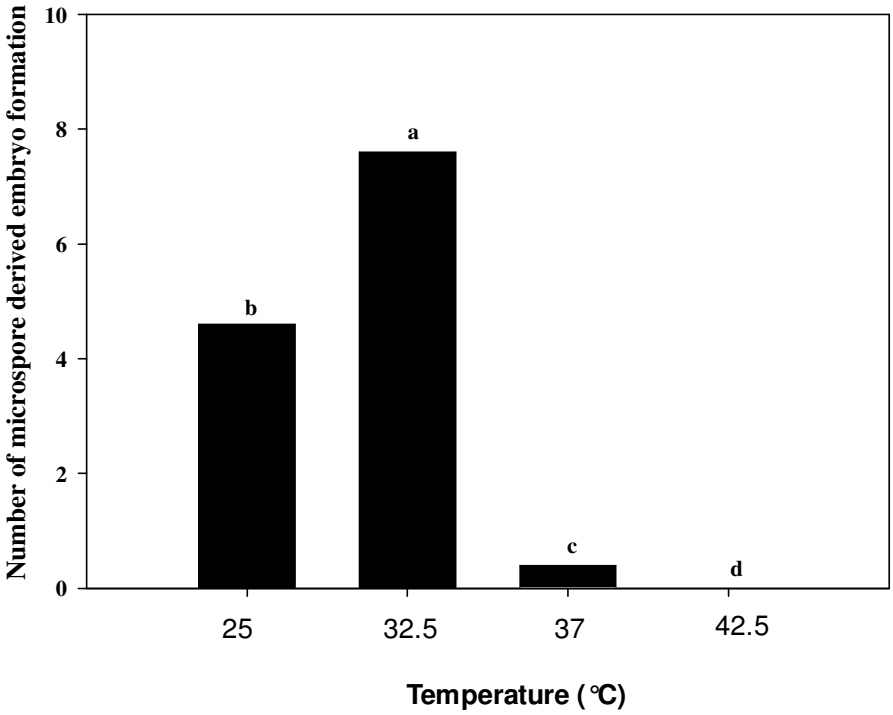

Figure 7. Microspore derived embryo yields (number of embryos/Petri-dish) of *Brassica oleracea* L.*var italica* of microspore cultures treated with various heat shock temperature for 24 h. Data was collected 30 days after culture. Each value is the average obtained from ten replications. Columns with the same letter are not significantly different by Duncan's multiple range tests at $P < 0.05$.

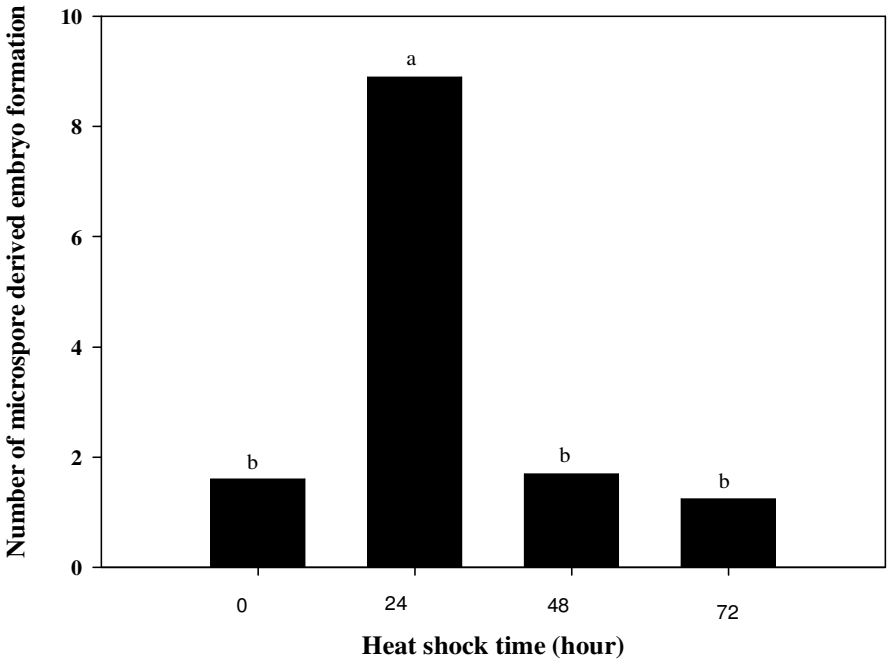

Figure 8. Microspore derived embryo yield (number of embryos/Petri-dish) of *Brassica oleracea* L.*var italica* of microspore cultures treated with various heat shock time at 32.5℃. Data was collected 30 days after culture. Each value is the average obtained from ten replications. Columns with the same letter are not significantly different by Duncan's multiple range tests at $P < 0.05$.

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