Interactive effects of heat shock and culture density on embryo induction in isolated microspores culture of *Brassica napus* L. cv. Global

Mohammad Reza Abdollahi, Ahmad Moieni and Mokhtar Jalali Javaran

Plant Breeding Department, Faculty of Agriculture, Tarbiat Modarres University, P.O. Box: 14115-336, Tehran, I.R. Iran.

Abstract

High yield and good quality embryos were obtained from cultures of isolated microspores of Brassica napus L. cv. Global. The donor plants were grown in a growth chamber at 15/10°C (day/night) with a 16/8h photoperiod. Microspores were isolated from whole buds of 2.5-3.5 mm in length containing late-uninucleate and early-binucleate microspores. Different heat shock treatments including, 30°C for 10, 14 and 18 days, 32°C for 2 and 3 days and 35°C for 18h followed by 30°C for 10 days and various culture densities including 60,000, 40,000 and 20,000 microspores per ml were used. Results showed significant differences among the heat shock treatments, the culture densities and their interaction for embryo induction. A large number of embryos were obtained from the microspores treated at 30°C for 18 days, 35°C for 18h followed by 30°C for 10 days and 30°C for 14 days with a density of 60,000 microspores per ml.

Keywords: *Brassica napus L., Microspore Culture, Embryogenesis, Heat Shock, Culture Density.*

INTRODUCTION

The most effective way to produce haploids has been through androgenesis by means of isolated microspores culture. This culture system could be utilized as a model system for somatic embryo induction, genetic manipulation and *in vitro* developmental studies (Huang *et al*., 1990). Since the first report of isolated microspore culture in *Brassica napus* (Lichter, 1982), there has been remarkable progress in developing this system. Numerous factors are required for high levels of embryogenesis from *Brassica* microspores. These factors include culture media, growing conditions of donor plants, genotype, microspores development stage (Gland *et al*., 1988; Hansen and Svinnset, 1993), and the incubation of microspores at elevated temperature during the early stages of culture (Keller *et al*., 1987). Culture density is another factor that may influence microspore culture in Brassica. A wide range of densities has been used, such as more than 100,000 microspores per ml (Polsoni *et al*., 1988), 50,000 microspores per ml (Fan *et al*., 1988) and 16,000 to 24,000 microspores per ml (Pechan and Keller, 1988). In this study, we examined the effect of culture density and heat shock on efficiency of microspore embryogenesis in *Brassica napus* cv. Global.

MATERIALS AND METHODS

Plant growth conditions: A spring cultivar of rapeseed (*Brassica napus* cv. Global) was examined in this study. Seeds were kindly provided by Oilseed Research and Development Co. Tehran, Iran. Donor plants were grown in a growth chamber with 16/8h photoperiod, and a day/night temperature of 15/10°C.

Microspore culture: Buds were selected on the basis of size (2.5-3.5 mm), placed in baskets and surface sterilized in 5.25% sodium hypocholorite for 10 min on a shaker followed by two 5 min washes with sterile

Correspondence to: **Ahmad Moieni, PhD**
Tel: +98 21 4196522, Fax: +98 21 4196524
E-mail: moieni_a@modares.ac.ir

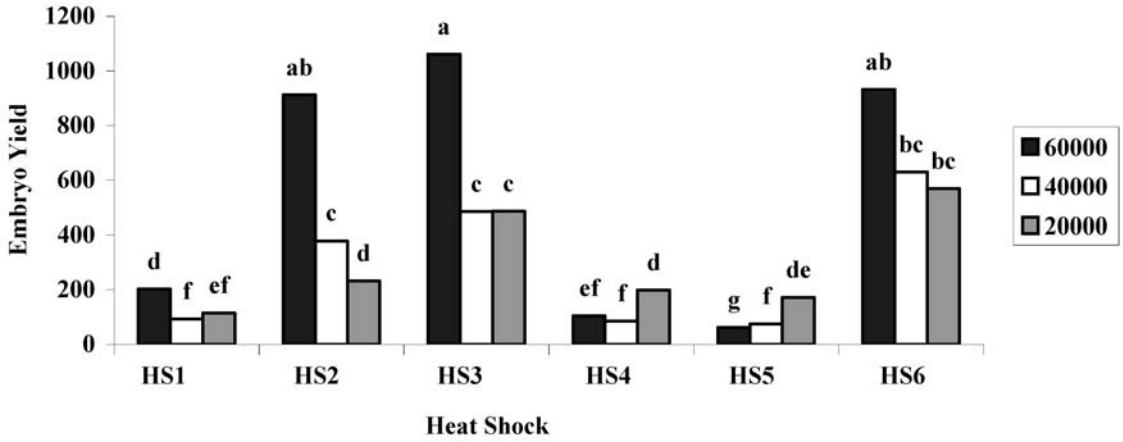

Figure 1. Mean performance of heat shocks and culture densities interaction for embryo production in microspore culture of *Brassica napus* cv. Global. **HS**: Heat Shocks. **HS1**: 30°C for 10 days. **HS2:** 30°C for 14 days. **HS3**: 30°C for 18 days. **HS4**: 32°C for 2 days. **HS5**: 32°C for 3 days. **HS6**: 35°C for 18h followed by 30°C for 10 days. Comparison of means was carried out on based of Duncan method.

water. Up to 100 buds, majority of which were at late-uninucleate and early-binucleate stage, were blended with a cool blender in 30 ml of cold microspore isolation solution containing 13% sucrose pH 6 (Feletcher *et al*., 1998). The crude suspension was filtered through a 106 µm metal mesh followed by a 53 µm mesh, both cups and meshes were rinsed, and a total of 50 ml was collected into two 50 ml centrifuge tubes and the microspore suspension was centrifuged at 200 ×g (1270 rpm) for 4 min, the supernatant removed and 25 ml of microspore isolation solution was added to each tube. This step was repeated twice, then 4-5 ml of filter-sterilized and modified NLN-13 liquid medium, (Lichter, 1982) supplemented with 13% sucrose but free of potato extract and growth regulators, was added to microspores. Then, the culture density was determined by a hemacytometer to achieve the desired density. Ten ml of microspore suspension was dispensed into each sterile glass Petri dish (120×20 mm). Cultures were incubated in the dark and then transferred to 25°C and darkness on a shaker (40 rpm). In this study, three densities, 20,000, 40,000 and 60,000 microspores per ml and six heat shocks, 10, 14 and 18 days at 30°C and darkness, 2 and 3 days at 32°C and darkness and 18h at 35°C followed by 30°C for 10 days were used.

A factorial experiment on basis of complete randomized design (CRD) with 2 factors and 8 replications (each glass Petri dish was a replication) was used in this study. The first factor (A) was the culture density with 3 levels and the second factor (B) was heat shock with 6 levels. After 30 days, the number of embryos was determined.

RESULTS

The analysis of variance showed significant differences between culture densities, heat shocks and their interaction for embryogenesis. The embryo yield differed according to the initial heat shock and culture density (Fig. 1). Globular stage embryos were observed after 10 days of culture (Fig. 2A). These embryos continued to develop respectively to heart stage, torpedo stage and cotyledonary embryos (Figs.

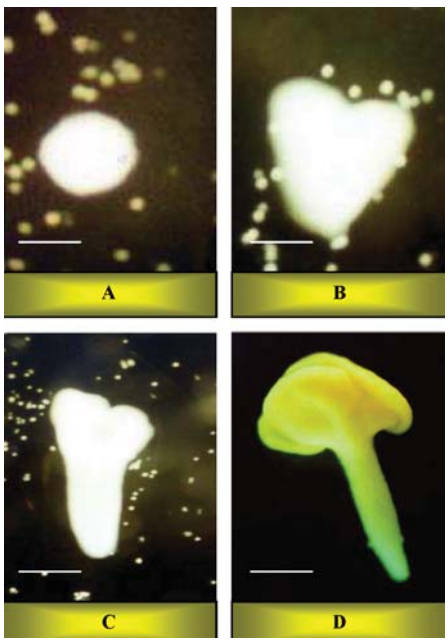

Figure 2. Different stages of embryogenesis in microspore culture of rapeseed (*Brassica napus* cv. Global). Globular stage (A), Heart stage (B), Torpedo stage (C), Cotyledonary stage (D), Bar: 1000 µm, Magnification:40X.

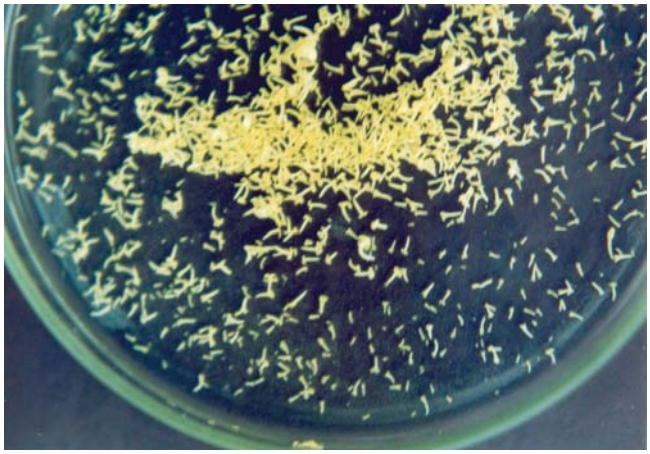

Figure 3. Mass production of embryos in microspore culture of *Brassica napus* cv. Global

2B, 2C, 2D). Mass production of embryos was obtained in this study is showed in figure 3.

A large number of embryos were obtained from the microspores treated at 30°C for 18 days, 35°C for 18h followed by 30°C for 10 days and 30°C for 14 days for density of 60,000 microspores per ml. Incubation of microspores at 35°C for 18h followed by 30°C for 10 days was the best temperature with densities of 40,000 and 20,000 microspores. Also 2 and 3 days incubation of microspores at 32°C and 10 days at 30°C with density of 40,000 microspores per ml were less effective heat shock for embryo production. Other heat shock treatments were also effective for embryo induction, but the yields were not as high as heat shocks described above.

DISCUSSION

A stress treatment of 32°C for at least 8 h was able to change the gametophytic program of the microspore, switching it to embryogenesis in *Brassica napus*, an interesting model for studying this process *in vitro*. After induction, some microspores started symmetric divisions and became haploid embryos after a few days, whereas other microspores, not sensitive to induction, followed their original gametophytic development (Segui-Simarro *et al*., 2003). It has been suggested that elevated temperature inhibits protein synthesis, leading to sporophytic induction. Alternatively, heat-shock may also cause destruction of pre-existing post-transcriptional control apparatus in the microspore. The destruction of these post transcriptional control mechanisms may contribute to reprogramming of microspore development towards embryogenesis.

Recently, the distribution and ultrastructural localization of two heat-shock proteins (Hsp70 and Hsp90) throughout the key stages before and after embryogenesis induction were studied. The results of a previous work (Segui-Simarro *et al*., 2003) have determined the differential appearance and distribution of two Hsp proteins between two completely different systems, embryogenic development (induced cells) and gametophytic development (non-induced cells) , as well as their association at specific stages. The data indicate that stress proteins are involved in the induction process of microspore embryogenesis. Also, in another study (Testillano *et al*., 2000) on three different plant species, *Brassica,* tobacco, and pepper, the switch of embryogenesis has been induced by stress (heat shock) at the very responsive stage of the microspore, which is the vacuolation stage. As a result, the cell nucleus undergoes striking structural changes with regard to late gametophytic development, including alterations of biosynthetic activities and proliferative activity. An enrichment in HSP70 heat-shock protein and in the presence of Ntf6-MAP kinase was observed after inductive treatment in the nuclei during early embryogenesis. This apparently reflected possible roles of these proteins, specifically the protective role of HSP70 for the nuclear machinery, and signal transduction of Ntf6-MAPK for the entry of cells into proliferation. This is important to note that, the observed nuclear changes were similar in the three species investigated and represented convenient markers for early monitoring of embryogenesis and selection purposes for obtaining double-haploid plants in plant breeding.

In this study, the observed differences between the responses of microspores to heat shocks are consistent with the findings of Yang *et al*., (1992) and Takahata and Keller (1991). Previous studies on *Brassica napus* also showed that a heat shock treatment for 18h at 35°C followed by 30°C for 10 days (Gland *et al*., 1988) and culture temperature of 30°C for 14 days (Feletcher *et al*., 1998) increased the number of embryos per bud considerably and our results are consistent with these studies. The studies show that, in *Brassica* species, an initial culture period of elevated temperature is required for embryogenesis (Takahata and Keller, 1991; Baillie *et al*., 1992). Concerning the culture density in microspore culture, Huang and coworkers (1990) found that in microspore culture of *Brassica napus* cv. Topas embryo yields increased with culture density up to about 40,000 microspores per ml. Also, the minimum density for induction of

embryogenesis was 3000 microspores per ml. According to Huang *et al*. (1990) a much higher density (100,000 microspores per ml) appeared to be inhibitory to embryogenesis . Kott *et al*., (1988) suggested that the reduction of embryogenesis in higher densities is due to a toxin which was released from microspores themselves. Furthermore, Duijs *et al*., (1992) found no clear effect of the plating density except when there was a high proportion of binucleate pollen in cultures. In the present study, interaction of culture density with heat shock was significant. Hence, the embryo yields with various culture densities differed according to the heat shock used.

Acknowledgement

The authors thank the Oilseed Research and Development Co.,Tehran, Iran for supporting this work.

References

Baillie AMR, Epp DJ, Keller WA and Hutcheson D. (1992) *In vitro* culture of isolated microspores and regeneration of plants in *Brassica campestris*. *Plant Cell Rep*. 11: 234-237.

Duijs JG, Voorrips RE, Visser DL and Custers JBM. (1992) Microspore culture is successful in most crop types of *Brassica oleracea* L. *Euphytica*. 60: 45-55.

Fan Z, Armstrong KC and Keller WA. (1988) Development of microspores *in vivo* and in vitro in *Brassica napus* L. *Protoplasma*. 147: 191-199.

Feletcher R, Coventry J and Kott LS. (1998) Doubled Haploid Technology for Spring and Winter *Brassica napus*. *Technical Bulletin, OAC Publication*, Canada.

Gland A, Lichter R and Schweiger HG. (1988) Genetic and exogenous factors affecting embryogenesis in isolated microspore culture of *Brassica napus*. *Plant Physiology*. 132: 613-617.

Hansen M and Svinnset K. (1993) Microspore culture of Swede and the effects of fresh and conditioned medium. *Plant Cell Rep*. 12: 496-500.

Huang B, Bird S, Kemble R, Simmonds D, Keller WA and Miki B. (1990) Effects of culture density, conditioned medium and feeder cultures on microspore embryogenesis in *Brassica napus*. *Plant Cell Rep*. 8: 594-597.

Keller WA, Arnison PG and Cardy BK. (1987) Haploids from gametophytic cells, recent development and future prospects. In: Green CE, Somers DA, Nackett WP and Biesbore DD (eds) *Plant Tissue and Cell Culture* (pp 223-241). Alan Liss Inc, NY.

Kott LS, Polsoni L, Ellis B and Beversdorf WD. (1988) Autotoxicity in isolated microspore cultures of *Brassica napus*. *Can. J. Bot*. 66: 1665-1670.

Lichter R. (1982) Efficient yield of embryoids by culture of isolated microspore of different Brassicacea species. *Plant Breeding*. 103: 119-123.

Pechan PM and Keller WA. (1988) Identification of potentially embryogenic microspores in *Brassica napus*. *Physiol Plant*. 74: 377-384.

Polsoni L, Kott LS & Beversdoref WD. (1988) Large-scale microspore culture technique for mutation/selection studies in *Brassica napus*. *Can. J Bot*. 66: 1681-1685.

Segui-Simarro JM, Testillano PS and Risueno MC. (2003) Hsp70 and Hsp90 change their expression and subcellular localization after microspore embryogenesis induction in *Brassica napus* L. *J Struct Biol*. 142: 379 –391.

Takahata Y and Keller WA. (1991) High frequency embryogenesis and plant regeneration in isolated microspore culture of *Brassica oleracea* L. *Plant Sci*. 74: 235-242.

Testillano PS, Coronado MJ, Segu JM, Domenech J, Gonzalez- Melendi P, Raska I and Risueno MC. (2000) Defined nuclear changes accompany the reprogramming of the microspore to embryogenesis. *J Struct Biol*. 129**:** 223– 232.

Yang Q, Chauvin JE and Herve Y. (1992) A study of factors affecting anther culture of cauliflower (*Brassica oleracea). Plant Cell Tiss Org Cult*. 28: 289-296.