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Effects of gibberellin, abscisic acid and embryo desiccation on normal plantlet regeneration, secondary embryogenesis and callogenesis in microspore culture of *Brassica napus* L. cv. PF_{704}

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Abstract

Most of the microspore-derived embryos can not regenerate normally in rapeseed. The effects of gibberellins (GA_3), abscisic acid (ABA), and embryo desiccation on normal plantlet regeneration were studied. The donor plants were grown in a growth chamber at day/night temperatures of 15/10˚C with a 16/8h photoperiod, respectively. Microspores were isolated from whole buds of 2.5-3.5mm in length, containing late-uninucleate and early-binucleate microspores, and cultured in modified NLN-13 liquid medium. After 30 days, cotyledonary embryos were transferred to B5 medium. The study of GA_3 concentrations (0, 0.05, 0.1, 0.15, 0.2 mg/l) showed that the use of 0.1 and 0.15 mg/l of filter sterilized GA_3 were the optimum treatment for normal plantlet production (50% and 44%, respectively). Among the various time periods of embryos desiccation (0, 3, 5, 10, 15, 20 min.), air drying of embryos for 10 min. produced the highest normal plantlets (60%). In the third experiment, 9 desiccation-ABA treatments (T_1-T_9) were tested. T_6 (no desiccation) or T_7 (5 min-desiccation) treatments with 40 µM ABA in B5 medium exhibited the highest number of normal plantlets (68% and 63%, respectively).

Keywords: ABA; *Brassica napus*; Embryo Desiccation; GA_3; Plantlet Regeneration; Rapeseed

Introduction

Doubled haploids (DH) are presently used in breeding of a number of crop species. This method enables breeders to develop homozygous genotypes from heterozygous parents in a single generation. The possibility to produce haploids in *B.napus* from anther culture (Wenzel et al., 1977), and later from microspore culture (Lichter, 1982) provided a new tool for breeding improved cultivars. The advantages of using doubled haploids in oilseed rape breeding consist primarily in the substantially reduced time to produce and release cultivars. Normally, 8–10 years are required to develop a new cultivar using traditional

procedures. The DH method reduces that time by about 2–4 years but the cost is sometimes twice higher than in conventional breeding. Besides, the probability of selecting progeny exceeding the performance of parents is higher than other breeding methods. Except significant shortening of the breeding cycle, this method is fully compatible with other biotechnological approaches such as *in vitro* selection, mutation, cry preservation and gene manipulation techniques. 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 important for high levels of embryogenesis from *Brassica* microspores, e.g. growing conditions of donor plants, genotype (Kuginuki et al., 1997), length of buds (Takahata et al., 1993; Li and Guan, 2003), developmental stage of microspores (Fan et al., 1988), microspore density, culture media (Takahata, 1997), and heat shock treatment (Pechan et al., 1991). For plant breeding and genetic engineering prospects, normal plant regeneration from microspores is essential, but it was reported that the majority of embryos were not able to regenerate normally (Huang et al., 1991). Some factors, for instance, developmental stage (Takahata and Keller, 1991), maturation and size of embryo are effective on normal plant regeneration from microspore-derived embryos. Among rapeseed cotyledonary embryos, 35-day-old embryos regenerated more normally than other embryos (Kott and Beversdorf, 1990) and, activated charcoal (AC) seems to have no effects on increasing embryogenesis and embryo development, but it is desirable for plant regeneration (Gland et al., 1988). In this study, we examined the effects of GA_3, ABA and embryo desiccation on normal plantlet regeneration, callogenesis and secondary embryos production in microspore culture of rapeseed (*Brassica napus* cv. PF_{704}), using three independent experiments. Our results revealed that the use of 0.1 and 0.15 mg/l of filter sterilized GA_3 and 40 µM ABA in B5 medium produced the highest number of normal plantlets.

Material and Methods

Plant growth conditions

A spring cultivar of rapeseed (*Brassica napus* cv. PF_{704}), which had relatively good response to microspore culture among different genotypes tested in our several preliminary experiments, was used in this study. Donor plants were grown under sodium lamps in a growth chamber with a 16-h/day photoperiod (300 $\mu E/m^2/s^1$) under 15/10 $^{\circ}C$ day/night temperatures, respectively.

Microspore culture

About 100 buds were selected on the basis of size (2.5-3.5 mm), placed in the basket and surface-sterilized in 5.25% (v/v) sodium hypochlorite for 10 min. followed by two 5 min. washes with sterile distilled water. Up to 100 buds, the majority of which were at late-uninucleate and early-binucleate stage, were blended with a prechilled blender in 30 ml of cold microspore isolation solution with 13% sucrose and pH adjusted to 6.0 (Fletcher 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. The microspore suspension was centrifuged at 200 g for 4 min., the

supernatant removed and 25 ml of microspore isolation solution was added to each tube. This procedure was repeated twice, and then 4-5 ml of filter-sterilized and modified NLN-13 liquid medium (Lichter, 1982) supplemented with 13% sucrose but free from potato extract and growth regulators, was added to microspores. The culture density was determined by a haemocytometer to achieve a density of 40000 microspore per ml. 12.5 ml of microspores suspension was dispensed into 100×15 mm sterile glass plate .Cultures were incubated in darkness at 30˚C for 14 days. The cultures were transferred to 25˚C in the dark on a shaker (40 rpm). After 30 days, cotyledonary embryos, 4-5 mm in length (Figure 4A), were transferred to B5 regeneration medium (Gamborg et al., 1968), containing 0.1 mg/l GA_3, 20 mg/l sucrose and 8 mg/l agar, pH 5.7 (10 embryos placed in each 90×15 mm plastic plates). Cultured embryos were treated in darkness at 4˚C for 10 days, and then were transferred to 25˚C with 16h/8h photoperiod.

Effect of GA_3 concentrations and sterilization methods on normal plantlet regeneration

The effect of different concentrations of GA_3 in B5 medium and its sterilization method on normal plantlet regeneration was studied. A complete randomized design (CRD) with factorial arrangement, consisting of two treatments, each with five replications was used. The first factor (A) was the concentration of GA_3 ($C_{19}H_{22}O_6$, 90+% purity, Sigma Chemical Co.) with 5 levels (0, 0.05, 0.1, 0.15 and 0.2 mg/l) and the second factor (B) was sterilization method of GA_3 with 2 levels, autoclave (121˚C and 1.2 bar for 20 min. in B5 medium) and filter sterilization (0.22 µm sterile filter with a sterile syringe).

Effect of embryos desiccation

The effect of embryos desiccation, embryos were placed on a nylon mesh (Nitex) in an open sterile glass plate in the air stream of a laminar flow hood, at 6 time periods including 0, 3, 5, 10, 15 and 20 min. on normal plantlet regeneration, callogenesis and secondary embryogenesis was studied. A CRD with six treatments and five replications was used.

Interactions between embryo desiccation and ABA

Interactions between embryos desiccation and the addition of ABA in B5 medium on normal plantlet regeneration, callogenesis and secondary embryogenesis were investigated. This experiment was carried out on basis of a CRD with nine treatments and five replications. These treatments consist of:
T_1: Transfer of embryos to B5, free of ABA (C_{15} $H_{20}O_4$, 99+% purity, Sigma Chemical Co.), and without desiccation treatments, T_2: Transfer of embryos to B5 containing 10 µM ABA, T_3: Transfer of embryos to B5 containing 10 µM ABA for one week and then sub culturing on to B5 without ABA, T_4: Embryos were transferred to B5 containing 10 µM ABA for one week and then air drying of embryos for 5 min. to the laminar-air flow cabinet and were sub culturing on to B5 without ABA, T_5: Transfer of embryos to B5 containing 40 µM ABA, T_6: Transfer of embryos to B5 containing 40 µM ABA for one week and then sub culturing on to B5 without ABA, T_7: Embryos were transferred to B5 containing 40 µM ABA for one week and then air drying of embryos for 5 min. to the laminar-air flow cabinet and were subculture

on to B5 without ABA, T_8: Transfer of embryos to B5, free of ABA, for one week without desiccation treatment, then transferring to B5 containing 40 µM ABA, T_9: Transfer of embryos to B5, free of ABA, without desiccation treatment for one week, then relocated them to B5 containing 10 µM ABA.

In all of the experiments, each glass plate containing 10 embryos was considered as one replication. Data was analyzed by SPSS software. If necessary, data were transformed with arcsin√x.

Results and Discussion

Effect of GA_3 concentrations and sterilization methods on normal plantlet regeneration

Normal plantlets percentage (including normal shoots, leaves and roots, Figure 4B, 4C) 30 days after transferring to regeneration medium showed significant differences between various concentrations of GA_3, methods of GA_3 sterilization and their interaction. Utilization of 0.1 and 0.15 mg/l filter sterilized GA_3 were the best effective concentrations on normal plantlets production (50% ±5.10 and 44%±2.45, respectively). The medium without GA_3 produced the lowest number of normal plantlets (16%±0.58) (Figure 1). GA_3 improved plant regeneration via elongation of the embryos axis and by acceleration in the embryo maturation. Filter sterilization had the most profound effect on plantlets regeneration via conservation of efficiency and quality of GA3. Our results are in agreement with those obtained in other studies. The effect of GA_3 on plant regeneration from hypocotyls and cotyledons of androgenic embryos of *B. napus* was reported (Bagniewska et al., 2001).

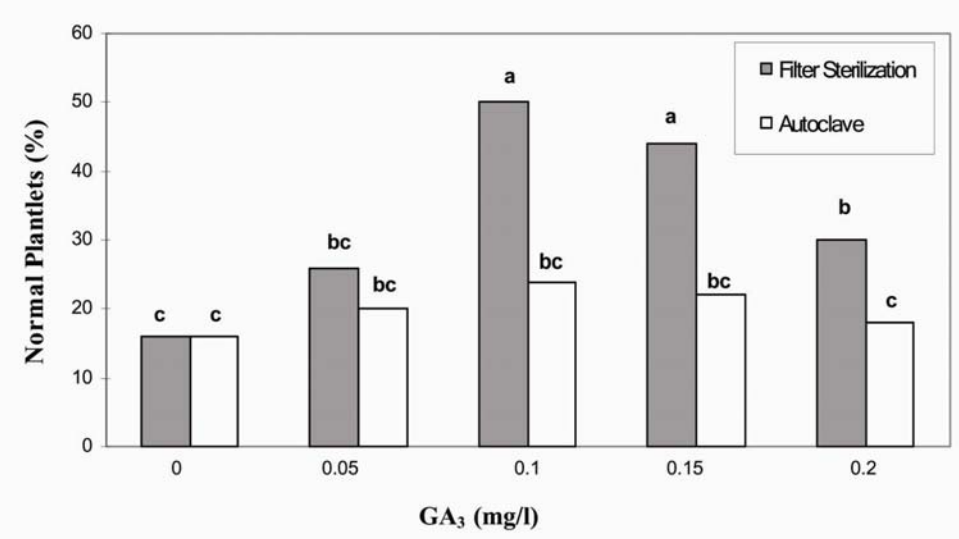

Figure 1. Effect of various concentrations of GA_3 and its sterilization methods on normal plantlets regeneration. Mean separation by the LSD test, P≤0.05.

Effect of embryos desiccation

The results revealed that the time of desiccation had a significant effect on normal plantlet regeneration, callogenesis and secondary embryogenesis. Desiccation for 10 and 15 min. Were optimum treatments for normal plantlet regeneration (60%±4.47 and 52%±4.90, respectively) while control (not desiccated) produced the lowest number of normal plantlets (22%±2.00) (Figure 2A). Calli were formed either on embryos (Figure 4E), secondary embryos or at the bottom of plantlet hypocotyls. The results revealed that increase of desiccation time period lead to reduction of callogenesis. Desiccation of embryos for 10, 15 and 20 min. showed the least effect on the abnormal plant regeneration (8%, 12% and 12%±4.90 callogenenesis, respectively) while the control produced the highest callogenesis (64%±7.48) (Figure 2B). Secondary embryogenesis was another trait that was investigated in this experiment (Figure 4F, 4G). This trait has been reported as an undesirable trait in plant regeneration (Keller et al., 1987; Huang et al., 1991). Secondary embryogenesis decreased when the desiccation time was increased. Desiccation for 3 min. was the best treatment on secondary embryos production (56% ± 4.00). Desiccation for 5, 10, 15 and 20 min. had the least effect on secondary embryogenesis (36%±4.00, 26%±4.00, 36%±2.45 and 26%±8.00, respectively) (Figure 2C).

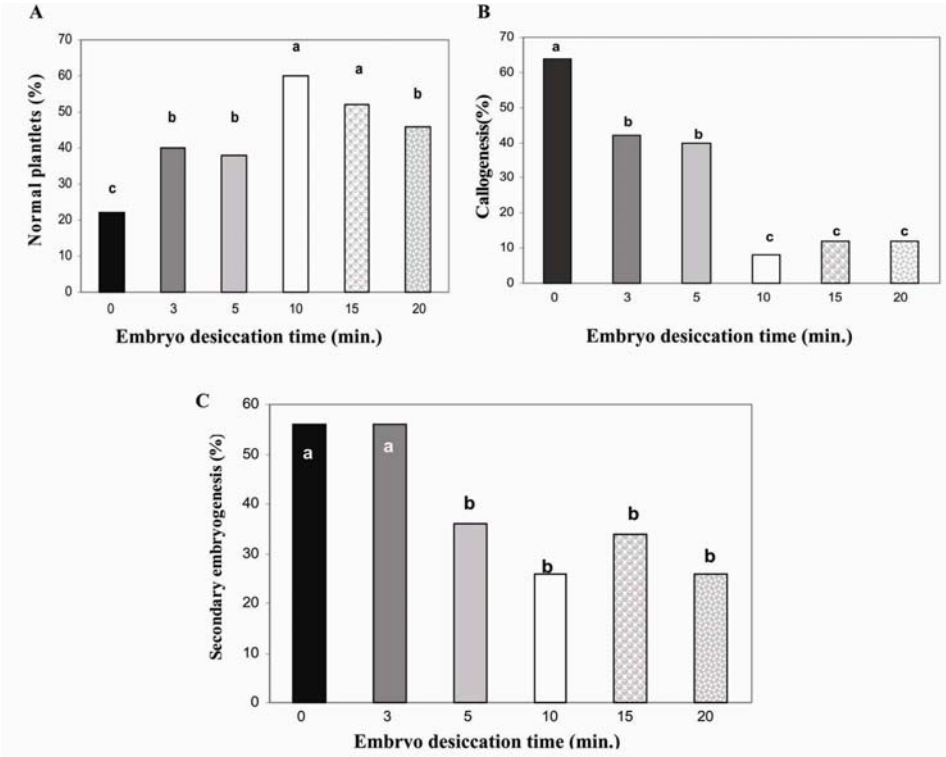

Figure 2. Effect of embryo desication at different time periods on (A) normal plantlets regeneration; (B) callogenesis; (C) secondary embryogenesis. Bars with different letters represent significantly different means (P≤0.05), using LSD test.

Embryo desiccation and ABA interaction

T_6 and T_7 treatments produced the largest number of normal plantlets (68%±6.32 and 63%±4.00, respectively) (Figure 3A). In T_6, embryos were transferred to B5 containing 40 µM ABA for one week whereas in T_7 treatment after culture of embryos on B5 containing 40 µM ABA for one week, and dried for 5 min. in the laminar-air flow cabinet. In both treatments, embryos were subculture on B5 free of ABA.

Concerning abnormal regeneration, T_1 and T_8 produced the highest callogenesis (64%±7.48 and 60%±3.16, respectively) (Figure 3B). In T_1, embryos were transferred to B5, free of ABA and without desiccation treatment, and in T_8, first, embryos were placed on regeneration medium free of ABA and without desiccation, and then subculture on to B5 containing 40 µM ABA. T_6 and T_7 treatments were the best treatments on the reduction of callogenesis.

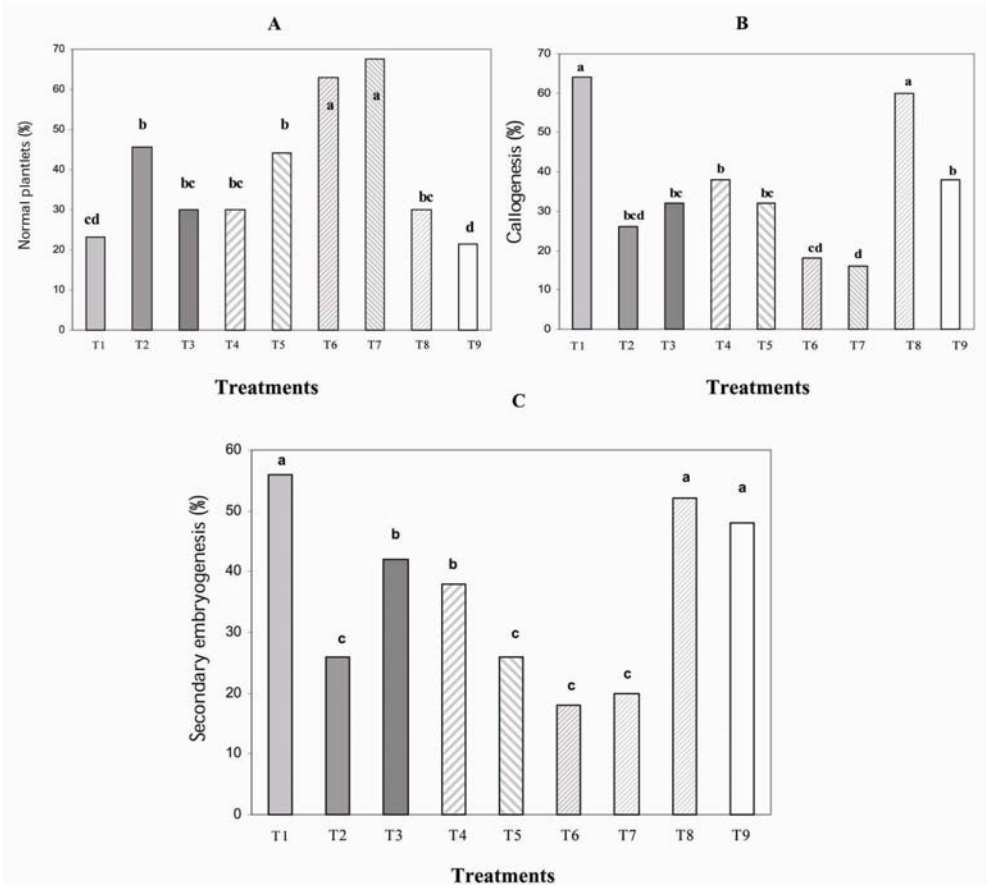

Figure 3. Effect of different treatments on (A) normal plantlets regeneration; (B) callogenesis; (C) secondary embryogenesis. Mean separation by the LSD test, P≤0.05.

Application of ABA for the first week of transferring embryos on B5 was more effective on reducing secondary embryogenesis than later weeks. T_1, T_8 and T_9 treatments produced higher secondary embryogenesis (56%±6.78, 52%±3.47 and 48%±3.47, respectively) and T_6, T_7, T_5 and T_2 produced the lowest number of secondary embryos (18%±2.00, 20%±3.16, 26%±2.45 and 26%±2.45, respectively) (Figure 3C). Direct inoculation of embryos with 10 and 40 µM ABA, then transfer to B5 without ABA did not show plantlet regeneration and were turned to a white color.

The desiccated embryos treated with 40 µM ABA were able to directly germinate and regenerate into normal plants in vermiculate and perlite moistened with B5 basal salts (Figure 4D). ABA and desiccation treatment increased the plant regeneration in the following ways:

1) Acceleration in embryo maturation: ABA can increase expression of maturation genes in embryos, including the *Em* genes (Early methionine-labeled), which may be important for desiccation tolerance (Morris et al., 1990).

2) Increase in desiccation tolerance: The desiccation tolerance of somatic embryos has been reported to be enhanced by the exogenous application of ABA (Brown et al., 1990; Lida et al., 1992). Molecular analysis revealed that the *Lea* genes (Late embryogenesis abundant) expressed in the embryos treated with ABA resulted in increase in desiccation tolerance (Wakui and Takahata, 2002) and consequently to normal regeneration.

Many factors such as endosperm content of embryos (Kermod and Bewley, 1988), embryo size and developmental stage, proline (Park et al., 1988; Janick et al., 1989) and heat and chilling stress (Brown et al., 1993) affecting the induction of desiccation tolerance and normal plantlet regeneration. The optimum concentration of ABA was different among the species of *Brassica.* Previous studies demonstrated that 100 µM ABA was most effective for broccoli (*B. oleracea*), and 10 µM ABA for Chinese cabbage (*B. campestris*) to induce a complete desiccation tolerance (Takahata et al., 1992). We found that concentrations over 100 µM ABA in B5 exerted a deleterious effect and embryos turned black. This finding is similar to that of Senaratna et al., (1991).

3) Wakui et al. (1999) showed that although no visual differences in morphological appearance were observed between the embryos treated and untreated with ABA, scanning electron microscopy observation revealed that the external surface of desiccated embryos treated with ABA was uniformly shriveled due to severe dehydration, and their internal tissue system was well preserved. It is also recognized that ABA raised the compatibility of plants to various environmental stress (Skriver and Mundy, 1990).

4) Wakui et al. (1994) showed that proteins of embryos treated with ABA analyzed by SDS-PAGE accumulated proteins with molecular weights ranging from 20 to 32 kD as well as less than 7 kD, that almost corresponded to the major seed storage proteins of Chinese cabbage and were also similar in size to cruciferin (12S) napin (1.7S) of rapeseed. Recovery of plantlets from desiccated embryos treated with ABA under soil condition is required for practical use as artificial seeds. The present results demonstrating that the dry embryos treated with ABA were able to germinate and regenerate into plants in perlite. Accumulation of storage proteins in the microspore-derived embryos which was promoted by ABA may induce plant regeneration ability of embryos in soil.

5) ABA pretreatment promoted dry matter accumulation such as proline, glycine- betaine, cyclitols and soluble carbohydrates and increased the concentration of K^+ and Na^+ ions in

explants (Wang et al., 2002) thus inducing desiccation tolerance and normal plant regeneration in somatic embryos. In another experiment, results showed that napin and oleosin gene expression in microspore-derived embryos of *B. napus* can be regulated by 10 µM ABA and 30 µM jasmonic acid (Wilen, 1992).

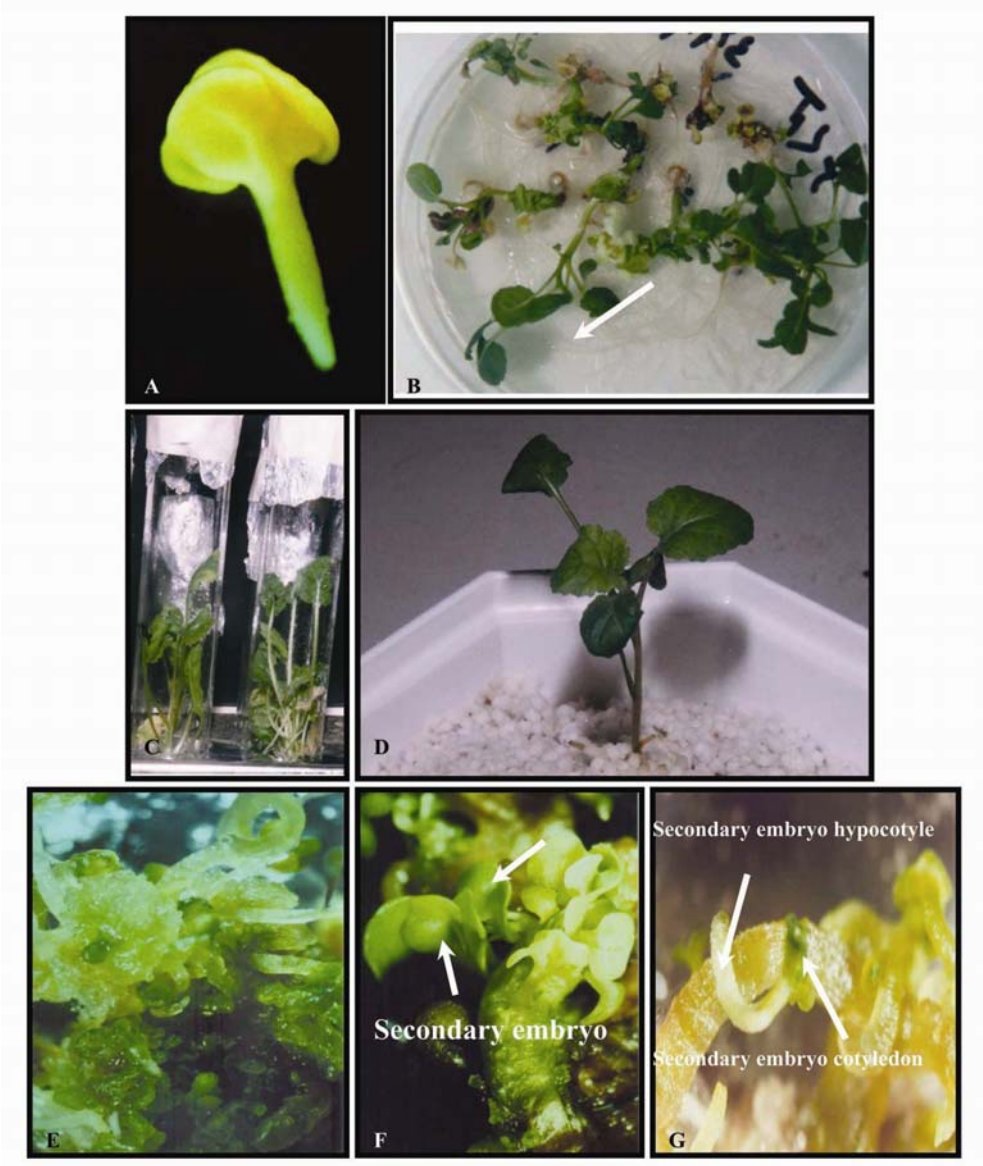

Figure 4. Isolated microspore culture of rapeseed (*Brassica napus* L. cv. PF_{704}). (A) Cotyledonary stage embryo; (B) 30 days after haploid plant regeneration; (C) Transfer of plantlets to test-tubes; (D) Direct plant regeneration from haploid embryo treated with ABA; (E) Callus formation on the surfaces of embryos; (F-H) Secondary embryos production.

In on study addition of suspension containing 1% activated charcoal and 0.5% agarose to each plate at the time of microspore culture resulted in more desirable plant regeneration from embryos (Gland et al., 1988). In another study, transferring embryos to a filter paper placed on agar medium led to increasing normal plant regeneration to 60%, this was 1.5 times more than embryos placed directly on agar medium (Zhao et al., 1996).

In particular, thousands of embryos from microspore cultures of rapeseed (*B. napus*) can be regenerated in a single experiment. Besides having the capacity to regenerate into plants, these embryos contain embryogenic or pre-embryogenic cells which in response to induction signals have the capacity to develop directly into secondary embryos, avoiding callus phase. The evident potential for practical applications of secondary embryos, such as selection and screening of novel disease-resistant plants. Clonal propagation of specific genotypes exploiting the embryos` high regeneration rates in comparison with other *in vitro* regeneration systems, and transformation, support further studies to obtain an efficient and reproducible procedure for induction and culture of secondary embryos in *Brassica* species (Nehlin et al., 1995)

In future studies, molecular markers such as RAPD combined with determination and sequencing of genes involved in plant regeneration (Zhang et al., 2003) can be used for transfer of these genes into non responsive genotype or recalcitrant cultivars by classic or genetic engineering methods.

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References

Bagniewska, Z.A., Cegielska, T.T., Zenkteler, M., 2001. Organogenesis initiation and plant regeneration from hypocotyls and cotyledons of androgenic embryos of *Brassica napus* L. Acta Biologica Cracoviensia Series Botanica 43:51-57.

Brown, D.C.W., Singh, J., Takahata, Y., Watson, E.M., 1990. Artificial seeds: induction of desiccation tolerance in canola microspored-derived embryos. *In ``Abstracts VII$^{th}$ International Congress on Plant Tissue and Cell Culture, Amesterdam `` Nijkamp , H. J. J. (eds.)*, Kluwer Academic Publishers, Dordrecht, The Netherlands, 184.

Brown, D.C.W., Watson, E.M., Pechan, P.M., 1993. Induction of desiccation tolerance in microspore-derived embryos of *Brassica napus* . *In Vitro Cell. Dev. Biol* 29: 113 -118.

Fan, Z., Armstrong, K.C., Keller, W.A., 1988. Development of microspores *in vivo* and *in vitro* in *Brassica napus* l. Protoplasma 147: 191-199.

Fletcher, R., Coventry, J., Kott. L.S., 1998. *Doubled haploid technology for spring and winter Brassica napus*, Technical Bulletin, OAC Publication, Canada.

Gamborg, O.L., Miller, R.A., Ojiwa, K., 1968. Nutrient requirements of suspension culture of soybean root callus. Experimental Cell Research 50:151-158.

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

Huang, B., Swanson, E.B., Baszczynski, C.L., Macrae, W.D., Bardour, E., Armavil, V., Wohe, L., Arnoldo, M., Rozakis, S., Westecott, M., Keats, R.F., Kimble, R., 1991. Application of microspore culture to canola improvement. In: *The Proceeding of. 8th International. Rapeseed Congres*, ed., McGregor, D.I., Saskatoon, Canada, pp: 298-302.

Janick, J. , Kitto, S. L. Dim, V.H., 1989. Production of synthetic seed by desiccation and encapsulation . In Vitro Cell. Dev. Biol 25: 1167-1172 .

Keller, W.A., Arnison, P.G., Cardy, B.K., 1987. Haploids from gametophytic cells recent development and future prospects. In: *Plant Tissue and Cell Culture*, Green, C.E., Somers, D.A., Nackett W.P. and Biesbore, D.D. (Ed.). pp: 233-241. Allan R. Liss, NewYork, USA.

Kermod, A.R., Bewley, J.D., 1988. The role of maturation drying in the transition from seed development to germination. Responses of the immature castor bean embryos to isolation from the whole seed a comparison with premature desiccation. J. Exp. Bot. 39: 487-498.

Kott, L., Beversdorf, W.D., 1990. Enhanced plant regeneration from microspore-derived embryos of *Brassica napus* by chilling, partial dessication and age selection. Plant Cell Tissue and Organ Culture 23: 187-192.

Kuginuki, Y., Nakamura, K., Hida, K., Yoshikawa, H., 1997. Varietal differences in embryogenic and regenerative ability in microspore culture of Chinese cabbage (*Brassica rapa* L. ssp. Pekinensis). Breed Sci. 47:341-346.

Li, X., and Guan, C., 2003. Studies of microspore culture and doubled haploid breeding on rapeseed: plant regeneration from microspore derived embryos of F_1 hybrids between *Brassica napus* and *Brassica juncea*. In: *The Proceeding of The 11th International Rapeseed Congress*, pp.132.

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

Lida, Y., Watanabe, K., Kamada, H., Harada, H., 1992. Effects of abscisic acid on induction of desiccation tolerance in carrot somatic embryos. J. Plant Physiol. 140: 356-360.

Morris, P.C., Kumar, A., Bowles, D.J., 1990. Osmotic stress and abscisic acid induce expression of the weat E_m genes. Eur. J. Biochem 190: 625-630.

Nehlin, L., Mollers, C., and Glimelius, K., 1995. Induction of secondary embryogenesis in microspore-derived embryos of *Brassica napus* L. Plant Science 1: 219-227.

Park, H.B., Kim, Y.H., Whipkey, A., 1988. Proline and ABA increase survival of desiccated somatic embryos of celery. Hortscience 23:754.

Pechan, P.M., Bartels, D., Brown, D.C.W., Schell, J., 1991. Messengers RNA and protein changes associated with induction of *Brassica* microspore embryogenesis. Planta 184: 161-165.

Senaratna, T., Kott, L., Beversdorf, D.W., McKersie, D., 1991. Desiccation of microspore derived embryos of oilseed rape (*Brassica napus* L.). Plant Cell Reports 10: 342-344.

Skriver, M., and Mundy, J., 1990. Gene expression in response to abscisic acid and osmotic stress. The Plant Cell 2: 503-512.

Takahata, Y., Wakui, K., Kaizuma, N., 1992. A dry artificial seed system for *Brassica* crops. Acta Horticulturae 1: 317-322.

Takahata, Y., 1997. Microspore culture. In: *Recent Advances in Oilseed Brassica*, eds. Kalia, H. R. and Gupta, S. K., pp. 160-181. Kalyani Publishers, Ludhiana.

Takahata, Y., and Keller, W.A., 1991. High frequency embryogenesis and plant regeneration in isolated microspore culture of *Brassica oleracea* L. *Plant Science* 74: 235-242.

Takahata, Y., Brown, D.C.W., Keller, W.A., and Kaizuma, N., 1993. Dry artificial seeds and desiccation tolerance induction in microspore-derived embryos of broccoli. *Plant Cell, Tissue and Organ Culture* 35: 121-129.

Wakui, K., Takahata, Y., and Kaizuma, N., 1994. Effect of abscisic acid and high osmoticum concentration on the induction of desiccation tolerance in microspore-derived embryos of Chinese cabbage (*Brassica campestris* L.). *Breeding Science* 44: 29-34

Wakui, K. , Takahata, Y., and Kaizuma, N., 1999. Scanning electron microscopy of desiccation-tolerant and -sensitive microspore-derived embryos of (*Brassica napus* L.). *Plant Cell Reports* 18: 595-600.

Wakui, K., and Takahata, Y., 2002. Isolation and expression of *Lea* gene in desiccation-tolerant microspore-derived embryos in *Brassica* spp. *Physiologia Plantarum* 116: 223-230.

Wang, X., Loh, C., Yeoh, H., and Sun, W., 2002. Drying rate and dehydrin synthesis associated with abscisic acid-induced dehydration tolerance in *Spathoglottis plicata* protocorms. *Journal of Experimental Botany* 53: 551-558.

Wenzel, G., Hoffman, F., and Thomas, E., 1977. Anther culture as a breeding tool in rape. *Z. Pfl.- Zücht*. 78: 149–155.

Wilen, R.W., 1992. Gene expression in microspore embryos. Ph.D. Thesis. University of Calgary, Calgary, Alberta, Canada.

Zhang, F., Aoki, S., and Takahata, Y., 2003. RAPD markers linked to microspore embryogenic ability in *Brassica* crops . *Euphytica* 131: 207-213.

Zhao, J.P., Simmonds, D.H., and William, N., 1996. High frequency production of doubled haploid plants of *Brassica napus* cv. Topas derived from colchicines-induced microspore embryogenesis without heat shock. *Plant Cell Reports* 15: 668-671.