Acta Universitatis Latviensis, 2005, Vol. 691, Biology, pp. 137–143

Induction of somatic embryos on *in vitro* cultured zygotic embryos of spring *Brassica napus*

Natalija Burbulis*, Ramune Kupriene

Department of Crop Science and Animal Husbandry, Lithuanian University of Agriculture, Studentu 11, Akademija, Kaunas distr. LT-53067, Lithuania
*Corresponding author, E-mail: natalija@nora.lzua.lt

Abstract

Mature and immature zygotic embryos of three *Brassica napus* doubled haploid lines were examined for their ability to produce somatic embryos. Seedlings from mature seeds did not produce somatic embryos in all of the tested media. Induction of somatic embryogenesis directly from immature seeds of doubled haploid lines was obtained on plant growth regulator-free medium. There was no callus phase, thus, the time of induction was very short. Age of zygotic embryos was the most significant factor influencing somatic embryos induction and development into plantlets. Immature zygotic embryos at the age of 20 to 21 days after pollination were most suitable for direct somatic embryogenesis in the tested rapeseed doubled haploid lines.

Key words: *Brassica napus,* zygotic embryos, somatic embryogenesis.

Introduction

The oilseed *Brassica* species (*B. napus*, *B. rapa* and *B. juncea*) are among the most important vegetable oil and protein-rich meal crops producers in the world. Cultivation of oilseed *Brassica* has increased tremendously during the last decade and, by now, it is the second largest contributor to the world supply of vegetable oil. This success is largely attributed to continuous and intensive breeding efforts (Zhou 2001). Oilseed *Brassica napus* L. was the first crop species in which breeding was achieved by both traditional as well as modern methods.

Various plant tissues have been used for regeneration of *B. napus* shoots, including hypocotyls (Khehra et al. 1992), cotyledons (Narasimhulu et al. 1988), root segments (Sharma et al. 1989), stem and leaf segments of one-month old seedlings (Ovesna et al. 1993). The creation of novel germplasm through techniques of tissue culture and gene transfer holds great potential for improving the quality and agronomic characters of rapeseed. For this, *in vitro* regeneration of plants via organogenesis or embryogenesis is a prerequisite. Regeneration of somatic embryos from plant tissue has been reported for many plant species (Bajaj 1995). Mature and immature zygotic embryos have been used to initiate a regenerable culture in many plants, including *Arabidopsis thaliana* (Luo, Koop 1997), *Pisum sativum* (Tetu et al. 1990), and *Dalbergia latifolia* (Rao, Sita 1996), however there have been only a few reports on the induction of direct somatic embryogenesis in *B. napus* with seeds as the starting material (Graves et al. 1991; Koh, Loh 2000).

The aim of the present paper was to induce direct somatic embryogenesis from zygotic

embryos of spring rapeseed of different doubled haploid lines.

Materials and methods

The investigation was carried out with doubled haploids (DH) 07-133, 07-152, 07-196 of spring rape (*Brassica napus* L.). Donor plants were grown in the growth room with a light intensity of 5000 lx and a 16-h photoperiod. the age of zygotic embryos was recorded as the number of days after pollination (DAP). Both mature and immature seeds (age 14 to 29 DAP) were used as explants for producing somatic embryos.

Seed pods of various age were surface-sterilised in 70 % ethanol for 2 min and rinsed three times with autoclaved water. Seeds (10 per dish) were aseptically germinated in 90 mm Petri dishes containing 25 ml of Murashige and Skoog (1962; MS) basal medium supplemented with 2 % sucrose and solidified with 0.8 % Difco-Bacto agar. The MS medium was adjusted to pH 3.5, 4.0 or 5.0 before the addition of agar and autoclaving at 115 °C for 30 min.

The Petri dishes were sealed with Parafilm and incubated at 25 ± 2 °C temperature, a 16-h photoperiod, and 5000 lx light intensity. After three days the seedlings were transferred to fresh MS medium of the same pH and cultivated 28 days under similar lighting and temperature conditions.

For each treatment, 30 seeds were cultured and the experiment was repeated three times. The percentage of seeds forming somatic embryos and the mean number of somatic embryos per embryogenic seedling was recorded. Mean values and SE for each genotype were calculated.

Results

Many of the seedlings developed swollen hypocotyls within two weeks after germination. Seedlings from matured seeds did not produce somatic embryos in all of the tested media (data not shown).

Immature zygotic embryos cultured on media with various pH formed white nodular structures without an intervening callus phase. The white nodular structures developed into globular somatic embryos after four weeks of culture, indicating that the nodular structures were somatic embryos at an early developmental stage. The frequency of somatic embryo formation was significantly affected by the genotype, zygotic embryo age and the pH of the medium.

Seedlings that developed from zygotic embryos at the age of 14 to 15 DAP cultivated on pH 3.5 medium did not form any somatic embryos (Table 1). Higher medium pH (4.0) stimulated somatic embryogenesis of lines 07-196 and 07-133, however increasing the medium pH to 5.0 increased the embryogenic potential only of line 07-133. Seedlings developed from zygotic embryos of age 20 to 21 DAP had underwent somatic embryogenesis in all of the tested media. Explants of lines 07-196 and 07-133 exhibited the greatest frequency of somatic embryo formation at pH 5.0 medium, while a pH of 4.0 was more suitable for line 07-152. Zygotic embryos isolated 25 to 26 DAP positively responded on all tested media, except explants of line 07-152 cultivated at pH 3.5 medium. For lines 07-196 and 07-152 the highest somatic embryos induction rates were observed at pH 5.0 of medium, while a lower medium pH (4.0) raised somatic embryogenesis for line

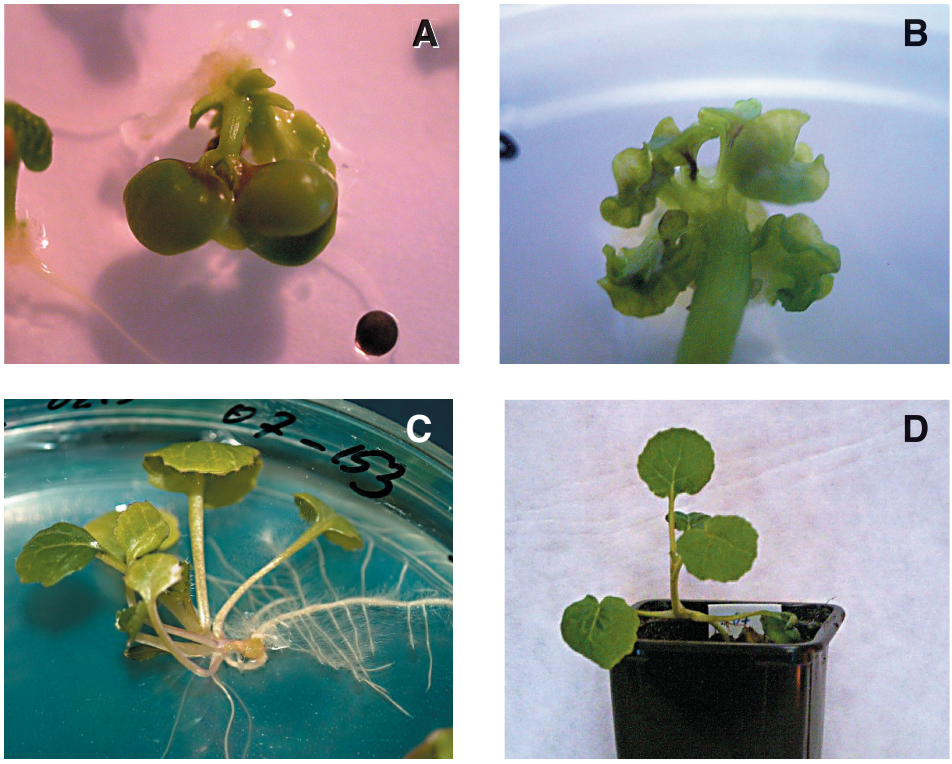

Fig. 1. Somatic embryogenesis of *Brassica napus*: A, somatic embryos on the hypocotyls; B, cotyledonary somatic embryos after four weeks of culture; C, plantlets in regeneration medium; D, rapeseed seedlings in greenhouse.

07-133 explants. Immature zygotic embryos at the age of 28 to 29 DAP had the greatest embryogenic potential at medium pH 5.0 (lines 07-196 and 07-152) and pH 4.0 (line 07-133).

Age of zygotic embryos was the most significant factor influencing somatic embryo development into plantlets. Zygotic embryos isolated 20 to 21 DAP showed the highest number of somatic embryos per explant (Table 2).

The frequency of somatic embryo formation decreased with an increase in zygotic embryo age. This suggests that zygotic embryos of 20 to 21 DAP are most suitable for direct somatic embryogenesis in the tested lines. The number of somatic embryos per embryogenic seedling also varied with the pH of medium. Culture medium of pH 4.0 appeared to be the best for somatic embryo formation, as it supported the highest number of somatic embryos per explant for DH 07-196 (18.2) and DH 07-152 (7.8), while DH 07-133 produced the highest number of SE per embryogenic seedling cultivated at pH 5.0 of medium.

Most somatic embryos were formed on the hypocotyls (Fig. 1A) of immature zygotic embryos and a few somatic embryos were formed on the cotyledon. No somatic embryos

Table 1. Effect of of zygotic embryo age and medium pH on percentage of somatic embryogenesis of *Brassica napus* doubled haploid DH lines

Genotype	Time after pollination (days)	Seedlings with somatic embryos (%)		
		pH 3.5	pH 4.0	pH 5.0
DH 07-196	14 - 15	0.0	4.7 ± 0.9	3.2 ± 0.4
DH 07-133	14 - 15	0.0	4.3 ± 1.1	12.3 ± 2.1
DH 07-152	14 - 15	0.0	0.0	0.0
DH 07-196	20 - 21	13.1 ± 2.3	4.6 ± 0.7	14.7 ± 2.3
DH 07-133	20 - 21	6.7 ± 1.1	9.1 ± 1.9	13.3 ± 1.9
DH 07-152	20 - 21	2.3 ± 0.4	14.8 ± 2.5	8.1 ± 1.6
DH 07-196	25 - 26	29.2 ± 3.6	25.2 ± 3.1	34.4 ± 4.2
DH 07-133	25 - 26	4.7 ± 0.9	31.3 ± 3.8	13.2 ± 2.3
DH 07-152	25 - 26	0.0	6.7 ± 1.5	30.7 ± 3.5
DH 07-196	28 - 29	47.3 ± 4.9	27.6 ± 3.2	89.8 ± 6.9
DH 07-133	28 - 29	4.7 ± 0.8	37.7 ± 4.1	11.1 ± 2.2
DH 07-152	28 - 29	0.0	0.0	14.3 ± 2.7

were formed on the radicle. Globular embryos derived from immature zygotic embryos developed into cotyledonary embryos after an additional four weeks of culture. Embryos and accessory cotyledons formed in a ring around the top of the hypocotyls, and on some embryos one to two accessory root poles were also initiated lower down near the original root pole. Most somatic embryos possessed two cotyledons, however, some had three or more cotyledons (Fig. 1B). Upon transfer to B_5 basal medium, cotyledonary embryos developed into plantlets (Fig. 1C) at a frequency of approximately 55 %. Plantlets were acclimatized to greenhouse conditions and resembled true rapeseed seedlings in growth habit (Fig. 1D).

Discussion

Direct somatic embryogenesis from hypocotyls of immature zygotic embryos has been reported for *Brassica campestris* (Maheswaran, Williams 1986), *Linum usitatissimum* (Pretova, Williams 1986), *Brassica napus*, *Brassica rapa* and *Brassica juncea* (Graves et al. 1991), and *Rosa hybrida* (Kim et al. 2003). Factors controlling the initiation process have been reviewed by Williams and Maheswaran (1986) and further discussed by Pretova et al. (2000). It has been suggested that initiation involves a weakening of the cell-cell interaction gradient, which coordinates normal bipolar development of the embryo. In the presence of a continuing stimulus for mitotic divisions, cells which are relatively undifferentiated and retain their internal pre-determination for embryo morphogenesis may escape from overall group control to re-initiate the embryogenic pathway independently as somatic embryoids. The external culture medium is believed to be permissive rather than determinative for somatic embryogenesis in this system.

It has been hypothesized that the addition of 6-benzylaminopurine in the process of

Table 2. Effect of zygotic embryo age and medium pH on intensity of somatic embryogenesis of *Brassica napus* doubled haploid (DH) lines

Genotype	Time after pollination (days)	Number of somatic embryos per embryogenic seedling		
		pH 3.5	pH 4.0	pH 5.0
DH 07-196	14 - 15	0.0	1.1 ± 0.1	8.4 ± 1.2
DH 07-133	14 - 15	0.0	3.2 ± 0.8	6.3 ± 1.3
DH 07-152	14 - 15	0.0	0.0	0.0
DH 07-196	20 - 21	1.1 ± 0.2	18.2 ± 2.7	10.3 ±2.4
DH 07-133	20 - 21	4.3 ± 0.9	6.1 ± 1.8	8.5 ± 2.1
DH 07-152	20 - 21	2.2 ± 0.7	7.8 ± 1.9	6.7 ± 1.1
DH 07-196	25 - 26	3.8 ± 1.1	4.4 ± 0.8	5.8 ± 0.9
DH 07-133	25 - 26	2.3 ± 0.8	3.2 ± 0.7	1.5 ± 0.1
DH 07-152	25 - 26	0.0	3.1 ± 0.5	3.3 ± 1.2
DH 07-196	28 - 29	0.2 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
DH 07-133	28 - 29	1.2 ± 0.3	0.3 ± 0.1	5.5 ± 0.8
DH 07-152	28 - 29	0.0	0.0	1.4 ± 0.2

direct somatic embryogenesis is responsible for preservation of the mitotic stimulus in the hypocotyl cells of the immature zygotic embryo (Pretova et al. 2000). The best embryogenic response in *Brassica napus* cultivar 'Regent' was obtained on a medium supplemented with 0.05 mg l^{-1} 6-benzylaminopurine, while somatic embryo production from 'Westar' zygotic embryos was improved two to three fold by decapitating the shoot apical meristem (Graves et al. 1991).

Auxin has been found to be essential for somatic embryo induction in many plant species (Merkle et al. 1995). However, immature zygotic embryos of some species require a relatively high concentration of cytokinin, which can reverse the arrest of pre-embryogenic cells, resulting in somatic embryogenesis (Norgaard, Krougstup 1991; Laurrain et al. 1996; Kim et al. 2003).

Unlike the results of many previous experiments, the induction and development of somatic embryos from immature zygotic embryos of *Brassica napus* in our study did not require an exogenous supply of plant growth regulators in the culture medium. Thus, we suggest that the immature zygotic embryos of the tested rapeseed lines are comprised of pre-embryogenic cells, which begin division in response to medium pH to differentiate into induced embryogenic determined cells. It has been hypothesized that low external pH may simulate auxin action during the induction of somatic embryos by lowering the cytosolic pH (Koh, Loh 2000). Another possible effect of low medium pH could be enhancement of the uptake of nutrients such as Fe compounds or NO_3^- (Polowick, Sawhney 1991), which could affect the subsequent development of the Brassica napus culture.

The procedure for somatic embryo induction from immature zygotic embryos described in this paper is efficient and can rapidly produce normal intact plants. This system may be of considerable potential for the study of plant embryogenesis, as it excludes the use of exogenous phytohormones and other complicated culture manipulations.

References

Bajaj Y.P.S. 1995. Somatic embryogenesis and its application for crop improvement. In: Bajaj Y.P.S. (ed) *Biotechnology in Agriculture and Forestry. Vol. 30. Somatic Embryogenesis and Synthetic Seed*. Springer, Berlin Heidelberg New York, pp. 105–125.

Graves A.J., Hemphill J.K., Ram R. 1991. Somatic embryogenesis in oilseed *Brassica*. Proceeding of 8th International rapeseed Congress "Rapeseed in a changing world", Saskatoon, Saskatchewan, Canada, 6: 1801–1808.

Khehra G. S. Mathias R. J. 1992. The interaction of genotype, explant and media on the regeneration of shoots from complex explants of *Brassica napus*. *J. Exp. Bot.* 43: 1413–1418.

Kim S.W., Oh S.C., Liu J.R. 2003. Control of direct and indirect somatic embryogenesis by exogenous growth regulators in immature zygotic embryo culture of rose. *Plant Cell Tissue Organ Cult.* 74: 61–66.

Koh W.L., Loh C.S. 2000. Direct somatic embryogenesis, plant regeneration and *in vitro* flowering in rapid-cycling *Brassica napus*. *Plant Cell Rep.* 19: 1177–1183.

Laurain D, Chenieux J.C., Tremouillaux-Guiller A. 1996. Somatic embryogenesis from immature zygotic embryos of *Ginkgo biloba*. *Plant Cell Tissue Organ Cult.* 44: 19–24.

Luo Y., Koop H.-U. 1997. Somatic embryogenesis in cultured immature zygotic embryos and leaf protoplast of *Arabidopsis thaliana* ecotypes. *Planta* 202: 387–396.

Maheswaran G., Williams E.G. 1986. Primary and secondary direct somatic embryogenesis from immature zygotic embryos of *Brassica campestris*. *J. Plant Physiol.* 124: 455–463.

Merkle S.A., Parrott W.A., Flinn B.S. 1995. Morphogenic aspects of somatic embryogenesis. In: Thorpe T.A. (ed.) In vitro *Embryogenesis in Plants*. Kluwer, Dordrecht, pp. 155–203.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco cultures. *Physiol Plant.* 15: 473–497.

Narasimhulu S. B., Chopra V. L. 1989. Species specific shoot regeneration response of cotyledonary explants of Brassicas. *Plant Cell Rep.* 7: 104–106.

Norggard J.V., Krogstup P. 1991. Cytokinin-induced somatic embryogenesis from immature embryos of *Abies nordmanniana* Lk. *Plant Cell Rep.* 9: 509–513.

Ovesna J., Placek L., Opatrny Z. 1993. Factors influencing the regeneration capacity of oilseed rape and cauliflower in transformation experiments. *Biol. Plant.* 35: 107–112.

Polowick P.L., Sawhney V.K. 1991. *In vitro* floral development of oilseed rape (*Brassica napus* L.): The effect of pH and plant growth regulators. *J. Exp. Bot.* 42: 1583–1588.

Pretova A., Hajduch M., Obert B. 2000. Some characteristics of flax embryo development *in situ* and *in vitro*. *Acta Biol. Cracov.* 42: 45–53.

Rao M.M., Sita G.L. 1996. Direct somatic embryogenesis from immature embryos of rosewood (*Dalbergia latifolia* Roxb.). *Plant Cell Rep.* 15: 355–359.

Sharma K. K., Thorpe T. A. 1989. *In vitro* regeneration of shoot buts and plantlets from seedling root segments of *Brassica napus* L. *Plant Cell Tissue Organ Cult.* 18: 129–134.

Tetu T., Sangwan R.S., Sangwan-Norreel B.S. 1990. Direct somatic embryogenesis and organogenesis in cultured immature zygotic embryos of *Pisum sativum* L. *J. Plant Physiol.* 137: 102–109.

Williams E.G, Maheswaran G. 1986. Somatic embryogenesis: Factors influencing coordinated behaviour of cells as an embryogenic group. *Ann. Bot.* 57: 443–462.

Zhou W. J. 2001. Oilseed rape. In: Zhang G. P., Zhou W. J. (eds) *Crop Cultivation*. Zhejiang University Press, Hangzhou, China, pp. 153-178.

Organoģenēze *Linum usitatissimum* kallusa kultūrā

Natalija Burbulis*, Ramune Kupriene

Augkopības un lopkopības departaments, Lietuvas Lauksaimniecības universitāte, Studentu 11, Akademija, Kauņas raj. LT-53067, Lietuva
*Korespondējošais autors, E-pasts: natalija@nora.lzua.lt

Kopsavilkums

Pārbaudīja nobriedušu un nenobriedušu *Brassica napus* trīs dažādu dubulto haploīdo līniju zigotisko embriju spēju veidot somatiskos embrijus. Dīgsti no nobriedušām sēklām neveidoja somatiskos mebrijus nevienā no pārbaudītajām barotnēm. Somatiskā embrioģenēze inducējās tieši no dubulto haploīdu sēklām barotnē bez augšanas regulatoriem. Tā kā attīstība notika bez kallusa fāzes, indukcijas laiks bija ļoti īss. Zigotisko embriju vecums bija būtiskākais faktors, kas ietekmēja somatisko embriju indukciju un to attīstību par mikroaugiem. Pārbaudītajām rapšu dubulto haploīdu līnijām vispiemērotākais nenobriedušo zigotisko embriju vecums tiešās somatiskās embrioģenēzes norisei bija 20 līdz 21 dienu pēc apputeksnēsanas.