SOMATIC EMBRYOGENESIS IN OILSEED BRASSICA

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INTRODUCTION

Embryogenic response in somatic tissues can be categorized into two groups: 1) Indirect somatic embryogenesis, where somatic embryos are produced in a callus derived from explant tissue, and 2) Direct somatic embryogenesis, where somatic embryos originate directly from a cell or a group of cells within the explant. In the present paper we discuss the somatic embryogenesis phenomenon in three important oilseed Brassica species: B. napus, B. rapa and B. juncea.

The genus Brassica comprises monogenomic species such as B. rapa, B. oleracea and B. nigra, and digenomic species such as B. napus, B. juncea and B. carinata. A wealth of information is available on the induction of embryogenesis in isolated microspore cultures and anther cultures of Brassica (Kott et al. 1990). However, there are considerably fewer reports on somatic embryogenesis from diploid tissue sources of oilseed Brassicas (Li and Kohlenbach 1982; Klimaszewska and Keller 1986; Gupta et al. 1990; Maheshwaran and Williams 1986; Pua 1990). In microspore cultures, significant genotypic influence on haploid embryo induction has been observed (E.J. Eikenberry, Agrigenetics Company, Madison. personal communication). In the present study we report considerable differences among various genotypes in their capability to produce somatic embryos.

MATERIALS AND METHODS

Plant Materials:

Digenomic species, B. napus and B. juncea and monogenomic species B. rapa were used in the present study. Seed of B. napus and B. rapa were kindly provided by Agriculture Canada, and seeds of B. juncea were provided by (the late) Dr. P.F. Knowles (University of California, Davis).

Tissue for callus initiation and indirect somatic embryogenesis was obtained from 5-7 days old, dark grown seedlings of B. napus cvs. Reston, Regent, Westar and Profit. For direct embryogenesis, pods of B. napus cvs. Reston, Westar and Regent and B. rapa cvs. Tobin, Candle and R-500, 14-17 days post pollination, were surface sterilzed in 20% bleach. Immature zygotic embryos were aseptically isolated and plated on secondary embryo induction media (see below). Protoplasts were isolated from hypocotyl tissues of 5-7 days old dark

grown seedlings of B. juncea.

Culture Conditions:

Unless otherwise specified, indirect embryogenesis experiments used MS (Murashige and Skoog, 1962) basal medium, modified Nitsch and Nitsch (1967) vitamins, 2% sucrose and 0.2 % Gelrite®. Hormones were filter sterilized and added to cooled autoclaved media except for 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA). Seed germination medium for callus intiation experiments included, in addition to MS basal medium, 0.1 mg/l indolebutyric acid (IBA). Callus cultures were maintained in diffused light (0.4 lux); somatic embryo germination and plant regeneration were conducted in bright light (5.5 lux) for 16 h. Callus induction and maintenance media were supplemented with an amino acid mixture, containing glutamine (0.8 g/l), methionine (0.35 g/l), alanine (1.0 g/l), asparagine (0.2 g/l) and arginine (0.87 g/l). For direct embryogenesis from zygotic embryos, B5 (Gamborg et al. 1968) basal medium containing 2% sucrose and 0.2% gelrite was used for culture initiation. Cultures for secondary embryo induction were maintained in continuous light (5.5 lux). All cultures were maintained at 25 ± 2 °C.

Protoplasts were isolated from hypocotyl segments by overnight digestion in 1% Cellulase R-10 and 0.1% Macerozyme R-10 (Onozuka) prepared in MS major salt solution without ammonium nitrate, 12% sucrose and buffered with 976 mg/l MES (pH 5.6). Protoplasts were separated by floatation on isolation medium and washed 2 to 3 times in rinse medium (Shepard 1980) prior to culturing in CL medium (Shepard 1980), modified to include 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and solidified by the addition of 0.6% agarose. Protoplasts were cultured at 100,000 cells/ml in agarose bead cultures, partially submerged in CL liquid medium. Well developed embryoids were germinated on cytokinin-rich medium (2.0 mg/l each of kinetin and zeatin) and root development occurred on B5 medium containing 0.1-0.5 mg/l IBA.

RESULTS

Indirect Embryogenesis:

In this system, plant regeneration from callus-derived somatic embryos involved several steps, each of which involved media manipulations:

Explant → Preconditioning → Callus → Somatic embryo → Embryo maturation → Embryo germination → Root development → Plantlet

Seeds of B. napus cvs. Reston, Regent, Westar and Profit were germinated in dark on MS medium containing 0.1 mg/l IBA. Seedling tissues 5-7 days old were preconditioned on germination medium for an additional seven days prior to transferring

to callus induction medium. Preconditioned hypocotyl and cotyledonary segments were then placed on media containing 2,4-D (0, 0.5, 1.0, 5.0, and 10.0 mg/l) and a mixture of amino acids, for embryogenic callus induction. Cultures were maintained in diffused light.

When using 5.0 mg/l 2,4-D for three weeks and diffused light culture conditions, embryogenic callus was produced in Reston. The cultivar Profit produced a better embryogenic response than Reston at 10 mg/l 2,4-D for one week, with 42% of hypocotyl segments and 23% cotyledons producing embryogenic callus (Table 1).

Table 1. Embryogenic callus production from seedling tissues of B. napus cultivars.

Genotype	Tissue	Initiated	Responded	% Response
Profit	Hypocotyl	409	170	41.6
	Cotyledon	226	52	23.0
Reston	Cotyledon	164	13	7.9

The embryogenic callus had smooth texture as noted in other crops such as corn, and is distinguishable from the non-embryogenic callus. Occasionally, the embryogenic callus also showed the presence of globular embryos. To maintain the embryogenic callus, several media combinations with decreasing concentrations of auxin and with or without supplemental abscisic acid (ABA) and osmoticum (mannitol) were tested. The embryogenic callus in Reston could be maintained for several months in diffused light on MS medium containing 1.0 mg/l 2,4-D, 1.5 mg/l indoleacetic acid (IAA), 0.26 mg/l ABA, and amino acid mixture without any loss of embryogenic potential (Fig.1a). However, embryogenic callus in Profit could not be maintained for more than four weeks on this medium.

To induce plant regeneration, somatic embryos produced on maintenance medium required an embryo maturation step. This allowed proper development of dicotyledonary structures with well defined root and shoot meristems. Several embryo maturation media were tested containing elevated levels of osmoticum produced by the addition of sucrose, sorbitol or mannitol. Additionally, ABA alone or in combination with the above described sugars were tested. Plating on medium containing 10% sucrose with no other additions for one week provided adequate conditions for Reston somatic embryos (Fig. 1b). However, in the case of Profit, addition of 0.026 mg/l ABA to a medium containing 2% sucrose for 1-3 weeks provided the best observed maturation conditions for somatic embryos. Additionally, the germination rate of Reston somatic embryos was increased 7-8 fold (from 4% to 30%) by transfer to a maturation medium containing charcoal (1%).

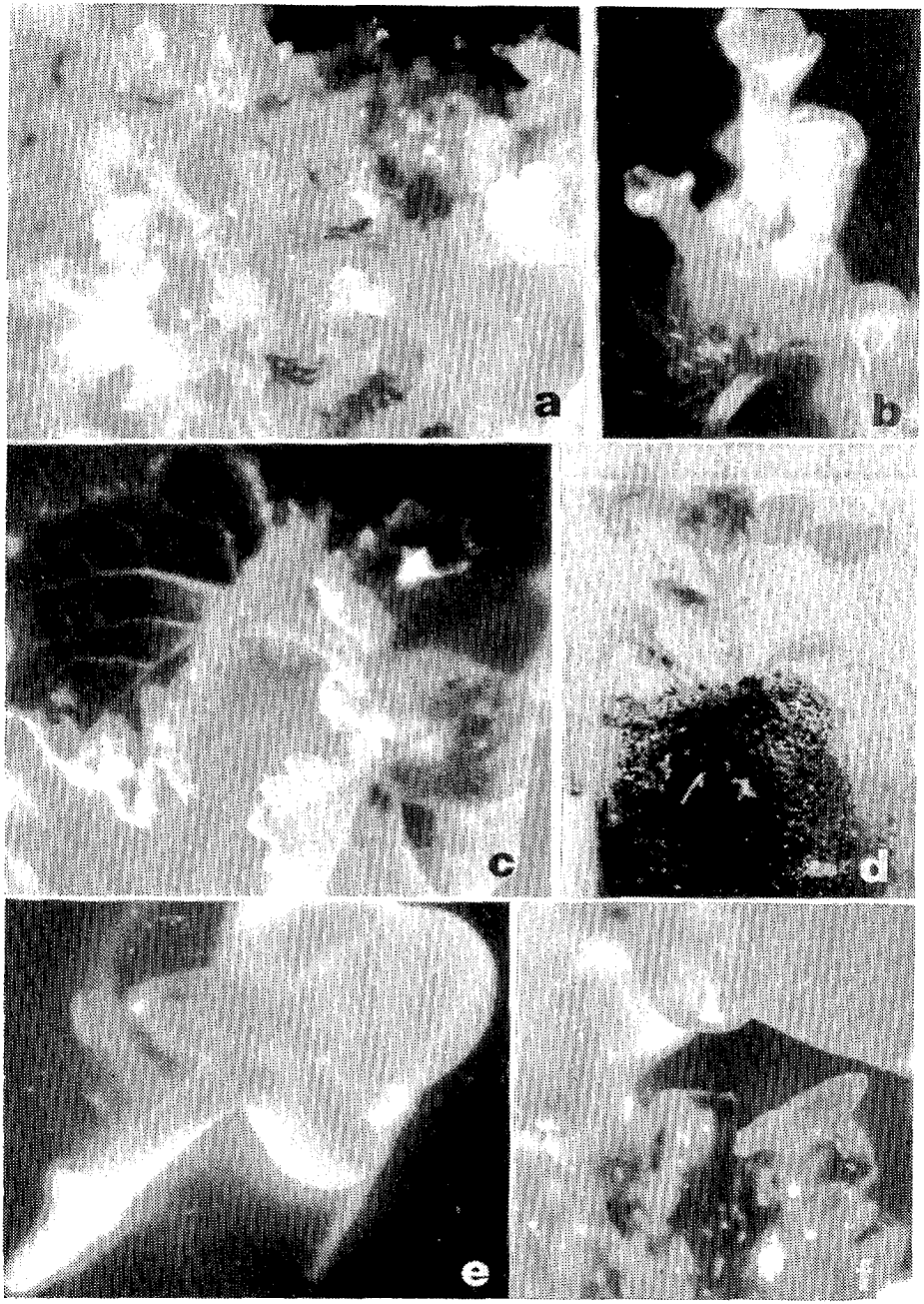

Fig. 1. Indirect somatic embryogenesis in B napus Reston (a-d): a) Embryogenic callus, b) Somatic embryo maturation, c) Somatic embryo germination, d) Plantlet in peat pellet. Direct embryogenesis in B. rapa Tobin (e,f): e) immature zygotic embryo, f) secondary embryogenesis.

As a next step, several germination media were tested to obtain plantlets from the somatic embryos (Fig. 1c). These consisted of B5 basal salts with the addition of various cytokinins such as BAP, kinetin, zeatin and thidiazuron, alone or in several combinations. For the cultivar Reston, germination rates as high as 52% were obtained in media supplemented with 0.6 mg/l zeatin. Whereas for the cultivar Profit, 33% germination was observed on MS medium supplemented with 0.5 mg/l NAA and 4.0 mg/l BAP. Higher levels of BAP or zeatin resulted only in shoot growth; root growth was suppressed.

Germinated somatic embryos were cultured in B5 basal medium for another three to six weeks, and about 30% produced plantlets with healthy roots. The addition of hormones and vitamins was deleterious to root growth. Plantlets were transferred to peat pellets (Fig.1d), and then to soil in the greenhouse. Somatic embryos which failed to germinate often produced secondary embryos.

Direct Embryogenesis:

Zygotic embryos (Fig.1e) isolated 14-17 days post pollination were plated on B5 basal medium with or without 3% sorbitol and with the addition of ABA or BAP. Tissue responded positively on all media by producing secondary embryoids on the surface of hypocotyls (Fig.1f). However, the best response was obtained in the cultivar Regent on a medium supplemented with 0.05 mg/l BAP and maintained in continuous light (Table 2). Somatic embryo production from Westar zygotic embryos was improved two to three fold by decapitating the shoot apical meristem.

Table 2: Secondary embryogenesis in zygotic embryos of Brassica.

Species	Cultivar	Embryos plated	S.embryos produced	% Response
B. napus	Regent	185	383	207
	Westar	50	29	58
B. rapa	R-500	11	4	36
	Tobin	120	133	111
	Candle	115	21	18

After three to five weeks in culture, secondary embryos were isolated from the hypocotyl region of the zygotic embryo and transferred to a B5 basal medium for germination. Secondary embryos germinated at a rate of 25-33% and produced plantlets. After meristem differentiation, these shoots were transferred to fresh B5 medium for root development. Somatic embryos which failed to germinate, often produced secondary embryos.

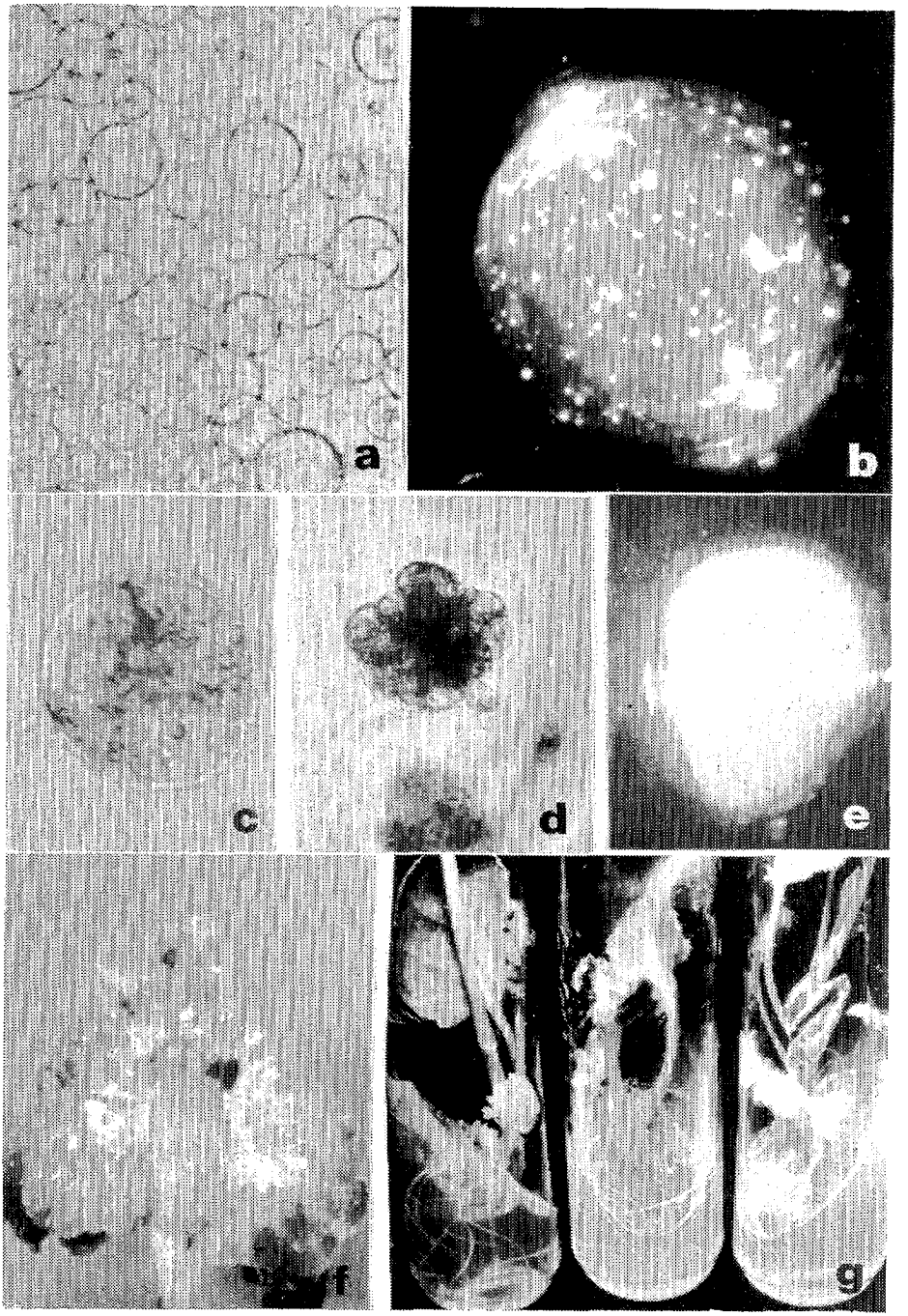

Fig. 2. Direct embryogenesis in B. juncea protoplast cultures. a)isolated protoplasts, b)protoplasts in bead culture, c)early divisions, d-e)multicellular embryogenic stages, f)germination , g)rooted plantlet.

Direct embryogenesis also occurred in B. juncea protoplast cultures. Protoplasts isolated from hypocotyl segments underwent wall formation and first division within 72 hours. Subsequent divisions resembled zygotic divisions leading to the formation of globular embryo-like structures (Fig.2a-e). These developed into bipolar heart and torpedo shaped embryos. Torpedo stage embryos were removed from agarose beads and plated on a cytokinin medium for germination (Fig.2f). Initial steps in germination resembled normal development. However, in the later stages only shoot growth persisted. Root developement required a separate medium containing IBA (Fig.2g).

DISCUSSION

Somatic embryogenesis via embryogenic callus formation is not common in Brassica. The only species that have been reported to produce embryogenic callus are B. oleracea (Pareek and Chandra 1978), B. nigra (Klimaszewska and Keller 1986) and B. juncea (Gupta et al. 1990). In our experiments, the embryogenic callus was induced in a high auxin medium (2,4-D), in a short period of time. However, the high auxin concentrations were deleterious for the maintenance of callus. Preconditioning of the seedling tissue in low auxin, and induction of callus in high auxin, are probably important for a high degree of response.

Embryo maturation was an important step for proper germination of somatic embryos and further plantlet development. Optimized maturation media generally contained high osmoticum and/or ABA. Considerable work has been done to establish in vitro conditions for isolated zygotic embryos which simulate the in vivo biochemical developmental pattern (Finkelstein and Crouch 1987). Similarities in developmental stages of somatic embryos to their counterpart zygotic embryos can be brought about by manipulating media components, thereby enhancing the germination potential of the somatic embryos.

Somatic embryos in most cases appear to originate from single cells. Particularly in the case of direct embryogenesis from protoplast cultures, we observed no evidence of callus formation. Therefore, somatic embryogenic systems, direct or indirect, may play a significant role not only in understanding the ontogeny of embryos, but also in producing non-chimeric mutants and transformants.

Induction of somatic embryos in Brassica appears to be easier with microspore and anther cultures than with diploid tissues. With haploid systems, considerable genotypic influence in the culture response is seen; B. napus being more amenable to culture than B. rapa. Similarly in our experience with diploid systems, B. rapa is more difficult to culture than B. napus lines. Even among the various B. napus cultivars only Profit and Westar responded by producing somatic embryos from callus cultures.

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REFERENCES

FINKELSTEIN, R.R. and CROUCH, M.L. 1987. Hormonal and osmotic effects on developmental potential of maturing rapeseed. HortSci. 22: 797-800.

GAMBORG, O.L., MILLER, R.A. and OJIMA, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

GUPTA, V., AGNIHOTRI, A., and JAGANNATHAN, V. 1990. Plant regeneration from callus and protoplasts of Brassica nigra (IC 257) through somatic embryogenesis. Plant Cell Rep. 9: 427-430.

KLIMASZEWSKA, K. and KELLER, W.A. 1986. Somatic embryogenesis in cell suspension and protoplast cultures of Brassica nigra (L) Koch. J. Plant Physiol. 122: 251-260.

KOTT, L.S., ERICKSON, L.R. and BEVERSDORF, W.D. 1990. The Role of Biotechnology in Canola/Rapeseed Research. In: Canola and Rapeseed:Production, Chemistry, Nutrition and Processing Technology. F. Shahidi (ed.). Van Nostrand Reihold, New York. pp. 47-78.

LI, L., and KOHLENBACH, W. 1982. Somatic embryogenesis in quite a direct way in cultures of mesophyll protoplasts of Brassica napus L. Plant Cell Rep. 1: 209-211.

MAHESHWARAN, G. and WILLIAMS, E.G. 1986. Primary and secondary direct somatic embryogenesis from immature zygotic embryos of Brassica campestris. J. Plant Physiol. 124: 455-463.

MURASHIGE, T. and SKOOG, F. 1962. A revised medium for rapid growth and bioassay with tobacco cultures. Physiol. Plant. 15: 473-492.

NITSCH, J.P. and NITSCH, C. 1967. The induction of flowering in vitro in stem segments of Plumbago indica L. I. The production of vegetative buds. Planta 72: 355-370.

PAREEK, L.K. and CHANDRA, N. 1978. Somatic embryogenesis in leaf callus from cauliflower (Brassica oleracea var. Botrytis). Plant Sci. Lett. 11: 311-316.

PUA, E-C. 1990. Somatic embryogenesis and plant regeneration from hypocotyl protoplasts of Brassica juncea (L.) Czern & Cross. Plant Sci. 68: 231-238.

SHEPARD, J. 1980. Mutant selection and plant regeneration from potato mesophyll protoplasts. In: Genetic Improvement of Crops. I. Rubenstein, B. Gengenbach, R.L. Phillips and C.E. Green (eds.). Univ. Minnesota. pp. 185-217.