

Maintenance of male sterile germplasm in *Brassica rapa* by *in vitro* propagation

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An efficient tissue culture system for plant regeneration, from mature cut branches, was established to maintain male sterile material in *Brassica rapa* L. The new-growth immature pods from the cut branches were used as explants; they gave better results in callus initiation (37 calli from 25 explants) and shoot formation (17 shoots from 75 explants) than flower buds and branch stems. Auxin [2,4-dichlorophenoxyacetic acid (2,4-D), 2 to 5 mg l⁻¹] and cytokinin [6-benzylaminopurine (BA), 2 to 4 mg l⁻¹] were essential in callus and shoot formation, respectively. Callus initiation and shoot regeneration capacities were genotype dependent. The regenerated plants were male sterile and were used in breeding programs.

Key words: *Brassica rapa* L., callus, *in vitro* micropropagation, plant growth regulators, shoots

Introduction

Plant regeneration from somatic cells is possible either via shoot/root organogenesis or via somatic embryogenesis. Both pathways have resulted in regenerants from numerous species (Lörz et al. 1988). One of the most useful applications of plant tissue culture lies in the possibility of cloning a particular genotype. This can

be especially important if the desired plant is a hybrid or an allogamous species, due to the fact that the sexually-derived progeny of these types are no longer identical to the original genotype (Linacero and Vazquez 1990). Clonal propagation of plants is based on the concept of totipotency. It is a prerequisite for most applied uses of plant tissue cultures. Effective plant tissue culture techniques have been developed for several of the economically important species of

Brassica from explants of leaves, hypocotyls, petioles, stem layers and protoplasts (Bhattacharya and Sen 1980, Li and Kohlenbach 1982, Eapen et al. 1989, Kirti and Chopra 1990).

Turnip rape (*Brassica rapa* L.) is the most important oil crop in Finland. Cytoplasmic male sterility was used in turnip rape breeding work. This cytoplasmic male sterility (cms), originally found by Ogura (1968), was transferred to *B. oleracea* and *B. napus*. Male sterile *B. napus* cybrids were then produced through protoplast fusion (Pelletier et al. 1983) to generate male sterile lines with highly stable male sterility, improved nectar secretion and a high productivity (Pelletier et al. 1987). This OGU-INRA cytoplasmic male sterility was transferred to *Brassica rapa* by back-crossing. The *in vitro* propagation method was used to save the male sterile breeding material in this study. This method facilitated recycling of the same sterile A-line (cms) genome and the crossing of it with certain R-lines (fertility restoring) in descending generations in order to study the maternal impact on F1 hybrids.

Brassica rapa is more recalcitrant in tissue culture than other *Brassica* species. In this study, cut branches were collected, cold treated and forced to induce new buds. Old stems and new-growth tissues such as flower buds and new-growth immature pods were then used as explants for *in vitro* propagation. The best results were achieved from new-growth immature pods of the harvested male sterile plants in callus initiation and shoot regeneration tests. Complete plants were regenerated. Male sterile breeding material was thus maintained via *in vitro* propagation using immature pods as explants.

Material and methods

Cut branches from 121 different male sterile breeding line plants, under cross-testing in the greenhouse, were used as explant sources. The greenhouse growth condition for donor plants

was 20°C/17°C day/night temperature and a 16-h photoperiod at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplemented by fluorescent lamps. The breeding lines included 91451-A, 92549-A, 4001-A, 4002-A and 4003-A, all Mildola's female breeding lines, from *Brassica rapa* hybrid program. Parkland, an old Canadian variety, was used as a control in this program. All test lines, except for the control, have Ogura radish (OGU-INRA) cytoplasmic male sterility.

Vegetative cut branches were stored with stalks in water in a 4°C cold room with 8-h photoperiod at 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for one month, then, these cut branches were transferred to a greenhouse to induce new growth. The growth condition for inducing flower buds and immature pods was 25°C and a 16-h photoperiod at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplemented by fluorescent lamps. The branch stalks were in tap water and water changed every three days.

Branch stems, flower buds and new-growth immature pods of the harvested branches were surface sterilized in 70 (v/v) ethanol for 1 min, followed by 5 min in a 10% sodium hypochlorite solution containing one drop of Tween-20 and then rinsed three times with sterile distilled water. The following explants were used to initiate cultures on semi-solid medium: the suitable size of flower bud was between 3 and 6 mm; branch stem explants were 3 mm thick transverse sections; 5–10 mm long immature pods containing embryos, were cut into 4 to 5 mm explants.

The explants were plated on semi-solid callus induction media. The culture medium consisted of B₅ (Gamborg et al. 1968) macro- and micro-nutrients, inositol 100 mg l⁻¹, nicotinic acid 1 mg l⁻¹, pyridoxine-HCl 1 mg l⁻¹, thiamine-HCl 5 mg l⁻¹, L-glutamine 2 mg l⁻¹, L-alanine 10 mg l⁻¹, casein hydrolysate 200 mg l⁻¹, glycine 2 mg l⁻¹, sucrose (3%, w/v) and Phytigel™ (0.3%, w/v). All media used in these experiments were sterilized by autoclaving at 1.1 kg/cm² and 120°C for 20 min. The explants were cultured in 9 cm Petri dishes containing 35 ml of sterile medium in darkness at 27°C. The effect of growth regulators on callus induction was investigated by culturing explants on induction media supple-

mented with different concentrations of auxin [2,4-dichlorophenoxyacetic acid (2,4-D), 1–6 mg l⁻¹] combined with 0.1 mg l⁻¹ kinetin (6-furfurylaminopurine). Each treatment consisted of three replicates each with 25 explants.

After a four week induction period, the calli were subcultured onto the same medium for three to four weeks. Calli between 5 and 10 mm in size, were transferred to shoot regeneration medium consisted of B₅ basal components containing 6-benzylaminopurine (BA) (1–5 mg l⁻¹) in combination with one of three low levels of auxin, naphthaleneacetic acid (NAA) 0.2 mg l⁻¹, 3-indoleacetic acid (IAA) 0.2 mg l⁻¹ or 2,4-D 0.1 mg l⁻¹. Shoot regeneration was maintained at a light intensity of 50 μmol m⁻² s⁻¹ and a 16-h photoperiod supplemented by fluorescent lamps and a temperature of 25°C. For rooting, regenerated shoots were transferred to half-strength, hormone-free B₅ medium at conditions same as that for shoot regeneration. The rooted plantlets were transferred into 15 cm plastic pots containing peat/sand mixture to a greenhouse under controlled conditions employing 25°C/18°C, 16 h photoperiod. The light intensity was about 150 μmol m⁻² s⁻¹ supplemented by fluorescent lamps. Plantlets were covered with glass beakers during the first week of acclimatization to prevent desiccation of plants.

Results and discussion

Explant sources

There were differences in callus induction and shoot propagation capacities among the three sources of explants (Table 1). The callus initiated from new-growth immature pods was solid, compact and grew rapidly (Fig. 1a). The callus from flower buds grew slowly, and was loose. The callus from branch stems grew slowly, turned brown in colour and died after several subcultures. Green shoots could not be obtained from branch stem derived callus. Callus induc-

tion and shoot formation was much better from immature pod explants than from flower bud and stem explants.

Explant source	No. callus ± SD*	No. shoot (total)
Immature pod	37 ± 9	17
Flower bud	16 ± 2	5
Branch stem	7 ± 3	0

* SD = standard deviation.

2,4-D = 2,4-dichlorophenoxyacetic acid

Kinetin = 6-furfurylaminopurine

BA = 6-benzylaminopurine

tion and shoot formation was much better from immature pod explants than from flower bud and stem explants.

The explant source acts an important role in micropropagation of *Brassica* species. In *Brassica rapa* ssp. *Pekinensis*, the successful explant for micropropagation was the cotyledon (Kuo and Tsay 1977), it represented a significant improvement in regeneration of Chinese cabbage in comparison with axillary buds that were cultured. Young hypocotyl explants were used for somatic embryogenesis and plant regeneration in six *Brassica* species (Dietert et al. 1982, Yang et al. 1991, Narasimhulu et al. 1992). Hypocotyl segments, stem and leaf explants from seedlings were used in somatic embryogenesis in *Brassica nigra* (Narasimhulu et al. 1992). In *Brassica napus*, *in vitro* plant formation was induced from internodal segments (Kartha et al. 1974), cotyledon, hypocotyls and stem sections (Khehra and Mathias 1992), axillary buds (Polowick and Sawhney 1991) and thin cell layer explants (Klimaszewska and Keller 1985, Shu and Loh 1991). In the present study, branch stems, flower buds and immature pods were used as explants in micropropagation due to old and wilting donor materials. The immature pods gave the best results for callus induction and plant regeneration.

The age of the donor plant (explant) is a crit-

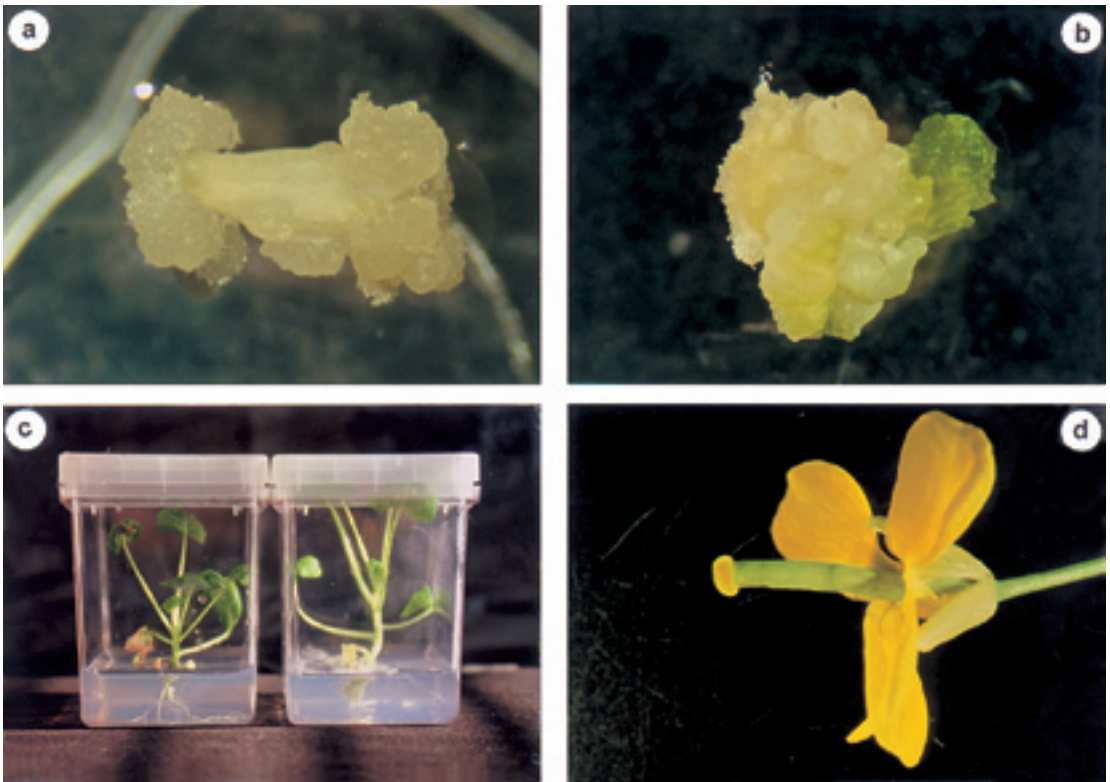


Fig. 1. Callus initiation and shoot regeneration in *Brassica rapa* male sterile material (4003A-6) using immature pods. The modified B₅ medium containing 4 mg l⁻¹ 2,4-D and 0.1 mg l⁻¹ kinetin was used in callus induction from immature pod. In shoot regeneration, B₅ medium with 2 mg l⁻¹ BA and 2,4-D 0.1 mg l⁻¹ was employed. Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid, Kinetin = 6-furfurylaminopurine, BA = 6-benzylaminopurine.

- (a) Callus initiation from an immature pod explant after two weeks of culture (8 x).
 (b) Shoot formation from callus in regeneration medium (8 x).
 (c) Well developed plantlet from immature pod propagation.
 (d) A flower from regenerated plant with male sterile character.

ical factor affecting callus initiation and shoot regeneration in *Brassica* species (Yang et al. 1991). Generally, young tissue is used as an optimal explant, the old explants are always avoided in plant tissue culture.

In the present study, 121 male sterile breeding line plants were used as explant sources. These donor plants were under cross-testing in the greenhouse at the time that the branches were cut. Only a few breeding lines were selected for micropropagation as determined by their agronomic characteristics and crossing ability in de-

scending generations in order to study the maternal impact on F1 hybrids. Therefore, the cut branches were stored (for one month at 4°C) awaiting greenhouse test results. Using this method, time and labour could be saved. However, these cut branches were old and conventional micropropagation techniques could not be applied.

After branches were moved to the greenhouse at 25°C, the new-growth immature pods and new-growth flower buds became usable. These new-growth organs with active metabolism

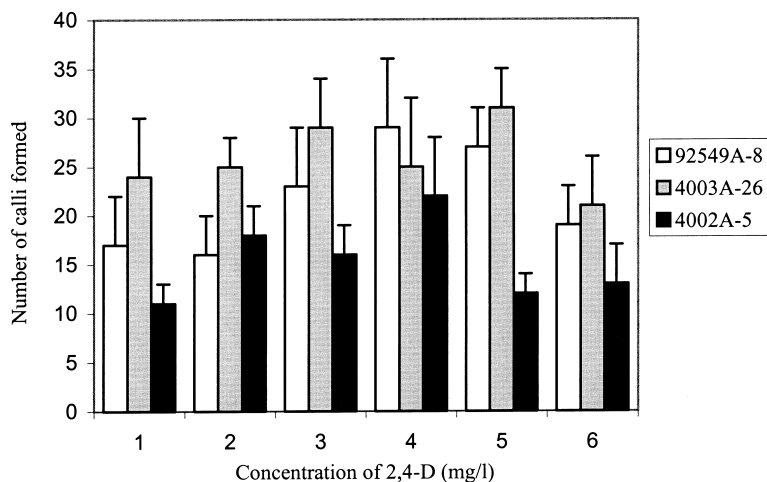


Fig. 2. Effect of auxin (2,4-D = dichlorophenoxyacetic acid) concentration combined with 0.1 mg l⁻¹ kinetin (6-furfurylaminopurine) on callus initiation from immature pod of turnip rape *in vitro* propagation. Four replicates, 25 explants per replicate. Vertical bars indicate the standard error of the mean.

yielded calli and shoots with a relatively high regularity.

Influence of 2,4-D concentration on callus initiation

B₅ basal medium (Gamborg et al. 1968) was used as callus induction and shoot regeneration medium in this study. Auxin and cytokinin are essential in callus induction and shoot formation, respectively. In this study, 2,4-D was used as the auxin at 1 mg l⁻¹, 2 mg l⁻¹, 3 mg l⁻¹, 4 mg l⁻¹, 5 mg l⁻¹, 6 mg l⁻¹ for callus induction, combined with a low level of cytokinin (kinetin 0.1 mg l⁻¹). Concentrations of 2,4-D between 2 and 5 mg l⁻¹ gave the best result for callus induction. However, there was a genotype difference. For clone 92549A-8, 4–5 mg l⁻¹ 2,4-D promoted the callus initiation effectively whereas for clones 4003A-26 and 4002A-5, the best dosages of 2,4-D were 5 mg l⁻¹ and 4 mg l⁻¹, respectively (Fig. 2). The importance of 2,4-D in callus initiation and growth in *Brassica* species has been recognized (Bajaj and Nietsch 1975, Dietert et al. 1982, Shu and Loh 1991). Dietert et al. (1982) reported that a high level of 2,4-D may inhibit callus growth, but the presence of low levels of this auxin is

required for callus proliferation and for inhibition of root development in certain cultivars of *B. oleracea* and *B. napus*.

Influence of BA on shoot formation

The function of BA in *Brassica* species micropropagation has been reported. Deng et al. (1991) described that in Chinese cabbage (*B. rapa* ssp. *Pekinensis*), the concentration of BA which promoted the highest rate of shoot induction showed clonal variation and was in the range of 44.4 to 177.6 μM. In *B. napus*, BA alone (5 μM) induced multiple shoot formation on stem explants (Kantha et al. 1974). In this work, the combinations of auxin (NAA 0.2 mg l⁻¹, IAA 0.2 mg l⁻¹ and 2,4-D 0.1 mg l⁻¹) and cytokinin (BA 1–5 mg l⁻¹) were investigated. In a shoot regeneration experiment using clone 4003A-8, and in the presence of NAA 0.2 mg l⁻¹, IAA 0.2 mg l⁻¹ or 2,4-D 0.1 mg l⁻¹, the optimal BA concentrations were 3 mg l⁻¹, 4 mg l⁻¹ and 2 mg l⁻¹, respectively (Fig. 3).

Shoots could be obtained after 4 to 6 weeks when calli were cultured on regeneration medium (Fig. 1b). When the shoots were 1 cm high, they were cut from callus and subcultured to the

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Table 2. Genotype effect on callus and shoot formation in *B. rapa* male sterile breeding materials propagation. Immature pods were used as explants in callus induction experiments. The modified B₅ medium containing 4 mg l⁻¹ 2,4-D and 0.1 mg l⁻¹ kinetin was used in callus induction. In shoot regeneration, B₅ medium with 2 mg l⁻¹ BA and 0.1 mg l⁻¹ 2,4-D was employed. Means ± standard deviation are from three replicates, 25 explants per replicate.

Breeding lines	No. callus/dish	Shoot formation (total)
91451A	-1	31 ± 3*
	-4	25 ± 3
	-5	17 ± 3
	-6	34 ± 4
	-11	20 ± 4
	-12	15 ± 5
	-14	16 ± 4
	-16	24 ± 4
	-19	24 ± 6
	-20	26 ± 8
	-21	24 ± 2
	-23	39 ± 1
	-24	24 ± 5
	-25	9 ± 3
	-28	21 ± 6
92549A	-5	17 ± 4
	-6	15 ± 3
	-8	33 ± 3
	-9	25 ± 2
	-11	37 ± 3
	-12	20 ± 6
	-14	18 ± 1
	-15	25 ± 9
	-16	34 ± 6
	-19	25 ± 7
Parkland	-3	23 ± 5
	-4	7 ± 3
	-5	14 ± 4
	-6	30 ± 3
	-7	25 ± 5
4001A	-8	33 ± 4
	-9	24 ± 6
	-10	28 ± 7
	-13	24 ± 3
	-14	20 ± 3
	-15	33 ± 4
	-16	34 ± 6
	-1	25 ± 7
	-3	24 ± 4
	-5	25 ± 4
4002A	-2	35 ± 3
	-3	31 ± 8
	-4	27 ± 8
	-5	25 ± 4
	-6	37 ± 2

continued on next column

continued from preceding column

Breeding lines	No. callus/dish	Shoot formation (total)
	-7	33 ± 5
	-10	19 ± 3
	-14	23 ± 3
	-15	18 ± 1
	-17	20 ± 4
	-18	31 ± 3
	-20	28 ± 6
	-22	27 ± 9
4003A	-1	25 ± 3
	-5	23 ± 9
	-6	31 ± 8
	-7	17 ± 4
	-8	25 ± 9
	-10	25 ± 5
	-14	24 ± 1
	-16	13 ± 3
	-17	35 ± 3
	-18	23 ± 3
	-19	26 ± 2
	-20	15 ± 3
	-21	31 ± 4
	-22	23 ± 6
	-25	34 ± 3
-26	35 ± 2	

* SD = standard deviation.

2,4-D = 2,4-dichlorophenoxyacetic acid

Kinetin = 6-furfurylamino-purine

BA = 6-benzylamino-purine

same regeneration medium. The shoots were transferred to a culture jar containing half-strength, hormone-free B₅ medium for rooting when they were over 2.5 cm high (Fig. 1c). The regenerated plants grew normally and had male sterile character (Fig. 1d). All the clones showed a high degree of phenotypic uniformity within the clone.

Genotype effect on callus and shoot formation

Among 121 branches, callus was initiated from 66 and shoots from 48 branches which indicates a genotype difference in propagation ability of

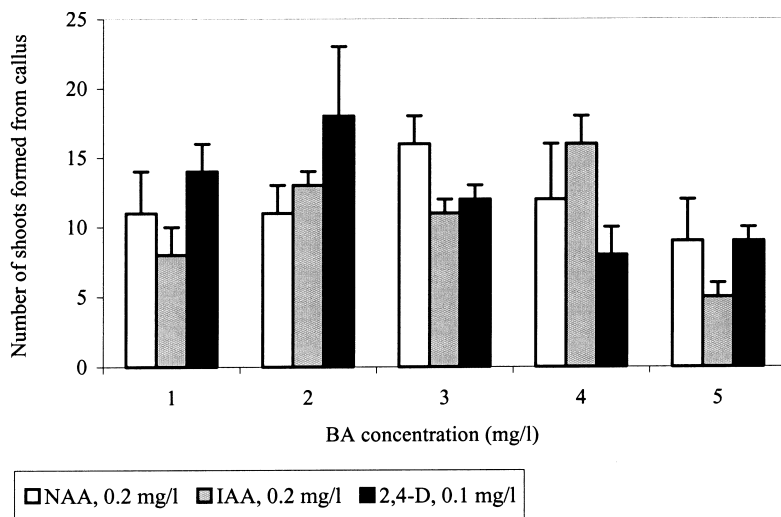


Fig. 3. Effect of hormone combinations on shoot formation in *B. rapa* (4003A – 8) in vitro propagation. Means are from four replicates, 25 explants per replicate. Vertical bars indicate the standard error of the mean.

B. rapa (Table 2). The callus initiation and shoot regeneration capacities were genotype dependent in this study.

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SELOSTUS

Isästeriilin kevätrypsin geeniaineksen ylläpito *in vitro* mikroviljelyllä

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Rypsin (*Brassica rapa*) solukkoviljely on ristikkukaisten kasvien solukkoviljelyistä vaikeinta. Tämän tutkimuksen tarkoituksena oli pelastaa *in vitro* mikroviljelyllä valinnassa olevat OGU-INRA isästeriilit CMS-kevätrypsit takaisin valintakiertoon. Tutkimuksessa yritettiin *in vitro* mikroviljellä tuleentuneen sadon tuottaneiden ja kylmäkäsitellyn saaneiden isästeriilien kasvien lehdistä ja varsista uusia, vihreitä kasveja. Tehtävä epäonnistui, koska tutkittava materiaali ei kyennyt uusiutumaan ikänsä ja mikrobi- ja sienisaastantansa vuoksi. Jatkotutkimuksessa sama, lähes eloton, tutkimusmateriaali siirrettiin kasvihuoneen

optimiolosuhteisiin ja nesteravintoviljelyyn. Kasvit pakotettiin kukintaan, joista edelleen epäkypsät lidut saatiin kasvattamaan kallusta, joka voitiin regeneroida isästeriiliksi lähtömateriaaliksi. Sekä kalluksen kehittyminen että regenerointi todettiin genotyypiriippuvaisiksi. Kehitetty menetelmä mahdollistaa arvokkaan CMS-materiaalin uudelleenkäytön kypsää satoa vaativan valintaprosessin jälkeen. Ennen kaikkea menetelmä mahdollistaa CMS-äidin uudelleen ja uudelleen testaamisen useiden tekijöiden suhteen, mikä muutoin ristikkukaisella kasvilla olisi mahdollista.