Comparsions of developmental stages of microspore by bud size and embryogenesis from its microspore in *Brassica* species

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Abstract

The purpose of this experiment is to search for the identifying method that separate buds with embryogenic potential from collected buds for increasing the efficiency of embryogenesis by microspore culture in *Brassica species*.In *Brassica napus*, Lisandra of spring type showed bud-size of 3.2-4.1mm which belongs to late-uninucleate stage as embryogenic. Hallyuchae of winter type showed bud-size of 3.1-3.6mm which belongs to late-uninucleate stage as embryogenic microspores. Two genotypes of *B. juncea* were nearly same as bud-size of Youngsanpohwanggyeja were 2.9-4.0mm and bud-size of Hwanggyeja were 2.9-4.1mm. Also, two genotypes of *B. campestris* were nearly same as bud-size of Hyakusai were 2.9-3.4mm and bud-size of Sosongchae were 2.9-3.2mm.In the embryo yield per 100,000 microspores by isolated microspore culture from the buds with embryonic microspores, Youngsanpohwanggyeja of *B. juncea* were 36 embryos and Hwanggyeja were 19 embryos. *B. campestris* of Hyakusai were 253 embryos and Sosongchae were 104 embryos. However in *B. napus* was showed much difference by the growth habits, the spring type of Lisandra were 4,128 embryos of which higher about 8.8 times than those of winter type Hallayuchae.

Key words: *Brassica species*, microspore culture, embryogenesis

Introduction

Brassica species have high productivity, good yield, and good agronomic characteristics. Many of these species are used for food, as oils, as biodiesel and as animal feed. Breeding programs have involved innovative techniques to assist the release of new cultivars.

Microspore culture has been successful in over 250 species including cereals, oilseeds, ornamentals, and vegetable crops. Several *Brassica* species have also shown success. These include *B. oleracea*, *B. napus*, *B. campestris*, and *B. carinata*. Microspore culture of rapeseed is an established tool for the production of homozygous lines in breeding program. For this purpose, fertile doubled haploid (DH) plants at a high and genotype-independent frequency are required. Usually, 70-90% of the microspore-derived plants are haploid (Licher, 1985, Chen and Beersdorf 1992). Doubled haploids (DH) are presently used in breeding of a number of crops species. This method enables breeders to develop completely homozygous genotypes from heterozygous parents in one single generation.

Microspore culture is used as an alternative to conventional breeding and is used in many *Brassica* breeding programs. There are several advantages and applications of microspore culture. Microspore culture can yield up to ten times more embryos than anther culture (Siebel and Pauls, 1989; Jang et al., 1997). This also reduces the number of plants that need to be screened, and because recessive traits are not masked, smaller populations are needed (Rajhathy, 1976; Ferrie et al., 1995). In plant breeding, microspore culture produces true breeding lines which can be used for studies of inheritance patterns (Evans et al., 1998). The time about 3 or 4 years of which a new cultivar is released is also decreased which frees up economic resources to be focused on other aspects of the breeding program (Ulrich et al., 1984). Selection is more efficient because no masking by dominant genes occurs and recessive traits are easily identified.

Other applications include mutagenesis, transformations, and biochemical studies. Mutagenesis has led to the development of *B. napus* lines with a thinner seed coat, higher oil and protein content, and low fibre content as well as other developments (Henderson and Pauls, 1992). Mutation techniques have also improved yield and resistance to diseases and pests in *B. carinata*. Gene linkage and interactions have been studied in *B. campestris* through the use of haploid plants (Guo and Pulli, 1996). Genetic engineering has been used with microspore culture in Agrobacterium transformations (Goll, 2000) and with the use of haploid technologies novel cultivars can be developed with a wide range of adaptations allowing crops to be farmed on marginal lands (Ferrie et al., 1994; Izquierdo, 1999). Embryos have can be used in *B. napus* to investigate biochemical pathways and screen end products (Wiberg et al., 1991).

Microspore culture is the culture of the male gametophytic cell into haploid embryos. These embryos are haploid and may spontaneously double into doubled haploid plants or in many species, a chromosome doubling treatment with colchicine may be needed. To have seed double haploid plants are necessary because haploid plants are sterile. Embryogenesis will only occur if the proper conditions are met. These factors may include genotype, donor plant conditions, development stage of the pollen grains, media composition, and post culture incubation and are discussed below. The figures below show the process and stages of embryogenesis.

The aim of the present study was to establish a standard procedure for microspore culture and haploid plant regeneration of *Brassica* species so as to select regenerative genotypes for mutagenesis and transformation experiments.

Materials and methods

Plant materials were used 'Lisandra' of spring type and 'Hallayuchae' of winter type in *Brassica napus*, 'Youngsanpohwanggyeja' and 'Hwanggyeja' of winter type in *B. juncea*, and Hyakusai and Sosongchae of *B. campestris*.

They were sowed in greenhouse at October 10th in 2004. From December to beginning flower, it was controlled 20-30°Cof diurnal temperature, 8-10°Cof night temperature. It was irrigated by interval of 2-3days during growth period.

It was collected 30 of flower buds at the stage of beginning 6-9 of flowers. After separated microspores from anther, it was measured bud length, petal length, anther length, and the rate of petal/anther, respectively. The microspores were isolated as described by Jang et al., (1992). The microspores were cultured at a density of $5x10^4$/ml in a modified NLN medium (Lichter, 1982), with 13% (w/v) sucrose. Aliquots of 2ml microspore suspension were plated in 6cm tissue culture Petri dishes. It was cultured during 3 or 5 days of initial culture at 32.5°C in darkness. And then it was cultured with 25°C continuously in darkness. After cultured during 24days, it was investigated shape of embryo.

The developmental stages represented in the initial microspore populations were scored as early uninucleate(EU), mid uninucleate(MU), late uninucleate(LU), vegetative and generative nuclei(VG), and vegetative and generative sperm nuclei(VSS), according to similar stages described by Kott et al.(1988).

Results

Haploid breeding is essential to to promote efficiency of embryogenesis from isolated microspores. It is need simple method which is distinguishable flower buds including embryonic microspores. It was investigated bud size, anther length, and rate of petal/anther of buds with embryonic microspores.

In Lisandra(*B. napus* L.) of spring type, the development stage of the microspores was mid uninucleate stage from 2.5mm of bud size to 3.0mm of that. It was appeared late uninucleate stage from 3.3mm of bud size to 4.3mm of that (Table 1).

Table 1. Relationships between bud size and petal/anther ratio of microspores in Lisandra (*Brassica napus* L.) with spring type

Bud size(mm)	Anther length(mm)	Petal/anther ratio	Pollen stage
2.1-2.4	1.5-1.7	1/4-1/3	EU
2.5-2.9	1.8-2.0	1/3-1/3	MU
3.0-3.3	2.2-2.3	1/3-1/2	MU-LU
3.4-3.7	2.4-2.6	2/3-2/3	LU
3.8-4.2	2.7-2.8	3/4-3/4	LU
4.3-4.6	2.9-3.1	1/1-1/1	LU+ - VG

Note: EU; early uninucleate stage, MU; mid uninucleate stage, LU; late uninucleate stage, LU+; most spores in LU, but some division present, VG; vegetative and generative nuclei present.

In case of Hallayuchae(*B. napus* L.) of winter type, the development of microspores was very fast according to extend bud size. It was appeared already late uninucleate stage in 3.0mm of bud size. When bud size was became 3.6mm, the development stage of microspores was vegetative and generative nuclei. It was appeared only late uninucleate stage from 3.0mm of bud size to 3.4mm of that (Table 2).

Table 2. Relationships between bud size and petal/anther ratio of microspores in Hallayuchae (*Brassica napus* L.) with winter type

Bud size(mm)	Anther length(mm)	Petal/anther ratio	Pollen stage
2.0-2.4	1.6-1.7	1/4-1/3	EU
2.5-2.7	1.8-2.0	1/3-1/3	MU
2.8-3.0	2.1-2.3	1/3-1/2	MU-LU
3.1-3.4	2.4-2.7	1/2-2/3	LU-LU+
3.6-3.9	2.8-2.9	2/3-3/4	VG
4.1-4.4	3.0-3.3	1/1-1/1	VG - VSS

Note: VSS; vegetative and two sperm nuclei present.

In case of *Brassica juncea*, the development stage of microspores was not the difference between two varieties of Youngsanpohwanggyeja and Hwanggyeja according to elongate bud size because of the same of growth habit. The late uninucleate stage of Youngsanpohwanggyeja was shown the extent with 3.0-4.0mm of bud size. Their petal/anther ratio was the rang of 1/2-3/4 (Table 3). The late uninucleate stage of Youngsanpohwanggyeja was shown the extent with 2.9-4.0mm of bud size.

Table 3. Relationships between bud size and petal/anther ratio of microspores in Youngsanpohwanggyeja (*Brassica juncea* L.).

Bud size(mm)	Anther length(mm)	Petal/anther ratio	Pollen stage
2.0-2.3	1.6-1.7	1/4-1/3	EU
2.5-3.0	1.8-2.0	1/3-1/2	MU-LU
3.1-3.3	2.2-2.5	1/2-1/2	LU
3.5-4.1	2.6-2.9	2/3-3/4	LU-LU+
4.2-4.5	3.2-3.9	3/4-1/1	VG

Table 4. Relationships between bud size and petal/anther ratio of microspores in Hwanggyeja (*B. juncea*).

Bud size(mm)	Anther length(mm)	Petal/anther ratio	Pollen stage
2.0-2.3	1.6-1.7	1/4-1/3	EU
2.5-2.9	1.8-2.0	1/3-1/2	MU-LU
3.0-3.4	2.1-2.4	1/2-1/2	LU
3.5-4.0	2.6-2.8	2/3-3/4	LU-LU+
4.1-4.4	3.1-3.6	3/4-1/1	VG

In *B. campestris*, two varieties of Hyakusai and Sosongchae was local variety. The results of the microspore development as bud elongation and the rate of petal/anther were shown in Table 5 and 6. In case of Hyakusai, it was appeared only the late uninucleate stage in the range with 2.9-3.4mm size of buds. The microspores with 3.8mm of bud size was the development stage which vegetative and generative nuclei is present. In case of Sosongchae, the bud sizes including the development of late uninucleate stage was the range with 2.8-3.3mm of size. It was appeared the development stage which vegetative and generative nuclei is present in 3.5mm of bud size.

Table 5. Relationships between bud size and petal/anther ratio of microspores in Hyakusai (*Brassica campestris* L.).

Bud size(mm)	Anther length(mm)	Petal/anther ratio	Pollen stage
2.1-2.4	1.5-1.7	1/3-1/2	EU
2.6-2.9	1.9-2.1	1/2-2/3	MU-LU
3.0-3.3	2.2-2.4	2/3-3/4	LU-LU+
3.4-3.8	2.5-2.7	3/4-1/1	LU+ - VG

Table 6. Relationships between bud size and petal/anther ratio of microspores in Sosongchae (*Brassica campestris* L.).

Bud size(mm)	Anther length(mm)	Petal/anther ratio	Pollen stage
2.0-2.4	1.5-1.8	1/3-1/2	EU
2.5-2.8	2.0-2.2	1/2-2/3	MU-LU
3.0-3.3	2.4-2.7	2/3-3/4	LU-LU+
3.5-3.8	2.9-3.3	1/1-1/1	VG-VSS

It was compared embryogenesis through the culture of isolated microspores from flower buds which is belongs to the development stage of mid uninucleate to late uninucleate in *Brassica* species (Table 7). The frequency of embryogenesis was very high in *B. napus*. The second was *B. campeatris*. The embryo production of *B. juncea* was very low. In *B. napus*, the produced embryos of Lisandra were 4,123 and that of Hallyuchae were 468. The embryo production of Lisandra was higher about about 8.8 times than those of Hallayuchae. In *B. juncea*, the produced embryos of Youngsanpoheanggyeja were 36 and that of Hwanggyeja were 19. In *B. campestris*, the produced embryos of Hyakusai and Sosongchae were 253 and 104, respectively. It was remarkable difference between two varieties of *B. napus* because of different growth habit. It was some difference between two varieties of *B. campestris* because of adaptation difference. Particularly, the rate of embryo production of *B. juncea* was appeared very low because was problem in the process of isolating microspores and collecting flower buds.

Table 7. Comparsion of embryo formation from isolated microspores among *Brassica* species.

Species	Variety	Growth habit	Range of bud size (mm)	Embryo yield*
Brassica napus	Lisandra	Spring type	2.3-4.2	4,128
	Hallayuchae	Winter type	2.8-3.4	468
Brassica juncea	Youngsanpoheanggyeja	Winter type	2.5-4.1	36
	Hwanggyeja	Winter type	2.5-4.0	19
Brassica campestris	Hyakusai	-	2.6-3.4	253
	Sosongchae	-	2.5-3.3	104

* No. of embryos per 5.0×10^4 microspores cultured.

Discussion

Haploid breeding is very desirable way to select homozygous line within short period. It is important to investigate precise stage of the microspore development in order to promote the efficiency of embryo production through microspore culture. And also it is need to extract flower buds including the microspore which is late nuinucleate or early binucleate stage with high potentials of embryogenesis from collected flower buds.

In this experiment, we showed remarkable difference the embryo production among *Brassica* species. It was the difference of growth environment among *Brassica* species such as *B. napus*, *B. juncea*, and *B. campestris. In vivo*, the development stage of microspores was appeared considerable difference between *Brassica* species or genotypes of the same variety.

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