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RESEARCH PAPER

Effect of cold pre-treatment on another culture in different *Brassica* genotypes

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Stress is an essential component to enhance callus induction in anther culture. Cold pretreatment has been used in cereal crops but very seldom attempted in *Brassica* anther culture. In order to assess the effect of cold pretreatment (4°C) of flower buds subjected to a liquid medium for 5, 10 and 12 days for callus induction in different genotypes of *Brassica*, three varieties (GSL1, DGS1 and RSPN25) of *B. napus* and three varieties of *Brassica juncea* (RSPR01, Varuna and Kranti) were evaluated. The appropriate duration of cold pre-treatment of flower buds was found to be 10 days which stimulated the maximum callus induction. Cold pretreatment was also able to promote development including the improvement of embryo quality and acceleration of embryogenesis.

Key words : Haploids, *Brassica napus*, *Brassica juncea*, Embryogenesis, Organogenesis

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INTRODUCTION

Brassica is the most economically important genus in the Brassicaceae family (syn. Cruciferae). Brassica occupy a prominent place in world's agrarian economy as vegetables, oilseeds, feed and fodder, green manure and condiments. They also contain a large number of novel photochemical, some of which are anti-carcinogenic (Steinmetz and Potter, 1996). The genus *Brassica* consists of over 150 species of annual or biennial herbs. Several of these are cultivated as oil seeds or vegetable crops. These comprise three diploid species, *B. nigra* (L.) Koch (2n = 16), *B. oleracea* L. (2n = 18) and *B. campestris* (Rapa) L. (2n = 20). The other three commonly cultivated species, *B. napus* L. (2n = 38), *B. juncea* (L.) *Czern* (2n = 36) and *B. carinata Braun* (2n = 34), are amphidiploids of the diploid species. *B. napus*, *B. campestris* and *B. juncea* are extensively cultivated for edible oil (Babbar *et al.*, 2004). Commercial oilseed varieties of *B. napus* and *B. rapa* known as canola have low erucic acid and low glucosinolate levels. The crop is grown both in subtropical and tropical countries.

Anther culture is an efficient way of producing doubled haploid plants in *Brassica species*. Compared with the traditional production of genetically stable homozygous lines, microspore culture dramatically speeds up breeding process and facilitates the selection of recessive traits (Henderson and Pauls, 1992). Microspore culture gives less response in *B. rapa* (Guo and Pulli, 1996) compared with *B. napus* (Zhou *et al*., 2002; Gu *et al*., 2004). The frequency of haploid embryos obtained using microscope culture has made it a suitable and important tool in selection in *Brassica* breeding programs (Palmer *et al*., 1996), mutagenesis and selection (Polsoni *et al*., 1988; Swasnson and Erickson, 1989) and genetic transformations (Huang, 1992). However, all these applications largely depend upon the efficient microspore embryogenesis and embryo development. There are various factors influencing microspore embryogenesis including donor plant genotype, donor plant physiology, microspore development stage, culture conditions, culture environment and pretreatments (Dunwell, 1996). Among these factors in *Brassica* microspore culture, a short heat shock treatment is basically required to stimulate microspore embryogenesis although it may be replaced by other stresses such as low level of gamma radiation, ethanol and colchicines (Pechan and Keller, 1989; Zhao *et al.*, 1996). When compared to heat stress, cold pretreatment is less frequently used in *Brassica* species inspite of its critical role in cereal microspore culture (Touraev *et al.*, 1997; Pechan and Smykal, 2001). Effective results from cold pretreatment of flower buds or inflorescence

were achieved in promoting microspore embryogenesis in *Brassica napus* (Lichter, 1982), *Brassica oleracea* although showing genotype dependency (Osolnik *et al.,* 1993), *B. rapa* (Sato *et al.,* 2002), and even by very temperature of isolated microspores in *B. napus* (Charne *et al.,* 1988). However negative results were also reported in *B. napus* (Dunwell *et al.,* 1985) and *B. rapa* (Sopory and Munshi, 1996).

In the present paper, different genotypes of *Brassica* were used to evaluate the effect of cold pretreatment of flower buds subjected to liquid medium on embryogenesis.

RESEARCH METHODOLOGY

Donor plants:

Experimental material comprised of anthers (explant) got from three varieties of *B. juncea* (RSPR01, Varuna and Kranti) and three varieties of *B. napus* (GSL1, DGS1 and RSPN 25). All the genotypes were collected from the Division of Plant Breeding and Genetics at Sher-e-Kashmir University of Agricultural Sciences and Technology (J), Chatha, Jammu.

Closed flower buds of 2-2.5 mm size of all selected genotypes, were collected at appropriate stage and time. Disease free flower buds, mostly from uninucleate to binucleate stage were collected during morning hours between 8:00 AM to 10:00 AM and evening hours between 4:00 PM to 6:00 PM. It is this time that the development stage of microspores would just before the nuclear mitosis stage.

Cold pretreatment:

The flower buds were surface sterilized in sodium hypochlorite (1%) for 8-10 minutes + fungicide (capt of 0.1%) + antibiotic cefatoxin (0.25%) which was followed by thorough rinsing with sterile distilled water. The flower buds were then put into test tubes containing a liquid medium with 13 per cent sucrose. The test tubes were placed for 5, 10 and 12 days at 4°C in the dark.

Anther culture:

Twenty to thirty flower buds, for each genotype were cut on a sterile Petri plate provided with lining of sterilized filter paper inside, under laminar airflow bench. Individual flower buds were cut at the base with sharp surgical sterilized scissors to free anther from the filament. With the help of sterilized pointed forceps the floret were picked at the apex and tapped on the rim of test tubes containing MS-medium (3% sucrose, 0.8% agar, pH 5.8) such that anthers fell on the surface of the medium. About 20-25 anthers per test tube were inoculated under aseptic conditions in laminar airflow cabinet. Tubes and flasks after inoculation were plugged with cotton plug and sealed with parafilm and incubated in dark at 25±2°C.

Callus induction and plant regeneration:

The calli induced were sub-cultured for further proliferation on the same media in which callus induction had take place. Large compact pieces of one month old calli were cut into small pieces (2-3 mm diameter) with sterilized blade inside laminar air flow cabinet. With the help of pointed forceps, they were picked and placed inside the test tubes containing semisolid regeneration media. The culture tubes were sealed with parafilm and incubated under cool white florescent light (2500 lux) in dark/light conditions for 16/8 h, respectively at 25±2°C. Observations with regard to number of plant regenerated were made within 5±1 weeks of inoculations. Approximately three weeks later, shoots were developed from calli. The fully formed plantlets were taken out from culture vessels. The agar was removed from the roots and plantlets shifted to MS basal medium supplemented with various concentration of auxin in large size test tube for proper development of roots and shoot and incubated for 15 days under light/dark period of 16/8 h, respectively, at 25±2°C.

Observation recorded:

For each genotype, anthers in aseptic cultures were counted and observations with respect to response of anthers to callusing were recorded. Data with respect to callus induction frequency (%) was worked out following Otani *et al.* (2005).

Callus induction frequency :

$$\frac{\text{No. of calli induced}}{\text{No. of anthers cultured}} \text{x100}$$

Data recorded for different parameters were subjected to Completely Randomized Design (CRD). Statistical analysis based on mean values per treatment was made using analysis of variance (ANOVA) technique of CRD.

RESEARCH FINDINGS AND ANALYSIS

The results obtained from the present investigation as well as relevant discussion have been summarized under the following heads :

Influence of cold pretreatment on callus induction:

To determine the effect of cold pretreatment of flower bud on callus induction, three varieties of *B. napus* (GSL1, DGS1 and RSPN 25) and three varieties of *B. juncea* (RSPR01, Varuna and Kranti) were given 4°C treatment (cold treatment) for different time duration. From the result (Table 1) it is evident that buds of different genotypes differed significantly in their capability for callus induction. It was seen that explants of DGS1 showed higher response to callus induction (38.56 %) followed by GSL1 (36.85 %). Remaining genotypes showed relatively lower percentage of callus induction capacity.

Influence of cold treatment duration on callus induction:

To determine the proper cold treatment duration of flower buds for callus induction frequency, each genotype from *Brassica napus* and *Brassica juncea* was tested for treatments of 5, 10 and 12 days in the experiment. All flower buds from late uninuleate to early binucleate stage were selected from donor plants and were distributed randomly for each temperature duration. Maximum percentage of callus induction frequency (44.17%) was observed when the anthers were given chilling treatment for 10 days. All other showed relatively lower per cent of callus induction frequency. Maximum percentage of callus induction frequency was observed in DGS1 (50.52%) when buds were given 4°C treatment for 10 days.

In *Brassica* genotypes, 10 days of cold pretreatment increased callus induction frequency significantly. The highest callus induction frequency (44.17) was obtained from 10 days cold pretreatment. There was a reduction in anther culture frequency by longer duration of cold pretreatment, suggesting a negative effect on callus induction if longer cold pretreatments than 10 days.

The switch of cultured microspore from gametophytic to sporophytic mode of development has been induced by various applied stresses (reviewed by Touraev *et al.,* 1997). To date four stresses widely used for the induction of microspore embryogenesis are cold (Sunderland and Xu, 1982; Gaillard *et al*., 1991, Gustafson *et al*., 1995; Gu *et al.,* 2004), heat (Duijs *et al.,* 1992; Custers *et al.,* 1994; Touraev *et al.,* 1996a, b), carbon starvatation (Kyo and Harada, 1986) and colchicine (Zhao *et al*., 1996). Some other stresses, such as ethanol and gamma irradiation (Pechan and Keller, 1989), have not been applied widely.

Cold pretreatment has known to improve microspore embryogenesis in barley (Davies and Mortan, 1988; Kasha *et al.,* 2001), wheat (Indrianto *et al.,* 1999), maize (Gaillard *et al*., 1991), and other important crops. In those cases, cold pretreatment were used alone or in combination with mannitol or starvation. With *Brassica*, inconsistent results for the effects of cold pretreatment on microspore embryogenesis have been reported. Cold pretreatments improved microspore embryogenesis in *B. napus* (Lichter, 1982; Gu *et al.,* 2004), *B. juncea* (George and Rao, 1982), and *B. rapa* (Sato *et al*., 2002). However, cold pretreatment of the buds inhibited microspore embryogenesis in *Brassica napus* (Dunwell *et al.*, 1985) and *Brassica rapa* (Sopory and Munshi, 1996). In previous studies, flower buds inflorescence or isolated microspore were treated by a piece of wet cotton (Osolnik *et al.,* 1993) or in a liquid medium (Charne *et al*., 1988; Sato *et al*., 2002) held at cold temperature before or after microspore isolation. In this study, using a short period of cold pretreatment, sterilized flower buds were treated in a liquid culture medium containing 13 per cent sucrose.

The present study was an attempt to evaluate the effect of cold pretreatment on callus induction in *B. juncea* and *B. napus*. A distinct enhancement of embryogenesis by cold pretreatment of flower buds was confirmed in *B. juncea* and *B. napus* under present experimental conditions. This promoting effect of cold pretreatment is in agreement with studies in *B. napus* (Lichter, 1982), *B.oleracea* (Osolnik *et al.,* 1993) and the *B.rapa* (Sato *et al.,* 2002, Gu *et al*., 2004). Maximum embryogenic callus was obtained when flowers buds were cold pre-treated at 4°C for about 10 days, similar response was reported earlier by Zhou *et al.,* 2002 and Zheng *et al*., 2006. In *B. rapa* as well as in other cold pre treated crops (Sopory and Munshi, 1996; Sato *et al*., 2002), increased percentage of bicellular stage microspores with two equal nuclei which was supposed to be necessary developmental stage for embryogenesis induction. In present study, anther pretreatment on callus induction appeared to be genotype dependent, as maximum callus was observed in the genotypes of *B. napus* than *B. juncea*. Similar response on callogenesis as reported by Natalija *et al*. (2004) is in conformity with present finding. With its positive potential to *Brassica* microspore culture, the cold pretreatment method will probably have its wide utilization for efficient doubled

Table 1 : Effect of cold pretreatment on anthers callus induction frequency in different *Brassica* genotypes

Sr. No.	Explant genotypes		5 Days			10 Days			12 Days			Mean
			AC	AR	ACF (%)	AC	AR	AC (%)	AC	AR	ACF (%)	
1.		GSL1	289	83	28.72	243	121	49.79	281	90	35.03	36.85
2.		DGS1	188	56	29.79	289	146	50.52	181	64	35.36	38.56
3.	*B.napus*	RSPN25	281	77	27.40	188	86	45.74	289	82	28.37	33.84
4.		Kranti	241	57	23.65	181	73	40.33	291	56	19.24	27.74
5.		Varuna	181	45	24.86	281	117	41.63	243	56	23.04	29.84
6.	*B.juncea*	RSPR01	281	61	21.71	289	107	37.02	189	33	17.46	25.40
		Mean			26.02			44.17			26.41	
		C.D. (P=0.05)			2.75			2.89			2.86	
		S.E. ±			0.92			0.96			0.96	

AC: No of anthers culture AR: No of anthers showing response to callusing ACF: Anther culture frequency

haploid production in *Brassica* breeding programme.

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