

**ANDROGENESIS MEDIATED GENERATION  
AND CHARACTERIZATION OF DOUBLED  
HAPLOIDS IN INDIAN MUSTARD  
(*Brassica juncea* (L.) Czern. and Coss.)**

**THESIS**

*By*

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*Submitted to*



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*IN*

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*OF*

**MASTER OF SCIENCE IN AGRICULTURE  
(AGRICULTURAL BIOTECHNOLOGY)**

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*Dedicated  
to  
my  
Revered  
Parents*

**Dr. R.K. Kapila**  
*Plant Breeder*

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## **CERTIFICATE – I**

This is to certify that the thesis entitled, "**Androgenesis mediated generation and characterization of doubled haploids in Indian mustard (*Brassica juncea* (L.) Czern. and Coss.)**", submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology** of Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, is a bonafide research work carried out by **Mr. Satinder Singh (Admission No. A-2003-30-08)** son of **S. Harinder Singh** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.



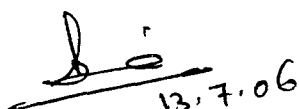
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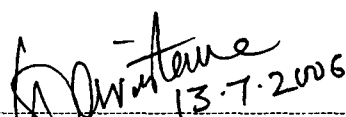
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This is to certify that the thesis entitled, "**Androgenesis mediated generation and characterization of doubled haploids in Indian mustard (*Brassica juncea* (L.) Czern. and Coss.)**", submitted by **Mr. Satinder Singh** son of **S. Harinder Singh** to Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, in partial fulfilment of the requirements for the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology**, has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

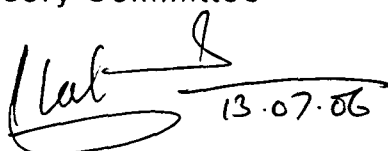
  
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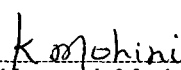
  
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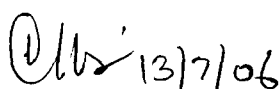
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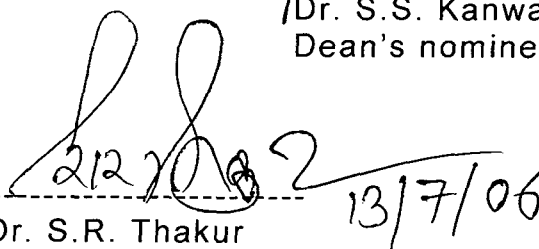
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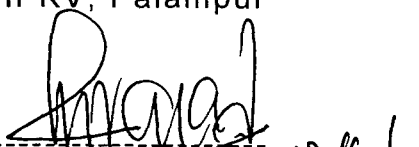
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*I owe entire responsibilities for all errors and omissions.*

Place: Palampur

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(Satinder Singh)

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## **ABBREVIATIONS USED**

°C	degree calcius
µl	microlitre
ml	millilitre
L	litre
ha	hectare
g	grams
mg	milligrams
cm	centimetres
ppm	parts per million
Sec	second(s)
min	minute(s)
hrs	hours
EDTA	ethylene diamine tetra acetic acid
HCl	hydrochloride
NaCl	sodium chloride
HgCl <sub>2</sub>	mercuric chloride
mM	milli molar (conc.)
M	molar (conc.)
Conc.	concentration
pH	puissance de hydrogen (ion conc.)
V	volts
UV	ultraviolet
cv(s)	cultivar(s)
Vs	versus
2,4-D	2,4-dichlorophenoxy acetic acid
NAA	napthalene acetic acid
TIBA	2,3,5-triiodo benzoic acid
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA
Taq	<i>Thermus aquaticus</i> DNA polymerase
UPGMA	Unweighted pair group method with averaging

# ***Introduction***

## INTRODUCTION

---

Oilseeds play a vital role in Indian agricultural economy. Being a rich source of fats and vitamins, these occupy very important position in our daily diet. India is one of the major oilseeds producing country after United States of America and China, with 21 per cent share in world area and 15 per cent in total world production (Reddi and Patti, 1998). However, in spite of all this, India is not able to meet its domestic edible oil needs, which is growing at the rate of 5 per cent per annum. The per capita requirement of oil in balanced diet is 52 g per day whereas per capita availability is 25 g per day (Sharma *et al.*, 1998).

Among oilseeds, rape seed and mustard group is the second most important group in India after groundnut. It occupies an area of 5.06 million hectares with a total production of 5.83 million tones. Average productivity of the country is 1152 kg per hectare (Anonymous, 2004a). In Himachal Pradesh, Rapeseed and Mustard account for about 57 per cent of the total oil seed area, and 60 per cent of total oilseed production of the state. Total area and production under Rapeseed and Mustard are 8.9 thousand hectares and 3.2 thousand tones, respectively with an average productivity of 365 kg per hectare which is very low (Anonymous, 2004b).

The low productivity of these crops under Indian sub-continent as a whole can partly be linked to the adoption of low yielding genotypes and their cultivation on marginal and less productive land. As such, there is little scope

to bring additional area under oilseed crops due to stiff competition from cereals, shrinkage of land holding and use of land for urbanization and industrialization etc. Hence, the most suitable alternate way to increase productivity is by adoption of high yielding, input responsive genotypes having resistance against biotic stresses with high stability index under *specific environmental conditions*.

Among *Brassicaceae*, Mustard (*Brassica juncea* (L.) Czern. and Coss.), also known as Raya, occupies majority of the area. It is a self-pollinated crop that yields comparatively better oil quality due to absence of rancid smell and comparatively low erucic acid contents (Anand, 1975).

Of the various diseases limiting the *Brassica juncea* productivity, white rust caused by *Albugo candida* (Lev.) Kuntze, is also a constraint in realizing full yield potential of mustard cultivars. In Indian mustard (*Brassica juncea*), it can cause a yield loss to the tune of 89.9 per cent (Varshney *et al.*, 2004). This disease is endemic to the most mustard growing areas of Himachal Pradesh due to conducive environments during crop seasons. Therefore, considerable efforts are being directed towards developing white rust resistant cultivars as a mean to achieve low cost, eco-friendly and sustainable management of this disease.

Development of crop varieties using conventional breeding methods take more than 6-7 years of continuous efforts to get true breeding stable lines that can be considered for testing in evaluation trials. Recent advances in plant cell techniques offer Plant Breeders, the method for rapidly producing large number of homozygous plants. *In vitro* techniques like anther culture of F<sub>1</sub>s can be used to facilitate regeneration of stable lines

through spontaneous or induced doubling in 1-2 years, saving a lot of time and resources. Besides induction of the doubled haploids (DHs) through anther culture of  $F_1$ s, their precise characterization is also important to identify promising DHs that show high genetic similarity with the agronomically elite parent while retaining the disease resistance of the donor genotype. Selection based on classical methods relies heavily on visible phenotype or morphological descriptors which are limited in number, and are highly influenced by environmental factors. However, the advent of molecular markers has opened new avenues for precise molecular characterization of newly developed breeding material.

Among molecular markers, polymerase chain reaction (PCR) based technique of DNA fingerprinting, known as Random Amplified Polymorphic DNA (RAPD), offers rapid and cost effective means of detecting variation at the DNA level (William *et al.* 1990). This technique has an advantage of being sensitive for discovering polymorphism which is randomly distributed throughout the target genome.

Therefore, the present study entitled "Androgenesis mediated generation and characterization of doubled haploids in Indian mustard (*Brassica juncea* (L.) Czern. and Coss.)", has been undertaken with the following broad objectives:

- 1 To generate doubled haploids of *Brassica juncea* by *in vitro* culturing of anthers of  $F_1$  involving white rust resistant parents,
- 2 to characterize doubled haploids morphologically and using DNA markers, and
- 3 to identify white rust resistant doubled haploids.

***Review of  
Literature***

## REVIEW OF LITERATURE

---

Guha and Maheshwari (1964) were the pioneer researchers who observed embryos developing from *in vitro* cultured anthers of *Datura innoxia* and subsequently demonstrated that the developed embryoids were derived from pollen. These studies with *Datura* were later confirmed and extended by several workers in other crops like tobacco (Tanaka and Nakata, 1969; Burk, 1970) and lotus (Niizeki and Grant, 1971). Since then, the technique has been successfully used to produce pollen plants in many species including crop plants such as *Solanum tuberosum* (Dunwell and Sunderland, 1973), *Hordeum vulgare* (Clapham, 1971, 1973, Malepszy and Grunewaldt, 1974), *Oryza sativa* (Niizeki and Oono, 1968; Anon, 1974), *Triticum aestivum* (Ouyang *et al.*, 1973; Craig, 1974), *Triticale* (Wang *et al.*, 1973) and *Secale cereale* (Thomas and Wenzel, 1975). In *Brassicaceae*, androgenesis was first reported by Kameya and Hinata (1970) in *Brassica oleracea*. Subsequently production of pollen plants/doubled haploids was reported in *Brassica juncea* also by various workers (Yadav *et al.*, 1988; Goyal *et al.*, 1990; Agarwal and Bhojwani, 1993; Purnima and Rawat, 1997; Roy and Saha, 1997; Singh and Sachan, 1999).

### **2.1 Androgenesis – a supplementary technique in conventional breeding programme**

Production of doubled haploids (DHs) through anther culture is a rapid method to achieve homozygosity essential to develop varieties in self pollinated crops. Genetic recombinants as a consequence of hybridization in

F<sub>1</sub> gametes, can be instantly fixed in one generation through androgenesis reducing the time of homozygous line development to one generation from 6-7 generations of selfing required, in general. The savings of time and resources are more in long duration crop species than in short duration crop species (Baenziger *et al.*, 1984).

In addition, doubled haploids are increasingly being used for the rapid development of mapping population and construction of genetic linkage maps. The haploids have been used to express several recessive traits in *Brassica napus* by Henderson and Pauls (1992). Simultaneously, haploids can also be useful to detect and fix desirable recessive traits induced/introduced through mutation or hybridization and also provide an opportunity to fix rare gene combinations which otherwise may not be possible to isolate in the segregating population through conventional means (Gosal *et al.*, 1997).

## **2.2 Factors affecting *in vitro* anther culture response**

Various factors have been shown to affect androgenic response of *in vitro* cultured anthers in different crops. These include genotype (Gresshoff and Doy, 1972a, b; Guha-Mukherjee, 1973), physiological status of the donor plant (Sunderland, 1971, 1974), pretreatment of anthers, developmental stage of microspores (Sunderland and Wicks, 1971; Clapham *et al.*, 1971; Ouyang *et al.*, 1973), culture medium (Sharp *et al.*, 1971; Clapham *et al.*, 1973; Nitsch, 1974), growth regulators and sucrose. The relevant literature pertaining to these in *Brassica* species is presented hereunder:

### 2.2.1 Plant genotype

In *Brassica juncea*, significant effect of genotype on response of *in vitro* cultured anthers/microspores has been reported in many studies (Yadav *et al.*, 1988; Goyal *et al.*, 1990; Roy and Saha, 1997; Singh and Sachan, 1999; Prem *et al.*, 2004). Yadav *et al.* (1988) found greater response in anthers of hybrid plants as compared to parental genotypes and concluded that appreciable differences existed among genotypes within species. Hiramatsu *et al.* (1995) in a study on microspore embryogenesis in leaf mustard (*Brassica juncea*) observed that mean embryo yield differed between cultivars and between individual donor plants of same cultivar. Maximum yield of 155 embryos per 100 anthers was reported in a plant of cv. 'Hsueh Le Hung'. Similar was demonstrated by Singh and Sachan (1999) who revealed that cultivar 'Divya' produced embryoids from cultures *in vitro* while Kranti did not produce any embryo.

Variable callus induction and plant regeneration in different cultivars of *Brassica juncea* has also been reported (Roy and Saha, 1997). Cultivar RLM-198 showed a higher percentage of callus formation and regeneration potential as compared to RJ-15. Malik *et al.* (2001), in anther culture of two wide hybrids (*Diplotaxis erucoids* × *Brassica campestris*) × *Brassica Juncea* and (*Diplotaxis berthautii* × *Brassica campestris*) × *Brassica juncea*, observed a range of responses in CMS lines and the parent species highlighting thereby the importance of the genotype.

Genotype of the donor plant is reported to have significant effect on embryoid yield and percentage responsive anthers in *Brassica oleracea* also (Ockendon, 1985; Phippen and Ockendon, 1990). Ockendon (1985) compared embryoid yields of six F<sub>1</sub> hybrid cultivars with that of highly responsive F<sub>1</sub> cultivar 'Gower' in *Brassica oleracea*. On the basis of about 1400 anthers cultured for each cultivar, 'Dorema' was classed with 'Gower' as highly responsive followed by 'Lunet' as moderately responsive while the others were unresponsive. Phippen and Ockendon (1990) reported a mean embryoid yield ranging from 82.2 embryoids per 100 anthers cultured for cultivar 'Dova' to 0.6 embryoids for cultivar 'Serano' in *Brassica oleracea*. They also found that autumn and winter maturing cauliflowers were generally more responsive than summer types.

### **2.2.2 Physiological status of the donor plants**

Many studies in different crops have reported considerable impact of physiological status of donor plants, at time of anther excision, on *in vitro* response (Sunderland, 1971,1974; Roulund *et al.*, 1990; Agarwal and Bhojwani, 1993). Plants usually tend to conserve resources under non-optimal growth condition often resulting in pollen abortion. Therefore, it may be essential to have controlled growth conditions of the donor plants to ensure better quality of the microspores for their *in vitro* culture.

Majority of studies conducted on effect of physiological status of donor plants on anther/pollen culture in *Brassica oleracea* have revealed plants grown under controlled environment conditions more suitable as

compared to the field grown plants (Arnison and Keller, 1990; Yang *et al.*, 1992; Gorecka, 1998). In contrast to it, Roulund *et al.* (1990) reported better embryo formation from anthers of field grown donor plants of head cabbage (*Brassica oleracea* L. convar. *capitata* (L.)) than plants grown in the green house. However, Arnison and Keller (1990) studying several varieties of *Brassica oleracea* reported that the utilization of anther culture was possible even without controlled environmental facilities. Yang *et al.* (1992) while studying culture condition of donor plants for the anther culture of cauliflower (*Brassica oleracea* var. *botrytis*) reported best response from spring season plants grown in glass house where temperature was maintained at 10-20° C. In the study, it was also concluded that the winter and spring seasons were more suitable than summer and early autumn for anther culture establishment. Gorecka (1998) observed effect of flowering phase and the time of anther excision also on the anther culture response of head cabbage (*Brassica oleracea* L. var. *capitata* L.). Vyvadilova *et al.* (1998a) achieved the best anther culture results in *Brassica oleracea* from most of the genotypes of donor plants that were grown in heated glass house during February-March.

In *Brassica juncea* also, Hiramatsu *et al.* (1995) reported maximum yield of embryoids from anthers of a variety "Hsueh Li Hung" when grown under controlled environment. Yadav *et al.* (1988) reported a response of 0.2 to 20.3 per cent from cultured anthers of *Brassica juncea* grown at 25° C without temperature treatment. However, Agarwal and Bhojwani (1993)

reported that the anthers derived from plants of *Brassica juncea* cv PR-45 sown about two months after the normal sowing period showed 18 per cent androgenesis as compared to 3.5 per cent in control. Microspore embryogenesis was studied by growing three Indian *B. juncea* varieties under field, controlled conditions and both conditions by Prem *et al.* (2005) and found bud size most suitable parameter under controlled conditions but not under field conditions.

### **2.2.3 Culture medium**

Culture medium is an important component for induction of embryos/callus from *in vitro* cultured anthers/microspores. Water, carbohydrates, minerals, vitamins, amino acids and growth regulators comprise the *in vitro* culture medium. These components as well as others (like pH) can be manipulated to influence the embryogenesis/callusing response of *in vitro* cultured anthers.

#### **2.2.3.1 Basal medium**

Gamborg's (B<sub>5</sub>), Murashige and Skoog's (MS) and Keller's media have been successfully used to induce *in vitro* callusing/embryogenesis in cultured anthers of *Brassica juncea* (Yadav *et al.*, 1988; Goel *et al.*, 1990; Agarwal and Bhojwani, 1993; Prabhudesai and Bhaskaran, 1993; Roy and Saha, 1997; Singh and Sachan, 1999). Majority of studies reported successful use of MS media for *in vitro* callusing/embryogenesis in cultured anthers of *Brassica juncea*. However, Gamborg's medium (B<sub>5</sub>) and its modifications have also been reported to be successful for anther culture of

### 2.2.3.2 Growth regulators

Pollen embryogenesis is known to be induced on simple mineral sucrose medium in plants like tobacco yet, for complete androgenesis, addition of certain growth regulators is required. The choice of plant growth regulators in *Brassica juncea* anther culture medium has been guided by considering the regenerability of anther derived calli and/or embryoid formation and germination rather than the anther culture response.

Scanning of literature on *Brassica juncea* anther culture indicated reports of pollen grain embryogenesis on a basal modified Keller's medium supplemented with glutathione, but, devoid of any growth regulators (Agarwal and Bhojwani, 1993). However, most *Brassica* species are reported to require an auxin and a cytokinin for direct pollen embryogenesis (George and Rao, 1982; Lillo and Hansen, 1987; Goel *et al.*, 1990; Paksoy *et al.*, 1995; Chang *et al.*, 1996; Purnima and Rawat, 1997). George and Rao (1982) obtained *in vitro* shoot bud formation from cultured anthers on a medium supplemented with NAA and BA. Roy and Saha (1997) reported better response in the microspore culture of *Brassica juncea* on a modified B<sub>5</sub> medium supplemented with 2mg/l 2,4-D or NAA. The plant regeneration efficiency of callus induced on 2,4-D was reported to be less as compared to that of NAA supplemented medium. However, Prem *et al.* (2005) reported antagonistic effect of NAA and BA for microspore embryogenesis of *Brassica juncea* while silver nitrate, added in NLN medium, had significant synergistic effect. Agarwal and Bhojwani (1993) reported poor germination of pollen

embryos induced on modified Keller's medium when subcultured on another medium (B<sub>5</sub>) containing GA<sub>3</sub>. However, ABA was reported to promote normal germination of these embryos.

In *Brassica oleracea* also, medium containing both auxins as well as cytokinins has been reported to be suitable for anther/microspore culture (Bagga *et al.*, 1982; Chang *et al.*, 1996). However, Lillo and Hansen (1987), and Paksoy *et al.* (1995) reported production of embryoids in anthers/microspore culture of *Brassica oleracea* on media supplemented with one or more auxins alone. Ockendon and McClenaghan (1993) reported significant effect of 2,4-D alone on anther culture of *Brassica oleracea* var. *gemmifera*.

### 2.2.3.3 Effect of organic substances

Organic substances can be potential additives *in vitro* for achieving higher/better response in cultured anthers/microspores, and sometimes can also be judiciously used to alter the mode of androgenic development. Activated charcoal has most commonly been used in many species of *Brassica* to achieve better response of *in vitro* cultured anthers/microspores (Lee *et al.*, 1987; Lichter, 1989; She *et al.*, 1999; Silva and da Silva, 1999; Prem *et al.*, 2004, 2005). Generally, majority of studies have reported significant effect of activated charcoal on *in vitro* anther/microspore embryogenesis of *Brassica* species (Lichter, 1989; She *et al.*, 1999; Silva and da Silva, 1999; Prem *et al.*, 2004). Leelavathi *et al.* (1987) reported better rooting of plants of *B. nigra* produced via anther culture on MS media

containing IAA and activated charcoal. However, Lee *et al.* (1987) and Prem *et al.* (2005) found activated charcoal not suitable for achieving efficient embryoid induction in *B. napus* and *B. juncea*, respectively. Similarly, coconut water was also reported to have no effect on germination of embryoids in anther culture of cabbage (Lillo and Hansen, 1987), but was reported beneficial in callus differentiation in broccoli (Quazi, 1978).

Casein hydrolysate added in Nitsch medium along with IAA (2ppm) and Kinetin (6-10ppm) lead to formation of profused callus in cultured anthers of *B. oleracea* (Bagga *et al.*, 1982). Similarly, yeast extract in MS medium with amino acids and sucrose has been reported to enhance callusing from cultured anthers of broccoli cv. Gem.

#### **2.2.4 Carbohydrate source and levels**

Sucrose, the main carbohydrate source is essential for androgenesis. Besides source of nutrition, it is also involved in osmotic regulation. It influences both callus/embryogenesis induction as well as green plantlet regeneration. Majority of studies conducted on *in vitro* anther/microspore culture in *Brassica juncea* have found sucrose as a better source of carbohydrate in the media (Chen and Evans, 1986; Agarwal and Bhojwani, 1993; Roy and Saha, 1997; Prem *et al.*, 2004). Generally, higher sucrose contents (13%) has been reported to be more effective in achieving callusing/embryogenesis in cultured anthers/microspores of *Brassica juncea* (Agarwal and Bhojwani, 1993; Purnima and Rawat, 1997; Singh and Sachan, 1999; Lionneton *et al.*, 2001; Prem *et al.*, 2004). Roy and Saha (1997)

achieved callusing in cultured anthers of *Brassica juncea* on a medium containing 12 per cent sucrose whereas for regeneration of shoots and roots, medium containing 4 and 2 per cent sucrose, respectively was found suitable. Agarwal and Bhojwani (1993) used three concentrations of sugar viz. 7, 10 and 13 per cent for anther culture of *Brassica juncea*. They reported lower response of *in vitro* cultured anthers at low concentration of sucrose which increased with increasing sugar up to 13 per cent. However, Goyal *et al.* (1990) reported no significant effect of sucrose levels on caulogenesis as well as embryogenesis in cultured flower buds of *Brassica juncea*. Chen and Evans (1986) described a procedure for embryogenesis in anther culture of *Brassica* species including *Brassica juncea*. The procedure involved inoculation of anthers into a liquid medium containing 20 per cent sucrose initially for 3-4 days thereafter, transferring them to new liquid medium containing 8-10 per cent sucrose along with hormone combination. The culture was transferred to solid hormone free medium containing 2 per cent sucrose after 10-20 days.

In *Brassica napus*, a comparatively low sucrose contents (8%) has been reported to be appropriate for anther/microspore culture (Lichter, 1981; Singh and Sachan, 1999). However, many studies in different subspecies of *Brassica oleracea* reported still higher sucrose contents ( $\geq 14\%$ ) appropriate for anther/microspore culture (Roulund *et al.*, 1991; Yang *et al.*, 1992; Gorecka *et al.*, 1997; Gorecka, 1998). Roulund *et al.* (1990) studied effect of 7, 10 and 13 per cent sucrose concentration in the medium for anther culture

response in *Brassica oleracea* and found increased response with the increment in the sucrose concentration. With maltose, generally considered inferior to sucrose as a carbohydrate source for anther culture, the response did not increase with increasing concentration of it above 10 per cent. Ferrie *et al.* (1999) while screening nineteen *Brassica oleracea* accessions for their suitability for microspore culture, reported that embryogenesis was improved from 10.5 per cent to 21 per cent of genotypes by initially culturing on NLN medium with 17 per cent sucrose and reducing it to 10 per cent after 48 hours.

### **2.3 Developmental stage of microspore**

Microspore developmental stage is most critical factor affecting frequency of pollen embryos/calli formation in anther/microspore culture. Generally, bud size is used as an index of pollen stage. However, size of the bud enclosing pollen at optimum stage may vary with growing conditions and age of plant (Takahata *et al.*, 1993). Therefore, the success depends upon accuracy in selecting floral buds containing appropriate stage of the microspore. The pollen of most *Brassica* species has been reported to be most vulnerable for embryogenic division at the late uninucleate stage (Leelavathi *et al.*, 1984; Dunwell, 1985; Sharma and Bhojwani, 1985; Yadav *et al.*, 1988; Agarwal and Bhojwani, 1993; Vyvadilova *et al.*, 1998b; Zhang *et al.*, 1999; Prem *et al.*, 2004, 2005). Successful cultures of microspores at uninucleate stage leading to pollen embryogenesis have been reported in *B.*

*juncea* by taking different size floral buds (Chen and Evans, 1986; Yadav *et al.*, 1988; Prabhudesai and Bhaskaran, 1993). Available literature indicated that flower buds, of size ranging from 2-3 mm, were having microspores at uninucleate stage (Yadav *et al.*, 1988; Agarwal and Bhojwani, 1993; Prabhudesai and Bhaskaran, 1993). Singh and Sachan (1999) in a study on embryogenesis in 3 *Brassica* species including *B. juncea* obtained best response of flower buds (1 to 1.5 mm size) at uninucleate stage of microspore whereas Prem *et al.* (2005) obtained highest frequency of microspore embryogenesis in *B. juncea* from late uninucleate microspores contained in bud sizes of 3.1 to 3.5 mm. In *Brassica napus*, best results were reported when anthers were excised from 2.1-2.5 mm long buds with pollens at mid uninucleate stage of development.

In *Brassica oleracea*, best results of pollen embryogenesis were reported when majority of cultured microspores were at late uninucleate to binucleate stage (Vyvadilova *et al.*, 1998b; Zhang *et al.*, 1998c). Zhang *et al.* (1999) reported production of 37.08 embryoids of broccoli per cultured bud when microspores were obtained from buds of 4.1-5.0 mm in length containing majority of microspores at late uninucleate and early binucleate phases. However, another study of Vyvadilova *et al.* (1998a) on androgenic embryogenesis in Brussels sprouts, Kohlrabi, Cauliflower and Cabbage, reported non-significant effects of the development stage of isolated microspores on pollen embryogenesis.

## 2.4 Pre – treatment

The normal gametophytic pathway of pollen can be altered to sporophytic pathways by subjecting anthers to certain physical and chemical treatments prior to their transfer to standard conditions. Various studies conducted on the effects of different pretreatments on androgenic embryogenesis/organogenesis in *Brassica* spp. are reviewed below:

A high temperature shock (30-35° C) for initial 1-4 days of culture has been reported to be beneficial for inducing androgenesis in most *Brassica* species (Keller and Armstrong, 1981; Sharma and Bhojwani, 1985; Hamaoka *et al.*, 1991; Purnima and Rawat, 1997; Zhang *et al.*, 1998 a, b; Singh and Sachan, 1999; Prem *et al.*, 2004, 2005). In *Brassica juncea*, effects of both cold and heat shocks have been reported on *in vitro* androgenesis. George and Rao (1982) reported better development of embryoids by subjecting *Brassica juncea* anthers to cold shock (10° C) along with temperature shock of 31 and 37° C. Sharma and Bhojwani (1985) reported that the preliminary incubation of microspores/anthers at 35° C for 1-5 days or at 5° C for 3 days prior to their maintenance at 25° C stimulates embryogenesis from microspores of *B. juncea*. Chen and Evans (1986) and Roy and Saha (1997) have reported cold treatments of 4-10° C for 3-6 days and 8-10° C for 4 days, better to achieve induction of embryoids/calli and plant regeneration in anthers of *B. juncea*. Yadav *et al.* (1988) also reported a cold pretreatment of 1, 3 and 5 days before culturing followed by incubation at 37° C for 2 days in *B. juncea* to be the best for androgenic

callus induction. Most of other reports have indicated beneficial effects of heat shock treatment (32-35° C) for 1-4 days on androgenic callus/embryoid induction in *B. juncea* (Ohkawa *et al.*, 1988; Goyal *et al.*, 1990; Hiramatsu *et al.*, 1995; Purnima and Rawat, 1997; Singh and Sachan, 1999; Prem *et al.*, 2005). Germination of androgenic embryoids of *B. juncea* has been reported to improve by subjecting embryoids to cold treatment of 4° C for 6-10 days before maintaining them at 25° C (Agarwal and Bhojwani, 1993; Prem *et al.*, 2004).

In *B. oleracea*, reports invariably have indicated an initial high temperature shock ranging from 32-35° C for a period ranging from 16 hours to 48 hours to be essential to induce androgenesis (Keller and Armstrong, 1981; Ockendon, 1984; Fabijanski *et al.*, 1991; Zhang *et al.*, 1998 a, b; Ferrie *et al.*, 1999; Achar, 2002). It has further been reported that longer duration of such thermal shocks may lead to decrease in androgenesis rate even up to no response sometimes (Ferrie *et al.*, 1999; Achar, 2002). Similar to *B. oleracea* and *B. juncea*, heat shock treatments ranging from 33-35° C for 1-6 days have also been reported to induce pollen embryogenesis in *B. campestris* sub species (Arora *et al.*, 1988; Hamaoka *et al.*, 1991; She *et al.*, 1999; Sato, 2000) and *B. napus* (Lee *et al.*, 1987; Kwon *et al.*, 1989).

## **2.5 Characterization of doubled haploids**

Characterization of doubled haploids (DHs) raised through anther culture of F<sub>1</sub>s is crucial to identify the desirable ones. The earlier approach of characterization based on morphological markers which are limited in

number and prone to environmental variation, was not very precise and reliable. However, recent advancements in DNA markers technology have provided more reliable characterization of diversity in regenerated DHs at molecular level. This can give realistic estimates of generated variability at genetic level and the relevant literature on these aspects is reviewed head-wise as below:

### **2.5.1 Agro-morphological evaluation**

Characterization of generated DHs for agro-morphological and other economic traits is an important step for their exploitation in crop improvement programme. Doubled haploids of *Brassica* spp. have been evaluated for different agro-morphological traits in different generation after regeneration to assess the spectrum of variability generated (George *et al.*, 1987; Abraham *et al.*, 1988; Tiwari *et al.*, 1988a; Agarwal and Bhojwani, 2004; Lionneton *et al.*, 2004). Generally, the advance generations of DHs have been reported to be used for characterization at agro-morphological and molecular level, however, few reports have used R<sub>0</sub> generation also to infer about spectrum of variability generated in androgenic lines derived from anther/pollen culture of F<sub>1</sub>s of *Brassica* spp. including *B. juncea* (Kaminski *et al.*, 1999; Agarwal and Bhojwani, 2004). Androgenic lines of *B. juncea* have shown variation for most of traits studied including plant height, days to maturity, seed yield, oil content and fatty acid composition (George *et al.*, 1987; Abraham *et al.*, 1988; Tiwari *et al.*, 1988a; Adamska *et al.*, 2001; Burbulis *et al.*, 2001; Agarwal and Bhojwani, 2004; Lionneton *et al.*, 2004). Agarwal and Bhojwani (2004) screened 27 androgenic lines of *B. juncea* for

plant height, time to maturity, pod number per plant, seeds per pod, seed mass, biomass and harvest index, and identified two dwarf lines (18-20%) having remarkable early maturity and three lines with 27-31 per cent higher yield than parent cultivars. Rudolf (2002) reported 17 lines with favorable morphological traits *viz.*, head weight, head shape coefficient, percentage of internal stump and head firmness in cabbage. He reported high heterogeneity in fifty four doubled haploid lines of cabbage studied. Burbulis *et al.* (2001) compared oil quality traits *viz.*, fatty acid profiles and glucosinolate levels among DH lines, their donor plants and parent plants in *Brassica napus*. The results revealed that the level of oil in parents was 65-68%, in F<sub>1</sub> the level was nearly 66%, whereas in the DH population it ranged from 58-70%. Kucera *et al.* (2002) obtained doubled haploid lines of winter oil seed rape (*Brassica napus*) having traits like yield parameters and resistance to important diseases from both parents. However, in majority of studies, regenerated DHs have been reported to perform at par with donor parents for seed yield (George *et al.*, 1987; Abraham *et al.*, 1988), and content and quality of oil (George *et al.*, 1987; Abraham *et al.*, 1988; Tiwari *et al.*, 1988a). Abraham *et al.* (1988) have also concluded that it was essential to regenerate large number of androgenic doubled haploids to get genotypes more productive than better parent in these crops.

In literature, comparative studies on segregation pattern in microspore derived progenies of F<sub>1</sub> *in vitro* and traditionally selfed plants have reported conflicting results. Thiagarajah and Stringam (1993) compared inheritance of two qualitative markers *viz.*, seed colour and leaf hairiness

using traditional segregation and microspore derived populations. The segregation of genes controlling seed colour and leaf hairs in doubled haploid progeny did not differ significantly from that expected under random assortment indicating that doubled haploids can be used in this species for genetic studies. However, Roy *et al.* (2000) compared microspore derived progenies ( $A_2$ ) with the  $F_2$  population of a reciprocal cross between two cultivars of *Brassica juncea* (RLM-198 and RJ-15). Study concluded that  $F_2$  population showed simple Mendelian ratio whereas in case of  $A_2$  plants non-Mendelian segregation was observed.

### **2.5.2 Characterization using molecular markers**

Molecular markers including RAPDs and AFLPs have been routinely used to assess genetic diversity in indigenous as well as exotic genotypes of *B. juncea* (Jain *et al.*, 1994; Ren *et al.*, 1995; Rabbani *et al.*, 1998; Qiao *et al.*, 1998a,b; Srivastava *et al.*, 2001; Warwick and Soleimani 2001; Dan *et al.*, 2003; Burton *et al.*, 2004) but no such reports are available on doubled haploids. However, Kaminski (2001) has characterized 118 androgenic plants of *B. oleracea* L. var. *capitata* using 14 polymorphic RAPDs primers.

Sakova and Curn (1998) reported screening of 40 RAPD primers for classification studies of selected *Brassica* species, and found 8 primers informative, out of which OPB06 was reported to be most informative. Warwick and Soleimani (2001) separated 2 main subclusters of vegetables vs seed oil use in *B. juncea* using 7 RFLP primer pairs. Srivastava *et al.*

(2001) grouped 30 *Brassica juncea* lines into 3 distinct clusters using 21 AFLP primer pairs that generated 778 polymorphic bands. Thormann *et al.* (1994) reported use of RAPD markers for evaluating phylogenetic relationship among 18 accessions from 6 cultivated species including *B. juncea*. They reported similar results for intraspecific genetic relationship using RAPD and RFLP markers. An *et al.* (2000) also analysed Chinese mustard, black mustard, xiniang wild mustard, yellow mustard and oilseed and vegetable accessions of *B. rapa* by RAPD markers and reported that Chinese and black mustard had similar genetic distance.

## **2.6 Ploidy status/level**

Haploid tissues are quite susceptible to changes in ploidy level during cell proliferation and growth *in vitro*. For obtaining homozygous lines, it is essential to analyse androgenic plants obtained (derived through anther culture) for their ploidy. Mostly the regenerated plants of *B. juncea* have been analysed following cytological studies particularly root tip cytology (Sharma and Bhojwani, 1985; Goel *et al.*, 1990; Prabhudesai and Bhaskaran, 1993; Roy and Saha, 1997; Prem *et al.*, 2004). The floral morphology has also been used to assess the ploidy level of anther derived plants in *B. juncea* (Goyal *et al.*, 1990) as well as in *B. napus* (Keller and Armstrong, 1978; Jain *et al.*, 1989). Lionneton *et al.* (2001) used a flow cytometer (Partec CA II) to determine ploidy level of *in vitro* microspore culture derived plants in *B. juncea*.

Reports have indicated that the regenerated androgenic plants are mostly mixture of haploids, diploids and higher ploidy levels. George and Rao (1982) reported 46 per cent haploid plants in 54 normal plantlets regenerated through anther culture of *B. juncea* whereas Roy and Saha (1997) reported 30-35 per cent haploid plants from microspore callus of mustard (*B. juncea*). Flow cytometer analysis of ploidy level in regenerated plantlets obtained through *in vitro* microspore culture in *B. juncea* indicated 0-22 per cent spontaneous diploids (Lionneton *et al.*, 2001) whereas Prem *et al.* (2004) reported a range of 0-12 per cent spontaneous diploids in population of microspore derived plants. However, Sharma and Bhojwani (1985) and Prabhudesai and Bhaskaran (1993) in their respective populations of plants derived from microspore culture in mustard, reported all haploid plants. Similarly Semova and Anokhin (1990) did cytological analysis of several embryoids derived from anther culture of *B. oleracea* and reported haploid population having  $n=9$ . Leelavathi *et al.* (1987) in their study on production of androgenic haploid plants in *Brassica nigra* (L.) Koch reported 41 per cent plants to be haploids ( $n=8$ ).

## **2.7 Screening for white rust**

White rust caused by fungus *Albugo candida* (Lev.) Kuntze is a serious disease of *Brassica juncea* and it can cause yield losses upto the extent of 89.9 per cent (Varshney *et al.*, 2004). A number of resistant varieties and cultivars have been reported in literature (Kolte, 1986; Bhardwaj and Sud, 1993; Khetmalas *et al.*, 1994; Velazhahan and

Thiyagarajan, 1994; Yadav *et al.*, 1999). Bhardwaj and Sud (1993) screened germplasm of *Brassica* species against individual and mixed isolates of *A. candida* from Kangra Valley, Himachal Pradesh and found 26 cultivars of *B. juncea* resistant to both isolates. Among the 37 genotypes tested by Velazhahan and Thiyagarajan (1994) for reaction to white rust under field conditions, 13 genotypes were found resistant, eight were moderately resistant and 16 were susceptible or highly susceptible. However, Khetmalas *et al.* (1994) screened 12 varieties of Indian mustard and none was found resistant to white rust. Yadav *et al.* (1999) evaluated 74 *B. juncea* lines for resistance to white rust under irrigated field conditions and found no completely resistant genotype to this disease.

The genetics of resistance to white rust in *B. juncea* has also been studied by various workers including Thukral and Singh (1986), Tiwari *et al.* (1988b), Paladhi *et al.* (1993), Sridhar and Raut (1998) and Bansal *et al.* (1999). They reported that resistance in *B. juncea* against *A. candida* is controlled by single gene. Thukral and Singh (1986) in their study on inheritance of resistance to *A. candida* in crosses EC 12749 x Parkash and EC 12749 x Varuna, reported dominance to be important along with additive epistatic effects in the inheritance of resistance. However, many later studies also have reported resistance to *A. candida* to be controlled by a single gene (Tiwari *et al.*, 1988b; Paladhi *et al.*, 1993; Sridhar and Raut, 1998). Bansal *et al.* (1999) studied the inheritance of partial resistance to race 2 of *A. candida* and reported it to be controlled by a single dominant gene that has variable expression.

Anther/microspore culture derived doubled haploid lines of *B. juncea* have also been used to identify the markers linked to white rust or to develop the linkage maps (Cheung *et al.*, 1998; Prabhu *et al.*, 1998; Varshney *et al.*, 2004). Cheung *et al.* (1998) reported RFLP markers linked to white rust gene (*Acr*) in mustard using 119 F<sub>1</sub> derived doubled haploid lines of a cross between white rust susceptible (J90-4317) and resistant (J90-2733) lines. Similarly, Prabhu *et al.* (1998) identified RAPD markers linked to white rust resistance using an F<sub>1</sub> derived doubled haploid population originating from a cross between white rust resistant and susceptible line of *B. juncea*. Varshney *et al.* (2004) also used doubled haploid population originating from a cross between exotic resistant accession of mustard (BEC-144) and Varuna to develop RAPD markers for mapping of locus *Ac2(t)*.

Apart from intraspecific sources, interspecific hybridization has also been employed in *B. juncea* to transfer white rust resistance from *B. napus* to *B. juncea* (Pal *et al.*, 1999, Somers *et al.*, 2002).

***Materials and  
Methods***

## MATERIALS AND METHODS

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The present investigation was carried out in the Advanced Centre of Hill Bioresources and Biotechnology, CSK Himachal Pradesh Krishi Vishvavidyalya, Palampur. The materials used and the methodology adopted to achieve the objectives of the present investigation are given here under.

### 3.1 Experimental Materials

The material used for anther culture comprised  $F_1$ s of the following three crosses involving white rust resistant and susceptible cultivars/lines.

1. Varuna x Hira
2. Domo x BJ-1058
3. Hira x Zem-1

Main features of five *Brassica juncea* lines/varieties involved in the aforementioned crosses is tabulated in Table 3.1.

### 3.2 Methods

#### 3.2.1 Plant material for anther culture

Sufficient numbers of  $F_1$  plants of aforementioned three crosses were raised in pots as well as in the cage house during normal season to obtain the explants for anther culture. In order to have availability of anthers over a longer period of time,  $F_1$  plants of all three crosses were sown in four lots at a gap of 15 days interval.

**Table 3.1: Main features of five lines/varieties involved in F<sub>1</sub> crosses used for anther culture**

Variety/ line	Important features
<b>Varuna</b>	It is a high yielding released variety of mustard at national level, suitable for cultivation in Himachal Pradesh (low hills). The variety is susceptible to white rust.
<b>Hira</b>	Hira has been obtained by crossing Zem-1/Zem-2 with improved mustard. The strain is known for its oil quality as it is '000' type having low erucic acid, low glucosinolates and low fibre. It is tall, sparsely branched and late in flowering and maturity. Hira is resistant to white rust, however, is poor in yield.
<b>Domo</b>	It is a selection from broad based <i>Brassica juncea</i> population having more number of branches, high seed yield and is resistant to white rust.
<b>BJ-1058</b>	It is an improved strain of mustard with low erucic acid, high seed yield but, is susceptible to white rust.
<b>Zem-1</b>	A strain of <i>Brassica juncea</i> obtained by Kirk and Oram (1981) in Australia by attempting repeated selfing followed by selection of individual seed having <2 per cent erucic acid. This strain is also susceptible to white rust.

### 3.2.2 Anther culture

#### 3.2.2.1 Stage of explant

Florets from aforementioned F<sub>1</sub> plants were clipped carefully when the size of bud was about 2–4 mm. The size was established on the basis of presence of majority of the microspores at late uninucleate to early

binucleate stage, studied by squashing of anthers in a drop of 1 per cent acetocarmine. The florets of appropriate sizes were collected in the test tubes containing distilled water.

### **3.2.2.2 Anther plating in callus/embryoid induction media**

The florets collected at aforementioned stages were dipped in ethanol for 10-15 seconds under aseptic conditions in the laminar air flow cabinet. Then the florets were surface sterilized with 0.1 per cent mercuric chloride for 2–3 minutes with intermittent shaking followed by three washings of the florets with sterilized distilled water. With the help of sterilized forceps, the florets were opened under aseptic conditions and the anthers were taken out carefully without causing any injury to anther wall. Atleast forty anthers were plated in each pre-sterilized petri plate containing 25-30 ml of different callus induction media in each replication. The experiments on different media for callusing/embryo induction were replicated thrice in two sets of experiments involving different media, sugar contents, plant growth regulators and organic additives. First set of experiment involved all possible combinations of four different media and four sugar levels, whereas in the second experiment, 15 different PGR combinations along with three additives constituted treatments to study the response of cultured anthers (Table 3.2 and Table 3.3). Anthers of all three different crosses were plated in a replicated fashion in both the experiments. In case of contamination in any of petri plate of a particular treatment, replating of anthers was done immediately to complete the experiment under uniform conditions.

**Table 3.2: Media containing different sugar contents used for callus induction in anthers of *Brassica juncea* crosses**

Medium**	Medium designation	Conc. of sucrose (%) used as carbon source
Keller	KB <sub>5</sub>	5
Keller	KB <sub>8</sub>	8
Keller	KB <sub>11</sub>	11
Keller	KB <sub>14</sub>	14
MS	MS <sub>5</sub>	5
MS	MS <sub>8</sub>	8
MS	MS <sub>11</sub>	11
MS	MS <sub>14</sub>	14
N <sub>6</sub>	N <sub>5</sub>	5
N <sub>6</sub>	N <sub>8</sub>	8
N <sub>6</sub>	N <sub>11</sub>	11
N <sub>6</sub>	N <sub>14</sub>	14
B <sub>5</sub>	B5 <sub>5</sub>	5
B <sub>5</sub>	B5 <sub>8</sub>	8
B <sub>5</sub>	B5 <sub>11</sub>	11
B <sub>5</sub>	B5 <sub>14</sub>	14

Media used were Keller medium (Keller *et al.*, 1975), MS (Murashige and Skoog, 1962), N<sub>6</sub> medium (Chu *et al.*, 1975), B5 medium (Gamborg *et al.*, 1968)

\*\*All media were supplemented with 0.2 mg/l 2,4-D, 0.2mg/l NAA and 0.8% agar.

All petri plates containing anthers were sealed with parafilm and kept in dark at 35° C initially for three days and then were transferred to 25±1° C in dark until induction of callus.

### 3.2.2.3 Regeneration of plantlets from calli

Calli started to appear after 15-20 days after plating the anthers. All developed calli of about >3mm diameter were transferred to 250 ml culture flasks containing 100 ml of regeneration media [B5 + NAA (0.2mg/l) + Kinetin (2mg/l) + sucrose (2%)]. The callus containing flasks were incubated at 25±1° C under 18/6 light/dark cycle for plantlets regeneration.

**Table 3.3: Media containing different combinations of plant growth regulators and organic additives used for callus induction**

Medium* designation	2,4 D	NAA	TIBA	Organic additives**	
				CAH	AC
K <sub>0</sub> 0	-	-	-	-	-
K <sub>0</sub> CAH	-	-	-	0.5%	-
K <sub>0</sub> AC	-	-	-	-	0.5%
K <sub>1</sub> 0	0.1	-	-	-	-
K <sub>1</sub> CAH	0.1	-	-	0.5%	-
K <sub>1</sub> AC	0.1	-	-	-	0.5%
K <sub>2</sub> 0	-	0.1	-	-	-
K <sub>2</sub> CAH	-	0.1	-	0.5%	-
K <sub>2</sub> AC	-	0.1	-	-	0.5%
K <sub>3</sub> 0	0.2	-	-	-	-
K <sub>3</sub> CAH	0.2	-	-	0.5%	-
K <sub>3</sub> AC	0.2	-	-	-	0.5%
K <sub>4</sub> 0	-	0.2	-	-	-
K <sub>4</sub> CAH	-	0.2	-	0.5%	-
K <sub>4</sub> AC	-	0.2	-	-	0.5%
K <sub>5</sub> 0	0.1	0.1	-	-	-
K <sub>5</sub> CAH	0.1	0.1	-	0.5%	-
K <sub>5</sub> AC	0.1	0.1	-	-	0.5%
K <sub>6</sub> 0	0.2	0.2	-	-	-
K <sub>6</sub> CAH	0.2	0.2	-	0.5%	-
K <sub>6</sub> AC	0.2	0.2	-	-	0.5%
K <sub>7</sub> 0	-	1.0	-	-	-
K <sub>7</sub> CAH	-	1.0	-	0.5%	-
K <sub>7</sub> AC	-	1.0	-	-	0.5%
K <sub>8</sub> 0	1.0	-	-	-	-
K <sub>8</sub> CAH	1.0	-	-	0.5%	-
K <sub>8</sub> AC	1.0	-	-	-	0.5%
K <sub>9</sub> 0	-	2.0	-	-	-
K <sub>9</sub> CAH	-	2.0	-	0.5%	-
K <sub>9</sub> AC	-	2.0	-	-	0.5%
K <sub>10</sub> 0	2.0	-	-	-	-
K <sub>10</sub> CAH	2.0	-	-	0.5%	-
K <sub>10</sub> AC	2.0	-	-	-	0.5%
K <sub>11</sub> 0	1.0	1.0	-	-	-
K <sub>11</sub> CAH	1.0	1.0	-	0.5%	-
K <sub>11</sub> AC	1.0	1.0	-	-	0.5%
K <sub>12</sub> 0	2.0	2.0	-	-	-
K <sub>12</sub> CAH	2.0	2.0	-	0.5%	-
K <sub>12</sub> AC	2.0	2.0	-	-	0.5%
K <sub>13</sub> 0	-	-	0.1	-	-
K <sub>13</sub> CAH	-	-	0.1	0.5%	-
K <sub>13</sub> AC	-	-	0.1	-	0.5%
K <sub>14</sub> 0	-	-	0.2	-	-
K <sub>14</sub> CAH	-	-	0.2	0.5%	-
K <sub>14</sub> AC	-	-	0.2	-	0.5%

\*All media were supplemented with 0.8% agar, 11% sucrose

\*\*Organic additives added were 0 (No additive), CAH (Casein Acid Hydrolysate) and AC (Activated Charcoal)

K-Keller's medium (Keller *et al.*, 1975)

#### **3.2.2.4 Rooting of the plantlets**

The green shoots regenerated were subsequently separated and subcultured in 250 ml flask/50 ml culture tubes containing rooting media [ $\frac{1}{2}$  MS + IAA (2mg/l) + Sucrose (2%)]. The green shoots subcultured were incubated at  $25\pm 1^\circ$  C under 18/6 light/dark cycle till the plantlets developed steady shoots and well developed roots.

#### **3.2.2.5 Hardening and raising anther culture derived plants**

Initially about 30-40 well developed plants were hardened by transferring them directly to plastic pots (20 cm diameter) containing mixture of soil : sand :: 1 : 1 but, devoid of organic manure without colchicine treatment in order to study ploidy status of regenerated plants. The pots were covered by perforated polythene bags and were kept at room temperature for hardening. After about 15-20 days, the polythene bags were removed and plants observed for wilting. The plants showing normal turgidity without polythene bags were then transferred to bigger plastic pots and were kept in cage house until maturity.

#### **3.2.2.6 Colchicine treatment**

All regenerated plants were hardened without colchicine treatment and later studied/observed for ploidy status/ sterility. All surviving plants which were showing sterility and haploid status were given 0.4 per cent colchicine treatment by placing a cotton swab on growing buds of the haploid plants for 3-4 days continuously. Further, plants still exhibiting sterility/haploid status were again given a colchicine treatment by uprooting

the plant and dipping it in 0.25 per cent colchicine solution for 1-1.5 hrs. Later, plants were washed thoroughly with water and replanted after trimming of foliage.

#### **3.2.2.7 Cytological analysis**

For ascertaining ploidy level, cytological analysis of root tips was carried out. Root tips were excised from the plantlets while transferring regenerated plantlets for hardening. Excised root tips were fixed in Cornoy's solution for 24 hours and then stored in distilled water at 4° C. For ploidy analysis, root tips were hydrolyzed with 1N HCl at 60° C for 20 minutes followed by washings with H<sub>2</sub>O. Roots were then squashed in a drop of 1 per cent acetocarmine and observed under microscope (Nikon E800). In those cases where counting of chromosomes was not possible, meiotic analysis was also attempted. For meiotic analysis of anther culture derived plants, young buds were fixed in acetoalcohol (1:3) and then transferred to distilled water after 24 hours of fixation and preserved at 4°C till their study. The PMCs of anther culture derived plants were studied by squashing anthers in 1 per cent acetocarmine under the microscope. Later at flowering, the ploidy status of plants was confirmed by morphological observation on seed/siliqua set.

#### **3.2.3 Characterization of anther culture derived plants**

The anther culture derived plants were characterized agro morphologically as well as using DNA markers.

### 3.2.3.1 Agro morphological characterization

Following data on all surviving anther culture derived plants and their parents in  $R_0$  generation and 11 DH lines along with five parents in  $R_1$  generation were recorded. In  $R_0$  generation, data were recorded on single regenerated plant whereas in  $R_1$  generation, data were recorded on five randomly taken plants in each row of the lines grown in field at SAREC, Kangra and mean calculated.

1. **Seed yield/plant (g):** Total seed yield was weighed and recorded in g.
2. **Pods/plant:** Total number of pods was counted at the time of harvesting the plants and recorded.
3. **Seeds/pod:** Ten pods were selected at random to record the total number of seeds and mean calculated.
4. **Pod length (cm):** Length of ten randomly selected pods was recorded and mean calculated.
5. **Branches/plant:** Total number of branches was counted at the time of harvesting and recorded.
6. **Plant height (cm):** Plant height was measured at time of harvesting of plants in centimeters and recorded.

### 3.2.3.2 Molecular characterization

#### 3.2.3.2.1 Isolation of plant genomic DNA

DNA was isolated by using CTAB method of Murray and Thompson (1980). Young leaves were excised, rinsed with deionized water and dried on tissue paper. About 300 mg of fresh leaf tissue was ground to fine powder

The final reaction mixture was placed in a Thermal Cycler (Biometra) programmed at initial cycle of 94° C for 5 minutes, 37° C for 1 minute and 72° C for 2 minutes. Further amplification was repeated for 40 cycles consisting of denaturation at 94° C for 1 minute, annealing at 37° C for 1 min and extension at 72° C for 2 minutes. Final extension of 5 minutes at 72° C was carried out before rapid cooling to 4° C.

#### **3.2.3.2.3 Analysis of PCR products**

Ten µl of each PCR product mixed with gel loading dye, bromophenol blue (0.25% bromophenol blue and 40% sucrose) was electrophoresed using 1.4 per cent agarose gel in 0.5X Tris acetate-EDTA (TAE) buffer (1 M Tris, 1 M Acetic acid, 0.5 mM EDTA, pH 8.0). The gel was run at a constant voltage of 100 volts for 2 hours. The stain, ethidium bromide was added in the gel at a concentration of 0.5 µg/100ml. The resolved PCR products were visualized over an ultraviolet transilluminator and a photograph of gel was stored in Gel-Doc (Bio-Rad) for further data recording.

#### **3.2.3.2.4 Primers**

In all, 106 primers were screened for polymorphism among parents and uniform amplification. Six random primers (Operon Technologies) were finally selected and used for RAPD profiling of 28 plants. The base sequences of the primers used in the present study are given in the Table 3.4.

### 3.2.3.2.5 Analysis of RAPD profiles/DNA fingerprints

The RAPD profiles generated by different primers were compared to determine relatedness among plants. The presence or absence of each RAPD band of a particular molecular weight in all genotypes was scored manually. A binary data matrix with '1' indicating presence of a particular molecular weight band and '0' indicating its absence was generated separately for each primer. The binary data was used to generate a similarity matrix using Jaccard's coefficient  $[(J_{ij}=C_{ij}/(n_i+n_j-c_{ij}))]$ , where  $C_{ij}$  is the number of positive matches between two genotypes while  $n_i$  and  $n_j$  is the total number of bands in the genotype  $i$  and  $j$ , respectively] in SIMQUAL programme of NT SYS-PC package (Rohlf, 1993). Cluster analysis of DH lines based on similarity values was done by unweighted pair group arithmetic mean method (UPGMA) in SAHN program of NT SYS-PC package.

**Table 3.4 Base sequences of primers used for DNA fingerprinting of DHs and parents**

S. No	Primer	Sequence
1.	OPB 11	5'-GTAGACCCGT-3'
2.	OPD 11	5'-AGCGCCATTG-3'
3.	OPF01	5'-ACGGATCCTG-3'
4.	OPF09	5'-CCAAGCTTCC-3'
5.	OPX05	5'-CCTTTCCCTC-3'
6.	OPX13	5'-ACGGGAGCAA-3'

### 3.2.4 Screening of doubled haploids for white rust reaction

#### 3.2.4.1 Inoculum

All inoculation studies were conducted using *Albugo candida* zoospores from naturally infected *Brassica* leaves, obtained from farmer's fields at Kangra, Himachal Pradesh. To prepare inoculum, zoospores from rust pustules on fresh leaves, were suspended in distilled water in a conical flask and stirred with a glass rod to disperse zoospores. The conical flask was then placed in a incubator for 3-4 hours at 16° C.

#### 3.2.4.2 Artificial inoculation and incubation

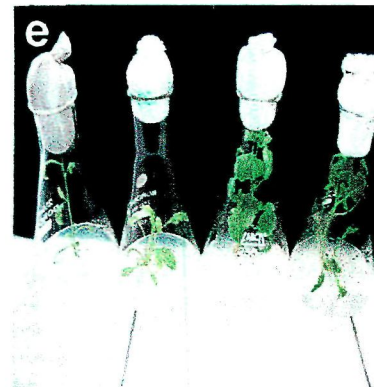
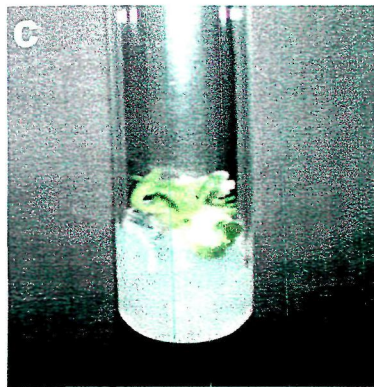
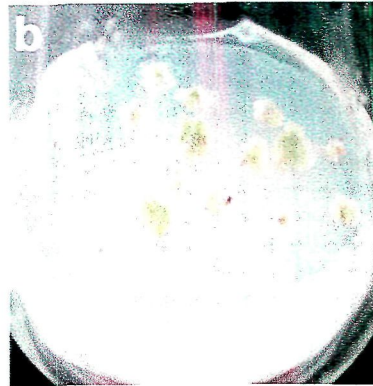
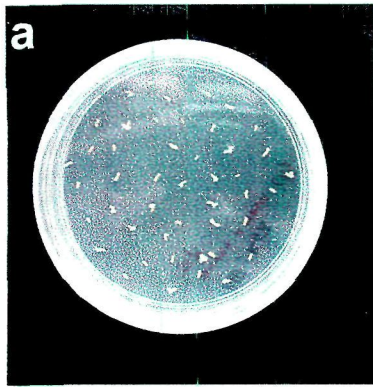
Doubled haploids (DHs) along with five parents (Varuna, Hira, Zem-1, Domo and BJ-1058) were raised in field at Shivalik Agricultural Research and Extension Centre (SAREC), Kangra. Plants at the age of about one month were inoculated in the evening by spraying the zoospore suspension to run-off. Inoculated plants were incubated for three days under a polythene sheet to maintain high humidity. Three days after inoculation, the polythene sheet was removed.

#### 3.2.4.3 Disease assessment

Fifty days after inoculation, disease severity was recorded. Data on disease severity were recorded on 20 leaves collected randomly from each line and rated as per Conn *et al.* (1990) scale given below:

Rating scale	Area covered by spots (%)
0	No symptoms on leaf
1	About 1 per cent of leaf area infected i.e. small necrotic spots on leaves
2	About 5 per cent of leaf area infected
3	About 10 per cent of leaf area infected
4	About 20 per cent of leaf area infected
5	About 30 per cent of leaf area infected
6	50 per cent or more leaf area infected

# ***Results***



**Plate I Stages of *in vitro* androgenesis in intervarietal crosses of *Brassica juncea***

- a. **Callus initiation on cultured anthers**
- b. **Calli starting regeneration**
- c. **Green regenerating *shoots***
- d. **Regenerated shoot kept on rooting medium**
- e. **Complete plantlets ready for hardening**
- f. **Established plants after hardening**

induction frequency (%). All interactions *viz.*, sugar x media, sugar x crosses and media x crosses were significant whereas interactions amongst all three factors (media x sugar x crosses) had non-significant effect on callus induction frequency (%).

**Table 4.1 ANOVA for androgenic callus induction frequency (%) in intervarietal crosses of *B. juncea* involving different treatments**

Source	d.f.	M.S.	F. ratio	CD (5%)	C.V.
A (media)	3	423.70 <sup>*†</sup>	13.78	2.59	
B (sugar)	3	1317.90 <sup>*†</sup>	42.85	2.59	
AB	9	109.55 <sup>*</sup>	3.56	5.19	
C (crosses)	2	84.06	2.73	NS	
AC	6	72.80 <sup>*</sup>	2.37	4.49	
BC	6	117.99 <sup>*</sup>	3.84	4.49	
ABC	18	45.02	1.46	NS	
Error	96	30.75			14.85

<sup>\*</sup>Significant at  $P \leq 0.05$  against error

<sup>†</sup> Significant at  $P \leq 0.05$  against interaction A x B

Significant treatments (tested against error), when tested against the respective interactions (taken as error) revealed that treatments (sugar and media) had pronounced effect in comparison to their respective interactions depicting thereby the importance of the individual factors (treatments) in comparison to their interactions.

#### 4.1.1.2 Effect of media

Effect of media and crosses (genotypes) on callus induction frequency (%) are presented in Table 4.2. Of the four media tested, anthers of three crosses plated on Keller's and N<sub>6</sub> media recorded highest mean callus formation to the extent of 42.28 and 42.10 per cent, respectively, followed by MS (34.90%) and B<sub>5</sub> (30.61%) (Plate Ib). Callus induction frequency (%) in Keller's and N<sub>6</sub> was at par statistically whereas MS exhibited better callus induction frequency (%) than B<sub>5</sub>. Media x crosses interaction also significantly affected callus induction frequency (%). Overall, callus induction frequency ranged from 29.29 per cent (D x BJ on B<sub>5</sub> medium) to the extent 46.84 per cent (D x BJ on N<sub>6</sub> medium). Best callusing of cultured anthers was recorded for the cross D x BJ on N<sub>6</sub> medium (46.84%) followed by D x BJ on Keller (46.08%), V x H on Keller's medium (44.00%) and H x Z on N<sub>6</sub> (42.31%) whereas D x BJ exhibited lowest response on B<sub>5</sub> medium (29.29%).

**Table 4.2** Effect of media and crosses (genotypes) on androgenic callus induction frequency (%) in intervarietal crosses of *B. juncea*

Media	Crosses			Mean	CD (P ≤ 0.05)
	V x H	H x Z	D x BJ		
Keller	44.00 (41.16)	36.76 (36.36)	46.06 (42.57)	<b>42.28</b> <b>(40.03)</b>	2.59
MS	30.36 (33.12)	37.78 (37.57)	36.57 (37.07)	34.90 (35.92)	
N <sub>6</sub>	37.16 (37.34)	42.31 (40.35)	<b>46.84</b> <b>(43.12)</b>	42.10 (40.27)	
B <sub>5</sub>	30.91 (33.46)	31.65 (33.48)	<b>29.29</b> <b>(32.53)</b>	<b>30.61</b> <b>(33.15)</b>	
Mean	35.60 (36.27)	37.12 (36.94)	39.69 (38.82)		
CD (P ≤ 0.05)	NS				
CD Interaction (A x C)	4.49				

Values in parentheses are arc sine transformed values

V x H – Varuna x Hira, H x Z – Hira x Zem-1, D x BJ – Domo x BJ-1058

#### 4.1.1.3 Effect of sugar levels

Effect of sugar levels and crosses (genotypes) on callus induction frequency (%) are presented in Table 4.3. Mean callus induction frequency (%) over three crosses ranged from 24.50 per cent (14% sugar) to a highest of 45.55 per cent (5% sugar). Of the four sugar levels tested, the anthers plated on 5 per cent and 8 per cent sugar levels recorded highest mean callus formation of 45.55 and 43.83 per cent, respectively which was statistically at par. Overall, 5 per cent and 8 per cent sugar level exhibited significantly better callusing frequency as compared to 11 per cent sugar which in turn was better than 14 per cent sugar.

**Table 4.3 Effect of sugar levels and crosses (genotypes) on androgenic callus induction frequency (%) in intervarietal crosses of *B. juncea***

Crosses Sugar levels (%)	V x H	H x Z	D x BJ	Mean	CD (P ≤ 0.05)
5	40.25 (39.24)	<b>51.75</b> <b>(46.01)</b>	44.65 (41.84)	<b>45.55</b> <b>(42.36)</b>	2.59
8	40.96 (39.55)	42.62 (40.65)	47.91 (43.75)	43.83 (41.30)	
11	32.65 (34.56)	36.07 (36.60)	39.36 (38.69)	36.02 (36.61)	
14	28.59 (31.73)	<b>18.06</b> <b>(24.50)</b>	26.86 (31.00)	<b>24.50</b> <b>(29.07)</b>	
Mean	35.61 (36.27)	37.12 (36.94)	39.69 (38.82)		
CD (P ≤ 0.05)	NS				
CD Interaction (B x C)	4.49				

Values in parentheses are arc sine transformed values

V x H – Varuna x Hira, H x Z – Hira x Zem-1, D x BJ – Domo x BJ-1058

**Table 4.4 Plantlet regeneration frequency (%) and anther culture response (%) of calli obtained on different media and sugar levels for three intervarietal crosses of *B. juncea***

Media	Sugar level (%)	Callusing frequency (%)	Calli transferred	Plants regenerated	Regeneration frequency (%)	Anther culture response (%)
Keller	5	57.00	225	29	12.88	7.34
	8	53.57	216	24	11.11	5.95
	11	36.69	147	41	27.89	10.22
	14	21.84	83	13	15.66	3.42
MS	5	39.55	153	8	5.22	2.05
	8	43.12	173	1	0.57	0.24
	11	32.94	133	0	0.00	0.00
	14	24.17	93	0	0.00	0.00
N <sub>6</sub>	5	50.90	205	8	3.90	1.98
	8	41.22	159	6	3.77	1.55
	11	41.59	157	21	13.37	5.56
	14	34.69	133	0	0.00	0.00
B <sub>5</sub>	5	34.90	130	0	0.00	0.00
	8	37.23	140	1	0.71	0.26
	11	32.89	123	28	22.76	7.48
	14	17.27	65	13	20.00	3.45

#### 4.1.2 Effect of different plant growth regulators (PGR) and organic additives on androgenic callus induction (%) and plant regeneration

##### 4.1.2.1 Analysis of variance

Data obtained on callus induction frequency (%) in anthers of three different crosses cultured *in vitro* on Keller's medium supplemented with 14 different combinations of different plant growth regulators and two additives, viz., casein acid hydrolysate and activated charcoal alongwith control were analyzed in CRD. ANOVA revealed that except crosses (genotypes) x organic additive interactions (X x Z), mean sum of squares due to all other factors were significant revealing thereby significant effects of crosses (genotypes), plant growth regulators and organic additives when tested against error (Table 4.5). Crosses x plant growth regulators, plant growth

**Table 4.5 ANOVA for *in vitro* callus induction frequency (%) in intervarietal crosses of *B. juncea* involving different treatments of crosses (genotypes), plant growth regulators (PGR) and organic additives**

Source	d.f.	M.S.	F. ratio	CD (5%)	C.V.
X (crosses)	2	87.34*	5.20	0.98	
Y (PGR)	14	1191.22*+	70.98	2.21	
XY	28	98.33*	5.86	3.83	
Z (Organic additive)	2	9262.75*+	551.93	0.98	
XZ	4	20.53	1.22	NS	
YZ	28	339.21*	20.21	3.83	
XYZ	56	57.95*	3.45	6.63	
Error	270	16.78			26.89

\*Significant against error at  $P \leq 0.05$

+ Significant against respective interaction at  $P \leq 0.05$

regulators x organic additives and crosses x plant growth regulator x organic additive interactions were also significant. Treatments when tested against their respective interactions indicated that mean sum of squares due to plant growth regulators and organic additives were significant revealing pronounced effect of these treatments against their respective interactions. However, mean sum of squares due to crosses (genotypes) was tested non-significant against crosses x PGR interactions (X x Y).

#### **4.1.2.2 Effect of plant growth regulators and crosses**

Data on effect of plant growth regulators and crosses (genotypes) on androgenic callus induction frequency (%) revealed that all the crosses (genotypes) tested responded on all hormone combinations, except on medium K<sub>0</sub>, K<sub>13</sub> and K<sub>14</sub> PGR combinations wherein none of the crosses in general exhibited callusing (Table 4.6). Mean *in vitro* androgenic callusing frequency (%) over different crosses (genotypes), ranged from 0 to 19.25 per cent in 15 different combinations of PGRs including control (K<sub>0</sub>). The highest mean callusing frequency (%) was recorded on PGR combination K<sub>12</sub> (19.25%), followed by K<sub>4</sub> (15.86%), K<sub>11</sub> (15.80%) and K<sub>6</sub> (15.78%) whereas no callusing was recorded in K<sub>0</sub> (control), K<sub>13</sub> (all crosses) and K<sub>14</sub> (all crosses). On the other hand, mean callusing frequency (%) in three different crosses over different PGR combinations did not exhibit much variation and ranged from 9.09 per cent (V x H) to 10.82 per cent (D x BJ). Overall, callusing frequency (%) ranged from 0 to 25.10 per cent for all the crosses (genotypes) tested on Keller media with different PGR combinations. The

**Table 4.6** Effect of plant growth regulators and crosses (genotypes) on androgenic callus induction frequency (%) in intervarietal crosses of *B. juncea*

Crosses	PGR*														Mean	CD (P≤0.05)		
	K <sub>0</sub>	K <sub>1</sub>	K <sub>2</sub>	K <sub>3</sub>	K <sub>4</sub>	K <sub>5</sub>	K <sub>6</sub>	K <sub>7</sub>	K <sub>8</sub>	K <sub>9</sub>	K <sub>10</sub>	K <sub>11</sub>	K <sub>12</sub>	K <sub>13</sub>			K <sub>14</sub>	
V x H	0 (5.70)	5.49 (12.29)	0 (5.70)	5.75 (11.89)	13.72 (19.51)	13.48 (19.56)	13.63 (19.53)	11.85 (18.07)	14.48 (20.12)	13.30 (19.40)	9.21 (14.92)	15.08 (20.81)	19.52 (23.60)	0 (5.70)	0 (5.70)	0 (5.70)	9.09 (14.83)	0.98
H x Z	0 (5.70)	2.19 (8.50)	0.27 (6.07)	5.03 (11.28)	8.76 (15.40)	5.79 (12.54)	16.44 (21.63)	13.08 (19.31)	21.03 (24.54)	11.29 (17.84)	15.04 (19.82)	13.56 (19.70)	24.55 (26.67)	0 (5.70)	0 (5.70)	0 (5.70)	9.13 (14.69)	
D x BJ	0 (5.70)	16.26 (21.32)	1.14 (7.14)	1.56 (7.80)	25.10 (27.51)	9.32 (16.09)	17.28 (21.96)	17.93 (22.55)	8.59 (15.41)	17.29 (22.35)	15.39 (21.34)	18.78 (23.07)	13.68 (18.65)	0 (5.70)	0 (5.70)	0 (5.70)	10.82 (16.15)	
Mean	0 (5.70)	7.98 (14.03)	0.47 (6.30)	4.11 (10.33)	15.86 (20.80)	9.53 (16.06)	15.78 (21.04)	14.28 (19.97)	14.70 (20.02)	13.96 (19.86)	13.21 (18.69)	15.80 (21.19)	19.25 (22.47)	0 (5.70)	0 (5.70)	0 (5.70)	9.68 (15.22)	
CD (P≤0.05)																		2.21
CD Interaction (X x Y)																		3.83

\* Values in parentheses are arc sine transformed values

\*

Medium designation	2,4-D	NAA	TIBA	Carbon source	Medium* designation	2,4-D	NAA	TIBA	Carbon source
K <sub>0</sub>	-	-	-	Sucrose 11%	K <sub>8</sub>	1.0	-	-	Sucrose 11%
K <sub>1</sub>	0.1	-	-	Sucrose 11%	K <sub>9</sub>	-	2.0	-	Sucrose 11%
K <sub>2</sub>	-	0.1	-	Sucrose 11%	K <sub>10</sub>	2.0	-	-	Sucrose 11%
K <sub>3</sub>	0.2	-	-	Sucrose 11%	K <sub>11</sub>	1.0	1.0	-	Sucrose 11%
K <sub>4</sub>	-	0.2	-	Sucrose 11%	K <sub>12</sub>	2.0	2.0	-	Sucrose 11%
K <sub>5</sub>	0.1	0.1	-	Sucrose 11%	K <sub>13</sub>	-	-	0.1	Sucrose 11%
K <sub>6</sub>	0.2	1.0	-	Sucrose 11%	K <sub>14</sub>	-	-	0.2	Sucrose 11%
K <sub>7</sub>	-	-	-	Sucrose 11%					

cross D x BJ recorded highest callusing frequency on K<sub>4</sub> PGR combination (25.10%) followed by H x Z on K<sub>12</sub> PGR combination (24.55%) and H x Z on K<sub>8</sub> PGR combination (21.03%).

#### 4.1.2.3 Effect of organic additives and PGRs

Effect of organic additives and PGRs on callus induction frequency (%) are presented in Table 4.7. The perusal of table revealed that mean *in vitro* androgenic callusing frequency (%) over different organic additives in the 15 different PGR combinations including control ranged from 0 to 19.25 per cent whereas amongst three organic additives over 15 PGR combinations, it ranged from 0.19 per cent (AC) to 16.52 per cent (control). Highest mean callusing frequency (%) amongst different organic additives over PGR combinations was observed for control (16.52%), followed by CAH (12.34%) and AC (0.19%) whereas highest mean callusing over organic additives was observed on K<sub>12</sub> PGR combination (19.25%), followed by K<sub>4</sub> (15.86%), K<sub>11</sub> (15.81%) and K<sub>6</sub> (15.78%). No callusing was recorded in K<sub>0</sub> (control) as well as K<sub>13</sub> and K<sub>14</sub>. Callusing frequency (%) was also affected by PGR x organic additive interactions. Overall, callusing frequency (%) ranged from 0 per cent (K<sub>0</sub>0, K<sub>0</sub>CAH, K<sub>0</sub>AC, K<sub>1</sub>AC, K<sub>2</sub>CAH, K<sub>3</sub>CAH, K<sub>3</sub>AC, K<sub>5</sub>AC, K<sub>6</sub>AC, K<sub>7</sub>AC, K<sub>8</sub>AC, K<sub>11</sub>AC, K<sub>12</sub>AC, K<sub>13</sub>0, K<sub>13</sub>CAH, K<sub>13</sub>AC, K<sub>14</sub>0, K<sub>14</sub>CAH and K<sub>14</sub>AC) to 29.91 per cent (K<sub>6</sub> PGR combination without additives).

**Table 4.7 Effect of organic additives and plant growth regulators on androgenic callus induction frequency (%) in intervarietal crosses of *B. juncea***

Organic additive	PGR														Mean	CD (P <sub>≤</sub> 0.05)	
	K <sub>0</sub>	K <sub>1</sub>	K <sub>2</sub>	K <sub>3</sub>	K <sub>4</sub>	K <sub>5</sub>	K <sub>6</sub>	K <sub>7</sub>	K <sub>8</sub>	K <sub>9</sub>	K <sub>10</sub>	K <sub>11</sub>	K <sub>12</sub>	K <sub>13</sub>			K <sub>14</sub>
0	0 (5.70)	16.32 (21.80)	1.14 (7.14)	12.35 (19.58)	22.09 (27.37)	14.67 (20.92)	29.91 (33.11)	24.01 (28.65)	27.55 (30.90)	24.61 (29.36)	23.95 (28.33)	21.86 (27.83)	29.47 (32.72)	0 (5.70)	0 (5.70)	16.52 (21.61)	0.98
CAH	0 (5.70)	7.63 (14.61)	0 (5.70)	0 (5.70)	24.94 (28.54)	13.92 (21.57)	17.45 (24.31)	18.86 (25.58)	16.56 (23.48)	16.79 (23.83)	15.14 (21.25)	25.57 (30.06)	28.28 (30.50)	0 (5.70)	0 (5.70)	12.34 (18.14)	
AC	0 (5.70)	0 (5.70)	0.27 (6.07)	0 (5.70)	0.55 (6.50)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0.48 (6.40)	0.55 (6.56)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0.19 (5.87)	
Means	0 (5.70)	7.98 (14.03)	0.47 (6.30)	4.11 (10.32)	15.86 (20.80)	9.53 (16.06)	15.78 (21.04)	14.29 (19.97)	14.70 (20.02)	13.96 (19.86)	13.21 (18.69)	15.81 (21.19)	19.25 (22.97)	0 (5.70)	0 (5.70)	9.67 (15.20)	
CD (P <sub>≤</sub> 0.05)		2.21															
CD Interaction (Y x Z)		3.83															

Values in parentheses are arc sine transformed values

0 – Without any organic additive  
 CAH – Casein acid hydrolysate  
 AC – Activated charcoal

#### **4.1.2.4 Effect of crosses (genotypes) x PGRs x organic additives interactions**

The effect of crosses (genotypes) x PGRs x organic additives on callus induction frequency (%) are presented in Table 4.8. The data in table revealed maximum callusing frequency (40.86%) for cross H x Z on K<sub>12</sub> combination of PGR with additive casein acid hydrolysate followed by cross D x BJ on K<sub>4</sub> with additive casein acid hydrolysate (38.86%) and H x Z on K<sub>8</sub> without organic additives (37.59%). For the cross V x H, maximum callusing frequency (%) was observed on K<sub>12</sub> with additive casein acid hydrolysate (36.02%) whereas for H x Z and D x BJ, maximum callusing was observed on K<sub>12</sub> with CAH (40.86%) and K<sub>4</sub> with CAH (38.86%), respectively. The anther plated on PGR combinations with activated charcoal did not exhibit callusing in all the treatments in three crosses tested except H x Z on K<sub>2</sub>, D x BJ on K<sub>4</sub>, D x BJ on K<sub>9</sub> and D x BJ on K<sub>10</sub>.

#### **4.1.2.5 Effect of PGRs and organic additives on regeneration frequency (%) and anther culture response (%)**

Data on regeneration frequency and anther culture response presented in Table 4.9 indicated that the plantlet regeneration frequency for calli induced on different PGR combination without any organic additives (control) ranged from 0 to 9.90 per cent (Plate 1c and d). No regeneration was observed for calli induced on K<sub>0</sub> (control), K<sub>1</sub>, K<sub>2</sub>, K<sub>3</sub>, K<sub>8</sub>, K<sub>9</sub>, K<sub>10</sub>, K<sub>11</sub>, K<sub>12</sub>, K<sub>13</sub> and K<sub>14</sub> PGR combinations whereas for calli developed on K<sub>6</sub> PGR combination, it was maximum (9.90%). Overall anther culture response in this combination varied from nil to 2.96 per cent. The response was maximum for calli developed on K<sub>6</sub> PGR combination (2.96%), followed by K<sub>5</sub> PGR combination (1.10%) and K<sub>4</sub> PGR combination (1.06%).

**Table 4.8** Effect of crosses (genotypes), plant growth regulators and organic additives on androgenic callus induction frequency (%) in intervarietal crosses of *B. juncea*

	V x H			H x Z			D x B J		
	0	CAH	AC	0	CAH	AC	0	CAH	AC
K <sub>0</sub>	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)
K <sub>1</sub>	8.25 (15.56)	8.23 (15.61)	0 (5.70)	6.58 (14.11)	0 (5.70)	0 (5.70)	34.14 (35.73)	14.66 (22.53)	0 (5.70)
K <sub>2</sub>	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0.83 (6.83)	3.42 (10.03)	0 (5.70)	0 (5.70)
K <sub>3</sub>	17.27 (24.48)	0 (5.70)	0 (5.70)	15.10 (22.45)	0 (5.70)	0 (5.70)	4.68 (12.01)	0 (5.70)	0 (5.70)
K <sub>4</sub>	16.87 (23.62)	24.30 (29.22)	0 (5.70)	14.62 (22.47)	11.66 (18.04)	0 (5.70)	34.79 (36.04)	<b>38.86 (38.38)</b>	1.66 (8.11)
K <sub>5</sub>	24.02 (29.14)	16.43 (23.84)	0 (5.70)	5.00 (11.39)	12.39 (20.54)	0 (5.70)	15.01 (22.24)	12.96 (20.33)	0 (5.70)
K <sub>6</sub>	28.05 (31.95)	12.86 (20.94)	0 (5.70)	28.92 (32.49)	20.41 (26.70)	0 (5.70)	32.76 (34.89)	19.10 (25.29)	0 (5.70)
K <sub>7</sub>	19.38 (24.91)	16.19 (23.60)	0 (5.70)	20.59 (26.70)	18.66 (25.55)	0 (5.70)	32.06 (34.35)	21.73 (27.61)	0 (5.70)
K <sub>8</sub>	30.07 (33.24)	13.39 (21.44)	0 (5.70)	37.59 (37.64)	25.52 (30.28)	0 (5.70)	14.99 (21.83)	10.78 (18.72)	0 (5.70)
K <sub>9</sub>	21.27 (27.17)	18.65 (25.34)	0 (5.70)	19.47 (25.93)	14.41 (21.91)	0 (5.70)	33.10 (34.99)	17.33 (24.26)	1.45 (7.81)
K <sub>10</sub>	25.33 (30.17)	2.32 (8.90)	0 (5.70)	23.08 (25.89)	22.05 (27.87)	0 (5.70)	23.46 (28.93)	21.06 (27.00)	1.66 (8.11)
K <sub>11</sub>	22.11 (28.02)	23.15 (28.73)	0 (5.70)	23.78 (29.16)	16.91 (24.26)	0 (5.70)	19.69 (26.32)	36.66 (37.19)	0 (5.70)
K <sub>12</sub>	22.54 (28.31)	<b>36.02 (36.81)</b>	0 (5.70)	32.80 (34.92)	<b>40.86 (39.39)</b>	0 (5.70)	33.08 (34.95)	7.97 (15.31)	0 (5.70)
K <sub>13</sub>	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)
K <sub>14</sub>	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)	0 (5.70)

CD (P≤0.05) 6.63

**Table 4.9 Plantlet regeneration frequency (%) and anther culture response (%) for calli induced on Keller's medium with different PGR combinations and organic additives in three intervarietal crosses of *B. juncea***

Organic additives	Medium	Callusing frequency (%)	Calli transferred	Plants regeneration	Regeneration frequency (%)	Anther culture response (%)
0	K <sub>0</sub>	0.00	0	0	0.00	0.00
	K <sub>1</sub>	48.97	61	0	0.00	0.00
	K <sub>2</sub>	1.14	4	0	0.00	0.00
	K <sub>3</sub>	12.35	46	0	0.00	0.00
	K <sub>4</sub>	22.09	83	4	4.81	1.06
	K <sub>5</sub>	14.67	53	4	7.54	1.10
	K <sub>6</sub>	29.91	111	11	<b>9.90</b>	<b>2.96</b>
	K <sub>7</sub>	24.01	87	3	3.44	0.82
	K <sub>8</sub>	27.55	103	0	0.00	0.00
	K <sub>9</sub>	24.61	93	0	0.00	0.00
	K <sub>10</sub>	23.95	92	0	0.00	0.00
	K <sub>11</sub>	21.86	83	0	0.00	0.00
	K <sub>12</sub>	29.47	110	0	0.00	0.00
	K <sub>13</sub>	0.00	0	0	0.00	0.00
K <sub>14</sub>	0.00	0	0	0.00	0.00	
CAH	K <sub>0</sub>	0.00	0	0	0.00	0.00
	K <sub>1</sub>	7.63	28	0	0.00	0.00
	K <sub>2</sub>	0.00	0	0	0.00	0.00
	K <sub>3</sub>	0.00	0	0	0.00	0.00
	K <sub>4</sub>	24.94	93	2	2.15	0.53
	K <sub>5</sub>	13.92	52	2	3.84	0.53
	K <sub>6</sub>	17.45	64	4	<b>6.25</b>	<b>1.09</b>
	K <sub>7</sub>	18.86	70	3	4.28	0.80
	K <sub>8</sub>	16.56	64	0	0.00	0.00
	K <sub>9</sub>	16.79	62	0	0.00	0.00
	K <sub>10</sub>	15.14	57	0	0.00	0.00
	K <sub>11</sub>	25.57	95	0	0.00	0.00
	K <sub>12</sub>	28.28	106	0	0.00	0.00
	K <sub>13</sub>	0.00	0	0	0.00	0.00
K <sub>14</sub>	0.00	0	0	0.00	0.00	
AC	K <sub>0</sub>	0.00	0	0	0.00	0.00
	K <sub>1</sub>	0.00	0	0	0.00	0.00
	K <sub>2</sub>	0.27	1	0	0.00	0.00
	K <sub>3</sub>	0.00	0	0	0.00	0.00
	K <sub>4</sub>	0.55	2	0	0.00	0.00
	K <sub>5</sub>	0.00	0	0	0.00	0.00
	K <sub>6</sub>	0.00	0	0	0.00	0.00
	K <sub>7</sub>	0.00	0	0	0.00	0.00
	K <sub>8</sub>	0.00	0	0	0.00	0.00
	K <sub>9</sub>	0.41	2	0	0.00	0.00
	K <sub>10</sub>	0.55	2	0	0.00	0.00
	K <sub>11</sub>	0.00	0	0	0.00	0.00
	K <sub>12</sub>	0.00	0	0	0.00	0.00
	K <sub>13</sub>	0.00	0	0	0.00	0.00
K <sub>14</sub>	0.00	0	0	0.00	0.00	

The regeneration frequency (%) for calli developed on different PGR combinations with organic additive casein acid hydrolysate ranged from nil to 6.25 per cent. It was nil on K<sub>0</sub> (control), K<sub>1</sub>, K<sub>2</sub>, K<sub>3</sub>, K<sub>8</sub>, K<sub>9</sub>, K<sub>10</sub>, K<sub>11</sub>, K<sub>12</sub>, K<sub>13</sub> and K<sub>14</sub> whereas calli of K<sub>6</sub> PGR combination also recorded maximum regeneration frequency (6.25%) in this combination. The overall anther culture response ranged from nil to 1.09 per cent. Highest anther culture response was for calli developed on K<sub>6</sub> PGR combination (1.09%) followed by K<sub>7</sub> PGR combination (0.80%), and K<sub>4</sub> and K<sub>5</sub> PGR combinations (0.53%) each. No regeneration was recorded on PGR combination with activated charcoal.

#### 4.1.3 Hardening and survival

Data on total number of androgenic plantlets regenerated and their survival (%) in three crosses viz., V x H, H x Z and D x BJ are presented in table 4.10 (Plate 1e and f). In all, 123 plants of cross V x H were regenerated out of which 32 survived and flowered with a survival frequency of 26.01 per cent. In H x Z, five plants were regenerated, out of which only one plant survived and flowered, and exhibited 20.00 per cent survival. However, in the cross D x BJ, survival (%) was high (34.69%) as 34 plants out of a total 98 regenerated, survived and flowered.

**Table 4.10 Total number of androgenic plantlets regenerated and their survival (%) in three crosses of *Brassica juncea***

Cross	No. of plants regenerated	No. of plants survived and flowered	Survival (%)
V x H	123	32	26.01
H x Z	5	1	20.00
D x BJ	98	34	34.69
Total	226	67	29.64

Overall 226 plants of three crosses, *viz.*, V x H, H x Z and D x BJ were regenerated and 67 of these survived and flowered with an overall survival of 29.64 per cent.

## **4.2 Ploidy status**

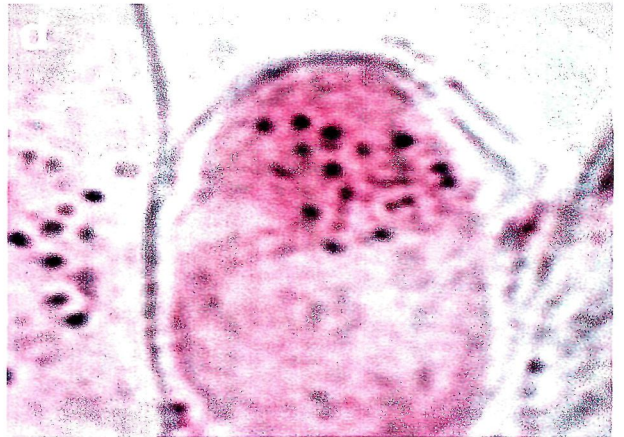
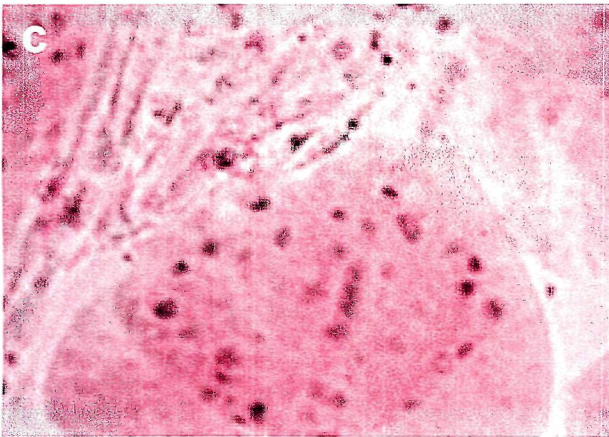
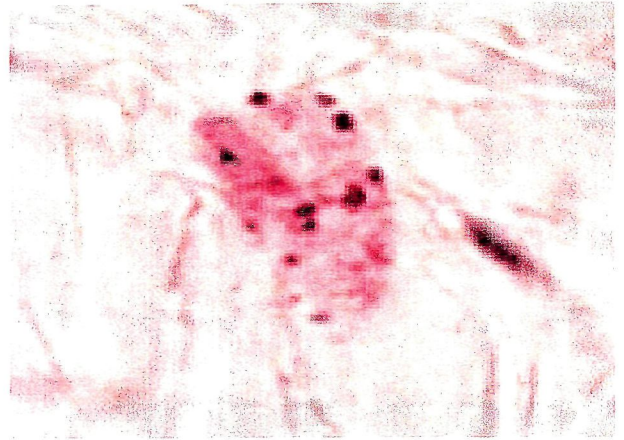
In order to assess the ploidy status of the regenerated plants, both the mitotic and meiotic analysis of root tips and floral buds as well as morphological observations on fertility status/siliqua set were carried out (Plate II, III and IV). In all, 20 plants were selected at random for mitotic and meiotic studies (Table 4.11). The results of the mitotic and meiotic analysis confirmed the presence of both haploid ( $2n=18$ ) as well as diploid ( $2n=36$ ) plants (Plate II and III). Out of 20 plants analyzed, seven were found to be haploid and they subsequently exhibited sterility.

During further growth of the anther culture derived plants in  $R_0$  generation, regenerated plants were also observed morphologically for siliqua/seed set to infer their ploidy status (Plate IV). Overall, on 18 plantlets of the cross V x H, seed set did not take place whereas in few other plants, seedless siliqua were also observed (Table 4.12). However, in other two crosses *viz.*, H x Z and D x BJ, all regenerated plants exhibited normal siliqua/seed set.

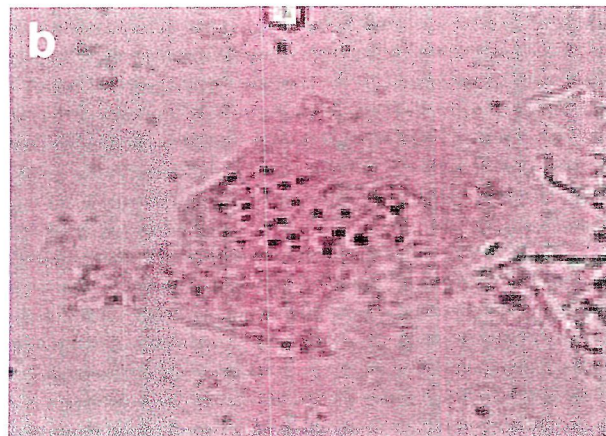
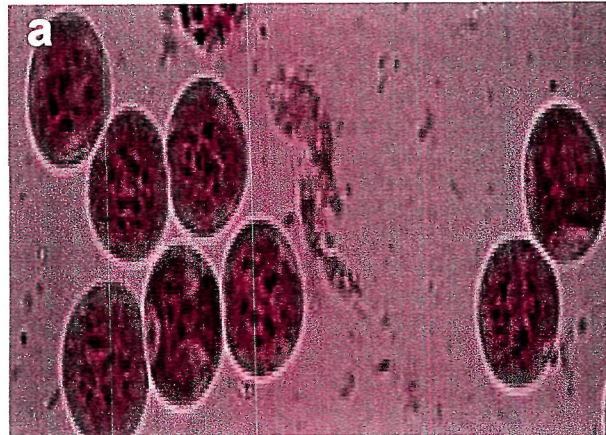
## **4.3 Characterization of doubled haploids**

### **4.3.1 Agro-morphological evaluation**

The parents as well as the DHs were studied for six morphological characters in  $R_0$  and  $R_1$  generations under cage house and field conditions at Palampur and Kangra, respectively (Plate V). Agro-morphological data for parents *viz.*, Varuna, Hira, Domo, BJ-1058 and Zem-1, and their DHs are presented in Appendix-II and Table 4.13. Appendix-II depicts single



**Plate II**      **Mitotic analysis of root tips of anther culture raised plants**  
**a, b Haploid cells ( $2n=18$ )**  
**c, d Diploid cells ( $2n=36$ )**



**Plate III**      **Meiotic analysis of floral buds of anther culture derived plants**  
**a Haploid cells**  
**b Diploid cells**



**Plate IV. Regenerated plants showing sterility in form of complete siliqua drop after pollination/formation of seed less siliquae**



**Plate V. Agro-morphological variability in regenerated plantlets**

**Table 4.11 Ploidy status of the regenerated plants as inferred by cytological analysis and fertility**

S. No.	DH/line	Cytological evaluation	Siliqua/Seed set
1.	VH, 2	Diploid	Fertile
2.	VH, 3	Diploid	Fertile
3.	VH, 5	Diploid	Fertile
4.	VH, 6	Diploid	Fertile
5.	VH, 9	Diploid	Fertile
6.	VH, 28	Haploid	Sterile
7.	VH, 37	Haploid	Sterile
8.	VH, 44	Diploid	Fertile
9.	VH, 45	Haploid	Sterile
10.	VH, 51	Haploid	Sterile
11.	VH, 58	Haploid	Sterile
12.	VH, 59	Haploid	Sterile
13.	VH, 63	Haploid	Sterile
14.	DB, 35	Diploid	Fertile
15.	DB, 36	Diploid	Fertile
16.	DB, 47	Diploid	Fertile
17.	DB, 50	Diploid	Fertile
18.	DB, 51	Diploid	Fertile
19.	DB, 54	Diploid	Fertile
20.	DB, 58	Diploid	Fertile

VH – Varuna x Hira derivative

DB – Domo x BJ-1058 derivative

**Table 4.12 Overall fertility status of regenerated plants ( $R_0$ ) in three crosses of *Brassica juncea***

Cross	Total survived plants that flowered	Sterile plants	Fertile plants	Sterile plants (%)
V x H	32	18	14	56.25
H x Z	1	0	1	0.00
D x BJ	34	0	34	0.00
Total	67	18	49	26.86

**Table 4.13 Performance of parents and their DHs ( $R_1$ ) of *B. juncea* for agro-morphological traits under field conditions at Kangra (2005-06)**

Plant	Plant height (cm)	Branches/plant	Pods/plant	Pod length (cm)	Seeds/pod	Seed yield/plant (g)
Varuna	174.8	5.2	138.5	5.18	14.00	4.14
Hira	194.0	2.4	94.4	4.68	12.36	2.22
Domo	168.0	6.4	108.4	5.50	12.46	3.86
BJ-1058	168.4	4.2	132.5	5.54	14.12	3.99
Zem-1	165.6	4.2	109.0	4.77	10.14	3.20
VH, 2	160.8	5.8	98.2	4.80	11.00	2.93
VH, 3	191.2	6.2	132.4	4.63	11.90	4.61
VH, 5	155.4	4.6	88.6	4.69	12.28	2.45
VH, 6	177.4	4.6	76.0	4.56	11.64	2.57
VH, 9	190.4	6.2	109.8	4.60	11.92	3.43
DB 1	189.8	4.2	64.0	4.37	10.88	1.82
DB 17	158.8	1.8	26.0	5.25	10.98	1.03
DB 35	194.4	3.8	70.4	5.51	13.30	3.37
DB 36	203.2	4.4	76.2	5.30	12.08	3.85
DB 47	183.0	3.4	49.4	4.97	12.26	1.55
HZ 1	153.8	6.2	98.6	5.50	13.50	4.27

plant data on eight doubled haploids of V x H cross and 31 doubled haploids of D x BJ cross in R<sub>0</sub> generation in cage house. Three doubled haploids of V x H cross viz., VH 41, VH 47 and VH 50 exhibited seed yield close to the parent 'Varuna', whereas rest of doubled haploids had comparatively lesser seed yield. Overall the height of the plants in R<sub>0</sub> was very less as compared to both of the parents. Number of branches ranged from three (VH 110) to a maximum of 15 branches (VH 50), with pods per plant ranging from six (VH 110) to the maximum of 152 pods per plant (VH 50). However, in case of doubled haploids of D x BJ cross, seed yield/plant ranged from 1.19 g to a maximum of 12.47 g. Seed yield was recorded maximum for DB 61 to the tune of 12.47 g, followed by DB 27 (9.13 g) and DB 93 (7.90 g). Number of branches ranged from five (DB 53, DB 56, DB 60, DB 90, DB 94) to a maximum of 16 (DB 58). On the other hand, pods per plant were minimum in DB 55 (60 pods) and maximum in DB 27 (407 pods).

Performance of parents and their DHs (R<sub>1</sub>) under field conditions at SAREC, Kangra during *rabi* 2005-2006, presented in table 4.13, reflects mean of five plants data, selected randomly from a line. Out of five doubled haploid lines of the cross V x H evaluated, VH 3 recorded maximum seed yield (4.61 g) followed by VH 9 (3.43 g) and VH 2 (2.93 g). Rest of the doubled haploid lines, viz., VH 5 and VH 6 yielded lesser than Varuna (4.14 g) but better than the other parent 'Hira' (2.22 g). Pods per plant ranged from 76.0 (VH 6) to a maximum of 132.4 (VH 3) in five DH lines of V x H. Maximum pods were recorded on VH 3 (132.4) which was closer to better

parent 'Varuna' (138.5) followed by VH 9 (109.8) and VH 2 (98.2), whereas rest of the doubled haploid lines, viz., VH 5, and VH 6 had 88.6 and 76.0 pods, respectively. The pod length was recorded less in DH lines as compared to parents and it ranged from 4.56 cm (VH 6) to a maximum of 4.80 cm (VH 2) in DH lines of V x H. Seeds per pod were maximum in VH 5 (12.28) followed by VH 9 (11.92), VH 3 (11.90) and VH 6 (11.64). Parent 'Varuna' had more seeds per pod (14.00) as compared to all the DHs. VH 3 and VH 9 were taller having 191.2 cm and 190.4 cm height, respectively, whereas rest of lines had comparable height with dwarf parent 'Varuna'. The branches per plant ranged from 4.6 (VH 5, VH 6) to a maximum of 6.2 (VH 3, VH 9) whereas in case of the parents 'Varuna' and 'Hira' had 5.2 and 2.5 branches/plant, respectively.

In five doubled haploid lines of cross D x BJ, seed yield/plant ranged from 1.03 g (DB 17) to a maximum of 3.85 g (DB 36). It was maximum for DB 36 (3.85 g) followed by DB 35 (3.37 g) and DB 1 (1.82 g). All DHs had lesser yield than both of the parents i.e. Domo (3.86 g) and BJ-1058 (3.99 g). Pods/plant was also recorded lesser than both of the parents and it ranged from 26.0 (DB 17) to 76.2 (DB 36). Similarly, mean pod length ranged from 4.37 cm (DB 1) to a maximum of 5.51 cm (DB 35) in the DH lines of D x BJ, whereas in case of parents, the pod length of BJ-1058 and Domo was 5.54 cm and 5.50 cm, respectively. Seeds per pod was maximum in DB 35 to the tune of 13.30 seeds per pod, followed by DB 47 (12.26), DB 36 (12.08) and DB 17 (10.98), whereas in the parents, 'BJ-1058' and 'Domo'

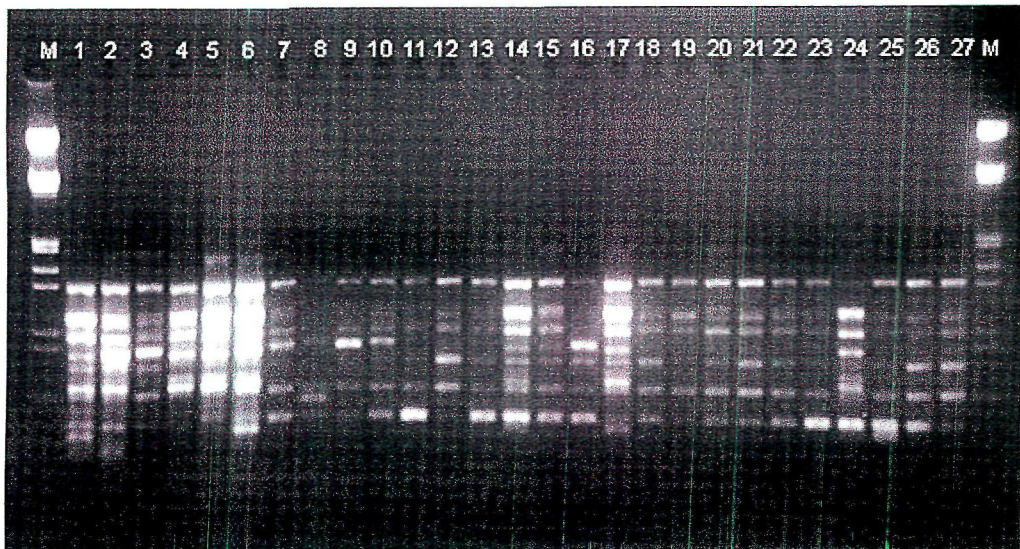
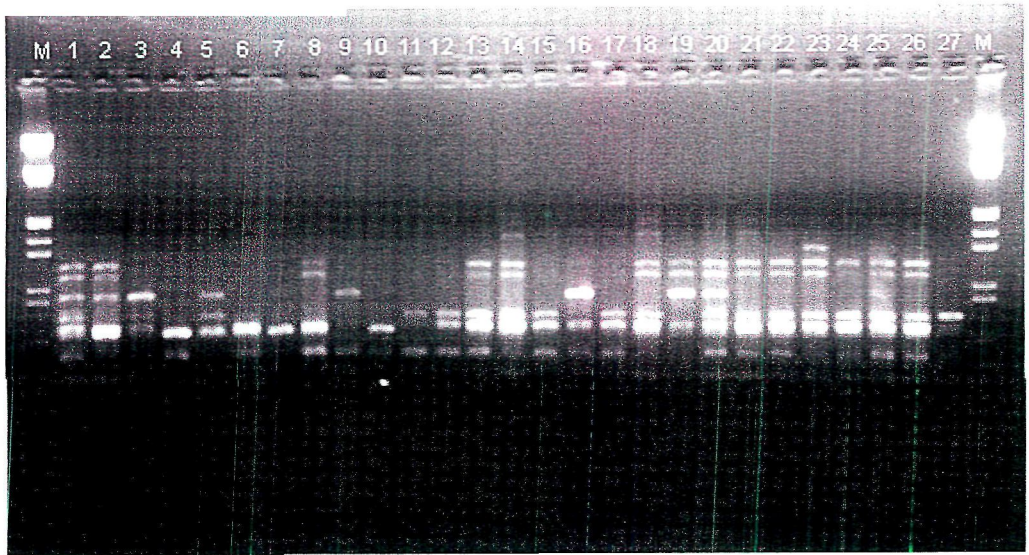
it was 14.12 and 12.46 seeds per pod, respectively. Both the parents had almost same height (168 cm) and its DH lines exhibited a wide range from 158.8 cm (DB 17) to a maximum of 203.2 cm (DB 36). Four out of five DH lines, viz., DB 1, DB 35, DB 36 and DB 47 of D x BJ cross were taller than both of the parents. Branches per plant ranged from 1.8 (DB 17) to 4.4 (DB 36) which were lesser than the better parent 'Domo' (6.4).

Only regenerated DH line of H x Z cross, i.e. HZ1, recorded higher seed yield (4.27 g) as compared to both of the parents, 'Zem-1' (3.20 g) and 'Hira' (2.22 g). It had intermediate pods per plant (98.6) and longer siliqua length (5.50 cm) as compared to both of the parents. Seeds per pod was also better (13.50) than both of the parents. However, HZ1 was dwarfer as compared to parents. Branches/plant varied from 2.4 to 4.2 in Hira and Zem-1, respectively whereas HZ1 had 6.2 branches per plant.

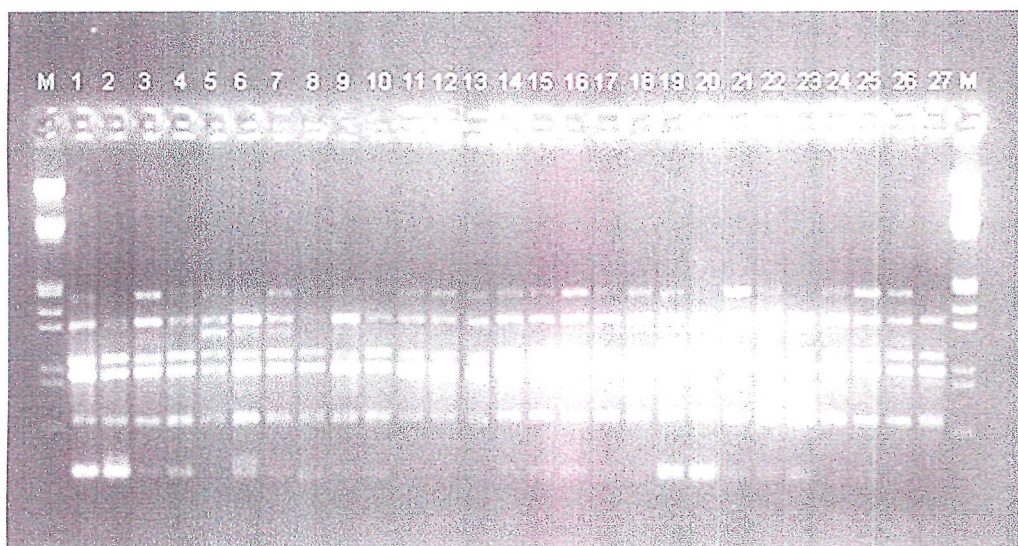
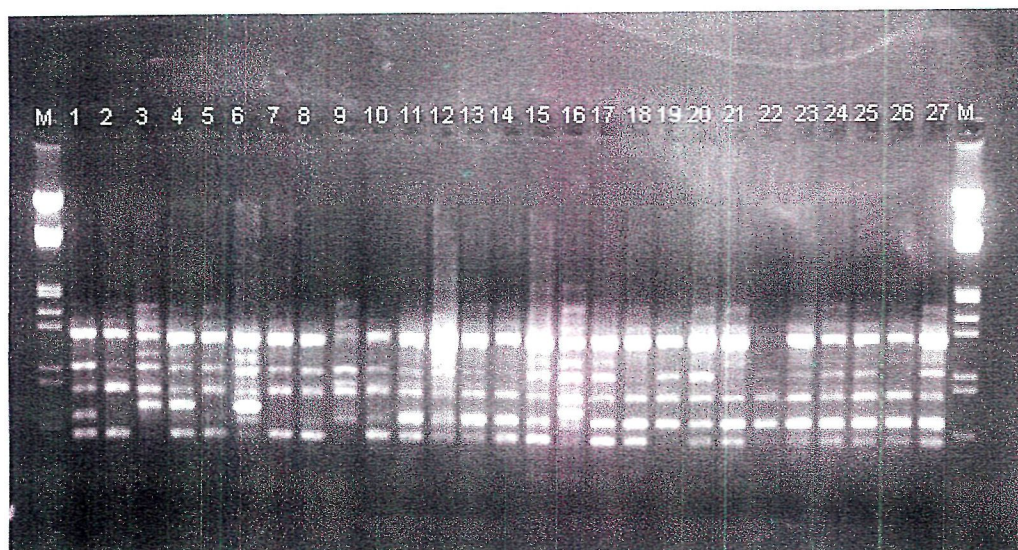
#### **4.3.2 Molecular characterization**

RAPD analysis of 23 anther culture derived lines of two intervarietal crosses of Varuna x Hira and Domo x BJ-1058 was done using six primers. Cross-wise number of scorable bands and polymorphic bands obtained with each primer are given in Table 4.14. The RAPD profiles generated using primers OPF01, OPF09, OPX05 and OPX13 are presented in Plates VI and VII.

RAPD profiling of the 16 anther culture derived lines of the cross V x H generated a total of 44 bands of which 36 bands were polymorphic. The bands were used to construct a dendrogram. The cluster analysis on the



**Plate VI** RAPD profile generated by primer OPX13 and OPF09. Lane 1-2: Varuna, Hira; Lane 3-18: doubled haploids of V x H; 19-20: Domo, BJ-1058 and 21-27: doubled haploids of Domo/BJ-1058. Lanes marked M are molecular weight marker

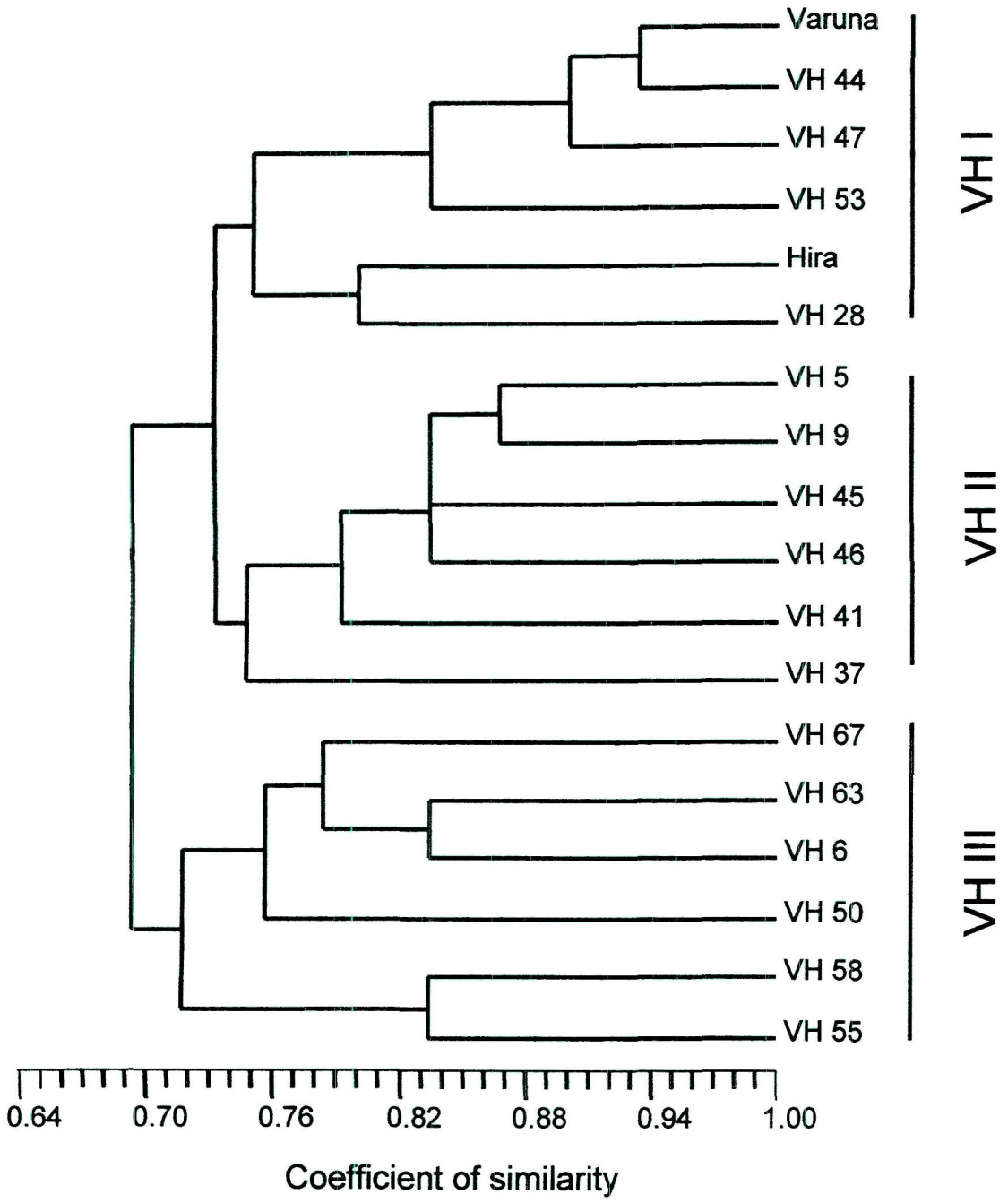


**Plate VII RAPD profile generated by primer OPF01 and OPX5. Lane 1-2: Varuna, Hira; Lane 3-18: doubled haploids of V x H; 19-20: Domo, BJ-1058 and 21-27: doubled haploids of Domo/BJ-1058. Lanes marked M are molecular weight marker**

basis of 44 bands lead to the formation of three groups, namely, VHI, VHII, and VHIII (Fig. I). Group VHI comprised four doubled haploids (VH 28, VH 47, VH 53 and VH 44) and two parents 'Varuna' and 'Hira'. VH 44 was closest genetically to 'Varuna' to the tune of 93 per cent. Similarly, 'Hira' and VH 28 comprised another sub cluster at 79 per cent similarity, whereas VH 44 and 'Varuna' exhibited 82 per cent genetic similarity with the sub cluster of VH 53 and VH 47. Overall, VHI was 73 per cent genetically similar to cluster VHII that comprised six doubled haploids viz., VH 5, VH 9, VH 37, VH 45, VH 46 and VH 41. VH 5 and VH 9 constituted sub cluster at 86.5 per cent similarity and joined sub cluster of VH 45 and VH 46 at 83 per cent similarity. Overall, the group VHII exhibited 74 per cent genetic similarity. On the whole group VHI and VHII exhibited 73 per cent genetic similarity among themselves. The third group/cluster VH III, comprised of six doubled

**Table 4.14** Number of scorable and polymorphic RAPD bands obtained in the PCR amplified DNA of doubled haploids of *B. juncea*

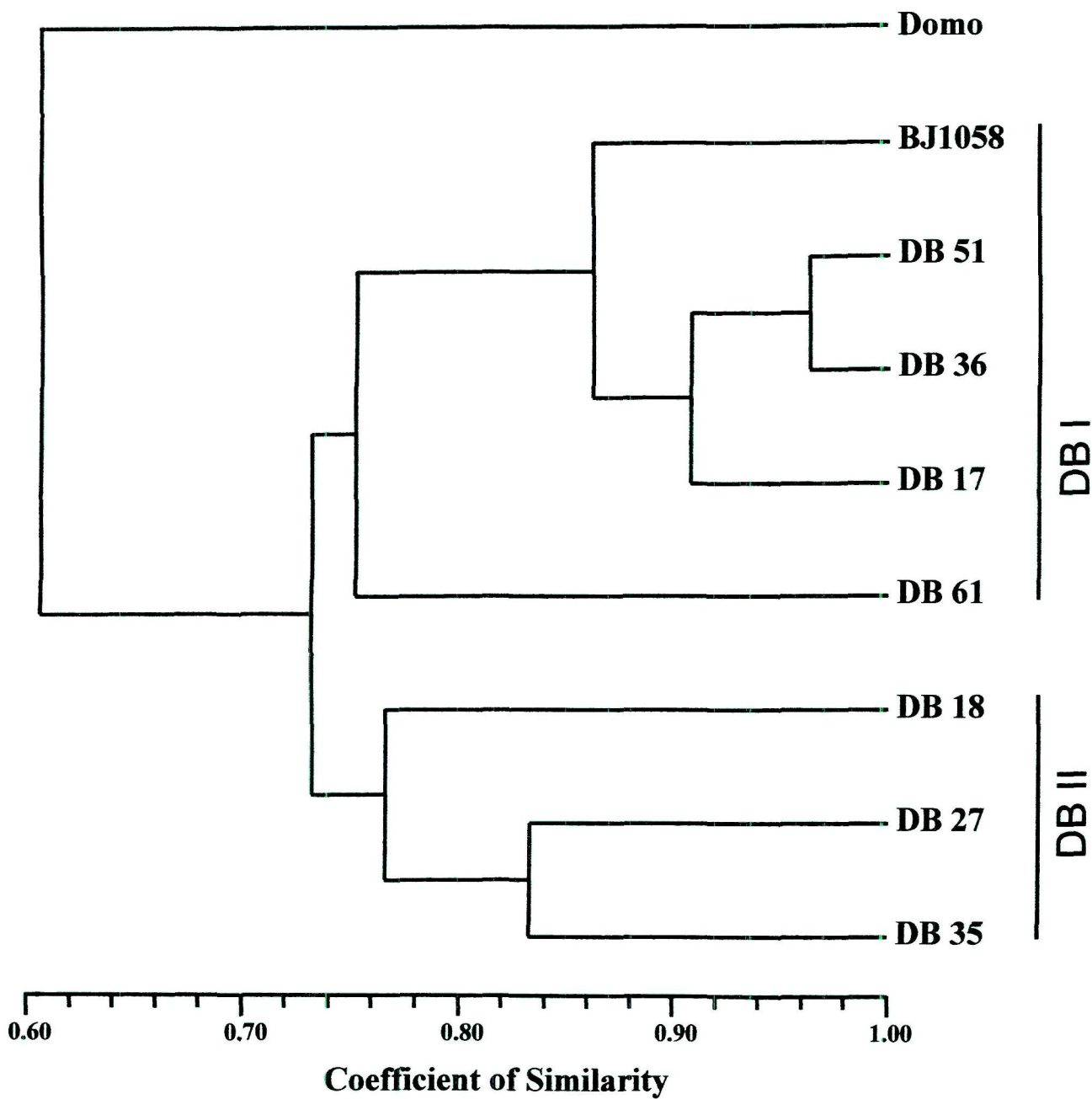
Primers	Scored bands		Polymorphic band	
	V x H	D x BJ	V x H	D x BJ
OPB11	4	3	4	2
OPD11	7	8	7	8
OPF01	7	6	4	4
OPF09	11	10	9	4
OPX05	8	6	5	2
OPX13	7	8	7	6
Total	44	41	36	26



**Fig. 1 Dendrogram of 16 doubled haploids (V x H) and the parents based on cluster analysis of RAPD data  
VH - Varuna x Hira derivatives**

haploids, viz., VH 67, VH 63, VH 6, VH 50, VH 58 and VH 55. VH 63 and VH 6 formed one sub cluster at 83 per cent genetic similarity. Similarly, VH 58 and VH 55 constituted another sub cluster at same genetic similarity and joined remaining lines at 71 per cent genetic similarity. VH 67 exhibited 78 per cent similarity with first sub cluster. On the whole, group VHIII was 71.5 per cent genetically similar. Overall, all three clusters viz., VHI, VHII and VHIII exhibited 69 per cent genetic similarity.

RAPD profiling of seven doubled haploids of D x BJ cross generated 41 bands of which 26 were polymorphic. The dendrogram generated through cluster analysis of 41 bands exhibited formation of two major clusters named DBI and DBII (Fig. II). The cluster DBI consisted of a main sub cluster having three DHs, viz., DB51, DB 36 and DB 17. The DHs DB 51 and DB 36 were 96 per cent similar and further exhibited 91 per cent genetic similarity with DB 17. Overall, the DHs of this sub cluster of DBI exhibited 86 per cent similarity with the parent 'BJ-1058'. Line DB 61 constituted another sub cluster of DBI and exhibited 75 per cent genetic similarity with other sub clusters. The cluster DBII comprised three DHs, viz., DB 18, DB 27 and DB 35. Of these, DB 27 and DB 35 exhibited 83 per cent genetic similarity and joined DB 18 at 76.5 per cent genetic similarity. On the whole, the two clusters had 73 per cent genetic similarity. On the basis of the primers screened in the present investigation, all the DHs of D x BJ including parent (BJ-1058) were approximately 60 per cent genetically similar to the other parent 'Domo' which did not cluster in the two main groups formed (DBI and II).



**Fig. II** Dendrogram of 7 doubled haploids (D x BJ) and the parents based on cluster analysis of RAPD data  
 DB – Domo x BJ-1058 derivatives

#### 4.4 White rust reaction of doubled haploids under field condition

Eleven DHs of the  $F_1$  of three different crosses viz., Varuna x Hira, Domo x BJ-1058 and Hira x Zem-1 alongwith their parents were screened for white rust reaction by artificially inoculating the plants under field conditions. The disease reaction was recorded on 0-6 scale and was converted into per cent disease (Plate VIII). Plants with less than 10 per cent disease index were rated as resistant while the plants with more than 10 per cent disease index were categorized as susceptible.

**Table 4.15** Per cent disease index and disease reaction of parents and their DHs of *B. juncea* evaluated for white rust under field conditions

Cultivar/DH	Per cent disease index	Reaction
Varuna	55.83	S
Hira	1.66	R
Domo	25.00	S
BJ-1058	37.50	S
Zem-1	30.00	S
VH 2	0.00	R
VH 3	6.66	R
VH 5	5.00	R
VH 6	8.33	R
VH 9	16.66	S
DB 1	11.66	S
DB 17	45.00	S
DB 35	49.16	S
DB 36	27.77	S
DB 47	16.66	S
HZ1	0.00	R

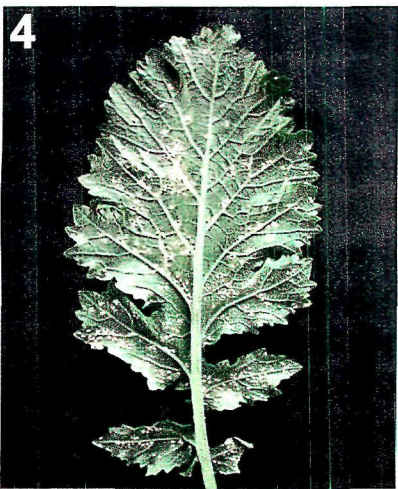
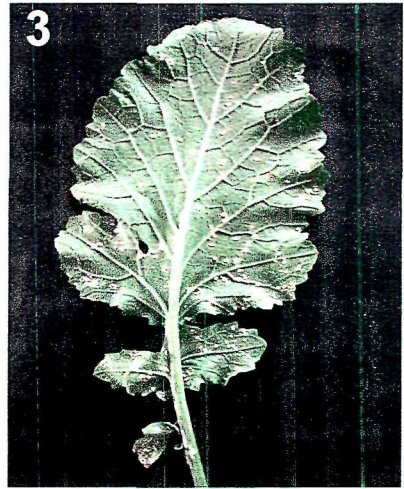


Plate VIII. White rust reaction on a scale of 0-6

Data on per cent disease index and disease reaction presented in Table 4.15 revealed that out of five parents screened against white rust, 'Hira' was found resistant and the others were susceptible to the white rust disease. Per cent disease index ranged from 0 (VH 2 and HZ 1) to 55.83 per cent (Varuna). Of the eleven DHs (five of V x H, five of D x BJ and one of H x Z) screened for the disease reaction, five were categorized to be resistant for the white rust and remaining six were susceptible. Five resistant DHs included four derived from cross V x H and one from H x Z. All the DHs of the cross D x BJ were found susceptible to white rust isolates prevalent in the region.

# ***D*iscussion**

## DISCUSSION

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Induced androgenesis was reported in *Datura innoxia* by Guha and Maheshwari (1964) for the first time and subsequently this technique has been extended to many crop plants of commercial importance in order to exploit advantages of this technique in generating haploids having multiple uses in genetics and breeding. In *Brassicas*, application of the anther technique was reported by Kameya and Hinata (1970) in *Brassica oleracea*. Further, with improvement in the protocols, induction of haploids through anther culture has been reported in *Brassica juncea* also (Yadav *et al.*, 1988; Goyal *et al.*, 1990; Roy and Saha, 1997; Singh and Sachan, 1999; Agarwal and Bhojwani, 1993; Purnima and Rawat, 1997).

Seed yield, oil quality and resistance against important prevalent diseases of the area, are some of the main breeding objectives of *B. juncea* crop improvement programmes. White rust caused by *Albugo candida* (Lev.) Kuntze, is one of the important disease of *B. juncea* in the area and deployment of resistant cultivars is the most effective and economical way of controlling white rust disease. However, transfer of white rust resistance alongwith other desirable traits including oil quality in the elite genetic background through conventional hybridization and selection requires long period of time (7-8 years) to obtain homozygous lines. By following induced androgenesis in  $F_1$ s, instant white rust resistant, high yielding and quality oil homozygous lines can be generated in a minimum possible time.

In the present study, anthers of  $F_1$ s of three crosses *viz.*, V x H, H x Z and D x BJ involving elite lines in combination with white rust resistance and quality oil donor parents were cultured *in vitro* on four media with four different sucrose concentrations to generate haploids. In a separate set of experiment, anthers of the same three crosses were also plated on 14 different PGR combinations with organic additives *viz.*, casein acid hydrolysate and activated charcoal to study their effect on androgenic haploid induction. Regenerated androgenic lines were characterized at the morphological and molecular level using RAPD markers and subsequently screened for reaction to white rust disease to identify white rust resistant promising lines. The results obtained on the various aspects of the investigations are discussed here under:

### **5.1 Generation of doubled haploids**

In the present study, media and sucrose levels had significant effect on callus induction whereas crosses (genotypes) exhibited non significant effects on androgenic callus induction frequency. All interactions *viz.*, sugar x media, sugar x crosses and media x crosses were significant whereas interactions amongst all three factors (media x sugar x crosses) had non significant effect on callus induction frequency (%). In other set of experiment, crosses, plant growth regulators and organic additives had significant effects on the callus induction frequency. Interactions involving crosses x plant growth regulators, plant growth regulators x organic additives and crosses x plant growth regulators x organic additives were also significant.

Maximum callus induction in cultured anthers of three  $F_1$ s *in vitro* was recorded on Keller's medium (42.28%) which was at par with  $N_6$  (42.10%) whereas MS exhibited better callus induction (34.90%) than  $B_5$  medium (30.61%). Plantlet regeneration frequency and the overall anther culture response was also maximum for the calli obtained on Keller's medium (11% and 14% sucrose) and  $B_5$  medium (11% and 14% sucrose). Yadav *et al.* (1988) and Roy and Saha (1997) have also reported successful androgenic callus induction on Keller's medium. Apart from Keller's medium, Gamborg ( $B_5$ ) and Murashige and Skoog (MS) media have also been successfully used to induce *in vitro* callusing/embryogenesis in cultured anthers of *Brassica juncea* by various workers (Goel *et al.*, 1990; Agarwal and Bhojwani, 1993; Prabhudesai and Bhaskaran, 1993; Singh and Sachan, 1999). Keller's medium is basically a modification of Gamborg's ( $B_5$ ) medium wherein concentration of macronutrient  $CaCl_2 \cdot 2H_2O$  is higher to the tune of 750 mg/l whereas in  $B_5$  medium it is 150 mg/l. Higher concentration of this compound might be promoting softening of anther wall tissues leading to more availability of microspores for callusing/embryogenesis.

Sucrose was used as carbohydrate source in four different concentrations *viz.*, 5, 8, 11 and 14 per cent in the present investigation. Maximum mean callusing was recorded on 5 per cent sucrose level and it reduced in the media with increasing sucrose concentrations. However, regeneration and anther culture response was higher in case of calli obtained on 11 per cent sucrose media as compared to the ones induced on

lower sucrose media. Use of higher sucrose (13%) has been reported to be more effective in achieving better anther culture response in *B. juncea* (Agarwal and Bhojwani, 1993; Purnima and Rawat, 1997; Singh and Sachan, 1999; Lionneton *et al.*, 2001; Prem *et al.*, 2004). Roy and Saha (1997) also achieved maximum callusing in anthers of *B. juncea* plated on a medium with 12 per cent sucrose whereas for regeneration, medium containing four per cent sucrose concentration has been reported to be used. However, contrary to present and other aforementioned findings, Goyal *et al.* (1990) has reported no significant effect of sucrose concentration in the medium on callus proliferation as well as embryogenesis. Generally, high sucrose concentration apart from providing energy as carbon source, also acts as an osmoticum in the medium. Sucrose not only specifically induces the division of pollen cells, but also inhibits the growth of anther wall and other somatic tissues including filament (Keller *et al.*, 1975). Particularly at lower levels of sucrose, chances of calli proliferation from the somatic tissues specifically from anther wall tissues are more and can subsequently increase the overall callusing frequency whereas on the other hand, at higher sucrose contents, chances of the proliferation of calli from somatic tissues are minimized that can reduce overall callusing frequency.

Under present investigation, three PGRs *viz.*, 2,4-D, NAA and TIBA were used in different concentrations and combinations with and without organic additives to induce callusing and/or embryogenesis. Maximum callus induction frequency was observed on K<sub>12</sub> medium (Keller's medium + 2 mg

2,4-D + 2 mg NAA and 11% sucrose per litre). No response was obtained in anthers plated on control (basal medium) and medium containing TIBA (0.1 mg/l and 0.2 mg/l). Regeneration frequency and overall anthers culture response was recorded maximum for the calli induced on K<sub>6</sub> medium (Keller's medium + 0.2 mg 2,4-D + 0.2 mg NAA and 11% sucrose per litre). Roy and Saha (1997) have also reported highest percentage of callus induction on a medium with 2 mg/l 2,4-D and NAA each. However, Prem *et al.* (2005) have reported antagonistic effect of NAA and BA for microspore embryogenesis of *Brassica juncea*. Although callusing increased with the increase in the concentration of growth regulators (2,4-D and NAA), plantlet regeneration and overall anther culture response was less / negligible at higher doses of the growth regulators. Being strong auxins, 2,4-D and NAA may hinder organ/embryo regeneration in callus cultures at such higher doses (Roy and Saha, 1997).

Two organic additives tested *viz.*, casein acid hydrolysate and activated charcoal did not exhibit any synergistic effect on callusing as well as overall anther culture response. Maximum callusing was observed on the medium with PGRs but devoid of these organic additives, followed by medium containing casein acid hydrolysate and activated charcoal. Prem *et al.* (2005) have also reported that activated charcoal may not be suitable for achieving embryoid induction in microspores of *Brassica juncea*. As far as effects of these additives on callusing are concerned, no report is available in literature specifically on *Brassica juncea*. Low callusing response with use of activated charcoal in the present study might have been due to its

antagonistic effect as charcoal is known to bind with other constituents in the medium. In the present study also, it might be inhibiting the activity of growth hormones by binding to them and making them unavailable for activity.

In this study, both haploids as well as diploid plants were found in the regenerated plant population. From a total of 67 regenerated plants, 18 were found haploid (26.86%). Meiotic and mitotic analysis confirmed the presence of haploids as well as diploid plants in regenerated population of androgenic plants. Further, the morphological observations on regenerated plants for siliqua/seed set, to infer the ploidy status, confirmed the presence of sterile and fertile plants in the population. Goyal *et al.*, (1990) have also reported regeneration of mixed ploidy status androgenic plants in *B. juncea*. George and Rao (1982) obtained 46 per cent haploids in regenerated androgenic plants through anther culture of *B. juncea* whereas Roy and Saha (1997) reported 30-35 per cent haploid plants in regenerants from microspore callus of *B. juncea*. However, Prem *et al.* (2004) reported low (0-12%) recovery of spontaneous diploids in the population of microspore derived plants. Sharma and Bhojwani (1985) and Prabhudesai and Bhaskaran (1993) have also reported all haploid plants in their microspore culture derived population in mustard.

## **5.2 Characterization of doubled haploids**

### **5.2.1 Agro-morphological evaluation**

Androgenic plant progenies of the  $F_1$ s are expected to represent genetic recombination in form of different possible combinations of both the parental traits. Therefore, the androgenic population of different  $F_1$ s was

evaluated using agro-morphological traits and molecular markers, in order to select those combining the desirable traits of both the parents. Parents as well as the DHs ( $R_0$  and  $R_1$ ) were evaluated for six agro-morphological traits of economic importance under present investigation. Of 39 plants evaluated in  $R_0$  generation, eight were derived from V x H cross and 31 were derived from D x BJ cross. In case of eight plants ( $R_0$ ) of V x H cross, three DHs exhibited seed yield close to better parent (Varuna) in  $R_0$  generation. For pods per plant (6 to 152) and branches/plant (3 to 15) greater variation was evident amongst DH lines whereas there was sufficient variation for plant height of DHs in  $R_0$  generation. On the other hand, seed yield in case of DHs of D x BJ cross was in the range of 1.19 g to 12.47 g and number of pods per plant ranged from 60 to 407. Plant height ranged from 40 cm to a maximum of 210 cm in the DHs. Goyal *et al.* (1990) and Agarwal and Bhojwani (2004) have also reported ample variation in  $R_0$  generation of pollen plants derived from anther/pollen culture of *Brassica juncea*. However, much inference can not be drawn from the single plant data obtained from  $R_0$  generation because  $R_0$  generation also represents the variation arising due to various effects and/or disturbances that might occur during *in vitro* culture and subsequently during their hardening and establishment in the greenhouse (Goyal *et al.*, 1990).

In present study, seed progenies of eleven  $R_0$  plants were grown in single rows for further evaluation in  $R_1$  generation under field conditions. One DH of V x H cross yielded more than better parent 'Varuna' whereas for

the remaining, lesser yields than better parent (Varuna) were recorded. However, yield in all these DHs was more than the other parent 'Hira'. In  $R_1$  generation, pods per plant ranged from 76 to a maximum of 132.4. Branches per plant ranged from 4.6 to a maximum of 6.2 branches per plant. Two DH lines were taller and rest of lines had a comparable height with dwarfer parent 'Varuna'. In case of DHs of D x BJ cross, seed yield ranged from 1.03 g to a maximum of 3.85 g which was less than both of the parents. Pods per plant ranged from 26 to 76 which was also less than both of the parents. In case of branches per plant, it ranged from 1.8 to 4.4 which was less than the better parent 'Domo'. Four, out of five DHs of D x BJ cross, were taller than both of the parents. However, in case of HZ1 (derivative of third cross H x Z) yield was more than both of the parents with intermediate pods per plant. HZ1 has more branches per plant as compared to both of the parents and it was dwarfer compared with both parents. Overall, in all the three crosses, lines superior, intermediate and inferior to the parents have been observed for almost all the traits studied. In case of V x H cross, all DHs regenerated were having seed yield more than the inferior parent 'Hira' with one DH line outyielding the better parent 'Varuna'. Of the five DHs of D x BJ cross, all were inferior to both of parents compared for yield and pods per plant. The DH line HZ 1 was superior in yield to both parents and had intermediate pods per plant. In literature, sufficient variation has been reported among androgenic lines in the advanced generations ( $R_1$ ,  $R_2$  etc.) for most of the

traits studied including plant height, days to maturity, seed yield, oil content and fatty acid composition (George *et al.*, 1987; Abraham *et al.*, 1988; Tiwari *et al.*, 1988a; Adamska *et al.*, 2001; Burbulis *et al.*, 2001; Agarwal and Bhojwani, 2004; Lionneton *et al.*, 2004). George *et al.* (1987) reported seven DH lines statistically at par with parents and 11 inferior to parents. Similarly, Agarwal and Bhojwani (2004) also reported two lines shorter (18-20%) than control plants and three lines showing 27-31 per cent higher yield than the parent cultivars. However, agro-morphological traits are not enough and completely reliable to understand overall contribution of the parental genomes towards the genotypes of the androgenic plants due to limited number of characters evaluated. Limited agro-morphological traits studied represent only a very small fraction of the genome and may also be highly influenced by environmental factors including photoperiod, light intensity, temperature, growing season and nutritional status. Therefore, such characterization could be misleading, non-reliable and can better be supplemented with molecular tools.

### **5.2.2 Characterization using molecular markers (RAPD)**

Many of the complications of agro-morphological evaluation can be overcome by characterization of genotypes using molecular markers which resolve whole of the genome provided sufficient markers are used for the purpose. Of the various PCR based markers, RAPD is most commonly used method for discovering polymorphism which is randomly distributed throughout the genome (Williams *et al.*, 1990).

In the present investigation, 23 anther culture derived plants of intervarietal crosses of Varuna x Hira and Domo x BJ-1058 were characterized using six 10-mer random primers. RAPD profiles revealed a total of 44 and 41 bands for V x H and D x BJ cross, of which 36 and 26 bands were polymorphic, respectively. The dendrogram of 16 DHs of V x H alongwith parents revealed formation of three distinct groups VHI, VHII and VHIII. VHI and VHII groups were 73 per cent genetically similar whereas VHIII was 71.5 per cent genetically similar. On the whole, all three groups exhibited 69 per cent genetic similarity. Group VHI comprised four DHs and two parents viz., Varuna and Hira. The other two groups VHII and VHIII comprised six doubled haploids each. VH 44 was 93 per cent genetically similar to parent 'Varuna' whereas VH 53 and VH 47 exhibited 82 per cent genetic similarity with sub-cluster of VH 44 and Varuna. Similarly, Hira and VH 28 comprised another sub-cluster at 79 per cent similarity. On the other hand, dendrogram of seven doubled haploids of D x BJ cross along with parents formed two groups, DB I and DB II. Three DHs of main sub-cluster of DB I group exhibited 86 per cent similarity with parent BJ-1058 while line of the sub-cluster comprised of single DH 'DB 61' joined at 75 per cent similarity with main cluster. The cluster DB II comprised three DHs of which two were 83 per cent genetically similar and joined with third at 76.5 per cent similarity. All the DHs and parent 'BJ-1058' were approximately 60 per cent genetically similar to the other parent 'Domo'. Morphologically, all the DHs were inferior to both of the parents. Two DHs viz., DB 35 and DB 36 had

yield closer to both parents but had less number of pods as well as branches. Both were taller than the parents and clustered in different groups on the basis of molecular characterization. Therefore, apparently there was not much agreement in two characterizations based on agro-morphological traits and molecular markers. This can be due to environmental effects on agro-morphological traits and limited number of markers studied for molecular characterization under the present investigation.

### **5.3 White rust reaction of doubled haploids**

Eleven doubled haploid lines along with parents were screened for white rust under field conditions. Among parents, only 'Hira' was found resistant whereas five out of eleven DH lines were categorized as the white rust resistant. Of five resistant lines, four were the derivatives of V x H cross and one of H x Z cross whereas none of the DHs derived from D x BJ cross was found resistant to white rust. In literature, as such there is no report available on screening of the DHs at field level for white rust in *Brassica juncea*. However, on the basis of screening of germplasm, a number of resistant genotypes and/or cultivars have been reported in literature (Kolte, 1986; Bhardwaj and Sud, 1993; Khetmalas *et al.*, 1994; Velazhahan and Thiyagarajan, 1994; Yadav *et al.*, 1999). Anther/microspore culture derived doubled haploids lines of *Brassica juncea* have also been used to identify the markers linked to white rust and/or to develop the linkage maps (Cheung *et al.*, 1998; Prabhu *et al.*, 1998; Varshney *et al.*, 2004). Cheung *et al.* (1998) used 119 F<sub>1</sub> derived doubled haploid lines to develop RFLP markers linked

to white rust gene (*Acr*). Prabhu *et al.* (1998) also identified RAPD markers linked to white rust resistance using an  $F_1$  derived doubled haploid population of *B. juncea*. Similarly, Varshney *et al.* (2004) used RAPD markers for mapping of locus *AC2(t)* using doubled haploid mapping population originating from a cross between an exotic resistant accession of mustard (BEC-144) and Varuna.

The DH lines developed during the present investigation can be supplemented with more such lines to have sufficient size for mapping work. Besides, lines exhibiting resistance to white rust along with other desirable economic traits can be subjected to further field evaluations in order to identify potential ones that can be directly used as cultivars and/or involved in future breeding programmes.

***S*ummary**

## SUMMARY

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The present investigation was undertaken to generate doubled haploids (DHs) of *Brassica juncea* through anther culture as well as to characterize regenerated DHs along with parents for agro-morphological traits and using Random Amplified Polymorphism DNA (RAPD) markers, and to identify white rust resistant doubled haploids.

Anthers of three crosses, viz., V x H, H x Z and D x BJ were cultured on four different media (Keller, B<sub>5</sub>, N<sub>6</sub> and MS) and four sucrose levels viz., 5, 8, 11 and 14 per cent, to induce callusing in first set of experiment. In the second set of experiment, Keller's medium with 14 PGR combinations and one control along with two organic additives, casein acid hydrolysate and activated charcoal were also used to induce callusing. The calli obtained from callusing media were transferred to regeneration medium [B<sub>5</sub> + NAA (0.2mg/l) + Kinetin (2mg/l) + Sucrose (2%)] and subsequently the shoots were transferred to rooting medium [ $\frac{1}{2}$  MS + IAA (2mg/l) + sucrose (2%)]. Regenerated green plantlets having shoots and well developed roots were hardened for 15-20 days at room temperature and then transferred to big pots which were kept in the cage house till maturity.

Data on six agro-morphological traits were recorded in R<sub>0</sub> generation on all regenerated plants. Eleven DH lines (R<sub>1</sub>) along with parents were also characterized for six agro-morphological traits under field

conditions at SAREC, Kangra during *rabi* 2005-06. Total genomic DNA of parents along with 23 DHs was amplified using six 10-mer random primers (OPB 11, OPD11, OPF01, OPF09, OPX05 and OPX13) and band data was used to generate dendrogram using cluster analysis. Besides, eleven DH lines were also screened for white rust under field conditions. Lines were inoculated by spraying the zoospore suspension upto run off and scored after 50 days for their reaction against white rust as per the scale (0-6) given by Conn *et al.* (1990).

Of the four media tested, all the three crosses recorded highest mean callusing on Keller's medium which was at par with N<sub>6</sub> medium whereas MS exhibited better callusing than B<sub>5</sub>. However, in the four sugar levels tested, crosses exhibited maximum callus induction frequency on 5 per cent sucrose and an inverse relation with callus induction frequency was observed as increasing sugar concentration lead to progressive decrease in overall callusing. But regeneration frequency and anther culture response was more in the calli obtained on Keller's medium with 11 per cent sucrose followed by calli induced on B<sub>5</sub> medium with 11 per cent sucrose. Generally, regeneration frequency and overall anther culture response was more in calli obtained on 11 per cent sucrose for all the media tested, except on MS medium.

Fourteen combinations of PGR tested for the callus induction frequency revealed highest mean callusing on K<sub>12</sub> medium [Keller medium + 2,4-D (2 mg/l) + NAA (2mg/l) + sucrose (11%)]. Two organic additives tested did not exhibit any synergistic effect on callusing and overall anther culture

response as maximum callusing was observed in the anthers plated on control (without any organic additives) followed by medium containing casein acid hydrolysate. Callusing was very less on the medium containing activated charcoal. Overall regeneration frequency and the anther culture response was best for the calli obtained on K<sub>6</sub> medium without any organic additive [Keller's medium + 2,4-D (0.2 mg/l) + NAA (0.2 mg/l) + 11 per cent sucrose]. However, in case of medium containing organic additive casein acid hydrolysate, it was best for calli induced on K<sub>6</sub> medium [Keller's medium + 2,4-D (0.2 mg/l) + NAA (0.2 mg/l) + casein acid hydrolysate (0.5%) + 11 per cent sucrose].

Ploidy status of the regenerated plants assessed both by mitotic and meiotic analysis of root tips and floral buds, respectively as well as on the basis of morphological observations on the fertility status revealed that seven of 20 plants selected randomly for mitotic and meiotic analysis were haploids and subsequently exhibited sterility also. Overall 67 plants survived and flowered, of which 18 were sterile exhibiting 26.86 per cent sterility/haploid's frequency.

DHs of *B. juncea* regenerated, were characterized for six morphological traits. Eleven DH lines evaluated in field at Kangra revealed sufficient variation in yield. One DH line of V x H cross (VH 3) yielded more than better parent 'Varuna' whereas rest of lines had less yield than better parent (Varuna), but more than the other parent 'Hira'. There was ample variation for pods per plant as it ranged from 76 to a high of 132.4. However, overall pods per plant were less than better parent. Two DH lines (VH3 and VH9) were taller whereas rest had comparable height with dwarfer parent

'Varuna'. On the other hand, DHs of D x BJ cross had lesser yield than both parents, except two DHs (DB35 and DB36) that yielded closer to parents. Pods per plant ranged from 26 to 76 that was less than both of the parents. Four DH lines of D x BJ cross (DB1, DB35, DB36 and DB47) were taller than both of the parents. Single regenerated DH of the third cross, H x Z, had the superiority over the both of the parents *viz.*, Hira and Zem-1 for the important economic traits.

RAPD based DNA fingerprinting of 23 DHs and their parents with six primers revealed 44 and 41 bands for V x H and D x BJ DHs, out of which 36 and 26 were polymorphic, respectively. The dendrogram generated from band data of the parents and DHs of V x H cross, lead to the formation of 3 main clusters/groups VH I, VH II and VH III. VH I cluster was comprised of four DHs and two parents i.e. Varuna and Hira and it had overall 73 per cent genetic similarity. The group VH II comprised six DHs. The third group VH III comprised of six DHs and was 71.5 per cent genetically similar. Overall, all the three clusters *viz.*, VH I, VH II and VH III exhibited 69 per cent genetic similarity among themselves. The dendrogram generated from parents and seven DHs of F<sub>1</sub> of D x BJ cross formed two groups i.e. DB I and DB II. The cluster DB I was 86 per cent genetically similar and comprised three DHs and parent BJ-1058. The other sub-cluster of DB I comprised single DH that joined main cluster at 75 per cent similarity. The second cluster DB II exhibited 76.5 per cent genetic similarity and was comprised of three DHs. On the whole, all the DHs of D x BJ including parent BJ-1058 were approximately 60 per cent genetically similar to the other parent 'Domo'.

Among five parents screened for white rust, only 'Hira' was found resistant to white rust under field conditions at Kangra. Screening of DHs for white rust in field along with parents revealed five lines resistant against white rust. Among five resistant DHs, four DHs were the derivatives of V x H cross (VH2, VH 3, VH 5 and VH 6) and one was the derivative of H x Z cross (HZ 1). All DHs of D x BJ cross were found susceptible to white rust isolate prevalent in the region.

## **CONCLUSION**

From aforementioned results and discussion, it can be concluded that Keller's medium with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 11 per cent sucrose is best for overall anther culture response in *B. juncea*. Organic additives tested did not promote anther culture response in the present case. Regenerated doubled haploids exhibited variable ploidy level and sufficient variation for agro-morphological traits also. Five (VH 2, VH 3, VH 5, VH 6 and HZ 1) out of the eleven DH lines screened were identified resistant to white rust, out of which VH 3 and HZ 1 also recorded promising yield on the basis of five plant data. Few more such lines are expected among DHs that are still in the R<sub>0</sub> generation and will be evaluated later. All such stable white rust resistant lines can be further evaluated in big plots for yield and their quality traits to identify future potential commercial genotypes and/or potential germplasm for hybridization programmes.

***L*iterature  
*C*ited**

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# ***Appendices***

**Appendix-I : Effect of media, sugar levels and crosses (genotypes) on callus induction frequency (%) in anther culture of intervarietal F<sub>1</sub>s of *B. juncea***

Crosses	Media	Sugar levels (%)				Mean
		5	8	11	14	
V x H	Keller	49.63 (44.76)	56.46 (48.72)	40.28 (39.35)	29.65 (31.81)	<b>44.00</b> <b>(41.16)</b>
	MS	32.79 (34.83)	41.23 (39.91)	25.45 (30.11)	22.00 (27.66)	<b>30.36</b> <b>(33.12)</b>
	N <sub>6</sub>	44.59 (41.86)	26.30 (30.66)	37.32 (37.48)	40.43 (39.38)	<b>37.16</b> <b>(37.34)</b>
	B <sub>5</sub>	33.90 (35.52)	39.37 (38.92)	27.58 (31.33)	22.29 (28.09)	<b>30.91</b> <b>(33.46)</b>
	<b>Mean</b>	<b>40.25</b> <b>(39.24)</b>	<b>40.96</b> <b>(39.55)</b>	<b>32.65</b> <b>(34.56)</b>	<b>28.59</b> <b>(31.73)</b>	<b>35.60</b> <b>(36.27)</b>
H x Z	Keller	54.82 (47.76)	50.34 (45.10)	32.27 (34.55)	9.63 (18.03)	<b>36.76</b> <b>(36.36)</b>
	MS	47.31 (43.43)	42.73 (40.71)	39.30 (38.53)	21.78 (27.62)	<b>37.78</b> <b>(37.57)</b>
	N <sub>6</sub>	64.02 (53.17)	40.03 (39.15)	35.27 (35.98)	29.93 (33.10)	<b>42.31</b> <b>(40.35)</b>
	B <sub>5</sub>	40.85 (39.69)	37.39 (37.64)	37.44 (37.35)	10.93 (19.27)	<b>31.65</b> <b>(33.48)</b>
	<b>Mean</b>	<b>51.75</b> <b>(46.01)</b>	<b>42.62</b> <b>(40.65)</b>	<b>37.07</b> <b>(36.60)</b>	<b>18.06</b> <b>(24.50)</b>	<b>37.12</b> <b>(36.94)</b>
D x BJ	Keller	66.56 (54.72)	53.93 (47.28)	37.52 (37.65)	26.24 (30.65)	<b>46.06</b> <b>(42.57)</b>
	MS	37.97 (38.01)	45.42 (42.33)	34.09 (35.56)	28.80 (32.40)	<b>36.57</b> <b>(37.07)</b>
	N <sub>6</sub>	44.11 (41.57)	57.35 (49.23)	52.19 (46.23)	33.71 (35.47)	<b>46.84</b> <b>(43.12)</b>
	B <sub>5</sub>	29.97 (33.09)	34.95 (36.19)	33.66 (35.34)	18.59 (25.51)	<b>29.29</b> <b>(32.53)</b>
	<b>Mean</b>	<b>44.65</b> <b>(41.84)</b>	<b>47.91</b> <b>(43.75)</b>	<b>39.36</b> <b>(38.69)</b>	<b>26.86</b> <b>(38.82)</b>	<b>39.69</b> <b>(38.82)</b>

Values in parentheses are arc sine transformed values

V x H – Varuna x Hira, H x Z – Hira x Zem-1, D x BJ – Domo x BJ-1058

**Appendix II : Performance of parents and their DHs ( $R_0$ ) of *B. juncea* for various agro-morphological traits studied under cage house conditions at Palampur**

Plant	Plant height (cm)	Branches/ plant	Pods/ plant	Pod length (cm)	Seeds/ pod	Seed yield/ plant (g)
Varuna	174	5	138	5.18	14.0	4.14
Hira	194	2	94	4.68	12.3	2.22
Domo	168	6	108	5.50	12.4	3.86
BJ-1058	168	4	132	5.54	14.1	3.99
Zem-1	165	4	109	4.77	10.1	3.20
VH 41	88	4	90	3.33	5.4	4.80
VH 44	85	6	45	4.68	6.9	1.35
VH 46	58	8	38	3.01	3.4	0.48
VH 47	57	9	92	3.43	4.3	3.59
VH 50	62	15	152	4.13	8.7	4.27
VH 53	92	11	98	4.56	10.0	2.61
VH 55	55	6	75	3.83	7.3	2.23
VH 110	24	3	6	2.50	2.2	0.03
DB 27	150	12	407	4.65	9.7	9.12
DB 35	135	15	258	4.16	12.3	7.10
DB 36	91	8	105	3.92	6.6	1.98
DB 47	112	8	203	4.13	9.0	4.76
DB 50	73	7	134	4.19	7.6	4.59
DB 51	70	7	147	3.84	8.4	3.08
DB 53	40	5	70	3.45	4.7	1.19
DB 54	147	7	90	3.80	6.5	3.86
DB 55	76	7	60	4.81	10.2	2.51
DB 56	80	5	110	2.49	4.1	3.30
DB 58	100	16	260	4.60	10.8	7.01
DB 59	95	11	185	4.05	4.9	6.94
DB 60	121	5	75	2.31	2.9	2.30
DB 61	81	12	203	4.37	8.3	12.47
DB 65	142	8	128	3.68	5.9	2.69
DB 69	97	7	85	4.65	5.2	1.34
DB 71	95	6	67	4.55	8.0	2.78
DB 72	148	7	180	3.67	6.2	4.55
DB 74	67	6	75	4.92	8.5	2.04
DB 77	110	13	220	3.98	5.3	7.22
DB 78	178	8	155	4.74	7.8	5.18
DB 83	110	10	197	6.07	13.5	5.44
DB 88	128	8	105	4.81	8.2	6.32
DB 89	135	7	130	3.77	9.5	5.09
DB 90	210	5	110	5.04	8.7	2.54
DB 91	104	6	115	4.34	6.2	4.17
DB 92	113	6	98	4.06	6.7	3.97
DB 93	124	11	210	5.12	9.6	7.90
DB 94	110	5	75	5.12	10.7	1.77
DB 97	117	8	127	3.74	6.6	5.92
DB 98	114	6	80	4.88	10.3	4.18