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Development of Alternaria blight resistant lines through interspecific hybridization between Indian mustard (*Brassica juncea* L.) and white mustard (*Brassica alba*) through embryo rescue

Ram C Yadav[1*], Dhiraj Singh[2], Asha Rani[1], Ajit Singh Rathi[2], Rajesh Arya[2], Rajinder Singh[2], Neelam R Yadav[1], Amit Singh[2] and Ramavtar[2]

[1]*Department of Molecular Biology, Biotechnology & Bioinformatics, CCS Haryana Agricultural University, Hisar 125004, Haryana, India*
[2]*Department of Genetics & Plant Breeding, CCS Haryana Agricultural University, Hisar 125004, Haryana, India*
**Corresponding author: rcyadavbiotech@yahoo.com*
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Abstract

Wide hybridization is an important technique for incorporation of desirable characters among related species of plants. In the present study, interspecific hybridization was carried out through *in vitro* culture of ovules from the crosses between *Brassica juncea* (cv. RH30, RH8812, RH270 and RH345) and *B. alba* for purpose of transferring Alternaria blight resistance from *B. alba* to *B. juncea.* Ovules were excised from siliquae and cultured on seven different MS modified media. In all crosses, the hybrid siliquae were shorter in length than their parent plants due to interspecific hybridization barriers. Pod setting in hybrid plants ranged from 39 to 48% and number of ovules per pod were also very few. Best germination response was observed in ovules used from 20 DAP on basal medium supplemented with Kin (2.5 mg/l) and CH (0.5 g/l) (for cv. RH30 x *B. alba*) and MS + BAP (2.5 mg/l) + CH (0.5 mg/l) (for other crosses). Six rooting media were tried for root formation in regenerated shoots. Maximum rooting response was on MS medium supplemented with IAA (0.5 mg/l) in all hybrids. The regenerated hybrid plants were transferred to a mixture of sand: soil in 1:1 ratio in pots and about 70 percent hybrid plants for all the crosses were survived. Successful hybrid plants were grown in field upto F_7 generations. Advanced generation progenies were also successfully confirmed using molecular marker analyses for Alternaria blight resistance character. In advanced generation, the hybrid plant's characteristics were comparable to their Indian mustard parent plants and they were highly resistant to Alternaria blight disease.

Key words: *Brassica juncea, brassica alba, embryo rescue, interspecific hybridization*

Introduction

Brassica belongs to family *Brassicaceae* and it is the third most important oilseed crops of the world after soybean and palm. The major rapeseed growing countries are India, China, Canada, and Germany. India is third largest rapeseed-mustard producing country with global contribution of 28.3% area and 19.8% production (Kumar *et al*., 2014). In India, four important oleiferous *Brassica* species (*B. juncea, B. napus, B. rapa and B. carinata*) are cultivated in about 6.45 million hectare area and produce 7.28 million tons (Mustard seed survey report, 2014-2015). They are winter season crop from September – October to February – March. The genus *Brassica* includes a number of important crop species which are used for various purposes e.g. as oilseed, vegetables, fodder & condiments.

Indian mustard (*B. juncea*) (2n=36) is one of the most important species in genus Brassica grown in tropical and subtropical region of the world. It yields important edible oil and also serves as raw material for industry & trade. *B. juncea* is an amphidiploid (AABB), apparently originated in Asia by hybridization between *B. rapa* (AA) and *B. nigra* (BB). Fungal diseases are the main biotic stresses which play a prominent role in reducing the yield of *B. juncea*. Alternaria blight (fungal disease) is caused by *A. brassicae* which is a major causal organism for Alternaria blight disease, results in 30-50% yield loss alone in the country (Meena *et al*., 2010; Saharan *et al*., 2016).

The family *Brassicaceae* consists of a large number of wild and weedy species which are an excellent reservoir of useful genes (Yao *et al*., 2010). *B. alba* (2n=24) is a weedy plant possesses resistance to Alternaria blight pathogen due to presence of relatively more epicuticular wax and higher concentration of phenolics in leaves than *B. juncea* (Aneja and Agnihotri, 2013; Kumar *et al.*, 2014). In spite of recent advances in applications of genetic engineering and molecular biology techniques for plant

breeding, interspecific hybridization still plays a crucial role in crop improvement. It is a simple and most direct method for the introgression of the valuable traits from wild species into cultivated crops for generating new ecotypes or species, broadening the genetic basis of crops (Malek, 2007; Yao *et al*., 2010).Though many investigations on hybridization based process have found that, the frequency of interspecific hybrids was very low by using conventional method of breeding since the crosses were incompatible (Kumar *et al*., 2001). However, by using various biotechnological approaches, especially the *in vitro* culture & embryo rescue, such interspecific crosses can be achieved in *Brassica* (Mohapatra & Bajaj, 1987; Kumar *et al*., 2001; Malek, 2007; Yadav *et al*., 2007). Embryo rescue technique was useful in obtaining wide hybrids where hybrid embryos abort in early stages of development. It was first used in *Brassica* by Nishi *et al*. (1959) for obtaining higher seed set. Ovary, ovule, and embryo culture are commonly used as embryo rescue techniques in interspecific crosses. Several investigators compared the efficiency of these techniques for the production of various interspecific hybrids in brassica and observed that ovule culture was superior to other techniques (Bennett *et al*., 2008; Wang *et al*., 2009). Therefore, ovule culture embryo rescue technique was used during the present work. Establishment of a culture system of ovules excised at the early stage of zygote development will make it possible to produce hybrids in cross combinations in which embryo aborts in the very early development stage. The present study reports the interspecific crossability between *B. juncea* and *B. alba*, to rescue the interspecific hybrid embryos through tissue culture, their performance in advanced generations and evidences for its successful establishment through molecular marker (resistance gene analog markers) assisted analyses. Recently, resistance gene analog markers (RGA) have been used to tag disease resistance genes in plants. These locus-specific markers may provide useful information in studying the genome of various species.

Materials and Methods

Plant materials and pollination

Four cultivars of *B. juncea* cv. RH8812, RH30, RH0270, RH0345 and *B. alba* were used in the interspecific hybridization programme. These were sown in the last week of September and took 2-3 months to flower. The crossing was done in the month of December–January. In all cultivars of *B. juncea*, emasculation was carried out a day before pollination & covered with paper bags. The emasculated buds were pollinated in between 10-12 a.m. with freshly collected pollens from *B. alba* & again tightly covered with paper bags.

Embryo rescue

The ovule culture technique was applied for rescue of the hybrid embryos and was performed in a laminar air flow hood under aseptic conditions. Swollen siliquae were excised 10-20 DAP (days after pollination) from the plants. Siliquae were first surface sterilized with teepol & then with mercuric chloride (0.1%) for 5-8 minutes followed by three washings with sterilized distilled water in a laminar hood. Sterilized siliquae were dissected aseptically using a sterile surgical blade, exposing developing ovules which were excised and counted. Ovules from 15-20 days after pollination were used as explants for embryo rescue from all the crosses. Excised developing ovules were cultured on modified MS media supplemented with different growth regulators (Table 2). All the cultures were incubated at 25±1°C under 16 h/ 8 h light/ dark photoperiod. After 20-30 days of inoculation, percent germination of ovules was observed. After then the germinated ovules were transferred to fresh basal medium for shoot elongation.

Plant regeneration and acclimatization

After three to four weeks, elongated shoots were transferred to MS modified media containing auxin for root formation e.g. MS + NAA (0.5mg/l), MS + NAA (1 mg/l), MS + IAA (0.5 mg/l), MS + IAA (1 mg/l), MS basal and MS half basal. Percent root response was observed. For hardening, the rooted plants were washed to remove the agar and transferred to small plastic bags/pots containing sterilized sand: soil (1:1) mixture. Well established interspecific plants were moved to the green house. Interspecific hybrids were maintained and transferred to field conditions in the Department of Genetics and Plant Breeding for advancement of generations (upto F_7 generations).

Statistical analysis

The means and standard errors were worked out using OPSTAT from replicate data (in percentages) obtained from various experiments. Each replicate consisted of a petri plate with five to seven immature ovules. For analysis of variance, the percentage data was transferred using Arc sine transformation to bring normal distribution.

Screening under field condition

Advanced F_7 progenies were screened for their resistance against Alternaria blight with artificial inoculation conditions in the field. These were spray inoculated with pure culture of *A. brassicae* (10^5 conidial suspension/ml distilled water) at initiation of flowering and siliquae development stage.

Table 1: Comparison between parent plants siliquae and their hybrids

Sr. No.	*B. alba* (cm)	RH30 (cm)	RH30 x *B. alba* (cm)	RH8812 (cm)	RH8812 x *B.alba* (cm)	RH270 (cm)	RH270x *B.alba* (cm)	RH345 (cm)	RH345 x *B.alba* (cm)
1	2	4.3	2.5	5.1	2.9	6.2	3.1	5	2.8
2	2.1	4.4	1.2	5.5	3.3	6.9	2.9	4.5	2.5
3	2.2	4.1	1.8	5.7	3.4	6.5	3.3	5.1	3.0
4	1.9	4.2	3	5.3	3.1	7.1	3.4	5.5	2.2
5	2	4.5	3	4.9	2.8	6.5	2.8	5.6	2.5

Table 2: *In vitro* response of cultured ovules on different media in cv. RH8812 X *Brassica alba*, cv. RH30 X *Brassica alba*, cv. RH270 X *Brassica alba* and cv. RH345 X *Brassica alba*

Media used	cv. RH30 x *B. alba* Shoot formation (%)	cv. RH8812 x *B. alba* Shoot formation (%)	cv. RH270 x *B. alba* Shoot formation (%)	cv. RH345 x *B.alba* Shoot formation (%)
MS + CH (0.5g/l)	55.6(48.2±0.92)	45.1(42.2±0.72)	51.1(49.2±0.97)	48.5(53.2±1.92)
MS + BAP (1mg/l) + CH (0.5g/l)	34.7(36.1±0.83)	27.5(31.6±0.92)	30.1(34.6±1.18)	32.2(29.8±0.99)
MS + BAP (2.5mg/l) + CH (0.5g/l)	36.4(37.1±0.62)	**45.6(42.5±1.94)**	**65.7(64.7±1.11)**	**60.2(65.8±0.98)**
MS + KIN (1mg/l) + CH (0.5g/l)	59.1(50.2±0.76)	39.2(38.7±1.11)	45.2(50.2±0.73)	40.2(38.7±1.07)
MS + KIN (2.5mg/l) + CH (0.5g/l)	**79.5(63.1±1.04)**	36.7(37.2±0.98)	48.1(45.2±0.86)	55.1(51.2±0.32)
MS + IAA (0.5mg/l) + KIN (1mg/l)	21.1(27.3±0.77)	27.9(31.8±1.17)	25.9(27.3±0.63)	30.7(31.8±1.01)
MS + IAA (0.5mg/l) + KIN (2.5 mg/l)	18 .0(25.1±0.86)	10.5(18.8±0.90)	11.1 (15.0±0.23)	15. 7(17.8±0.98)

Table 3: *In vitro* rooting response in cv. RH8812 X *B. alba*, cv. RH30 X *B. alba*, cv. RH270 X *B. alba* and cv. RH345 X *B. alba*

Medium used	cv. RH8812 x *B. alba* Root formation (%)	cv. RH30 x *B. alba* Root formation (%)	cv. RH270 x *B. alba* Root formation (%)	cv. RH345 x *B. alba* Root formation (%)
MS Basal	29.2 (32.6±1.32)	67.5 (54.7±1.02)	43.3 (38.0±1.72)	37.7 (40.8±1.22)
MS + IAA (1mg/l)	62.1 (52.0±0.88)	80.0 (63.5±1.66)	55.2 (62.0±0.98)	61.1 (55.0±0.36)
MS + IAA (0.5mg/l)	**100 (90.0±0.00)**	**100 (90.0±0.00)**	**98 (94.0±1.76)**	**95 (90.0±0.99)**
MS + NAA (1mg/l)	0.0 (0.0±0.00)	5.6 (13.6±1.37)	10 (6.0±0.32)	13.6 (9.1±1.57)
MS + NAA(0.5mg/l)	13.0 (21.0±1.54)	18.5 (25.4±1.36)	15.8 (20.1±0.84)	21.0 (24.3±1.86)
MS half basal	14.1 (20.0±1.08)	18.3 (25.3±1.25)	19.1 (25.0±1.38)	7.6 (10.7±1.19)

Molecular analysis in advanced generation

RGA primers were used for confirmation of presence of donor fragment in advanced interspecific cross progenies. The PCR amplification reactions were performed using 50 ng of genomic DNA, extracted by the procedure described by Saghai-Maroof *et al*. (1984), in a total volume of 20 µl containing 0.2 µM of each primer, 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, and 1.5 U Taq DNA polymerase. The PCR programme was optimized and is as follows: an initial step of 3 minutes at 95 °C, followed by 40 cycles of 1 minute at 94 °C, 1 minute at 48-57 °C, 1 minute at 72 °C, and a final extension step of 7 minutes at 72 °C. Amplification products were separated on 1.5% standard agarose gel using 1X TBE buffer. Fourteen degenerate primers were used for hybridity confirmation that was already available in the lab. These had been designed from the conserved kinase-1a (GVGKTT) and hydrophobic domains (GLPLAL) of known NBSLRR type R-genes and EST databases of mungbean (Mutlu *et al*., 2006) Degenerate primer sequences, melting temperatures and domains are shown in Table 6.

Results and Discussion

In a cross between *B. juncea* cv. RH30 as seed parent and *B. alba* as pollen parent, the hybrid siliquae length ranged from 1.2 to 3 cm whereas in parent plants of cv. RH30,

Fig. 1: Siliquae of *B. juncea* cv. RH8812 and hybrid siliquae of cross cv. RH8812 X *B. alba*

Fig. 2: Siliquae of *B. juncea* cv. RH30 and hybrid siliquae of cross cv. RH30 X *B. alba*

siliquae length was 4.1 to 4.5 cm. In crosses between *B. juncea* cv. RH8812 and *B. alba*, the hybrid siliquae ranged from 2.8 to 3.4 cm in length while in parent plants of cv. RH8812, the siliquae length was 4.9 to 5.7 cm. Similarly siliquae lengths were smaller for cross RH0345 x *B. alba* (2.2-3 cm) and RH0345 x *B. alba* (2.8-3.4 cm) than their recipient parent plants (RH0270: 6.2-7.1 cm; RH0345: 4.5-5.6 cm). *Brassica alba* siliquae length range was observed from 1.9 to 2.2 cm (Table 1). The siliquae obtained in all the crosses, at 20 DAP had only 2 to 4 ovules as compared to parents(18-20 ovules) (Fig. 1, 2). These ovules when cultured on different media formed seedlings (Table 2). In all crosses of *B. juncea* and *B. alba*, about 39-48% cross success was found (data not shown).In advanced generation (F_7), significant differences were observed in length of siliquae and number of ovules. In all crosses, siliquae length ranged from 4.7 to 6.0 cm and no. of ovules were 12 to 18.

Embryo rescue and shoot initiation

Developing siliquae were excised 10-20 DAP from the field grown plants. After surface sterilization, siliquae were dissected aseptically in a laminar hood exposing developing ovules and these were cultured on modified MS media supplemented with different growth regulators. It was observed that maximum ovule germination was from 20 DAP siliquae (Fig. 3, 4). Shoot initiation and shoot elongation was observed from ovules cultured on different media. Effective media and appropriate concentration of plant growth regulators were essential to induce ovule

Fig. 3: Germinating hybrid ovules of *B. juncea* cv. RH30 X *B. alba*

Fig. 4: Rooting in regenerated shoots derived from *B. juncea* cv. RH30 X *B. alba*

germination. Effects of basal medium and different concentration of growth regulators on cultured ovules in all crosses are summarized in Table 2. In a cross of cv. RH30 x *B. alba*, the maximum percent of *in vitro* embryo germination (79.5 %) was observed on MS medium containing 2.5 mg /l kin and 0.5 mg/l CH. For cv. RH8812 x *B. alba*, cv. RH0345 x *B. alba* and cv. RH270 x *B. alba*, best germination response was 45.6%, 60.2% and 65.7% respectively on MS medium supplemented with 2.5 mg / l BAP and 0.5 mg/l CH. MS basal media containing auxin (IAA) showed poor germination response in all the crosses. But only type of basal medium with different growth regulators did not determine the development of embryos. The effect of DAP was very remarkable for the development of embryos of *in vitro* cultured ovules. A very low frequency of the ovules germination were found if ovules were cultured at DAP other than 10-20 DAP which indicated that the hybrid ovules excised between 10-20 DAP were more efficient in *in vitro* culture.

Rooting and Hardening

Seedlings obtained above were proliferated and rooted successfully in different rooting media viz. MS Basal,

Table 4: Percent survival of regenerated hybrid plants transferred to soil condition

Hybrid plants	No. of plants transferred	No. of plants survived	Percent survival
RH30 x *B. alba*	10	8	80
RH8812 x *B. alba*	10	8	80
RH270 x *B. alba*	12	10	83
RH345 x *B. alba*	12	8	67

Fig. 5: The regenerated hybrid plants transferred to pots covered with paper bags

Fig.6: Hybrid plants successfully transferred to soil in the green house

MS medium with IAA (0.5 mg/l), MS medium with IAA (1 mg/l), MS medium + NAA (0.5 mg/l), MS medium + NAA (1 mg/l) and MS half basal. Best rooting was observed on the MS medium containing IAA (0.5 mg/l). Maximum rooting response was observed on MS medium supplemented with IAA (0.5 mg/l) in all hybrids and lowest root response was on medium MS + NAA (1 mg/l) (Table 3). On MS basal medium supplemented with NAA (1 mg/l), callus formation and multiple shoots were observed. Moreover, all regenerated hybrid plants were successfully hardened off under controlled conditions for a month. Later they were transplanted to the soil where they grew normally. The regenerated plants showed 80% survival under soil conditions in crosses i.e. cv. RH30 x *B. alba* hybrids and cv. RH8812 x *B. alba* hybrids. In cv. RH270 x *B. alba* and cv. RH345 x *B. alba*, 83 and 67 percent survival was observed (Table 4, Fig. 5 and 6).

Screening

In each generation, plants were screened under artificial inoculation conditions in the field. Resistant lines were selected and generations were advanced. In F_5 generation, sixty seven lines were screened for their resistance against Alternaria blight with artificial inoculation conditions in the field. Thirty six advanced cross progenies of RH 0345 x *B. alba*-4-11, RH0270 x *B. alba*-2, RH 30 x *B. alba*, RH8812 x *B. alba*-1, RH 30 x *B. alba*-10 and RH 30 x *B. alba*-15 were observed as promising rich donor source lines for Alternaria blight resistance. Thirty one lines were moderately resistant. The seed yield ranged from 98 to 832 g/paired rows of 3.8 m as compared to controls viz cv. RH 30 (470 g) and cv. RH8812 (510 g) per paired row (Data not shown). Similarly in F_6 and F_7 generation, the material was screened. Thirteen *B. juncea* x *B. alba* F_7 advanced progenies were evaluated for agronomic attributes and screened for their resistance against Alternaria blight. Two advanced cross progenies of interspecific crosses RH 1372 (RH 0270 x *B. alba*) and RH 1378 (RH 8812 x *B. alba*) were observed as promising rich donor source (resistant) lines for Alternaria blight resistance. Other lines were moderately resistant (MR) (Table 5). Fig. 7 shows F_7 progenies growing in the field conditions which were highly resistant to Alternaria blight disease.

Molecular analyses using RGA markers

RGA marker analysis of genomic DNA was carried out to confirm the presence of donor fragment in two advanced cross progenies of interspecific crosses RH 1372 (RH 0270 x *B. alba*) and RH 1378 (RH 8812 x *B. alba*). In the present study, thirty RGA markers were used. Out of these, four (K 07/HD 07, K 07/HD 03, K 07/HD 04 and K 01/HD 06) and one marker combination (K 07/HD 03) showed the presence of donor fragment from *B. alba* in RH 1372 (RH 8812 x *B. alba*) and RH 1378 (RH 8812 x *B. alba*) respectively which establishes the confirmation of successful interspecific hybrids development. With primer combination K 07/HD 07 and K 07/HD 03, a polymorphic band of about 600 bp was generated that appeared not only in Alternaria blight resistant cultivar (*B. alba*) but

Table 5: Performance of F_7 progeny derived from *B. juncea* x *B. alba* in SST (Small scale trial)

Genotype	Plant stand	Days to maturity	Seed yield (kg/ha)	1000 seed weight (g)	Oil content (%)	Oil yield (kg/ha)	Alternaria blight severity (%)
RH-1368 (RH-30 x *B.alba*)	89	146	2541	4.39	38.3	972	14.5
RH-1369 (RH-30 x *B.alba*)	85	148	2861	5.18	39.2	1121	8.9
RH-1370(RH-30 x *B.alba*)	84	147	2518	4.80	38.8	977	13.4
RH-1371(RH-30 x *B.alba*)	85	149	2731	4.62	38.7	1057	5.0
RH-1372(RH-0270 x *B.alba*)	**85**	**149**	**3191**	**5.01**	**38.1**	**1214**	**0.0**
RH-1373(RH-345 x *B.alba*)	87	148	2636	6.40	38.8	1021	15.6
RH-1374(RH-345 x *B.alba*)	86	146	2045	5.02	40.2	821	7.8
RH-1375(RH-345 x *B.alba*)	87	146	2896	4.71	40.1	1161	5.0
RH-1376(RH-345 x *B.alba*)	85	146	2317	4.96	39.8	922	7.8
RH-1377(RH-8812 x *B.alba*)	87	149	2612	5.33	38.2	998	3.2
RH-1378(RH-8812 x *B.alba*)	**83**	**149**	**2683**	**4.75**	**37.9**	**1016**	**0.0**
RH-1379(RH-8812 x *B.alba*)	86	147	2435	4.73	38.8	944	8.9
RH-1380(RH-8812 x *B.alba*)	87	149	2139	4.98	38.7	828	7.9
RH 8113	84	150	3180	4.63	38.7	1229	14.5
RH 0749	84	151	3404	6.30	39.0	1328	22.2
RH-30	85	147	2979	5.80	38.6	1150	22.0
GM	85	148	2679	5.05	38.9	1016	-
CD 5%	NS	0.78	471	0.41	0.99	-	-
CV %	6.94	0.24	9.12	3.71	1.71	-	-

Fig. 7 F_7 progenies grown in the research area

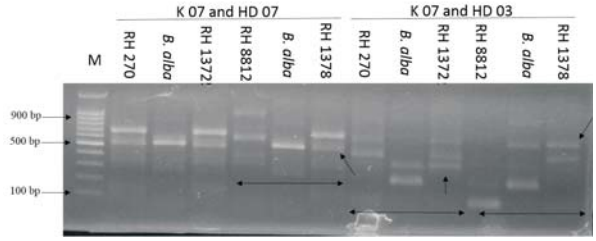

Fig. 8: RGA marker analysis of parents and their advanced cross progenies (RH 1372 for cross RH 270 x *B. alba* and RH 1378 for cross RH 8812 x *B. alba*) with different kinase and hydrophobic domain (HD) primer combinations (K 07, HD 07 and K 07, HD 03)

also in RH 1372 and RH 1378 (Fig. 8).

Several wild species possess desirable traits e.g. resistance to biotic or abiotic stresses (Abraham *et al*., 2004). The integration of these desirable traits into related cultivars using hybridization is of great practical benefit. In the present study, interspecific hybridization between *B. juncea* cv. RH30, cv. RH8812, cv. RH 0270, cv. RH 0345 and *B. alba* were attempted to transfer the Alternaria blight disease resistance from *B. alba* to *B. juncea*. The production of interspecific hybrids in *Brassica* is difficult due to certain crossability barriers such as abortion of hybrid embryos. It was reported earlier that, the frequency of hybridization success was very low in normal breeding program due to the occurrence of crossing barriers (Nishi *et al*., 1959; Wen *et al*., 2008). Application of *in vitro* culture techniques would appreciably enhance the number of hybrid production. Several investigators have focused on improving the interspecific hybridization efficiency within the family *Brassicaceae* (Rahman, 2004; Zhang *et al*., 2004; Yadav *et al*., 2007; Wen *et al*., 2008). These studies primarily based on the method of embryo rescue, time of harvest of hybrid embryos (DAP), and type of culture media. When embryo aborts at early stages or the embryo is too small to manipulate, the embryo can sometimes be rescued by culturing the ovary or ovule (Raghavan, 2007; Tang *et al*., 2009; Cheng *et al*., 2010; Deng *et al*., 2010a). Among the ovary and ovule culture,

Table 6: The sequences, melting temperatures (T_m), and degree of degeneracy of kinase-1a (K) (forward) and hydrophobic domain (HD) (reverse) degenerate primers used during research work

Markers	Sequence (DNA)	Tm (C)	Degeneracy	Amino acid
K 01	5'GGS GGG GTG GGG AAG ACS AC 3'	65.6	4	GGVGKTT
K 02	5'GGW GGG GTT GGG AAG ACW AC 3'	58.3	4	GGVGKTT
K 03	5'GGS GGS GTG GGT AAR ACD AC 3 '	60.9	24	GGVGKTT
K 07	5'GGV GGV YTN GGC AAR ACD AC 3'	59.9	432	GGLGKTT
HD 01	5'GAG GGC GAG GGG GAG GCC 3'	65.7	0	GLPLAL
HD 02	5'CCA ACG CCA ATG GAA GAC C 3'	57.3	0	GLPLAL
HD 03	5'AAG NCT AAR GGG AGG GCC 3'	57.1	8	GLPLAL
HD 04	5'GAG CGC CAR CGG GAG GCC 3'	65.8	2	GLPLAL
HD 05	5'GAG VGC GAA GGG GAG GCC 3'	62.6	3	GLPFAL
HD 06	5'GAG VGC CAR CGG NGA GCC 3'	63.3	24	GSPLAL
HD 07	5'GAG VGC CAR SGG RTG GCC 3'	63.4	24	GHPLAL
HD 08	5'GAG VGC CAR SGG YTT GCC 3'	61.6	24	GKPLAL
HD 09	5'GAG VGC CAR SGG RTT GCC 3'	61.2	24	GNPLAL

ovule culture was efficient in terms of production of various brassica interspecific hybrids (Mohapatra and Bajaj, 1987; Yadav *et al*., 2007; Bennett *et al*., 2008). Interspecific hybridization between *B. juncea* L. viz. cv. RH0345, cv. Varuna, cv. RH8812 and *B. alba* were carried out using ovary and ovule culture by Yadav *et al*. (2007). In ovary culture, callus induction was observed at the excised end of ovary and further no recovery of seeds was observed. Mohapatra and Bajaj (1987) obtained interspecific hybrids in an incompatible cross between *B. juncea* x *B. hirta* through the *in vitro* culture of hybrid ovules and ovaries. They reported the poor response (only 16.6% germination of hybrid seeds) from ovary culture. In both cases, there was better response from ovule culture. Based on these reports, we also applied ovule culture to rescue embryos to overcome the crossable barriers.

It has been observed that most of the interspecific crosses showed reduced seed production with short siliquae length and an increased proportion of nonviable seedlings like *B. alba* as compared to *B. juncea* species (Table 1). All hybrid plants had only 2-4 numbers of ovules per pod than their parent plants (18-20 numbers of ovules/pod). Only 39-48% cross success was found in all the crosses. Similar results were reported in a cross between *B. carinata* x *B. alba*. 46 siliquae were obtained from 200 crosses with very less no. of ovules (Sridevi and Sarla 2005). This poor performance of hybrid plants might be due to the prefertilization barriers during incompatible crosses (Bang *et al*., 1997; Bang *et al*., 1996a). In advanced generation (F_7), significant differences were observed in length of siliquae and number of ovules as compared to initial generation. In all crosses of advanced generation, siliquae length ranged from about 4.7 to 6.0 cm and no. of ovules were 12 to 18 which were close to *B. juncea*. Thus the plants in the F_7 generation were found stable and morphologically near to *B. juncea*.

Ovule germination

The excised ovules from the hybrid siliquae were cultured on different media during the present work. The result have shown that ovules excised at 20 DAP had a higher germination efficiency. No success was recorded in ovules of early and later due to pre and post fertilization barriers. Successful application of biotechnological method largely depends on the age of the embryo being rescued and cultured *in vitro* which were also supported by many investigators. Yadav *et al*. (2007) explained in a cross of *B. juncea* viz. cv. RH0345, cv. Varuna, cv. RH8812 and *B. alba* that ovules excised from less than 7 DAP siliquae did not form plants, however later stages (10-15 DAP) gave good success. The germination of hybrid ovules was observed after 20-30 days of culturing whereas in cv. RH30 and cv. RH8812, ovules germinated after 8 -10 days of culturing. Mohapatra and Bajaj (1987) also reported that the hybrid ovules required 25-30 days to germinate whereas parental ovules started to grow within two days of culture. Similarly, interspecific hybridization was carried out between *Lilium longiflorum* and *L. lophophorum* var. linearifolium using ovule culture by Wang *et al*. (2009) where maximum ovule generation was on 15-20 DAP. Different composition of media has proved to influence the efficiency of embryo rescue techniques in many crops. Effective media and appropriate concentration of plant growth regulators were essential to induce embryo germination in interspecific hybrids. BAP, KIN and IAA were used as plant growth regulators in the experiment. The ovules were germinated on all the media but the efficiency was very different. In cross cv. RH30 x *B.*

alba, MS medium supplemented with kinetin (2.5 mg/l) and CH (0.5 g/l) was more favorable for the development of ovules than other medium. In other crosses, highest ovule germination response was observed on MS + BAP (2.5 mg/l) + CH (0.5 mg/l) medium. Percent of shoot regeneration increased with the increased concentration of BAP and KIN. The effective use of BAP and Kinetin on adventitious shoot formation and growth of hybrids has been reported by many authors. In *in vitro* shoot regeneration from seedling root segments of *B. oleracea* and *B. napus* cultivars, BAP (2.5 mg/l) was the best cytokinin (Khan *et al*., 2009). Khan *et al*. (2003) revealed that BAP (2 mg/l) with IAA (0.5 mg/l) was the most appropriate combination to produce the multiple shoots during regeneration in *B. napus* through hypocotyl explants. Similar observations were also made by Mohapatra and Bajaj (1987). According to Yadav *et al*. (2007), maximum percent response for ovule germination was observed on MS medium containing Kin (2.5 mg/l). Sosnowska & Taras (2014) developed the interspecific hybrids of *B. oleracea* × *B. rapa* via application of *in vitro* placental pollination. Pollinated ovaries were cultured on modified Murashige and Skoog (MS) medium. After 24-d culture, the developing ovules showed best response onto MS medium supplemented with 100 µM kinetin. Thus the effect of growth regulators on shoot germination in the present research work is about same as reported earlier and it is recommended to use MS basal media supplemented with BAP or KIN at concentration of 2.5 mg/l for embryo rescue. The percent germination efficiency for all the genotypes was very low after addition of auxin (IAA) with Kinetin in media during the experiment. Similar observations were reported by Ravanfar *et al*. (2009). The additions of 0.5 and 1 mg/l NAA in the BAP containing media decreased the mean number of shoot produced in *B. oleracea*. Failure of this germination efficiency could probably be attributed to the fact that plant growth regulators, modify membrane permeability, alter the uptake of other hormones and thus affect the system adversely at the level of absorption. Moreover, it was seen that *in vitro* embryogenesis percent efficiency for all the genotypes of *B. juncea* were different and the main reason for the obtained results is probably due to the fact that the effects of genotype impose one of the greatest constraints on plant tissue regeneration. In some instances, observed responses have been related by identifiable genetic factors, nuclear genes, cytoplasmic genes and gene interactions (Zeynali *et al*., 2010).

Rooting and Hardening

Induction of root from regenerated shoots showed wide variations according to different concentration of auxins. Out of six rooting media, root formation was best on MS medium supplemented with IAA (0.5 mg/l) in all the crosses (Table 3). In the present study, higher level of auxin concentration (more than 0.5 mg /l) resulted in lower percent of germination. Khan *et al*. (2009) stated that increasing auxin concentration inhibited root formation and this may be due to imbalance of endogenous auxins present in the shoots and external auxins supplied in the media as reported in Brassica genotypes. Therefore, lower concentrations of auxins should be used for rooting in the culture media. The plants with well-developed shoots and roots system were obtained within 1-2 month. These plantlets were transferred to pots containing sterilized sand soil mixture and covered with polythene bags to retain the humidity. Gradually the regenerated plants were adapted to the soil.

Advanced generation screening

Successful hybrid plants were transferred to field conditions in the Department of Genetics and Plant Breeding, CCSHAU, Hisar, India till advanced generations (F_7) where 13 F_7 advanced progenies were screened for their resistance against Alternaria blight and also evaluated for their agronomic performances. Phenotypic observations were comparable to parent plants. Two advanced cross progenies of interspecific crosses RH1372 (RH 0270 x *B. alba*) and RH 1378 (RH 8812 x *B. alba*) were found highly resistant for Alternaria blight resistance (Table 5). Based upon above findings, it can be said that RH 1372 and RH 1378 were better donor source lines for Alternaria blight resistance.

In the last two decades, substantial amount of literally knowledge has been accumulated on the R genes. It is now well established that the naturally occurring plant disease resistance is mainly conferred by NBS-LRR class of genes. Within the NBS domain, the common motifs that are highly conserved across plant species have provided opportunities for isolating resistant gene analogues (RGAs) using PCR based approach with degenerate primers in a large number of plant species such as soybean, potato, rape, lettuce, cereals, peanut, sugar beet, cotton, and chestnut rose (Maiti *et al*., 2011). Therefore, in our study, for confirmation of alternaria blight disease resistant character in advanced cross progenies, we used degenerate primers from the conserved kinase-1a (GVGKTT) and hydrophobic domains (GLPLAL) of known NBSLRR type R-genes and EST databases of mungbean (Mutlu *et al*., 2006) that were already available

in the lab. In the present investigation, thirty RGA markers were used to confirm the presence of donor fragment in two advanced cross progenies of interspecific crosses RH 1372 (RH 0270 x *B. alba*) and RH 1378 (RH 8812 x *B. alba*). However, only four and one RGA marker was able for confirming donor fragment presence in RH 1372 and RH 1378 respectively. Similarly Saal and Struss (2005) used RGA- and RAPD markers for a *Brassica* B-genome introgression conferring resistance to blackleg in oilseed rape.

Conclusion

Interspecific hybridization was carried out between *B. juncea* (cv. RH30, RH8812, RH270 and RH345) and *B. alba* for transfer of Alternaria blight resistance from *B. alba* to *B. juncea*. Ovule culture technique was used to rescue the hybrid embryo. We found that ovules of 20 DAP showed best germination response. The use of BAP, Kin was of good advantages where the combination of BAP, Kin with MS basal determines the percent germination response for different genotypes.MS basal with IAA (0.5 mg/l) was the best medium for rooting. Successful interspecific hybrids were grown in field till F_7 generation. Artificial screening was done and finally we got Alternaria blight resistant lines [RH1372 (RH0270 x *B. alba*) and RH1378 (RH8812 x *B. alba*)] with promising yield. Their hybridity were also successfully confirmed by using molecular marker analyses (RGA markers). These will be utilized for developing Alternaria blight resistant cultivars. Incorporation of such useful traits was difficult by using conventional method of breeding, since the crosses are incompatible. By using various biotechnological approaches (*in vitro* culture, embryo rescue technique), such traits can be incorporated.

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