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Generation and Evaluation of Somaclones of *Brassica juncea* for Resistance to *Albugo candida* and *Alternaria brassicae*

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Callus cultures were established from hypocotyl and cotyledon explants of 6-10 days old seedlings obtained from non-irradiated and gamma-ray irradiated seeds of *Brassica juncea* cv Varuna on MS medium supplemented with various combinations and concentrations of growth hormones. Frequency of shoot bud formation from the calli obtained from non-irradiated seeds was 11.53 and 9.09% on MS_4 and MS_5 respectively, Shoot buds transferred onto MS medium devoid of growth hormones produced 8-10 shoots. Highest percentage of calli (69.23%) and shoot buds (33.33%) was obtained from calli initiated from 40kR gamma-ray irradiated seeds. For root induction in tissue-cultured shoots, IAA 2.5 mg/l gave best results with 81.81% rooting frequency. More than 90% plantlets established in the pots after 10-12 days of hardening under high humidity conditions. Out of 42 somaclones tested for their reactions to *Albugo candida* and *Alternaria brassicae*, six somaclones were rated as resistant and three somaclones were moderately resistant to *A. candida*. Seven somaclones were highly resistant to *A. brassicae*. Resistance to *A. candida* and *A. brassicae* in some of the somaclones was stable and heritable in R_2 generation under field conditions.

Key Words: ***Brassica juncea*, Somaclones, *Alternaria brassicae*, *Albugo candida***

Abbreviations: *IAA*, Indole acetic acid; *NAA*, Nepthalone acetic acid; *IBA*, Indole butyric acid; *2, 4-D*, 2, 4-Dichlorophenoxy acetic acid; *BAP-6*, Benzyl amino-purine; *Kin*, Kinetin; *MS*, Murashige and Skoog (1962)

Introduction

Generation of stable and heritable variation from cell cultures is a very promising area for the development of disease-resistant germplasm in crop plants. *In vitro* culture techniques have been used successfully to generate agriculturally useful variation in many crop species by various workers (Larkin & Scowcroft 1981, Evans et al. 1986). Somaclonal variation promises to be a valuable tool for the genetic modification of *Brassica juncea* which is highly susceptible to *Alternaria* blight and white rust diseases. There are several reports about plant regeneration in *Brassica* species using different media (Hui & Zee 1978, George & Rao 1980, Murata & Orton 1983, Singh & Chandra 1984, Zhou et al. 1985 and Surya Parkash et al. 1991). However, reports on plant regeneration in *B. juncea* are limited. Murata and Orton (1987) reported very low frequency of plant regeneration from cotyledon and hypocotyl-derived explants of *B. juncea*. *In vitro* multiple shoot formation in cotyledonary calli of *B. juncea* cv Parkash was reported by Jain et al. (1987). High frequency plant regeneration through somatic embryogenesis (Kirti & Chopra 1989) has also been reported in *B. juncea*.

Since the first prerequisite for the use of such technology is to optimise culture conditions for tissue culture mediated plant regeneration, the present communication reports high frequency plant regeneration and root induction from explants of non-irradiated and gamma-ray irradiated seeds of *B. juncea* cv Varuna and somaclonal variation for resistance to *Albugo candida* and *Alternaria brassicae.*

Materials and Methods

Plant material: Seeds of *Brassica juncea* cv Varuna were provided by Dr H L Thakur, Oilseed Breeder, Himachal Pradesh Krishi Vishvavidyalaya, Oil seeds Research Station, Kangra, India. Five hundred dry dormant and uniform sized seeds were exposed to 40, 60, 70 and 80 kR of gamma-rays, using a 5500C ^{60}Co gamma-rays chamber (4000 BARC, Bombay) at Jawahar Lal Nehru University, New Delhi.

Establishment of callus cultures and plant regeneration: Fifty seeds of *B. juncea* cv Varuna, both non-irradiated and gamma-ray irradiated, were surface-sterilized with aqueous solution of 0.1% $HgCl_2$ and two drops of Tween-20 for 5 min followed by 3-4 washings with sterlized distilled water under aseptic conditions. These seeds were dried on sterilized blotting papers and germinaterd on half strength MS medium in 250 ml erlenmeyer flasks. After 6-8 days cotyledonary leaves and hypocotyls were excised (0.5-1.0cm) and cultured on MS medium containing sucrose (3%), 2,4-D (0.2mg/l), Kin (0.2mg/l) and agar (0.8%). The *p*H of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. Cultures were incubated under 16 hr light/8 hr dark cycles at 25 ± 1°C. Calli were excised from undifferentiated explants, subcultured after every three weeks and maintained on the same medium for three months. Small pieces of callus (ca 5mm) were transferred onto MS medium supplemented with various concentrations of cytokinins and auxins (table 1) and scored for shoot bud formation. Shoot buds were transferred onto MS medium either devoid of growth hormones or supplemented with BAP (2.0 mg/l) for multiple shoot formation.

Root induction: For root induction, 3 week old shoots were excised from the calli and transferred to MS medium supplemented with IBA, NAA or IAA (2.5 mg/l). Ten ml liquid medium was poured in culture tubes (25 × 150 mm) and shoots were placed on filter paper bridges. After 12-15 days well rooted shoots were transferred to sterilized potting mixture (soil, sand and farmyard manure. 1:1:1, w/w) in 4" diam. plastic pots and covered with polythene bags to maintain high humidity for 12 days. The hardened regenerated plants (R_0) were transferred to large plastic pots (8" diam.) and kept on screen house benches for better plant growth.

Evaluation of somaclones: R_0 plants of *B. juncea* cv Varuna were observed for their reaction to *Albugo candida* and *Alternaria brassicae* during 1990-91. Severity of white rust was recorded on five randomly selected leaves of each plant under natural epiphytotic conditions using 0-5 scale. Disease severity on each plant was converted into percent disease index (PDI) using the following formula:

$$PDI(\%) = \frac{\text{Total sum of numerical ratings} \times 100}{\text{Max. rating} \times \text{No. of leaf samples}}$$

On the basis of PDI, plants were placed into following categories : 0 = highly resistant; 1-5% = resistant; 6-10% = moderately resistant; 11-20% = moderately susceptible; 21-40% = susceptible and above 40% = highly susceptible.

The somaclones were also evaluated for their reaction to *Alternaria brassicae* in detached leaf culture with standard spore suspension (2000-2500 spores/ml) as described by Sharma (1990) and grouped into different reaction categories on the basis of spot size, sporulation intensity and intensity of yellow halo. To study the inheritance and stability of resistance to *A. candida* and *A.*

brassicae, **self fertilized progenies of selected R_1 plants were grown under natural epiphytotic conditions during the rabi season of 1992. Data were recorded at pod filling stage on the presence or absence of any disease symptoms in case of *A. candida* and on the basis of spot size in case of *A. brassicae*.**

Results

Generation of somaclones: **The frequency of root and shoot formation from calli obtained from cotyledon and hypocotyl explants of *B. juncea* cv Varuna is given in table 1. It was found that 11.53% and 9.09% calli regenerated into shoot buds on MS_4 and MS_5 respectively. On transfer to MS medium devoid of growth hormones and MS + 20 g sucrose/l + BAP (2.0 mg/l), there was excessive brenching of shoots on media supplemented with BAP, which ranged from 20-30 shoots per shoot bud. However, the growth of shoots was not well organised. Shoot buds transferred to MS medium without any growth hormone produced 8-10 shoots which developed into well developed plantlets.**

Plantlets were also regenerated from hypocotyl and cotyledon explants obtained from gamma-ray irradiated seeds (table 2). Highest percentage of callus (69.23%) and shoot bud (33.33%) formation was obtained at 40 kR. However, with irradiation at 80 kR callus formation was high but shoot bud formation was limited. These shoot buds were placed on MS medium devoid of BAP for multiple and better shoot growth.

Root induction in plantlets : Plantlets obtained from hypocotyl and cotyledon explants through callus cultures were without roots. Supplementation of liquid MS medium with IBA/NAA/IAA at 2.5 mg/l resulted in hard calli formation in 6 to 8 days at the cut ends of shoots transferred to MS medium. Two to three thick roots were formed in the medium with IBA. However, fibrous roots were formed in the medium with NAA. The frequency of root formation in media with IBA and NAA was 33.33% and 27.27%, respectively. In both these cases plants were ready for transfer within 20-26 days. On the other hand highest frequency (81.81%) of root formation was obtained in medium with

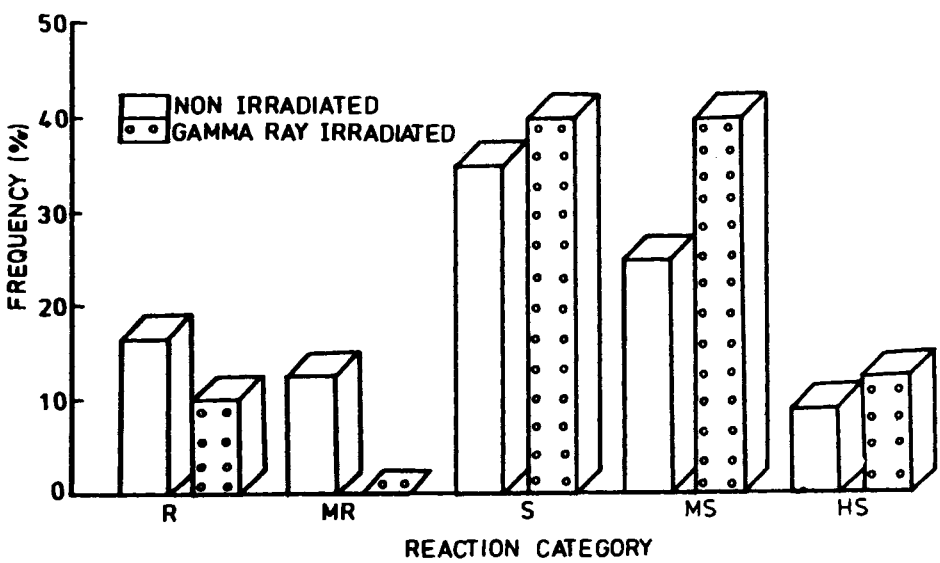

Figure 1 Frequency of somaclonal variants of *Brassica juncea* resistant to *Albugo candida*

Table 1 *Effect of combinations of growth regulators on root and shoot induction in calli of* ***Brassica juncea*** *on MS medium*

Medium	MS with growth regulators	Roots (%)	Shoots (%)
MS_0		48.78*	-
MS_1	Kin (0.1 mg/l)	9.00	-
MS_2	Kin (2.0 mg/l) + IAA (0.1 mg/l)	4.00	-
MS_3	BAP (2.0 mg/l) + IAA (0.1 mg/l)	26.60*	-
MS_4	BAP (0.5 mg/l) + 2,4-D (0.25 mg/l) + NAA (0.5 mg/l)	-	11.53
MS_5	BAP (0.75 mg/l) + 2,4-D (0.25 mg/l) + NAA (0.5 mg/l)	-	9.09

*Greening of calli

-Not formed

Table 2 *Callusing and differentiation in explants obtained from gamma-ray irradiated seeds of* ***Brassica juncea*** *cv Varuna on MS medium*

Radiation dose (kR)	Explants transferred	Calli (%)	Shoots formed (%)
40	78	69.23	33.33
60	57	43.36	21.05
70	81	48.14	30.76
80	69	60.86	21.42

Medium = MS + Kin (0.5 mg/l) + NAA (0.5 mg/l) + 2,4-D(0.2 mg/l)

Table 3 *Effect of auxins (2.5 mg/l) added to MS medium on root induction in tissue culture derived shoots of* ***Brassica juncea*** *cv Varuna*

Auxin	Shoots transferred (No.)	Roots (%)	Calli (%)	Remarks
IAA	33	81.81	18.19	Normal thick roots
IBA	27	33.33	66.67	Callusing at cut ends, fibrous roots
NAA	33	27.27	72.73	Callusing at cut ends, fibrous roots
MS	15	26.26	-	Long hairy roots

IAA (table 3). Ten to fifteen long thick roots emerged on the 6th day of transferring shoots onto rooting medium and plantlets were ready for transfer to pots within 10-12 days. Roots were also formed on basal MS medium after one month but only 1-2 roots were formed. More than 90% plantlets obtained from MS medium supplemented with IAA, established in the pots after hardening under highly humid conditions for 8-10 days in sterilized potting mixture.

Somaclonal variation for ***Albugo candida*** *and* ***Alternaria brassicae:*** Forty-two somaclones (R_0) derived from hypocotyl and cotyledon explants of gamma-ray irradiated and non-irradiated seeds were evaluated for their reaction to *A. candida* under natural epiphytotic conditions of regeneration. The frequency of variation for *A. candida* is depicted in figure 1. Maximum percentage (15.6%) of resistant somaclones was obtained in plants raised from calli derived from non-irradiated seeds. The frequency of moderately resistant somaclones was 12.5%. In case of somaclones regenerated from the calli initiated from seeds treated with differ-

Table 4 *Comparative reaction of somaclones of* ***Brassica juncea*** *cv Varuna to* ***Albugo candida*** *and* ***Alternaria brassicae*** *in R_0 generation*

Somaclone	*Albugo candida*		*Alternaria brassicae*[1]
	PDI (%)	Rating	
1	7.5	MR	R
2	3	R	S
3	2.5	R	S
4	5.5	R	S
5	3	R	S
6	20	MS	R
7	3	R	R
8	10	MR	-
9	22.5	S	-
*10	16	MS	-
*11	100	HS	-
*12	24	S	HR
13	12	MR	R
14	18	MS	-
15	12	MR	HR
16	18	MS	S
17	18	MD	-
18	30	S	HR
19	25	S	HR
20	100	HS	-
*21	30	S	R
22	12	MS	HR
23	25.7	S	S
24	18	MS	S
25	22	S	HR
26	27.7	S	-
*27	27.7	S	-
*28	20	MS	S
*29	18	MS	S
30	22	S	S
31	18	MS	-
32	100	HS	-
33	25.7	S	S
34	33.3	S	S
35	18	MS	R
36	30	S	R
37	28.6	S	R
38	20	MS	HR
39	20	MS	S
*40	33.3	S	HR
41	1.5	R	R
*42	1.5	R	-
Varuna	95	HS	S

[1]Tested in seedling stage
*Regenerated from the calli derived from gamma-ray irradiated seeds
HR Highly resistant
R Resistant
MR Moderately resistant
MS Moderately susceptible
S Susceptible
HS Highly susceptible
- Not tested

ent doses of gamma-rays, only 10 percent were resistant to *A. candida.*

Reaction of 42 somaclones to *A. candida* and *A. brassicae* is given in table 4. Seven somaclones viz., 2, 3, 4, 5, 7, 41 and 42 were rated as resistant to *A. candida.* However, none of the somaclones was free from *A. candida* infection. Seven somaclones were highly resistant to *A. brassicae* in detached leaf evalution at seedlings stage and nine were rated as resistant on the basis of spot size, sporulation and yellow halo (table 4). Somaclone 7 was resistant to both the diseases and 15 was moderately resistant to *A. candida* and highly resistant to *A. brassicae.*

Reaction of progenies of 12 R_1 somaclones (R_2 generation) under natural epiphytotic conditions to *A. candida* and *A. brassicae* showed that all the plants of somaclones 3, 18-2 and 15 were rated as highly resistant to *A. brassicae* on the basis of absence of any visible symptoms on the leaves. Other somaclones with small spot size of *A. brassicae* were placed in the resistant category (table 5). Somaclone 3 showed resistance to both the diseases in R_2 generation. High resistance to *A. candida* in somaclone 3 and *A. brassicae* in somaclone 15 was passed on from R_0 to R_2 generation in a stable manner. However, segregation for resistance to *A. brassicae* was observed in R_2 plant progenies of somaclone 7. Resistance to *A. candida* in somaclone 7 also did not remain stable in R_2 generation.

Discussion

Plant regeneration from single cells or protoplasts is the first step to obtain somaclonal variation in different crops. Although *in vitro* regeneration of plants from calli obtained from different explants of *Brassica* species has been reported earlier (Singh & Chandra 1984), yet there are few reports on the *in vitro* plant regeneration in *B. juncea,* that too with low frequency. The present results on high frequency plant regeneration from hypocotyl and cotyledonary

Table 5 *Field evaluation of somaclones of Brassica juncea cv Varuna against Albugo candida and Alternaria brassicae in R_2 generation*

Progeny of somaclone	Reaction to	
	Albugo candida	*Alternaria brassicae*
3	R	HR
5	S	HR
7-1	S	S
7-2	S	R
7-3	S	HR
7-4	S	HR
13	S	R
15	S	HR
18-1	S	R
18-2	R	R
18-3	S	R
40	S	R
41	S	R
43-1	S	R
43-2	S	R
44	S	R
45	S	R
46-1	S	S
46-2	S	R
46-3	S	R
Varuna	S	S

^{R}Resistant
^{S}Susceptible
^{HR}Highly resistant

explants of *B. juncea* and multiple shoot formation by using 2, 4-D, NAA and BAP or Kin in combinations are in agreement with those of Kirti and Chopra (1989). Jain et al. (1989) also reported multiple shoot formation in cotyledonary calli of *B. juncea* cv Parkash with high cytokinin (Kin & Zeatin) and low IAA concentrations.

Callus cultures obtained from gamma-ray irradiated seeds to increase the frequency of variation in tissue culture derived plants resulted in limited shoot bud formation although callus formation was enhanced. Similar results were obtained by George and Rao (1980) who reported that treatment of seeds with gamma-rays above 2 kR inhibited shoot regeneration. The frequency of shoot bud formation from the explants obtained from gamma-ray irradiated seeds in the present investigation was quite high. However, most of the plants were abnormal and could not survive under field conditions. Many of those which survived, did not bear flowers and thus could not be propagated further.

Effect of auxins on root induction in tissue cultured shoots has been reported by Murata and Orton (1987). They obtained best root formation on MS medium containing 2 mg/l IAA + 0.1 mg/l Kin. However, Jain et al. (1989) did not find significant effect of IAA, IBA and NAA on root induction and reported rooting of long term cultures of shoots on MS medium devoid of auxins. In the present study MS + 2.5 mg/l IAA gave best results with rapid root induction. Plantlets were ready for transfer to the pots in 10-12 days. However, IBA and NAA induced excessive callusing on cut ends of shoots which hindered root formation. Establishment of rooted plantlets in soil was significantly higher in the present study and more than 90% plantlets were successfully transferred to the pots in sterlized soil under high humidity conditions and subsequently transferred to large pots.

Our results demonstrate that variation for resistance to *A. candida* and *A. brassicae* was present in somaclones derived from a susceptible cultivar of *Brassica juncea*. Although frequency of resistant somaclones varied for the two diseases yet frequency of somaclones placed in the resistant category was quite high. In case of *A. candida* however, not a single somaclone was found free from infection in R_0 generation. On the other hand necrotic fleck type reaction to *A. brassicae* was obtained in 23.3% somaclones. One somaclone (Sc7) was resistant to both the diseases in R_0 generation. Resistance to *A. Candida* in somaclone 7 is R_0 generation was probably due to epigenetic changes and it did not remain stable in R_2 generation. There is only one report of selection of resistant somaclones of *B.*

juncea against *A. candida* and *A. brassicae* under field conditions (Katiyar & Chopra 1990).

In the present investigation, somaclones resistant to *A. candida* were obtained from calli derived from gamma-ray irradiated seeds. The frequency of resistant somaclones did not increase with the irradiation dose. However, high frequency of *A. candida* resistant somaclones was also observed from calli obtained from explants of non-irradiated seeds. Sacristan (1985) also found that plants resistant to *Phoma lingam* could be regenerated from calli and embryogenic cultures of *B.napus* previously treated with mutagens. Although, the mechanisms of high frequency cell culture induced variation have not been reported yet, Duncon and Widholm (1986) proposed various reasons for the chromosomal variation in *in vitro* culture.

In our study, resistant reaction to *A. candida* in somaclone 7 in R_0 generation was probably due to epigenetic changes as it did not remain stable in R_2 generation.

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