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Efficient Callus Induction and Regeneration in *Brassica juncea* for Environment Friendly Agriculture

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ABSTRACT

In the present study an efficient regeneration protocol has been established for mustard (Brassica juncea var RSPR 01, RSPR 03) using hypocotyls of in vitro grown seedlings for callus induction and regeneration. Different concentrations of phytohormones, auxin (NAA: Naphthalene acetic acid, 2,4-Dichlorophenoxyacetic acid) and cytokinins (BAP: Benzylaminopurine) were used for callus induction and plant regeneration. Formation of callus from hypocotyls explants were observed in the MS media supplemented with 3% sucrose and 2,4-Dichlorophenoxyacetic acid at different concentrations of (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) high response of about 57.14 to 95.24% was observed at 2.0 to 2.5 and no response was found at 0.5 and 1.0 mg/l of 2,4 –D. The regeneration frequency was observed with different concentrations of (MS+ 2,4-D (0.5mg/l)+BAP (1, 2, 3, 4 and 5 mg/l) the concentration of 2,4D were kept constant with the change in concentration of BAP from 1.0 to 5.0mg/l. Regeneration frequency (RF) of 100 per cent with variety RSPR 03 was observed in the media supplemented with 0.5mg/l 2,4-D +5.0 mg/l BAP while as the cultivar RSPR 01 showed regeneration frequency of 100 per cent when 0.5mg/l 2,4-D +4.0 mg/l BAP. This protocol can be further explored for transformation of mustard for incorporation of specific genetic traits for its improvement.

Key words: *Brassica juncea, Callus induction, Regeneration efficiency, Protocol.*

INTRODUCTION

Brassicas occupy a prominent place in world's agrarian economy as vegetables, oil seeds, feed and fodder, green manure and condiments. Oleiferous *Brassica* species *viz.Brassica napus, Brassica campestris* and *Brassica juncea* constitute the world's third most important source of edible oils[8]. India is the third largest rapeseed-mustard producer in the world after China and Canada with 12 per cent of world's total production.

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India holds a premier position in rapeseed-mustard economy of the world with 2nd and 3rd rank in area and production respectively. The oilseed (*Brassica* species) cultivation has increased tremendously from last few years and by now it is the second largest contributor to the world supply of vegetable oil. It has been reported from various studies that improvement of plants through conventional breeding method is relatively time consuming, slow and labor intensive. Conventional genetic improvement programmes based on plant tissue culture and molecular genetics are essential as a complement to standard breeding. Tissue culture technique can be used in combination with molecular techniques, which find to be a successful approach for incorporation of specific trait through gene transfer called DNA recombinant technology[4]. Unlike application of harmful pesticides, herbicides and other chemicals for developing pest and disease free produce, transgenic technology is a best alternative for producing varieties with inbuilt genetic resistance against insect pests and diseases. Therefore providing an environment friendly product resistant to biotic and abiotic factors. Development of a tissue culture based regeneration protocol is a base of every genetic engineering / transgenic technology. Regeneration in mustard is highly variable and genotype specific. Use of hypocotyls and / or cotyledons as an explants for in vitro plant regeneration has received considerable attention[1,3,9,5,12,13]. Success in plant tissue culture and plant transformation depends on two important factors, choice of explant and supplemented culture medium. Frequency of shoot regeneration is high when we use hypocotyls as an explants, it has been reported from several *Brassica* species for genetic transformation[7,19]. Advances in technologies such as transfer of foreign gene in plants have overcome several barriers to crop improvement[10]. As a consequence, both for agronomic improvement and genetic studies, callus induction and regeneration protocol are required. The present experiments were conducted to determine the callus formation and regeneration efficiency of hypocotyl segments of in vitro grown mustard seedling and find out the best medium for further tissue culture based crop management of mustard[2].

MATERIALS AND METHODS

Surface sterilization and inoculation of seeds in MS media: The certified seeds of mustard (*Brassica juncea* var RSPR 03 and RSPR 01) were procured from Division of Genetics and plant Breeding Sher-e-Kashmir University of Agricultural Sciences and Technology, Faculty of Agriculture, Chatha, Jammu. As the percent *in vitro* seed germination is reduced with high bacterial and fungal contamination, an efficient protocol for surface sterilization of seeds was standardized. The seed size is a considerable and significant factor in the germination and early stage of plant growth. Germination of seeds, shoot and root induction also affected by increasing salt concentration[6]. So concentration of all salts in MS medium should be balanced for appropriate regeneration. In the present study, a medium size seed has been taken for germination. The seeds were given treatment with Tween-20 for 10 min and then washed with distilled water. The seeds were washed in running tap water to remove traces of Bavistin and tween 20. Inside the Laminar air flow chamber seeds were surface sterilized by treatment with 0.1 per cent mercuric chloride ($HgCl_2$) for 3-5 minutes. The seeds were finally washed 3-4 times with sterilized distilled water in a laminar air flow cabinet, and inoculated on Murashige and Skoog full strength basal medium containing 3 per cent sucrose with pH 5.8. Ten to twenty days old seedlings were used as a source of explants. The seeds were washed in running tap water to remove traces of Bavistin and tween 20. Inside the Laminar air flow chamber seeds were surface sterilized by treatment with 0.1 per cent mercuric chloride ($HgCl_2$) for 3-5 minutes. The seeds were finally washed 3-4 times with sterilized distilled water in a laminar air flow cabinet, and inoculated on Murashige and Skoog full strength basal medium containing 3 per cent sucrose with pH 5.8. Twenty days old seedlings were used as a source of explants. About 8-10 seeds were

transferred aseptically with the help of forceps to each conical flask containing 50 ml of MS medium and incubated at 26 ± 2^0C under photoperiod of 16 hr light and 8 hr dark.

Callus formation and shoot regeneration: Hypocotyls were used as an explant from *in vitro* grown seedlings source in the present study. About 0.8-1 cm long pieces of hypocotyls of 10-20 days old mustard seedlings were cut with the help of sterile blades. Hypocotyls below the first true leaf from 3-4 weeks old in vitro seedlings were taken. Four pieces of hypocotyls were transferred to each of the conical flasks containing 50 ml of MS medium supplemented with various combinations of growth regulators (2,4-D). For callus induction and plant regeneration, the MS medium was supplemented with 3% sucrose and 2,4-D) at different concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L. To optimize the culture medium for shoot regeneration, the calli were divided into small pieces and cultured on modified MS medium supplemented with various combinations and concentration of phyto harmones *viz*. BAP, NAA and 2,4-D[17]. The relative response of cultivated crucifers for callusing and regeneration was observed for the following parameters as per the formulae followed by Moghaieb *et al*[14].

$$\textbf{Callus Induction Frequency (CIF)} = \frac{\textbf{No. of calli produced explant}}{\textbf{Total no. of explants in the culture}} \times 100$$

$$\textbf{Regeneration Frequency (RF)} = \frac{\textbf{No. of shoot initiated in the culture}}{\textbf{Total no. of calli used}} \times 100$$

RESULTS AND DISCUSSION

Brassica seeds started germination after 2-3 days after inoculation on the MS medium figure-1a. The matured seedlings with two leaf stages were arisen at 8-10 days after germination of the seeds. Similar results were also reported earlier Patil *et al*[17]., and Munir *et al*[15]. The hypocotyls of the *in vitro* grown seedlings were used as explants in the present study. Earlier, hypocotyls segment of *in vitro* grown Brassica seedlings have been used by Pushpa *et al*[18]. The hypocotyls of uniform size (1 cm) were taken in the present study. The number of explants inducing callus were supplemented with MS media at different concentrations of 2,4-D and NAA of (0.5,1.0, 1.5, 2.0 and 2.5 mg/l). The results revealed that all the cultivars of *Brassica juncea* responded to callus at more than 1.5 mg/l 2,4-D, data, figures are shown in table 1 and Fig. The response gradually increased with the increasing concentration of 2,4-D. The maximum CIF were recorded at 2.0 and 2.5 mg/l of 2,4-D. High levels of 2,4-D are expected to give more callusing, however, the studies revealed that callus induced at high 2,4-D mg/l (more than 2.5 mg/l) results into retarding regeneration. The low level of concentration of 2,4-D (0.5 to 1.0) seems to be inefficient in inducing callusing. This matches with the findings of Khan et al[11].,who reported 2mg/l as best calli producing concentration using only 2, 4-D for the callus induction in *Brassica napus* L. cultivar Oscar and found no significant difference between the different explants of a cultivar and among the cultivars for callus induction ($P<0.05$). The cotyledonary explants generally gave calli with more mean weight and mean length. The *same* results were obtained by Zhang and Bhalla[23]. Very high response of callus induction was observed in *Brassica juncea* cvs. RSPR-01 and RSPR-03 inoculated on MS media supplemented with 2,4-D at 2.0 and 2.5 mg/l. The 2,4-D has been reported to be a potent auxin for inducing callusing in *Brassica*. No response was observed on MS media supplemented with 2,4-D at 0.5 and 1.0 mg/l, medium response was shown at 1.5 mg/l of 2,4-D in both the varieties of *Brassica juncea*. Ullah *et al*[21]., reported that the concentration of 1.5 mg/l 2,4-D would be adequate for callus induction from hypocotyl segments of *Brassica napus* on MS medium.

Vyvadilova and Zelenkova[22] also observed that best callus induction from auxiliary and terminal meristems, leaves, hypocotyls, cotyledons and roots was on MS medium supplemented with 0.5 to 2.0 mg/l 2,4-D. Trivedi and Dubey[20] also reported that about 91.6- 100% callus formation from hypocotyls explants were observed in the MS media supplemented with BAP at 0.5-1.0 mg/L + NAA at 0.5-1.0 mg/L. In these conditions, the explants also produce more number of shoots ranging from 7 to 20 shoot lets/explants.

The regenerating media containing various concentrations of growth regulators i.e (MS+ 2,4-D (0.5mg/l)+BAP 1-5 mg/l)) showed that the cultivars showed significant differences in the number of regenerating plants data, figures are shown in table 2 and Fig 2. *Brassica juncia* (RSPR 01, RSPR 03) showed higher regeneration frequency (RF) at 0.5mg/l 2,4-D +5.0 mg/l BAP, while as, no regeneration frequency was observed at 0.5 mg/l 2,4-D + 1.0 mg/l BAP in both the cultivars. It was also observed by Trivedi and Dubey[20] (2014) that the MS medium with BAP at 2.0-2.5 mg/L + NAA at 0.5 mg/L, all the explants (100%) produced callus but shoot induction was found to be very less and few explants showed shoot formation with as less as 1-2 shoot lets per explant. Krishania *et al*., 2013, Burbulis *et al*[5]., reported maximum shoots formation from hypocotyl-derived callus was obtained earlier on a medium supplemented with 4.0 mg/ L of BAP and 0.05 mg/L of NAA. In the present study, increased number of shoot per hypocotyls derived callus was found in the medium with 0.5-1.0 mg/L of BAP + 0.5-1.0 mg/L of NAA.

Regeneration in Brassica is highly genotype dependent and has been reported in several species. In *Brassica napus*, there was a huge variation ranging from 0% to 91% in the 100 cultivars tested[16]. *Brassica napus* cultivar GSL 1 showed better regeneration efficiency than Westar (a standard cultivar for transformation) in one study. Chinese cabbage (*Brassica rapa*. ssp. *pekinensis*), a large variation was observed in regeneration frequency, ranging from 95% to 100%[23]. Thus, genotype specificity is a limiting factor in Brassica tissue culture and regeneration, which severely limits the germplasm that can be manipulated or improved.

Table 1. Callus induction frequency of *Brassica juncea* at different concentrations of growth regulators

Genotype	Concentration of MS+ 2,4-D (mg/l)	No. of cultured explants	Callus inducing explants	CIF (%)
RSPR 01	0.5	14	0.00	0.00 (1.00)
	1.0	14	0.00	0.00 (1.00)
	1.5	14	8.00	57.14 (7.59)
	2.0	14	12.66	90.48 (9.56)
	2.5	14	13.34	95.24 (9.80)
RSPR 03	0.5	14	0.00	0.00 (1.00)
	1.0	14	0.00	0.00 (1.00)
	1.5	14	10.00	71.43 (8.48)
	2.0	14	11.34	80.95 (9.02)
	2.5	14	13.34	95.24 (9.80)
C.D. (p=0.05)				0.65
S.E. m (±)				0.22
C.V.				9.24

Figures in parenthesis represent square root transformed values

Table 2. Effect of different combination of growth regulators on Shoot regeneration frequency of *Brassica juncea*

Genotype	Concentation of MS+ 2,4-D+BAP (mg/l)	No. of calli used	No. of shoot regenerated	No. of regenerated plants	RF (%)
RSPR 01	0.5+1.0	12	0.00	0.00	0.00 (1.00)
	0.5+2.0	12	6.00	4.00	50.00 (7.08)
	0.5+3.0	12	8.66	7.34	72.17 (8.55)
	0.5+4.0	12	12.00	10.66	100.00 (10.05)
	0.5+5.0	12	11.34	11.34	94.50 (9.76)
RSPR 03	0.5+1.0	12	0.00	0.00	0.00 (1.00)
	0.5+2.0	12	4.00	4.00	33.33 (5.54)
	0.5+3.0	12	9.34	6.66	77.83 (8.79)
	0.5+4.0	12	10.66	9.34	88.83 (9.44)
	0.5+5.0	12	12.00	11.34	100.00 (10.05)
C.D. (p=0.05)					1.24
S.E. m (±)					0.42
C.V.					14.51

Figures in parenthesis represent square root transformed values

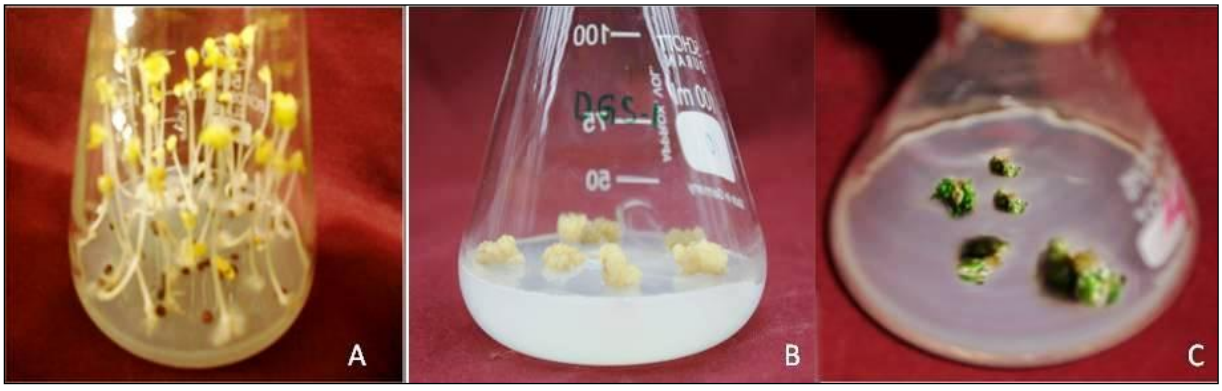

Fig. 1: Callus induction and shoot formation in inoculated hypocotyls of mustard var RSPR-01. A: germination of seeds in to seedlings, B: callus formation at MS medium supplemented with 1.5 mg/l to 2.5 mg/l of 2,4-D, C: shoot initiation at 0.5mg/l 2,4-D +4.0 mg/l BAP

Fig. 2: Callus induction and shoot formation in inoculated hypocotyls of mustard var RSPR- 03. A: germination of seeds in to seedlings, B: callus formation at MS medium supplemented with 1.5 mg/l to 2.5 mg/l of 2,4-D, C: shoot initiation at 0.5mg/l 2,4-D +5.0 mg/l BAP

CONCLUSION

On the basis of results it may be concluded that regeneration protocol developed in the present investigation for *Brassica juncea* var. RSPR-01, RSPR-03 is reliable and can be effectively utilized for genetic transformation of *Brassica* species. It was concluded that maximum CIF were recorded at 2.0 and 2.5 mg/l of 2,4-D and higher regeneration frequency (RF) at 0.5mg/l 2,4-D +5.0 mg/l BAP. No significant differences in callus induction and regeneration were observed among cultivars of *Brassica juncea*, species. It may be due to the fact that these are selections from common pedigree.

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