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Efficient callus Regeneration and Multiple shoot induction in *Brassica juncea* var. Pusa Jaikisan

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Abstract

In the present work an efficient regeneration protocol has been established for mustard (Brassica juncea var Pusa Jaikisan) using hypocotyls of in vitro grown seedlings for callus induction and multiple shoot induction. Different concentrations of phytohormones, auxin (NAA: Naphthalene acetic acid) and cytokinins (BAP: Benzylaminopurine) were used. For callus induction and plant regeneration, the MS medium was supplemented with 3% sucrose and BAP (6-benzylamino purine) and NAA (Naphthalene acetic acid) at different concentrations of 0.2, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L. About 91.6-100% formation of callus 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 shootlets/explant. It was also observed that at MS medium with BPA 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 about 8.3-33.3% explants showed shoot formation with as less as 1-2 shootlets/explant. This protocol can be further explored for transformation of mustard for incorporation of specific genetic traits for improvement of crops.

Keywords: *Brassica juncea*, Callus induction, multiple shoot induction.

Introduction

The oilseed (*Brassica* sp) cultivation has increased tremendously from last few years and by now it is the second largest contributor to the world supply of vegetable oil. Tissue culture technique can be use in combination with molecular techniques, which find to be a successful approach for incorporation of specific trait through gene transfer called DNA recombinant technology[1].

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. 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, 2, 3, 4].

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 sp* for genetic transformation[5]. Advances in technologies such as transfer of foreign gene in plants have overcome several barriers to crop improvement[6]. 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.

Material and Methods

Surface sterilization and inoculation of seeds in MS media: The seeds of mustard (*Brassica juncea* var Pusa Jaikisan) were procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi. 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[7]. Germination of seeds and shoot and root induction also affected by increasing salt concentration[8]. So concentration of all salts in MS medium should be balanced for appropriate regeneration. In the present study medium size seeds 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 then treated with Bavastin (Carbendazim at 0.25%, w/v) for 2 min followed by washing with distilled water. The seeds were further treated with 70% ethanol for 2 min followed by washing distilled water and further surface sterilized by immersing the seed in 0.1% mercuric chloride ($HgCl_2$) for 2-3 min, rinsed with sterile water. About 7-8 seeds were transferred aseptically with the help of forceps to each jam bottle (7-8 cm diameter) 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 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 jam bottle containing 50 ml of MS medium supplemented with different combinations of hormones (BAP and NAA) and kept in incubation chamber at 26 ± 2^0C temp^r maintaining white florescent lights under photoperiod of 16 hr light and 8 hr dark. For callus induction and plant regeneration, the MS medium was supplemented with 3% sucrose and BAP (6-benzylamino purine) and NAA (Naphthalene acetic acid) at different concentrations of 0.2, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L. Fourteen plant regeneration media, CRM1 to CRM14 were made and used for determination of regeneration capacity Table-1.

To get higher regeneration, the explants were sliced at an angle increasing surface and placed the explants horizontally dipping the basal end on medium. The regenerated explants were sub-cultured regularly at 10 days interval. The results were observed according to appearance of callus after 28 days of inoculation and percent plants regenerated was calculated.

Results and Discussion

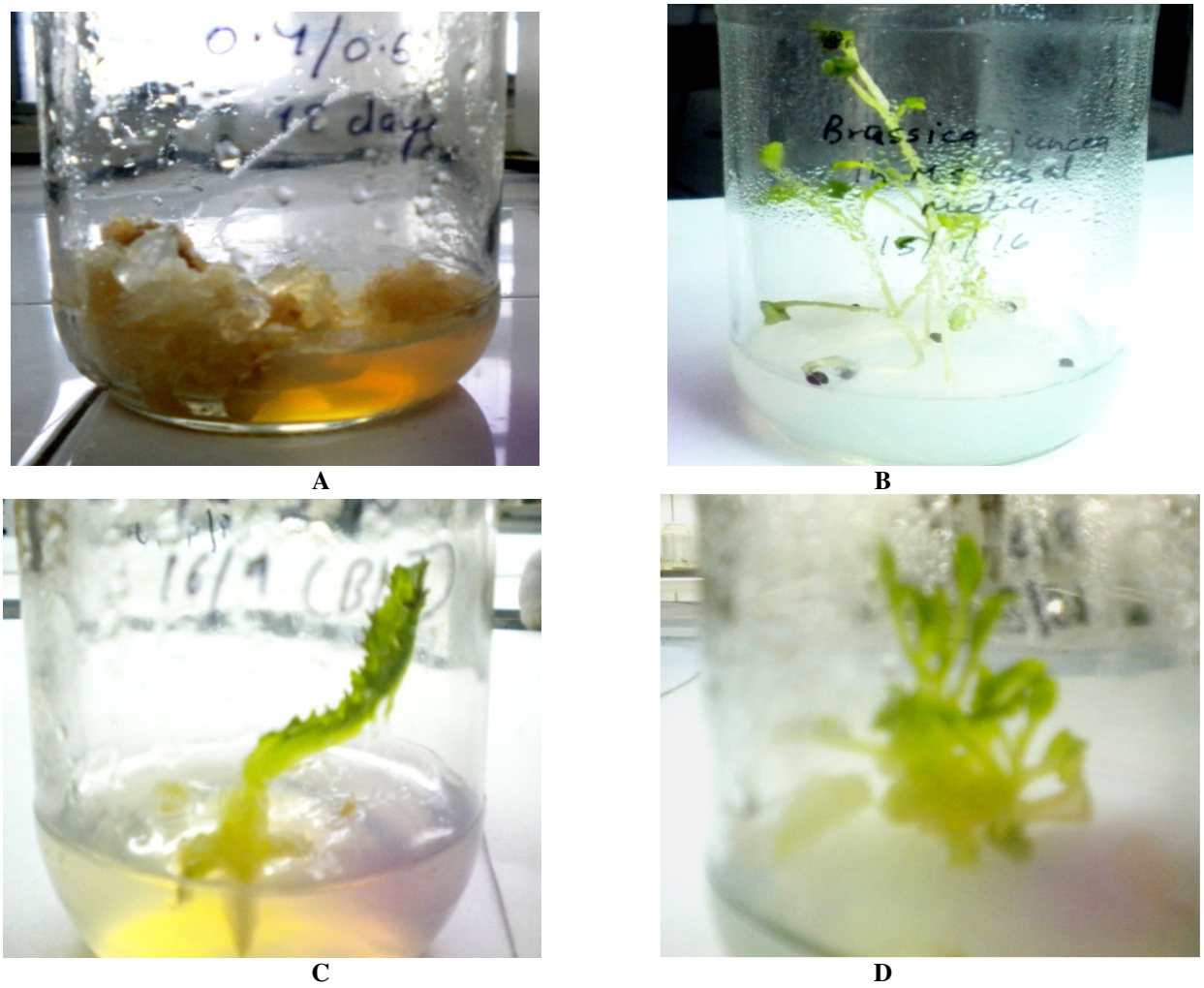
Brassica seeds started germination after 2-3 days after inocultion on the MS medium figure-1a. The matured seedlings with two-leaf stages were arisen at 8-10 days after germination of the seeds (data not shown). Similar results were also reported earlier[9,10]. The hypocotyls of the *in vitro* grown seedlings were used as explants in the present study. Earlier, hypocotyls segment of *in vitro* grown *Brassica* seedlings have been used[11]. The hypocotyls of uniform size (1 cm) were taken in the present study.

The number of explants inducing callus and producing shoots varied at different hormonal treatment Figure-1b,c. 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 shootlets/explants Figure-1d. It was also observed that at 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 about 8.3-33.3% explants showed shoot formation with as less as 1-2 shootlets per explant. 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[1]. 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. Minimum callus induction has been reported earlier in MS medium, supplemented with 2 mg/l BAP and 5 mg/l Kinetin[3]. Effect of heavy metals on callus induction and regeneration has also been studied earlier[12]. Previously, cent percent shoot regeneration has been reported in the MS medium supplemented with 2mg/L of BAP and 0.01mg/L of NAA[13]. Successful shoot regeneration up to 90% from hypocotyls of *Brassica* sp callus has been achieved at 2mg/L of BAP[4]. Regeneration of 67-82% shoots from hypocotyls derived callus at different concentrations of hormones BAP (2mg/L) and L Silver nitrate (5mg/L) in MS medium having 0.1mg/L NAA has been reported[14].

Table-1
Callus and shoot Induction of hypocotyls of *in vitro* grown seedling of *Brassica juncea* var Pusa Jaikisan

MS media supplemented with BAP and NAA	Callus induction*			Shoot induction after keeping the callus in the same medium**	
	No. of explants used for callus formation	No. of explant producing callus	Percent explants inducing callus formation (Frequency)	No. of explant indeuced formation	Percent explants inducing shoot formation (Frequency)
CRM1. 0.5 B+0.2 N	12	7	58.33	-	-
CRM2. 2.0 B+0.2 N	10	7	70	-	-
CRM3. 1.0 B+1.0 N	12	11	91.6	1(20)	8.3
CRM4. 1.0 B+0.5 N	12	12	100	1(10)	8.3
CRM5. 0.5 B+0.5 N	12	12	100	1(7)	8.3
CRM6. 0.5 B+1.0 N	12	12	100	1(15)	8.3
CRM7. 2.5 B+1.0 N	12	5	41.6	2 (1)	16.6
CRM8. 0.5 B+1.5 N	6	12	50	1 (1)	16.6
CRM9. 2.5 B+0.5 N	12	11	100	1 (1)	12.5
CRM10. 2.5 B+1.5 N	12	10	91.66	1 (1)	12.5
CRM11. 2.0 B+1.0 N	12	9	83.33	1 (1)	12.5
CRM12. 2.0 B+1.5 N	16	12	56.25	3 (2)	18.7
CRM13. 2.0 B+0.5 N	12	8	100	4 (2)	33.3
CRM14 0.5 B+0.1 N	8	-	100	1 (1)	12.5

B: BAP; N: NAA; Numerical denotes the concentration of the hormone/L; Parentesis denotes maximum number of shoot/explants produced; *: data taken after 4 weeks, **: data taken after six weeks



A **B**

C **D**

Figure-1 a-d

Callus formation and shoot Induction in inoculated hypocotyls of mustard var Pusa Jaikishan at different concentrations of BAP and NAA in MS medium; A: germination of seeds in to seedlings B:callus formation at MS medium supplemented with 1.0 mg/L +1.0 mg/L of BAP +NAA, shoot formation at 1.0 mg/L+1 mg/L of BAP +NAA (B/N), C: shoot initiation at 0.5 mg/L +1.0 mg/L of BAP +NAA, D: multiple shoot formation at 2.5 mg/L+0.5 mg/L of BAP +NAA

The present study revealed that MS medium supplemented with 0.5-2.5 mg/L of BAP along with 0.5-1.0mg/L of NAA gives good response to maximum callus induction and shoot regeneration for mustard. The increased amount of NAA (>1.5 mg/L) decreased the callus formation. As the effect a combination of growth regulators on callus induction was genotype-dependent, it is concluded that mustard var. Pusa Jaikisan is more responsive to *in vito* regeneration in minimum concentration of hormones. Genetic modification can be done in the enzymes which involve in phytohormones production pathways, which can change the architecture of plant, as earlier genetic modification in gibberelin oxidase has been done to change plant architecture[15]. Finally, it was concluded that the regeneration protocol developed in the present investigation is reliable and it would be effectively utilized for further genetic transformation of *Brassica* sp using different genetic traits of crop improvement.

Conclusion

On the basis of results it may be concluded that regeneration protocol developed in the present investigation for *Brassica juncea* var. Pusa Jaikisan is reliable and can be effectively utilized for genetic transformation of *Brassica* species.

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