Pak. J. Bot., 42(2): 963-969, 2010.

DEVELOPMENT OF AN EFFICIENT REGENERATION PROTOCOL FOR THREE GENOTYPES OF *BRASSICA JUNCEA*

RAISA BANO[1,2], MOHAMMAD HAROON KHAN[1,2], RAHAM SHER KHAN[1], HAMID RASHID[2*] AND ZAHOOR A. SWATI[1]

[1]*Institute of Biotechnology and Genetic Engineering, NWFP Agricultural University Peshawar, Pakistan*
[2]*Department of Bioinformatics, Mohammad Ali Jinnah University, Islamabad, Pakistan*

Abstract

Two phytohormones, auxins (Naphthalene acetic acid and Indole acetic acid) and cytokinins (Benzylaminopurine and Kinetin) with concentrations were used to develop an efficient regeneration protocol for 3 genotypes of *Brassica juncea* (UCD-635, RL-18 and NIFA RAYE). The explants were cultured on MS-medium supplemented with BAP 1.0 mgL^{-1}/NAA 0.1 mgL^{-1}, BAP 2.0 mg L^{-1}/NAA 0.2 mg L^{-1}, BAP 3.0 mgL^{-1}/NAA 0.3 mg L^{-1} and Kinetin 1.0 mg L^{-1}/IAA 0.1 mg L^{-1}, Kinetin 2.0 mg L^{-1}/IAA 0.2 mg L^{-1}, Kinetin 3.0mg L^{-1}/IAA 0.3 mg L^{-1}. Maximum callus production (65.55) was observed on MS medium containing with BAP 2.0 mgL^{-1}/NAA 0.2 mg L^{-}1. Maximum shooting (22.31) was observed BAP 3.0 mg L^{-1}/NAA 0.3 mg L^{-1} and KIN 3.0 mg L^{-1}/IAA 0.3 mg L^{-1}. Regeneration efficiency was found maximum (7.13) with BAP 3.0 mg L^{-1}/NAA 0.3 mg L^{-}1. The three genotypes were found significantly different at $p \leq 0.05$ in shoots production and regeneration efficiency.

Introduction

Brassica oilseed species hold the third position among oilseed crops. The oilseed Brassica species cultivation has increased tremendously during the last decade and by now it is the second largest contributor to the world supply of vegetable oil (Zhou, 2001). Due to the growing world population and increasing industrialization, the demand for edible oil and biofuels is increasing; thus cultivation of oilseed crops has gained great importance (Indrajit *et al*., 2008)

Pakistan has been facing a chronic shortage of edible oil, a large quantity of edible oil is imported annually from other countries to fill the gap between local production and consumption. In 2003~2004, the total consumption was estimated as 2.199 million tons and the local production was sufficient to meet merely 29% of the consumption; while the remaining 71% was met through imports (Anon., 2004). In addition, the import of edible oil is continuously increasing with an alarming rate of 13% annually (Razi, 2004).

Indian mustard [*Brassica juncea* (L.) Czern.] is a major oilseed crop throughout the world and is cultivated on more than 6 million hectares in the Indian subcontinent and some parts of Australia, Canada, China and Russia (Kumar 1999). Mustard has become an important target for crop improvement as productivity of this crop is constrained by several abiotic and biotic stresses (Grover & Pental 2003; Dutta *et al*., 2005).

Crosses between genetically divergent cultivars to produce hybrid *B. juncea* with enhanced productivity through traditional breeding are time consuming and labor-intensive. Additionally, suitable donor varieties tolerant to salt stress and resistant to insect attack are not available within the mustard germplasm. As a result, development of biotechnological approaches and deployment of transgenic plants has become increasingly important for improved and sustainable production of mustard (Indrajit *et al*., 2008).

*Corresponding author E-mail: drhamid@jinnah.edu.pk

Regeneration in Brassica is highly genotype dependent and has been reported in several species (Ono *et al*., 1994). The available information shows that regeneration through organogenesis has been accomplished from various tissues including cotyledons (Hachey *et al*., 1991; Ono *et al*., 1994), hypocotyls (Khehra & Mathias, 1992; Phogat *et al*., 2000), peduncle (Eapen & George 1997), leaves (Radke *et al*., 1988), thin cell layers of epidermis and sub epidermis (Klimaszewska & Keller, 2002), and protoplasts (Hu *et al*., 1999).

In most of the reports regarding tissue culture and genetic transformation of *Brassica* species, *B. juncea* has not yet been explored. An efficient regeneration protocol for *B. juncea* is needed to be established for its use in transformation experiments. The present study is an attempt to develop and standardize an efficient and high frequency regeneration system for different genotypes of *Brassica juncea*.

Materials and Methods

Three different genotypes of *Brassica juncea* (UCD-635, RL-18 and NIFA-RAYE) used in this study were provided by the Institute of Biotechnology and Genetic Engineering (IBGE), NWFP Agricultural University, Peshawar, Pakistan. For surface sterilization, Brassica seeds were washed with tap water and dipped in 70% ethanol for 30 seconds. Then the seeds were treated with Sodium hypochlorite solution (1% active chlorine) containing a few drops of TWEEN-20 for 20-25 minutes followed by rinsing 3-4 times with sterilized distilled water**.** After sterilization, seeds were cultured for germination on half strength MS medium (Murashige & Skoog, 1962) consisting of 2% sucrose and solidified with 0.8% agar. Explants were taken from 3-5 days old seedlings. The basal and apical portions of hypocotyls were discarded and the middle part was cut into 5 mm long segments. MS medium supplemented with 3% sucrose, different concentrations of auxins (indole-3-acetic acid, α-naphthalene acetic acid) and cytokinins (Benzyl amino purine and Kinetin) and solidified with 0.8% agar was used to induce calli and shoots from all the 3 genotypes.

Data on the following parameters were recorded:

- Days to callus formation.
- Number of explants forming roots.
- Days to roots formation.
- Number of explants producing shoots on MS medium supplemented with different concentrations of PGRs.
- Days to shoot production.
- Regeneration efficiency of shoots from, RE = Shoots produced x 100/Number of explants.
- Effect of different concentrations of PGRs on all the above mentioned parameters

After culturing explants on MS medium supplemented with different concentrations of plant growth regulators, cultures were incubated at 25^{o}C under 16 h light/8 h dark conditions and observed regularly for callus initiation and/or shoot formation. Rooted shoots were transferred to the soil in pots and acclimatized to the greenhouse conditions.

Results

1. Effect of BAP/NAA

Days to callus formation: Mean values for the days to callus formation by 3 *Brassica juncea* genotypes showed that the minimum number of days (10.043) taken by explants to produce callus was recorded on MS medium supplemented with BAP 2 mgL^{-1} and NAA 0.2 mgL^{-1}, whereas the maximum number of days (15.63) taken by explants to produce callus was noted on MS medium containing BAP 1 mgL^{-1} and NAA 0.1 mg L^{-1}. (Table 1) The least significant difference for days to callus formation was recorded to be 1.47 at $p \geq 0.05$.

Days to shoot initiation: The mean values recorded for days to shoot initiation showed that the 3 *Brassica juncea* genotypes responded differently on MS medium supplemented with different concentrations of BAP and NAA (Table 1). The two hormones with their different concentrations (BAP 1 mgL^{-1}/NAA 0.1 mgL^{-1}, BAP 2 mgL^{-1}/NAA 0.2 mgL^{-1} and BAP 3 mgL^{-1}/NAA 0.5 mgL^{-1}) significantly affected the number of days to shoots initiation. The minimum number of days recorded were 22.48 to produce shoots on MS medium supplemented with BAP 2 mgL^{-1} and NAA 0.2 mg L^{-1} while the maximum numbers of days (38.48) was recorded for shoots produced on MS medium supplemented with BAP 3 mgL^{-1} and NAA 0.5 mgL^{-1}. MS medium containing BAP 2 mgL^{-1} and NAA 0.2 mgL^{-1} caused shoot initiation earlier. The least significant differences were recorded to be 3.37 at $p \leq 0.05$.

Number of explants produced shoots: Mean values recorded for the number of explants produced shoots from the 3 *Brassica juncea* genotypes, on MS medium containing 3 different concentration of BAP and NAA (Table 1) showed that the minimum number of explants (6.29) produced callus on MS medium containing BAP 1 mgL^{-1} and NAA 0.1 mgL^{-1} while the maximum number of explants (22.31) produced callus was recorded on MS medium supplemented with BAP 3 mgL^{-1} and NAA 0.5 mgL^{-1}. The least significant differences observed for number of explants produced shoots was 9.3 at $p \leq 0.05$.

Regeneration efficiency: Mean values for regeneration efficiency i.e., % explants forming shoots of 3 *Brassica juncea* genotypes, showed that minimum value (2.06) was recorded in MS medium containing BAP 1 mgL^{-1} and NAA 0.1 mgL^{-1} whereas maximum regeneration efficiency (7.13) was recorded on MS medium containing BAP 3 mgL^{-1} and NAA 0.5 mgL^{-1} (Table 1). The least significant differences recorded for regeneration efficiency was 3.15 at $p \leq 0.05$. Whereas non-significant values were recorded for number of explants forming callus, number of explants forming shoots and days to roots formation for the three *Brassica juncea* genotypes. The effect of different hormone concentrations was found non-significant for aforementioned three parameters (Table 1).

2. Effect of Kinetin/IAA

Days to callus formation: Mean values for days to callus formation by 3 genotypes (UCD-635, RL-18 and N-R) of *Brassica juncea* cultured on 3 different concentrations of kinetin/indole acetic acid showed that minimum value (14.59) was recorded for the hormone concentration, kinetin 2 mgL^{-1}/indole acetic acid 0.2 mgL^{-1}, whereas the maximum value (18.33) was recorded for the hormone concentration, kinetin 1 mgL^{-1}/indole acetic acid 0.1 mgL^{-1} by the three genotypes of *Brassica juncea* (Table 1). Least significant differences (5.35) were recorded among these 3 hormonal concentrations at $p \leq 0.05$.

Table 1. Mean values for the parameters, XPC, DCF, EFR, DRF, DSI, NPS and RE of the three *Brassica juncea* genotypes, UCD-635, RL-18 and NIFA-RAYE affected by different combinations and concentrations of hormones.

Hormone	XPC	DCF	EFR	DRF	DSI	NPS	RE
	MS medium containing BAP/NAA						
H_1	60.27	14.00b	46.65	14.33	28.22b	6.29b	2.06b
H_2	65.55	15.63c	46.03	15.37	22.48a	15.28ab	4.83ab
H_3	60.42	10.04a	57.05	15.66	38.48c	22.31a	7.13a
LSD		**1.47**			**3.37**	**9.3**	**3.15**
	MS medium containing Kinetin/IAA						
H_4	46.16	18.33a	54.07	21.25	34.03c	5.38b	0.58a
H_5	48.29	14.59b	50.32	16.70	22.41a	11.85b	0.88a
H_6	50.79	16.30b	50.63	16.44	27.11b	22.31a	1.08a
LSD		**5.35**			**4.27**	**7.51**	**2.76**

Means within a column having the same letters are not significantly different ($p \geq .05$).
H_1 = BAP 1.0 mgL^{-1}/NAA 0.1 mgL^{-1} H_2 = BAP 2.0 mgL^{-1}/NAA 0.2 mgL^{-1} H_3 = BAP 3.0 mgL^{-1}/NAA 0.5 mgL^{-1}, H_4= Kinetin 1 mgL^{-1}/IAA 0.1 mgL^{-1} H_5 = Kinetin 2 mgL^{-1}/IAA 0.2 mgL^{-1}, H_6 = Kinetin 3 mgL^{-1}/IAA 0.5 mgL^{-1}
XPC= Number of explants producing callus, DCF= Days to callus formation, EFR= Number of explants forming roots, DRF= Days to roots formation, DSI= Days to shoots initiation, NPS= Number of explants forming shoots, RE= Regeneration efficiency

Days to shoots initiation: Mean values for days to shoots initiation for the 3 *Brassica juncea* genotypes on three different concentrations of hormones (kinetin/indole acetic acid), the minimum value (22.41) was observed at the hormone concentration, kinetin 2 mgL^{-1}/indole acetic acid 0.2 mgL^{-1}, whereas maximum value (34.03) was noted for the hormone concentration, kinetin 1 mgL^{-1}/indole acetic acid 0.1 mgL^{-1} (Table 1). Shoots initiation was efficient on hormone concentration kinetin 2 mgL^{-1}/indole acetic acid 0.2 mgL^{-1}. The LSD value (4.27) was recorded for the days to callus initiation at $p \leq 0.05$.

Number of explants producing shoots: Mean values for number of explants produced shoots by the 3 *Brassica juncea* genotypes on the 3different hormone concentrations of kinetin/indole acetic acid are given in (Table 1). The minimum value (5.38) was recorded for kinetin 1 mgL^{-1}/indole acetic acid 0.1 mgL^{-1} by the three genotypes collectively, whereas the maximum value (22.31) was observed for hormone concentration kinetin 3 mgL^{-1}/indole acetic acid 0.5 mgL^{-1}. Maximum number of shoots was produced on MS medium supplemented with kinetin 3 mgL^{-1}/indole acetic acid 0.5 mgL^{-1}. The least significant differences (7.51) were recorded among these three hormonal combinations for shoots production at $p \leq 0.05$.

Regeneration efficiency: It is the percentage of explants which produced shoots on 3 different hormonal combinations of kinetin/indole acetic acid (kinetin 1 mgL^{-1}/indole acetic acid 0.1 mgL^{-1}, kinetin 2 mgL^{-1}/indole acetic acid 0.2 mgL^{-1} and kinetin 3 mgL^{-1}/indole acetic acid 0.5 mgL^{-1}). Mean values for regeneration efficiency (Table 1), of the 3 *Brassica juncea* genotypes, show that the minimum value (0.58) was calculated for hormonal combination, kinetin 1 mgL^{-1}/indole acetic acid 0.1 mgL^{-1} while the maximum value (1.08) was recorded for the hormone concentration, kinetin 3 mgL^{-1}/indole acetic acid 0.5 mgL^{-1}. The regeneration efficiency of the 3 genotypes was higher at hormonal combination, kinetin 3 mgL^{-1}/indole acetic acid 0.5 mgL^{-1} as compared to the other two hormonal combinations. The least significant differences (2.76) were calculated for the three concentrations of kinetin/indole acetic acid at $p \leq 0.05$.

3. Effect of genotype

MS medium containing BAP/NAA, mean values regarding days to callus formation showed that the minimum value (13.04) was recorded for the genotype (G_2) (RL-18) on MS medium containing BAP 2 mgL^{-1} and NAA 0.2 $mgL^{-1}$and the highest value (17.07) was recorded for the genotype (G_3) (NIFA-RAYE) on MS medium containing BAP 3 mgL^{-1} and NAA 0.5 mgL^{-1}. The genotype, RL-18 was found more responsive to callus production than the other two genotypes, UCD-635 and NIFA-RAYE. The least significant differences recorded for the days to produce callus was 0.50 at $p \leq 0.05$.

Days to callus formation by the 3 different genotypes of *Brassica juncea* (UCD-635, RL-18 and NIFA-RAYE) on MS medium containing Kinetin/IAA were also counted. Mean values for days to callus formation showed that the minimum value (12.78) was recorded for genotype NIFA-RAYE while maximum value (15.11) was observed for genotype RL-18. Less number of days to initiate callus was recorded for NIFA-RAYE, while genotype RL-18 took more number of days to initiate callus. The least significant differences (5.35) for these three genotypes of *Brassica juncea* were calculated at $p \leq 0.05$.

The mean values recorded for the number of explants producing callus, number of explants forming roots, days to roots formation, days to shoots initiation, number of explants produced shoots and their regeneration efficiency were found statistically non-significant for the three genotypes (Table 2).

Discussion

In this study effort was made to develop an efficient regeneration protocol for 3 genotypes of *Brassica juncea* (UCD-635, RL-18 and NIFA-RAYE) using two types of plant growth regulators with their three levels of concentrations. The explants were cultured on MS medium supplemented with different concentration of hormones and incubated at 25^{o}C under 16 h light/8 h dark conditions and observed regularly for callus initiation and/or shoot formation.

Table 2. Mean values of the parameters, XPC, DCF, EFR, DRF, DSI, NPS and RE of the three *Brassica juncea* genotypes.

Genotype	XPC	DCF	EFR	DRF	DSI	NPS	RE
	MS medium containing BAP/NAA						
G_1	58.89	16.56b	53.50	14.33	24.14	15.64	5.06
G_2	64.55	13.04a	49.68	15.37	25.07	14.90	4.74
G_3	62.80	17.07c	46.55	15.66	24.96	13.33	4.21
LSD		**0.50**					
	MS medium containing Kinetin/IAA						
G_1	47.08	13.33b	51.31	16.81	23.88	13.98	4.64
G_2	47.06	15.11a	51.66	21.51	25.22	11.87	4.36
G_3	51.10	12.78ab	52.05	16.07	24.44	13.70	4.54
LSD		**5.35**					

Means within a column having the same letters are not significantly different ($p \geq 0.05$).
G1= UCD-635, G2= RL-18, G3= NIFA-RAYE
XPC= Number of explants producing callus, DCF= Days to callus formation, EFR= Number of explants forming roots, DRF= Days to roots formation, DSI= Days to shoots initiation, NPS= Number of explants forming shoots, RE= Regeneration efficiency

The explants exhibited an initial swelling followed by callus formation within two weeks of incubation. It was noted that callus proliferation started from cut ends of the explants cultured on MS media containing different concentration of auxins and cytokinin and eventually extended all over the explants. Approximately, 3 weeks after culturing, the explants almost completely converted into callus. In general, a high percentage of explants formed callus. Similar results have been described in other reports (Muhammad *et al*., 2002: Moghaieb *et al*., 2006). However, no significant differences in callus induction between the cultivars were observed (Tables 1). Callus from the cotyledon was found more regenerative than that from the other two explants. *Brassica* explants also produced white, hairy and dense adventitious roots. Adventitious root production from the cotyledon explants was found more profusely.

The response of different genotypes to callus formation varied considerably. Also the time taken by the explants of the three genotypes to produced callus was found different. Such results can be attributed to the genetic differences of the genotypes. Genotypic influence on *In vitro* morphogenesis in *Brassica* has been documented previously (Dietert *et al*., 1982; Fazekas *et al*., 1986; Khehra & Mathias, 1992). Such reports indicate that the developmental processes reflected by *in vitro* response are genetically controlled. Total number of shoots regenerated varied significantly among the three genotypes. During this study a maximum number of shoots (> 40) were produced from UCD-635. However, mean separation by MSTATC showed that the three genotypes of *Brassica juncea* were not statistically different in their shoot regeneration capacity. The genotype, NIFA-RAYE showed a poor response to shoot regeneration (Table 2).

References

Anonymous. 2004. *Economic Survey Report 2003~2004.Government of Pakistan, Finance Division. Islamabad*.

Dietert, M.F., S.A. Barron and O.C. Yoder. 1982. Effects of genotype on *In vitro* culture in the genus *Brassica*. *Plant Sci Let*., 26: 233-240.

Dutta, I., P. Majumder, P. Saha, K. Ray and S. Das. 2005. Constitutive and phloem specific expression of *Allium sativum* leaf agglutinin (ASAL) to engineer aphid (*Lipaphis erysimi*) resistance in transgenic Indian mustard (*Brassica juncea*). *Plant Sci*., 169: 996-1007.

Eapen, S. and L. George. 1997. Plant regeneration from peduncle segments of oil seed *Brassica* species: influence of Silver nitrate and Silver thiosulfate. *Plant Cell Tiss. Organ Cult*., 51: 229-232.

Fazekas, G.A., P.A. Sedmach and M.V. Palmer. 1986. Genetic and environmental effects on *In vitro* shoot regeneration from cotyledonary explants of *Brassica juncea. Plant Cell Tiss Organ Cult*., 6: 177-180.

Grover, A. and D. Pental. 2003. Breeding objectives and requirements for producing transgenics for major field crops of India. *Curr. Sci*., 84: 310-320.

Hachey, J.E., K.K. Sharma and M.M. Moloney. 1991. Efficient shoot regeneration of *Brassica campestris* using cotyledon explants cultured *In vitro*. *Plant Cell Rep*., 9: 549-554.

Hu, Q., S.B. Anderson and L.N. Hansen. 1999. Plant regeneration capacity of mesophyll protoplasts from *Brassica napus* and related species. *Plant Cell Tiss. Organ Cult*., 59: 189-196.

Indrajit, D., S. Prasenjit and D. Sampa. 2008. Efficient *Agrobacterium*-mediated genetic transformation of oilseed mustard [*Brassica juncea* (L.) Czern.] using leaf piece explants. *In Vitro Cell. Dev. Biol. Plant*, 44: 401-411.

Khehra, G.S. and R.J. Mathias. 1992. The interaction of genotype, explant and media on the regeneration of shoots from complex explants of *Brassica napus* L. *J. Exp. Bot*., 43: 1413-1418.

Klimaszewska, K. and K. Keller. 2002. High frequency plant regeneration from thin cell layer explants of *Brassica napus*. *Plant Cell Tiss. Organ Cult*., 4: 183-197.

Kumar, P.R. 1999. *Rapeseed mustard research in India: 21st century strategies*. 10th International Rapeseed Congress, Canberra, Australia.

Moghaieb, R.E.A., M.A. El-Awady, R.G.E. Mergawy, S.S. Youssef and A.M. El-Sharkawy. 2006. A reproducible protocol for regeneration and transformation in canola (*Brassica napus* L.). *Afric. J. Biotechnol*., 5(2): 143-148.

Muhammad, R.K., H. Rashid and A. Quraishi. 2002. Effects of various growth regulators on callus formation and regeneration in *Brassica napus* Cv. Oscar. *Pakistan J. Biol. Sci*., 5: 693-695.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*., 15: 473-379.

Ono, Y., Y. Takahata and N. Kaizuma. 1994. Effect of genotype on shoot regeneration from cotyledonary explants of rapeseed (*Brassica napus* L.). *Plant Cell Rep*., 14: 13-17.

Phogat, S.K., P.K. Burma and D. Pental. 2000. High frequency regeneration of *Brassica napus* varieties and genetic transformation of stocks containing fertility restorer genes of two cytoplasmic male sterility systems. *J. Pl. Biochem. Biotechnol*., 9: 73-79.

Radke, S.E., B.M. Andrews, M.M. Moloney, M.L. Crouch, J.C. Krid and V.C. Knauf. 1988. Transformation of *Brassica napus* L., using *Agrobacterium tumefaciens*: developmentally regulated expression of a reintroduced napin gene. *Theor. Appl. Genet*., 75: 685-694.

Razi, R. 2004. Edible oil worth Rs-31 billion imported. *Business News. Daily The NEWS*, Islamabad-Pakistan. July 26, pp. 08.

Sharma, K.K., S.S. Bhojwani and T.A. Thorpe. 1990. Factors affecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. *Plant Sci*., 66: 247-253.

Yang, M.Z., S.R. Jia and E.C. Pua. 1991. High frequency of plant regeneration from hypocotyls explants of *Brassica carinata* A.Br. *Plant Cell Tiss. Organ Cult*., 24 :79-82.

Zhang, Y. and P.L. Bhalla. 1998. Shoot regeneration potential from seedling explants of Australian cultivars of oil seed rape (*Brassica napus* L.). 10th International Rapeseed Congress, Canberra, Australia.

Zhou W.J. 2001. Oilseed rape. In: *Crop Cultivation*. (Eds.): G.P. Zhang and W.J. Zhou. Zhejiang University Press, Hangzhou, China, 153-178.

(Received for publication 20 November 2008)