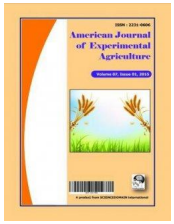

American Journal of Experimental Agriculture
8(6): 342-349, 2015, Article no.AJEA.2015.178
ISSN: 2231-0606

SCIENCEDOMAIN *international*
www.sciencedomain.org

Somatic Embryo Formation from Co-cultivated Protoplasts of *Brassica rapa* & *B. juncea*

Md Jasim Uddin[1], Arif Hasan Khan Robin[1*], Sharif-Ar Raffi[1] and Sabrina Afrin[1]

[1]Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

Authors' contributions

This work was carried out in collaboration between all authors. Author AHKR planned and designed the experiment. Author MJU and SA executed lab work. Author MJU analyzed data and prepared primary manuscript. Authors AHKR and SAR supervised the experiment and provided editorial comments. All authors read and approved the final version of the manuscript.

Article Information

DOI: 10.9734/AJEA/2015/17696
Editor(s):
(1) Lanzhuang Chen, Laboratory of Plant Biotechnology, Faculty of Environment and Horticulture, Minami Kyushu University, Miyazaki, Japan.
Reviewers:
(1) Anonymous, Sunchon National University, South Korea.
(2) Funda Eryilmaz Acikgoz, Department of Plant and Animal Production, Namik Kemal University, Turkey.
(3) Anonymous, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1080&id=2&aid=9632

Original Research Article

Received 25th March 2015
Accepted 28th April 2015
Published 6th June 2015

ABSTRACT

Aims: The objectives of this experiment was to establish initial steps of somatic embryogenesis from the fused protoplasts between two *Brassica* species: *Brassica rapa* L. (var. Agrani, AA, 2n=20) and *Brassica juncea* L. (var. BINA Sharisha-7 and var. BINA Sharisha-8, AABB, 2n=36).
Study Design: The experiment was carried out in completely randomized design with three replications for each hormone treatment of each variety combination.
Place and Duration of Study: An experiment was conducted in growth room of tissue culture laboratory at the Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh during the period from April, 2013 to May, 2014.
Methodology: Mesophyll protoplasts were isolated from the young leaves of aseptically grown plants. Variety combinations for protoplasts fusion were: Agrani+BINA Sharisha-7 and Agrani+ BINA Sharisha-8. 100% polyethylene glycol-400 was used as a fusogen. Pellieter different compositions of liquid media were used for protoplasts cultured, microcalli, calli and somatic embryo formation.
Results: Fused products were cultured in a modified Pellieter B liquid media with different

**Corresponding author: E-mail: gpb21bau@bau.edu.bd; gpb21bau@gmail.com;*

combinations of auxins and cytokinin for callus induction. A medium having 1 mg L^{-1} NAA + 0.5 mg L^{-1} 2,4-D produced micro-calli of 518 µm diameter on 15 days after dark treatment. The same hormone combinations produced 1.6 mm visible calli after 24 days of incubation. After 82 days of culture, a medium composition of 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA resulted in 76% somatic embryo formation. Agrani+BINA Sarisha-7 produced significantly higher somatic embryos compared to Agrani+BINA Sarisha-8.

Conclusion: Our results might be helpful for developing a complete and efficient protocol of somatic hybridization in *Brassica* species.

Keywords: Protoplast fusion; somatic embryo formation; Brassica rapa; Brassica juncea; polyethylene glycol.

1. INTRODUCTION

Brassica rapa (AA, 2n=20) and *B. juncea* (AABB, 2n=36) are the two prime oil producing mustard species in Bangladesh. *B. juncea* is a hybrid between *B. rapa* (n=10, A genome) and *B. nigra* (n=8, B genome) and all of them are U triangle species [1-3, Fig. 1]. In Bangladesh, approximately 70% edible oil comes from mustard and rest is covered by other oil producing crops [4]. Majority of the cultivated mustard varieties in Bangladesh are low yielding approximately 1.0 t ha^{-1} compared to European varieties that yield more than 2 t ha^{-1} [5]. Furthermore, existence of low range of variation for those mustard species make the situation more worse for improving mustard varieties through conventional breeding. Due to cross incompatibility among the U triangle species the scope of creating variation and resynthesizing *Brassica* through conventional breeding is limited to some extent [6,7]. Therefore, application of different available biotechnological tools is required to increase variability in mustard germplasm in Bangladesh. Somatic hybridization is a potential tissue culture technique might be used to overcome interspecific cross incompatibility [8] with a view to increase variability in mustard germplasm. This technique is capable of overcoming sexual incompatibility among the U- triangle species, i.e. *B. juncea* and *B. rapa* [9]. Considering the available advantages of somatic hybridization of overcoming incompatibility barriers [8,9], an experiment was planned to establish initial steps of somatic hybridization between *B. rapa* and *B. juncea* which includes evaluation of suitability of different hormone combinations for calli induction and subsequently somatic embryo formation from the fused cells.

2. MATERIALS AND METHODS

Two varieties of *B. juncea* namely BINA Sarisha-7 and BINA Sarisha- 8 and one genotype of *B. rapa* namely Agrani were selected for this study. Thus the fusion combinations were: i) BINA Sarisha-7 x Agrani and ii) BINA Sarisha-8 x Agrani. The seeds of all three varietiess were collected from the Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. These varieties were used as the donor plants for protoplast fusion. Seeds were surface-sterilized for 3 min in 70% ethanol and 2 min in a 0.1% mercuric chloride ($MgCl_2$) solution and then rinsed three times with sterile distilled water. Seeds were placed in vials containing Pelletier A media (Table 1) supplemented with 30 gL^{-1} sucrose and solidified with 9 g L^{-1} agar (pH 5.8, adjusted before autoclaving) for germination [10]. The vials were kept under controlled conditions (Tem. 22±2ºC, 16 hour photoperiod and fluorescent light, 50 µ mol m^{-2} s^{-1}, see Fig. 2a).

2.1 Protoplast Isolation

Approximately 0.5 g broad young leaves from 22 days old plants were segmented to 1.0 mm slices and placed separately to 60 mm glass Petri dishes with 5 mL of an enzymatic solution (mixture of 1% Cellulase, Sigma-Aldrich and 0.1% pectinase, Sigma-Aldrich in modified B media (Table 1). Plasmolisation in the osmoticum was carried out on a shaker with 20 rpm at room temperature in the dark, for 16 to 18 h (Fig. 2b). The protoplast mixture was filtered through a 100 µm cotton mesh and distributed into four 15 mL conical centrifuge tubes for each varieties, 10 mL mixture per tube (Fig. 2c). Protoplasts were pelleted by centrifugation with 1000 rpm, 5 min and the supernatant was discarded. The protoplasts were washed by re-suspending the pellet in the W5 wash solution [11] containing 16% sucrose and again centrifuged two times at 800 rpm for 7 min. After the final rinse with W5 wash solution and repeating final centrifugation at 800 rpm for 7 min, pellets of viable protoplasts were separated by discarding the supernatant, and observed under a microscope. Before fusion, 2 mL of modified B media (Table 1) was

added onto the pellets of the protoplasts and those were suspended to prepare a mixture of 10^4-10^5 protoplasts per mL [10]. The size of five isolated protoplasts from each variety (Agrani, BINA Sarisha-7 and BINA Sarisha-8) was measured with an eye-piece micrometer (µm) scale.

2.2 Protoplasts Fusion

About 80 µL of protoplast suspension of each of two different varieties were pipetted onto the bottom of a petri dish and placed side-by-side. Eighty microliters of fusogen containing 100% PEG (Mn 400, Sigma), 0.15% (w/v) $CaCl_2.2H_2O$ and 0.01% (w/v) KH_2PO_4 (pH 5.8) was added gently in between two protoplast drops of two varieties. After 10 min, 160 µ1 of solution containing 5% (w/v) glucose, 0.735% (w/v) $CaCl_2.2H_2O$, (pH 10.5) was poured to enhance protoplast fusion process.

2.3 Culturing Fused Protoplasts towards Micro-Calli Formation

Fused protoplasts were cultured in 60 mm petri dishes in 4.0 ml of liquid culture medium B (Table 1) supplemented with 0.5 mg L^{-1} NAA+ 0.5 mg L^{-1} 2,4-D+ 0.5 mg L^{-1} BAP and 3% sucrose [10]. The cultures were kept at 22±2ºC in dark condition. After 7 days 2 mL modified Pelletier C media (Table 1) which supplemented with three different hormonal combinations a) 0.5 mg L^{-1} NAA+ 0.5 mg L^{-1} 2,4-D, b) 1.0 mg L^{-1} NAA+ 0.5 mg L^{-1} 2,4-D or c) 0.5 mg L^{-1} NAA+ 0.5 mg L^{-1} 2,4-D+ 0.5 mg L^{-1} BAP was added to each petri-dish [10]. Culture medium was refreshed after 11 days with same composition for further development of micro-calli. There were three replicates for each hormone combination meaning that there were a total of six replicates for each varieties combination (fusion combination) × hormone combination and the experiment was conducted following a completely randomized design. Cultures were maintained in dark condition over 15 days under controlled temperature (25±2ºC) for micro-callus induction. Star-shaped micro-calli developed within 15 days of culture as observed under a light microscope. Diameter of micro-calli was measured using an eye-piece micrometer scale.

2.4 Somatic Embryogenesis from Micro-Calli

When the micro-calli became visible (0.5-1.0 mm) they are removed aseptically from the culture vessels and placed onto a sterilized petri dish inside the laminar airflow cabinet. The micro-calli were placed into modified Pelletier D media (Table 1) containing two different hormonal combinations a) 0.5 mg L^{-1} NAA+ 0.5 mg L^{-1} BAP and b) 0.5 mg L^{-1} NAA+ 1 mg L^{-1} BAP. The cultures were kept at 23ºC under fluorescent light (50 µ mol m^{-2} s^{-1}) in a 16/8 h day/night regime. Repeated sub-culturing was done at interval of 10 days. Micro-calli were maintained to get visible calli in modified Pelletier C media (Table 1). Visible callus induction was studied through two quantitative traits *viz.* size of visible callus and days to callus induction. Colour of calli was visually scored. Whitish, yellowish and greenish coloured calli were respectively scored numerically as 1, 2 and 3. The data were recorded for days to somatic embryogenesis and percentage of somatic embryo. All data related to calli and somatic embryos were recoded for three replicates of each hormone treatment from each fusion combination. Data were statistically analyzed with MSTAT-C software and least significant difference (*lsd*) test was carried out for a significant treatment difference.

3. RESULTS

3.1 Protoplast Size

Size of protoplasts varied significantly among three varieties. The variety Agrani had the largest size (82 µm) and BINA Sarisha-8 had the smallest size (56 µm) of protoplasts (Figs. 3a and 4).

3.2 Size of Micro-Calli

Protoplasts fusion process was observed under a light microscope as shown in Fig.3b. The fused protoplasts started to divide and formed micro-calli. Size of micro-calli was significantly different under three different treatment combinations (Fig. 5) and that ranged from 393 µm to 518 µm. The largest size (518 µm) of callus (Fig. 3c) was observed in modified Pellieter B media supplemented with 1 mg L^{-1} NAA and 0.5 mg L^{-1} 2,4-D whereas the smallest size of callus was found in Pellieter B media supplemented with 0.5 mg L^{-1} NAA; 0.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP (Fig. 5).

3.3 Calli Traits

Three different hormone combinations brought significant variations in different traits of calli: size of callus, days to calli induction and colour of callus (Table 2). Size of calli ranged between 1.6 mm to 0.983 mm (Table 2). The largest size (1.6

mm) of callus was observed in Pellieter C media supplemented with 0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} NAA (Fig. 3d) and the smallest size (0.983 mm) of callus was found in the media supplemented in 0.5 mg L^{-1} 2,4-D; 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP.

Days required for induction of visible callus ranged from 24 to 32 days for different treatment combinations (Table 2). The Pellieter C media supplemented with 0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} NAA took the least days (24) for callus induction. The media supplemented with 0.5 mg L^{-1} 2,4-D; 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP took maximum days (32) for callus induction (Table 2).

Table 1. Compositions (mg L^{-1}) of the different modified Pelletier A, B, C and D media used for somatic embryogenesis from the fused mesophyll protoplasts of mustard species [10]

Category of elements	Name of the elements	A medium for Seed germination	B medium for callus induction	C medium for nourishment of micro calli	D medium for somatic embryogenesis
Macro nutrients	NH_4NO_3	1650		1650	1650
	KNO_3	1900	2400	1900	1900
	$(NH_4)_2SO_4$	---	140	---	---
	NaH_2PO_4. H_2O	170	130	170	170
	$CaCl_2.2H_2O$	440	800	440	440
	$(Mg)_2SO_4.7H_2O$	370	200	370	370
Micro nutrients	H_3BO_3	12.4	2.4	12.4	12.4
	$MnS0_4.4H_2O$	33.6	10	33.6	33.6
	$ZnSO_4.7H_2O$	21	3	21	21
	KI	2.00	1	2.00	2.00
	$Na_2MO_42H_2O$	0.5	0.25	0.5	0.25
	$CuSO_4.5H_2O$	0.05	0.03	0.05	0.25
	$CoCl_2.6$ H_2O	0.05	0.03	0.05	0.25
Iron source	FeSO4.$7H_2O$	27.8	27.8	27.8	27.8
	Na_2EDTA. 2 H_2O	37.3	37.3	37.3	37.3
Vitamin		1	1	1	1
Sucrose		10,000	30,000	30,000	30,000
D-mannitol			50,000	---	---
Agar		9.0	----	9.0	9.0
pH		5.8	5.8	5.8	5.8

Table 2. Effects of media combination on size of calli, days to calli and colour of visible callus induced *in vitro* from fused protoplasts of *Brassica* species. Each data point is the average of six observations

Pelletier B media+ 2,4-D+ NAA +BAP mg L^{-1}	Size of callus	Days to callus induction	Colour of callus
0.5 + 0.5	1.0 b	30.1 a	3.0 a
0.5 + 1.0	1.60 a	24.5 b	1.1 c
0.5 + 0.5 + 0.5	0.983 b	32.8 a	2.0 b
$LSD_{0.01}$	0.367	5.7	1.0
Level of significance	Significant at I% level of probability		
CV%	10.52	7.62	9.86

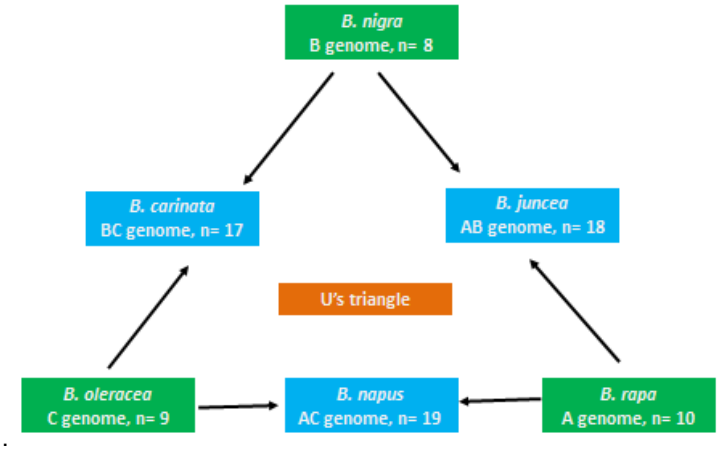

Fig. 1. The genetic relationship between *Brassica* species of the 'Triangle of U' [1]. Diploid species are indicated by green box, allotetraploid (amphidiploid) species by blue box

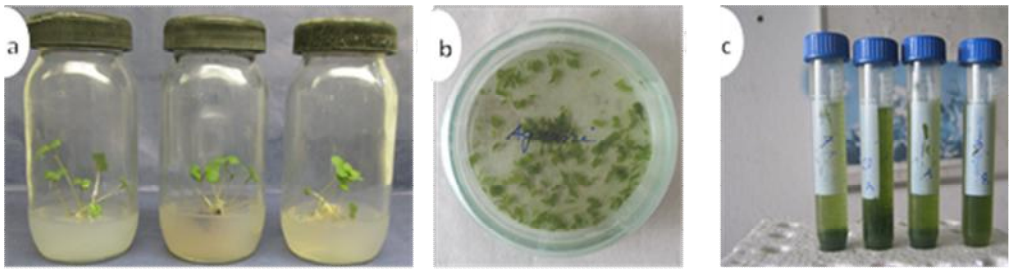

Fig. 2. a) Raising of seedlings in Pelletier A media., b) Plasmolisation of cutting leaves with 5% sorbitol containing 0.05 M $CaCl_2.2H_2O$ for 1 hour., c) Protoplasts were pelleted by centrifugation (1000 rpm, 5 min.) and the supernatant was discarded

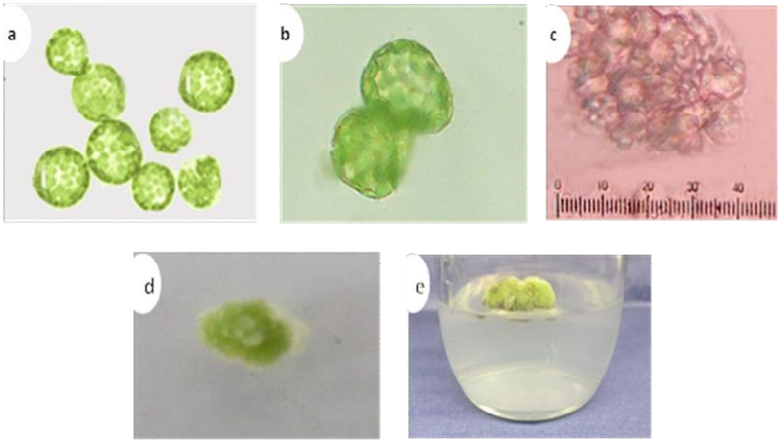

Fig. 3. a) Isolated protoplasts Agrani + BINA Sarisha-7 (400x)., b) Fused protoplasts Agrani + BINA Sarisha-7., c) Size of micro calli on 15 days after protoplasts fusion., d) Calli from fused protoplast in Pellieter C media + 1 mg L^{-1} NAA and 0.5 mg L^{-1} 2,4-D (8x), e) Somatic embryo formation from fused protoplast derived callus of Agrani+BINA Sarisha-7 in Pellieter D media supplemented with 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA after 82 days of callus

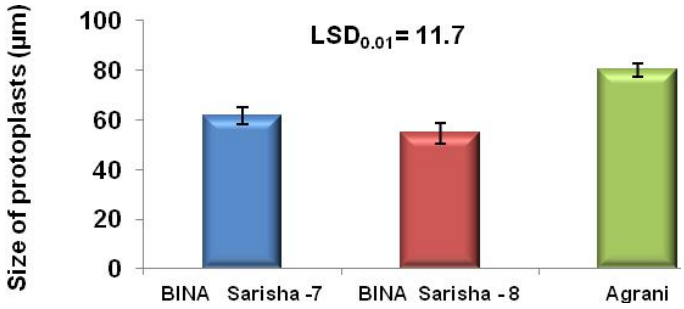

Fig. 4. Bar graph showing the size of protoplasts of different *Brassica* varieties. Each data point is the average of five observations. Vertical bars indicating standard error of mean. LSD pointing out least significant differences between size of protoplast at 1% level of significance

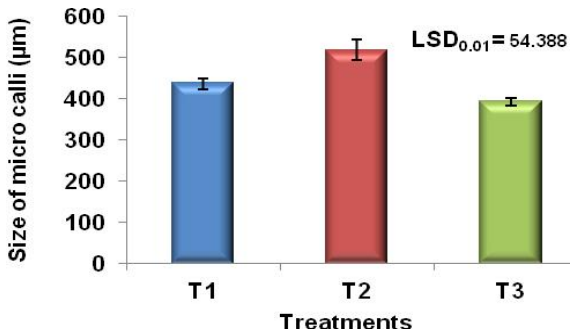

Fig. 5. Bar graph showing the size of micro-calli on 15 days after protoplasts fusion in Pellieter B media supplemented with different hormone combinations T1= 0.5 mg L^{-1} NAA and 0.5 mg L^{-1}2,4-D, T2= 1 mg L^{-1}NAA and 0.5 mg L^{-1}2,4-D and T3= 0.5 mg L^{-1}, NAA; 0.5 mg L^{-1}, 2,4-D and 0.5 mg L^{-1}BAP. Each data point is the average of six observations. Vertical bars indicating standard error of mean. LSD pointing out least significant differences between treatments at 1% level of significance

The greenish callus (3) was seen in Pellieter C media supplemented with 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA. The media supplemented with 0.5 mg L^{-1} 2,4-D+ 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BAP produced yellowish callus (2) and also with 0.5 mg L^{-1} 2,4-D + 1 mg L^{-1} NAA produced whitish callus (1).

3.4 Formation of Somatic Embryos (Green Tissues) on Calli

Effect of hormone combinations for percentage of somatic embryo formation was significant. The composition of Pellieter D media supplemented with 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA induced the highest percentage (75.6) of somatic embryo and media with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA induced the lowest percentage (43.6) of somatic embryo.

The Pellieter D media containing 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA took minimum days (82) for somatic embryogenesis while 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA took maximum days (90) for somatic embryogenesis (Fig. 6).

For days to somatic embryogenesis, the interaction Agrani+BINA Sarisha-7 x Pellieter D media supplemented with 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA took minimum days (81) whereas Agrani+BINA Sarisha-7 x Pellieter D media supplemented with 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA took maximum days (95). The interactions Agrani+BINA Sarisha-8 x Pellieter D media + 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA took the minimum days (84) for somatic embryogenesis whereas 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA took maximum days (86) (Fig. 6).

4. DISCUSSION

Apparently Agrani was more vigorous than BINA Sarisha-7 and BINA Sarisha-8 at the seedling stage. The size of the protoplasts was

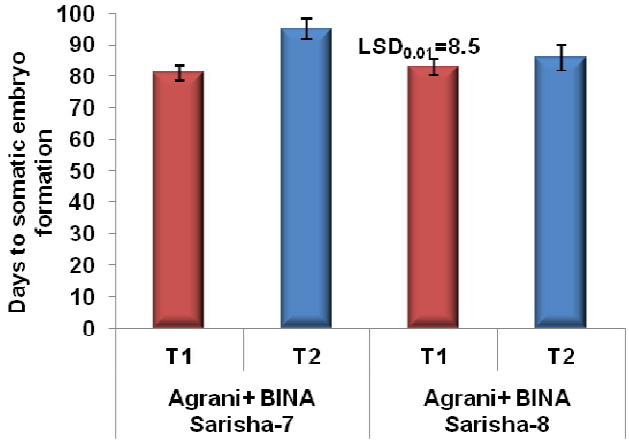

Fig. 6. Days to somatic embryo formation for two different fusion combinations (Agrani + BINA Sarisha7 and Agrani+ BINA sarisha8) in Pellieter D media supplemented with two different hormones of T1= 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and T2= 1 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. Each data point is the average of six observations. Vertical bars indicating standard error of mean. LSD indicates least significant difference between treatments at 1% level of significance

proportionate to the vigorous growth of seedlings even though seedling vigour was not scored. In the present study, PEG 400 (100%) was used to increase the rate of protoplasts fusion (Fig. 3b). Whereas, previous studies documented the use of PEG 30, 40 and 50% of PEG 8000, 6000 and 1540 MW respectively for protoplasts fusion [12-14]. The higher concentration of PEG with low molecular weight increases the rate of protoplast fusion [15].

Both micro calli and visible calli produced at a comparatively higher concentration of NAA and low concentration of BAP. Zhang et al. [16] used only 0.2 mg L^{-1} BAP and reported similar results. BAP helps increase division of the fused cells of protoplasts in dark condition [16]. Kaur et al. [17] suggested that prolonged dark period is essential for the stability of protoplasts and hence for the formation of micro calli.

A lower dose of BAP induced higher percentage of somatic embryos and also decreased the total days required for the formation of somatic embryos from protoplast derived calli of Agrani + BINA Sarisha-7 and Agrani + BINA Sarisha-8. The similar results was reported by Wang et al. [18] suggested that lower concentration of BAP helps to increase the number of somatic embryo and decrease the days to somatic embryogenesis. Primard et al. [19] documented similar findings and mentioned that embryogenesis is accelerated under lower concentration of BAP. As reported in Jiang et al. [20] a combination of lower concentration of BAP and NAA has similar effect on somatic embryogenesis.

5. CONCLUSION

A comparatively higher dose of NAA induced larger micro-calli and subsequently induced larger calli compared to a lower dose of NAA. On the other hand a lower dose of BAP induced higher percentage of somatic embryos. The results are supplementary in developing an efficient and complete somatic hybridization protocol for Brassica species.

ACKNOWLEDGMENTS

The authors thank Plant Breeding Division of Bangladesh Institute of Nuclear Agriculture for providing seeds.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. UN. Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of

fertilization. Japanese J. Bot. 1935;7:389-452
2. Frandsen KJ. The Experimental Formation of *Brassica juncea Czern. Et Coss:* Dansk botanisk arkiv (preliminary Report). Munksgaard, 1943.
3. Axelsson T, Bowman CM, Sharpe AG, Lydiate DJ, Lagercrantz U. Amphidiploid *Brassica juncea* contains conserved progenitor genomes. Genome. 2000; 43(4):679-688.
4. Gazi M, Hussain A, Islam MZ, Hussain MA, Khalequuzzaman M. Effect of some insecticides on mustard aphid, *Lipaphis erysimi* (Kaltenbach) in field and net house conditions. J. Bio. Sci. 2001;1(11):1031-1033.
5. FAO; 2013. Avaliable:http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor
6. Nishiyama I, & Inomata N. Embryological studies on cross-incompatibility between 2x and 4x in Brassica. Japanese J. Genet. 1966;41(1):27-42.
7. Hiscock SJ, Dickinson HG. Unilateral incompatibility within the Brassicaceae: further evidence for the involvement of the self-incompatibility (S)-locus. Theor. Appl. Genet. 1993;86(6):744-753.
8. Power JB, Berry SF, Chapman JV, Cocking EC. Somatic hybridization of sexually incompatible petunias: *Petunia parodii*, *Petunia parviflora*. Theor. Appl. Genet. 1980;57(1):1-4.
9. Beranek M, Bechyně M, Klima M. Protoplast isolation and fusion between *Brassica carinata* Braun. and *Brassica rapa* L. Agricultura Tropica and Subtropica. 2007;40(1):1-6.
10. Pelletier G, Primard C, Vedel F, Chetrit P, Remy R, Renard M. Intergeneric cytoplasmic hybridization in Cruciferae by protoplast fusion. Mole. Gen. Genet. 1983; 191(2):244-250.
11. Menczel L, Nagy F, Kiss ZR, Maliga P. Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum*+ *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. Theor. Appl. Genet.1981;59(3):191-195.
12. Liu F, Ryschka U, Marthe F, Klocke E, Schumann G, Zhao H. Culture and fusion of pollen protoplasts of *Brassica oleracea* L. var. italica with haploid mesophyll protoplasts of *B. rapa* L. ssp. pekinensis. Protoplasma. 2007;231(1-2):89-97.
13. Pelletier G. Somatic hybridization. In Plant Breed. 1993;93-106. Springer Netherlands.
14. Kao KN, Michayluk M A method for high-frequency intergeneric fusion of plant protoplasts. Planta. 1974;115(4):355-367.
15. Klima M, Abraha E, Vyvadilova M, Bechyn M. Protoplast culture and fusion between *Brassica carinata* and *Brassica napus*. Agricultura Tropica and Subtropica. 2009; 42(1):34-45.
16. Zhang C, Yang Z, Gui X, Liu Y, Mao X, Xia G, Lin L. Somatic hybridization between *Brassica napus* and *Eruca sativa* mill. Chinese J. Biotech. 2008;24(5):793-802.
17. Kaur ND, Vyvadilova M, Klíma M, Bechyně M. A simple procedure for mesophyll protoplast culture and plant regeneration in *Brassica oleracea* L. Czech J. Genet. Plant Breed. 2006;42(3):103-110.
18. Wang YP, Sonntag K, Rudloff E. Development of rapeseed with high erucic acid content by asymmetric somatic hybridization between *Brassica napus* and *Crambe abyssinica*. Theor. Appl. Genet. 2003;106(7):1147-1155.
19. Primard C, Vedel F, Mathieu C, Pelletier G, Chevre AM. Interspecific somatic hybridization between *Brassica napus* and *Brassica hirta* (*Sinapis alba* L.). Theor. Appl. Genet. 1988;75(4):546-552.
20. Jiang JJ, Zhao XX, Tian W, Li TB, Wang YP. Intertribal somatic hybrids between *Brassica napus* and *Camelina sativa* with high linolenic acid content. Plant Cell, Tiss. Organ Cult. 2009;99(1):91-95.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=1080&id=2&aid=9632