Plant Tissue Cult. & Biotech. **32**(2): 237-246, 2022 (December) **ISSN 1817-3721, E-ISSN 1818-8745**
DOI: https://doi.org/10.3329/ptcb.v32i2.63557

PTC&B

Establishment of an Efficient *in vitro* Regeneration Protocol for *Brassica campestris* L. Var. Tori-7

Tamanna Islam Toma[1], Ava Biswas, Saima Akhter, Tahmina Islam, Md. Imdadul Hoque and Rakha Hari Sarker*

Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

Key words: Brassica, Tori-7, *in vitro* regeneration

Abstract

The aim of the present investigation was to develop an efficient and reproducible *in vitro* regeneration protocol for *Brassica campestris* L. var. Tori-7. *Brassica* is one of the most popular and major oil yielding crop plants around the world including Bangladesh. For *in vitro* regeneration of this crop plant, MS medium supplemented with various concentrations of 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) were used. The highest frequency of callus and shoot regeneration was obtained on MS medium supplemented with 3.0 mg/l BAP and 0.2 mg/l NAA from both cotyledonary leaf with petiole and hypocotyl explants. Elongation of shoots was also optimized on the same media composition. For inducing roots from the excised *in vitro* derived shoots, half strength of MS medium supplemented with 0.3 mg/l indole-3-butyric acid (IBA) was found to be more effective than other hormonal supplements applied. Following the development of effective roots, *in vitro* raised plantlets were successfully transplanted into soil where they produced flowers and fertile seeds. The plants of R_1 generation was successfully established in natural environment. *In vitro* regeneration protocol of *Brassica campestris* var. Tori-7 established in this study could be used for future plant genetic transformation experiments.

Introduction

Agriculture has been playing a pioneering role in maintaining growth and stability of national economy of Bangladesh (Akhi et al. 2021). The main agricultural commodities of Bangladesh are rice, wheat, pulses, jute and oilseeds (Sharmin et al. 2018). Among oilseeds, *Brassica* is considered as the major crops in Bangladesh which ranked the world's third most important vegetable oils after soybean and palm (Paul et al. 2020,

*Author for correspondence: <rhsarker2000@yahoo.co.uk>. [1]Department of Botany, Jagannath University, Dhaka-1100, Bangladesh

Hossain et al. 2015). Oil yielding *Brassica* is commonly known as rapeseed and mustard and seeds of *Brassica* contain 40-45% oil and 20-25% protein. It is one of the best cooking oils particularly for heart patient because it has omega-3 and 6 fatty acid compositions. Several other fatty acids named palmitic acid, stearic acid, erucic acid, linoleic acid and linolenic acid etc. are also present in mustard oil which is important for meeting calorie requirement (Sharafi et al. 2015). It is rich in natural antioxidants and vitamin E. It has also antibacterial and various medicinal properties that can fight against infections (Mollika et al. 2011). In Bangladesh mustard oil is mainly used for cooking purposes, salad dressings and to marinate several food stuffs before cooking it (Mortuza et al. 2018).

Nowadays, *Brassica* plants have gained extensive scientific interest due to their nutritional value and agricultural importance across the world (Farooq et al. 2019, Goswami et al. 2020). However, the productivity and quality of these crops are globally affected by various climatic variability. *Brassica* ranked first position and occupies 78% of land among the oilseed crops grown in this country in respect to area and production (BBS 2018, Goswami et al. 2020). Although, average yield per hectare of *Brassica* in Bangladesh is still alarming due to different weather variability along with the unavailability of high yielding and short duration varieties (Mamun et al. 2014, Miah et al. 2017). As most of the farmers of Bangladesh usually cultivate *Brassica* in the gap between transplanted aman and boro rice which is comparatively a shorter period of time (80-90 days). Therefore, short duration varieties of *Brassica* are required (Miah et al. 2017). Apart from that, several factors including drought, salinity, high temperature and waterlogging also hampered the *Brassica* cultivation in Bangladesh (Mollika et al. 2011). Previously, both the conventional and modern crop improvement strategies have been taken to improve the quality of mustards and rapeseeds. Nevertheless, conventional breeding was not a good choice due to unavailability of suitable germplasm and cross-pollinating nature of *Brassica*. It is also time consuming and takes eight to ten generations to develop a new variety (Cardoza and Stewart 2004).

However, a breeding program associated to biotechnological tools largely depends upon the efficacy of *in vitro* plant regeneration system (Abu-El-Heba et al. 2008). Significant progress has been achieved in developing *in vitro* regeneration system for many species of *Brassica* where *Brassica campestris*, has consistently been found more difficult to regenerate under *in vitro* condition (Dietert et al. 1982, Glimelius 1984, Goswami et al. 2020). Although, there are many reports available on the use of *in vitro* techniques like *in vitro* plant regeneration, organogenesis and somatic embryogenesis in different *Brassica* spp. (Antonio et al. 1987, Jain et al. 1988, Ono et al. 1994, Koh and Loh 2000, Khan et al. 2002). Therefore, this study aims at to establish an efficient regeneration protocol for *Brassica campestris* var. Tori-7 that would be further used for developing transformation protocol with desired characteristics.

Materials and Methods

The farmer's popular mustard variety, *Brassica campestris* var. Tori-7 was chosen in this study due to its higher yield, short life cycle and minimal water requirement. The seeds of this variety used in this experiment were obtained from the Oil Seed Division of the Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur.

The surface sterilized seeds were inoculated on to full strength of MS medium with 3% sucrose and 0.8% agar for germination and seedling development. The cultured seeds were kept in dark condition till the germination and then transferred to 16 hrs light condition at 25±2ºC in growth room. Generally, germination took place within 2-3 days of seed inoculation. Two types of explants such as cotyledonary leaf with petiole and hypocotyl from five days of old seedlings were used for *in vitro* regeneration. Isolated explants were cultured on MS media containing BAP and NAA singly or in combinations for regeneration. For further multiplication, *in vitro* regenerated shoots were sub-cultured regularly in a fresh medium at an interval of 12-15 days. About 2-3 cm long shoots were separated and cultured on a rooting medium containing half strengths of MS with different concentrations of IBA for root formation. The plantlets with sufficient effective root system were transplanted to small plastic pots with sterilized soils. Initially the pots were covered with transparent perforated polythene bags and were maintained in the growth room. After proper hardening, plantlets were transferred to natural environment.

Results and Discussion

The entire experiment was conducted through Complete Randomized Design (CRD). The data were collected, on the basis of mean values and values of standard deviation and analyzed in Microsoft Excel 2010. The significant difference was measured by Duncan's Multiple Range Test (DMRT) at 5% probability level.

For callus induction, six different concentrations of BAP and four different combinations of BAP and NAA were used. Among the combinations tested, cotyledonary leaf with petiole explants showed the highest callus initiation frequency (74.54%) on MS + 3.0 mg/l BAP + 0.2 mg/l NAA and the lowest (9.72%) on MS + 5.0 mg/l BAP whereas hypocotyl explants showed the highest callus initiation frequency (46.83%) on MS + 3.0 mg/l BAP + 1.0 mg/l NAA combination and the lowest (5.56%) on MS + 4 mg/l BAP (Table 1). It was observed that the responses from both explants were not significant without NAA intervention and found to be best on MS + 3.0 mg/l BAP + 0.2 mg/l NAA (Table. 1). A significant difference was observed between cotyledonary and hypocotyl explants on callus initiation frequency. Cotyledonary leaf with petiole explants showed better performance than hypocotyl explants in terms of callus initiation in the same concentrations except MS + 1.0 mg/l BAP, MS + 5.0 mg/l BAP and MS + 3.0 mg/l BAP + 0.5 mg/l NAA (Table. 1). Dina et al. (2019) also found highest callus initiation frequency using MS media with BAP and NAA in *Brassica napus.*

Table 1. Frequency of callus initiation of *B. campestris* L. var. Tori-7

Hormone combination	Callus initiation frequency	
	Hypocotyl	Cotyledonary leaf with petiole
0.5 mg/l BAP	0 ± 0 f	0 ± 0 f
1.0 mg/l BAP	14.287 ± 14.28 cd	0 ± 0 f
2.0 mg/l BAP	9.52 ± 16.49 de	23.82 ± 41.24 cd
3.0 mg/l BAP	10.32 ± 9.01 de	15.87 ± 16.72 de
4.0 mg/l BAP	5.56 ± 9.62 de	19.45 ± 4.81 de
5.0 mg/l BAP	26.39 ± 12.03 bc	9.72 ± 8.67 ef
3.0 mg/l BAP + 0.2 mg/l NAA	38.89 ± 28.80 ab	74.54 ± 9.95 a
3.0 mg/l BAP + 0.5 mg/l NAA	40.48 ± 10.91 ab	34.92 ± 7.28 c
3.0 mg/l BAP + 1.0 mg/l NAA	46.83 ± 34.69 a	55.56 ± 11.98 b
3.0 mg/l BAP + 2.0 mg/l NAA	25.39 ± 9.91 bc	35.71 ± 12.37 c

Frequency of callus initiation from 5 days old cotyledon and hypocotyl explants cultured on the full strength of MS medium comprised with several strengths of BAP and NAA. The mean values were compared by DMRT. Mean ± SD followed by similar letters aren't significantly diverse at P = 0.05.

Shoot bud formation started from callus tissue after two to three weeks of explants inoculation. Both the calli and calli with shoot buds were transferred to shoot induction media for obtaining complete shoot buds (Fig. 1a & 1b). Similar media compositions which used for callus induction were used as shoot regeneration media. The highest shoot regeneration frequency (75.0 %) was obtained from cotyledonary leaf with petiole explants on MS media supplemented with 4.0 mg/l BAP whereas the same concentration showed very low (13.89%) in case of hypocotyl explants. On the other hand, MS supplemented with 3.0 mg/l BAP and 0.2 mg/l NAA showed best shoot regeneration frequency for both cotyledon (50%) and hypocotyl (49.2%) explants (Table 2). Khan et al. (2010) and Dina et al. (2019) found enhanced shoot regeneration frequency from cotyledon than hypocotyl explant. However, hypocotyl explants showed variable responses towards regeneration than cotyledonary leaf with petiole in certain combinations optimized in the present study (Fig. 1c-1f). It was also noticed that regeneration frequency of both explants was decreased with the increasing concentration of NAA (Table. 2). Hachey et al. (1991), Mollika et al. (2011) had reported efficient regeneration in *B. campestris* with BAP in combination with NAA. Goswami et al. (2018) also found responses on shoot regeneration of *B. juncea* in combination of BAP and NAA using hypocotyl as explants. It was observed that 18-20 days were required to shoot initiation in case of both the explants.

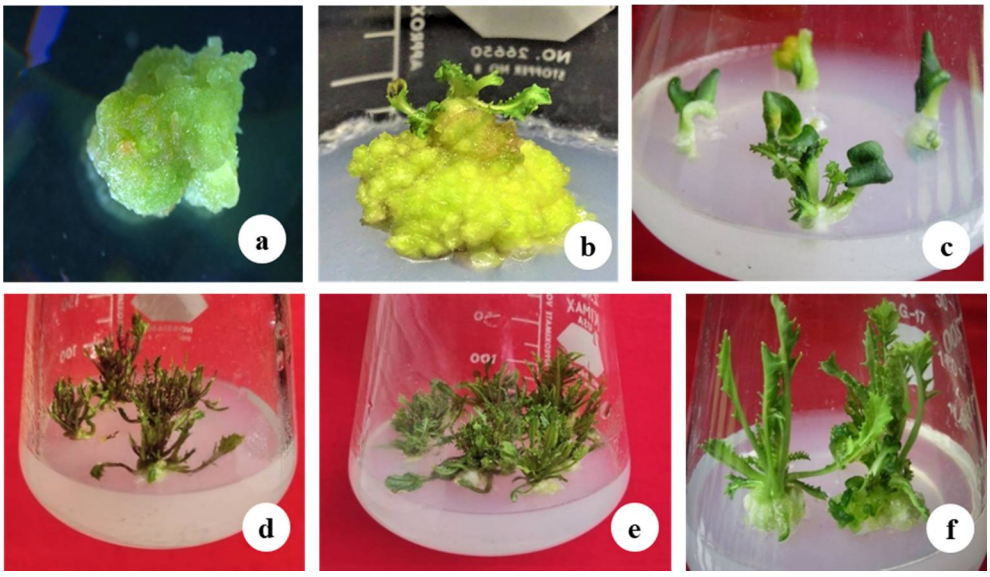

Fig. 1 (a-f): Different stages of *in vitro* shoot regeneration on MS medium supplemented with 3.0 mg/l BAP and 0.2 mg/l NAA. (a) Stereomicroscopic view of callus from hypocotyl explants of Tori-7; (b) Induction of shoots from callus derived from hypocotyl; (c) Newly formed multiple shoots from the cotyledonary leaf with the petiole of Tori-7; (d) and (e) Proliferation of several shoots following sub-culture of initially developed shoots; (f) Elongated developing shoots.

Table 2. Frequency of shoot initiation of *B. campestris* L. var. tori-7

Hormone combination	Shoot initiation frequency	
	Hypocotyl	Cotyledonary leaf with petiole
0.5 mg/l BAP	0 ± 0 i	0 ± 0 g
1.0 mg/l BAP	44.44 ± 11.98 abc	16.67 ± 15.28 ef
2.0 mg/l BAP	48.4 ± 28.39 ab	24.61 ± 15.85 cdef
3.0 mg/l BAP	10.32 ± 9.02 gh	34.92 ± 7.28 c
4.0 mg/l BAP	13.89 ± 12.73 fg	75 ± 8.33 a
5.0 mg/l BAP	34.72 ± 2.41 cd	33.33 ± 28.87 cd
3.0 mg/l BAP + 0.2 mg/l NAA	49.2 ± 19.82 a	50 ± 7.14 b
3.0 mg/l BAP + 0.5 mg/l NAA	30.15 ± 2.74 de	25.39 ± 9.91 cde
3.0 mg/l BAP + 1.0 mg/l NAA	23.81 ± 21.82 def	0 ± 0 g
3.0 mg/l BAP + 2.0 mg/l NAA	0 ± 0 i	0 ± 0 g

Frequency of shoot initiation from 5 days old cotyledonary leaf with petiole and hypocotyl explants cultured on the full strength of MS medium comprised with several strengths of BAP and NAA. The mean values were compared by DMRT. Mean ± SD followed by similar letters aren't significantly diverse at P = 0.05.

Age of the explant was found to play a significant role towards *in vitro* shoot regeneration. To find out the suitable explant, explant of different ages (3 to 7 days) were used. It was observed that cotyledonary leaf with petiole explants from five days old seedlings showed the highest (74.54%) callus regeneration frequency and from seven days old seedlings showed the lowest (15%) callus regeneration frequency on the best callus induction medium (MS + 3.0 mg/l BAP + 0.2 mg/l NAA) (Fig. 2). There was no significant difference between callus regeneration frequencies of hypocotyl explants from 5 days (38.89%), 6 days (38%) and 7 days (40%) old seedlings. However, the number of the shoot(s) per explants was higher in the case of 6 days old explants. A steady reduction in shoot regeneration frequency was observed in case of cotyledonary leaf with petiole explants excised from 6 days to 7 days old seedlings (Fig. 2). This result indicates that age of seedling affects the callus and shoot regeneration frequency and most range of shoot is produced from 6 days old seedling from both explants. Many researchers have reported that explants excised from 4-6 days old seedlings produced optimal shoot regeneration rates (Cardoza and Stewart 2004, Mollika et al. 2011, Khalil et al. 2022). It was also narrated that younger explants exhibited greater morphogenic potential than older explants as they might have more metabolically active cells with hormonal and nutritional conditions that are responsible for increased organogenesis.

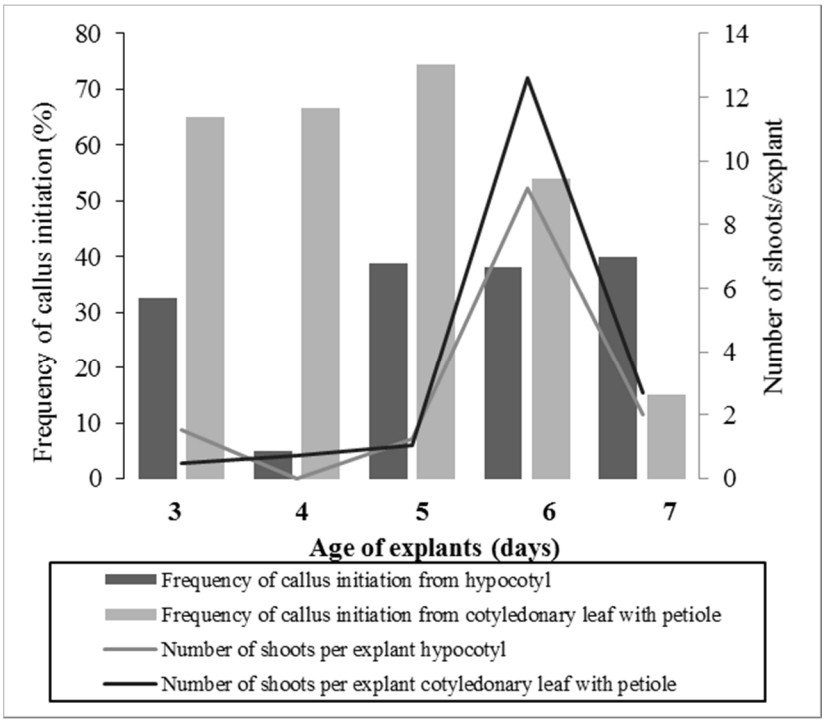

Fig. 2 Effects of explant age on shoot regeneration of *B. campestris* L. var. Tori-7 from cotyledonary leaf with petiole and hypocotyl explants.

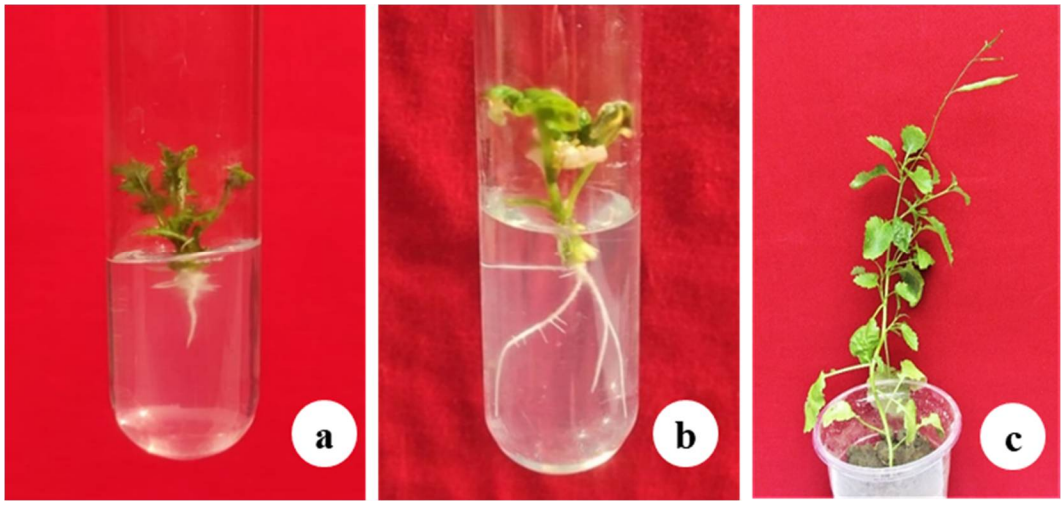

Fig. 3 (a-c): Induction of roots from excised regenerated shoots of Tori-7 on half strength of MS medium with 0.3 mg/l IBA. (a) Initiation of roots; (b) fully developed roots; (c) One of the *in vitro* raised plantlets with developing silique.

For root induction, MS as well as half strength of MS medium supplemented with different concentrations of IBA was used. Best rooting (80%) was found of the regenerated shoots on half strength of MS media with 0.3 mg/l IBA. About 16-18 days were required for root induction and the number of roots per plant was recorded to be 3-5 (Fig. 3a & 3b). Similar results were observed by Mollika et al. (2011). Interestingly, it was recorded that the *in vitro* regenerated shoots of Tori-7 produced *in vitro* flowers on regeneration media. These *in vitro* flowers were relatively smaller than those of *in vivo* produced flowers. This result revealed that regenerated shoots may synthesize flower inducing hormone in their body and induced flowering hormones spontaneously. Similar results on *in vitro* flowering of *B. campestris* and cauliflower was also reported by Verma and Sing (2007), Vandana et al. (1995) and Mollika et al. 2011. Plantlets of Tori-7 were successfully transplanted into small plastic pots after adequate development of roots and the mean percentage of survival rate of the transplanted plantlets was 38.40 (Fig. 3c). Flowering occurred within 1-1.5 months and they flowered throughout the season. Seeds formation took place after 2-2.5 months and was harvested from *in vitro* regenerated plants. The germination rate of the seeds of R_1 generation was recorded to be about 90% which was identical with the mother plant (Fig. 4a & 4b). The flower buds of R_1 plants appeared within one month and complete flowering took place within 45 days. Siliques formation took place after pollination and seeds were collected after maturation (Fig. 4c-4i).

Through this investigation, a successful *in vitro* regeneration protocol for *Brassica campestris* var. Tori-7 was developed and can be utilized for genetic transformation of *Brassica* species using agronomically important genes.

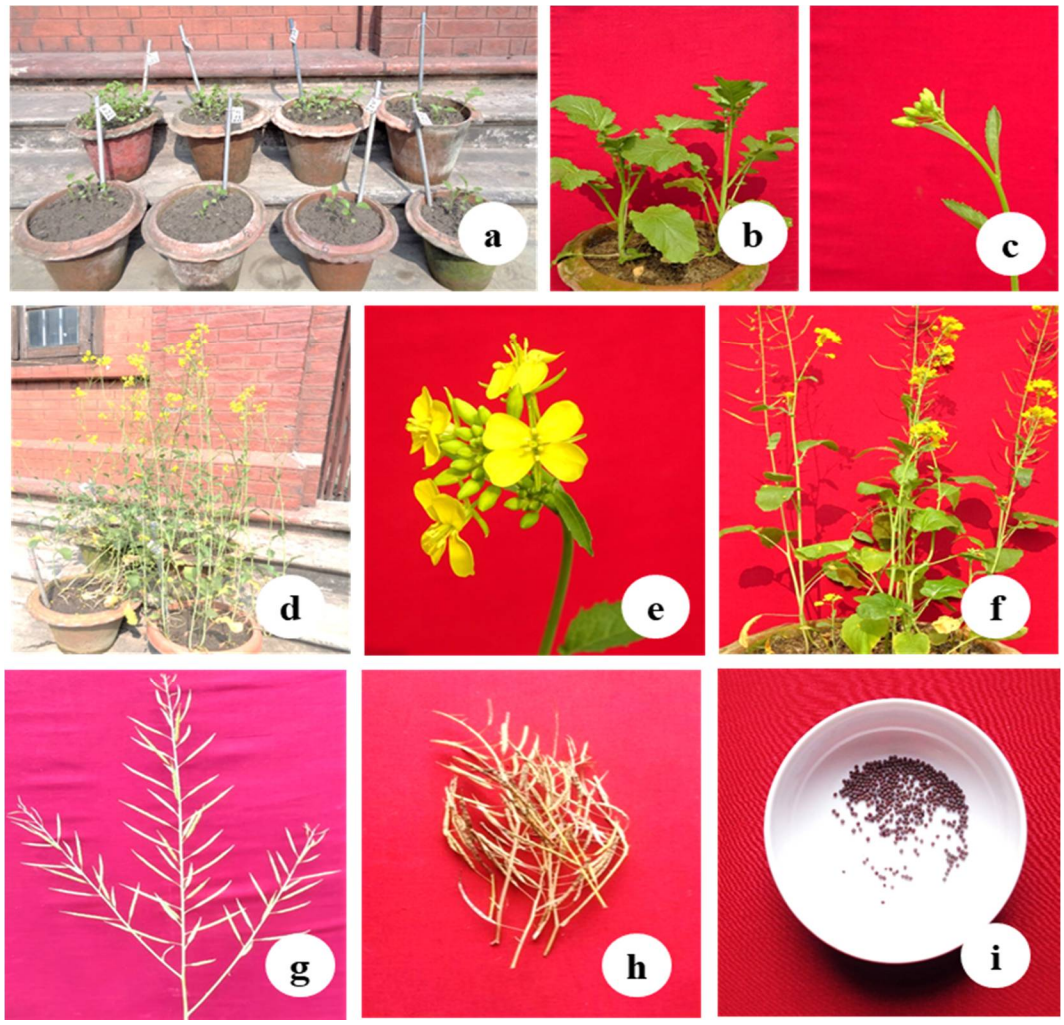

Fig. 4 (a-i): Establishment of R1 generation. (a) Development of R_1 seedlings in large clay pots; (b) One of the healthy R_1 plantlets; (c) Induction of flower bud within one month of plantation; (d) Plants at amaximum flowering stage within 1.5 months of plantation; (e) Magnified view of flowers with several flower buds; (f) Flowers as well as siliques formation on the plants; (g) Maturesiliques of one of the R_1 plants; (h) Harvested siliques developed from one of the R_1 plants; (i) Seeds developed from one of the R_1 plants.

Acknowledgement

Authors acknowledge Centennial research grant from University of Dhaka for conducting this study.

Reference

Abu-El-Heba GA, Hussein GM and **Abdalla NA** (2008) A rapid and efficient tomato regeneration and transformation system. Landbauforschung Volkenrode. **58**(1/2): 103.

Akhi K, Sultana N and **Sharmin S** (2021) Production behavior and Forecasting of some selected winter vegetables of Bangladesh. J. Bangladesh Agril. Univ. **19**(2): 251-260

Antonio BA, Namai H and **Kikuchi F** (1987) Tissue culture ability of vegetative organs from different cultivars of Brassica. SABRAO Journal. **19**(2): 73-79.

Bangladesh Bureau of Statistics (BBS) (2018) Statistical Yearbook of Bangladesh, Statistics Division. Ministry of Planning, Govt. People's Republic. Bangladesh.

Cardoza V and **Stewart CN** (2004) Brassica biotechnology: progress in cellular and molecular biology. In Vitro Cell. Dev. Biol. **40**(6): 542-551.

Chapman LA and **Goring DR** (2010) Pollen-pistil interactions regulating successful fertilization in the Brassicaceae. J. EXP. Bot. **61**(7): 1987-1999.

Dietert MF, Barron SA and **Yoder OC** (1982) Effects of genotype on *in vitro* culture in the genus Brassica. Pl. Sci. Lett. **26**: 233-240.

Dina MMA, Sultana S and **Bhuiyan MSU** (2019) Development of high frequency in vitro plant regeneration protocol of *Brassica napus*. J Adv Biotechnol Exp Ther. **2**(3): 114-119.

Distefano G, Gentle A and **Herrero M** (2011) Pollen-pistil interactions and early fruiting in parthenocarpic citrus. Ann. Bot. **108**(3): 499-509.

Dutta SK, Srivastav M, Chaudhury R, Lal K, Patil P, Singh SK and **Singh AK** (2013) Low temperature storage of mango (*Mangifera indica* L.) pollen. Sci. Hortic. **161**: 193-197.

Farooq N, Nawaz MA, Mukhtar Z, Ali I, Hundleby P and **Ahmad N** (2019) Investigating the *in vitro* regeneration potential of commercial cultivars of *Brassica*. Plants **8**(12): 558.

Glimelius K (1984) High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. Physiol. Plant. **61**(1): 38-44.

Goswami B, Hoque MI and **Sarker RH** (2018) *Agrobacterium*- mediated-enetic Transformation of oilseed *Brassica juncea* (L.) J. Nat. Sci. Res. **8**: 2224-3186.

Goswami B, Hoque MI, Khan S and **Sarker RH** (2020) *In vitro* regeneration of three varieties of *Brassica campestris* L. grown in Bangladesh. BJSIR. **55**(3): 181-188.

Hachey JE, Sharma KK and **Moloney MM** (1991) Efficient shoot regeneration of *Brassica campestris* using cotyledon explants cultured in vitro. Plant Cell Rep. **9**: 549-554.

Hossain M, Ahmed K, Chowdhury F, Roksana K, Islam S and **Barman A** (2015) Experimental study on grain weight, moisture, ash, carbohydrates, protein, oil, total energy and minerals content of different varieties of rapeseed and mustard (*Brassica* spp.). Int J Sci Res Publ. **5**: 394-400.

Jain RK, Chawdhury JB, Sharma DR and **Friedt W** (1988) Genotypic and media effects on plant regeneration from cotyledon explant cultures of some Brassica species. Plant Cell. Tiss. Org. Cult. **14**(3): 197-200.

Khalil MI, Mitra S, Toma TI, Hoque MI and **Sarker RH** (2022) *In Vitro* Regeneration and overexpression of pea DNA helicase 45 (PDH45) gene through *Agrobacterium*-mediated genetic transformation in oilseed *Brassica* Spp. Dhaka Univ. J. Biol. Sci. **30**(3): 345-358.

Khan MR, Rashid H and **Quraishi A** (2002) Effect of various growth regulators on callus formatio n and regeneration in *Brassica napus* cv. Oscar. Pak. J. Biol. Sci. **5**: 693-695.

Khan MMA, Robin ABM AHK, Nazim-Ud-Dowla MAN, Talukder SK and **Hassan L** (2010) *In vitro* regeneration potentiality of oil seed *Brassica* genotypes in differential growth regulator. Bangladesh J. Agril. Res. **33(**2): 189-191.

Koh WL and **Loh CS** (2000) Direct somatic embryogenesis, plant regeneration and *in vitro* flowering in rapid cycling *Brassica napus*. Plant Cell Rep. **19(12)**: 1177-1183.

Mamun F, Ali MH, Chowdhury IF, Hasanuzzaman M and **Matin MA** (2014) Performance of rapeseed and mustard varieties grown under different plant density. Sci. Agric. **8**(2): 70-75.

Miah MAM and **Mondal MRI** (2017) Oilseeds sector of Bangladesh: challenges and opportunities. SAARC J. Agric. **15**(1): 161-172.

Mollika SR, Sarker RH and **Hoque MI** (2011) *In vitro* Plant Regeneration in *Brassica* spp. Plant Tissue Cult Biotechnol. **21**(2): 127-134.

Mortuza MG, Dutta PC and **Das ML** (2006) Erucic acid content in some rapeseed/mustard cultivars developed in Bangladesh. J. Sci. Food Agric. **86**: 135-139.

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

Paul M, Islam T, Hoque MI and **Sarker RH** (2020) Analysis of genetic diversity in oilseed Brassica germplasm through ISSR markers and isozyme profiling. Bangladesh J. Bot. **49**(1): 147-158.

Sharafi Y, Majidi MM and **Goli SAH** (2015) Oil content and fatty acids composition in *Brassica* species. Int. J. Food. Prop. **18**(10).

Sharmin S, Mitra S and **Rashid M** (2018) Production, yield and area growth of major winter vegetables of Bangladesh. J Bangladesh Agril Univ. **16**(3): 492-502.

Vandana AK, Kumar A and **Kumar J** (1995) *In vitro* flowering and pod formation in cauliflower (*B. oleracea* var. botrytis). Curr. Sci. **69**(6): 543-545.

Verma R and **Singh RR** (2007) Regeneration and *in vitro* lowering in *Brassica campestris* (L.) Var. Bhavani. Nature **5**(1): 21-24.

Zhang FL, Takahata Y and **Xu JB** (1998) Medium and genotype factors influencing shoot regeneration from cotyledonary explants of Chinese cabbage (*Brassica campestris* L. ssp. pekinensis). Plant Cell Rep. **17**(10): 780-786.

(Manuscript received on 12 December, 2022; revised on 18 December, 2022)