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In vitro regeneration of three varieties of *Brassica campestris* L. grown in Bangladesh

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Abstract

A reproducible *in vitro* regeneration system was developed for three varieties of *Brassica campestris* L. namely Agrani, BINA Sarisha-10 and BINA Sarisha-6 using hypocotyl and cotyledonary leaf with petiole as explants. MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA was found to be the best for the multiple shoot formation for all the three varieties. Among three varieties, BINA Sarisha-6 showed best response in terms of shoot regeneration as well as number of shoots per explant (9.0) using hypocotyls as explants. In case of Agrani and BINA Sarisha-10 highest number of shoot per explants were found 8.2 and 7.0, respectively. Interestingly *in vitro* regenerated shoots of BINA Sarisha-6 and BINA Sarisha-10 were produced *in vitro* flowers on shoot regeneration media. Best root induction in BINA Sarisha-6, Agrani and BINA Sarisha-10 was achieved on MS media supplemented with 0.5 mg/l IBA. After proper hardening, the *in vitro* regenerated plantlets were successfully transplanted into soil.

Keywords: *Brassica campestris;* Regeneration; Hypocotyl; *In vitro* flowering; Rooting etc.

Introduction

Genus *Brassica* belongs to the family Brassicaceae L. (syn. Cruciferae). Rapeseed (*Brassica campestris* L. and *B. napus* L.), mustard (*B. juncea* L. Czern and Coss.), turnip (*B. rapa*), cabbage and broccoli (*B. oleracea*) are the economically important crop plants of this genus. The oilseed *Brassica* are found within *B. rapa*(syn. *B. campestris*) *B. juncea, B. carinata* and *B. napus* collectively and are commonly called rapeseed and mustard (Cardoza and Stewart, 2004). Due to their agricultural importance, *Brassica* plants have been the subject of much scientific interest.

Oilseed *Brassica* is one of the most important sources of edible vegetable oil, industrial oil and protein-rich product in the world. The oil possesses a number of properties which has some significant human health benefit. Species of *Brassica* contain 40-45% oil and 20-25% protein. It is one of the best cooking oil particularly for heart patient because of the presence of omega 3 and fatty acid compositions (linolic and alpha linolic acid, respectively) in it.

The worldwide annual production of vegetable oil is approximately 160.59 mio MT, consisting mainly soybean, oilpalm, rapeseed and sunflower. Among these crops, *Brassica napus*, *B. rapa* and *B. juncea* cover approximately 11 mio hectares of the world's agricultural land and provide over 8% of the major oil grown area under a variety of climatic conditions (Downey and Robbelen, 1989). Oilseed *Brassica* ranks third after soybean and palm oil in the global production (Canola Council of Canada, 2006).

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Bangladesh has a remarkable demand for edible oil. Among the oilseed crops grown in this country, *Brassica* occupies first position in respect to area of production. Bangladesh consumes around 1.73 mio MT of oils per year of which about 1.6 mio MT fully met up by import. Hence, it is necessarily important to increase the production of *Brassica* which can save the country from the huge import pressure (Alam *et al*., 2013).

The productivity and quality of *Brassica* oilseed crops in Bangladesh are affected by various biotic and abiotic stresses. Biotic stresses that reduce the production of oil crops include infestation by insects, damage due to bacterial and fungal pathogens. Rapeseed and mustard suffer from 14 diseases in Bangladesh. Among these diseases leaf blight, downy mildew and the parasitic plant are important. Parasitic flowering plant *Orobanche* sp. is very common in the Northern region of Bangladesh causing serious crop damage. Farmers store their seeds in traditional methods, where heavy infestation of different fungi occurs. The most seed borne pathogens are *Alternaria brassicae, A. brassicola, A. raphani, Botrytis cinerea, Fusarium avenaceum* and *Phoma lingam* (Fakir, 1976). There are many other reasons for lower yield and low production of oil crops in our country. Low yield of traditional varieties and higher yielding varieties(HYVs) due to inadequate management, non-availability of HYVs seed and good quality seed, longer duration of HYVs and poor adjustment in existing cropping patterns, lack of pest and disease resistant varieties, narrow seeding time and growing period, extreme land competition in winter season, problem in oil extraction and processing, etc.

Although a number of new varieties have been released from different research institutes of Bangladesh, none has come up to the expectation of breeders, because the released varieties are not resistant to the diseases and pests. Conventional breeding programmes alone were not successful enough in *Brassica* due to high degree of segregation upon cross pollination and unavailability of suitable germplasm. Conventional breeding of *Brassica* is labor and resource intensive and time consuming. It takes several generations to develop a new variety. In this context, an alternative to trait improvement especially disease resistant variety would be evolved by applying biotechnological approaches. Tissue culture is an important tool of biotechnology, which can be used to improve productivity of crop via rapid availability of superior planting stock (Bhatia and Ashwath, 2004). However, a breeding program associated to biotechnological tools depends upon the development of an efficient *in vitro* plant regeneration system (Abu-El-Heba *et al.,* 2008). Nowaday' sgenetic transformation is an important technique to insert important genes and development of abiotic and biotic stress tolerant plants. However, before embarking upon such a programme it is necessary to establish *in vitro* plant regeneration system of that particular plant. Considering the importance of *Brassica* spp. in Bangladesh and limitation in improving yield and quality through conventional breeding techniques, it is necessary to improve *Brassica* varieties by incorporating gene of desired character within the local varieties grown in Bangladesh.

Significant progress has been achieved in developing *in vitro* regeneration system for many species of *Brassica. Brassica campestris*, in contrast to other species of *Brassica*, has consistently been proved more difficult to regenerate under *in vitro* condition (Dietert *et al*., 1982; Glimelius, 1984). Bangladesh Institute of Nuclear Agriculture (BINA) has developed and released some varieties of *B. campestris* namely var. Agrani, BINA Sarisha-10 and BINA Sarisha-6. These varieties has some unique characteristics. Agrani is a yellow seeded mustard variety which has thick pod coat. BINA Sarisha-10 is an early maturing black seeded mustard variety. BINA Sarisha-6 is a salt tolerant (up to 13 dS/m) yellow seeded variety. Seeds of all the three varieties contain 42-44% oil. Therefore, using biotechnological tool if a suitable and reproducible regeneration protocol is achieved for these local rapeseed varieties, it will bring desired genes into our local *B. campestris* as well as other *Brassica* species. So, the aim of the present study was to develop reproducible *in vitro* regeneration protocol for three varieties of *B. campestris* (var. Agrani, BINA Sarisha-10 and BINA Sarisha-6) growing in Bangladesh.

Materials and methods

Three varieties of *Brassica campestris* namely, Agrani, BINA Sarisha-10 and BINA Sarisha-6 were used as plant materials for this investigation. Seeds were collected from Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. For the preparation of explants surface sterilized procedure was followed (Goswami *et al*., 2018). Four to five day old seedlings were used as the source of explant. Cotyledonary leaf with petiole and hypocotyl explants were excised from *in vitro* grown seedlings and used as explants. Explants were selected from the previous report of Mollika *et al.* (2011). Isolated explants were cultured on MS (Murashige and Skoog, 1962) containing BAP, NAA, IAA and Kn singly or in combinations for regeneration. *In vitro* regenerated shoots were sub-cultured regularly to fresh medium at an interval of 12-15 days for further multiplication. About 2-3 cm long shoots were separated and cultured on rooting medium containing full and half strengths of MS without hormonal supplement or with different concentrations of IBA. The plantlets with sufficient root system were then transplanted to small plastic pots containing sterilized soil for hardening.

Results and discussion

Regeneration experiments of the present investigation were mainly conducted using the hypocotyl and cotyledonary leaf with petiole explants. Explants from 4-5 days old *in vitro* grown seedlings were found to be suitable for shoot regeneration. Most researchers have reported that explants excised from 3-5 day-old seedlings gave optimal shoot regeneration rates (Cardoza and Stewart, 2004). Similar findings were also supported by Hachey *et al*. (1991) in *B. rapa.*

the best medium in terms of percentage of shoot regeneration and the number of shoots per explants.Shoot initiation was found to occur after 15-21 days of culture from hypocotyl explants in this combination (Table I). Shoot initiation was found to occur after 18-23 days of inoculation using cotyledonary leaf with petiole explants (data not shown). Shoot primordial were originated from the cut end of the petiole of cotyledonary leaf with petiole explants. Shoot initiation from hypocoytl and cotyledonary leaf with petiole explants of BINA Sarisha-6 and BINA Sarisha-10 on MS with 2.0 BAP mg/l and 0.2 mg/l IAA are presented in Fig. 2(a, b), respectively.

Hypocotyl explants showed 80, 84 and 90% regeneration for Agrani, BINA Sarisha-10 and BINA Sarisha-6 respectively (Table I). The number of shoot per explants was found 9.0

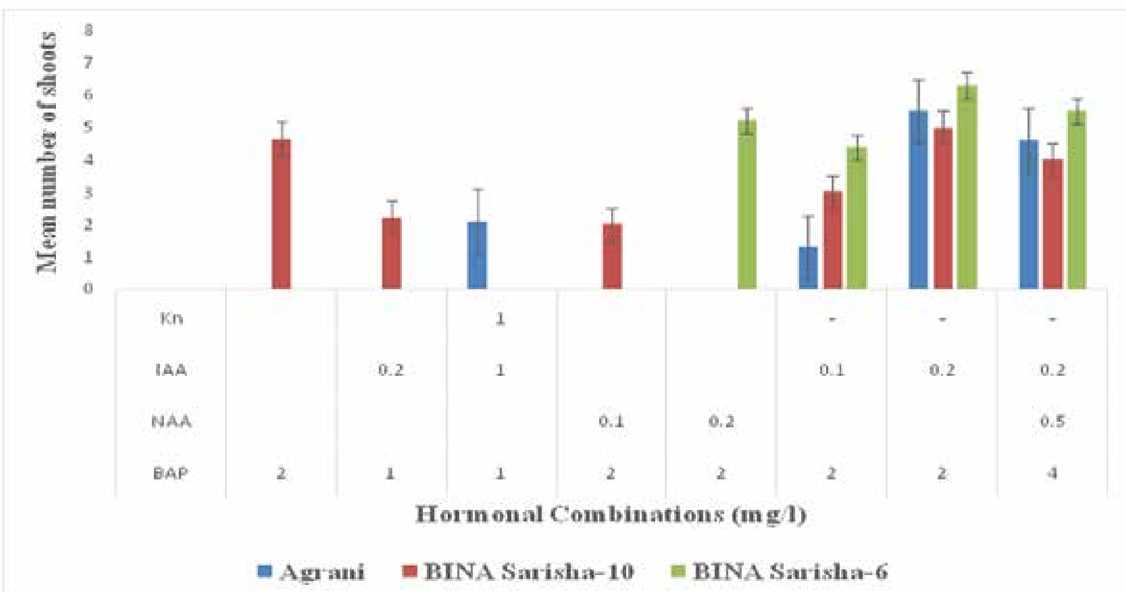

Fig. 1. Responses of three varieties of *B. campestris* var. Agrani, BINA Sarisha-6 and BINA Sarisha-10on shoot regeneration from cotyledonary leaf with petiole explants on MS medium supplemented with BAP, IAA, NAA and Kn (mg/l)

Different concentrations and combinations of BAP, IAA, Kn and NAA were used in MS medium to determine the optimum media composition for initiation and development of multiple shoots from explants of *B. campestris* namely Agrani, BINA Sarisha-10 and BINA Sarisha-6. Among all the hormonal combinations, MS media supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA was found to be the most suitable for multiple shoot regeneration from hypocotyls (Table I) and cotyledonary leaf with petiole explants (Fig.1) for three varieties of *B. campestris* namely Agrani, BINA Sarisha-10 and BINA Sarisha-6 used in this study. This was

(Fig 2c) in BINA Sarisha-6. Proliferation of multiple shoots of BINA Sarisha-6 is presented in Fig.2d. The mean number of shoot was 7.0 in BINA Sarisha-10 (Fig. 2e) and it was 8.2 in Agrani (Table I). The development of multiple shoots of Agrani are presented in Fig. 2f. Alam *et al.* (2009) had also reported efficient haploid plant regeneration in *B. campestris* with BAP in combination with IAA. This finding is also supported by the earlier report of Singh *et al.* (2011). The second highest multiple shoot regenerating medium was found MS medium supplemented with1.0 mg/l BAP + 1.0 mg/l Kn +1.0 mg/l IAA using hypocotyl explants (Table I) for all three varieties of *B. campestris*.

Table I. Effect of different hormonal combinations on shoot regeneration of *Brassica campestris* var. Agrani, BINA Sharisha-10 and BINA Sarisha-6 from hypocotyl explants

Varieties	Hormonal supplements (mg/l)				% of responsive explants	Mean no. of shoots/ explants	Days to shoot initiation
	BAP	NAA	IAA	Kn			
	1	-	-	-	-	-	-
Agrani	**1.0**	**-**	**1.0**	**1.0**	**50.00**	**7.05**	15-18
	2.0	0.1	-	-	35.00	3.00	15-16
	2.0	**0.2**	**-**	**-**	**60.00**	**5.00**	17-20
	2.0	**-**	**0.1**	**-**	**66. 66**	**4.00**	19-20
	2.0	**-**	**0.2**	**-**	**80.00**	**8.20**	**15-18**
	3.0	0.2	-	-	13.00	2.00	15-16
	4.0	**-**	**0.5**	**-**	**15.00**	**2.0**	17-20
BINA Sarisha-10	1	-	-	-	-	-	-
	1.0	**-**	**1.0**	**1.0**	**60.00**	**5.60**	18-21
	2.0	0.1	-	-	52	4.00	16-20
	2.0	**0.2**	**-**	**-**	**65**	**6.50**	17-19
	2.0	**-**	**0.1**	**-**	**58.00**	**3.00**	18-20
	2.0	**-**	**0.2**	**-**	**84.00**	**7.00**	**18-21**
	3.0	0.2	-	-	19.00	2.00	20-21
	4.0	**-**	**0.5**	**-**	**27.00**	**3.06**	15-17
BINA Sarisha-6	1	-	-	-	**53.33**	**6.25**	17-21
	1.0	**-**	**1.0**	**1.0**	39.00	3.60	20-21
	2.0	0.1	-	-	**76**	**5.8**	20-21
	2.0	**0.2**	**-**	**-**	**74.00**	**4.00**	21-23
	2.0	**-**	**0.1**	**-**	**90.00**	**9.00**	**15-18**
	2.0	**-**	**0.2**	**-**	16.00	2.00	21-23
	3.0	0.2	-	-	**30.00**	**3.57**	18-21
	4.0	**-**	**0.5**	**-**	**53.33**	**6.25**	21-23

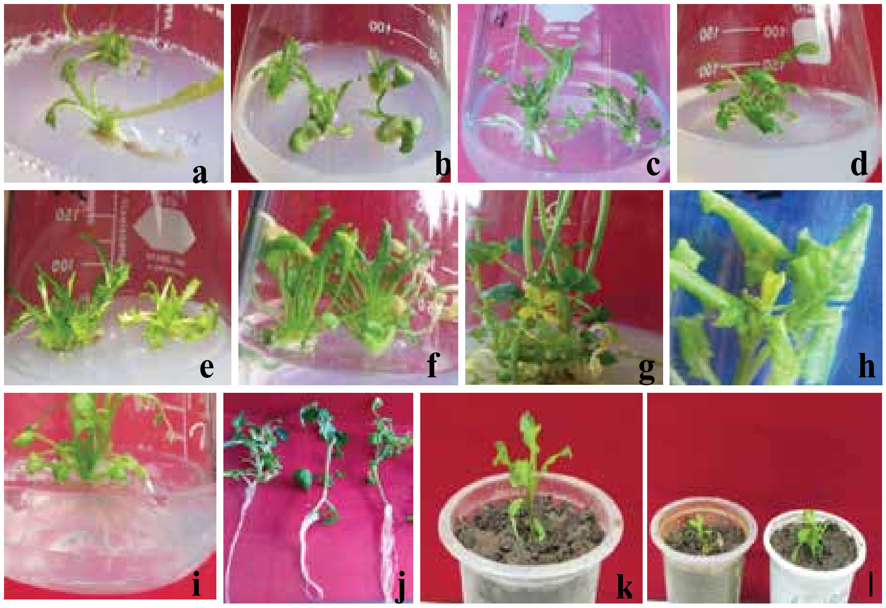

Fig. 2(a-l). Different Stages of shoot regeneration of three varieties of *B. campestris*. a. Initiation of shoots from hypocotyl explants of BINA Sarisha-6 on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA, b. Initiation of shoots from cotyledonary leaf with petiole explants of BINA Sarisha-10 on the same media mentioned above, c. Multiple shoot regeneration from hypocotyl explants in BINA Sarisha-6 on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA. d. Proliferation of multiple shoots of BINA Sarisha-6 on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA. e. Multiple shoot formation from hypocotyl explants of BINA Sarisha-10 on the same media mentioned above. f. Development of multiple shoots of Agrani from hypocotyls explants on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA. g. In vitro flowering of BINA Sarisha-10 on shoot regeneration media, h. In vitro flowering of BINA Sarisha-6 on MS media supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA. i. Rooting of in vitro shoots of BINA Sarisha-6 on MS medium supplemented with 0.5 mg/l IBA. j. Root formation from in vitroshoots of BINA Sarisha-10 on MS medium supplemented with 0.5 mg/l IBA. k. Regenerated plantlets of BINA Sarisha-6 on plastic pot l. Full plantlets of Agrani transferred to soil in small plastic pots

Many researchers (Verma and Singh, 2007; Singh *et al.,* 2011) also reported similar findings. Banu *et al.* (2017) also observed better response in *Gynura procumbens* on MS with BAP and Kn supplemented medium. Combinations of MS with 2.0 mg/l BAP and 0.2 mg/l NAA also showed good response for multiple shoot formation using hypocotyl as explants (Table I). 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.

On the other hand, a small number of shoot regeneration was observed when cotyledonary leaf with petiole was used as explants (Fig. 1). Under this condition, the mean number of shoot was found 5.5 in case of Agrani and 5.0 for BINA Sarisha-10 (Fig. 1) on MS with 2.0 mg/l BAP and 0.2 mg/l IAA. In case of BINA Sarisha -6 the response was found good and mean number of shoot was found 6.3 (Fig. 1). Khan *et al.* (2018) also found shoot regeneration on MS with BAP and IAA using leaf explants in *Rawolfia*. George and Rao (1980) also observed maximum regeneration from cotyledon explants in *B. juncea* with combinations of BAP. Cotyledonary leaf with petiole explants shows comparatively less responses towards the development of shoots (Fig.1). This finding is also supported the view that considerable variation in shoot regeneration from cotyledon explants was observed both between and within *Brassica* species (Tang *et al*., 2003). Narasimhulu and Chopra (1987) reported that *B. rapa* has the lowest frequency of regeneration from cotyledon among three basic diploid species, *B. rapa, B. oleracea*, *B. nigra* and their amphidiploids, *B. juncea, B. napus* and *B. carinata.*

Table II. Effect of half- and full- strength of MS medium with the supplements of IBA on root induction from the in vitro regenerated shoots for *Brassica campestris* var. Agrani, BINA Sharisha-10 and BINA Sharisha-6

Varieties	Hormonal supplements	% of No. of responsive shoots in rooting	Number of roots per shoot	Days to root induction
Agrani	Half strength of MS	-	-	-
	Hormone free MS	50	10	10-12
	MS+0.25mg/l IBA	70	8	10-12
	MS+0.5mg/l IBA	83	12	8-10
BINA Sarisha -10	Half strength of MS	-	-	-
	Hormone free MS	30	4	10-12
	MS+0.25mg/l IBA	50	8	10-12
	MS+0.5mg/l IBA	80	13	8-9
BINA Sarisha -6	Half strength of MS	-	-	-
	Hormone free MS	75	7	15-18
	MS+0.25mg/l IBA	80	9	10-12
	MS+0.5mg/l IBA	87	17	7-10

A considerable variation on shoot regeneration was observed in all the three varieties of *B. campestris.* Between two explants studied, hypocotyl explants were found to be the most responsive for all three varieties of *B. campestris* (Table I). It may be mentioned that hypocotyl as a suitable explants for regeneration of shoots was observed by Goswami *et al.* (2018), Khan *et al*. (2010) and Maheshwari *et al*. (2011). It was noticed that *B. campestris,* BINA Sarisha-6 showed maximum percentage (90%) of response in terms of shoot regeneration as well as highest multiple shoots using hypocotyl as explants (Table I). Multiple shoots of all the varieties of *B. campestris* used in this study are separated and sub-culutred with the same media combination for elongation. *In vitro* flowering was recorded in BINA Sarisha-10 (Fig. 2g) and BINA Sarisha-6 (Fig. 2h) on regeneration media (MS+2.0 mg/ BAP+0.2 mg/l IAA) when the shoots get elongated in this media. But these *in vitro* flowers were smaller than *in vivo* ones. It revealed that regenerated shoots synthesized flower inducing hormone in their body and induced flowering spontaneously without any external flowering hormonal supplement. The *in vitro* flowering of *B. campestris* was also reported by Verma and Singh (2007). Sarker *et al*. (2012) reported the formation of *in vitro* flowers and seeds in case of lentil.

For induction and development of root, about 3-4 cm long shoots were separated and cultured on freshly prepared full and half strength of MS medium as well as MS supplemented with different concentrations (0.25 and 0.5 mg/l) of IBA. In the present study, the best response towards root induction was observed on MS with 0.5 mg/l IBA for the all varieties of *B.campestris* (Table II) used in this study. The number of roots per shoot was found 10-17 in this combination. Rooting of *in vitro* shoots of BINA Sarisha-6 and BINA Sarisha-10 on MS medium supplemented with 0.5 mg/l IBA are presented in Fig. 2i and 2j, respectively. Some studies indicate that IBA was most effective for root initiation of *Brassica* spp. (Alam *et al.* 2009, 2013). In this combination 90% regenerated shoots formed well developed roots and they required 7-12 days for root induction. Full strength of MS medium also showed root induction (Table II). Following sufficient development of roots, plantlets were successfully transplanted into small plastic pots containing soil. Using this method the survival rate of the transplanted plantlets was 80% (data not shown). These plants are successfully established into soil. The plantlets of BINA Sarisha-6 and Agrani are presented in Fig. 2 (k, l), respectively.

Conclusion

Through this investigation, a successful *in vitro* regeneration protocol for *Brassica campestris* var. Agrani, BINA Sarisha-6 and BINA Sarisha-10 was developed. The protocol is applicable for the biotechnological approach like genetic transformation of locally grown *B.campestris* varieties using agronomically important genes.

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