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Efficient *in vitro* Regeneration of *Brassica napus* L. Var. BARI Sorisha-18

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Abstract

Brassica is one of the most nutritious and significant oil yielding crop plants around the world, including in Bangladesh. However, the development of a smart variety with a short maturity duration and high yield is a need of the present day. To achieve that, both genetic transformation and genome editing can be promising technologies for improvement, and establishment of suitable regeneration systems is a pre-requisite to deploy such technique. For this reason, an efficient and reproducible *in vitro* regeneration protocol for *Brassica napus* L. var. BARI Sorisha-18 was developed using MS medium supplemented with various concentrations and combinations of 2, 4-D, BAP and NAA. The highest frequency of *in vitro* regeneration of multiple shoots was obtained on MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA from both cotyledonary leaf attached with petiole and hypocotyl explants. The elongation of shoots was also achieved with the same media composition. Half strength of MS medium supplemented with 0.2 mg/l IBA was found to be more effective in producing effective roots than other hormonal supplements applied. Following the development of effective roots, *in vitro* raised plantlets were successfully transplanted into the soil.

Introduction

Brassica is one of the important oilseed crops in Bangladesh and is considered the world's third most important vegetable oil after soybean and palm (Paul et al. 2020, Hossain et al. 2015). Both mustard (*B. campestris*) and rapeseed (*B. napus*) are extensively used as traditional mustard oil in Bangladesh. For Bengalis, this oil has a rich cultural past. It also has a high nutritional value because it includes 20-25% proteins, various natural antioxidants, vitamin E, antimicrobial properties, and fatty acids like omega-3 and omega-6 (Mollika et al. 2011). Therefore, popularity and demand for mustard oil are

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increasing, and it is predicted to be 2.1 lakh MT in 2030 from 1.48 lakh in 2021 (Bokhtiar et al. 2023). Among the cultivated oilseeds, only rapeseed and mustard are used for edible oil production in Bangladesh, which contributes about 1.2 million metric tons to meet only 10% of the country's edible oil requirements. The remaining 90% is imported as crude oil or as oilseeds (USDA 2022). In fiscal year 2020-21, about 48 lakh tons of oil crops had to be imported at a cost of Tk. 25,910 crore (Parvez 2022). As a result, Bangladesh has to spend huge amounts of foreign currency to meet the deficit of oils and oilseeds. The average yield of *Brassica* is also very poor in this country compared to other oil producing countries (Mamun et al. 2014). The major reasons are cross pollinating nature, climate change, and the unavailability of high yielding and short duration varieties. In Bangladesh, *Brassica* spp. is cultivated between T. Aman and boro rice, with an 80-90 days gap. Therefore, we need *Brassica* varieties with quick maturation periods (Miah et al. 2017). Moreover, in Bangladesh, climate changes including drought, salinity, high temperatures, and water-logging also hamper cultivation (Mollika et al. 2011). As a result, $^{1}/_{5}$th of the cultivated land became un-arable. Therefore, it is crucial to create high-yielding varieties in order to boost production and meet our needs. Moreover, rapeseed-mustard oil is undesirable due to the presence of glucosinolates and erucic acid, even though it includes trace amounts of harmful saturated fatty acids, which can lead to serious health problems (Dina et al. 2019, Kumar et al. 2009). So, in order to increase mustard production on restricted land, we must use high-yielding varieties with low glucosinolate and erucic acid contents.

Under these circumstances, it is imperative to develop a smart crop that can minimize these problems, improve yield and quality, and control genetic variability. Plant breeders in the past several decades have used interspecific breeding to develop better hybrid varieties of mustard. However, sexual incompatibility and unavailability suitable germplasm are the major hindrances to the success of this process. Although these attempts resulted in a few new varieties, unfortunately, none of these were able to meet breeders' expectations (Goswami et al. 2020). The development of a new crop variety usually takes eight to ten generations under a conventional breeding program. Recently, genetic transformation techniques have been utilized to develop crop plants with desirable characteristics. Before beginning such a genetic transformation program, it is important to create an *in vitro* plant regeneration system for that specific plant. *B. napus* has become a valuable object of breeding and tissue culture studies as it is a healthy and high-yielding oil source due to its rich canola quality (Rahman et al. 2022). Tissue culture techniques can play an important role in improving genetic variability by initiating variation (somaclonal variation) or mutation at an unusually high rate (Krishna et al. 2016). Considering the above, the present work aims to develop an efficient *in vitro* regeneration system for *B. napus* in order to enhance crop quality through biotechnological manipulation.

Materials and Methods

Brassica napus var. BARI Sorisha-18 was used in this study due to its higher yield and oil content. 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.

For germination and seedling development, the surface-sterilized seeds were inoculated onto full strength MS medium containing 0.8% agar and 3% sucrose. The seedlings were cultivated in complete darkness until they germinated, after which they were moved to a growth environment with 16 hours of light and a temperature of 25 ± 2 ^{o}C. Once the seeds were inoculated, germination usually occurred in two to three days. Two types of explants, such as cotyledonary leaf with petiole and hypocotyl from five to eight days old seedlings, were used for *in vitro* regeneration. Isolated explants were cultured on MS media containing various hormones such as 2, 4-D, BAP, and NAA singly or in combinations for regeneration. *In vitro* regenerated shoots were routinely subcultured in a fresh medium every 12 to 15 days in order to facilitate continued multiplication. About 2-3 cm long shoots were separated and cultured on a medium that contained half strengths of MS and varying amounts of IBA to promote the growth of roots. The plantlets with a sufficient effective root system were transplanted into small plastic pots with sterilized soil. Initially, the pots were covered with transparent perforated polythene bags and were maintained in the growth room. After proper hardening, plantlets were transferred to the natural environment.

Results and Discussion

In this study, different concentrations and combinations of BAP, NAA, and 2, 4-D were used in MS medium to determine the optimum media composition for the initiation and development of multiple shoots of BARI Sorisha-18. Two types of explants, such as the cotyledonary leaf with petiole and hypocotyl, were found to be the most responsive in terms of the percentage of responsive explants as well as the number of shoots per explant. Among all the hormonal combinations, MS media supplemented with 1.0 mg/l BAP + 0.1 mg/l NAA was found to be the most effective for multiple shoot regeneration (Table 1) for both cotyledonary leaf with petiole (63.33%) and hypocotyl (43.33%) explants (Fig. 1c and 1d). It took 12-16 days for the induction of shoots. The maximum number of shoots per explant (4.84) from the cotyledonary leaf with petiole was found from the same medium composition, while MS + 4.0 mg/l BAP showed the best result for hypocotyl (1.85) explants (Table 1). Mollika et al. (2011) also reported excellent regeneration on MS media combined with 3.0 mg/l BAP and 0.2 mg/l NAA in *B. campestris*, whereas Hachey et al. (1991) found the best responses on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA. Goswami et al. (2018) also found shoot regeneration from *B. juncea* in combination with 2.0 mg/l BAP and 0.2 mg/l NAA using hypocotyl as an explant. Among the single concentrations of BAP, cotyledonary leaf with petiole showed the highest regeneration frequency (60%) on MS + 3.0 mg/l BAP and the lowest (30%) regeneration frequency on MS + 5.0 mg/l BAP (Table 1). On MS medium supplemented with 0.4 mg/l NAA, cotyledonary leaves with petioles showed the maximum regeneration frequency (50.00%), whereas hypocotyl had the lowest frequency (13.33%). It was also noticed that the majority of the explants developed

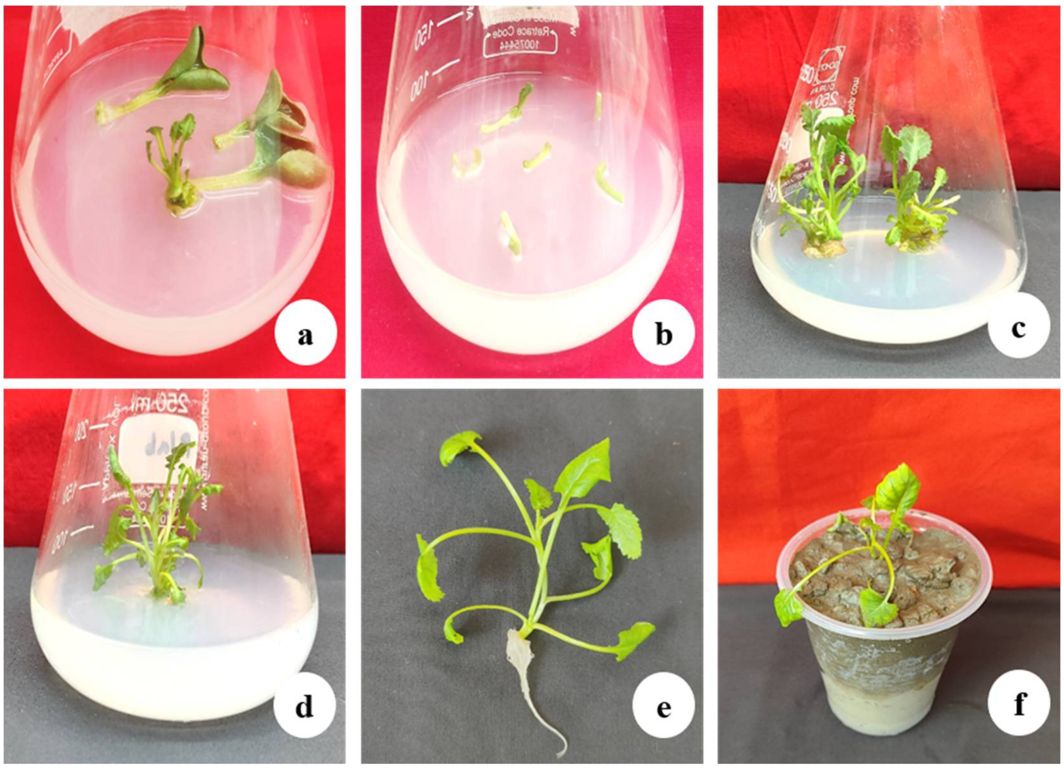

Fig. 1(a-f): Different stages of *in vitro* regeneration of *Brassica napus* var. BARI Sorisha-18. (a) and (b) Induction of shoots from 7 days old cotyledonary leaf with petiole and hypocotyl explants on MS media supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA; (c) Elongation of developing shoots; (d) Proliferation of shoots on the same media; (e) Fully developed roots from excised regenerated shoots of BARI Sorisha-18; (f) Regenerated plantlets of BARI Sorisha-18 transferred to soil in small plastic pots.

roots as the NAA concentration rose. On the other hand, no regeneration was observed in MS media supplemented with 2, 4-D for *in vitro* regeneration. Cotyledonary leaf with petiole showed better responses than hypocotyl in all media combinations in terms of percentage of responsive explants and number of shoots/explant. Khan et al. (2010) and Dina et al. (2019) also found enhanced shoot regeneration frequency from cotyledonary leaf with petiole compared to hypocotyl explants. It was observed that regenerated shoots were found to elongate within four weeks of culture.

Table 1. Effect of different concentrations and combinations of 2, 4-D, BAP and NAA on MS medium for induction of shoots from cotyledonary leaf with petiole and hypocotyl explants of *B. napus* var. BARI Sorisha-18.

Media combination with hormonal Supplement	Explant type	Responsive explants	No. of shoots/explant
MS + 0.5 mg/l 2, 4-D	CP	0.00%	0.00
	H	0.00%	0.00
MS + 1.0 mg/l 2, 4-D	CP	0.00%	0.00
	H	0.00%	0.00
MS + 1.0 mg/l BAP	CP+	40.00%	4.41
	H+	40.00%	1.08
MS + 2.0 mg/l BAP	CP++	47.00%	4.21
	H+	23.33%	0.70
MS + 3.0 mg/l BAP	CP++	60.00%	2.55
	H+	40.00%	1.75
MS + 4.0 mg/l BAP	CP++	37.00%	3.54
	H+	23.33%	1.85
MS + 5.0 mg/l BAP	CP++	30.00%	3.50
	H+	16.67%	1.80
MS + 0.1 mg/l NAA	CP++	40.00%	4.16
	H+	33.33%	0.50
MS + 0.2 mg/l NAA	CP++	40.00%	2.58
	H+	30.00%	0.55
MS + 0.3 mg/l NAA	CP++	33.00%	3.80
	H+	13.33%	0.50
MS + 0.4 mg/l NAA	CP++	50.00%	3.73
	H+	13.33%	0.75
MS + 0.5 mg/l NAA	CP++	20.00%	2.66
	H+	16.67%	0.60
MS + 1.0 mg/l BAP + 0.1 mg/l NAA	CP++	63.33%	4.84
	H+	43.33%	1.38
MS + 1.0 mg/l BAP + 0.3 mg/l NAA	CP++	53.00%	3.06
	H+	16.67%	1.44
MS + 2.0 mg/l BAP + 0.1 mg/l NAA	CP++	43.00%	3.07
	H+	36.67%	0.54
MS + 2.0 mg/l BAP + 0.3 mg/l NAA	CP++	33.00%	2.80
	H+	20.00%	1.16

CP- Cotyledonary leaf attached with petiole; H- Hypocotyl

+/++ - Magnitude of response

The explant's age had a big impact on *in vitro* regeneration. Four to eight-day-old explants were employed to determine which explant was best. It was observed that explants excised from four-day-old plants were very small and showed no results towards *in vitro* regeneration. Cotyledonary leaf with petiole from seven-day-old seedlings showed the highest regeneration frequency (63.33%), and from five-day-old seedlings, it showed the lowest (6.66%) regeneration frequency on the best shoot induction medium (MS + 1.0 mg/l BAP + 0.1 mg/l NAA) (Fig. 2). Moreover, hypocotyl segments from seven-day-old seedlings showed the highest frequency (43.33%) of regeneration, whereas five-day-old explants showed no response. There was no significant difference between regeneration frequencies of cotyledonary leaf with petiole explants from six-day (23.3%) and eight-day (36.6%) old seedlings (Fig. 2).

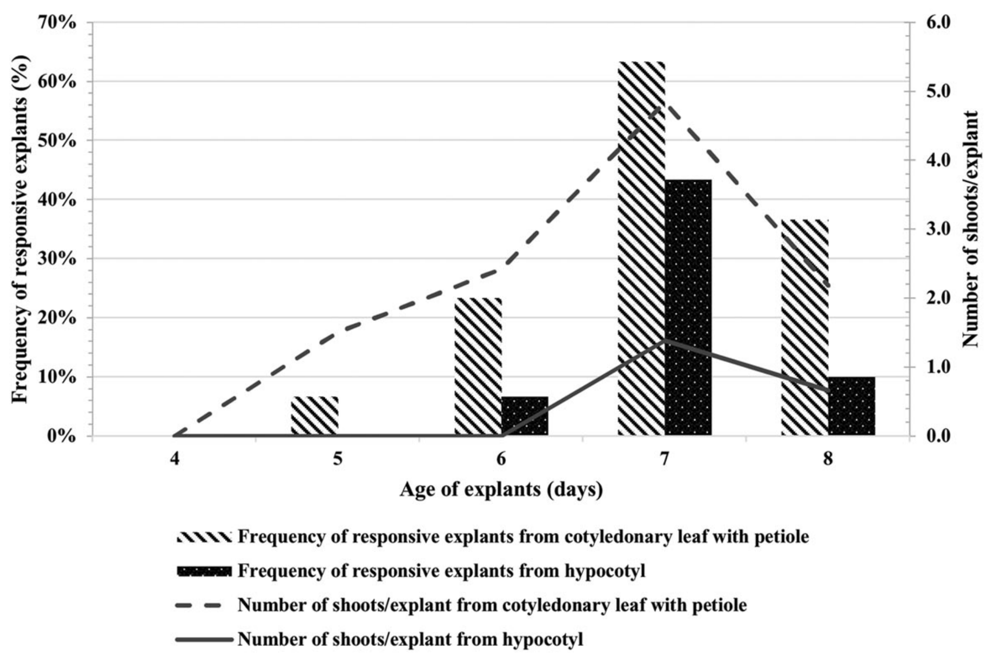

Fig. 2. Effects of explant age on *in vitro* regeneration of *B. napus* L. var. BARI Sorisha-18 from cotyledonary leaf with petiole and hypocotyl explants.

However, the number of shoots per explant was higher in the case of seven-day-old explants (Fig. 2). According to numerous studies, explants aged between four and six days were shown to provide the best rates of shoot regeneration (Cardoza and Stewart 2004, Mollika et al. 2011, Khalil et al. 2022). In this study, most shoots are produced from seven to eight-day-old seedlings from both explants, which indicates that the effects of seedling's age on regeneration frequency vary from explant to explant.

For induction of roots, half-strength MS medium was used singly and supplemented with different concentrations of IBA (0.1, 0.2, 0.3 and 0.4 mg/l). The highest (80%) root formation frequency was found in half-strength MS medium fortified by 0.2 mg/l IBA and the lowest root initiation (20%) occurred in half-strength MS medium with 0.3 mg/l (Fig. 3). Half-strength MS medium alone and supplemented with 0.4 mg/l showed no response towards root induction (Fig. 3). About 18-20 days were required for root induction and the number of roots per plant was recorded to be 3-5 (Fig. 1e and 1f). It is found that root formation frequency varies with the different concentrations of IBA.

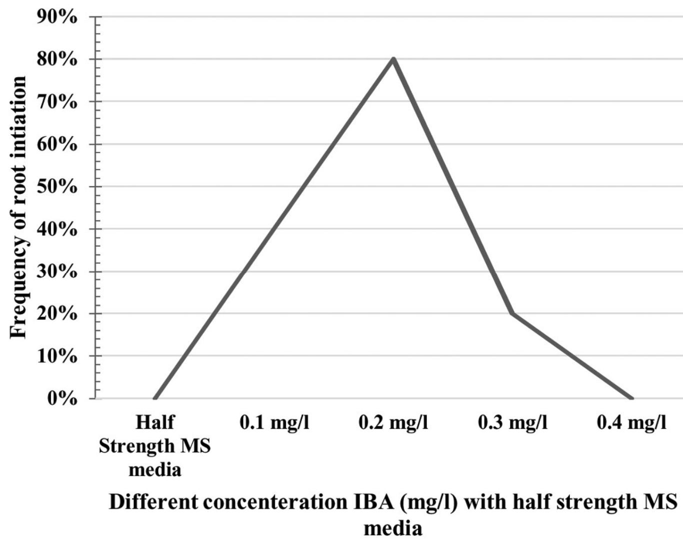

Fig. 3. Root development at the base of regenerated shoots of *B. napus* var. BARI sarisha-18.

Many studies revealed that IBA was most successful in inducing roots in *Brassica* spp. (Alam et al. 2013, Goswami et al. 2020). Following sufficient development of roots, plantlets were successfully transplanted into small plastic pots containing soil. The acclimatized plantlets were grown successfully in a natural environment.

A successful *in vitro* regeneration methodology for *Brassica napus* var. BARI Sorisha-18 was developed through this investigation, and it can be used to genetically change the *Brassica* species employing genes that are crucial to agronomy.

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