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Development of high frequency *in vitro* plant regeneration protocol of *Brassica napus*

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ABSTRACT: Various factors like plant growth regulator combinations, explant type and explant age were examined for establishing a convenient protocol for high frequency plant regeneration of *Brassica napus*. Cotyledon and hypocotyl explants of *B. napus* cv. BARI sarisha-8 were cultured on Murashige and Skoog (MS) medium fortified by different strengths of 6-Benzylaminopurine (BA), 2,4-Dichlorophenoxy Acetic Acid (2,4-D) and α-Napthalene Acetic Acid (NAA) to determine the suitable callus induction and shoot regeneration media. MS medium supplemented with 0.5 mg/L NAA and 3.0 mg/L BA was the best combination as regeneration medium because of showing highest frequency for both callus (80% for cotyledon and 53.33% for hypocotyl explants) and shoot initiation (73.33% for cotyledon and 40% for hypocotyl explants). Four days old cotyledon explants showed the highest (73.33%) frequency of shoot regeneration and highest shoot number to each explant (3.13) when 3-7 days old cotyledon cultured. Among the four tested genotypes of *B. napus*, BARI sarisha-8 showed the highest shoot regeneration frequency (73.33%) with maximum shoot number to each explant (3.13) while the lowest frequency of shoot regeneration was found in BINA sarisha-4 (46.66%) with minimum shoot number per explant (1.66). The ideal rooting medium was MS media comprised with 0.1 mg/L NAA that offered the maximum frequency (100%) of rooting. The regenerated plantlets were shifted to pot soil, acclimatized and grown until maturity in natural conditions. All plants were fertile and morphologically identical with the source plants. This protocol for high frequency regeneration of *B. napus* could be used for genetic transformation experiments.

KEYWORDS: *Brassica napus*, tissue culture, organogenesis, cotyledon, plant growth regulators.

INTRODUCTION

Brassica is an important oil-yielding crop under Brassicaceae family. Among the vegetable oil crops, the ranking of *Brassica* is 3rd following palm oil and soya bean oil in the world. It also ranks the 5th position among economically essential crops, after rice, wheat, maize and cotton [1-3]. It is the second leading source of protein meal [3]. *Brassica* seeds contain 40-45% oil [4] and 20-25% protein [5]. Rapeseed and mustard are being cultivated in 0.325 million hectares in Bangladesh that is about 60% of total oilseed crop cultivation area [6, 7]. About 70% of the total oil production in Bangladesh is covered by mustard [7]. Oilseed production of Bangladesh is about 0.254 million tons, which is 39- 40% of the country's need [5, 8]). The production of edible oil is being decreased because of delayed harvesting of high yielding transplant Aman rice, enhanced plantation of boro rice, lack of land and oil processing industry. But the demand for oil is increasing day by day with the increasing population. The present per capita oil consumption is only 10 g/day, but need is 22 g/day [9]. So, it is very essential to develop high yielding variety to ensure more production and to fulfill our need.

The mustard cultivars contain high erucic acid and glucosinolates that makes it lower market choice. Although rapeseed-mustard oil contains two essential

fatty acids like linoleic and linolenic and trace amount of unhealthy saturated fatty acids, the existence of erucic acid and glucosinolates makes it undesirable. Erucic acid causes health complications and high glucosinolates are not wished in the oil cake of animal feed [10]. So we have to promote mustard production with limited land by using high yielding variety that contains low erucic acid and glucosinolates.

For developing high yielding crop variety of *B. napus* with low erucic acid and glucosinolates, conventional breeding program is not convenient because it is laborious and very time-consuming method. Development of a new crop variety by conventional breeding program needs eight to ten generations [11]. Recently, genetic transformation techniques have been utilized to develop desired character containing crop plants. However, it is essential to develop an *in vitro* plant regeneration system of that individual plant before starting such a program on genetic transformation. *B. napus* has become an object of extensive tissue culture studies and breeding. Tissue culture techniques can play an important role for improvement of genetic variability by initiating variation (somaclonal variation) or mutation at an unusually high rate [12-14].

Considering the above, this study was carried out to set up an effective and high frequency regeneration system of *B. napus* plants for further crop improvement program by biotechnological manipulation and to optimize this system for regeneration of a number of *B. napus* cultivars.

MATERIALS AND METHODS

Plant materials

Four *B. napus* cultivars namely BARI sarisha-8, BARI sarisha-13, BINA sarisha-4 and BINA sarisha-8) were collected from Regional Agriculture Research Station (RARS), Jamalpur and Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. Among these cultivars BARI sarisha-8 was used to standardize the regeneration protocol for *B. napus* and other cultivars were used to evaluate their plantlet regeneration potentiality.

Explant and media preparation, and culture method

The seeds of *B. napus* were surface sterilized by submerging them into 70% ethyl alcohol (MERCK, Germany) for 2 minutes, 10% Clorox (Sodium hypochlorite, The Clorox Company, Oakland, USA) for 10 minutes and then seeds were rinsed for three minutes by sterilized distilled H_2O for 3 times. For raising *in vitro* seedlings, the seeds were then placed on germination medium comprising 1/2 strength of MS (Murashige and Skoog) salts and vitamins [15], 3% sucrose and 1% agar with a density of 10 seeds per callus and incubated in 25±2°C temperature under 16/8 hours (light/dark) photoperiods provided by 144 W white fluorescent lamps (culture condition).

Cotyledon and hypocotyl explants were prepared from four days aged *in vitro* seedling of *B. napus* and they were cultured on MS medium supplemented with several strengths BA (99%, Duchefa Biochemie, the Netherlands) (1.0, 2.0 and 3.0 mg/L), 2,4-D (96%, Duchefa Biochemie, the Netherlands (0.1, 0.5 and 1.0 mg/L) and NAA (98%, Duchefa Biochemie, the Netherlands) (0.1, 0.5 and 1.0 mg/L) to determine optimal medium for callus initiation. Cotyledons along with 1-2 mm petioles were very carefully excised from the hypocotyl and apical shoot meristems of seedlings. The hypocotyls were then discarded from the root tip and cut into 4-5 mm length segments. The whole procedure was carried out in laminar airflow cabinet. Ten explants were placed on each callus containing 50 ml callus induction media. Cotyledons along with petioles were placed in upward direction with the petiole in contact with the media whereas hypocotyl segments were cultured on top of the media horizontally (Fig. 1 a & b).

The cultured vessels were then marked with permanent marker to indicate specific treatment after sealing with Parafilm. After that the callus were incubated in previously discussed culture condition at culture room. After 14 days of incubation of explants, when the calli became proper size, then these were excised from explants inside the laminar airflow cabinet. Then, calli were cultured in callus having freshly prepared shoot regeneration medium (MS salts and vitamins, 1% agar, 3% sucrose and several strengths of BA (1.0, 2.0 and 3.0 mg/L), 2,4-D (0.5 and 1.0 mg/L) and NAA (0.1, 0.5 and 1.0 mg/L). When the shoot grew properly and became 2-3 cm long, these were separated from the callus aseptically within the laminar airflow cabinet and again cultured in callus containing freshly prepared root induction medium (half strength MS salts and vitamins, 1% agar, 3% sucrose and various strengths of NAA (0.1, 0.2 and 0.5 mg/L).

Hardening and transplantation

When the plantlets developed enough root system and became 5-7 cm in length, these were removed from the callus without disturbing rooting system and gently washed in tap H_2O to get rid of agar medium and sucrose traces to reduce the risk of infection by fungal contamination. The plantlets were then transplanted to

moistened soil in pots and covered with moist polythene bags to prevent desiccation and put in culture room for 4-5 days. Then acclimatized plants were shifted to natural environment and allowed to grow until maturity.

Statistical analysis

The research was operated in Completely Randomized Design (CRD) with three replications. Data were recorded on the percentage of callus initiation, shoot regeneration percentage and shoot number in each explant and statistically analyzed to confirm the significance of the experimental results. The standard deviation and mean for all treatments were calculated by using MS Excel 2010. The significance and difference between means were evaluated by Dunkan's Multiple Range Test (DMRT) at 5% significance level by R software [16].

Table 1. Frequency of callus initiation of *B. napus* cv. BARI sarisha-8

Media combinations	Callus initiation frequency	
	Cotyledon	Hypocotyl
MS	0.00 ± 0.0 g	0.00 ± 0.0 i
MS + 0.1 mg/L 2,4-D	0.00 ±0.0 g	0.00 ± 0.0 i
MS + 0.5 mg/L 2,4-D	6.66 ± 0.58 g	3.33 ± 0.58 hi
MS + 1.0 mg/L 2,4-D	13.33 ± 0.58 fg	10.00 ± 1.00 ghi
MS + 0.5 mg/L 2,4-D +1.0 mg/LBA	26.66 ± 0.58 ef	16.66 ± 0.58 fgh
MS + 0.5 mg/L 2,4-D + 2.0 mg/L BA	30.00 ± 1.00 e	23.33 ± 0.58 defg
MS + 0.5 mg/L 2,4-D + 3.0 mg/L BA	40.00 ± 1.00 de	33.33 ± 1.15 bcde
MS + 1.0 mg/L 2,4-D + 1.0 mg/L BA	33.33 ± 0.58 e	23.33 ± 0.58 defg
MS + 1.0 mg/L 2,4-D + 2.0 mg/L BA	36.66 ± 1.53 e	26.66 ± 0.58 cdef
MS + 1.0 mg/L 2,4-D + 3.0 mg/L BA	40.00 ± 1.00 de	30.00 ± 1.00 bcdef
MS + 0.1mg/ L NAA + 1.0 mg/L BA	33.33 ± 0.58 e	20.00 ± 1.00 efg
MS + 0.1 mg/L NAA + 2.0 mg/L BA	40.00 ± 1.00 de	26.66 ± 0.58 cdef
MS + 0.1 mg/L NAA + 3.0 mg/L BA	53.33 ± 0.58 cd	30.00 ± 1.00 bcdef
MS + 0.5 mg/L NAA + 1.0 mg/L BA	56.66 ± 0.58 c	23.33 ± 0.58 defg
MS + 0.5 mg/L NAA + 2.0 mg/L BA	73.33 ± 0.58 ab	36.66 ± 1.53 abcd
MS + 0.5 mg/LNAA + 3.0 mg/L BA	80.00 ± 1.00 a	53.33 ± 0.58 a
MS + 1.0 mg/L NAA + 1.0 mg/L BA	53.33 ± 0.58 cd	36.66 ± 0.58 abc
MS + 1.0 mg/L NAA + 2.0 mg/L BA	60.00 ± 1.73 bc	40.00 ± 1.00 abc
MS + 1.0 mg/L NAA + 3.0 mg/L BA	63.33 ± 0.58 bc	43.33 ± 0.58 ab

Frequency of callus initiation from 4 days old cotyledon and hypocotyl explants of *B. napus* cv. BARI sarisha-8 cultured on MS medium comprised with several strengths of 2,4-D, BA and NAA. Data consist of three replication and 10 explants were used for each replication. The mean values were compared by DMRT. Mean ± SD followed by similar letters aren't significantly diverse at P = 0.05.

RESULTS AND DISCUSSION

The optimal medium for callus induction

For inducing callus of *B. napus* cv. BARI sarisha-8, total 19 combinations of BA, 2, 4-D and NAA were used. These combinations were grouped into three classes like (a) MS + 2,4-D (b) MS + BA + 2,4-D and (c) MS + BA + NAA (Table 1). Typically, the explants had swollen after 3-4 days of culture and calli appeared within a week (Fig. 1 c & d), however, explants cultured on hormone-free basal medium (control) did not produce any callus and died after a few days. Among the combinations tested, cotyledon explants showed the highest 80% callus initiation frequency in MS + 0.5 mg/L NAA+ 3.0 mg/L BA combination and the lowest 6.66% in MS + 0.5 mg/L 2,4-D combination whereas hypocotyl explants showed the highest 53.33% callus initiation frequency in MS + 0.5 mg/L NAA + 3.0 mg/L BA combination and the lowest 3.33% in MS + 0.5 mg/L 2,4-D combination (Table 1). There was a significant difference between cotyledon and hypocotyl explants on callus initiation frequency. Cotyledon explants showed better performance than hypocotyl explants in terms of callus initiation in the same concentrations. Similar trends in callus initiation were also reported previously that cotyledon explants produced higher frequency of calli than the hypocotyls in *B. napus* [17], *B. juncea* [18], and *B. campestris* [19].

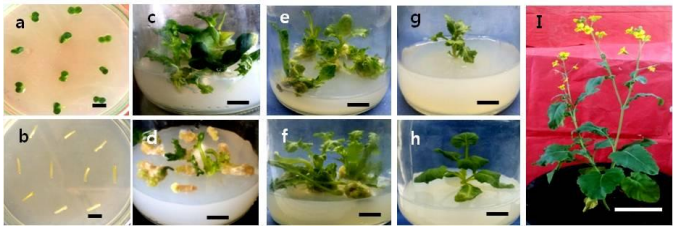

Figure 1. *In vitro* organogenesis of *B. napus* cv. BARI sarisha-8. (a) cotyledon explants at 1st day of culture, (b) hypocotyl explants at 1st day of culture, (c) callus and shoot induction from cotyledon explants after 14 days of culture on regeneration media (MS + 0.5 mg/L NAA + 3.0 mg/L BA), (d) callus and shoot induction from hypocotyl explants after 14 days of culture on regeneration media (MS + 3.0 mg/L BA + 0.5 mg/L NAA), (e) shoot elongation on shoot induction medium from cotyledon explants, (f) shoot elongation from hypocotyl explants on shoot induction medium, (g, h) root induction on MS + 0.1 mg/L NAA in regenerated shoots obtained from cotyledon and hypocotyl explants, respectively, (i) flowered plants in natural environment. *Scale bars* represent 5mm (a & b), 1cm (c, d, e, f, g & h), 10cm (i).

The optimal medium for shoot induction

Shoot bud formation started from the calli after two weeks of explants incubation. Both the calli and calli with the shoot buds were cultured on shoot induction media to obtain complete shoot buds (Fig. 1 e & f). From a total of 17 mixtures of BA, 2,4-D and NAA tested, both cotyledon and hypocotyl explants did not

produce any shoot in MS + 0.5 mg/L 2,4-D, MS + 1.0 mg/L 2,4-D combinations (Table 2). This result indicates that shoot does not regenerate without BA. So, we must use BA in combination with 2,4-D or NAA. For cotyledon explants, the maximum 73.33% and the minimum 13.33% shoot formation frequency were obtained in MS + 0.5 mg/L NAA + 3.0 mg/L BA and MS + 1.0 mg/L 2,4-D + 1.0 mg/L BA combinations, respectively (Table 2). On the other hand, the highest 40% and lowest 6.66% shoot regeneration frequency for hypocotyl explant were found in same media as cotyledon.

It was observed that cotyledon explants showed more regeneration frequency than the hypocotyl explants in all the combinations used. This agrees with previous reports that stated that cotyledon explants showed greater shoot regeneration ability than that of hypocotyl [19, 20]. In previous report, the maximum 31.42% shoot regeneration frequency was observed in *B. napus* [21]. However, we observed maximum 73.33% shoot regeneration frequency from cotyledon explants in this study.

Table 2. Frequency of shoot regeneration

Media combinations	Shoot regeneration frequency	
	Cotyledon	Hypocotyl
MS + 0.5 mg/L 2,4-D	0.00 ± 0.00 g	0.00 ± 0.00 e
MS + 1.0 mg/L 2,4-D	0.00 ± 0.00 g	0.00 ± 0.00 e
MS + 0.5 mg/L 2,4-D + 1.0 mg/L BA	26.66 ± 1.15 def	13.33 ± 0.58 cde
MS + 0.5 mg/L 2,4-D + 2.0 mg/L BA	40.00 ± 1.00 cd	20.00 ± 1.00 bcd
MS + 0.5 mg/L 2,4-D + 3.0 mg/L BA	46.66 ± 0.58 bc	26.66 ± 1.15 abc
MS + 1.0 mg/L 2,4-D + 1.0 mg/L BA	13.33 ± 1.15 fg	6.66 ± 0.58 de
MS + 1.0 mg/L 2,4-D + 2.0 mg/L BA	20.00 ± 1.00 ef	13.33 ± 0.58 cde
MS + 1.0 mg/L 2,4-D + 3.0 mg/L BA	26.66 ± 0.58 def	20.00 ± 1.00 bcd
MS + 0.1 mg/L NAA + 1.0 mg/L BA	40.00 1.00 cd	13.33 ± 1.15 cde
MS + 0.1 mg/L NAA + 2.0 mg/L BA	46.66 ± 0.58 bc	26.66 ± 0.58 abc
MS + 0.1 mg/L NAA + 3.0 mg/L BA	60.00 ± 1.00 ab	33.33 ± 0.58 ab
MS + 0.5 mg/L NAA + 1.0 mg/L BA	40.00 ± 1.00 cd	20.00 ± 1.00 bcd
MS + 0.5 mg/L NAA + 2.0 mg/L BA	66.66 ± 0.58 a	26.66 ± 0.58 abc
MS + 0.5 mg/L NAA + 3.0 mg/L BA	73.33 ± 1.15 a	40.00 ± 1.00 a
MS + 1.0 mg/L NAA + 1.0 mg/L BA	20.00 ± 1.00 ef	13.33 ± 0.58 cde
MS + 1.0 mg/L NAA + 2.0 mg/L BA	26.66 ± 1.15 def	20.00 ± 1.00 bcd
MS + 1.0 mg/L NAA + 3.0 mg/L BA	33.33 ± 0.58 cde	26.66 ± 1.15 abc

Frequency of shoot regeneration from 4 days old cotyledon and hypocotyl explants of *B. napus* cv. BARI sarisha-8 cultured in MS media complemented with various strengths of 2,4-D, NAA and BA. Data consist of three replications and 10 explants were used for each replication. The mean values were compared by DMRT. Mean ± SD followed by similar letters aren't significantly diverse at P = 0.05.

Influence of explants age

To inspect the impact of age of explant supply (seedling) on shoot regeneration, at first cotyledon explants of different ages (3 to 7 days) were cultivated on the optimum callus initiation medium (MS + 0.5 mg/L NAA + 3.0 mg/L BA) followed by the best shoot induction media same as the callus induction media. Explants excised from 2 days aged plants were very small and that's why these were not used in this experiment. After two weeks of callus culture, cotyledon explants collected from seed plant of four days old seedlings showed the highest (73.33%) shoot regeneration frequency and explants from seven days aged seedlings showed the lowest (26.66%) shoot regeneration frequency (Fig. 2). There was no noticeable significant difference between shoot regeneration frequencies of explant from 4 days (73.33%) and 5 days (60%) old seedling, but a steady reduction in shoot regeneration frequency was observed in the explants used from 4 days to 7 days old seedlings (Fig. 2). So, the result indicates that seedling age affects the shoot regeneration frequency and most range of shoot is produced from 4 days old seedling explants. This investigation is identical with the previous reports on *B. napus* [22], *B. juncea* [18], *B. campestris* [19] and *Solanum sisymbriifolium* [23]. 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.

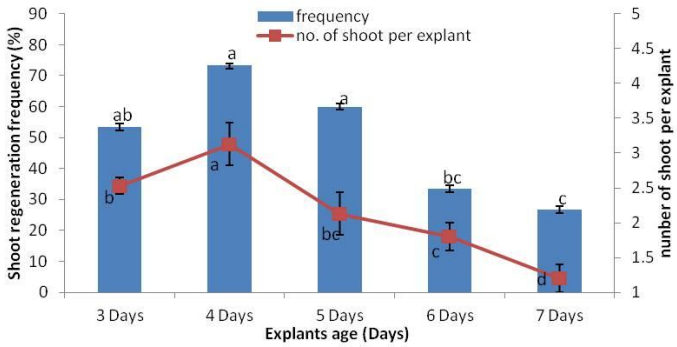

Figure 2. Effects of explant age on shoot regeneration of *B. napus* cv. BARI sarisha-8 from cotyledon explant. Data consist of three replications and 10 explants were used for each replication. *Bars* represent SD of means. Values with distinct letters are significantly diverse at P = 0.05 (DMRT).

Genotypic variation

To investigate shoot regeneration capacity, Cotyledon explants excised from 4 days old seedlings of four genotypes of *B. napus* were cultured on the best regeneration media (MS + 0.5 mg/L NAA + 3.0 mg/L BA). Shoot regeneration frequency was 73.33%, 70%, 46.66%, 63.33% in BARI sarisha-8, BARI sarisha-13, BINA sarisha-4 and BINA sarisha-8, respectively. Again, shoot number in each explant was 3.13, 2.73, 1.66 and 2.06 in BARI sarisha-8, BARI sarisha-13,

BINA sarisha-4 and BINA sarisha-8, respectively (Fig. 3).

It is clear from the above results that the frequency of shoot regeneration and shoot number per explants were affected by the genotypic variation. It is also found that BARI sarisha-8 showed the highest (73.33%) frequency of shoot growth and maximum shoot number in each explant (3.13) while BINA sarisha-4 had the lowest (46.66%) frequency of shoot regeneration and lowest shoots number per explant (1.66) (Fig. 3).

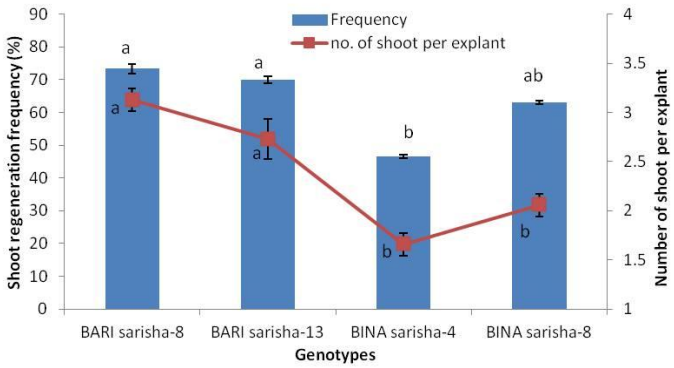

Figure 3. Influence of genotypes on shoot regeneration from 4 days old cotyledon explants of *B. napus*. Data consist of three replications and 10 explants were used for each replication. *Bars* represent SD of means. Values with distinct letters are significantly diverse at P = 0.05 (DMRT).

Initiation of roots

For root initiation the reproduced shoots were placed into ½ MS medium, MS medium and MS medium complemented with various combination of NAA (0, 0.1, 0.2 and 0.5 mg/L). Within 5 days root formation started (Fig. 1 g & h). The highest (100%) root formation frequency was found in MS medium fortified by 0.1 mg/L NAA and the lowest root initiation (40%) was occurred in ½ MS medium (Fig. 4). It is found that root formation frequency varies with the different concentrations of NAA. This result is identical to previous report found in *B. juncea* [18]. Plantlets produced well developed root system in 10 to 15 days and then plantlets were acclimatized. The acclimatized plantlets were grown successfully in natural environment until maturity.

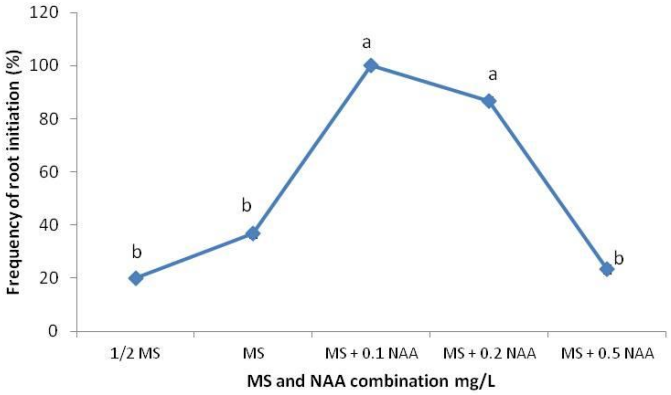

Figure 4. Root development of regenerated shoots from cotyledon explants of *B. napus* cv. BARI sarisha-8. Data consist of three replications and 10 regenerated plants were used for each replication. *Bars* represent SD of means. Values with distinct letters are significantly diverse at P = 0.05 (DMRT).

CONCLUSIONS

From the above results it can be concluded that cotyledon explant from four days old seedlings is better for callus initiation and subsequent shoot development than hypocotyl. Age of explants and genotypes of *B. napus* have great influence on shoot regeneration. MS medium fortified by 3.0 mg/L BA and 0.5 mg/L NAA is the best medium for both callus and shoot induction. The best rooting medium for *B. napus* is MS medium comprised with 0.1 mg/L NAA. This protocol can be used for future research such as developing transgenic *B. napus* plants with desired genes.

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AUTHOR CONTRIBUTIONS

Mst Maiful Akter Dina and Mohammed Shafi Ullah Bhuiyan designed the experiment and draft the manuscript. Mst Maiful Akter Dina and Sayeda Sultana carried out the experiments and analyzed the data. Mohammed Shafi Ullah Bhuiyan and Sayeda Sultana supervised the research work and finalized the manuscript. The final manuscript was carefully revised and approved by all authors.

CONFLICTS OF INTEREST

The authors declare that no conflict of interest exists.

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