Original Research Article

Development of an efficient plant regeneration system of field mustard (*Brassica campestris*)

ABSTRACT

Aims: The present study was conducted with a view to develop an efficient protocol for high frequency plant regeneration of *Brassica campestris* for further crop improvement program by biotechnological manipulation and to optimize this system for regeneration of a number of *B. campestris* genotypes.
Study design: Completely Randomized Design.
Place and Duration of Study: This experiment was carried out in the Genetic Engineering Laboratory of the Department of Genetics and Plant Breeding, Sylhet Agricultural University, Bangladesh during the period of July 2013 to June 2014.
Methodology: Cotyledon and hypocotyl explants of *B. campestris* cv. BARI sarisha-12 were cultured on MS medium supplemented with different concentration of 6-Benzyaminopurine (BAP) and α-Naphthaleneacetic acid (NAA) for callus initiation and shoot regeneration. Later on subsequent subculturing is done for shoot elongation and multiplication. MS medium supplemented with various concentrations of NAA were used for root formation.
Results: From a total of 15 different combinations of BAP and NAA tested, the combination of 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA gave the highest frequency of callus initiation (94.44%) as well as shoot regeneration (63.89%) in case of cotyledon explants where as hypocotyl explants showed 47.62% callus initiation and 19.04% shoot regeneration frequency. Four days old cotyledon explants showed the highest shoot regeneration frequency (72.22%) and higher number of shoots per explant (3.94) than those from older seedling. The shoot regeneration frequency markedly enhanced to 83.33% by the addition of 2.0 mg L^{-1} $AgNO_3$ to the MS medium supplemented with 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and this combination also showed the maximum number of shoots per explant (6.86). Shoot regeneration potentiality of five *B. campestris* genotypes were investigated and indicated that this system would be widely applicable to all the genotypes. The regenerated shoots were easily rooted on MS medium supplemented with 0.2 mg L^{-1} NAA and the whole plants were transferred to pot soils and grown to maturity.
Conclusion: MS medium supplemented with 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} $AgNO_3$ is more efficient for multiple shoot regeneration by using cotyledon explants and it may be utilized for *in vitro* improvement program of *B. campestris.*

Keywords: Brassica campestris; Phytohormons, Cotyledon; Hypocotyl; Regeneration.

1. INTRODUCTION

Brassicaceae is a family having about 3,000 species grouped into 350 genera including several types of edible plants [1]. The genus *Brassica* comprises commercially important vegetables and oilseed crops that are the good source of nutrients and health promoting phytochemicals [2]. High intake of these crops lessen the risk of age-related chronic illnesses such as cardiovascular health and other degenerative diseases [3] and also reduces the risk of several types of cancer [3-5]. Among the oilseed crops, *B. campestris* has a wide spread global distribution and mostly cultivated as vegetable and oilseed crops in Europe, Canada and Indian subcontinents.

In Bangladesh, *B campestris* is one of the most important oilseed crops. The climatic and edapic factors of Bangladesh are quite favorable for the cultivation of rapeseed and mustard. The total cultivated area under rapeseed and mustard cultivation is 0.532 million hectares which produces 0.657 million tons of mustard per year covering only 40% of domestic need [6]. As a result the country is continuously facing a huge shortage of oils and oilseed and spending huge amount of foreign currency to meet the country's demand [7]. The poor yield condition of mustard in Bangladesh might be due to the lack of high yielding variety, poor cultural and management practices and plant protection measures for raising the crop. As our land is limited but we have to increase our mustard production within limited land, so that it is necessary to develop high yielding as well as biotic and abiotic stress resistant *B. campestris* crop varieties to fulfill the domestic need.

Conventional breeding programs alone were not successful enough to develop high yielding crop variety of *B. campestris* due to high degree of segregation upon cross pollination and unavailability of suitable germplasm as well as it is labor and resource intensive and time consuming [8]. On the other hand, recent techniques in plant genetic engineering have opened new avenues for crop improvement by developing transgenic. In this regards a high frequency plant regeneration system is crucial. *In vitro* techniques have been applied in *Brassica* from different point of views and organogenesis, somatic embryogenesis and regeneration were achieved [9-14]. During last decades, considerable efforts have been made to develop *in vitro* technique for regeneration of *Brassica* spp. During these attempts a wide variety of explants have been used such as leaves [15]; roots [16]; anther [17-18]; filament [19]; cotyledon [11]; hypocotyls [20] and protoplasts [21]. However, it is proved that, *B. campestris* is one of the recalcitrant members of Brassicaceae in tissue culture by studying shoot regeneration from callus [22], leaf discs [23], cotyledons [10], and from isolated protoplasts [24]. Moreover, various explants like cotyledons [25-27], hypocotyls [27], stem and leaf segments [28], shoot tips [26], and filaments and anthers [29] have been used for *in vitro* regeneration of *B. campestris*. However, no report has been found on *in vitro* plant regeneration of *B. campestris* genotypes grown in Bangladesh except *B. campestris* cv. Tori-7 which showed low regeneration frequency.

Considering the above, this study was carried out to establish an efficient protocol for high frequency plant regeneration of *B. campestris* genotypes grown in Bangladesh, which is prerequisite for genetic transformation and to evaluate the genotypic variation for plantlet regeneration potentiality of *B. campestris*.

2. MATERIAL AND METHODS

This experiment was carried out in the Genetic Engineering Laboratory of the Department of Genetics and Plant Breeding, Sylhet Agricultural University, Bangladesh during the period of July 2013 to June 2014. Five *B. campestris* genotypes namely Tori-7, BARI sarisha-6, BARI sarisha-9, BARI sarisha-12 and BARI sarisha-15 [collected from Bangladesh Agriculture Research Institute (BARI)] were used to fulfill the objectives of the present investigations. Among these varieties BARI sarisha-12 was used to standardize the plant regeneration protocol for *B. campestris* and other genotypes were used to observe their plantlet regeneration potentiality.

The seeds were sterilized in the solution of 70% ethyl alcohol (MERCK, Germany) for 2 min and 10% Clorox (Sodium hypochlorite, The Clorox Company, Oakland, USA) for 10 min followed by four rinses in sterile distilled water. The seeds were then placed on germination medium comprising half strength MS [30] salts and vitamins, 3% sucrose and 1% agar with a density of 15 seeds per culture vessels and incubated in 25±2ºC temperature under 16 hours photoperiod provided by 144W white fluorescent lamps (culture condition).

Five days old cotyledon and hypocotyl explants of *B. campestris* cv. BARI sarisha-12 was cultured on MS media supplemented with different concentration of BAP (0.5, 1.0, 2.0, 3.0 and 4.0 mg L^{-1}) and NAA (0.1, 0.2 and 0.5 mg L^{-1}) to determine optimal medium for callus

initiation. Cotyledons alone with 1-2 mm petioles were very carefully excised from the hypocotyl and apical shoot meristems of seedlings (3 to 7 days old 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 to 15 excised cotyledons alone with petioles and hypocotyl segments were placed on each culture vessels containing 50 ml regeneration media. Cotyledons alone with petioles were placed in upward direction with the petiole in contact with the media whereas hypocotyl segments were placed horizontally on the surface of the media (Fig. 1a & b). The culture vessels were sealed with parafilm and marked with permanent marker to indicate specific treatment and incubated in culture condition.

Regeneration media comprised MS salts and vitamins, 3% sucrose, 1% agar and various concentrations of 6-Benzylaminopurine (BAP) (0.5, 1.0, 2.0, 3.0 and 4.0 mg L^{-1}) and α-Naphthalene acetic acid (NAA) (0.1, 0.2 and 0.5 mg L^{-1}). To investigate the effect of $AgNO_3$ on shoot regeneration various concentration of $AgNO_3$ (1.0, 2.0, 3.0, 4.0 and 5.0 mg L^{-1}) were added with the regeneration media. *In vitro* regenerated shoots were subcultured regularly to fresh media at an interval of 12-15 days for further multiplication. About 2-3 cm elongated shoots were separated and cultured on rooting medium containing MS salts and vitamins, 3% sucrose, 1% agar and different concentration of NAA (0, 0.1, 0.2 and 0.5 mg L^{-1}) for root formation. When the rooted plantlets became 5-7 cm in length with sufficient root system, these were taken out very carefully from the culture vessels with undisturbed rooting system and washed gently in tap water to remove agar medium and sucrose traces to discourage infection by fungal contamination. The plantlets were then transplanted to moistened soil in pots containing sterilized soil and covered with moist polythene bags for preventing desiccation. After proper hardening, the plantlets were transferred to natural environment.

The experiment was arranged in Completely Randomized Design (CRD) with 3 replications. Data were recorded on the percentage of callus initiation, percentage of shoot regeneration and number of shoots per explant and statistically analyzed to ascertain the significance of the experimental results. The mean and standard deviation for all treatments were calculated by using MS Excel 2007. The significance and difference between means were evaluated at 5% level of significance by Duncan's Multiple Rang Test [31] using MSTATC statistical software [32].

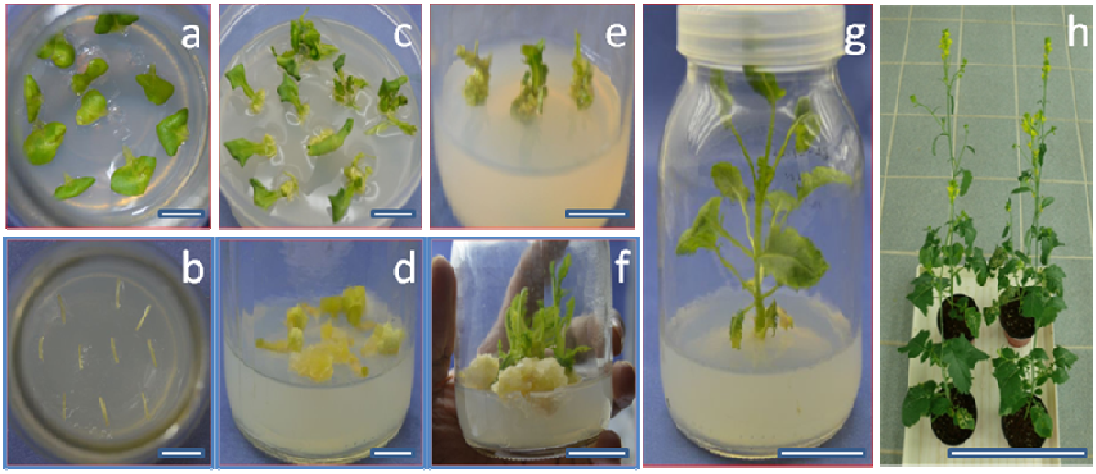

Fig. 1. The regeneration process of *B. campestris* cv. BARI sarisha-12. (a) cotyledon explants, (b) hypocotyl explants, (c and d) shoot regeneration in MS medium supplemented with 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} $AgNO_3$ from cotyledon and hypocotyl explants respectively, (e and f) shoot elongation on 1.0 mg L^{-}

[1] BAP, 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} $AgNO_3$ medium (g) root induction of regenerated shoot on MS medium supplemented with 0.2 mg L^{-1} NAA, (h) flowering of regenerated plants. *Scale bars* represent 5 mm (a, b, c, d), 1 cm (e, f), 2 cm (g), and 10 cm (h).

3. RESULTS AND DISCUSSION

3.1 Optimal media for callus introduction

From a total of 15 different combinations tested, cotyledon explants showed the highest (94.44%) callus initiation frequency in MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA combination and the lowest (33.33%) in MS + 4.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA combination whereas hypocotyl explants showed the highest (47.62%) callus initiation frequency in MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA combination and the lowest (11.9%) in MS + 4.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA combination (Table 1). A significant difference was found in callus initiation frequency between cotyledon and hypocotyl explants and it is clear that cotyledon explants showed better performance than the hypocotyl explants. Similar trend in callus initiation was also reported previously that cotyledon explants produced higher frequency of celli than the hypocotyls [27].

Table 1. Frequency of callus initiation from 5 days old cotyledon and hypocotyl explants of *B. campestris* cv. BARI sarisha-12 on MS media supplemented with various concentrations of BAP and NAA. Data consist of three replicates, each comprising 12 explants. The mean values were compared by DMRT. Mean ± SD followed by same letters are not significantly different at *P* = .05.

Treatments	Callus initiation frequency (%)	
	Cotyledon	Hypocotyl
MS + 0.5 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	58.33±1.0efg	30.95±0.5cde
MS + 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	75.00±1.0bc	33.33±0.5bcd
MS + 2.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	50.00±1.0fgh	23.81±0.5defg
MS + 3.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	44.40±0.5hi	16.67±0.5gh
MS + 4.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	33.33±1.0i	11.90±0.5h
MS + 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	72.22±0.5bcd	23.81±0.5defg
MS + 1.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	80.50±0.5b	42.86±2.0ab
MS + 2.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	55.56±1.1efgh	21.43±1.0efgh
MS + 3.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	61.11±0.5def	19.05±0.5fgh
MS + 4.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	47.22±1.1gh	16.67±0.5gh
MS + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	83.33±1.0ab	40.48±1.5abc
MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	94.44±0.5a	47.62±1.1a
MS + 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	66.67±1.0cde	33.33±0.5bcd
MS + 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	63.88±0.5cde	30.95±0.5cde
MS + 4.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	61.11±0.5def	28.57±1.0def

3.2 Optimal media for shoot regeneration

After two weeks of explants culture, shoot bud formation started from the calli. Cotyledon explants showed shoot regeneration frequency in all the combinations, but in case of hypocotyl explants some combinations did not produce any shoot (Table 2). The highest (63.88%) and the lowest (13.88%) shoot formation frequency were obtained by using 5 days old cotyledon explants in MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and MS + 4.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA combinations respectively. On the other hand hypocotyl explants showed the highest (19.04%) shoot regeneration frequency in MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA combination and the lowest (2.38%) in MS + 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA, MS + 2.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA and MS + 2.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA combinations. It was observed that when BAP concentration increased up to 1.0 mg L^{-1} along with same NAA concentration showed the highest shoot regeneration frequency and further increase of BAP (2.0, 3.0 and 4.0 mg L^{-1}) concentration decreased the shoot regeneration frequency. From the above results, it is determined that the use of cotyledon explants showed more shoot regeneration frequency than the use of hypocotyl explants cultured on MS medium supplemented with 15 combinations of BAP and NAA. This agrees with previously reported result that is frequency of shoot formation from cotyledon explants was generally higher than the frequency of hypocotyls explants [33]. The maximum frequency (63.88%) of shoot regeneration of *B. campestris* from cotyledons cultured on MS medium supplemented with 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA was somewhat different from the previously reported result of maximum shoot regeneration frequency of *B. campestris* from cotyledons cultured on MS medium supplemented with 2.0 mg L^{-1} BAP, 0.5 mg L^{-1} and 3 mg L^{-1} $AgNO_3$ [25] and MS medium added with 3.0 mg L^{-1} BAP, 0.1 mg L^{-1} NAA and 5.0 mg L^{-1} $AgNO_3$ [34]. A maximum of 46.66% shoot regeneration frequency of *B. campestris* cv. Tory-7 was reported previously on 3.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA combination [35].

Table 2. Frequency of shoot regeneration from 5 days old cotyledon and hypocotyl explants of *B. campestris* cv. BARI sarisha-12 on MS media supplemented with various concentrations of BAP and NAA. Data consist of three replicates, each comprising 12 explants. The mean values were compared by DMRT. Mean ± SD followed by same letters are not significantly different at *P* = .05.

Treatment	Shoot Regeneration frequency (%)	
	Cotyledon	Hypocotyl
MS + 0.5 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	30.56±1.1defg	2.38±0.5ef
MS + 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	41.67±1.0bcd	7.14±0.8cde
MS + 2.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	25.00±1.0efgh	2.38±0.5ef
MS + 3.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	22.22±0.5fgh	00±0.0f
MS + 4.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	13.89±0.5h	00±0.0f
MS + 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	38.89±0.5bcde	9.52±0.5cd
MS + 1.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	52.78±1.5ab	16.66±0.5ab
MS + 2.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	27.78±0.5defgh	2.38±0.5ef
MS + 3.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	25.00±1.0efgh	00±0.0f
MS + 4.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	19.44±1.5gh	00±0.0f
MS + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	47.22±1.5bc	11.9±0.5bc
MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	63.89±1.1a	19.04±1.0a
MS + 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	36.11±0.5cdef	4.76±0.5def
MS + 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	30.56±0.5defg	00±0.0f
MS + 4.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	25.00±1.0efgh	00±0.0f

3.3 Effect of explant age

In order to investigate the effect of age of explants, cotyledon explants of different ages (3 to 7 days) were cultured on shoot regeneration media (MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA) followed by callus initiation media (MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA). Explants from 2 days old seedling were too small and were not used in this experiment. Cotyledon explants of 4 days old seedlings showed the highest (72.22%) shoot regeneration frequency and explants of 7 days old seedlings showed the lowest (19.44%) shoot regeneration frequency after two weeks of explant incubation. However, the shoot regeneration frequency of 3 days (55.56%) and 5 days (63.89%) old seedling showed no significant difference, but a steady decrease in shoot regeneration frequency was observed in the explants derived from 4 days to 7 days old seedlings. The result indicates that the frequency of shoot regeneration is affected by seedling age and maximum number of shoot is produced from 4 days old seedling explants (Fig. 2). This observation is compliant with the previous works of *B. juncea* [14] and *B. napus* [36].

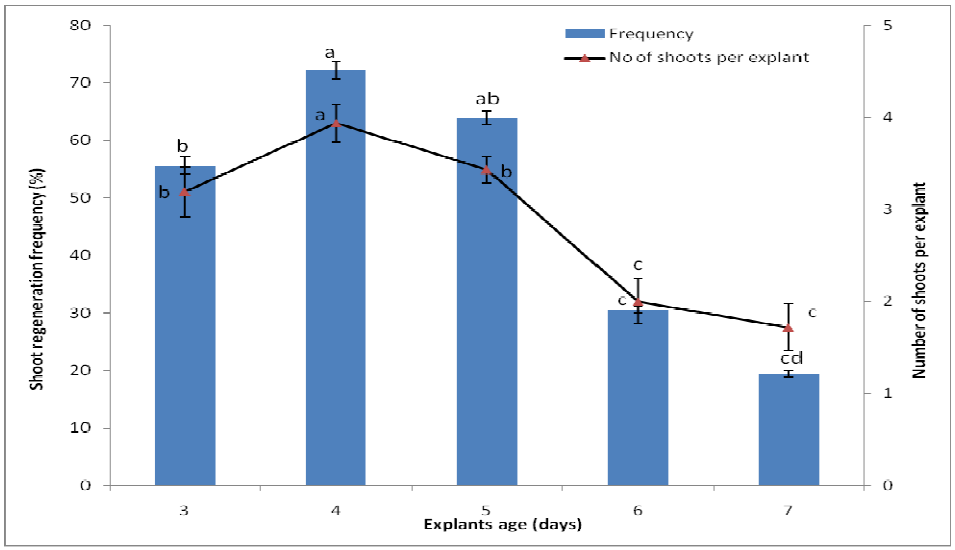

Fig. 2. Effect of explant age on shoot regeneration from cotyledon explants of *B. campestris* cv. BARI sarisha-12. Data consist of three replications and 12 explants were used for each replication. Bars represent SD of means. Values with different letters are significantly different at P = .05 (DMRT).

3.4 Influence of $AgNO_3$

To investigate the effect of $AgNO_3$ on shoot regeneration and number of shoots per explant, 4 days old cotyledon explants of *B. campestris* cv. BARI sarisha-12 were cultured on shoot regeneration media (MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA) supplemented with different concentration of $AgNO_3$ (1.0, 2.0, 3.0, 4.0 and 5.0 mg L^{-1}) (Fig. 3). The highest (83.33%) shoot regeneration frequency and the highest number of shoots per explant (6.86) was observed in shoot regeneration media supplemented with 2.0 mg L^{-1} $AgNO_3$ and the lowest (25.0%) shoot regeneration frequency and the lowest number of shoots per explant (3.23) was observed in shoot regeneration medium supplemented with 5.0 mg L^{-1} $AgNO_3$. The shoot regeneration frequency and number of shoots per explant producing capacity enhanced with the increase of $AgNO_3$ concentration up to 2 mg L^{-1} but further increase of $AgNO_3$ concentration decreased the regeneration frequency and shoot producing capacity. The shoot regeneration frequency and number of shoots per explant is markedly enhanced with the addition of ethyelene biosynthesis inhibitor $AgNO_3$. It is observed that the level of enhancements of shoot regeneration and number of shoots per explant depends on the level of concentrations of $AgNO_3$. By adding $AgNO_3$ maximum 80% of shoot regeneration frequency was reported previously in case of *B. campestris* cv. Tory-7 [34]. The positive effect of $AgNO_3$ was consistent with the previous results from the cotyledon explants of *B. rapa* spp. *oleifera* [37], *B. campestris* spp. *pekinensis* [38, 39] and hypocotyls of *B. juncea* [40].

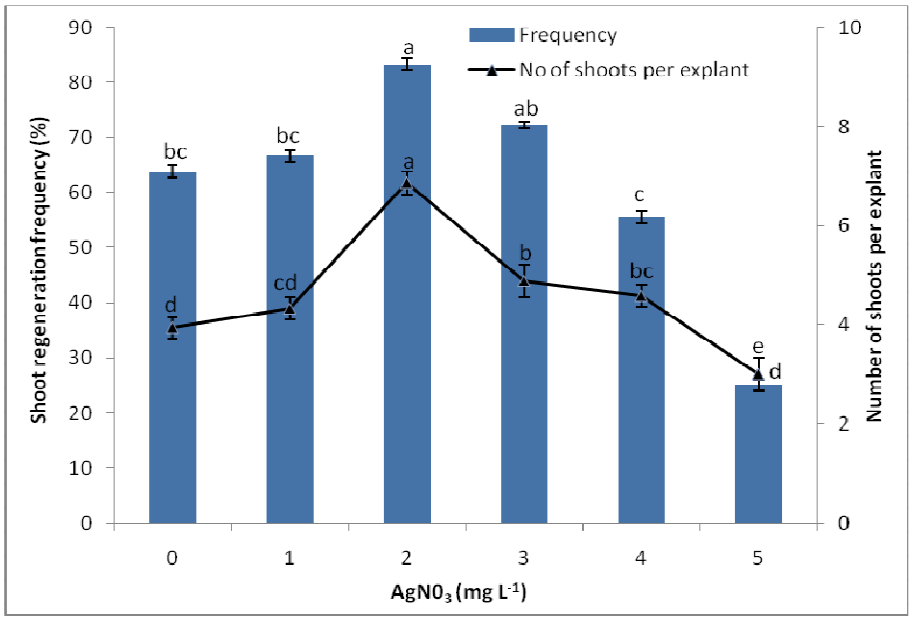

Fig. 3. Effect of $AgNO_3$ concentrations on shoot regeneration from 4 days old cotyledon explants of *B. campestris* cv. BARI sarisha-12. Data consist of three replications and 12 explants were used for each replication. Bars represent SD of the means. Values with different letters are significantly different at P = .05 (DMRT).

3.5 Genotypic variation

Cotyledon explants from 4 days old seedlings of five *B. campestris* genotype namely Tori-7, BARI sarisha-6, BARI sarisha-9, BARI sarisha-12 and BARI sarisha-15 were cultured on shoot regeneration medium (MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA) in addition with 2 mg L^{-1} $AgNO_3$ to determine their shoot regeneration ability and number of shoots per explants. Shoot regeneration frequency is 83.33%, 77.78%, 66.67%, 61.11% and 52.78% in BARI sarisha-12, BARI sarisha-9, Tori-7, BARI sarisha-6 and BARI sarisha-15, respectively (Fig. 6). The number of shoots per explant is 6.85, 6.11, 4.2, 3.88 and 3.23 in BARI sarisha-12, BARI sarisha-9, Tori-7, BARI sarisha-6 and BARI sarisha-15, respectively (Fig. 4).

Result indicated that shoot regeneration frequency and number of shoots per explants were greatly influenced by the genotypic variation. From the above result, it is found that BARI sarisha-12 showed the highest (83.33%) shoot regeneration frequency and maximum number of shoots per explant. On the other hand BARI sarisha-15 showed the lowest (52.78%) shoot regeneration frequency and least number of shoots per explant (Fig. 4).

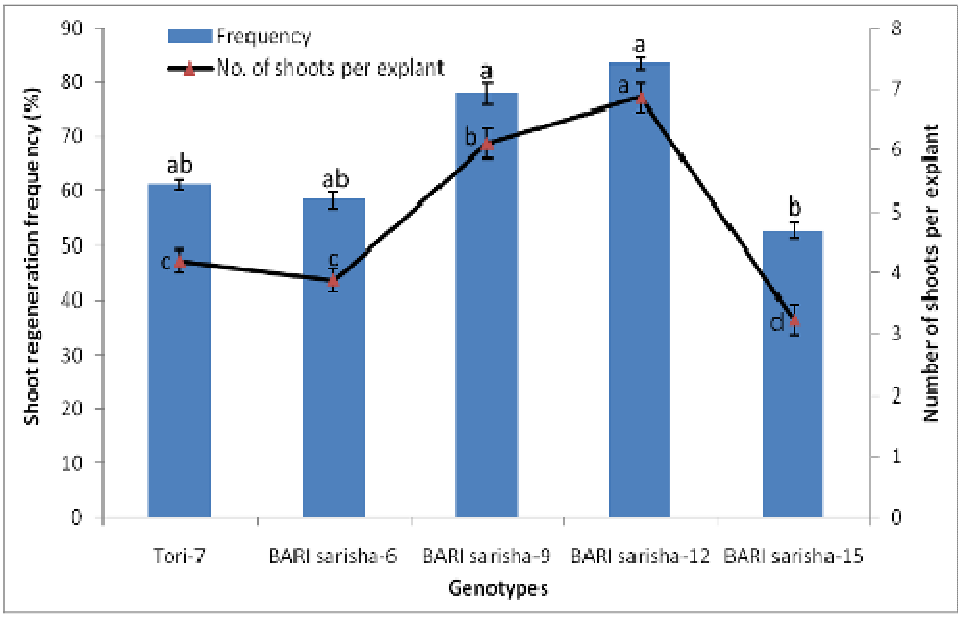

Fig. 4. Influence of genotypes on shoot regeneration from 4 days old cotyledon explants of *B. campestris*. Data consist of three replications and 12 explants were used for each replication. Bars represent SD of means. Values with different letters are significantly different at P = .05 (DMRT).

3.6 Initiation of roots

Root formation frequency varies with the different concentrations of NAA. The highest (100%) root formation frequency was observed in MS medium supplemented with 0.2 mg L^{-1} NAA and the lowest (47.22%) were observed in MS medium and MS + 0.5 mg L^{-1} NAA combination (Table 3). Plantlets produced well developed root system within 10 to 12 days.

Table 3. Influence of NAA concentrations on rooting of regenerated shoots from cotyledon explants of *B. campestris* cv. BAPRI sarisha-12. Data consist of three replicates, each comprising 12 plants. The mean values were compared by DMRT. Mean ± SD followed by same letters are not significantly different at *P* = .05.

Treatment	Root formation frequency (%)
MS	47.22±2.0b
MS + 0.1 mg L^{-1} NAA	91.67±1.0a
MS + 0.2 mg L^{-1} NAA	100±0.0a
MS + 0.5 mg L^{-1} NAA	47.22±2.0b

4. CONCLUSION

It is apparent from these results that, cotyledon explants showed the higher callus and shoot regeneration frequency compared with hypocotyl explants of *B. campestris*. Age of explants and $AgNO_3$ have great influence on shoot regeneration and multiplication of *B. campestris*. MS medium supplemented with 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} $AgNO_3$ is more efficient medium for multiple shoot regeneration by using 4 days old cotyledon explants and it may be utilized in *in vitro* improvement program of *B. campestris*.

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