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Research Article

Callus Induction and Regeneration Response of Mustard (*Brassica* spp.)

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ABSTRACT

Mustard (*Brassica* spp.) is the third most important edible oil crop belonging to the family Brassicaceae (Cruciferae) and the genus *Brassica*. Due to the growing world population and increasing industrialization, the demand for edible oil and biofuels is increasing worldwide. Stress-tolerant mustard variety development by transformation or genome editing, as well as varietal *in vitro* regeneration responses, are critical challenges. The objective of this study was to design a tissue culture protocol for better use of explants by using hypocotyls and cotyledons to observe the *in vitro* response to callus induction and regeneration of three mustard genotypes, namely BARI Sarisha-14, Binasarisha-4, and Binasarisha-9. Explants were cultured on Murashige and Skoog medium (MS) supplemented with different concentrations and combinations of NAA (10 mg mL^{-1}), BAP (10 mg mL^{-1} for callus induction and 2.5 mg mL^{-1} for shoot initiation), $AgNO_3$ (50 mg mL^{-1}) GA_3 (2 mg mL^{-1}) and IBA (10 mg mL^{-1}) for callus induction, shoot initiation, shoot outgrowth, and root initiation. Binasarisha-4 showed the best performance with hypocotyls 76.67% callus induction, 70.03% shoot initiation, 92.23% shoot outgrowth, and 40% root initiation followed by BARI Sarisha-14 (71.67%, 58.23%, 73.33% and 23.33%, respectively) and Binasarisha-9 (54.17%, 38.87%, 45.83% and 11.11%, respectively). It is recommended that *in vitro* hypocotyls culture of Binasharisha-4 can be used for frequent and successful genetic transformation in Mustard.

Introduction

Brassica is a member of the Brassicaceae family, which includes many economically important vegetables, condiments, forage, and oil seed crops. It contains vitamins, minerals, and phytochemicals that protect against carcinogenesis (Raboanatahiry *et al.*, 2021; Steinmetz and Potter, 1996)., potentially providing great health benefits in preventing illnesses and medical conditions such as breast cancer, colon cancer, stomach tumors, lung cancer, bladder cancer, ovarian cancer, and prostate cancer (Han *et al*., 2014; Hanschen *et al*., 2015; Odongo *et al*., 2017; Rebolla *et al*., 2013).

In vitro regeneration is an important part of biotechnological research and can be used to overcome the incompatibility barrier through the fusion of protoplasts from vegetative cells of inter-specific, inter-generic, and inter familiar groups (Rao and Chadha, 1986; Rao, 1985). During the last decades, efforts have been made to develop *in vitro* techniques for the regeneration of *Brassica*. Shoot regeneration, rooting, and survival of plants were high in plants regenerated from cotyledon and hypocotyls explants of Indian mustard cultivars (Bhalla et al., 2001). Shoot tip explants of *Brassica* were reported to be effective for initiating shoots and roots (Zhang et al., 1989).

In vitro plant regeneration of *Brassica* using different explants such as cotyledons (Guo *et al.,* 2005; Al-Naggar *et al.,* 2010; Akmal *et al.,* 2011; Abrha *et al.,* 2013), hypocotyls (Phogat *et al.,* 2000; Burbulis *et al.,* 2008; Abrha *et al.,* 2013; Dubey and Gupta, 2014; Lone *et al.,* 2017), apical meristem (Shanti et al., 2006; Abrha *et al.,* 2013), epicotyls (Lone *et al.,* 2017; Daud *et al.,* 2015), leaves (Guo *et al.,* 2005; Daud *et al.,* 2015), petioles (Daud *et al.,* 2015), roots (Xu *et al.,* 1982; Bhuyian *et al.,* 2009; Bano *et al.,* 2010; Daud *et al.,* 2015), microspores (Sayem *et al.,* 2010; Keller and Armstrong, 1977), protoplast (Hu *et al.,* 1999), and immature zygotic embryos were popular. *In vitro* regeneration is greatly influenced by environmental factors, culture medium composition, explants source and genotype (Zhang *et*

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al., 1998; Bano *et al.,* 2010). Therefore, the present study has taken under consideration to observe the *in vitro* response regarding callus induction and regeneration of selected mustard varieties.

Materials and Methods

The experiment was conducted from January 2022 to June 2022 at the Tissue Culture Laboratory, Biotechnology Division, Bangladesh Institute of Nuclear Agriculture, Mymensingh. Three varieties of mustard (BARI Sarisha-14, Binasarisha-4, and Binasarisha-9) were used as experimental materials. These three varieties were chosen because they are considered mega mustard varieties in Bangladesh. Seeds of Mustard varieties were collected from Bangladesh Agricultural Research Institute (BARI), Gazipur, and Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. Aseptically grown seedlings on MS media of mustard varieties were used as the source of explants. Explants were collected from 6-7 days old seedlings. Cotyledon and hypocotyls were used as explants.

Chemical compounds such as MS power (Duchefa Biochemie), sugar, agar, phytagel, ethanol, sodium hypochlorite, vitamins, amino acids, and growth regulators were used in this study. Sterilizing agents such as 2-3% Sodium Hypochlorite and 70% ethanol were used.Plant growth regulators (PGRs) included two types of Auxins; Indole Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA), two types of Cytokinins, 6-Benzylaminopurine (BAP), and AgNO3, Gibberellic acid (GA_3), Adenine hemisulphate, and Polyvinylpyrrolidone (PVP). Carbenicillin and Kanamycin were used as bactericidal antibiotics. Gelling and solidifying agents included Phytagel and sugar was used as a carbon source.

Preparation of culture media (500 mL)

The steps required to prepare culture media are the most crucial information in this work. 20 grams of sugar were dissolved in 350 milliliters of distilled water and added to a 1 liter beaker while being stirred on a magnetic stirrer. 2.25 gm of MS power was added into the beaker and the volume was made up to 500mL. With the use of a digital pH meter and where needed, 0.1 N HCl or 40% NaOH, the medium's pH was brought down to 5.8. To make the medium more solid, 2g of Phytagel was added. The medium was secured with aluminum foil before being autoclaved at 121°C for 30 minutes at 15 psi. The sterile medium was then added to sterile Petri plates, both with and without the addition of hormones. All instruments were autoclaved for 30 minutes at 15 pressure and 121^{0}C to disinfect them. By turning on the UV light for 30 minutes and cleaning the work surface with 70% ethanol, the airflow cabinet was sanitized.

Preparation of stock solution

For media preparation, separate stock solutions of plant growth regulators (PGRs) were prepared.10 mg NAA was dissolved in 100 µL of 1 N NaOH, and then 900 µL of sterile distilled water was added to make NAA (10 mg mL^{-1}).10 mg BAP was dissolved in 100 µL of 1 N NaOH and diluted in 900 µL of sterile distilled water for BAP (stock 1; 10 mg mL^{-1}). Similarly, 2.5 mg BAP was dissolved in 100 µL of 1 N NaOH and 900 µL of sterile distilled water was added to get the desired concentration to make BAP (stock 2; 2.5 mg mL^{-1}). To make $AgNO_3$ (50 mg mL^{-1}), 50 mg $AgNO_3$ was dissolved in 1 mL of sterile distilled water, and to make GA_3 (2 mg mL^{-1}): 2 mg GA_3 was dissolved in 1 mL of 50% ethanol. Lastly, IBA (10 mg mL^{-1}) was prepared by dissolving 10 mg IBA in 100 µL of 1 N NaOH. and then adding 900 µL of sterile distilled water.

Media used for tissue culture

Murashige and Skoog (1962) medium supplemented with phytohormones was used for callus induction, shoot initiation, shoot multiplication, and root induction. The stock solution was prepared at the beginning and stored at 4^{o}C. MS media was used as a Seed germination medium (500mL). Media composition with growth regulator concentration were used according to Bhalla & Singh (2008). Callus induction medium (500mL) was made by supplementing MS media with 50 µL $AgNO_3$, 37.5 µL BAP (10 mg mL^{-1}), 10 µL NAA and 2.5 µL GA_3. Shoot initiation medium (500mL) was made by supplementing MS media with 50 µL $AgNO_3$, 150 µL BAP (10 mg mL^{-1}), 10 µL NAA and 2.5 µL GA_3.MS media was supplemented with 20 mg adenine hemisulfate, 250 mg PVP and 5 µL BAP (2.5 mg mL^{-1}) to make Shoot outgrowth medium (500mL). In root induction medium (500mL), MS media (half strength) was supplemented with 50 µL IBA.

In vitro culture method

Fresh, mature, and dried seeds of mustard varieties were washed with double distilled water, kept in 70% ethanol for 1 min, then washed three times with distilled water after ethanol treatment. 2-3% NaOCl was added to the seeds and shaken, then treated with Tween-20 for 3 minutes. Again, the seeds were washed three to four times. The seeds were poured on sterile tissue paper and placed on Petri dishes containing MS media and wrapped with laboratory paraffin. The Petri dishes were stored in the culture room for 10-20 days at 24±2^{o}C for seed germination and explants collection.

The hypocotyls (1-2 cm) and cotyledons (3-5 cm with 2 mm petioles) were cut from 6-7 days old seedlings and

placed horizontally and vertically on callus induction medium respectively in Petri dishes. The culture was maintained under a 16h photoperiod (3000 lux, approx.) regime at 24±2oC temperature. After 1-2 weeks of incubation, explants started to produce callus, and subculture was done after 3-4 weeks carrying out subsequence observation to note the explant response. Then calli (2-3 cm) were cut into small pieces if required and transferred into the shoot induction medium in a conical flask and were incubated in the same culture condition. After that, Calli were sub-cultured at a 2-3 weeks interval to avoid nutrient deficiency and space competition among the explants carrying out observations regularly.

Finally, when the regenerated shoots reached 3-4 cm in length with 3-4 well-developed leaves (after 25-30 days of callus culture), they were rescued aseptically from the conical flasks and separated from each other. Only the shoots from hypocotyls explants were removed from the media and placed individually on the rooting medium and kept under the same culture condition.

Experimental Design

The experiment was carried out in a Completely Randomized Design (CRD) with three replications. To investigate the effects of different treatments, the data were recorded on the following parameters:

Days required for callus induction

The observation of explants on callus media started from 7th days of inoculation and continued up to 35 days. The percentage of callus initiation was noted after 35 days of explant culture using the following formula:

$$\% \text{ callus induction} = \frac{\text{Number of explants induced calli}}{\text{Numberof explants incubated}} \times 100$$

Days required for shoot initiation

The observation of culture media started from 21th day of inoculation and continued up to 60 days to record the days of shoot initiation.The following of shoot initiation was noted after 35 days of callus culture by using the following formula:

$$\% \text{ shoot initiation} = \frac{\text{Number of calliwith shoots}}{\text{Number of callicultured on shooting media}} \times 100$$

Days required for Root initiation

The observation of shoots on rooting media started from 14th day of inoculation and continued up to 45 days.The data on root initiation was noted after 45 days of shoot culture by using the following formula:

$$\% \text{ root initiation} = \frac{\text{Number of shoots with roots}}{\text{Number of shoots inculated on rooting media}} \times 100$$

Statistical analysis

The data were statistically analyzed using Minitab 17 software and MS Excel 2010.

Results and Discussion

This study investigated the callus induction ability and regeneration potentiality of three varieties of *Brassica* spp. (BARI Sarisha-14, Binasarisha-4 and Binasarisha-9) with two type of explants such as hypocotyls and cotyledon. Investigations of *in vitro* regeneration of three genotypes were accomplished with callus induction, maintenance of Calli, shoot induction, and root initiation.

Seed sterilization and germination of seed

The viability of seed was decreased after exceeding the sterilization period by more than 2 minutes for 70% ethanol and more than 5 minutes for 2-3% sodium hypochlorite. So, the seed contamination and seed germination depend on these disinfectants (Kumar et al., 2017). Average seed germination percentage of BARI Sarisha-14 was (73.3%), for Binasarisha-4 was (74.13%) and for Binasarisha-9 was (67.53%).

Callus induction

Cotyledon and hypocotyls segments from 6-7 days old seedlings were used as explants on MS medium supplemented with 2 mg mL^{-1} gibberellic acid (GA_3), 10 mg mL^{-1} auxin (NAA) and cytokinin (10 mg mL^{-1} BAP and 10 mg mL^{-1} $AgNO_3$) to observe their callus inducing potentiality. BARI Sarisha-14 showed that hypocotyls required 7-10 days and cotyledon explants required 8-13 days for callus induction. Cotyledons showed better performance than hypocotyls in terms of callus induction, with 78.33% and 71.67% respectively even it took a longer period to get the calli. Hypocotyls required 5-10 days and cotyledon explants required 6-10 days for callus induction in Binasarisha-4, while callus obtained after 8-13 days of hypocotyls culture and cotyledon explants required 11-15 days in Bisasarisha-9. Hypocotyls showed better callus induction performance for Binasarisha-4 (76.67% whereas cotyledon is 50.83%). where cotyledon explants showed better callus induction in Bisasarisha-9 (54.17% where hypocotyls 30.00%). BARI Sarisha-14 showed the best performance in terms of callus induction using both hypocotyls and cotyledon explants, while Binasarisha-9 was the lowest callus producing genotype using both. However, binasarisha-4 showed the best callus induction ability using hypocotyls explants (Figure 1). The present

experimental findings showed similar observations obtained by Dubey and Gupta (2014) who also reported hypocotyls as the best explants for callus induction (94%). Biswas et al., 2017 experimented with both cotyledon and stem explants of four mustard genotypes; among these explants, the stem was found to be better responsive in terms of callus induction than cotyledon. Among the genotypes used, Binasarisha-4 induced the highest percentage (100%) of callus from stem explants cultured on MS containing 2.0 mgL^{-1} BAP, 0.5 mgL^{-1} NAA, and 2.0 mgL^{-1} $AgNO_3$.

Table 1. Response of three *Brassica* genotypes towards callus induction

Genotypes	Explants	Replications	No. of explants	No. of explants showing callus induction	% of callus induction	Average % of callus induction ± SE	Days required for callus initiation
BARI Sarisha-14	Hypocotyl	1	40	29	72.5	71.67±0.83	7-8
		2	40	28	70.0		7-8
		3	40	29	72.5		7-10
	Cotyledon	1	40	29	72.5	78.33±3.00	8-9
		2	40	32	80.0		9-11
		3	40	33	82.5		9-13
Binasarisha-4	Hypocotyl	1	40	30	75.0	76.67±3.00	6-8
		2	40	33	82.5		5-10
		3	40	29	72.5		5-6
	Cotyledon	1	40	23	57.5	50.83±3.63	6-7
		2	40	18	45.0		7-9
		3	40	20	50.0		6-10
Binasarisha-9	Hypocotyl	1	40	20	50.0	54.17±4.17	8-9
		2	40	20	50.0		9-13
		3	40	25	62.5		9-11
	Cotyledon	1	40	10	25.0	30.00±6.29	11-15
		2	40	17	42.5		11-13
		3	40	09	22.5		11-13

SE = Standard Error

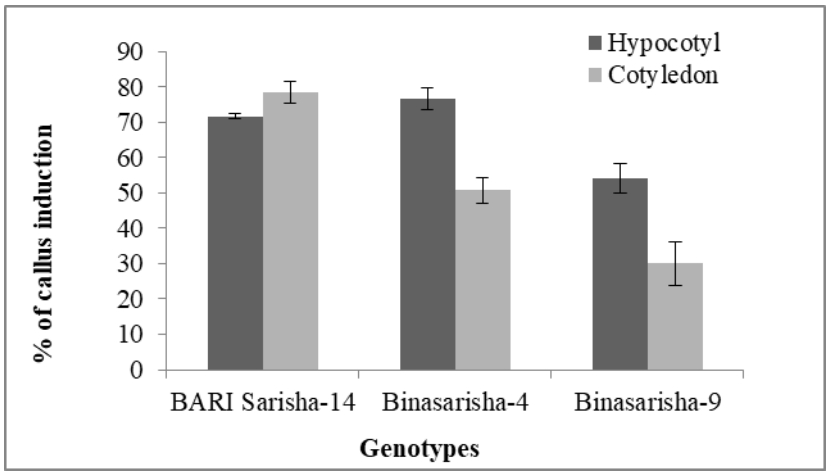

Figure 1. Responses of three varieties of *Brassica* to percent callus induction using hypocotyl and cotyledon explants cultured on callus induction media

Maintenance of callus for shoot initiation

Both hypocotyls and cotyledon explants took 3-4 weeks and 3 weeks to shoot initiation in BARI Sarisha-14 and Binasarisha-4, respectively (Table 2), with BARI Sarisha-14's hypocotyls having higher shoot initiation (58.23%) compared to cotyledon (11.67%) and the hypocotyls showing much better shoot initiation (70.03%) for Binasarisha-4 than the cotyledon (4.45%).In the case of Binasarisha-9, both hypocotyls and cotyledon explants required 4 weeks for shoot initiation, with cotyledon showing a very lower percentage of shoot initiation (2.77%) than hypocotyls (38.87%). Calli from hypocotyls explants were better in shoot initiation than cotyledon in all three varieties, but Binasarisha-4 was the best performer (Figure 3). It was found that the concentrations of BAP and $AgNO_3$ were less in this study but shoot initiation percentage was higher than the result found by Goswami et al. (2020), where the day

requirement for shoot induction was less. Mollika et al. (2011) 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 a combination of BAP and NAA using hypocotyls as explants.

Table 2. Responses of three *Brassica* genotypes towards shoot initiation

Genotypes	Explants	Replications	No. of callus set for shoot initiation	No. of shoot initiation	% of shoot initiation	Average % of shoot initiation ± SE
BARI Sarisha-14	Hypocotyl	1	28	17	60.7	58.23±4.23
		2	25	16	64.0	
		3	26	13	50.0	
	Cotyledon	1	20	2	10.0	11.67±1.67
		2	20	3	15.0	
		3	20	2	10.0	
Binasarisha-4	Hypocotyl	1	27	17	63.0	70.03 ±3.96
		2	30	23	76.7	
		3	27	19	70.4	
	Cotyledon	1	15	0	0.0	4.46±2.23
		2	15	1	6.7	
		3	15	1	6.7	
Binasarisha-9	Hypocotyl	1	18	8	44.4	38.87±3.20
		2	18	7	38.9	
		3	21	7	33.3	
	Cotyledon	1	8	0	0.0	2.77±2.77
		2	12	1	8.3	
		3	8	0	0.0	

SE = Standard Error

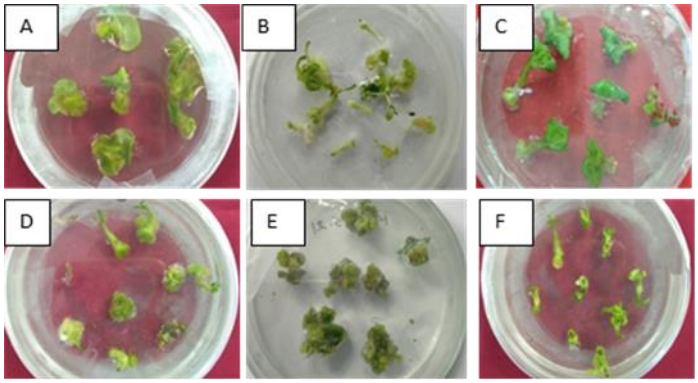

Figure 2. Callus response of BARI Sarisha-14 from cotyledon (A) and hypocotyl (B); Binasarisha-4 from cotyledon (C) and hypocotyl (D); Binasarisha-9 from cotyledon (E) and hypocotyl (F) explants

Shoot regeneration

BARI Sarisha-14 shoots from hypocotyls (73.33%) showed better shoot growth than cotyledon (55.56%). Hypocotyls showed better performance than cotyledon in shoot regeneration from shoot initials in Binasarisha-4 and Binasarisha-9 (Table 3). The shoot outgrowth of hypocotyls explants showed better performance than cotyledon explants in all studied varieties. Some replicates of cotyledon explants did not show any shoot growth, indicating high standard error values (Table 3) and large error bars (Figure 5). But the previous studies showed lower results due to the use of different growth hormones. Biswas et al., 2017 reported that the highest percentage of shoot regeneration (58.34%) from stem explants of Binasarisha-4 was observed in MS medium supplemented with a combination of hormone and silver nitrate. The MS media supplemented with 0.5 mgL^{-1} BAP and 2.5 mgL^{-1} NAA showed better shooting (Nasrin et al., 2017). The MS medium with 2.0-2.5 mgL-1 BAP + 0.5 mgL-1 NAA was the suitable medium for callus induction as all the explants produced callus on this medium but shoot induction was very poor (Trivedi and Dubey, 2014). So, it can be assumed that a combination of adenine hemisulfate, PVP, and BAP is better than other growth hormones.

Table 3. Responses of three *Brassica* genotypes towards shoot outgrowth

Genotypes	Explants	Replications	No. of shoot set for outgrowth	No. of outgrowth shoot	% of outgrowth shoot	Average % of outgrowth shoot ± SE
BARI Sarisha-14	Hypocotyl	1	13	11	80.0	73.33±3.33
		2	14	11	70.0	
		3	10	7	70.0	
	Cotyledon	1	2	1	50.0	55.56±5.57
		2	3	2	66.7	
		3	2	1	50.0	
Binasarisha-4	Hypocotyl	1	13	12	92.3	92.23±1.27
		2	20	18	90.0	
		3	18	17	94.4	
	Cotyledon	1	0	0	0.0	66.66±33.33
		2	1	1	100	
		3	1	1	100	
Binasarisha-9	Hypocotyl	1	8	3	37.4	45.83±5.87
		2	7	4	57.1	
		3	7	3	42.9	
	Cotyledon	1	0	0	0.0	33.33±33.33
		2	1	1	100	
		3	0	0	0.0	

SE = Standard Error

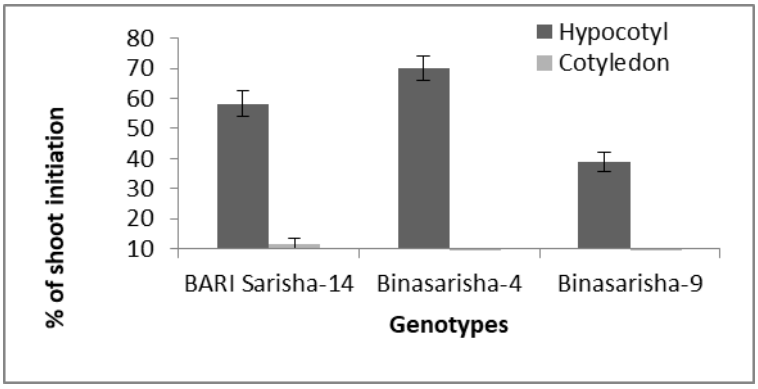

Figure 3. Responses of three varieties of *Brassica* to percent shoot initiation cultured on shoot initiation media

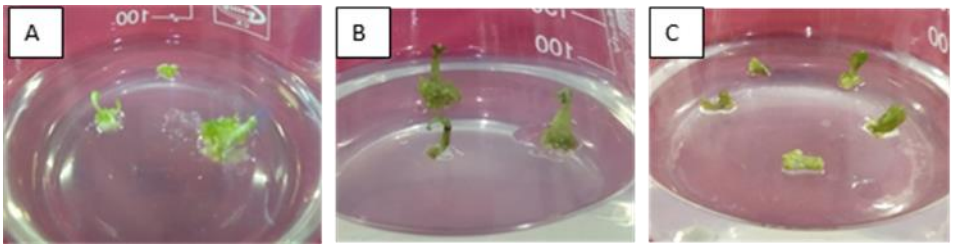

Figure 4. Callus of BARI Sarisha-14 with shoot initials (A); Binasarisha-4 with shoot initials (B) and Binasarisha-9 with shoot initials (C) after 3-4 weeks of culture on shooting media

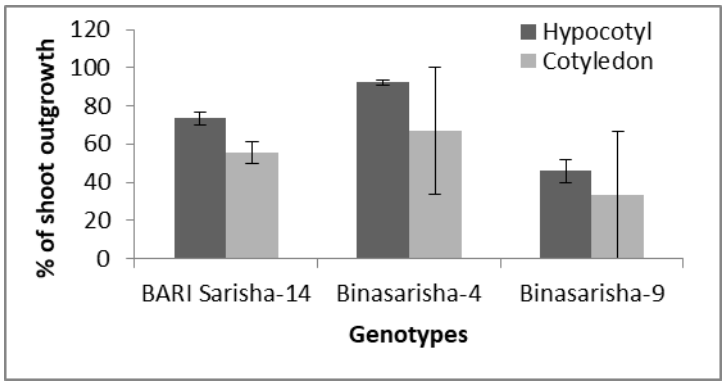

Figure 5. Responses of three genotypes of *Brassica* on percent shoot outgrowth from shoot initials cultured on shoot outgrowth media

Figure 6. Shoot outgrowth of BARI Sarisha-14 (A); Binasarisha-4 (B) and Binasarisha-9 (C) from shoot initials cultured on shoot outgrowth medium

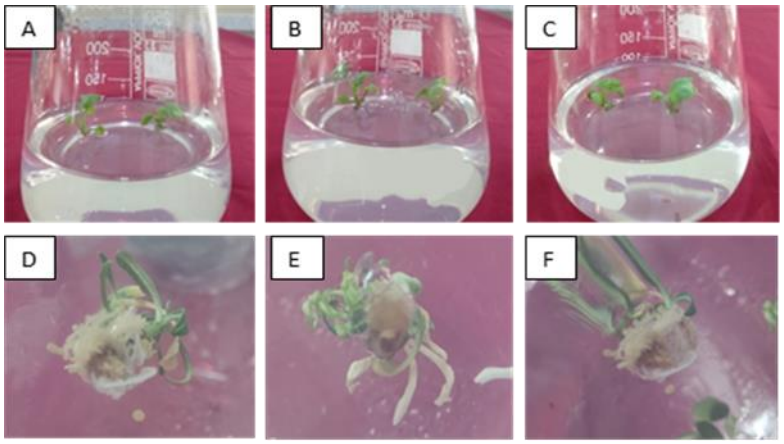

Figure 7. Regenerated shoot of BARI Sarisha-14 (A); Binasarisha-4 (B) and Binasarisha-9 (C) set on rooting medium. Root initiation of BARI Sarisha-14 (D); Binasarisha-4 (E) and Binasarisha-9 (F)

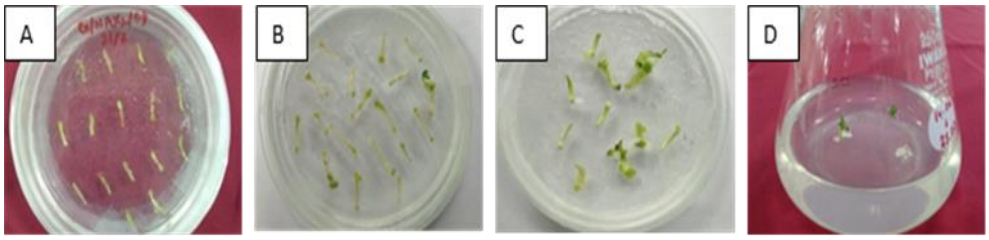

Figure 8. Steps of shoot induction of *Brassica* spp. Nodal part on callus induction medium (A); callus on callus induction medium (B); callus with shoot initials on shoot induction medium (C) and shoot initials cultured on shoot outgrowth medium (D) of Binasarisha-4

Root induction

Well-developed elongated shoots were excised and transferred in MS medium (half strength) containing IBA. Root initiation was recorded after 28-35 days of culture in root induction medium. BARI Sarisha-14 showed root initiation in 35 days, while Binasarisha-4 and Binasarisha-9 took 28 and 29 days respectively. Binasarisha-4 showed the highest root initiation (40%), followed by BARI Sarisha-14 (23.33%) and Binasarish-9 (11.11%), (Table 4). A similar result was found in several previous studies. Nasrin et al. (2017) found that the medium supplemented with MS + 1 mgL^{-1} IBA was the best medium for root formation within 20-24 days. In this study, the days require for root initiation were mostly varied with genotypes. The best root initiation was observed when shoots were rooted on MS medium supplemented with 1 mgL^{-1} NAA (George and Rao, 1980). Bhuyian et al. (2009) also found that in *Brassica juncea* the highest frequency of rooting was reported in MS media supplemented with 0.1 mgL^{-1} NAA. Increasing the concentration of NAA dramatically decreased the frequency of rooting. But surprisingly Aoun et al. (2008) experimented that regenerated shoots were rooted on MS basal medium without any growth regulators and that induced 99% roots per shoot.

Table 4. Responses of three *Brassica* genotypes towards root initiation

Genotypes	Explants	Replications	No. of shoot set in rooting media	No. of shoot produced root initials	% of root initiation	Average % of root initiation ± SE
BARI Sarisha-14	Hypocotyl	1	10	2	20	23.33±3.33
		2	10	3	30	
		3	5	1	20	
Binasarisha-4	Hypocotyl	1	10	3	30	40±2.89
		2	10	4	40	
		3	10	5	50	
Binasarisha-9	Hypocotyl	1	2	0	0	11.11±11.11
		2	3	1	33.33	
		3	2	0	0	

SE = Standard Error

Table 5. Response of Binasarisha-4 towards callus induction and shoot initiation

Genotype	Explant	Replications	Callus induction (%)	Average callus induction (%)	Day required for callus induction	Shoot initiation (%)	Average % of shoot initiation
Binasarisha-4	**Hypocotyl**	1	6.4	**7.35**	10	2.3	3.15
		2	8.3		12	4	

Conclusion

Through this experiment a detailed investigation was carried out to study the callus induction ability and a subsequent regeneration of three *Brassica* genotypes. According to the findings, *in vitro* Binasarisha-4 hypocotyls culture has the potential to be employed for frequent and effective genetic transformation in Mustard. This experiment outlined a suitable protocol for *in vitro* regeneration and successful genetic transformation of *Brassica*, which is the prerequisite for the development of stress-tolerant *Brassica* through genetic transformation.

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