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EFFICIENT *IN VITRO* REGENERATION OF MUSTARD VIA ORGANOGENESIS FOR GENETIC TRANSFORMATION APPLICATIONS

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Abstract

The third-most significant edible oil crop is mustard (*Brassica spp.*), which is a part of the genus *Brassica* and the family Brassicaceae (Cruciferae). Global demand for edible oil and biofuels is rising as a result of rising world population and industrialization. An efficient *in vitro* regeneration protocol was developed for *Brassica spp.* through direct and indirect organogenesis from hypocotyl, cotyledon and cotyledonary nodal segment. Murashige and Skoog medium (MS) medium with different concentrations and combinations of plant growth regulators were used for multiple shoot regeneration in three genotypes of mustard namely BARI Sarisha-14, Binasarisha-4 and Binasarisha-9. Explants were inoculated on supplemented with various concentrations and combinations of NAA, BAP, $AgNO_3$, GA_3, and IBA for callus induction, shoot initiation, shoot outgrowth, and root initiation. For *In vitro* regeneration, cotyledonary nodes performed the best with 100% in Binasarisha-4, 98.33% in BARI Sarisha-14 and 91.67% in Binasarisha-9. Binasarisha-4 demonstrated 76.67% callus induction, 70.83% shoot initiation, 91.82% shoot outgrowth, and 40% root initiation with hypocotyl, followed by BARI Sarisha-14 (76.67%, 58.33%, 75.00%, and 23.33%, respectively) and Binasarisha-9 (52.78%, 47.78%, 49.04%, and 11.11%). After proper hardening the *in vitro* regenerated plantlets were successfully transplanted into soil. Interestingly some of the *in vitro* regenerated shoots produced early flowers on rooting media.

Keywords: Callus induction, Hypocotyl explants, Micropropagation, Plant growth regulators, Shoot organogenesis.

Introduction

After soybean and oil palm, rapeseed-mustard (*Brassica* spp.) is the third-most significant oilseed crop in the world. Round, average-sized mustard seeds have a nutty, sweeter, and slightly acrid flavor. These seeds have a high percentage of oil (29-40%), protein (23-30%) and carbohydrates (12-18%) (Zargar *et al.*, 2016). With 0.61 million hectors and 822 thousand metric tons of production, respectively, mustard ranks first among the oilseed crops cultivated in Bangladesh in the fiscal year 2021-22 (Rahman *et al.*, 2024; BBS, 2022).

Farmers are more driven to raise their output since mustard oil is more expensive than both soybean and palm oil. It is one of the greatest cooking oils, especially for heart patients, as it contains both linolic acid and alpha linolic acid, which are omega 3 and omega

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6 fatty acids. In addition to these fatty acids, mustard oil also contains stearic acid, palmitic acid, arachidic acid, behenic acid, lignoceric acid, oleic acid, eicosenic acid, erucic acid, and linolenic acid. Vitamin E and other natural antioxidants are abundant (Kaur *et al*., 2019). Additionally, it has a number of medical qualities, is naturally antibacterial, and prevents infections. Due to the presence of substances like glucosinolate, mustard oil has antifungal effects. The oil meal (oil cake), which is a byproduct of the extraction of oil from the seeds, is utilized as a protein supplement in dairy, beef, and poultry rations and is known for its consistent quality as well as its value (Shaaban, 2020). In addition to this, certain *Brassica campestris* cultivars are cultivated for their high erucic acid content. This oil is also used in plastics, lubricants, lacquers, and detergents.

Almost 20 distinct kinds of mustard have been released from Bangladesh Agricultural Research Institute (BARI) and Bangladesh Institute of Nuclear Agriculture (BINA) and Department of Agricultural Extension (DAE) is pushing the increase of rapeseed and mustard production through its extension operations (USDA, 2022). The degree of farm-level productivity is still uninspiring because most farmers disregarded the research organizations' farming advice. The percentage of area brought under mustard cultivation in the fiscal year 2020-21 has been steadily declining but has recently been growing significantly. Better HYV seeds and contemporary farming techniques have improved mustard yield as well. From the year 2020–21, the cultivated area increased by 0.36%, and the yield climbed by 2.87%. Due to producers of mustard receiving acceptable prices over the past couple of years, cultivation area has also risen continuously (Arafat, 2022; BBS, 2021). This is quite optimistic for Bangladesh's goal of achieving food independence.

To achieve self-sufficiency in edible oils, the Bangladeshi government has placed a high priority on oilseed crop research and development (R&D) and has made large financial investments. The BARI and BINA have made a number of improved oilseed cultivars available (Miah *et al.*, 2015b; Miah *et al*., 2015c). The adoption rate of these enhanced varieties is encouraging at the farm level, and it has had a positive impact and saved the country foreign exchange (Miah *et al*., 2015a).

For crop development, conventional breeding techniques are most frequently applied. However, in real-world applications, these methods need to be complemented with plant tissue culture techniques, either to boost their effectiveness or to accomplish goals that can't be accomplished with the conventional approaches. The standard breeding methods were used to enhance the agronomically significant traits in *Brassica*, but they were not very successful because of the high degree of segregation through cross-pollination and the lack of adequate wild *Brassica* germplasm. Additionally, a typical breeding program requires careful selection of desirable features and is time-consuming and labor-intensive. *In vitro* regeneration and transformation may be able to meet breeding requirements (Khan *et al*., 2010). The plant *Brassica napus* has been the subject of in-depth research and breeding using tissue culture.

Plant regeneration from tissue culture is a significant and necessary part of biotechnological research and is occasionally necessary for the genetic engineering of plants. A lot of work has been put into developing an *in vitro* approach for *Brassica* regeneration during the past few decades. *Brassica* species have successfully regenerated plants utilizing a variety of explants, including petiole, cotyledon, stem, and shoot tips. In plants grown from cotyledon and hypocotyl explants of Indian mustard cultivars, shoot regeneration, rooting, and plant survival were all high (Bhalla *et al*., 2001). Shoot tip explant of *Brassica* were reported to be effective for initiating shoots and roots (Zhang *et al*., 1998).

Due to the rapid climate change and consistent expansion in world population, plants' essential economic features must be improved quickly. Aiming to improve plant genotypes through rapid multiplication, micropropagation of disease-free plants, manufacture of plant-derived metabolites, and gene transformation, *in vitro*-based biotechnology technologies are now used in breeding (Hesami *et al*. 2021). One of the most important biotechnological strategies for enhancing plant performance is genetic engineering. There are direct and indirect ways to change a plant's genetic makeup (Nasrin *et al*., 2017). Genetic transformation of crop plants is quickly taking over as the preferred method for creating new agricultural types. For use in genetic transformation experiments, an effective regeneration protocol for selected germplasms of *Brassica* is very essential in order to yield transgenic with the desired properties. Therefore, the present study was designed in order to develop and standardize an effective protocol for *in vitro* callus induction and regeneration of selected *Brassica* cultivars for further genetic transformation.

Materials and Methods

The experiment was conducted between January 2023 and September 2023 in the Tissue Culture Laboratory of the Biotechnology Division at the Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. Three mustard cultivars, namely BARI Sarisha-14, Binasarisha-4, and Binasarisha-9, were selected as experimental materials. The seeds were sourced from the Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, and BINA, Mymensingh. The seeds were sterilized and plated onto Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Explants, including cotyledons, cotyledonary nodes, and hypocotyls, were collected from aseptically grown seedlings aged 6-7 days.

Nutrient media preparation and culture conditions

The MS medium was supplemented with essential nutrients, vitamins, amino acids, and plant growth regulators (PGRs) obtained from Sigma Aldrich and Phytotech (USA). The growth regulators used included auxins (Indole Butyric Acid - IBA, and Naphthalene Acetic Acid - NAA), cytokinins (6-Benzylaminopurine - BAP, and $AgNO_3$), and gibberellic acid (GA3). The medium was solidified using gelrite, and sucrose was used as the carbon source. Cultures were maintained in a controlled environment with a 16-hour photoperiod under 500 lux cool white fluorescent light at 24 ± 2°C.

To prepare 500 mL of MS medium, 20 g of sucrose was dissolved in 350 mL of distilled water, followed by the addition of 2.25 g of MS powder. The volume was adjusted to 500 mL after thorough mixing. The pH was adjusted to 5.8 using 0.1 N HCl or 40% NaOH, and 3 g of gelrite was added for solidification. The medium was covered with aluminum foil and autoclaved at 121°C under 15 psi pressure for 30 minutes. The sterile medium was dispensed into Petri dishes inside a laminar airflow cabinet under aseptic conditions.

Sterilization protocols and stock solutions

The sterilization process involved treating materials with 70% ethanol, 2–3% sodium hypochlorite, and 0.1% mercuric chloride, while hormones were sterilized using a 0.25 µm filter. The sterilization of beakers, test tubes, conical flasks, pipettes, forceps, scalpels, and other metal equipment was carried out using an autoclave at 121°C and 15 psi for 30 minutes. Forceps, scissors, and scalpels were flame-sterilized before dipping in ethanol. The culture room was sanitized by washing with detergent, wiping with 70% ethanol, and sterilizing the laminar airflow cabinet with UV light for 30 minutes.

Stock solutions of PGRs were prepared separately: NAA (10 mg/mL) was dissolved in NaOH and diluted with sterile distilled water. BAP (10 mg/mL and 2.5 mg/mL) was prepared similarly, while $AgNO_3$ (50 mg/mL) was dissolved directly in sterile distilled water. GA_3 (2 mg/mL) was dissolved in 50% ethanol, and IBA (10 mg/mL) followed the same procedure as NAA.

Tissue culture procedures

Four types of media were used: seed germination, callus induction, shoot initiation, and root induction media. The callus induction medium contained MS supplemented with 5mg/l $AgNO_3$, 3.0 mg/l BAP, 1.0 mg/l NAA and 0.2 mg/l GA_3. Shoot initiation medium contained the same composition. Root induction medium used half-strength MS supplemented with1.0 mg/l IBA and 1.2 mg/l NAA (Verma *et al*., 2016).

For *in vitro* culture, mustard seeds were surface-sterilized by rinsing with double-distilled water, followed by immersion in 70% ethanol for one minute. After ethanol treatment, seeds were rinsed thrice in distilled water, then shaken in 2–3% NaOCl for 10 minutes with Tween-20. Excess sterilant was removed with additional rinses before plating seeds onto MS medium in Petri dishes, which were sealed with parafilm and incubated at 24°C for 10-20 days.

Explants, including hypocotyls (1-2 cm), cotyledonary nodes (1-2 cm), and cotyledons (3-5 cm), were excised from 6-7-day-old seedlings using a sterile scalpel. Hypocotyls and cotyledonary nodes were placed horizontally on callus induction media, while cotyledons with 2 mm petioles were positioned vertically. The cultures were maintained at 24°C under a 16-hour photoperiod with 3000 lux light. Callus formation was observed within 1-2 weeks, and subculturing was performed after 3-4 weeks.

Calli (2-3 cm) were sectioned and transferred to shoot induction media. Subculturing was conducted every 2-3 weeks to prevent nutritional depletion and crowding. Shoot growth was monitored regularly, and once regenerated shoots reached 3-4 cm with 3-4 fully developed leaves (after 25-30 days), they were aseptically transferred to rooting media.

Experimental design and data collection

The experiment followed a Completely Randomized Design (CRD) with three replications. Data were collected on key parameters, including the time required for callus induction (observed from the 7th to the 35th day), percentage of callus initiation (calculated as the number of explants forming callus divided by total explants inoculated × 100), shoot initiation (observed from the 21st to the 60th day), percentage of shoot initiation (number of calli producing shoots divided by total calli cultured × 100), root initiation (observed from the 14th to the 45th day), and percentage of root initiation (number of shoots with roots divided by total shoots transferred × 100).

Statistical analysis

The recorded data were statistically analyzed using Microsoft Statistical (MSTAT) software and Microsoft Excel. Analysis of variance (ANOVA) was performed following the CRD method, and means were compared using the Least Significant Difference (LSD) test at a 5% probability level. Results were ranked accordingly to assess significant differences among treatments.

Results and Discussion

Effect of genotype on seed germination and callus formation

The study assessed seed germination rates among three genotypes of *Brassica* spp.: BARI Sarisha-14, Binasarisha-4, and Binasarisha-9. A total of 112 seeds were plated, with BARI Sarisha-14 exhibiting the highest germination rate (74.67%), followed by Binasarisha-4 (71.67%), while Binasarisha-9 had the lowest germination rate (41.33%) (Table 1). These findings align with prior research indicating that genotype significantly influences germination efficiency (Dubey and Gupta, 2014). Although BARI Sarisha-14 had the highest germination percentage, Binasarisha-4 required the shortest germination time (6.00 days), whereas BARI Sarisha-14 and Binasarisha-9 took 10.33 and 12.67 days, respectively.

Callus formation efficiency also varied significantly among genotypes. BARI Sarisha-14 exhibited the highest callus formation rate (48.70%), followed by Binasarisha-4 (42.04%) and Binasarisha-9 (27.96%) (Table 2). Binasarisha-4 initiated callus formation in the shortest time (9.22 days), whereas Binasarisha-9 required the longest (11.83 days). These findings are consistent with previous studies demonstrating genotype-specific variations in callus induction efficiency (Biswas *et al*., 2017).

Table 1. Effect of genotype on seeds germination of *Brassica* spp.

Genotypes	No. of inoculated seeds	No. of germinating seed	Seed germination (%)	Days required for seed germination
BARI Sarisha-14	112	74.67 a	66.66 a	10.33 b
Binasarisha-4	112	71.00 a	63.39 a	6.000 c
Binasarisha-9	112	41.33 b	36.90 b	12.67 a
Level of sig.	–	**	**	**
LSD(0.05)	–	7.40	6.61	0.93
CV (%)	–	5.24	5.24	4.22

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance; **= Significant at 1% level of probability

Table 2. Effect of different genotypes on callus formation

Genotypes	No. of inoculated explant	No. of callus explant^{-1}	Callus induction (%)	Days required for callus initiation
BARI Sarisha-14	60	29.22 a	48.70 a	10.22 b
Binasarisha-4	60	25.22 b	42.04 b	9.222 c
Binasarisha-9	60	16.78 c	27.96 c	11.83 a
Level of sig.	–	**	**	**
LSD(0.05)	–	3.26	5.44	0.75
CV (%)	–	13.88	13.88	7.27

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance; **= Significant at 1% level of probability

Influence of explant type and genotypic variability on callus formation, shoot initiation, and shoot outgrowth

Effect of explants on callus formation

Among the explants tested, hypocotyls demonstrated the highest callus induction rate (65.93%), outperforming cotyledons (52.78%) and cotyledonary nodes (0%) (Table 3). Hypocotyls also required the shortest duration for callus induction (8.11 days), consistent with findings by Dubey and Gupta (2014), who reported higher callus induction from hypocotyl explants.

Table 3. Effect of different explant on callus formation

Genotypes	No. of inoculated explant	No. of callus explant^{-1}	Callus induction (%)	Days required for callus initiation
Hypocotyl	60	39.56 a	65.93 a	8.111 c
Cotyledon	60	31.67 b	52.78 b	10.00 b
Cotyledonary nodes	60	0.000 c	0.000 c	13.17 a
Level of sig.	–	**	**	**
LSD(0.05)	–	3.26	5.44	0.75
CV (%)	–	13.88	13.88	7.27

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance; **= Significant at 1% level of probability

Effect of genotype and explant interactions on callus formation

Callus induction was significantly influenced by genotype-explant interactions. BARI Sarisha-14's cotyledons exhibited the highest callus induction (76.67%) in 9.83 days, whereas Binasarisha-4's hypocotyls demonstrated a similarly high callus induction rate (75.56%) in just 6.67 days (Table 4). Conversely, Binasarisha-9's cotyledons exhibited the lowest callus induction rate (31.11%), corroborating Bano *et al*. (2010), who reported that genotype and explant type significantly affect callus induction efficiency.

Table 4. Effects of interaction between genotype and explant on callus formation

Genotypes	Explants	No. of explant	No. of callus explant^{-1}	Callus induction (%)	Days required for callus initiation
BARI Sarisha-14	Hypocotyl	60	41.67 a	69.45 a	7.833 c
	Cotyledon	60	46.00 a	76.67 a	9.833 b
	Cotyledonary nodes	60	0.000 d	0.000 d	13.00 a
Binasarisha-4	Hypocotyl	60	45.33 a	75.56 a	6.667 c
	Cotyledon	60	30.33 b	50.56 b	7.500 c
	Cotyledonary nodes	60	0.000 d	0.000 d	13.50 a
Binasarisha-9	Hypocotyl	60	31.67 b	52.78 b	9.833 b
	Cotyledon	60	18.67 c	31.11 c	12.67 a
	Cotyledonary nodes	60	0.000 d	0.000 d	13.00 a
Level of sig.		–	**	**	**
LSD(0.05)		–	5.65	9.42	1.30
CV (%)		–	13.88	13.88	7.27

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance; **= Significant at 1% level of probability

Effect of genotype on shoot initiation from callus

Both BARI Sarisha-14 and Binasarisha-4 demonstrated superior shoot initiation (32.92%), significantly higher than Binasarisha-9 (25.56%) (Table 5). This result aligns with prior studies indicating that genotype influences shoot initiation (Goswami *et al*., 2020).

Table 5. Effect of genotype on shoot initiation from callus

Genotypes	No. of callus inoculated for shoot initiation	No. of shoots callus^{-1}	Shoot formation (%)
BARI Sarisha-14	40	13.17 a	32.92 a
Binasarisha-4	40	14.83 a	37.64 a
Binasarisha-9	40	7.333 b	25.56 b
Level of sig.	–	**	**
LSD(0.05)	–	2.22	6.62
CV (%)	–	14.98	16.41

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance; **= Significant at 1% level of probability

Effect of explants on shoot initiation from callus

Hypocotyl explants exhibited the highest shoot initiation rate (58.98%) compared to cotyledons (5.09%) (Table 6). This observation supports previous research highlighting the superiority of hypocotyls in shoot initiation (Biswas *et al*., 2017).

Table 6. Effect of explants on shoot initiation from callus

Genotypes	No. of callus inoculated for shoot initiation	No. of shoots callus^{-1}	Shoot formation (%)
Hypocotyl	40	22.00 a	58.98 a
Cotyledon	40	1.556 b	5.093 b
Level of sig.	–	**	**
LSD(0.05)	–	1.81	5.40
CV (%)	–	14.98	16.41

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance; **= Significant at 1% level of probability

Effect of genotype and explant interactions on shoot initiation

Genotype-explant interactions further demonstrated that hypocotyl explants consistently showed superior shoot initiation across all genotypes (Table 7). Binasarisha-4's hypocotyl explants exhibited the highest shoot initiation rate (70.83%). This finding is in line with Goswami *et al*. (2020), who found that hypocotyls were more efficient than cotyledons in shoot initiation for *Brassica* species.

Table 7. Effects of interactions between genotype and explant on shoot initiation from callus

Genotypes	Explants	No. of callus inoculated for shoot initiation	No. of shoots callus^{-1}	Shoot formation (%)
BARI Sarisha-14	Hypocotyl	60	23.33 B	58.33 B
	Cotyledon	60	3.000 d	7.500 d
Binasarisha-4	Hypocotyl	60	28.33 a	70.83 a
	Cotyledon	60	1.333 d	4.447 d
Binasarisha-9	Hypocotyl	60	14.33 c	47.78 c
	Cotyledon	60	0.333 d	3.333 d
Level of sig.		–	**	**
LSD(0.05)		–	3.14	9.35
CV (%)		–	14.98	16.41

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance; **= Significant at 1% level of probability

Effect of genotype on shoot outgrowth

BARI Sarisha-14 exhibited the best shoot outgrowth (76.30%), followed by Binasarisha-4 (86.16%), while Binasarisha-9 exhibited the lowest (Fig. 1). These results suggest that shoot outgrowth is genotype-dependent, with Binasarisha-4 demonstrating superior performance in this phase of regeneration.

Effect of Explants on Shoot Outgrowth

Cotyledonary nodes demonstrated the highest shoot proliferation rate (96.67%), followed by hypocotyls (71.95%) and cotyledons (51.85%). These results underscore the importance of selecting the appropriate explant for optimal shoot regeneration in mustard tissue culture.

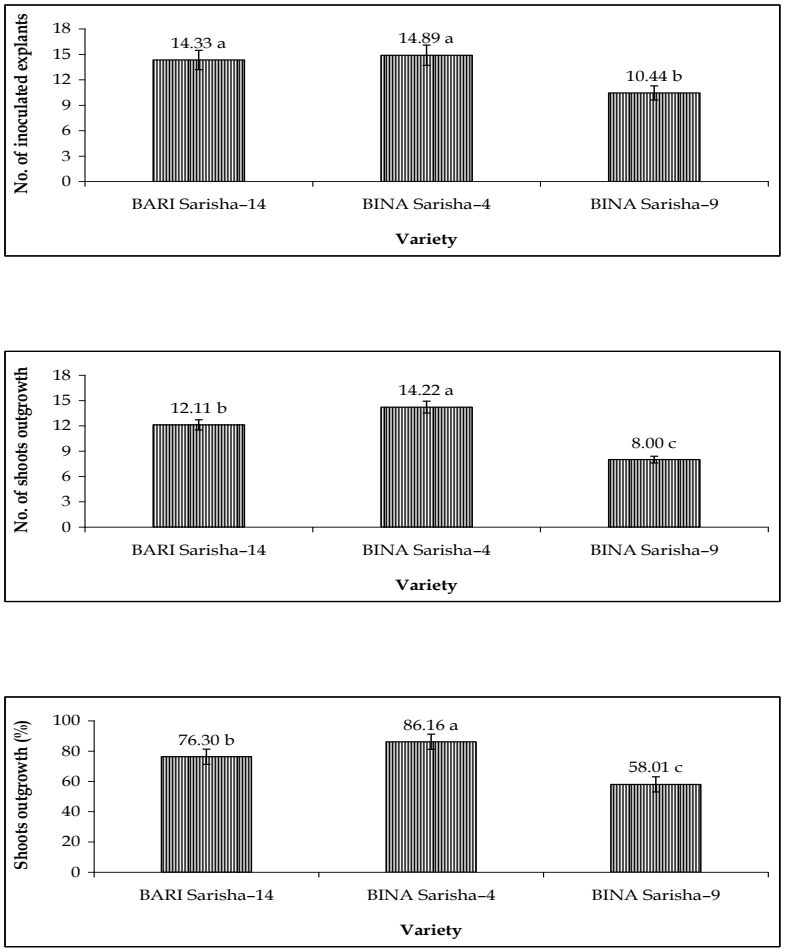

Fig. 1. Effect of genotype on shoot outgrowth.

Effect of Genotype and Explant Interactions on Shoot Outgrowth

The interaction between genotype and explant revealed that cotyledonary nodes were the most effective in shoot outgrowth for Binasarisha-4 (100%), followed by BARI Sarisha-14 (98.33%) and Binasarisha-9 (91.67%) (Table 8). These findings support prior research indicating that cotyledonary nodes and hypocotyls are efficient explants for shoot regeneration (Biswas *et al*., 2017).

Table 8. Effects of interactions between genotype and explant on outgrowth of shoot

Genotypes	Explants	No. of explants inoculated	No. of inoculated explants	No. of shoots outgrowth	Shoot outgrowth (%)
BARI Sarisha-14	Hypocotyl	40	20.00 b	15.00 d	75.00 b
	Cotyledon	40	3.000 d	1.667 f	55.56 c
	Cotyledonary nodes	40	20.00 b	19.67 b	98.33 a
Binasarisha-4	Hypocotyl	40	24.00 a	22.00 a	91.82 a
	Cotyledon	40	0.667 e	0.667 fg	66.67 b
	Cotyledonary nodes	40	20.00 b	20.00 b	100.0 a
Binasarisha-9	Hypocotyl	40	11.00 c	5.333 e	49.04 c
	Cotyledon	40	0.333 e	0.333 g	33.33 d
	Cotyledonary nodes	40	20.00 b	18.33 c	91.67 a
Level of sig.		–	**	**	**
LSD(0.05)		–	1.36	1.10	10.06
CV (%)		–	6.0	5.83	7.97

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance.
**= Significant at 1% level of probability

Genotypic and explant-specific variability in root formation

Effect of explants on root formation

Hypocotyl explants exhibited the highest root formation rate (24.81%), while cotyledonary nodes exhibited the lowest (18.89%) (Table 9). This observation is consistent with earlier studies indicating that hypocotyls provide favorable conditions for root initiation in mustard tissue culture (Ahmad and Spoor, 1999).

Table 9. Effect of explants on root formation

Genotypes	No. of shoots set in rooting media	No. of roots shoot^{-1}	Root formation (%)
Hypocotyl	6.889 b	2.111	24.81 a
Cotyledonary nodes	10.00 a	1.889	18.89 b
Level of sig.	**	ns	*
LSD(0.05)	1.23	0.49	4.91
CV (%)	14.20	23.57	21.87

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance.
**, *= Significant at 1 and 5%, respectively level of probability and ns= non-significant

Effect of genotype and explant interactions on root formation

The interaction between genotype and explant confirmed that hypocotyls of Binasarisha-4 were the most effective for root formation (40.00%), whereas Binasarisha-9 exhibited the lowest root formation from hypocotyl explants (11.11%) (Table 10). These findings further highlight the role of genotype and explant type in root induction responses, aligning with the findings of Ahmad and Spoor (1999).

Table 10. Effects of interactions between genotype and explants on root formation

Genotypes	Explants	No. of shoots set in rooting media	No. of roots shoot^{-1}	Root formation (%)
BARI Sarisha-14	Hypocotyl	8.333 a	2.000 b	23.33 b
	Cotyledonary nodes	10.00 a	1.667 b	16.67 bc
Binasarisha-4	Hypocotyl	10.00 a	4.000 a	40.00 a
	Cotyledonary nodes	10.00 a	2.333 b	23.33 b
Binasarisha-9	Hypocotyl	2.333 b	0.333 c	11.11 c
	Cotyledonary nodes	10.00 a	1.667 b	16.67 bc
Level of sig.		**	**	**
LSD(0.05)		2.14	0.84	8.51
CV (%)		14.23	23.57	21.87

In a column means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of significance; **= Significant at 1% level of probability

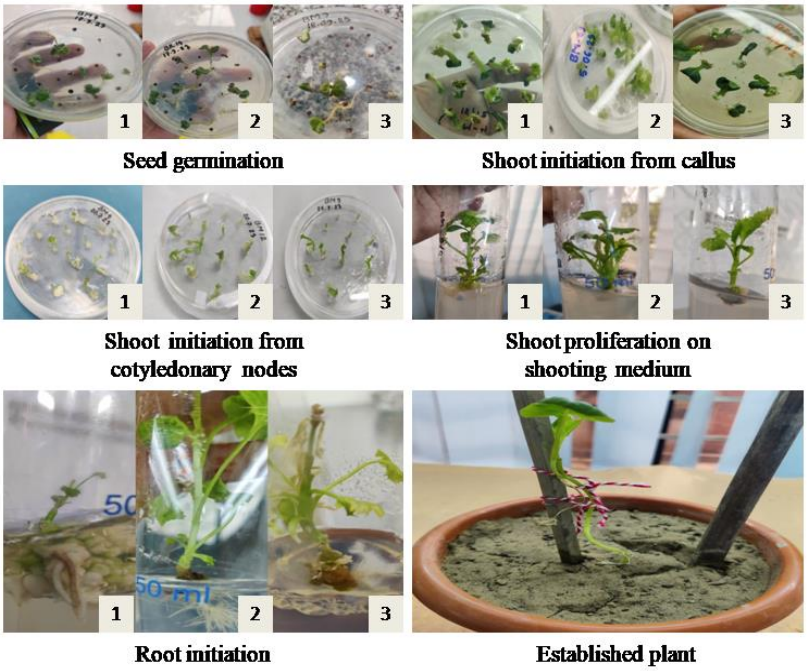

Fig. 2. *In vitro* regeneration of three mustard genotypes (1. BARI Sarisha-14, 2. Binasarisha-4 and 3. Binasarisha-9)

This study demonstrated significant genotypic and explant-specific variations in seed germination, callus formation, shoot initiation, shoot outgrowth, and root formation in *Brassica* spp. Binasarisha-4 exhibited superior performance across multiple regeneration stages, particularly in hypocotyl explants (Fig. 2). These results provide valuable insights into mustard tissue culture, contributing to future advancements in genetic improvement and plant regeneration techniques.

Conclusion

The findings of this study highlight the significant influence of genotype and explant type on *in vitro* regeneration of *Brassica* spp. Binasarisha-4 demonstrated the highest efficiency in callus induction, shoot initiation, and root formation, particularly from hypocotyl explants. The study also revealed spontaneous *in vitro* flowering in Binasarisha-9, suggesting the potential for endogenous hormone synthesis. These results provide valuable insights for future genetic transformation and breeding programs aimed at improving mustard varieties. The established regeneration protocol can serve as a foundation for further advancements in *Brassica* tissue culture and biotechnology research.

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