Establishment of Tissue Culture Protocol of *Brassica juncea* var. BARI Sarisha-11

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Declaration

It is hereby declared that

1. The thesis submitted was our original work while completing a degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Ethics Statement

We, Hridi Prova Saha, Alisa Jawad, Samiha Nujhat Zaman, and Asmita Roy, hereby certify that the following criteria are fulfilled for the manuscript "Establishment of Tissue Culture Protocol of *Brassica juncea* var. BARI Sarisha-11".

1. This content is our own original work and has not been previously published.
2. All sources used are appropriately acknowledged through accurate citations and justified references.

Abstract

Brassica sp., commonly known as mustard, holds significant economic importance as a crop with high oil content, particularly in Bangladesh. Despite widespread demand, obstacles in production such as insufficient germination and various biotic and abiotic factors persist, biotechnological methods, including tissue culture, gene transfer, and genome editing systems, are increasingly employed to enhance this crop quality. This study focuses on improving *Brassica juncea*, specifically the variety BARI Sarisha-11, a vital mustard variety in Bangladesh. The primary objective is to refine a tissue culture-mediated regeneration procedure, laying the groundwork for future applications in micropropagation. In this study, to achieve high-frequency, uncontaminated germination, a seed sterilization protocol was established. Various combinations and durations of 70% ethanol, 0.1% mercury chloride, and 10% Clorox solution were tested. Among them, the highest optimal uncontaminated germination was obtained when seeds were sterilized with 70% ethanol for one minute and 0.1% mercury chloride for ten minutes. For shoot regeneration, 7 days old- cotyledonary leaves with petiole explants were used in nutrient media enriched with plant growth regulators, specifically 6-benzylaminopurine (BAP), 6-furfurylaminopurine (Kinetin/Kn), and naphthalene acetic acid (NAA). The highest regeneration (75%) was achieved with 1.0 mg/L BAP, 0.1 mg/L NAA, and 0.5 mg/L Kn. Rooting was examined using hormone less half-strength MS media, and acclimatization of plantlets was done in sterilized potted soil. Only one plantlet was produced following transplantation. In summary, given the crucial role of mustard as the primary oilseed crop in Bangladesh, this study represents a vital initial step in implementing mustard crop improvement techniques. The establishment of a practical, efficient, and replicable regeneration process, as demonstrated in this study, holds significant importance for the future of mustard cultivation.

Keywords: tissue culture; *Brassica juncea*; sterilization; germination; regeneration; rooting.

Dedication

This thesis is dedicated to those who have been our guiding lights throughout this academic journey.

To our family for their unwavering sacrifices, support, and love. Without you, it would not have been possible, and we are forever indebted to you for that.

To our supervisor, faculties, and all the mentors, we wholeheartedly appreciate your guidance and patience. You've made a big impact on our learning journey. We hope we can live up to your expectations in the future.

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We truly hope that we will be able to keep up with all of your expectations in the future and will utilize these experiences and knowledge in our further career and research work for the betterment of the people.

Hridi Prova Saha, Alisa Jawad, Samiha Nujhat Zaman, Asmita Roy

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List of Acronyms

The following abbreviations have been used throughout the text.

BAP	6-Benzylaminopurine
BARI	BARI Bangladesh Agricultural Research Institute
NAA	Naphthalene acetic acid
Kn	Kinetin
mg	Milligram
ml	Millilitre
L	Litre
MS	Murashige and Skoog (1962) medium
SD	Standard Deviation
SIM	Shoot Induction Media

Chapter 1

Introduction

Oilseed crops are primarily cultivated for the purpose of producing edible oil. There has been a recent surge in demand for oilseeds due to their nutritious vegetable oils, use as animal feed, production of pharmaceuticals, biofuels, and other industrial applications in the oleochemical industry (Gupta, 2016), which has led to increasing focus towards them. The usage of edible oil in Bangladesh is growing by the day. In Bangladesh, mustard (*Brassica sp*.) oil is widely utilized in culinary practices, serving as a cooking oil, salad dressing, and marinade for various food items. Additionally, it is employed as a preservative in pickling and chutney preparation. Mustard oil possesses several properties that offer notable health benefits, such as reducing blood pressure and cholesterol. Beyond culinary uses, mustard oil finds industrial applications in plastics, lubricants, lacquers, and detergents.

1.1 Significance of Mustard Production for Bangladesh's Economy:

In the fiscal year 2021–2022, mustard stood out as the leading oilseed crop in Bangladesh, occupying the top position in terms of both cultivated area and production output, covering an area of 6.10 lakh hectares. This amount only covers a small percentage of the nation's need for cooking oil. Hence, in 2021, Bangladesh spent 2.12 million US dollars importing mustard. Australia was the main supplier of mustard to Bangladesh, accounting for a 75% share of total imports, followed by Canada (18%), Ukraine (6%), and Malaysia (1%) in 2021 (Arafat, 2022).

Moreover, large amounts of sunflower and soybean oil were also imported to meet the country's cooking oil demand due to its shortage of cultivation. The nation's mustard cultivation has been declining since imported soybean oil is less expensive than domestic mustard oil. (Arafat, 2022) However, Bangladesh's agricultural industry is focusing on encouraging farmers to boost the production and cultivation of mustard to reduce import expenditures.

1.2 Challenges with Traditional Mustard Farming in Bangladesh

Bangladesh's mustard farming has several obstacles, most of which are caused by biotic and abiotic stresses that severely reduce crop yield and quality. Insect invasions, bacterial and fungal

infections, and parasitic plants cause significant harm to these crops. Examples of these diseases include leaf blight and downy mildew. Although there are several improved varieties of mustard available on the market, farmers face hardship since these types are not resistant to common diseases and pests. Moreover, there are certain inherent ingredients like erucic acid, long-chain fatty acids, glucosinolates, etc. Erucic acid has been linked to cardiac injury, while glucosinolate has been reported to exhibit goitrogenic effects (Sharma et al., 2022). These must be removed for better nutritional value, consumer safety, and appeal.

Mustard grows best in regions where the annual precipitation falls within the range of 500 to 4200 mm, the annual temperature varies from 6 to 27°C, and the soil pH ranges from 4.3 to 8.3 (Shekhawat, 2012). Therefore, *Brassica sp.* is sown from mid-October to mid-November and harvested from late January to mid-February. However, the average yield per hectare of *Brassica* in Bangladesh remains a concern due to unpredictable weather patterns and the absence of high-yielding, short-duration varieties (Miah et al., 2017). Typically, farmers in Bangladesh grow *Brassica d*uring the period between transplanting aman and boro rice, which spans around 80-90 days. This necessitates a type of mustard that matures more quickly to facilitate harvesting (Miah et al., 2017).

Traditional breeding programs face limitations due to restricted natural germplasm that arises due to *Brassica's* high degree of segregation after cross-pollination which results in the separation of allelic genes. This results in disease susceptibility, low yield, and poor adaptability to natural conditions. To address these challenges and develop plants with enhanced agronomic and qualitative traits, it is beneficial to augment genetic diversity through methods such as mutation, somaclonal variation, in vitro selection, and protoplast fusion (Mollika et al., 2011).The effective utilization of tissue culture techniques for enhancing crop traits relies on the capability to attain a high rate of plant regeneration from an explant (Shyam et al., 2021).

1.3 Origin of *Brassica juncea*:

Brassica juncea, widely recognized as oriental mustard, brown mustard, Indian mustard, or Chinese mustard, holds significant agricultural importance. This plant species is an allotetraploid, characterized by 36 chromosomes (AABB, 2n = 36). It was produced by combining *Brassica nigra* (BB, 2n = 16) with *Brassica rapa* (AA, 2n = 20), two diploid parent

plants (Liu *et al.* 2021). Across Asia, Europe, Africa, America, and Australia, *Brassica juncea* is a widely distributed crop that can be found as an invasive species, farmed crops, and native vegetation. In addition to being an important oilseed crop in China, India, Bangladesh, and Ukraine, *B. juncea* is also becoming more and more important in Canada and Australia. Based on biogeographic research, Afghanistan and its surrounding nations were identified as the origin for *B. juncea,* with Asia Minor, central/western China, and eastern India proposed as secondary sources of origination. (Kang. L). On the other hand, several scientists have proposed that *B. juncea* evolved in the Middle East, where *B. nigra* and *B. rapa*, its parent species, co-habit. It is debatable where *B. juncea* originated and if it is monophyletic or polyphyletic. Earlier morphological investigations suggested a single origin, in contrast to the polyphyletic origin reported by more recent studies using nuclear DNA markers, chemotaxonomy, and chloroplast (CP) genomic markers (Liu *et al.* 2021).

1.4 Taxonomy:

Scientific name

Brassica juncea

Taxonomic Classification

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Brassicales
Family	Brassicaceae
Genus	Brassica
Species	*Brassica juncea* (L.) Czern.

1.5 Reproductive Biology of *Brassica juncea:*

The leaves of a *B. juncea* plant emerge first, followed by the flowers. The flower is characterized by its superior ovary and absence of hypanthium. The reproductive organs of flowers include stamens and carpels, making them all synoecious which produce both pollen and seeds (*"Brassica juncea* (Chinese mustard)," n.d.).

All kinds of *B. juncea* exhibit an annual growth habit. The fertilization of ovules typically occurs through self-pollination, although there have been reports of interplant outcrossing rates ranging from 20-30% (Rakow and Woods, 1987). Bees serve as the main pollen vector due to the fact that the pollen is dense and adhesive, making it less likely to be transported over long distances by wind. Physical contact between flowering racemes can also lead to cross-pollination of neighboring plants

1.6 Significance of *Brassica juncea:*

Indian mustard (*Brassica juncea*) is an important oilseed crop in Bangladesh because the yield of the varieties of Indian mustard (*B. juncea*) is stable when it is planted late (Hoque, 2007). *Brassica juncea* has agronomic potential, particularly under rapid climate change, due to its tolerance to environmental stressors, such as drought and salinity, (Naveen *et al*. 2021) and its ability to tolerate diverse soil types. Moreover, pod-shattering resistance and herbicide resistance properties make *Brassica juncea* a compelling choice for commercially viable crops. Other than agriculture, *Brassica juncea,* also has applications in apiculture and biofumigation (Abdallah et al, 2020).

1.7 Types/Varieties of *Brassica juncea:*

There are many varieties of *Brassica juncea* available worldwide. In Bangladesh, *B. juncea* is one of the most essential oilseed crops, having six varieties named (Hasanuzzaman, n.d.) :

1. Daulat
2. Sambal
3. Rai

4. BARI Sarisha-10
5. BARI Sarisha-11
6. BARI Sarisha-16

1.8 BARI Sarisha-11:

The variety of *Brassica juncea* of interest for this study was BARI Sarisha-11. Seeds used in this study were collected from BARI, Joydebpur, and Gazipur. This variety of mustard is a Bangladesh Research Institute of Agriculture (BARI) mandated crop. This variety exhibits a distinctive combination of features from both native and exotic germplasm and was released in 2001. ("কৃষি পরামর্শক বাতায়ন," n.d., p. 82).The main characteristics of BARI Sarisha-11 include suitability for late planting after harvesting aman rice (Mia *et al.* 2017). The plants grow to a height of 120-130 cm and typically have 3-5 branches. These branches come out of the stem and slightly extend towards the ground. The leaves are light green with a texture. The flowers bloom downward from the axils and have a yellow color. BARI Sarisha-11 has a higher seed weight compared to the Rai sarisa varieties. It thrives during the Rabi season with the time to plant being, from mid-October to mid-November. It takes 105 to 110 days to reach maturity. According to Bangladesh Agriculture Research Council, BARI Sarisha-11 has an average yield of 1.8 tons per hectare, which is 20 to 25% higher than the Daulat variety, and an oil content of 42% per seed ("কৃষি পরামর্শক বাতায়ন," n.d., p. 82). Due to climate change, saline intrusion can potentially change the composition of soil and is a matter of concern for Bangladesh due to its extensive river network. *Brassica juncea,* being drought and salinity-tolerant, has the potential to adapt to the aftermath of climate change (Naveen *et al*. 2021).

1.9 Significance of this Study:

The crop BARI Sarisha-11 is popular among local farmers in Bangladesh. The conventional breeding techniques cannot fully solve the difficulties of improving mustard types since the techniques are time-consuming, laborious, and might give rise to heterozygous genes. A viable way to get around the drawbacks of traditional breeding is through genetic transformation, which provides a rapid and accurate way to build resistance to pests and diseases. Moreover, genome editing has been already employed in mustard to achieve consumer-favored characteristics. The same can be done in our mustard to make it more nutritious and consumer-accepted. But to do

so, the first step in solving these conventional issues is the establishment of tissue culture methods, such as setting up an *in vitro* plant regeneration protocol for conducting genetic improvements and genome editing experiments in order to improve the crop. More than a decade old in vitro regeneration system is available in BARI Sarisha-11. However, some important parameters have not been explored in that study. Moreover, due to strictly being a cross-pollinated crop the present variety might have a considerable change in genotype which might affect the regeneration response to the previously reported protocol. Under the circumstances, there is an utter need to establish an in vitro regeneration system following the evaluation of all relevant regeneration parameters to determine a sound, reproducible plant tissue culture protocol.

With this background, in the present study, the *Brassica juncea*, BARI Sarisha-11 has been explored to establish an robust and consistent tissue culture protocol. Therefore, the aim of the present study is-

I. To Standardize the seed sterilization procedure,
II. To determine the optimal age for an explant for regeneration,
III. To determine and optimize hormonal concentration for shoot regeneration,
IV. To establish root at the base of the regenerated shoots
V. Transplantation and establishment of plantlets in soil.

Chapter 2

Methodology

This research was conducted at the Plant Biotechnology and Biosafety Laboratory of BRAC University, Bangladesh. The subsequent sections outline the materials and methods employed in this investigation.

2.1 Materials:

2.1.1 Seeds:

In this current study, seeds from one mustard variety of *Brassica juncea*, namely BARI Sarisha-11, were used. The seeds of BARI Sarisha-11 were obtained from the Bangladesh Agriculture Research Institute (BARI). The seeds were brown, round shaped, and germinated into plants of height 120-130cm with 3-5 branches.

2.1.2 Sterilization Agents:

Sterilants, like 70% ethanol, 0.1% Mercury Chloride, and 10% Clorox solution were used.

2.1.3 Germination Media:

Germination of the seeds was carried out in autoclaved flasks containing 3% sucrose and 0.8% agar media.

2.1.4 Regeneration Media:

Murashige and Skoog (MS) medium (1962) in half strength along with 3% sucrose and 0.8% agar and different hormones were used for *in-vitro* regeneration. This study examined the impact of the plant growth regulators 6-benzylaminopurine (BAP), 6-furfurylaminopurine (Kinetin/Kn), and naphthalene acetic acid (NAA).

2.2 Methodology:

2.2.1 Literature Review:

For the first phase of the study, several articles regarding the tissue culture of *Brassica juncea* were reviewed. The databases used were Springer, International Journal of Agricultural Technology, Bangladesh Journals Online, Journal of Emerging Technologies and Innovative Research, Environmental and Experimental Biology, and the Bangladesh Association for Plant Tissue Culture and Biotechnology.

2.2.2 Preparation of Germination Media:

In order to prepare 1 liter of basal media containing agar and sucrose, the following steps were performed-

I. 30g of sucrose was precisely measured using a fine electronic balance (Startorius) and added to around 800 ml of distilled water in a beaker.

II. The beaker was placed on a magnetic stirrer and stirred till the sucrose dissolved.

III. The solution was poured into a measuring cylinder, and distilled water was added to a volume of up to 1000 ml, then again transferred to the beaker.

IV. The pH of the solution was measured over the magnetic stirrer and adjusted to pH 5.8 ± 0.2

IV. 8.5g of agar powder was measured using an electronic balance and added to the beaker.

V. The agar solution was melted in an electric oven and transferred to conical flasks.

VI. The conical flasks were autoclaved at 15 psi pressure at 121°C (Model: CL – 32L, ALP Co., Ltd). The media was cooled and stored at 25±2°C.

2.2.3. Preparation of Hormones for Shoot Regeneration:

To make the stock solutions of the growth regulators at a concentration of 1mg/ml, 30 mg of each hormone was measured using an electronic balance. A few drops of the 1N sodium hydroxide (NaOH) solvent were added to the hormone powder to dissolve, and the resulting

mixture was then adjusted to a volume of 30 ml by adding distilled water. The solution was vortexed to ensure a uniform solution. The solution was stored at -20°C and used for 1 month.

The hormones in appropriate amounts were added to basal MS media. The different combinations and amounts of stock hormone solution (1 mg/ml) to prepare 1L media are shown in Table 2.1.

2.2.4 Preparation of Media for Shoot Regeneration:

To prepare 1 liter of shoot regeneration media containing MS, sucrose, hormones and agar the following steps were performed-

I. Around 30g of sucrose was measured using an electronic balance and added to around 800 ml of distilled water in a beaker.

II. The beaker was placed on a magnetic stirrer and stirred till the sucrose was dissolved.

III. Around 4.4g of MS powder was measured using an electronic balance and added to the solution.

IV. The solution was poured into a measuring cylinder, and distilled water was added to a volume of up to 1000 ml and then again transferred to the beaker.

V. The beaker was placed on a magnetic stirrer and stirred till the MS powder dissolved.

VI. The correct volume of hormones, as mentioned in Table 2.1 are added to the media solution using sterile pipettes.

VII. The pH of the media was measured, making sure it was at 5.8±0.2

VIII. 8.5g of agar powder was measured using an electronic balance and added to the beaker.

IX. The agar solution was melted in an electric oven and transferred to conical flasks.

X. The conical flasks were autoclaved at 15 psi pressure at 121°C (Model: CL – 32L, ALP Co., Ltd). The media was cooled and stored at 25±2°C.

Table 2.1: Volumes of hormones used in media for shoot regeneration.

Hormonal combinations in MS basal media	**Volume of Stock Solution added to 1L MS basal media (µl)**		
	BAP	NAA	Kn
MS + 1 mg/l BAP	1000	-	-
MS + 2 mg/l BAP	2000	-	-
MS + 3 mg/l BAP	3000	-	-
MS+ 1 mg/l BAP + 0.1 mg/l NAA	1000	100	-
MS + 1 mg/l BAP+ 0.1 mg/l NAA + 0.5 mg/l Kn	1000	100	500
MS + 1 mg/l BAP + 0.2 mg/l NAA	1000	200	-
MS + 1 mg/l BAP + 0.2 mg/l NAA +0.5 mg/l Kn	1000	200	500
MS + 2 mg/l BAP + 0.1mg/l NAA	2000	100	-
MS + 2 mg/l BAP + 0.2mg/l NAA	2000	200	-
MS + 2 mg/l BAP + 0.5mg/l Kn	2000	-	500

2.2.5 Culture Techniques:

A. Seed Surface Sterilization:

The seed sterilization experiment was a crucial procedure in our experiment. The combinations of sterilizing agents and their duration of use are shown in Table 2.2

Table 2.2: Summary of different sterilization treatments adopted, and duration of treatment, for sterilization of BARI Sarisha-11 seeds.

70% Ethanol	0.1% Mercury Chloride	10% Clorox
1min	-	-
-	1min	-
-	2mins	-
-	5mins	-
-	10min	-
-	-	5mins
-	-	10mins
1min	5min	-
	10min	-
2min	2min	-
	5min	-
	10min	-
	-	5mins
	-	10mins
5min	2min	-
	5min	-
	10min	-

I. Seeds were taken in conical flasks, washed with tap water, and rinsed five times.

II. The seeds, autoclaved media, and forceps were taken into the laminar flow.

III. 70% ethanol solution was added, and the flask was continuously agitated for 1, 2 or 5 minutes. After the time duration, the seeds were rinsed with distilled water five times.

IV. 0.1% mercury chloride solution was added, and the flask was continuously agitated for 1, 2, 5, or 10 minutes. After the time duration, the seeds were rinsed with distilled water five times.

V. 10% Clorox solution was added, and the flask was continuously agitated for 5 or 10 minutes. After the time duration, the seeds were rinsed with distilled water five times.

B. Seed Germination:

I. About 10 sterilized seeds were then inoculated in conical flasks using sterilized forceps in the laminar flow containing autoclaved agar-water media.

II. The sealed flasks were kept in a dark chamber. After 3 days, the flasks were incubated at 25±2℃ under a photoperiod of 6 days or more, depending on the age of the explants.

C. Explant Selection and Inoculation:

I. Cotyledonary leaves with petioles were used as explants in this experiment.

II. Cotyledons from 7-10 days aged *in vitro* seedlings, along with 1-2 mm petioles, were very carefully excised from the apical shoot meristems of seedlings using sterilized forceps and scalpel in the laminar flow.

III. Five explants were placed on each flask containing MS media along with different hormone combinations. Cotyledonary leaves with petioles were placed in an upward direction, with the petiole in contact with the media.

IV. The cultured vessels were then labeled using permanent markers to indicate specific treatment after sealing with Parafilm.

V. The sealed flasks were incubated at a temperature of 25±2°C provided by 144W white fluorescent lamps.

D. **Subculture:**

I. The *in vitro* regenerated shoots were subcultured regularly at an interval of 15-20 days in fresh medium with the same shooting hormonal combinations.
II. The flasks were incubated at a temperature of 25±2°C provided by 144W white fluorescent lamps.

E. Rooting:

I. Mature shoots were cut above the nodes and separated from other shoots. Also vitrified tissue was removed using a sterilized surgical blade.
II. The individual shoots were then transferred to the media with half-strength MS salts without hormones.

F. Acclimatization of Rooted Plantlets:

I. Shoots with strong, mature roots were carefully washed under tap water to remove the media.
II. Sterilized soil (autoclaved) was soaked with distilled water, and the prepared plantlet was planted.
III. Plantlets were covered in plastic bags, and water was sprayed to create a humid environment inside.
IV. The plantlets were kept under fluorescent light and supplemented with MS 3% sucrose solution and 1/10 MS solution daily.

2.2.6 Statistical Analysis

R programming language and RStudio interface were used in order to conduct the statistical analysis. At the $p<0.05$ significance level, a 1-way Analysis of Variance (ANOVA) test was used to ascertain if the differences were the result of treatment differences or random variation. A p-value of less than 0.05 was considered statistically significant for the outcome.

Chapter 3

Result

The aim of this study was to optimize an efficient *in vitro* regeneration protocol for the mustard variety BARI Sarisha-11. Seeds sourced from the Bangladesh Agricultural Research Institute (BARI) were used for the experiment. The optimization process involved subjecting the seeds to various chemical treatments, such as 70% ethanol, mercury chloride, and 10% Clorox, either individually or in combination, for different durations to optimize an ideal sterilization procedure. In a hormone-less agar medium, the sterilized seeds were subsequently germinated, spending an initial 3 days in a dark chamber before being transferred to a light chamber. To induce shoot regeneration, cotyledonary leaves with petiole explants were used. These explants were inoculated in MS media containing hormones with varying concentrations of cytokinins (BAP, Kn) and auxin (NAA), either alone or in combination. The resulting shoots were regularly subcultured and then subjected to the rooting process. Finally, the shoots that developed roots were transplanted into sterilized soil.

3. 1. Seed Sterilization and Germination:

The sterilization process was performed following treatment protocols obtained from literature reviews, single treatments of chemicals, and combination treatments for establishing a new protocol for a higher germination rate.

The seeds of BARI Sarisha-11 started to germinate within 3 to 4 days of incubation. The germination rate on the third day under all the treatments was within 30%, with 0.1% mercury chloride for 1 minute showing the highest rate of 30.91%. Although the seeds sterilized with 0.1% mercury chloride for 5 and 10 minutes initially showed a slow germination response of 24.29% and 20.00%, respectively, the germination rate of those seeds was the highest later on.

For the treatments derived from literature reviews, using 70% ethanol for 2 minutes followed by 10 minutes of 0.1% mercury chloride exhibited the best growth rate of 67.50%. (Table 3.1) Moreover, no contamination was observed in any of the treatments. Hence, chemicals were decided to be used singly for varying periods in order to attempt to reduce the time of use of the chemicals as well as to obtain a higher percentage of germination.

When 70% ethanol, 0.1% mercury chloride, and 10% Clorox were used singly, it was found that 0.1% mercury chloride for 5 minutes was most effective for sterilization as it showed a 75.7% (Table 3.2) (Figure 3.1A) germination rate. Moreover, 10 minutes of exposure to 0.1% mercury chloride also yielded noticeable germination rates of 70.0% (Table 3.2) (Figure 3.1 E). Besides, the highest germination frequency (61.4%) (Table 3.2) (Figure 3.1 D) using 70% ethanol was observed when used for 1 minute. However, germination frequency using 10% Clorox treatment was low for both 5 and 10 minutes and had high variability. (Table 3.2). Therefore, to establish a new protocol for maximum germination, combination treatments were used with 70% ethanol for 1 minute and 0.1% mercury chloride for 5 and 10 minutes to find the optimum treatment combination.

The impact of different sterilizing treatments on the germination frequency of BARI Sarisha-11 for each treatment using singly was evaluated using a single-factor Analysis of Variance (ANOVA). The findings demonstrate a statistically significant (p-value = $4.28e^{-06}$) effect of various sterilizing treatments on the frequency of germination for BARI Sarisha-11, with significance noted at the $p<0.05$ level. (Table 3.3)

For treatments with 70% ethanol and 0.1% mercury chloride, treating for 10 minutes showed better germination than for 5 minutes. The maximum germination rate was 69.2% (Table 3.4) (Figure 3.1 B) using 1 minute of 70% ethanol with 0.1% mercury chloride for 10 minutes. As a result, further experiments for the regeneration of *Brassica juncea* were performed with this treatment.

Table 3.1: Sterilization of BARI Sarisha-11 seeds using treatment protocol derived from literature review

70% EtOH (mins)	01% $HgCl_2$ (mins)	10% NaOCl (mins)	Percentage of the germinated seeds (%)
2	-	-	48.8
5	-	-	28.8
2	2	-	57.5
2	5	-	56.2
2	**10**	**-**	**67.5**
5	2	-	43.7
5	5	-	63.7
5	10	-	50.0
2	-	5	48.7
2	-	10	40.0

Table 3.2: Sterilization of BARI Sarisha-11 seeds using the reagents singly for different duration.

Treatment	Time (mins)	Percentage of the germinated seeds (%)
70% EtOH	**1**	**61.4**
0.1% $HgCl_2$	1	68.8
	2	67.5
	5	**75.7**
	10	**70.0**
10% NaOCl	5	53.3
	10	57.0

Table 3.3: ANOVA results for germination of BARI Sarisha-11 seeds using different reagents singly, as mentioned in Table 3.2.

Source of Variation	df	Sum Sq	Mean Sq	F value	Pr(>F)
Between Groups	8	8012	1001.5	6.783	**$4.28e^{-06}$**
Within Groups	53	7825	147.7		
Total	61	15837			

Table 3.4: Percentage of germination of BARI Sarisha-11 seeds obtained after sterilization with a combination of 70% ethanol for 1 minute and 0.1% mercury chloride for 5 and 10 minutes.

70% EtOH (mins)	0.1% HgCl2 (mins)	Percentage of the germinated seeds (%)
1	5	60.6± 2.62
1	**10**	**69.2± 3.23**

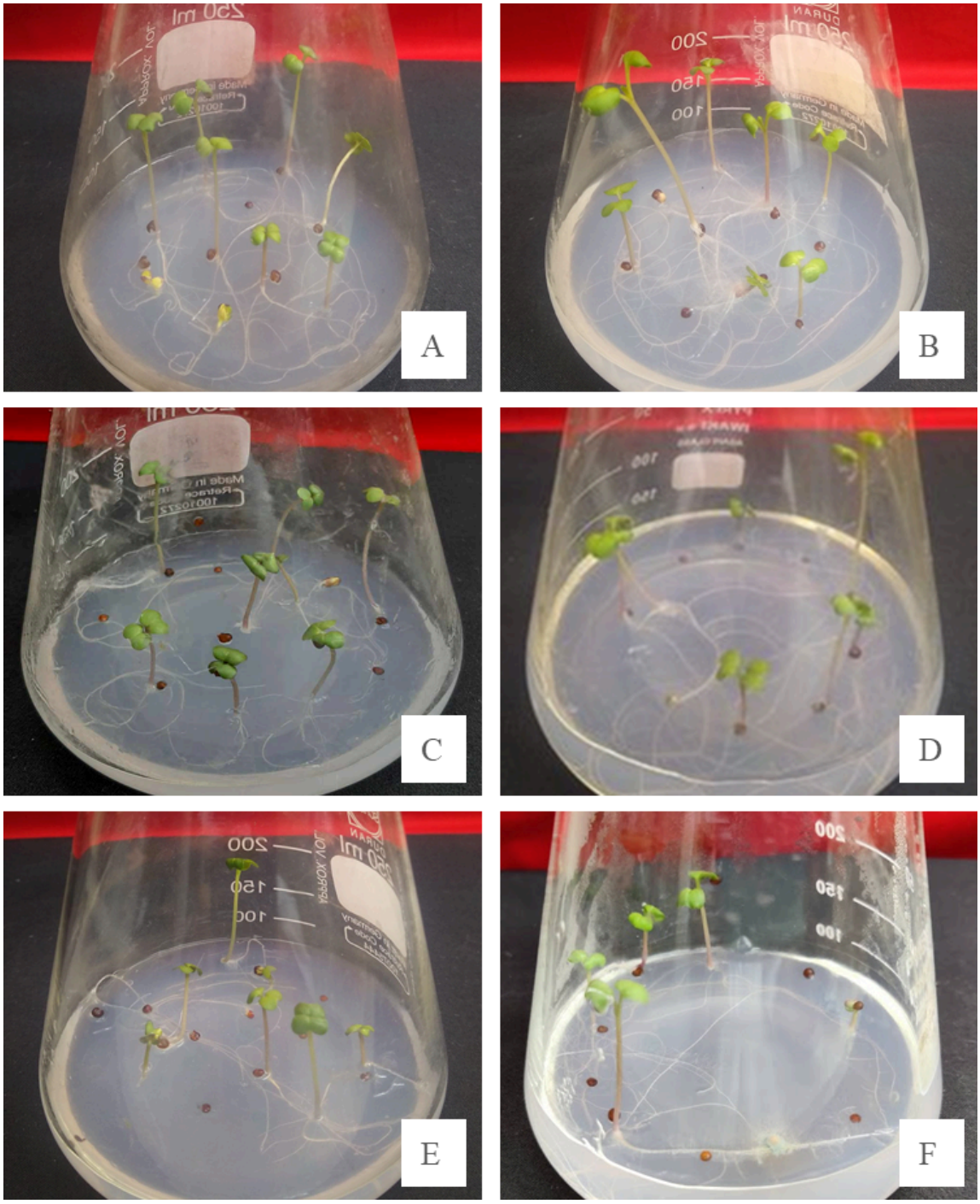

Fig 3.1: Germination of BARI Sarisha-11 seeds with different sterilization treatments. Photos were taken 7 days after treatment. A) 5 mins 0.1% $HgCl_2$ B) 1 min 70% Ethanol + 10 mins 0.1% $HgCl_2$ C) 2mins 0.1% $HgCl_2$ D) 1 min 70% Ethanol E) 10 min 0.1% $HgCl_2$ F) 1 min 70% Ethanol + 5 mins $HgCl_2$

3.2. Effect of Age of the Explants on Regeneration Response:

For the shoot induction and development, explants were excised from the petiole along with the cotyledonary leaves, which were then inoculated on media supplemented with full-strength MS + 2.0 mg/L BAP + 0.2 mg/L NAA + 0.5 mg/l Kn.

Cotyledonary leaves with petioles were used as the ideal choice of explant in this study. The explants were then placed on a medium supplemented with full-strength MS, 2.0 mg/L BAP, 0.2 mg/L NAA, and 0.5 mg/L Kn. To find the ideal age for shoot regeneration, explants at 6, 7, 8, 9, and 10 days were used in this study, results are shown in Figure 3.2. Seven-day-old explants showed the highest regeneration rate among them, with 6-day old explants having the second highest. With initiation taking 7–10 days, 7-day-old explants showed the best regeneration rate of 58.44% (Table 3.5). On the other hand, explants that were 8, 9, and 10 days old showed lower rates of regeneration and longer periods for initiation.

Table 3.5: Type of regeneration, days to shoot initiation, total number of regenerated explants, and percentage of responsive explants of BARI Sarisha-11 using media supplemented with full-strength MS + 2.0 mg/L BAP + 0.2 mg/L NAA + 0.5mg/L Kn.

Explant Age	Total no. of explant inoculated	Type of regeneration	Days to shoot initiation	Total No. of regenerated explants	% of responsive explant
Day 6	62	Direct	8-11	33	53.23%
Day 7	**77**	**Direct**	**7-10**	**45**	**58.44%**
Day 8	57	Direct	>15	23	40.35%
Day 9	41	Direct	9-11	17	41.46%
Day 10	51	Direct	>15	15	29.41%

Fig 3.2: Shoot regeneration of BARI Sarisha-11 observed with A) 6-day old explants, picture taken 17 days after inoculation B) 7-day old explants picture taken 15 days after inoculation C) 8 days old explants, picture taken 20 days after inoculation D) 9 days old explants picture taken 20 days after inoculation.

3.3 Multiple Shoot Regeneration Response Under Different Hormonal Combinations:

In the present study, cotyledonary leaves with petioles were used as a source of explants to create an effective shoot regeneration system for this particular mustard species. For shoot regeneration, the current study employed various hormone combinations (Table 3.6) to standardize maximum shoot regeneration in Bari Sarisha-11.

The explants of *Brassica juncea*, variety BARI Sarisha-11 from 7 days old seedlings showed the highest shoot regeneration yield when subjected to the media containing the hormonal combinations of 1 mg/L BAP, 0.1 mg/L NAA, and 0.5 mg/L Kn, out of the eight combinations examined generating an impressive 75% regeneration (Table 3.6) (Figure 3.3 C, D). The lowest regeneration rate 26.7% (Table 3.6) was found when explants were inoculated in media using MS media with 3 mg/L BAP.

The explants required a minimum of 9 days to initiate shoots in the MS media containing 2 mg/l BAP + 0.2 mg/l NAA. In contrast, the longest time for shoot initiation was 18 days after inoculation on the MS media supplemented with 2 mg/l BAP only.

Table 3.6: Combined effects of different concentrations and combinations of BAP, NAA, and Kn on MS medium for regeneration of multiple shoots *Brassica juncea* variety BARI Sarisha-11

Variety	Type of explant	Hormonal Supplement			No. of explants inoculated	No. of regenerated explants	% responsive explants	Days to shoot initiation	Mean no. of shoots/ explant
		BAP (mg/l)	NAA (mg/l)	Kn (mg/l)					
BARI Sarisha -11	Cotyledonary leaf with petioles	1	-	-	30	15	50.0 ±10	14-17	3.3
		1	0.1	-	30	14	45.0 ± 5	15-16	5.5
		1	0.2	-	30	15	44.0 ± 16	14-16	2.9
		1	**0.1**	**0.5**	**30**	**22**	**75.0 ± 5**	**16-17**	**5.2**
		1	0.2	0.5	30	16	45±10	12-14	2.5
		2	-	-	30	9	30.0 ±10	15-18	1.5
		2	0.1	-	30	17	55.0 ± 5	15-16	2.7
		2	0.2	-	30	11	33.6 ± 6.3	9-12	4.0
		2	0.1	0.5	30	12	37.2 ± 7.2	14-15	1.7
		2	0.2	0.5	30	12	43.3 ± 6.5	15-17	2.2
		3	-	-	30	8	26.7 ± 6.7	14-16	2.3

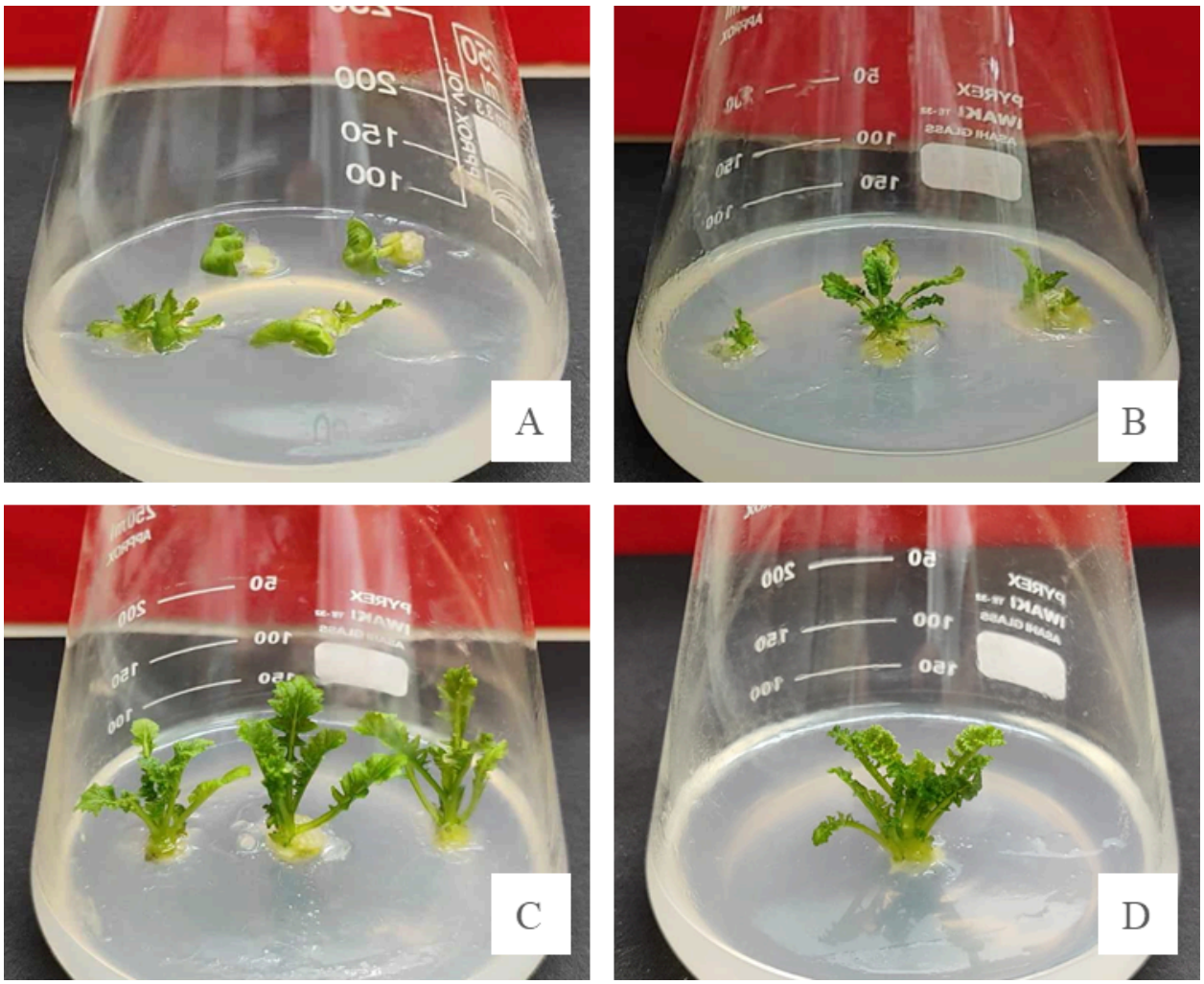

Fig 3.3: Photos of A) Initiation of BARI Sarisha-11 shoots in MS + 2 mg/l BAP + 0.1mg/l NAA taken after 16 days from inoculation B) Multiplication of BARI Sarisha-11 shoots in MS + 1 mg/l BAP + 0.2mg/l NAA taken after 18 days from inoculation C) Elongation of BARI Sarisha-11 shoots in MS + 1 mg/l BAP + 0.1mg/l NAA + 0.5mg/l Kn taken after 20 days from inoculation D) Subcultured BARI Sarisha-11 shoot in fresh media containing MS + 1 mg/l BAP + 0.1mg/l NAA + 0.5mg/l Kn taken after 25 days from inoculation.

3.4 Root Regeneration Response:

As soon as healthy shoots were observed, single regenerated shoots were collected and transferred to rooting media (1⁄2 strength MS media with 3% sucrose and 0.15% phytagel). A total of 6 explants were transferred, of which one shoot showed root regeneration in hormoneless media in 12 days(Figure 3.4). Thin fibrous root formation was observed.

Fig 3.4.: Bari Sarisha-11 plantlet with root and shoot in hormoneless rooting media (1⁄2 strength MS media with 3% sucrose and 0.15% phytagel). The picture was taken 12 days after inoculation.

3.5 Acclimatization of Plantlets:

Upon root induction, plantlets were transferred to pre-sterilized hydrated soil. The plantlet was kept under fluorescent light with a 16/8-hour light photoperiod inside sealed plastic bags. The soil was supplemented with a controlled amount of 3% sucrose solution and MS solution on daily. A total of four plantlets reached the acclimation stage (Figure 3.5). One of the four plants produced buds.

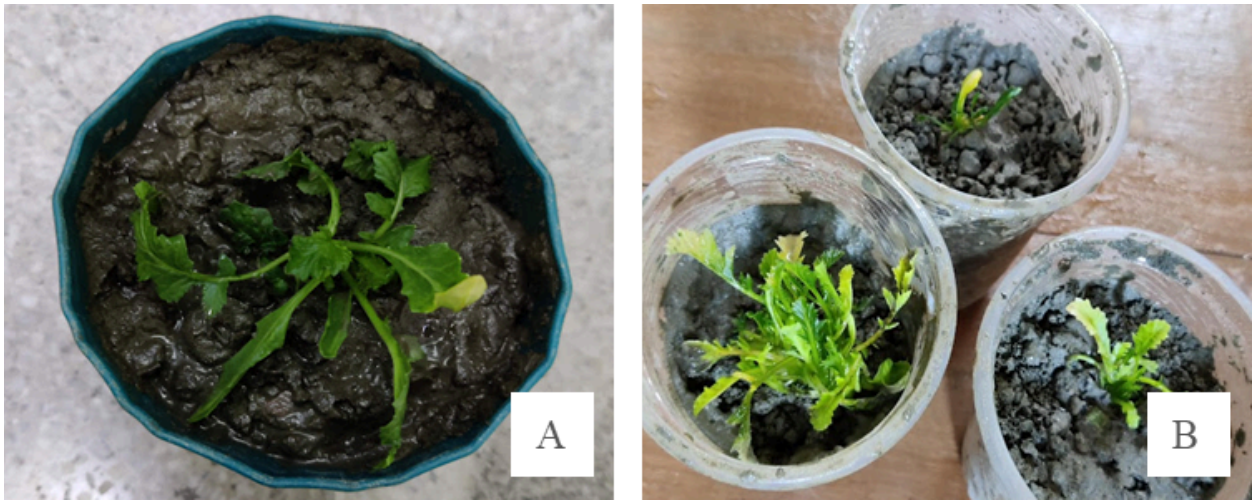

Fig 3.5: BARI Sarisha-11 plantlets transferred to soil in small plastic pots for acclimatization.

Chapter 4

Discussion

4.1 Sterilization:

Optimization of the seed sterilization protocol is the most important step for a successful *in vitro* culture establishment. In the current study, 70% ethanol, 0.1% mercury chloride, and 10% Clorox were used to find the most effective protocol for sterilization. The rate of contamination was below 1% for most of the combinations used, for which the fastest germination rate was the deciding factor for choosing the best sterilization treatment. Ethanol was used as a sterilant by Toma *et al*. (2022), Khan *et al*.(2009), Shyam *et al*. (2021), Bhuiyan *et al*. (2009), Gerszerbg *et al*. (2015), Guo *et al.*(2005), Basak *et al.* (2012), Bahamnia *et al.* (2019) for different *Brassica sp*. out of which Basak *et al.* (2012) and Bhuiyan *et al.* (2009) worked with *Brassica juncea*. Ethanol and similar alcohols rapidly eliminate bacteria when they are in their active, vegetative phase. They also have properties that can kill tuberculosis, fungi, and viruses, but they do not have the ability to eradicate bacterial spores. As ethanol is a potent and highly damaging sterilizing agent with strong phytotoxic effects, it is often used for a brief period (Sen *et al.* 2013). In the present study, the rate of germination was seen to decrease with the increasing duration of exposure to 70% ethanol. Our treatment utilized the least duration of 70% ethanol exposure of 1 minute, reducing the chances of phytotoxic effects while ensuring maximum germination rate. To enhance the efficiency of the sterilization process, ethanol is commonly utilized before the application of other substances. Another common sterilant for *Brassica sp.* is $HgCl_2$, which was used by Basak *et al.* (2012), Shyam *et al.*(2021), Pental *et al.* (1993), Khan *et al.*(2009), Zafar *et al.*(2016), George *et al.* (1980) and Bahamnia *et al.* (2019) out of which Basak *et al.* (2012), Shyam *et al.* (2021), and Bahamnia *et al.* (2019) used it for sterilizing *Brassica juncea* seeds. When used with 70% ethanol, the germination rate using 0.1% mercury chloride was seen to have increased with an increase in the duration of its exposure (5 and 10 min). It has been noted that Clorox is highly powerful against a wide variety of bacteria; micromolar doses are sufficient to drastically lower bacterial populations. Bhuiyan *et al. s*urface sterilized *Brassica juncea* seeds using 70% ethanol for 30 seconds, followed by 10% Clorox for 10 minutes. However, in our study, the rate of germination was found to be very low in both 5

and 10-minute treatments compared to the other chemicals. As a result, Clorox was excluded, and sterilization was carried out using 1 minute of 70% ethanol and 0.1% $HgCl_2$ for 5 and 10 minutes. Finally, the best percentage of germination (69.2%) was obtained using 1 minute of 70% ethanol and 10 minutes of 0.1% $HgCl_2$. (Fig. 3.1B). As the success of sterilization and germination is very much related to the amount of surface contamination and seed viability, our variation of the sterilization method might have been due to these reasons.

4.2 Culture Media for Shoot Regeneration:

We selected MS media as a shoot regeneration basal media because of its balanced nutrient composition, offering an ideal combination of macro, micro, and organic nutrients for most plant species. Additionally, our experiment involved the use of benzylaminopurine (BAP), either independently or in combination with kinetin or naphthaleneacetic acid (NAA), and sometimes both, at varying concentrations in order to ascertain the ideal medium composition for the initiation and development of numerous shoots from the mustard species. Benzylaminopurine, also known by various names such as benzyl adenine, BAP, or BA, is a first-generation cytokinin plant growth regulator. It plays a role in shaping plant development and growth, triggering flowering, and boosting fruit yield through the stimulation of cell division. On the other hand, a synthetic substance called kinetin, which resembles cytokinin, controls plant cell proliferation. (Cliniscience, n.d) A phytohormone auxin commonly employed in cell culture medium is naphthaleneacetic acid or NAA. (Bio-world (n.d.).) Auxins have a crucial role in controlling development and elongating shoots. It has previously been demonstrated that BAP, either by itself or in combination with auxin, is ideal for shoot regeneration and multiplication in a variety of *Brassica* species. The concentrations of growth regulators (both auxin and cytokinin), which are specific to genotype, and explant type are crucial for successful plant regeneration even though the macro and micronutrients of *in vitro* culture media may not differ significantly between species. (Ahmad *et al, 1999)* Additionally, it has been said that for the best development and differentiation, each species needed a certain hormone dosage. It is widely known from several studies by numerous researchers that auxin concentrations that are either very low or nonexistent in conjunction with strong cytokinin concentrations stimulate shoot regeneration.

4.3 Type of Explant

Over the course of various investigations, cotyledons, and hypocotyls have emerged as proficient explants for regeneration in numerous *Brassica* species. Mollika *et al*. (2011) conducted a study involving multiple varieties of *Brassica juncea*, revealing that the use of cotyledonary leaf without petiole as an explant resulted in a limited number of shoot regenerations (1.6 ‑ 10.0%). Notably, the presence of petiole was identified as a critical factor for successful shoot regeneration when cotyledon was employed as an explant. In their study among the tested varieties, BARI Sarisha-11 exhibited the most favorable regeneration response when cotyledonary leaf with petioles was utilized, ranking highest in terms of percentage of shoot development, with hypocotyls following closely as the second-highest responsive explant. Another investigation of *Brassica juncea* by Bhuiyan *et al.* (2009) reported that cotyledon explants surpassed hypocotyls in achieving the highest shoot regeneration frequency (56.67%). Guo *et al.* (2005) and Bahamnia *et al.* (2019) conducted a study wherein cotyledonary explants of *Brassica juncea* were utilized, leading to a notable increase in the frequency of shoot regeneration.

Considering the findings from these studies, we have chosen the cotyledonary leaf with petiole as the preferred explant for our research, as it consistently demonstrated a higher rate of regeneration response across experiments.

4.4 Age of Explant

The age of the explants in plant tissue culture has a major impact on both the ability for plant regeneration and the effectiveness of genetic transformation. Regeneration in the majority of *Brassica* species depends on the explants' age. It was also observed that younger explants have been demonstrated to produce preferable results than older explants. In their experiment, Mollika *et al.* (2011) used explants that were collected from 4-5 days old seedlings, whereas Shyam *et al.* (2021) used seedlings that were 10-15 days old to analyze the regeneration of *Brassica juncea*. Similarly, Bhuiyan *et al. (*2009) chose 4-day-old seedlings as their preferred explant, and Basak *et al.* (2012) used 6-7 day-old seedlings. Additionally, for their studies on the *in vitro* regeneration of *Brassica juncea*, Pental *et al.* and George *et al.* selected seedlings that were 5 and 8 days old. Observing the vast variation in explant collection timing from the aforementioned

studies, our research sought to determine the optimal explant age for BARI Sarisha-11 in order to establish an ideal regeneration protocol. After careful consideration, we determined that an explant of age between 6 and 10 days would yield the best results for our chosen variety, BARI Sarisha-11.

In order to find the ideal age of the explant, explants aged between 6 to 10 days old were taken and inoculated on medium with full-strength MS + 2.0 mg/L BAP + 0.2 mg/L NAA + 0.5 mg/L Kn to induce shoots. A similar hormonal combination was also found to be used in the study conducted by Mollika *et al.* (2011). The rates of adventitious shoot production from cotyledonary leaves with petioles produced from seedlings that were 6, 7, 8, 9, and 10 days old were similarly determined to be 53.23%, 58.44%, 40.35%, 41.46%, and 29.41%, respectively in this study (Table 3.5). As a result, day 7 explants were chosen as the optimal option for maximum regeneration in this experiment.

4.5 Shoot Regeneration

The primary objective of plant tissue culture is to generate numerous plantlets from explants. Here plant parts or explants are used to achieve *de novo* organogenesis which will ultimately result into plantlets regeneration under *in vitro* conditions. Explants are usually placed on a medium supplemented with cytokinins or a mixture of cytokinins and auxins to facilitate this regeneration process. Shoot initiation often needs culture on a cytokinin-enriched medium, such as, shoot induction media (SIM). Additionally, auxin also plays a role in mediating the effect of cytokinins on shoot induction, rather than only cytokinin alone. In this study, shoot regeneration was encouraged by high ratios of cytokinin to auxin, while root regeneration was favored by high ratios of auxin to cytokinin. Both cytokinin and auxin were used either separately or in combination during the investigation.

With a maximum regeneration response of 75%, 1 mg/L BAP + 0.1 mg/L NAA + 0.5 mg/L Kn was determined to be the most suitable combination for shoot initiation in terms of both the percentage of shoot regeneration and the number of shoots per explant in this variety. On the other hand, the lowest regeneration percentage was observed when the explants were inoculated in the MS media using only 3 mg/L BAP.

The regeneration response was first observed using three different concentrations of the cell division and growth-promoting hormone BAP. With the increase of BAP concentration (from 1mg/L to 3mg/L BAP), the regeneration response was found to decrease. When NAA was added to BAP, the combination showed a better response with a higher concentration combination.

According to Bhuiyan *et al.* (2009), the selection of the optimal medium for shoot regeneration involved culturing cotyledonary explants on MS medium containing vitamins, supplemented with various combinations of benzylaminopurine (BAP) ranging from 0.5 to 4 mg/L and naphthaleneacetic acid (NAA) from 0 to 0.4 mg/L. Their study indicated a significant improvement in shoot regeneration with the addition of NAA to BAP. Our investigation found that the addition of 2 mg/L BAP resulted in a 30% regeneration rate. However, when 0.1 mg/L NAA was combined with BAP, the rate increased to 55%. Contrarily, Bhuiyan et al.'s study discovered that the highest rate of shoot regeneration (56.67%) was achieved using MS media supplemented with 1 mg/L BA and 0.1 mg/L NAA. Nevertheless, our analysis showed a little reduced regeneration rate of 45%.

As reported by Mollika *et al.* (2011), optimal regeneration, reaching 83.33% for BARI Sarisha-11, was achieved when explants were cultured on MS media supplemented with 2 mg/L BAP, 0.2 mg/L NAA, and 0.5 mg/L Kinetin. While a similar hormonal combination was employed in this study, the results diverged, yielding a regeneration rate of only 43.3%. This change in regeneration response is very vital. Notably, this experiment revealed that the highest regeneration response of 75% was attained when both BAP and NAA concentrations were reduced to 1 mg/L and 0.1 mg/L respectively, contrasting with the findings of Mollika *et al.* A similarity between this study and Mollika *et al*. (2011) research was the observed enhancement in regeneration rates with the incorporation of 0.5 mg/L of Kn in each hormonal combination except when added to 1 mg/l BAP and 0.2 mg/l NAA.

A similar observation was noted in the study conducted by Guo *et al.* (2005) and George *et al.* (1980), where an increase in the concentration of BAP was found to inhibit shoot regeneration. This aligns with our findings, as using 1 mg/L BAP alone in MS Media resulted in a regeneration rate of 50.00%, (Table 3.6) whereas an increase to 2 mg/L led to a decreased regeneration rate of 30.00%.(Table 3.6) On the other hand, when BAP was again increased to 3 mg/L the lowest

regeneration rate 26.7% (Table 3.6) was observed. However, unlike George *et al.'s* experiment, the addition of kinetin did induce shoot formation in our study.

In this investigation, it was observed that the earliest onset of shoot initiation occurred on the 9th day when explants were cultured on MS media supplemented with 2 mg/L BAP and 0.2 mg/L NAA, a result consistent with the findings reported by Bano *et al.* (2010).

4.6 Rooting

To stimulate root induction, popular practice is to supplement auxin, such as, NAA or IAA into rooting 1/2 strength MS media, as demonstrated in the study by Thakur *et al.* (2013) and Bhuiyan *et al* (2009). In a study by Baskak *et al* (2012), root induction was achieved with hormone less ½ strength MS media (½ strength MS media, 3% sucrose, and Phytogel) for the mustard variety Shampad. In this study, rooting induction for BARI Sarisha-11 was achieved with hormone less ½ strength shooting media. This study indicated the potential of root induction without the need for external stimulation from auxin.

4.7 Acclimatization

Plantlets with roots were transferred to pre-soaked autoclaved soil and kept sealed in plastic bags. No fertilizer was administered, thus, the soil was supplemented with MS solution and sucrose solution to ensure the availability of nutrients. One of the four plantlets reached the budding stage.

Literature and the present study demonstrated that from sterilization to plant regeneration *Brassica juncea* have considerable differences. This variation can be due to various biological factors like genotype, type of explants, age of the explants, hormonal supplements etc. A very interesting observation was that the previous report on BARI Sarisha-11 varied from the present study. This may be due to the genetic variation that may have been incorporated in the present variety due to the strictly cross pollination nature of the crop. Moreover, the previous studies didn't examine critical factors like optimum sterilization and seedling age for explant collection. Both of these are very important to establish a reproducible regeneration system. These were checked in this study which makes it a more precise protocol to be used in future endeavors of genome editing and genetic engineering.

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