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RESEARCH ARTICLE

In vitro Micro Propagation of Soybean (*Glycine max*) BARI-5 Variety

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Abstract:

Introduction:

The present research work was undertaken with a view to developing a suitable protocol for *in vitro* plant regeneration of economically important plant (*Glycine max*) (Bangladesh Agricultural Research Institute BARI- 5) variety, *via* both direct and indirect organogenesis from *in vitro* grown seedlings.

Methods:

For micropropagation explants were cultured on MS and half strength Murashige and Skoog (MS) medium supplemented with various plant growth regulators (cytokinins and auxins). In the present study for inducting of callus, among 3 different hormone combinations, the suitable medium was 3.32 mg/L 2, 4-D containing MS medium and the callus was deep green in color. Different type of media like MS, 1/2 MS and MS with different (6-Benzyl Amino Purine) BAP concentration was used for seed germination of *Glycine max*. 100% of seed germination was observed in MS +1 mg/L BAP containing the medium.

Results:

In the present investigation, different concentration of cytokinins and auxins{BAP, 2, 4-D, and Naphthalene Acetic Acid (NAA)} were used individually or in combinations with MS medium to observe their effect on multiple shoot regeneration from the cotyledonary nodal segment. 100% shoot formation from cotyledonary nodal segment was recorded in 1.5 mg/L BAP and 0.15 mg/L BAP + 0.025 mg/L NAA containing MS medium, the best number of shoot was 10.9±2.0 found in MS + 1.5 mg/L BAP containing medium and highest length of shoot was 2 cm recorded in 1.5 mg/L BAP + 0.3 mg/L (different concentrations of Giberrellic acid) GA_3 containing MS medium. In addition, for root induction *in vitro* raised well developed and elongated shoots were excised and cultured on MS and 1/2 MS medium supplemented with various concentration of Indole-3-Butyric acid (IBA). It was observed that MS medium containing 0.1 mg/L IBA and 1/2 MS medium containing 0.25 mg/L IBA was optimal for root induction. In which 100% shoots rooted well within 13 days of culture. The highest average number of roots per shoot was 6 recorded in MS +0.5 mg/L IBA containing the medium and highest average length of root was 8 cm recorded in 0.1 mg/L IBA containing MS medium.

Conclusion:

The most effective surface sterilization treatment for explants of *Glycine max* has been found in 0.1% $HgCl_2$ solution for 15 minutes.

Keywords: Micro propagation, Regeneration, MS medium, *Glycine max*, Soybean, Seed germination.

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1. INTRODUCTION

Soybean (*Glycine max*) belonging to the family *Leguminosae* constitute one of the oldest cultivated crops of the tropical and sub-tropical regions, and one of the world's most important sources of protein and oil. Soybeans are probably the most important oilseed legume which has its origin in Eastern Asia, mainly China. The cultivar *Glycine max* is thought to be derived from *Glycine ussuriensis* and *Glycine tomentosa* which grow wild in China and can be found in great quantities in Asian countries such as Japan and Indonesia [1].

The seeds vary in shape and color depending on the cultivar. In shape, they can be spherical to flatten while the color varies from white, yellow and brown to black. Also, the chemical compositions of each variety of soybeans differ from each other. As a result of high protein content in soybean, it

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can be used as a substitute for expensive meat and meat products [2].

Soybean is particularly varied unique for a different reason and hence classified as a valuable and economical agricultural commodity. In the first instance, it possesses agronomic characteristics with its ability to adapt to a wide range of soil and climate and its nitrogen-fixing ability. This makes it a good rotational crop for use with high nitrogen consuming crops such as corn and rice. Secondly, soybean unique chemical composition on an average dry matter basis is about 40% of protein and 20% of the oil. This composition makes it rank highest in terms of protein content among all food crops and second in terms of oil content after peanut (48%) among all food legumes. Furthermore, soybean is a very nutritious food crop [3].

Furthermore, soybean seeds are also a major source of excellent health benefit products for example antioxidants, omega-3 fatty acids, amino acids, phytoestrogens, and foliate. Soybean is moreover utilized in a variety of processed nutritional food products (soy milk, soy cheese, yogurt, and ice cream) as well as considered as nutritionally comparable to meat. Soybean oil is utilized for lots of human food products as well as cooking oil and has many manufacturing industrial uses including soaps, oils, paints, cosmetics, plastics, clothing, and solvents. In recent times, soybean is also utilized as biofuel crop to run into worldwide energy demands putting important pressure on crop production for food. In addition, global climate change presents threats to our food supply.

Soybean is an allopolyploid with three rounds of whole genome duplication [4, 5]. In recent times, there has been a major work in thoughtful the genome of soybean by undertaking large-scale genome sequencing, microarray, expressed sequence tag sequencing, and transcriptome analyses [6, 7]. Moreover, comprehensive studies on synteny, Quantitative Trait Locus (QTL) mapping, and comparative genomics have improved our knowledge on soybean as well as closely related legumes of financial significance [8 - 11].

Soybean has widespread adaptability with a huge quantity of cultivars showing variability to flower in response to day length and temperature. Thus, soybean cultivars have been divided into thirteen maturity groups based on the photoperiod and temperature requirements for flowering.

Soybean, originating in China, at present is a most important crop in North and South America as well as Asia. Soybean production has improved fivefold during the past four decades making it the most important protein, as well as oil crop globally [4] and this growth are predicted to continue due to increasing demand for food, feed, and fuel. Significant efforts have been made to increase soybean through conventional breeding. However, classical breeding programs for soybean are limited by its self-pollination ability [12]. Therefore, there is an essential for transgenic approaches to its development. Efficient plant regeneration procedure is the prerequisite for successful application of genetic transformation technology. There has been an ongoing effort for enhancing the plant regeneration potential of soybean *via* either organogenesis or somatic embryogenesis. Organogenesis based regeneration is attractive due to an abundant and convenient supply of explants. Earlier reports have tried using different explants such as cotyledonary node [13, 14], whole cotyledonary node [15], epicotyl and primary leaves [16, 17], primary leaf nodes [18], and hypocotyls [12, 19–22].

We aim to develop genetic transformation technology for commercial soybean varieties by identifying the most optimal way to regenerate shoots from different soybean explants and evaluate whether shoots regeneration capacity is related to the maturity grouping of the soybean cultivars. It is well established that crop plant regeneration ability is tissue and genotype dependent. Hence, we compared the regeneration ability of three different soybean explants from nine soybean cultivars. This is the first report of *in vitro* plant regeneration of commercially grown Bangladesh soybean varieties. The protocol developed would assist in developing a genetic transformation system for these soybean varieties.

2. MATERIALS AND METHODS

The experiment was conducted at Biological Research Division of Bangladesh Council of Scientific and Industrial Research (BCSIR) Dhanmondi, Dhaka. Soybean Seeds (*Glycine max*) BARI-5 variety were collected from Bangladesh Agricultural Research Institute (BARI), Gazipur.

Seeds were washed thoroughly under running tap water, presoaked in liquid detergent for about 30 min, wiped with cotton and dipped in 70% (v/v) ethanol for 1 min. They were then surface sterilized with 0.1% (w/v) mercuric chloride for 5 min, followed by five times rinse with sterile distilled water under laminar air flow cabinet.

Cotyledonary nodal explants from aseptically grown mature seeds of *Glycine max* were cultured on MS medium [23] following normal *in vitro* culture procedures [24] for adventitious shoot regeneration. Half strength MS medium was used for *in vitro* rooting. All media were supplemented with 30 g/L sucrose, 7 g/L agar (Difco) and dispensed into 15x150 mm culture tubes and 250 ml conical flasks. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 20 min. The cultures were incubated for a 16 h photoperiod at 24 ± 20°C under a fluorescent light.

The surface sterilized explants were sized to 1.0 - 1.5 cm length containing a single node with an axillary bud or a shoot tip with an apical bud. Explants were placed vertically on the culture medium. The new shoots induced from the *in vitro* cultures were further used as explants for shoot regeneration. MS with different concentrations of hormone was used for shoot proliferation and shoot regeneration and half strength MS was used for *in vitro* root induction. All media were supplemented with 30 g/L sucrose, 7 g/L agar *In vitro* Shoot Proliferation and Plant Regeneration and dispensed into 15 × 150 mm culture tubes and 250 mL conical flasks. The pH of the media was adjusted to 5.8 before autoclaving at 1.9 kg/cm^2 pressure at 121°C for 20 min. The cultures were incubated for a 16 hr photoperiod at 24 ± 2°C under 1200 lux/m^2 fluorescent light. Shoot proliferation from shoot tip and nodal explants was obtained in two separate sets of experiments. In the first experiment 0.5 - 2.0 mg/L BAP and 0.5 - 2.0 mg/L Kn were

incorporated into MS to select the best cytokinin for the response of shoot induction. In the second set, combination of BAP (0.5 - 2.0 mg/L) with NAA (0.1 - 0.5 mg/L) and BAP (0.5 - 2.0 mg/L) with IAA (0.1 - 0.5 mg/L) were assessed for shoot multiplication. A number of new shoot proliferation of each culture was recorded after every week of inoculation. For *in vitro* rooting, individual shoots (3 - 5 cm long) were excised from the proliferated shoot cultures and implanted onto half strength MS media with different concentrations and combinations of NAA, IBA and IAA. The rooted plantlets were taken out from the culture tubes, washed to remove agar gel adhered to the roots and transplanted to plastic pots with soil and compost (1: 1) for hardening. The plantlets were kept in a poly chamber at 80% relative humidity, 32 ± 2°C for a 12 hr photoperiod under 1500 lux.

The materials and methods used throughout the investigation *in vitro* micropropagation are described below under the following heads and subheads.

2.1. Sterility and Surfactant

Ethyl alcohol (70%) was used to wipe surfaces of working areas, to rinse hand, to dip instruments with or without subsequent flaming, 0.1% Mercuric chloride ($HgCl_2$) was used to disinfect the plant tissues. Jet powders (detergent) were also used as detergent and disinfectant.

2.1.1. Sterilization

Finally, the culture media were sterilized by autoclaving at 15 Ibs/inch^2 pressure for 15 minutes at 121°C. Then the vessels with the medium were allowed to cool as vertically and then marked with a permanent marker pen to indicate specific hormonal supplements and stored in the culture room for ready to use.

2.2. Preparation of Plant Material and Sterilization of Seed

In the present investigation, the cotyledonary nodal segment of *in vitro* grown seedling of *Glycine max* (BARI-5 variety) was used as explants. For the primary establishment of *in vitro* culture, surface sterilization of seed of *Glycine max* (BARI – 5 variety) was essential because the nutrient media which are used in tissue culture techniques are most suitable for the growth of microbes also. That's at first freshly collected seeds were washed thoroughly detergent for 4-5 times under running tap water. After that, they are taken in laminar air flow cabinet and dipped in 0.1% $HgCl_2$ solution for 5 minutes with constant shaking to ensure contaminant-free cultures. Then seeds were washed with 70% ethanol for 1 minute. Lastly, the seeds were rinsed with autoclaved distilled water for 3-4 times in order to remove the last drop of $HgCl_2$. The explants were then put in a sterile conical flask for inoculation (Fig. **1**).

2.3. Inoculation of Explants

Before starting the inoculation programmed the laminar airflow's Floor was cleaned with 70% ethanol. After then it was put on for 30 minutes. All the inoculations and aseptic manipulations were carried out in front of laminar air flow cabinet. The sterilized dissecting instruments were brought to the laminar air flow and before every use; the above instruments were dipped into absolute alcohol and flamed over a spirit lamp several times. Other sterilized requirements like a petri dish, distilled water, blotting paper were also used for the procedure. Before starting inoculation hands were repeatedly washed thoroughly by soap and made sterile by spraying 70% ethanol. For surgical operations, sufficient care was taken as usual to obtain possible contamination-free conditions. Sterilized seeds were collected in a sterilized petri dish and remove the thin surface layer of seeds so that embryo can germinate properly and quickly. Then the prepared seeds were carefully inoculated in culture vessels containing agar gelled nutrient medium supplemented with different combination and concentrations of hormones. Special care was taken so that the part of seeds must penetrate in the medium but not deep into the medium. The mouth of the culture vessels plugged tightly inside laminar air flow cabinet in presence of spirit lamp and marked by a permanent marker with inoculation date. Then the flasks are ready for incubation and observation.

2.4. Incubation

The inoculated culture flasks were incubated in a growth chamber providing a special culture environment. The culture flasks were placed on the shelves of the cupboard in the growth chamber. Which was illuminated by 40 watts white fluorescent tubes fitted at a distance at 30- 40 cm from the culture shelves? The cultures were maintained at 25 ±21 °C with air cooler under the fluorescent light intensity of 28-34 molm^{-2}s-1. The photoperiod was maintained generally 16 hours light and 8 hours dark by an automated timer. The culture vessels were checked periodically to note the response.

2.5. Subculture for Shoot Multiplication and Elongation

After two weeks of inoculations, small plant was transferred aseptically to a sterile petri dish and was cut into small pieces of different part(like cotyledonary nodal segment having auxiliary buds, shoot tip node enter node *etc*.) by a sterile scalpel. Then cotyledonary nodal segment was transferred to freshly prepare medium containing different combinations of hormone supplement for multiplication and elongation of shoots. After every 3 weeks, multiple shoots were placed in root inducing media. Rest of the ex-plants with small multiple shoot buds and leafy structure were again cut into convenient sizes and inoculated in optimum hormonal supplemented media.

2.6. Subculture for Rooting

In this stage, the shoots were excised from the vessels of multiplication media and cultured individually in freshly prepared rooting media containing different concentrations and combinations of auxins.

3. RESULTS

In the present investigation, the cotyledonary nodal segment of *in vitro* grown seedling of *Glycine max* (BARI-5variety) was used as explants. Details of the result so far obtained from each of the experiments are described under following heads:

3.1. Standardization of Surface Sterilization of Explants

Surface Sterilization of explants was accomplished with an aqueous solution of mercuric chloride ($HgCl_2$). Considering the problem of contamination, different concentration of $HgCl_2$ was used and the explants were treated in different periods of time.

3.1.1. Surface Sterilization of Seed of Glycine Max (BARI-5 Variety)

A concentration of $HgCl_2$ solution up to 0.2% and the treatment period of 20 minutes did not harm viability due to the hard seed coat. Treatment with 0.05% $HgCl_2$ solution for 20 minutes, 0.1% $HgCl_2$ solution for 10 to 20 minutes and 0.2% solution for 08 to 20 minutes resulted in 100% contamination free seeds.Treatment with 0.05% $HgCl_2$ solution for 06 and 08 minutes showed 50% contamination free seeds.Whereas treatment with 0.05% and 0.1% $HgCl_2$ solution for less than 10 to 20 minutes showed 100% contamination free seeds. In the present experiment, the seeds were treated with the different concentration of $HgCl_2$. 100% of cultures were found contamination free without injury when treated with 0.1% $HgCl_2$ for 15 minutes. (The summarized results of these experiments are shown in Table **1**.

3.2. Effect of Different Medium on Seed Germination of *Glycine Max* (BARI-5 Variety)

In this experiment, a different type of media like MS, 1/2 MS and MS with different BAP concentration was used for seed germination of *Glycine max*. 100% seed germination was recorded in MS + 1 mg/L BAP containing a medium highest average length of shoot per culture was record 11cm in MS media.

Lowest percentage (70%) of seed germination was recorded in MS medium lowest average length of shoot per culture was recorded 2cm in MS + 0.5 mg/L BAP containing media. (The summarized results of these experiments are shown in Table **2**.

3.3. Shoot Multiplication

Soybean seeds were cultured in MS [23] medium supplemented with different concentration of growth regulators (Cytokinins and Auxins) either singly or in combination to find out a suitable medium for shoot multiplication. Data were taken for the percentage of cultures respond to the average number of shoot per culture, average length of shoot and root formation. All data were collected after 23 days of culture. The results were discussed under the following separate heads:

3.4. Effect of Different Concentration of BAP alone in MS Medium on Multiple Shoot Development of *Glycine Max* (BARI -5 Variety).

In this experiment, BAP was used to find out its effect on shoot development form the cotyledonary nodal segment of *Glycine max* (BARI -5 varieties. Percentage of culture responded to multiple shoot formation in a different level of BAP varied considerably.

100% of multiple shoot development was recorded in 1.5 mg/L BAP containing media, on the other hand, the lowest percentage of multiple shoot development was recorded in 0.5 mg/L BAP containing the medium. Highest average number of shoot per culture was recorded at 23 days of culture. The highest average number of shoot per culture was 10.9 ± 2.0 recorded in 1.5 mg/L BAP containing media and the highest average length of shoot per culture was recorded 1cm in 1.5 mg/L BAP containing media. On the other hand lowest average number of shoot per culture was recorded 5.3 ± 0.34 in 0.5 mg/L BAP containing media and the lowest average length of shoot per culture was recorded 0.5cm in 0.5 mg/L BAP containing media. (The summarized results of these experiments are shown in Table **3**.

3.5. Effect of Different Concentration of BAP in Combination with GA_3 in MS Medium on Multiplication and Elongation of the Shoot

The highest percentage (76%) of culture has recorded in media containing 1.5 mg/L BAP + 0.3 mg/L GA3. Whereas lowest percentage (47%) of was respond in 1.0 mg/L BAP + 0.2 mg/L containing medium. In the experiment, the highest average number of shoot per culture was 7.6 ± 20.56 in the medium containing 1.5 mg/L BAP + 0.3 mg/L GA_3. The highest average length of the shoot was 2cm observed in the same medium. As well as the lowest average number of shoot per culture was 4.7 ± 0.39 in 1.0 mg/L BAP + 0.2 mg/L GA_3 containing the medium. The lowest average lenth of the shoot was observed 0.5 cm in medium containing1.0 mg/L BAP + 0.2 mg/L GA_3. The most effective best media formulation 1.5 mg/L BAP + 0.3 mg/L GA_3. (The summarized results of these experiments are shown in) Table **4**.

3.6. Effect of Different Concentration of BAP in Combination with NAA in MS Medium on Induction Multiple Shoots

100% of culture has responded in medium containing 1.0 mg/L BAP + 0.025 mg/L NAA. Whereas, lowest percentage (32%) was observed in 0.05 mg/L BAP + 0.05 mg/L NAA containing medium. The highest average number of shoot per culture was observed 10 ± 0.56 in the medium containing 0.15 mg/L BAP + 0.025 mg/L NAA and the highest average lenth of shoot was 1cm found in medium containing 0.15 mg/L BAP + 0.025 mg/L NAA. As well as lowest average number of shoot per culture was 1.6 ± 0.36 found in medium containing 0.05 mg/L BAP + 0.05 mg/L NAA. As well as, the lowest average lenth of shoot was 0.5cm found in medium containing 0.05 mg/L BAP + 005 mg/L NAA. The most effective best media formulation was 0.15 mg/L BAP + 0.025 mg/L NAA. (The summarized results of these experiments are shown in) Table **5**.

3.7. The best Result from Seed and Shoots

From the above experiments, it was found that among all the growth regulators-

- MS + BAP 1 mg/L showed the 100% of seed germination.
- MS + BAP 1 mg/L and 0.15 mg/L BAP + 0.025 mg/L NAA containing medium showed the 100% of multiple shoot development.

- The highest average number of multiple shoots 10.9 ± 2.0 was observed in MS + 1.5 mg/L BAP containing the medium.
- The highest average length of the shoot was 2cm observed in MS + 1.5 mg/L BAP containing the medium.

3.8. Callus Induction

Induction of callus is a novel way to have soma clonal variants. Regarding this MS media supplemented with different kinds of hormones at different concentration were used to initiate callusing. Rapid induction of callus was seen in the seedling explants than those of mature plants. It was found that for best callus induction, proper contact of the explants with the medium was absulately essential.

3.9. Effect of Different Concentration of BAP alone in MS Medium on Callus Induction Fromcotylidonary Nodal Segment of *Glycine Max* (BARI -5 Variety).

In the present study, 90% callus induction response was obtained in MS medium supplemented with 2.0 mg/L BAP. 100% of callus was obtained in 1.5 mg/L BAP containing the medium. The color of callus was deep green. (The summarized results of these experiments are shown in) Table **6**.

3.10. Effect of Different Concentration of BAP in Combination with GA_3 in MS Medium on Callus Induction from the Cotyledonary Nodal segment of *Glycine Max* (BARI -5 Variety).

In the present study, 40% callus induction response was obtained in MS medium supplemented with 0.5 mg/L BAP + 0.2 mg/L GA_3. The highest percentage (70%) of callus is obtained in 1.0 mg/L BAP + 0.2 mg/L GA_3 containing the medium. The color of the callus was black. (The summarized results of these experiments are shown in) Table **7**.

3.11. Effect of Different Concentration of 2,4-D Alone in MS Medium on Callus Induction from the Cotyledonary Nodal Segment of *Glycine Max* (BARI -5 Variety).

The present study showed a 90% callus induction on MS medium supplemented with 0.73 mg/L 2,4-D. 100% of callus was obtained in MS + 3.32 mg/L 2,4-D containing the medium. The color of callus was deep green . The summarized results of these experiments are shown in Table **8**.

3.12. Effect of Different Concentration of 2,4-D in Combination with NAA in MS Medium on Callus Induction from the Cotyledonary Nodal Segment of *Glycine Max* (BARI -5 Variety).

In the present study, the highest percentage (25%) of callus is observed in 0.73 2,4, D + 0.69 mg/L NAA containing the medium. The color of callus was light green. (The summarized results of these experiments are shown in) Table **9**.

3.12.1. The Best Result from Callus

From the above experiments, it was found that among all the growth regulators-

- MS + 3.32 mg/L 2,4-D and MS + 1.5 mg/L BAP containing medium showed 100% of callus development.

3.13. Rooting of *in vitro* Regenerated Shoots

Shoot development as well as rooting of regenerated shoots is especially important for establishing tissue cultured derived plants.The regenerated shoots did not produce root spontaneously. For this purpose, *in vitro* raised well developed and elongated shoots were excised from the clamp of multiple shoots and they were cultured for rooting. In this experiments, for *in vitro* rooting MS and half strength MS medium was supplemented with different concentration of IBA.

3.14. Effect of Different Concentration of IBA Alone in MS Medium on Rooting of *in vitro* Regenerated Shoot of *Glycine Max* (BARI -5 Variety).

In this experiment, IBA was used in the concentration of 0.1-4.0 mg/L. It was observed that the best response for rooting was obtained in MS + 0.1 mg/L IBA containing the medium. In this composition 100%, excised shoots were rooted well within 13 days. The highest average number of roots per shoot was 6 found in MS + 0.5 mg/L IBA containing the medium. The highest average length of root was 8 found in MS + 0.1 mg/L IBA containing the medium. (The summarized results of these experiments are shown in) Table **10**.

3.15. Effect of Different Concentration of IBA Alone in 1/2 MS Medium on Rooting of *in vitro* Regenerated Shoot of *Glycine Max* (BARI -5 Variety).

In this experiment, IBA was used in the concentration of 0.1-0.5 mg/L. It was observed that the best response for rooting was obtained in 1/2 MS + 0.25 mg/L IBA was used. 100% excised shoots were rooted well within 13 days in the same medium. The highest average number of roots per shoot was 2 and the highest average length of root was 1.5cm observed in 1/2 MS + 0.25 mg/L IBA containing medium. (The summarized results of these experiments are shown in) Table **11**.

3.15.1. The Best Result from Root

From the above experiments, it was found that among all the growth regulators-

- MS + 0.1 mg/L IBA containing medium and 1/2 MS + 0.25 mg/L IBA containing medium showed 100% of root development from multiple shoots.
- The highest average number of root per multiple shoots was 6 recorded in1 MS + 0.5 mg/L IBA containing medium.
- The highest average length of the shoot was 8cm observed in MS + 0.1 mg/L IBA containing the medium.

3.16. Surface Sterilization of Seed of *GLycine Max* (BARI-5 variety).

The present investigation was aimed to develop a suitable protocol for *in vitro* regeneration of diseases free culture of

economically important plant *GLycine max (**BARI-5**variety).* $HgCl_2$ is widely applied sterilizing agent in the field of tissue culture because of its oxidizing nature to kill the microorganisms. Bacterial and fungal infections can be

Table 1. Surface sterilization of seed of *Glycine max* (BARI-5 variety).

$HgCl_2$ Treatment in Minutes	Number of Seed Sterilized	Concentration of Mercuric Chloride ($HgCl_2$)		
		0.05%	0.1%	0.2%
		No. of Concentration Free Seed	No. of Concentration Free Seed	No. of Concentration Free Seed
5	100	-	-	50
6	100	-	47	62
7	100	-	66	85
8	100	-	87	100
9	100	-	92	100
10	100	20	100	100
15	100	73	100	100
20	100	100	100	100

Table 2. Effect of different medium on seed germination of *Glycine max* (BARI-5 variety).

Media Used	No. of Seed Inoculated	% of Seed Germinated	Days Required for Germination	The Average Length of Shoot (cm)
MS	5	70	7	11
$_1/_2$MS	5	73	7	4
MS + BAP0.5mg/l	5	90	6	2
MS + BAP 1mg/l	**5**	**100**	**6**	**2.5**
MS + BAP1.5mg/l	5	95	6	2.3

Table 3. Effect of different concentration of BAP alone in MS medium on multiple shoot development of *Glycine max* (BARI-5 variety).

Media Content mg/l	No. of Cotylidonary Nodal Segment Inoculated	Average No. of Multiple Shoots (mean)±	The Average Length of Multiple Shoots (cm)	% of Multiple Shoots
MS + BAP0.5	10	5.3±0.34	0.5	53
MS + BAP 1.5	**10**	**10.9±2.0**	**1**	**100**
MS + BAP 2	10	**7**±1.34	0.5	70

Table 4. Effect of different concentration of BAP alone in combination with GA_3 in MS medium on multiplication and elongation of the shoot.

Media Content mg/L	No. of Cotyledonary Nodal Segment Inoculated	Average No. of Multiple Shoots (mean)± SE	The Average Length of Multiple Shoots (cm)	% of Multiple Shoots
BAP + $GA_3$1.0+0.2	10	4.7±0.39	0.5	47
BAP + $GA_3$1.0+0.3	10	5.4±0.56	1	54
BAP + $GA_3$1.5+0.3	**10**	**7**.6±**0.56**	**2**	**76**

Table 5. Effect of different concentration of BAP in combination with NAA in MS medium on induction of multiple shoots.

Media Content mg/L	No. of Cotylidonary Nodal Segment Inoculated	Average no. of Multiple Shoots (mean) ± SE	The average Length of Multiple Shoots (cm)	% of Multiple Shoots
BAP + NAA0.05+0.05	5	1.6±0.36	0.5	32
BAP + NAA 0.1+0.05	5	3±0.66	1	60
BAP + NAA0.15+0.05	5	2±0.18	0.5	40
BAP + NAA0.2+0.05	5	2±0.18	0.5	40
BAP + NAA0.05+0.025	5	2±0.18	0.5	40
BAP + NAA0.1+0.025	5	2±0.18	0.5	40
BAP + NAA0.15+0.025	**5**	**10**±0.56	**1**	**100**
BAP + NAA0.2+0.025	5	4±0.63	1	80

Table 6. Effect of different concentration of BAP alone in MS medium on callus induction from the cotyledonary nodal segment of *Glycine max* (BARI-5 variety).

Media Content mg/L	No. of Cotylidonary Nodal Segment Inoculated	Days Reruired for Callus Induction	% of Callus Induction	Color of Callus
MS + BAP0.5	5	28	50	Lightgreen
MS + BAP1.5	**5**	**28**	**100**	**Deep green**
MS + BAP2	5	28	90	Coffee

Table 7. Effect of different concentration of BAP in combination with GA_3 in MS medium on callus induction from the cotyledonary nodal segment of *Glycine max* (BARI-5 variety).

Media Content mg/L	No. of Cotylidonary Nodal Segment Inoculated	Days Required for Callus Induction	% of Callus Induction	Color of Callus
BAP + $GA_3$0.5+0.1	5	23	30	Yellow
BAP + $GA_3$0.5+0.2	5	23	40	Light green
BAP + $GA_3$1.0+0.2	**5**	**23**	**70**	**Black**

Table 8. Effect of different concentration of 2,4-D in combination in MS medium on callus induction from the cotyledonary nodal segment of *Glycine max* (BARI-5 variety)

Media Content mg/L	No. of Cotylidonary Nodal Segment Inoculated	Days Required for Callus Induction	% of Callus Induction	Color of Callus
MS + 2,4-D0.73	5	14	90	Green
MS + 2,4-D1.99	5	14	40	Light green
MS + 2,4-D3.32	**5**	**14**	**100**	**Deep green**
MS + 2,4-D4.64	5	14	50	Light green

Table 9. Effect of different concentration of 2,4-D in combination with NAA in MS medium on callus induction from the cotyledonary nodal segment of *Glycine max* (BARI-5 variety).

Media Content mg/L	No. of Cotylidonary Nodal Segment Inoculated	Days Required for Callus Induction	% of Callus Induction	Color of Callus
2,4D+NAA 0.73+0.69	5	12	25	Light green
2,4D+NAA 1.99+1.76	5	12	10	Light green
2,4D+NAA 3.32+2.83	5	12	5	Creamy

Table 10. Effect of different concentration of IBA alone in MS medium on rooting of *in vitro* regenerated shoot of *Glycine max* (BARI-5 variety).

Media Content mg/L	No. of Shoot Inoculated	% of Root Per Cultured Shoot	Days Required for Rooting	Average No. of Root Per Shoot	The Average Length of The Root (cm)
MS + IBA0.1	**5**	**100**	**13**	**3**	**8**
MS + IBA0.25	5	70	13	2	0.5
MS + IBA0.5	5	90	13	6	0.5
MS + IBA1	5	70	13	3	4
MS + IBA2	5	50	13	1	0.001
MS + IBA3	5	50	13	1	0.002
MS + IBA4	5	50	13	1	0.001

eliminated accurately by proper immersion time of explants in the $HgCl_2$ solution. Furthermore, the concentration of $HgCl_2$ solution is equally important to obtain sterile ex-plants. In the present experiment the seed of *GLycine max* (BARI-5 variety). Were treated with different conc. of $HgCl_2$ solution, 100% of cultures were found contamination free without injury when treated with 0.1% $HgCL_2$ for 15 minutes. Whereas [25] following a 5 min. Sterilization treatment of soybean seed in 0.2% aqucous swolution of $HgCl_2$.

Table 11. Effect of different concentration of IBA alone in ½ MS medium on rooting of *in vitro* regenerated shoot of *Glycine max* (BARI-5 variety).

Media Content mg/L	No. of Shoot Inoculated	% of Root Per Cultured Shoot	Days Required for Rooting	Average No. of Root Per Shoot	The average Length of The Root (cm)
$_1/_2$MS + IBA0.1	5	70	13	1	0.5
$_1/_2$MS + IBA0.25	**5**	**100**	**13**	**2**	**1.5**
$_1/_2$MS + IBA0.5	5	90	13	1.5	1
$_1/_2$MS + IBA1	5	60	13	1	1

Fig. (1) . Callus induction from the cotyledonary nodal segment of *Glycine Max* (BARI- 5 varieties) after culture.

3.17. Callus Induction

Callus inducting is a prerequisite on the way to regenerate soma clonal variation because during cell division of callus in artificial condition different types of genetic changes occur and this ultimately contributed to the regenerated plants. As a result, a lot of variation may be found in the plants regenerated from a single callus population. Callus is actively dividing nonorganized tissue of undifferentiated and differentiated cells often developing from injury (wounding) or in tissue culture [26]. Callus formed during *in vitro* culture has some similarity to in tissue arising *in vitro* injury plants (so called wound callus). However, there is a difference in morphology. Cellular structure growth and metabolism between callus derived through tissue culture and natural wound cells. Now it has been established that any tissue can be changed into a callus. If cultured on a suitable defined medium under controlled conditions [27] demonstrated hormonal control of differentiation and laid the foundation of clonal propagation of plants through tissue culture techniques. Exogenous supply of auxins and often in combination with cytokinins to medium is essential but many other factors such as light temperature. Humidity and others are also important for callus production [26]. in the present study. A variable amount of callus produced from cotylidonary nodal segment when they were cultured in the presence of BAP, 2.4-D, NAA singly or in combination. It was observed that callus was light green. White black deep green and coffee color. Callusing was best (100%) on medium supplemented with MS + 3.32 mg /L 2.4-D. (table-8). Thus indirectly organogenesis took place [28] produced callus from mature half seed' s nodal segment using different combinations of BAP and 2.4- bichlorophenoxy acetic acid (2.4-D). The callus produced a number of plants when cultured on Gamborg B5 medium supplemented with BAP while the addition of 2.4-D enhanced somatic embryo production (Fig. (**2**).

Fig. (2). Callus induction from the cotyledonary nodal segment of *Glycine Max* (BARI- 5 varieties) after 15 days.

3.18. Organogenesis of *Glycine Max*

The most important development in culture media was the incorporation of growth regulators like auxins and cytokinins. In present study MS and 1/2 strength of MS media were used for callus induction shoot multiplication and root formation from the cotyledonary nodal segment of Glycine max (BARI-5 variety). Various hormonal supplements namely BAP, NAA, 2.4-D IBA, GA_3 and their various combinations were used in these media.

3.19. Multiple Shoot Formation

In the present study, MS media supplemented with various hormones namely NAA, BAP, GA_3 and their various combinations were used for shoot multiplication. In this study, it was observed that the highest number of multiple shoots was 10.9 ±2.0 initiates in 1.5 mg/L BAP containing the medium and highest average length of multiple shoots 2cm was found in 1.5 mg/L BAP + 0.3 mg/L GA3 containing the medium. BAP provided smaller length of proliferated shoots in contrast to shoots number [29] found that 86.7% cotylidonary nodes induced shoots with an average of treatment for shoot induction was carried out in MS salts and B5 vitamins (MS B5) medium supplemented with 11.1Um BAP and 0.5Um a-NAA

[30] found that soybean cultivars ` Pungsannamulkong ` and `llpumgeomjeongkong` produced adventitious shoots when BAP was used in the media. Whereas `Alchangkong produced shoot when treated with zeatin and IAA [31] were able to get multiple shoot bud formation from cotyledonary nodes on medium containing high concentrations of 6-benzyl aminopurine (BAP) but bud growth improved when the cultures were transferred to low concentrations of BAP. However, the addition of thidiazuron (TDZ) [12, 32] with BAP has been reported as a better combination for embryo formation from cotyledonary nodal ex-plants Fig. (**3**).

Fig. (3). Multiple shoots regeneration from the cotyledonary nodal segment of *Glycine Max* (BARI- 5 varieties).

3.20. Rooting of *in vitro* Regenerated Shoot

Auxins are considered as the effective plant growth regulators which accelerate the processes of root induction and development by differentiation of vascular bundles. They promote rooting in the plants through changes in the biochemical system of the plants [33]. Among various auxins. IBA is known to stimulate rooting more effectively due to its week toxicity and greater stability for induction of roots where as [31] reported that Soybean plants were produced by rooting of tissue culture shoots on a basal medium without auxins. In the present study different concentration of IBA were used for *in vitro* rooting well-developed multiple shoots when attaining considerable height were shifted to half strength MS medium containing a different concentration of IBA. In the present study among the different concentration of hormone best rooting response was 100% obtained in MS medium containing 0.1 mg /L IBA within 13 days of inoculation. (Fig. **4**).In the present investigation good rooting response was visualized from nodal explants in MS medium and observed that highest number of roots per shoot was 6 initiates in MS + 0.5 mg/L IBA containing medium and highest average length of root per shoot was 8cm found in 0.1 mg/L IBA containing MS medium [34] found that root was induced from shoots on B5 medium with indole butyric acid (IBA. 14 7Um) [35] reported that thidiazuron (TDZ) functions as cytokinins (to produce the shoots) and auxins (to produce the roots) on various explants depending on the basal medium used (Fig. **5**).

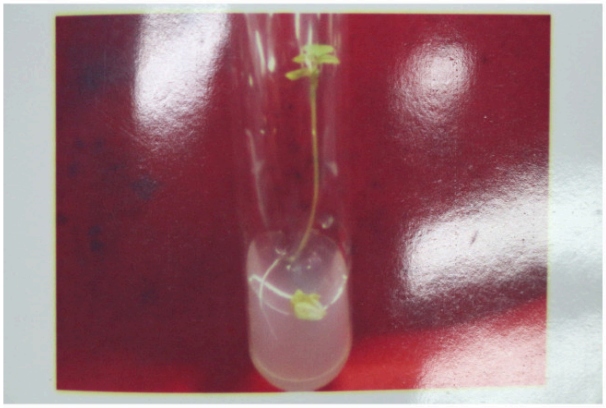

Fig. (4). Rooting of *in vitro* regenerated multiple shoots of *Glycine Max* (BARI- 5 varieties) on IBA containing MS medium.

Fig. (5). Rooting of *in Vitro* regenerated multiple shoots of ***Glycine Max*** **(BARI- 5 varieties) on IBA containing the 1/2MS medium.**

Fig. (6). *In vitro* micro plant establishment in a pot after 2.5 months.

CONCLUSION

In vitro micro propagation technique exploits the concept that detached plant (organ Tissue *etc*.) (Fig. **6**).Are totipotent

and capable of regenerating the whole plant and allows for a rapid low volume and high yield multiplication system under diseases free condition. It has been effectively used to conserve a variety of plant genotypes. Combination and concentration of plant growth regulators affect regeneration from *in vitro* cultures of soybean tissue culture has been reported using immature cotyledons hypocotyls and ovaries Primary leaf tissues hypocotyls and epicotyl and cotylidonary nodes of few days germinated seedlings. As demands increase for soybean oil and protein, the improvement of soybean quality and production through genetic transfor-mation and functional genomics becomes an important issue throughout the world. Modern genetic analysis and improvement of soybean heavily depend on an efficient regeneration and transformation process, especially commercially important genotypes. The transform-ation techniques developed until now till date do not allow high throughout analyses in soybean functional genomics; though significant improvements have been made in the particle bombardment of embryogenic culture and Agrobacetrium mediated transformation of the cotyledonary node over the past three decades. However, routine recovery of transgenic soybean plants using either of these two transformation systems has been restricted to a few genotypes with no reports of transformation on other locally available commercial genotypes. Therefore, development of an efficient and consistent transformation protocol for other locally available commercial genotypes will greatly aid soybean functional genomics and transgenic technology. Considerable and detailed knowledge has been gathered about commercial plant species and some genetic improvement has been achieved. However, in Bangladesh very little is known about variation patterns. Usefulness, and adaptability to this environmental condition of this plant.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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