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# *In vitro* Regeneration and Biohardening of Soybean (JS335) Variety

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**Abstract:** ***An efficient protocol for in vitro regeneration of half seed explant with embryonic axis of soybean and biohardening of in vitro regenerated plantlets were standardized. For organogenesis, MS medium supplemented with cytokinins BAP (15 µM) and TDZ (15 µM) in combination with NAA (1, 5 and 10 µM) or IBA (1, 5 and 10 µM). Among the concentrations, TDZ (15 µM) + NAA (10 µM) was found to be best for highest percentage (86%) of shoots as well as the maximum number of shoots (20.6±0.88) and length of shoots (9.0±0.78 cms). The best response for maximum number of roots (18.6±0.88) with root length (2.9±0.18 cms) was noticed in the combination of TDZ (15 µM) with IBA (5 µM). The plantlets were transferred into the papercups and inoculated with Bacillus subtilis to enhance the survival of regenerated plantlets. The maximum survival (85%) was recorded in the plantlets treated with B. subtilis (0.6) of Optical Density at 600 nm with maximum number of shoots (26.0±0.7) and length of shoots (21.0±0.5 cms) and maximum number of roots (12.3±0.4) and root length (2.7±.01 cms) when compared with untreated plantlets. This protocol may be used in mass multiplication of elite soybean genotypes through tissue culture and genetic transformation studies.***

**Keywords:** micropropagation, in vitro regeneration, biohardening, half seed with embryonic axis, *Bacillus subtilis*.

## 1. Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the most important crops in the world and is generally recognized as the most economical source of food protein. Plant Growth Regulators (PGR) have been used in regeneration of plants via organogenesis. Cytokinin, 6-benzylaminopurine (BAP) was commonly used PGR for miropropagation of plants. First study on organogenesis was reported from cotyledonary explants derived from germinated soybean seedling (1). BAP was efficient for multiple shoot formation from half seed explants (2) and TDZ was efficient for indirect organogenesis from half seed explants (3). BAP in combination with indole butyric acids (IBA) was used for improved regeneration frequency from embryonic axes (4) and cotyledonary node (5,6).

The transfer of *in vitro* raised plantlets to *ex vitro* conditions is one of the most critical factors in the micropropagation process and cause of higher production costs. High mortality is often observed upon transfer to *ex vitro* conditions as the cultured plants have non functional stomata, weak root system and poorly developed cuticle. In order to increase growth and reduce mortality in plantlets at the acclimatization stage, introduction of beneficial sterile endophytic bacteria was most ideal. Both Gram negative and Gram positive strains of endophytic bacteria can be applied in this system (7). Application of bacteria is probably most effective for endophyte-free materials, where niches can still be colonized by the introduced endophyte with relatively low competition from naturally present endophytes (7). Application of endophytes at the nursery stage on tissue cultured clones in order to allow the establishment of the microbes prior to transplanting to the field (8).

Biohardening has been reported to enhance the field survival by promoting growth of the host plant and formation of secondary metabolites related to plant defence (9). The biohardening of *in vitro* raised gladiolus plantlets (10) and banana (11) for enhanced survival. The previous reports were available where *Bacillus* bioformulation could survive up to one or more year in several bioformulations (12). Carrier based preparations of two PGPR, viz., *B. subtilis* and *Pseudomonas corrugata* developed in five formulations were also evaluated for their growth promotion, rhizosphere colonization and viability under storage (13). Four bacterial isolates, *Bacillus subtilis* and *Bacillus* sp. (associates of established tea rhizosphere), *Pseudomonas corrugata* 1 and *P. corrugata* 2 (associates of young tea rhizosphere) were used as test inoculants and the bacteria were selected on the basis of strong antifungal activity against several fungi including pathogens of tea (14). The present study was aimed to standardize the protocol for *in vitro* regeneration and for the enhancement of the survival of *in vitro* regenerated soybean through biohardening using *Bacillus subtilis*.

## 2. Materials and Methods

### 1) Source of Explants

Soybean variety of JS335 seeds were obtained from the IARI, New Delhi the half seed explant with embryonic axis was used to standardize. The seeds were surface sterilized by detergent and washed with sterile distilled water. Seeds were soaked in sodium hypochlorite solution (4%) for 4 hours and washed thrice with sterile distilled water.

### *2) In vitro* Regeneration

The half seed explants with embryonic axis were separated and inoculated on MS medium fortified with TDZ (15 µM) and BAP (15 µM) in combination of NAA (1, 5 and 10 µM) and IBA (1, 5 and 10 µM) for regeneration of plantlets. The cultures were incubated at 25±2°C with 16 h photoperiod.

### 3) Biohardening

Plantlets were transferred into the papercups with sterile sand and soil in the ratio of 1:1 for hardening. Before

hardening, plantlets were inoculated with *Bacillus subtilis* (0.6-1.0) of OD at 600 nm to enhance the survival of plantlets. After 20 days, growth of hardened plantlets was studied. The data were statistically analyzed by using one way Annova with three replicates.

## 3. Results and Discussion

Among the concentrations of NAA and IBA (1, 5 and 10 µM) in combination with BAP (15 µM) and TDZ (15 µM), the best response of regeneration frequency (86%), number of shoots (20.6±0.88) with length of 9.0±0.78 cms was noticed in the concentration of TDZ (15 µM) + NAA (10 µM) for multiple shoot induction (Table 1). In rooting, the highest number of roots (18.6±0.88) were noticed in the concentrations of TDZ (15 µM) with IBA (5 µM) with the mean length of roots (2.9±0.18 cms) (Fig. 1). TDZ is considered to be one of the most active cytokinin for shoot induction in plant tissue culture (15). TDZ was responsible for higher regeneration capacity and multiple shoot formation efficiency than BAP (15, 16). Regeneration frequency and number of shoots depends upon the cytokinin concentration and explant interaction, both BAP and TDZ are most effective cytokinins for shoot organogenesis in soybean (17).

The rooted plantlets were transferred into papercups containing sterile sand and soil in the ratio of 1:1 for hardening. The maximum survival of 85% of plantlets was recorded in the plantlets treated with *Bacillus subtilis* (0.6) of optical density at 600 nm as compared to the control (55%) (Table 2). The maximum number of shoots (26.0±0.7) with length of shoots (21.0±0.5 cms) and the number of roots (12.3±0.4) with root length (2.7±.01 cms) of biohardened plantlets was significantly higher than the controls (Fig. 2).

The ultimate success of micropropagation depends on the ability to transfer plants out of culture on a large scale at low cost with high survival rates. The *in vitro* grown plantlets are unable to compete with soil microbes and could not cope with the environmental conditions while transferred into the field, (18). The plant growth promoting rhizobacteria were used in the bio priming of the micro propagated banana showed increased growth of the root length, internode diameter and number of leaves (19). Likewise plantlets inoculated with *Glomus fasciculatum* and mixed AMF observed that flowering early and high yield in Chrysanthemum (20).

Application of the microbes is strongly recommended at the nursery stage on tissue-cultured clones in order to allow the establishment of endophytes prior to the transplanting to the field (21). In the present study, regeneration protocol may be highly useful for the production of planting material with short duration. The hardening process and plantlets for better establishment, survival, improved growth and yield of plants, which translates to higher profits for farmers. The results indicate that the biologically hardened plants were enhanced the survival of plants than the controls.

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**Table 1:** Effect of hormones on plantlet regeneration of halfseed explants with embryonic axis of soybean variety (JS335)

| Hormone Concentrations (µM) | Percentage of Response (%) | No. of Shoots | Length of Shoots (cms) | No. of Roots | Length of Roots (cms) |
|---|---|---|---|---|---|
| Control | 46d | 05.6±0.88$^{d}$ | 3.1±0.34 d | 6.6±1.20d | 1.3±0.14d |
| 1 | 76c | 14.6±2.02$^{b}$ | 4.8±0.20c | 11.6±1.20c | 1.6±0.15c |
| TDZ (15)+NAA | 83b | 15.0±1.52b | 5.5±0.15b | 13.6±1.20b | 2.3±0.17a |
| 5 | **86a** | **20.6±0.88a** | **9.0±0.78a** | 14.6±0.88a | 2.3±0.14a |
| **10** | | | | | |
| 1 | 63c | 11.0±0.57b | 6.4±0.17a | 15.0±1.15b | 2.7±0.14b |
| TDZ (15) +IBA | 66b | 12.0±1.15a | 5.3±1.21b | **18.6±0.88a** | **2.9±0.18a** |
| **5** | 83a | 11.0±1.15b | 4.2±0.23c | 14.3±2.33c | 2.6±0.11c |
| 10 | | | | | |
| 1 | 66a | 8.3±2.02b | 3.5±0.26c | 6.0±1.52a | 1.6±0.17a |
| BAP (15)+NAA | 60c | 11.6±3.28a | 5.3±0.06a | 2.0±0.00c | 1.5±0.27b |
| 5 | 63b | 8.3±2.96b | 4.6±0.08b | 5.6±0.33b | 1.5±0.24b |
| 10 | | | | | |
| 1 | 63b | 6.6±1.45b | 4.1±0.12b | 13.3±1.20b | 2.4±0.06a |
| BAP (15) +IBA | 70a | 8.0±1.15a | 5.3±0.13a | 15.6±2.33a | 2.0±0.30b |
| 5 | 70a | 6.6±1.20b | 3.7±0.20c | 11.3±0.88c | 1.7±0.12c |
| 10 | | | | | |

Data represented as the mean value ± standard error from the three experiments with 10 explants Mean followed by different letters (DMRT) in the same column differ significantly P<0.05.

**Table 2:** Effect of *Bacillus subtilis* inoculation on survival of *in vitro* raised plantlets of soybean (JS335) for biohardening.

| *Bacillus subtilis* Optical Density (600 nm) | Percentage of response (%) | Number of Shoots | | Length of Shoots (cms) | | No of Roots | | Length of Roots (cms) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Before treatment | After treatment | Before treatment | After treatment | Before treatment | After treatment | Before treatment | After treatment |
| Control | 55d | 9.3±0.8d | 15.0±0.5d | 3.2±0.4d | 3.5±0.3d | 2.0±0.0d | 5.3±0.4d | 1.2±0.1d | 1.6±0.1d |
| 0.6 | 85a | 13.3±0.4a | 26.0±0.7a | 9.6±0.8a | 21.0±0.5a | 6.0±0.5a | 12.3±0.4a | 2.4±0.3a | 2.7±.01a |
| 0.8 | 76b | 12.6±0.3b | 23.0±0.5b | 5.3±1.2b | 17.9±0.5b | 6.3±0.8b | 8.3±0.5b | 2.0±0.4b | 2.1±0.5b |
| 1.0 | 70c | 12.0±0.5c | 22.0±0.2c | 4.2±0.2c | 16.7±0.8c | 4.6±0.3c | 6.0±0.5c | 1.3±0.1c | 2.0±0.3c |

10 plantlets were taken and experiments were replicated thrice, after 20 days of treatment results were noticed and analysed the data by SPSS

**Figure 1:** Plantlet regeneration of soybean (JS335) variety by using half seed explant

a) Half seed explants inoculated in MS medium and shoot initiation after 10 days; b. Multiplication of shoot after 15 days; c. Multiplication of shoot after 20 days in the MS medium fortified with TDZ and IBA (10 µM); d. Multiplication of shoot after 20 days in the MS medium fortified with TDZ and NAA (10 µM); e. Root initiation in the MS medium fortified with TDZ and NAA (10 µM); f. Root initiation in the MS medium fortified with TDZ and IBA (10 µM); g. Hardening of Plantlets in papercups; h. Biohardened plantlets after application of *Bacillus subtilis* after 20 days.

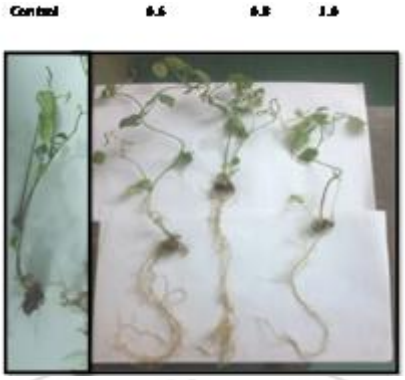


**Figure 2:** Effect of *Bacillus subtilis* inoculum for Biohardening.