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# Variation in *in vitro* Morphogenic Response to Growth Regulators in Soybean Genotypes from India and Bulgaria

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**Summary:** Soybean (Glycinae max (L.) Merrill.) is receiving great global importance due to its nutraceutical value but its cultivation suffers the problems of biotic/abiotic stress. To improve soybean germplasm biotechnological approach can be applied. The objectives of the experiments were to study the possibilities for establishment of in vitro cultures which can be used for genetic manipulations and modelling of stress. In vitro morphogeneic response of two Indian (Hardee and JS 335), one Bulgarian (Daniela) and one american (Hodson) soybean cultivars were studied using plant growth regulators. Using cotyledonary nodes as explants, high organogenic response was observed for cv Daniela and cv Hodson on media containig BAP and IBA. TDZ induced multiple shoot buds in all the cultivars, with varying degree of response and it was found to be genotype specific. A maximum of 8 shoot buds were obtained from cotyledonary node explants in presence of TDZ (0.5 mg/l) for the cv. Hardee. A negative correlation was observed between bud number and size for the Bulgarian cultivars. The results indicate the stimulating effect of TDZ on organogenesis and the interaction of genotype and culture media, which can be utilized for crop improvement using tissue culture techniques.

**Keywords**: Soybean, Glycine Max, In Vitro, Callogenesis, Organogenesis, Cotyledonary Node, Thidiazuron.

## 1. INTRODUCTION

Soybean (*Glycine max* (L.) Merrill.*)* is receiving great global importance due to its nutraceutical value and its application in functional foods. As an oil and protein rich plant it provides about 60% of the plant based protein in the world, and it is a source of more than 200 indusrtrial products [1]. Beneficial secondary metabolites of soybean include isoflavones, phenolic compounds and saponins. *Glycine max* is the largest cultivated pulse crop in the world. In India the area under soybean cultivation is on the rise and there is a huge demand for soy oil and other soy based products. However, its cultivation suffers the problems of biotic and abiotic stress. In order to improve this crop for modification in pre harvest and post harvest applications it is essential to improve the germplasm through biotechnological interventions [2]. Soybean plant breeding in Bulgaria is directed to development of varieties with higher disease resistance and drought tolerance [3]. In Bulgaria biotechnological approach is being recently applied in addition to the classical ones for genetic improvement [4]. *In vitro* cultures are the basis for plant biotechnology and manipulations. Induction and efficiency of callogenesis, organogenesis and regeneration of plants depends on many factors. But the genotype, the explant and culture media are the most important parameters. In this context, the *in vitro* morphogenetic potential of Indian and Bulgarian soybean cultivars were studied under the Indo-Bulgarian collaborative project.

The objectives of the experiments were to study the possibilities for establishment of *in vitro* cultures from Indian and Bulgarian genotypes which can be used for genetic manipulations and modelling of stress tolerance by *in vitro* culture methods.

## 2. MATERIALS AND METHODS

### A. Plant material:

Two varieties “Daniela” and “Hodson” were used in the experiments carried in the Institute of Genetics at Bulgaria. The first one was selected in the Institute of Soybean (at present Institute of Forage Crops – branch Pavlikeni, Bulgaria) and is characterized with higher drought tolerance and productivity. The latter is an internationally cultivated variety.

Two Indian cultivars *viz.*, Hardee and JS 335 procured from Gandhi Krishi Vigyana Kendra, University of Agricultural Sciences, Bangalore, India were also used to study their *in vitro* morphogenetic response.

B. Explant preparation:

Cotyledonary nodes were excised from soybean seedlings of varieties "Daniela" and "Hodson". Seeds were surface decontaminated in 70% ethanol for 1 min, followed by 30% v/v commercial bleach and rinsed three times in autoclaved distilled water. Basal media of Murashige and Skoog (MS) [5] was used for seed germination. Cotyledonary nodes were excised from 10-14 days old seedlings before the formation of the first leaf. Similarly the soybean seeds of Indian genotypes viz., Hardee and JS 335 were surface decontaminated and the *in vitro* seedlings were raised on MS basal medium. Cotyledonary node from 14 day old seedlings were used as explants.

C. *In vitro* cultivation

Two media were used for induction of organogenesis: (a) MSF – containing MS basal medium enriched with aminoacids [6], 0.4 mg/l benzylaminopurin (BAP) and 0.1 mg/l 3indolebutyric acid IBA; (b) MSFT – MSF medium supplemented with 0.2 mg/l thidiazuron (TDZ). Explants were cultivated following two schemes: (A) one month on MSF medium; (B) one month on MSFT medium (long-term treatment). At the end of these periods (totally 30 days) explants from the two groups were transferred on MSF medium. Regenerated plants were rooted in MS or B5 Gamborg's media [7]. The cotyledonary nodal explants from Indian genotypes viz., Hardee and JS 335 were cultured on media containing MS basal salts and vitamins with 30 g/l sucrose and 8.0 g/l of agar (pH 5.8) supplemented with varying concentrations of TDZ (0.2, 0.5, 1.0 and 2.0 mg/l) and BAP (0.5 and 1.0 mg/l) for shoot bud induction. After 4 weeks the explants with shoot buds were transferred to medium containing MS basal salts and vitamins supplemented with gibberellic acid ($GA_3$) (0.1, 0.2, 0.5, and 1.0 mg/l) and BAP (0.2 and 0.5 mg/l) for elongation. The regenerated plantlets were rooted in MS medium containing 1.0 mg/l IBA. All cultures were grown in phytotrone rooms at $26^{o}$ C, and 16/8 h photoperiod.

## 3. RESULTS AND DISCUSSIONS

Criteria for the level of organogenic potential were formation of adventitious buds, their size and number, formation of callus and its size. *In vitro* response of the varieties under investigation varied depending on the culture media (Table 1). On MSF medium (containing 0.4 mg/l BAP and 0.1 mg/l IBA) induction of bud formation from the meristematic tissue of the cotyledonary nodes was observed after one week. On the tenth day most of the explants formed buds with better performance of cv. Hodson (48%) compared to cv. Daniela (36%). However, induction of organogenesis continued with greater rate in cv. Daniela reaching 100% on the 30$^{th}$ day, while the values for Hodson were 86 %. Newly formed buds of 0.1-0.2 cm grew to 0.8-0.9 cm at the end of the cultivation period with slight difference between the two varieties (Table 1A, Fig.1). Number of buds per explant (which was another criterion for evaluation of the organogenic potential) slightly varied (2-4 buds) with no distinguishable genotypic difference. These experiments were considered as a control, as far as, medium MSF was previously used [6] to evaluate *in vitro* response of soybean explants.

Table. 1 (A and B). Effect of growth regulators on organogenesis from cotyledonary nodes of soybean seedlings.

1.A. Explants were cultivated for one month on MSF medium

| Genotype | Bud formation [%] | | | Buds size (min-max) [cm] | | |
|---|---|---|---|---|---|---|
| | MSF medium | | | MSF medium | | |
| | 10$^{th}$ d[1] | 20$^{th}$ d | 30$^{th}$ d | 10$^{th}$ d | 20$^{th}$ d | 30$^{th}$ d |
| Daniela | 36.0 | 86.0 | 100.0 | 0.2-0.3 | 0.4-0.5 | 0.7-0.9 |
| Hodson | 48.0 | 66.0 | 86.0 | 0.1-0.2 | 0.3-0.5 | 0.4-0.8 |

1.B. Explants were cultivated for one month on MSF medium supplemented with TDZ (long-term treatment)

| Genotype | Bud formation [%] | | | Buds size (min-max) [cm] | | |
|---|---|---|---|---|---|---|
| | MSFT medium | | | MSFT medium | | |
| | 10$^{th}$ d[1] | 20$^{th}$ d | 30$^{th}$ d | 10$^{th}$ d | 20$^{th}$ d | 30$^{th}$ d |
| Daniela | 55.0 | 60.0 | 100.0 | B. pr.[2] | 0.1 | 0.1-0.2 |
| Hodson | 34.0 | 40.0 | 67.0 | Bud primordia | | |

Legend: [1]d – day; [2]B.pr. – Bud primordia

Fig. 1. Formation of shoots and buds of cv Hodson seedlings cotyledonary nodes on MSF (left) and MSFT (right) media

In another set of experiments, where the plant growth regulator thidiazuron (TDZ), was added to MSF medium, stimulation of bud formation in Daniela (Table 1.B) was observed at the beginning of the cultivation. However, organogenesis observed in 60% of the explants remained the same for the next 10 days (on the 20$^{th}$ day of cultivation) and reached the maximum of 100% at the end of the tested period. It was observed that TDZ in combination with BAP in long-term treatment had suppressive effect in cv. Hodson what was more pronounced at the beginning of the cultivation period e.g. 10$^{th}$ - 20$^{th}$ (34%). Till the 30$^{th}$ day the number of explants forming buds increased to 67% but the values of organogenesis obtained in the control MSF medium were not reached. Growth of buds of the both varieties was retarded, too. However, if in Daniela bud primordia developed into small buds (though 0.1-0.2 cm) the numerous compact green structures on the Hodson explants did not change in the time. Explants which formed buds or small shoots were transferred on MSF medium for further development. *In vitro* rooting was achieved when shoots were transferred on Gamborg's basal medium [7] lacking plant growth regulators.

During the cultivation of the explants formation of callus tissue was observed in both variants of the culture media and in both plant varieties (Table 2). Usually green compact calli was formed at the basal part of the cotyledonary node which was dipped in the media. Strict correlation between callus growth from one side and genotype and culture media form the other could not be observed in these experiments.

Table 2. Formation of callus tissue on different media

| Genotype | Callus size expressed by the min/max diameter of the pieces [cm] | | | | | |
|---|---|---|---|---|---|---|
| | Scheme 1A | | | Scheme 1B | | |
| | MSF medium | | | MSFT medium | | |
| | $10^{th}$ d | $20^{th}$ d | $30^{th}$ d | $10^{th}$ d | $20^{th}$ d | $30^{th}$ d |
| Daniela | 0.6-0.9 | 1.0-1.4 | 1.8-2.2 | 0.4-0.8 | 1.0-1.6 | 1.7-2.0 |
| Hodson | 0.3-0.7 | 0.8-1.2 | 2.0-2.2 | 0.2-0.3 | 0.6-0.8 | 0.6-0.8 |

Table 3. Response of Indian cultivars on MS medium with TDZ and BAP

| Plant growth regulators [mg/l] | Hardee | | JS335 | |
|---|---|---|---|---|
| | Shoot bud formation [%] | No. of shoot buds | Shoot bud formation [%] | No. of shoot buds |
| TDZ 0.2 | 68 | 28-33 | 64 | 27-30 |
| 0.5 | 60 | 32-36 | 56 | 30-32 |
| 1.0 | 48 | 22-28 | 48 | 22-26 |
| 2.0 | 44 | 15-18 | 40 | 11-16 |
| BAP 0.5 | 52 | 18-21 | 44 | 15-18 |
| 1.0 | 60 | 20-24 | 64 | 16-21 |

Table 4. Effect of $GA_3$ and BAP on shoot bud elongation*

| Plant growth regulators [mg/l] | Hardee | | JS335 | |
|---|---|---|---|---|
| | No. of shoots/ explant | Shoot length [cm] | No. of shoots/ explant | Shoot length [cm] |
| $GA_3$ | | | | |
| 0.1 | 3.6±0.89 | 3.44±0.86 | 3.0±0.71 | 4.14±0.26 |
| 0.2 | 5.2±1.48 | 4.96±0.30 | 4.4±1.52 | 4.84±0.29 |
| 0.5 | 6.2±1.10 | 5.24±0.62 | 5.4±0.89 | 5.72±0.62 |
| 1.0 | 4.3±1.53 | 6.24±0.42 | 3.6±1.14 | 6.56±0.38 |
| BAP | | | | |
| 0.2 | 6.6±1.14 | 5.04±0.52 | 5.4±1.67 | 4.68±0.55 |
| 0.5 | 5.0±1.58 | 4.66±0.69 | 3.8±1.30 | 4.0±0.38 |

* Values are mean ± SD

a) b)

Fig. 2. (a) Multiple shoots from cotyledonary nodal explants of cv. Hardee; (b) Rooted plantlets of cv. Hardee.

In the experiments carried with Indian cultivars, out of the various growth regulators used, thidiazuron induced high level of multiple shoot bud formation. Hardee variety exhibited very good morphogenetic response compared to JS 335 (Table 3), wherein a maximum of 8 shoots per cotyledonary node explant (Fig. 2) were obtained on Murashige and Skoog medium containing thidiazuron (0.2 mg/l). Shoot bud elongation (Table 4) was best achieved in the presence of gibberellic acid ($GA_3$ at 0.5mg/l). *In vitro* rooting of regenerated shoots was achieved on medium with 1mg/l indole butyric acid (IBA) with varying degree of response in the cultivars Recently Shan et al [8] have reported the influence of pretreatment of cotyledonary node explants of soybean cv. 'White hilum' with 0.1mg/l TDZ for one week and its subsequent culture on 0.5 mg/l BA resulted in inducing multiple shoot buds. A similar response was reported for organogenesis from cotyledonary node explants in pea [9]. The response of the genotypes to TDZ in the present study is similar to the earlier reports, but the variation in shoot bud induction may be attributed to genotype differences.

## 4. CONCLUSIONS

The results presented here show a stimulating effect of the growth regulator thidiazuron for a higher organogenetic response where bud formation is lower and shoot growth is slower. Data confirms the interaction of the genotype and the culture media in eliciting morphogenetic response. However shoot bud growth is slower. These results form an excellent guideline to obtain *in vitro* response for further studies using plant tissue culture based improvement for genetic manipulation.

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