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Ambore AD
PG Students, Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV), Parbhani, Maharashtra, India

Honshette SM
PG Students, Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV), Parbhani, Maharashtra, India

Wankhade DJ
Senior Research Assistant, Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV), Parbhani, Maharashtra, India

Shaikh US
Project Assistant, Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV), Parbhani, Maharashtra, India

Chavan RL
Assistant Professor, Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV), Parbhani

VR Hinge
Assistant Professor, Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV) Parbhani, Maharashtra, India

Corresponding Author:
VR Hinge
Assistant Professor, Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV) Parbhani, Maharashtra, India

In vitro somatic embryogenesis studies in soybean [*Glycine max* (L.) Merr.]

Ambore AD, Honshette SM, Wankhade DJ, Shaikh US, Chavan RL and VR Hinge

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Abstract
Advancement in breeding technologies and plant tissue culture methods, in biotechnology such as somatic embryogenesis, are necessary for developing high yielding, stress-tolerant and disease-resistant varieties. This study aimed to evaluate the somatic embryogenesis potential of the high-yielding soybean genotype MAUS-158. An efficient protocol for *In vitro* somatic embryogenesis was developed using immature cotyledons as an explant of MAUS-158 genotype of Soybean (*Glycine max* (L.) Merr). Somatic embryos were induced directly from immature cotyledons on MS medium supplemented with different concentrations of 2,4-D (2.5,3.0,3.5,4.0,4.5,5.0 mg/L) and BAP (1.0,1.5,2.0,2.5,3.0,3.5 mg/L). Initially fast-growing greenish callus lines containing somatic embryos were established on initiation medium. Further embryo development and maturation was achieved on MS medium supplemented with different concentrations of 2,4-D (2.5,3.0,3.5,4.0,4.5,5.0 mg/L) and BAP (1.0,1.5,2.0,2.5,3.0,3.5 mg/L). The MS Medium supplemented with 4.5 mg/l 2, 4-D and 3.0 mg/l BAP was found the best treatment for somatic embryogenesis with 91.66% response and mean value of 7.33±0.882. The well developed and mature embryos were transferred to the shoot initiation medium supplemented with different concentrations of BAP (2.5,3.0,3.5,4.0,4.5,5.0 mg/L) and 2,4-D (1.0,1.5,2.0,2.5,3.0,3.5 mg/L). The highest numbers of shoots were induced in medium containing 3.5 mg/l BAP and 2.0 mg/l 2, 4-D with 75% of shoot initiation and Mean 6.00±0.577. The somatic embryogenesis results obtained in the present investigation will be utilized for developing faster and reliable *In vitro* regeneration protocol in soybean.

Keywords: *Glycine max*, somatic embryogenesis, 2,4-D, BAP, shoot initiation, immature cotyledons

1. Introduction
Soybean [*Glycine max* (L.) Merr.] is an important oilseed crop in the world which belongs to the family Leguminosae. It ranks first in oilseed crops in the world as well as in India. The world soybean production is 383.31 million metric tons in the year 2023. India's total soybean production is 11,875 million metric tons in the year 2023 which is 3% of the world's total soybean production. Soybean serves as a significant protein source for both human and animal consumption. The seeds of soybeans contain approximately 90% soluble proteins, predominantly globulins. Among these globulins, glycinin (G) (11S globulin) and β-conglycinin (βc) (7S globulin) constitute more than 70%. Glycinin is particularly notable for its relatively high content of sulphur-containing amino acids, such as methionine and cysteine (3-4.5%). This protein is mainly stored in the cotyledons of seeds, where it accumulates within protein bodies. It also contains 43% protein and 20% oil (Srilatha T *et al.* 2019) [14]. In soybeans, the primary components of seed protein content are the seed storage protein (SSP) families known as glycinin (11S) and β-conglycinin (7S). Among these, glycinin is the most prevalent, comprising approximately 33% of the total seed protein (E.A. Dean & J.J. Finer 2022) [2].
However, various biotic and abiotic stress factors affecting soybean production. The global climatic changes causing abiotic stresses such as drought, flood are major concerns to the soybean cultivation across the world, due to which the production and productivity is decreasing. Hence to overcome these constraints we need to develop various advanced technologies for developing new soybean varieties which may contains the genes of interest. There is another need to develop disease and virus free plants through various plant tissue culture interventions such as somatic embryogenesis and somaclonal variation, etc.

In order to boost the production of various crops, including soybean, a sustainable crop improvement is needed to overcome the challenges of biotic and abiotic stresses, such as salt, drought, water-logging, high and low temperatures, diseases, weeds and insect pests (Joyner, *et al.* 2010) [6]. Several protocols for regeneration of soybean plants via somatic embryogenesis have been documented (Komatsuda *et al.*, 1987) [8].

Hence the present study focuses on the *In vitro* somatic embryogenesis study in soybean genotypes MAUS-158. Soybean genotype MAUS 158 is a high yielding and early maturing soybean variety developed by the Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV), Parbhani. Plant Genetic transformation technology has increasingly become an important tool for both cultivar improvement and gene function studies. Most existing transformation protocols necessitate a dependable regeneration system to recover plants from cell or callus cultures. These cultures can be regenerate either through organogenesis or somatic embryogenesis. Hence the present study was undertaken to evaluate somatic embryogenesis potential in soybean genotype MAUS-158.

2. Material and Methods

2.1 Plant material

Seeds of soybean (*Glycine max* L.) genotype MAUS-158 were obtained from Soybean Research Centre, VNMKV, Parbhani. Further seeds were sown in greenhouse to obtain immature cotyledons. After 40-45 days immature pods were collected and outer cover of pods were removed. All the immature seeds were collected in a jar bottle and washed 3 times with running tap water and kept in laminar air flower for further process.

2.2 Preparation of Explant (Surface Sterilization & Inoculation of Immature Cotyledon Explant)

All aseptic preparations were conducted in a laminar air-flow chamber having a HEPA filter (0.22 µm). Glassware and other instruments were kept under UV light for 25 minutes in laminar air flow before use. In order to remove seed coat with ease the immature cotyledons were collected and submerged in water for 10 minutes. Decoated seeds were thoroughly rinsed with sterilized water twice for 1 minute and then treated with fungicide solution containing carbendazim (Bavistin BASF India) for 5 minutes. Traces of Bavistin were removed by rinsing the seeds two times with sterile water for 1 minute each. Further seeds were surface sterilized with [0.1% (w/v)] mercuric chloride ($HgCl_2$) for 3 minutes; followed by rinsed twice with sterile distilled water for 1 minute each to remove any traces of $HgCl_2$ and then seeds were further surface sterilized with Ethanol [70% (v/v)] for 1 minute. The final wash was then given three times with sterile water and the decoated seeds were air-dried on sterile tissue paper before inoculation. Decoated seeds were cut longitudinally along the embryonic axis with a sterile scalpel and utilized as explants for somatic embryogenesis study. The outer seed coat was peeled off by effecting a gentle cut on the seed coat with a sterile scalpel and immature embryos were removed aseptically with sterile forceps. The immature zygotic embryos measuring 2 to 3 mm in length were excised carefully and then cotyledons were cultured on somatic embryo induction medium with the abaxial side facing the medium. The immature cotyledonary explants were placed on the MS medium supplemented with various concentration of 2, 4-D and BAP. The culture conditions are maintained as 25 ± 2°C temperature with light intensity (1600 lux) was provided from fluorescent tubes (40 watt) with a 16/8-h light/dark cycle. The explants were grown into embryogenic calli after 2-3 weeks. The embryogenic calli were sub cultured onto fresh MS medium at 21-day intervals to promote further growth and development. The duration of calli induced was recorded and the percentage of embryogenic callus induction was calculated after 6 weeks. The number of somatic embryos formed per explant was recorded after four weeks of culture.

2.3 Preparation of Media for Somatic Embryogenesis

The MS basal medium was supplemented with various combinations of 2, 4-D [2.5 to 5.0mg/l] and BAP [1.0 to 3.5 mg/l]. The MS media was supplemented with 3% sucrose and pH was adjusted to 5.8 ± 0.2 with 0.1 N NaOH / 0.1 N HCl. For solidification MS Media was supplemented with agar powder (0.8%) and sterilized by autoclaving at 121 ºC under 15 psi pressure for 20 min. The sterilized warm culture medium was used for somatic embryogenesis studies.

2.4 Preparation of Shoot Initiation Media

The MS basal medium was supplemented with different types of growth regulators i.e., cytokinin [BAP 2.5 to 5.0mg/l] and auxin [2,4-D 1.0 to 3.5 mg/l] (Hi-media) in combination with varying concentrations for shoot initiation. Unless specified all combinations of culture media were added with 3% sucrose and pH was adjusted to 5.8 ±0.2 with 0.1 N NaOH / 0.1 N HCl solution. Media with added agar powder (0.8%) was sterilized by autoclaving at 121 ºC under 15 psi pressure for 20 min.

3. Results and Discussion

The development of somatic embryos in soybeans was significantly influenced by the type and concentration of plant growth regulators used in the culture medium. The present study investigated the effect of different concentrations of 2,4-D and BAP (6-benzylaminopurine) on the development of somatic embryos in soybeans.

3.1 The effect of 2,4-D and BAP on the development of the somatic embryo in soybean genotype MAUS-158

The MS medium was supplemented with varying levels of auxin 2,4-D (2.5,3.0,3.5,4.0,4.5 and 5.0 mg/l) and cytokinin BAP (1.0,1.5,2.0,2.5,3.0, and 3.5 mg/l) for the somatic embryogenesis development studies (Table 1). In this study we found that with increase in concentration of auxin the response of explant towards somatic embryogenesis is also increased. In all the treatments under study maximum response of somatic embryogenesis was found in treatment T_5 (MS medium with 4.5 mg/l 2, 4-D and 3.0 mg/l BAP) with mean value 7.33±0.882 and 91.66% of somatic embryogenesis response followed by T_6 (MS medium with 5.0 mg/l 2, 4-D and 3.5 mg/l BAP) (Fig 1). The cotyledonary explants cultured with varying concentrations of 2,4-D (1.0-5.0 mg/L) exhibited swelling, general dedifferentiation, and developed friable callus within 8-10 days. Earlier reports where the highest number of somatic embryos per explant and the highest response rate for somatic embryo formation were documented at 3.0 mg/l of 2,4-D (Srilatha T *et al.* 2019) [14].

The 2,4-D and BAP played a crucial role in regulating cell division and differentiation during somatic embryogenesis. BAP, a cytokinin, promoted cell division and shoot development, while 2,4-D, an auxin, promoted somatic embryo development and maturation. The optimal combinations of 2,4-D and BAP in this study suggested that, the balance between cytokinin and auxin levels is essential for somatic embryo induction in soybeans.

Manipulating the culture medium with specific plant growth regulators facilitated direct organogenesis in soybean hypocotyl segments, resulting in high-frequency plantlet development and successful hardening with a 94.89% survival rate (Patel *et al.*, 2023) [11]. The combination of 6-benzylaminopurine (BAP) and thidiazuron (TDZ) in the medium enhanced regeneration efficiency in soybeans (Franklin *et al.* 2004) [3]. In the study of (Raza *et al.*, 2017) [13] somatic embryo induction was successfully carried out using immature cotyledons cultured in media with a 40 ppm concentration of 2,4-D. The average response for somatic embryogenesis reported earlier was 86.5% and duration was 25.1 days (Gustian *et al.* 2022) [4], (Huynh *et al.* 2015) [5]. Ebony *et al.* successfully induced callus from excised cotyledons and embryos of germinating seeds using 2,4-D at concentrations of 3-21 µM, achieving 100% callus induction. Following callus formation, transferring them to media with BAP and Kinetin resulted in root and shoot development, with 5 µM BAP being the most effective. Fully developed plants were transplanted to pots within three months and produced healthy seeds (Joyner *et al.* 2010) [6]. The effect of abscisic acid (ABA) on soybean somatic embryogenesis, development, and maturation was investigated at 1, 10, 50, 100, and 500 µM concentration. ABA promoted embryo growth and development when applied at the globular stage (Tian and Daniel , 2000).

In the present study optimum concentration of auxin 2, 4-D i.e. 4.5 mg/l and 3.0 mg/l (BAP 3.0 mg/l) was found optimum to induce somatic embryo in soybean cv. MAUS-158 among all treatments. The cytokinin to auxin ratio in the culture media was important and defined the type of somatic embryo development. These findings demonstrated the type and concentration of PGRs played a crucial role in the development of somatic embryos in soybean cv. MAUS-158 also highlighted the need to optimize PGR combinations and concentrations in soybeans for effective somatic embryogenesis.

Table 1: *In vitro* somatic embryogenesis response of immature cotyledon of soybean genotype MAUS-158 supplemented with MS Medium with various combinations of 2, 4-D and BAP

Treatment No	Medium MS+2,4-D (mg)/l+BAP (mg/l)	No of explants responded for SE (8 per replication Total 24 explants per treatment) R-I			Total No of explants responded to SE	SE induction%	Somatic embryo induced mean ± SE
		R-I	R-II	R-II			
T0	0.0+0.0	0	0	0	00	00	00
T1	2.5+1.0	4	3	3	10	41.66	3.33 ± 0.333^{d}
T2	3.0+1.5	4	3	4	11	45.83	3.66 ± 0.333^{cd}
T3	3.5+2.0	5	7	4	16	66.66	5.33 ± 0.882^{bc}
T4	4.0+2.5	6	7	5	18	75.00	6.00 ± 0.577^{ab}
T5	4.5+3.0	7	9	6	22	91.66	7.33 ± 0.882^{a}
T6	5.0+3.5	5	7	8	20	83.33	6.66 ± 0.882^{ab}
CV= 24.089, CD(0.01)=2.705, CD(0.05)=1.949							

Fig 1: *In vitro* somatic embryogenesis response of immature cotyledons.
a) Immature pods of MAUS 158 variety. b) Immature cotyledons placed on MS medium. c) Globular greenish somatic embryos formation. d) Maturation of somatic embryos on fresh medium of same composition e) matured somatic embryos

3.2 The effect of auxin (2, 4-D) and cytokinin (BAP) on shoot induction of matured somatic embryos of soybean genotype MAUS-158

In present study the immature cotyledon explants which showed the somatic embryogenesis response were transferred to the maturation medium whose composition is same as somatic embryogenesis medium (Table.2). Further these matured somatic embryos were transferred to the shoot induction medium with different concentrations of cytokinin (BAP) and Auxin (2, 4-D). The highest shoot induction was observed in treatment T_3 (3.5 mg/L BAP and 2.0 mg/L 2,4-D) after 25 days (Fig.2). The treatments showed the shoot induction response except control one which had not shown any shoot initiation response. Maximum explants showed shoot initiation response in treatment T_3 which contains 3.5 mg/l BAP and 2.0 mg/l 2,4-D with 75.00% of shoot initiation response with mean of 6.00±0.577(Table. 2). It was observed that with increase in the concentration of cytokinin (BAP) and Auxin (2.4-D) with combination the percentage of shoot initiation was also increased. But the highest shoot induction percentage was observed in treatment T_3. Many explants shown the multiple shoots also. In the study of they successfully used a two-step medium process for shoot induction and elongation in explants. Initially, explants cultured on SSIM with Gamborg B5 salts, myo-inositol, MES, BAP, and agar showed shoot induction. Subsequently, transfer to SSEM with MS salts, vitamins, various amino acids, silver nitrate, zeatin, gibberellic acid, IAA, MES, and agar facilitated effective shoot elongation. In a study of Kim *et al.* (1990) [7] reported reproducible protocol for regenerating soybean plants from 7-day-old seedling explants has been established. Using a modified MS agar medium enriched with higher levels of micronutrients, vitamins, proline, BA, and NAA, the protocol effectively induces adventitious shoot formation, outperforming standard MS and B5 media. Explants from the cultivar Peking yielded approximately 20 shoots per explant within 4-5 weeks. Key factors influencing shoot formation include medium composition, explant orientation, seedling age, and cultivar, with exogenous cytokinin (BA) and elevated micronutrients significantly enhancing shoot production. In the investigation of Phat *et al.* (2015) 1 mg L-1 BAP in shoot induction medium (SIM) were found to be the most efficient conditions for induction of soybean regeneration, both in callus development and shoot regeneration.

Srilatha T *et al.* (2019) [14] reported well-formed embryos germinated into complete plantlets on MS medium containing (3.0mg/l 2, 4-D + 1.0mg/l) TDZ. Individual shoots were aseptically excised and sub cultured in the same media for shoot elongation (Srilatha T *et al.*., 2019) [14].

Table 2: Response of explant on growth regulators for shoot induction from matured somatic embryos

Treatment No	Medium MS+BAP (mg)/L+2,4-D (mg/L)	No of explants responded for Shoot initiation among the 8 explant inoculated per replication (Total 24 explants inoculated in three replications			Total No of explants responded to shoot initiation	Percentage of shoot initiation	Shoot initiation response Mean ± SE
		RI	RII	RIII			
T0	0.0+0.0	0	0	0	0	00	00
T1	2.5+1.0	1	1	2	4	16.66	1.33±0.333^{cd}
T2	3.0+1.5	1	3	1	5	20.83	1.66±0.667^{c}
T3	3.5+2.0	6	7	5	18	75.00	6.00±0.577^{b}
T4	4.0+2.5	5	3	3	11	45.83	3.66±0.667^{a}
T5	4.5+3.0	5	3	4	12	50.00	4.00±0.577^{b}
T6	5.0+3.5	6	4	4	14	58.33	4.66±0.667^{ab}
CV=31.211, CD(0.01)=2.312, CD(0.05)=1.666							

Fig 2: *In vitro* shoot initiation response of matured somatic embryos. a) Shoot initiation starts after 25 days of subculture; b) shoot induction or initiation after 35 days; c) Formation of multiple shoots; d) Shoot elongation

4. Conclusion

This study on somatic embryogenesis in soybean genotype MAUS-158 highlighted the critical role of plant growth regulators, specifically 2,4-D and BAP, in promoting somatic embryo development and shoot induction. The optimal combination of 4.5 mg/l 2,4-D and 3.0 mg/l BAP yielded the highest somatic embryo induction (91.66%) in soybean. Additionally, a combination of 3.5 mg/l BAP and 2.0 mg/l 2,4-D produced the best shoot initiation response, with a 75% success rate. This study confirms the importance of optimizing the auxin-to-cytokinin ratio for effective plant regeneration. These findings can be utilized for improving soybean tissue culture protocols, offering potential applications in genetic transformation and crop improvement, especially in developing stress-resistant varieties.

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