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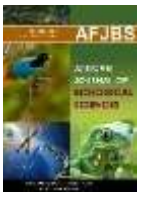

Deciphering Genotype-specific Hormonal Responses: Implications for Enhanced Callus Induction and Regeneration in Sorghum (*Sorghum bicolor* L. Moench).

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ABSTRACT

This study delves the challenges and potential solutions associated with the *in vitro* culture of *Sorghum bicolor*, a plant species that has historically been difficult to regenerate in a laboratory due to its recalcitrant nature. The primary objective of this research was to evaluate the impact of various hormonal treatments on the induction of callus - a mass of undifferentiated plant cells and the subsequent regeneration of the plant from the callus using tissue culture techniques. The research utilized two types of explant sources: immature embryo and mature seeds. These were subjected to a range of concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L) of the hormones 2,4-Dichlorophenoxyacetic acidIndole-3-acetic acid, 6-Benzylaminopurine, Kinetin (6-furfurylaminopurine) and α-Naphthaleneacetic acid. The aim was to induce callus formation and stimulate the regeneration of six distinct sorghum genotypes. These included IS 3477, IS 33095, IS 7005 (non-pigmented), IS 2898, IS 7115, and IS 1202 (pigmented). Results showed that callus induction frequencies varied significantly among the different genotypes. IS 3477 and IS 33095 exhibited the highest rates of callus induction, while IS 7115 and IS 1202 showed the lowest. The most significant embryogenic callus induction was observed in IS 3477 (92%) when treated with 2,4-D at 2.5 mg/L and KN at 0.5 mg/L. In contrast, IS 1202 displayed the lowest induction rate (48%). Further investigation into the effects of various cytokinin concentrations revealed that a regeneration medium containing 2.0 mg/L BAP resulted in high regeneration frequencies. This was particularly evident in the non-pigmented IS 3477 genotypes, which produced the highest number of shoots (20). Additionally, mature seed explants displayed varying embryogenic callus induction frequencies. IS 3477 exhibited the highest rate (76%) when treated with 2,4-D at 2.5 mg/L and BAP at 0.5 mg/L, while IS 1202 displayed the lowest induction rate (36%). Optimal regeneration responses were observed with ZN at 1.5 mg/L, with IS 3477 producing the highest shoot count (11). This study elucidated the genotype-specific responses to *in vitro* protocols across diverse sorghum-pigmented and non-pigmented lines. The findings offer valuable insights into the development of high-frequency response profiles, crucial for advancing impending genetic transformation efforts. This research underscores the importance of understanding different genotypes' specific requirements to optimize *in vitro* culture techniques for plant regeneration.

Keywords: Sorghum, 2,4-Dichlorophenoxyacetic acid, 6-Benzylaminopurin Kinetin, Murashige and Skoog Media, Callus, *In vitro* culture, regeneratio embryogenic callus.

1. INTRODUCTION

1.1 Sorghum: A Multifaceted Crop for Global Agriculture, Nutrition, and Renewable Energy

Sorghum (*Sorghum bicolor* L.) serves a crucial role as the world's fifth-largest cereal crop, contributing significantly to global agriculture and food security. With over 500 million individuals relying on it as a staple food, especially in African and Asian nations, sorghum's versatility and resilience to water deficiency make it a promising crop for climate change adaptation. Its classification as a C4 plant, characterized by efficient water usage and robust biomass production, further enhances its agricultural value. Additionally, sorghum serves as a cost-effective and environmentally friendly feedstock for biofuel production, particularly ethanol derived from sweet sorghum [1]. Renowned for its versatility and nutrient density, sorghum is rich in antioxidants, including phenolic compounds and flavonoids. These antioxidants play a vital role in combating oxidative stress, contributing to overall health and wellness. The high antioxidant content not only enhances sorghum's nutritional value but also positions it as a potential contributor to the development of health-promoting foods [2-4].

Sorghum's natural attributes, such as abiotic stress tolerance, a diverse genetic base, and high grain and sugar yields, position it as a promising bioenergy feedstock. Its potential for establishing an efficient and low-cost biofuel industry is underscored by its use in ethanol production. A study by Laopaiboon et al. demonstrated the utilization of sweet sorghum juice, supplemented with 0.5% ammonium sulfate, as a substrate for ethanol production by Saccharomyces cerevisiae. Fed-batch fermentation further improved the efficiency of ethanol production, highlighting sorghum's versatility in the realm of renewable energy [5, 6]. The field of proteomics has revolutionized the understanding of sorghum's protein content, revealing its high protein richness. Sorghum, traditionally known for its hardiness and nutritional value, emerges as a valuable source of protein. These findings not only confirm the nutritional importance of sorghum but also open avenues for addressing global nutritional challenges, contributing to health and well-being [7, 8]. Numerous studies have investigated sorghum regeneration through embryogenic callus formation, employing various explants. These include immature inflorescences, immature embryos, shoot tips, and meristems. While immature inflorescences and embryos exhibit higher regenerative capacity, practical challenges arise due to their limited production and availability. Immature inflorescences must be harvested before pollination, while immature embryos are collected after pollination. Our research aims to address these limitations and explore alternative explants for efficient sorghum regeneration [9-15].

The genetic transformation of sorghum has been extensively researched, leading to significant advancements. Raghuwanshi and Birch developed a repeatable transformation system for sweet sorghum, optimizing tissue culture conditions and implementing an effective selection regimen [16]. Howe et al. furthered this progress with a rapid and reproducible Agrobacterium-mediated transformation protocol [17]. Gurel et al. enhanced efficiency by introducing a heat treatment of immature embryos, significantly increasing transformation frequencies [18]. Liu and Godwin achieved a highly efficient transformation system, demonstrating the potential of auxins and copper sulphate in enhancing root proliferation and explant growth [19, 20]. Despite the known regenerative capabilities of sorghum, there remains a lack of understanding regarding the optimal

conditions for embryogenic callus formation across diverse genotypes, which this study aims to elucidate.

1.2 Addressing Challenges in Sorghum Regeneration:

Our research is adopted to refine sorghum tissue culture protocols through an exhaustive evaluation of the embryogenic potential among selected sorghum genotypes, namely IS 3477, IS 33095, IS 7155, IS 2898, IS 7005, and IS 1202. We aim to enhance regeneration outcomes by rigorously testing various hormone concentrations and combinations. Despite considerable progress in sorghum tissue culture, the need for a more efficient regenerative system persists, especially to address issues like tissue browning and polyphenol production. Our work is focused on overcoming genotype-dependent challenges, leveraging sorghum's inherent resistance, and establishing effective and repeatable regeneration protocols. By fine-tuning these protocols, we aspire to advance the genetic transformation of sorghum, thereby increasing crop yields and enhancing agricultural productivity. Employing a factorial experimental design, this study methodically examines the impact of hormone levels and their interactions on the embryogenic potential of the six sorghum genotypes, concentrating on mitigating the challenges of tissue browning and polyphenol production.

1.3 Objective of Sorghum Tissue Culture Study

This research aims to achieve several critical goals in sorghum tissue culture. Firstly, we seek to identify the optimal hormone concentrations and combinations that enhance embryogenic callus formation in specific sorghum genotypes. Secondly, we aim to develop a robust and universally applicable protocol for sorghum regeneration across diverse genotypes. Additionally, we strive to unravel the genotype-specific mechanisms underlying tissue culture responses. Furthermore, we address challenges related to tissue browning and polyphenol production during the process. Lastly, our study aims to enhance regeneration for future genetic transformation processes in sorghum, ultimately contributing to improved crop yields and agricultural productivity.

2. MATERIALS AND METHODS

2.1 Plant Material: Six distinct cultivars of *Sorghum bicolor* (L.) Moench, namely IS 3477, IS 33095, IS 7005 (non-pigmented), IS 2898, IS 7155, and IS 1202 (pigmented), were selected for the study. Seeds were procured from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in Patancheru, Hyderabad, Andhra Pradesh, India. All cultivars were preserved through self-pollination, safeguarded from cross-pollination using protective brown paper bags, and subsequently cultivated at the experimental farm of the Department of Botany, Andhra University, Visakhapatnam.

2.2 Sterilization Agents: Various sterilization agents were employed in the study, including Sodium hypochlorite (NaOCl) (70%), Mercuric chloride (0.1%), and ethanol (70%). These agents were used to sterilize the plant materials and culture apparatus to maintain an aseptic environment.

2.3 Preparation of Media: Murashige and Skoog (MS) medium was used as the base, supplemented with 3% sucrose (sigma) and 0.8% agar (sigma). Fresh stock solutions of vitamins, amino acids, plant growth hormones, and micro and macronutrients were prepared bi-monthly and stored at 4°C. The appropriate volumes of stock solutions were added to double-distilled water to create the final medium, adjusted to a pH of 5.8 using 1N HCl and 1N NaOH. Agar (0.8% w/v) was added to solidify the medium, followed by autoclaving at 120ºC and 15 lb/in² pressure for 15 minutes to ensure sterility.

2.4 Callus Induction and Regeneration:

2.4.1 Immature Embryos and Mature Seeds: For callus initiation, both immature embryos and mature seeds were chosen as the initial explants. These represent different developmental stages, offering flexibility in selecting the starting material.

2.4.2 Culture Medium Composition:

2.4.3 Auxins and Cytokinins: The base MS medium was enriched with a variety of plant growth regulators. These included 2,4-D (2,4-Dichlorophenoxyacetic acid), IBA (Indole-3-butyric acid), IAA (Indole-3-acetic acid), NAA (1-Naphthaleneacetic acid), KN (Kinetin), and ZN (Zeatin). Each of these hormones serves a pivotal role in the initiation and enhancement of callus formation. This strategic addition of hormones to the MS medium facilitates the optimal growth and development of the callus.

2.4.4 Hormone Concentrations: The concentrations of auxins and cytokinins ranged from 1.0 mg/L to 3.0 mg/L. This variation allows for testing different hormonal levels to optimize callus induction based on the specific requirements of the plant material and experimental goals.

2.4.5 Medium Sterilization:

Supplementation and Autoclaving: After the hormones were incorporated into the medium, it was supplemented accordingly. The medium, including hormones, was then poured into culture tubes, Petri plates, and baby jars. Subsequently, autoclaving was performed to sterilize the medium and eliminate any potential contaminants.

2.4.6 Quantification and Evaluation: The cultures were evaluated over 8 weeks to monitor the progress of callus formation. Both embryogenic and non-embryogenic callus quantities were assessed, providing insights into the effectiveness of the hormonal treatments.

2.4.7 Reproducibility:

Standardization: To enhance reproducibility, it is crucial to maintain standardization in factors such as medium composition, hormone concentrations, and culture conditions. Detailed documentation of the experimental setup, including medium preparation, autoclaving parameters, and culture vessel types, contributes to reproducibility.

2.4.8 Callus Evaluation: The embryogenic and non-embryogenic callus quantities were systematically evaluated at twelve weeks, specifically at the 3^{rd}, 6^{th}, 9^{th}, and 12^{th} weeks. This evaluation aimed to assess the development and characteristics of the induced callus, providing insights into the temporal dynamics of callus formation.

2.4.9 Regeneration Process: Regeneration was initiated by transferring a three-week-old calli to a specialized regeneration medium. This medium contained specific plant growth regulators, including BAP, KN, ZN, GA3, and TDZ, at concentrations ranging from 0.5 to 3.0 mg/L. The regeneration cultures were maintained under controlled conditions, specifically at a temperature of 25°C, and exposed to continuous fluorescent light it ranges 50 to 200 µmol/m²/s for 16 hours per day.

2.4.10 Regeneration Evaluation: Similar to the callus induction phase, the regeneration process was systematically evaluated over time. This assessment included monitoring the development of shoots and their characteristics. The objective was to enhance the efficiency of shoot development for improved regeneration capabilities.

2. 5. *In vitro* Characterization and Measurement of Callus Quantity:

2.5.1 Total Callus Quantity: The combined amount of embryogenic and non-embryogenic callus was quantified as the total callus quantity. After removal from the culture medium, the total callus was carefully blotted on sterile blotting paper to remove excess moisture. Subsequently, the callus was transferred into a sterile vial with a known weight. The vial containing the callus was weighed using an electronic balance after the 3rd, 6th, 9th, and 12th week of culture.

2.5.2 Embryogenic Callus Quantity: Distinguishing between embryogenic and non-embryogenic callus types was based on their distinct morphological characteristics. Embryogenic callus displayed a compact, well-organized structure, appearing white to pale white in color. In contrast, non-embryogenic callus exhibited a soft, creamy texture and lacked organization, displaying a creamy coloration.

Quantification of embryogenic callus was performed by evaluating the proportion of embryogenic callus area within the total callus mass. This parameter was expressed as the embryogenic callus quantity. This measurement was taken at the end of the 3rd, 6th, 9th, and 12th week of culture. The *In vitro* cultivation period allowed for the monitoring of changes in callus characteristics and quantities over time, providing valuable insights into the development and differentiation of callus cultures.

2.6 Rooting and Greenhouse Establishment:

2.6.1 Rooting Induction: After successful regeneration, the subsequent phase involved rooting induction for the regenerated shoots. The process commenced with the transfer of shoots to half-strength MS media supplemented with NAA (~~Naphthalene~~ ~~acetic~~ ~~acid~~) at varying concentrations ranging from 0.5 to 2.0 mg/L. This specific formulation was designed to stimulate the development of roots in the regenerated plantlets.

2.6.2 Conditions for Rooting: The rooting induction process was meticulously conducted under controlled environmental conditions. The culture vessels containing the shoots and rooting medium were maintained in a growth chamber with a regulated temperature set at 25°C. The photoperiod was fixed at 16 hours of continuous fluorescent light per day, providing an optimal environment for the rooting initiation of the regenerated plantlets.

2.6.3 Greenhouse Establishment: After successful rooting, the plantlets were transferred to small plastic containers with sterile sand, and covered with sealed plastic bags to control humidity. They were maintained under a 16-hour photoperiod at 26°C and 50-60% relative humidity until robust establishment, preparing them for transplantation.

2.7 Conditions for Greenhouse Establishment: The plantlets, now in their initial stages of establishment, were placed in a greenhouse. The greenhouse provided a conducive environment for the transition of the plantlets to more natural growing conditions. Throughout this phase, the plantlets were carefully monitored, and environmental conditions were controlled to ensure optimal growth. After achieving sufficient establishment in the greenhouse, the plantlets were further transplanted into larger pots with a sterile mix of compost and soil (1:1 ratio) before being introduced to the field for subsequent growth and development.

2. 8 Statistical Analysis for Callus Studies:

The evaluation of callus quantity underwent a robust statistical analysis, employing a Two-Way Analysis of Variance (ANOVA) methodology. This statistical approach was chosen to thoroughly investigate the influence of two distinct categorical independent variables on a dependent variable, while also considering potential interactions between these variables. In this study, the Two-Way ANOVA served as a powerful tool for analysing the impact of different weeks of evaluation as one categorical variable and the type of callus (total or embryogenic) as another categorical variable on the observed outcomes. The primary objective was to assess variations in callus quantity over time and discern how these variables interacted to influence the overall results.

3. RESULTS

3.1 Explants and Callus Initiation: Both mature seeds and immature embryos were employed as explants in this investigation. Callus initiation commenced approximately three weeks post-inoculation, with the emergence of bulging embryogenic structures becoming evident just three days after inoculation (Picture 1 and Picture 2). However, after one week, the explants exhibited signs of pigmentation, characterized by dark brown and purple leakage into the culture media from the cut ends.

3.2 Callus Induction in Immature Embryos: The callus derived from immature embryos typically manifested as a mixture of two distinct types: embryogenic and non-embryogenic. Embryogenic callus exhibited characteristics such as opaqueness, a white or light yellow hue, compactness, a globular shape with a smooth surface, and a morphogenic nature. In contrast, non-embryogenic calli appeared unorganized, soft, loosely packed, and displayed a pale yellow or dull creamy color. After approximately 15-18 days of explant inoculation, viable embryogenic calli with a globular structure became discernible. Through successive subcultures, changes in callus morphology occurred in both embryogenic and non-embryogenic calli. Embryogenic calli tended to become opaque, white, sometimes yellowish, compact, and modulated. Even when turning yellowish, they exhibited proliferation. Over time, they transitioned into cup or dish-shaped structures. Conversely, non-embryogenic calli took on a brown coloration and became more disorganized.

3.3 Effect of PGRs Concentration on Callusing: The impact of different concentrations (1.0, 1.5, 2.0, 2.5, and 3.0 mg/L) of various plant growth regulators (PGRs) such as 2,4-D, IBA, IAA, BAP, NAA, KN, and ZN was assessed for their influence on callus formation from immature embryo explants of six Sorghum genotypes . Callus initiation occurred across all concentrations of the tested PGRs, but the frequency of callus induction varied. Notably, 2,4-D at concentrations of 2.0-2.5 mg/L yielded the highest frequency (76-88%), followed by IAA at 2.0 mg/L and IBA at 2.0 mg/L, which produced a frequency of 72%. The combination of KN at 0.5 mg/L with 2,4-D at 2.5 mg/L in the callus induction medium was particularly effective, enhancing callus formation frequency by 4-15% **(Table 1).**

3.4 Callus Induction Frequency in Immature Embryos: Callus induction frequency ranged between 40% and 88% for immature embryos across different PGR types. Specifically, 2,4-D at a concentration of 2.0-2.5 mg/L exhibited the highest frequency (76-88%). Additionally, IAA at 2.0 mg/L and IBA at 2.0 mg/L resulted in a 72% frequency. The combination of KN at 0.5 mg/L with 2,4-D at 2.5 mg/L in the callus induction medium significantly enhanced callus formation frequency, with the highest frequency observed in the variety IS 3477 (92%) (**(Table 2)**. Statistical analysis confirmed that the differences in callus induction frequencies were significant ($p < 0.05$). The highest callus induction frequencies observed with 2,4-D at 2.0-2.5 mg/L and the combination of KN with 2,4-D were significantly greater than those obtained with other PGRs and their concentrations. This indicates a clear optimal concentration range for 2,4-D and the beneficial effect of KN in combination with 2,4-D for enhancing callus formation in Sorghum genotypes .

3.5 Weights of Total and Embryogenic Callus in Immature Embryos: Weight data for total callus and embryogenic callus were collected at intervals of 3^{rd}, 6^{th}, 9^{th}, and 12^{th} week. Both total and embryogenic callus weights were highest in IS 3477, followed by IS 33095, IS 7005, IS 2898, IS 7155, and IS 1202. The weight of the callus increased with successive subcultures, with variations observed among different genotypes . Statistical analysis revealed significant differences ($p<0.05$) in total callus weight and embryogenic callus weights among the genotypes (**Table 3 & 4**).

3.6 Regeneration in Immature Embryo: At the end of the third week, both embryogenic and non-embryogenic calli underwent segmentation into small fragments, which were then transferred to a regeneration medium. Notably, the non-embryogenic callus experienced necrosis upon transfer to the regeneration medium. Importantly, embryoid formation and regeneration were exclusive to the embryogenic calli across all explants.

Upon transfer to the regeneration medium, embryogenic calli demonstrated embryoid formation within nine days of culture transfer. Over the subsequent 12-16 days, clusters of rounded or oval structures emerged, with their basal ends embedded in the callus mass. These structures underwent a transformation into green-colored shoot buds, subsequently developing into shoots in the presence of light **(refer to Plate No. 2 E).**

3.7 Effect of PGR on Regeneration: Various concentrations of cytokinins were systematically tested for their impact on regeneration. The regeneration medium supplemented with 2.0 mg/L BAP exhibited a notably high frequency of regeneration **(Table 5)**. Generally, regeneration on media with higher concentrations of hormones displayed a less distinct appearance and exhibited a tendency to turn yellow without progressing further. The inclusion of TDZ, a phenylurea compound, in the medium acted as an inhibitor of callus formation. In instances where callus formation was inhibited, germination occurred in embryos without any callus formation or alteration of the medium.

The maximum number of shoots was observed in non-pigmented genotypes: IS 3477 (20), IS 33095 (18), IS 7005 (16), and pigmented genotypes : IS 2898 (13), IS 7155 (10), IS 1202 (8) per culture. Subsequently, shoots measuring 2-4 cm in height were transplanted to a rooting medium comprising half-strength MS medium with 1 mg/L NAA and 2% sucrose. Initial responses were favourable with varying concentrations of 0.5 mg/L NAA. The number of roots displayed an approximately proportional relationship to the shoot number across all genotypes , with the maximum number of roots observed in variety IS 3477 (39), followed by IS 33095 (32), IS 7005 (31), IS 2898 (29), IS 7155 (26), and IS 1202 (22) per culture **(Figure 1)**.

3.8 Callus Induction in Mature Seeds: Callus initiation from mature seeds was observed around ten days after inoculation with scutellum. Initially, the developing callus exhibited a friable and non-embryogenic nature. Subsequent subcultures conducted at weekly intervals led to the emergence of compact nodular embryogenic callus alongside the friable non-embryogenic callus. The embryogenic callus displayed distinct characteristics, being compact, highly nodular, and yellowish-white in appearance. In contrast, the non-embryogenic callus remained friable and exhibited a brown coloration. Upon transfer to regeneration media, the embryogenic calli initiated the development of white embryoids, which eventually differentiated into plantlets on the same medium. These plantlets were then transferred to MS basal medium for further establishment. Post-acclimatization, the fully developed plants were transplanted into greenhouse pots to complete their maturation **(Picture 2).**

3.9 Callus Induction Frequency in Mature Seeds: Across the tested cultivars, callus induction frequencies ranged from 36% to 76%. Among the PGRs, 2,4-D demonstrated the most robust response at 2.5 mg/L, exhibiting the highest callus induction frequency among all auxin types. Among the cultivars, IS 3477 exhibited the highest callus induction frequency (76%) when treated with 2,4-D at 2.5 mg/L, followed by IS 33095 (68%), IS 7005 (64%), IS 2898 (52%), IS 7155 (40%), and IS 1202 (36%). The combination of 2,4-D (2.5 mg/L) and BAP (1.0 mg/L) resulted in an increase of callus induction frequency across all cultivars, ranging from 8% to 24%. Among these, IS 3477 displayed the highest response (76%) to the 2, 4-D + BAP combination, while IS 1202 exhibited the lowest response (36%) (**Table 6).**

3.10 Study of Growth Rate in Total and Embryogenic Callus: To investigate the growth rate, weights of both total and embryogenic callus were recorded at intervals of 3rd, 6th, 9th, and 12th weeks. The overall trend revealed that the maximum growth rate, encompassing both total and embryogenic callus weights, was consistently observed in variety IS 3477. This was followed by IS 33095, IS 7005, IS 2898, IS 7155, and IS 1202. Across all genotypes , the general growth rate pattern indicated an increase in callus weight with each successive subculture. However, the

magnitude of this increase varied among the different genotypes . Importantly, statistical analysis confirmed that the differences in total callus weight and embryogenic callus weights among the genotypes were statistically significant ($p<0.05$) **(Table 7 and 8)**. This underscores the distinct growth characteristics exhibited by each sorghum variety in terms of callus development.

3.11 Effect of PGRs on Regeneration: The impact of different cytokinin concentrations on regeneration was examined. The regeneration medium supplemented with 2.0 mg/L BAP yielded a high frequency of shoot regeneration, displaying optimal results. Higher hormone concentrations generally resulted in less distinct shoot regeneration, with shoots often becoming yellow and not further developing. The use of the phenyl urea compound TDZ inhibited callus formation and directly triggered germination in embryos without callus formation. Shoot numbers varied among genotypes, with the non-pigmented genotypes IS 3477 (20), IS 33095 (18), and IS 7005 (16) as well as pigmented genotypes IS 2898 (13), IS 7155 (10), and IS 1202 (8) per culture exhibiting maximum shoot formation **(Table 9).** Subsequent rooting occurred in a medium supplemented with half-strength MS medium, 1 mg/L NAA, and 2% sucrose. Various concentrations of 0.5 mg/L NAA yielded favourable results, with root numbers generally corresponding to shoot numbers across all genotypes**.**

3.12 Regeneration in Mature Seeds: The embryogenic calli derived from mature seeds exhibited a compact, highly nodular appearance with a yellowish-white coloration. Following transfer to the regeneration medium, shoot formation commenced within 14-20 days (**Plate 2).** The most favourable response was observed with ZN at a concentration of 1.5 mg/L**.** Among the genotypes, IS 3477 displayed the highest shoot formation (11), followed by IS 33095 (10), IS 7005 (8), IS 2898 (7), IS 7155 (6), and IS 1202 (4) **(Table 10)**. Elongated shoots were subsequently transferred to a rooting medium containing 1.0 mg/L NAA. Root initiation occurred within 9-13 days. The number of roots was most prominent in the non-pigmented genotypes IS 3477 (26), IS 33095 (25), and IS 7005 (23), as well as the pigmented genotypes IS 2898 (20), IS 7155 (19), and IS 1202 (16) per culture **(Figure. 2).**

3.13 Acclimatization and Transfer of Plantlets to Soil: Following successful regeneration, the robust root systems of the regenerated Sorghum plantlets from various explants were carefully extracted, ensuring thorough rinsing with distilled water to eliminate any residual culture medium. These cleansed plantlets were then gently placed into small pots or plastic containers containing sterile soil. To maintain optimal humidity levels, the pots were covered with plastic bags, creating a controlled microenvironment for acclimatization. During this period, the plantlets were watered using sterile water three times weekly to promote healthy growth.

The transition from the controlled culture room to a greenhouse was gradual. Initially, the acclimatized plantlets were introduced to the culture room for a week before being moved to the greenhouse. In the greenhouse, they were positioned under scattered sunlight for approximately ten days, and the polythene bags that previously encased them were removed after the first two weeks. As they adapted to direct sunlight, the plantlets' growth continued in a manner similar to conventional seedlings. The transition from *In vitro* to greenhouse conditions facilitated the development of sturdy, green foliage. In terms of physical appearance, there was no discernible distinction between the plantlets grown *In vitro* and those nurtured outdoors.

Upon achieving a substantial growth size within the greenhouse, the regenerated plantlets were transferred to the field for further development. This final step marked the culmination of their journey from tissue culture to full-fledged, field-grown plants, ready to thrive and contribute to the continuation of their life cycle in a natural environmen

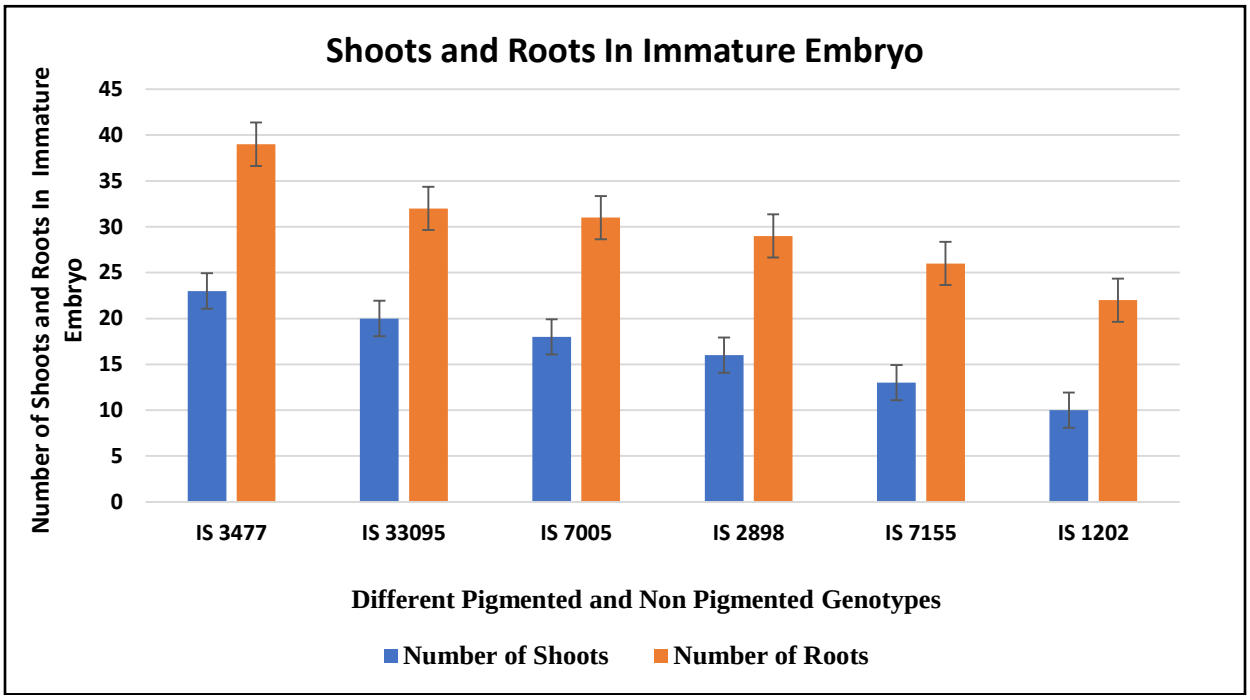


Figure 1. Number of shoots and roots in Immature embryo

Table 1. Effect of PGRs on callus induction in immature embryo culture (25 explants per treatment)

Concentrations of PGRs							No. of Explants responded	
2,4 D	IBA	IAA	NAA	BAP	KN	ZN	With E. Calli	E. Calli frequency (%)
1.0							15±1	60
1.5							16±2	64
2.0							**19**±1	**76**
2.5							**22**±1	**88**
3.0							14±2	56
	1.0						12±3	48
	1.5						10±1	40
	2.0						**18**±1	**72**
	2.5						11±1	44
	3.0						12±1	48
		1.0					13±2	52

		1.5					10±1	40
		2.0					**18**±1	**72**
		2.5					12±2	48
		3.0					14±1	56
			1.0				16±3	64
			1.5				14±1	56
			2.0				13±3	52
			2.5				12±2	48
			3.0				13±2	52
				1.0			10±2	40
				1.5			14±1	56
				2.0			13±1	52
				2.5			14±2	56
				3.0			10±3	40
					1.0		13±1	52
					1.5		15±2	60
					2.0		15±5	60
					2.5		14±1	56
					3.0		15±1	60
						1.0	12±1	48
						1.5	10±2	40
						2.0	9±1	36
						2.5	8±2	32
						3.0	7±3	28

Table 2. Comparative effect of 2,4-D, and KN and their combination in callus induction frequency from immature embryo explants

Variety	PGR concentration mg/l	Total no of explants inoculated	No. of explants with E. Calli	E. Calli frequency (%)
IS 3477	2,4-D 2.5mg/l	25	22±1	88
	KN 0.5mg/l	25	10±1	40
	2,4-D 2.5mg/l+KN 0.5mg/l	25	23±2	92
IS 33095	2,4-D 2.5mg/l	25	15±1	60
	KN 0.5mg/l	25	14±1	56
	2,4-D 2.5mg/l+KN 0.5mg/l	25	19±2	76
IS 7005	2,4-D 2.5mg/l	25	17±3	68
	KN 0.5mg/l	25	14±1	56
	2,4-D 2.5mg/l+KN 0.5mg/l	25	18±2	72
IS 2898	2,4-D 2.5mg/l	25	15±4	60
	KN 0.5mg/l	25	12±1	48
	2,4-D 2.5mg/l+KN 0.5mg/l	25	16±2	60
IS 7155	2,4-D 2.5mg/l	25	13±1	52
	KN 0.5mg/l	25	11±2	44
	2,4-D 2.5mg/l+KN 0.5mg/l	25	14±2	56
IS 1202	2,4-D 2.5mg/l	25	10±1	40
	KN 0.5mg/l	25	9±1	36
	2,4-D 2.5mg/l+KN 0.5mg/l	25	12±1	48

Table 3. Analysis of variance test for total callus weight of Immature embryo (ANOVA)

Source of variation	Sum of squares	Degrees of Freedeom	Mean sum of squares	Variance ratio
TSS	156325.1459	23		
CSS	106490.6657	3	35496.88856	36.10864602
RSS	35088.61215	5	7017.72243	7.138666665
ESS	14745.86802	15	983.0578678	

* Showing significant difference; $P_{(0.05;\ 3;\ 15)}$: 3.29; $P_{(0.05;\ 5;\ 15)}$: 2.90

Table 4. Analysis of variance test for Embryogenic callus weight of Immature embryo (ANOVA)

Source of variation	Sum of squares	Degrees of freedeom	Mean sum of squares	Variance ratio
TSS	145107.2575	23		
CSS	102491.4451	3	34163.81505	33.03323767
RSS	27102.43059	5	5420.486117	5.241106883
ESS	15513.38173	15	1034.225449	

* Showing significant difference; $P_{(0.05;\ 3;\ 15)}$: 3.29; $P_{(0.05;\ 5;\ 15)}$: 2.90

Table 5. Effect of different PGR concentrations on regeneration from Immature Embryo

BAP	KN	ZN	TAD	GA3	No. of Shoots and Roots	
					Shoots	Roots
1.0					17±1	34±2
1.5					18±2	36±2
2.0					**20**±1	**39**±1
2.5					16±1	32±5
3.0					15±1	30±5
	1.0				13±2	26±4
	1.5				14±1	29±6
	2.0				16±2	33±1
	2.5				12±3	20±2
	3.0				14±1	24±2
		1.0			12±1	20±2
		1.5			13±2	30±2
		2.0			11±2	22±5
		2.5			10±1	19±2
		3.0			11±1	20±2
			1.0		12±1	21±4
			1.5		13±2	26±4
			2.0		15±1	25±3
			2.5		10±2	20±4
			3.0		11±1	21±5
				1.0	7±2	14±2
				1.5	8±1	15±3
				2.0	6±1	13±3
				2.5	5±1	11±2

				3.0	3±0	8±3

Table 6. Effect of PGRs on callus induction in mature seed (25 explants per treatment)

Concentrations of PGRs							No. of Explants responded	
2,4 D	2,4,5-T	IBA	IAA	NAA	KN	ZN	With E. Calli	E. Calli freq. (%)
1.0							13±1	52
1.5							15±2	60
2.0							16±2	64
2.5							**18**±1	**72**
3.0							13±1	52
	1.0						14±2	56
	1.5						17±1	68
	2.0						14±1	56
	2.5						15±2	60
	3.0						13±3	52
		1.0					10±1	40
		1.5					14±1	56
		2.0					17±2	68
		2.5					16±1	64
		3.0					14±2	56
			1.0				10±1	40
			1.5				15±2	60
			2.0				18±1	72
			2.5				14±3	56
			3.0				11±1	44
				1.0			11±2	44
				1.5			13±1	52
				2.0			17±2	68
				2.5			14±1	56
				3.0			10±1	40
					0.2		5±1	20
					0.5		6±1	24
					1.0		14±1	16
					1.5		15±1	60
					2.0		11±2	44
						0.2	12±1	48
						0.5	14±2	56
						1.0	16±3	64
						1.5	14±1	56
						2.0	12±2	48

Table 7. Analysis of variance test for total callus weight of Mature seed (ANOVA)

Source of variation	Sum of squares	Degrees of freedom	Mean sum of squares	Variance ratio
TSS	136894.5203	23		23.43467443 4.148181835
CSS	90756.20418	3	30252.06806	
RSS	26774.65812	5	5354.931624	
ESS	19363.658	15	1290.910533	

*Showing significant difference; $P_{(0.05;\ 3;\ 15)}$: 3.29; $P_{(0.05;\ 5;\ 15)}$: 2.90

Table 8. Analysis of variance test for Embryogenic callus weight of Mature seed (ANOVA)

Source of variation	Sum of squares	Degrees of Freedom	Mean sum of squares	Variance ratio
TSS	73552.77733	23		13.8855666 8.462548599
CSS	30958.70683	3	10319.56894	
RSS	31446.26943	5	6289.253887	
ESS	11147.80107	15	743.1867378	

* Showing significant difference; $P_{(0.05;\ 3;\ 15)}$: 3.29; $P_{(0.05;\ 5;\ 15)}$: 2.90

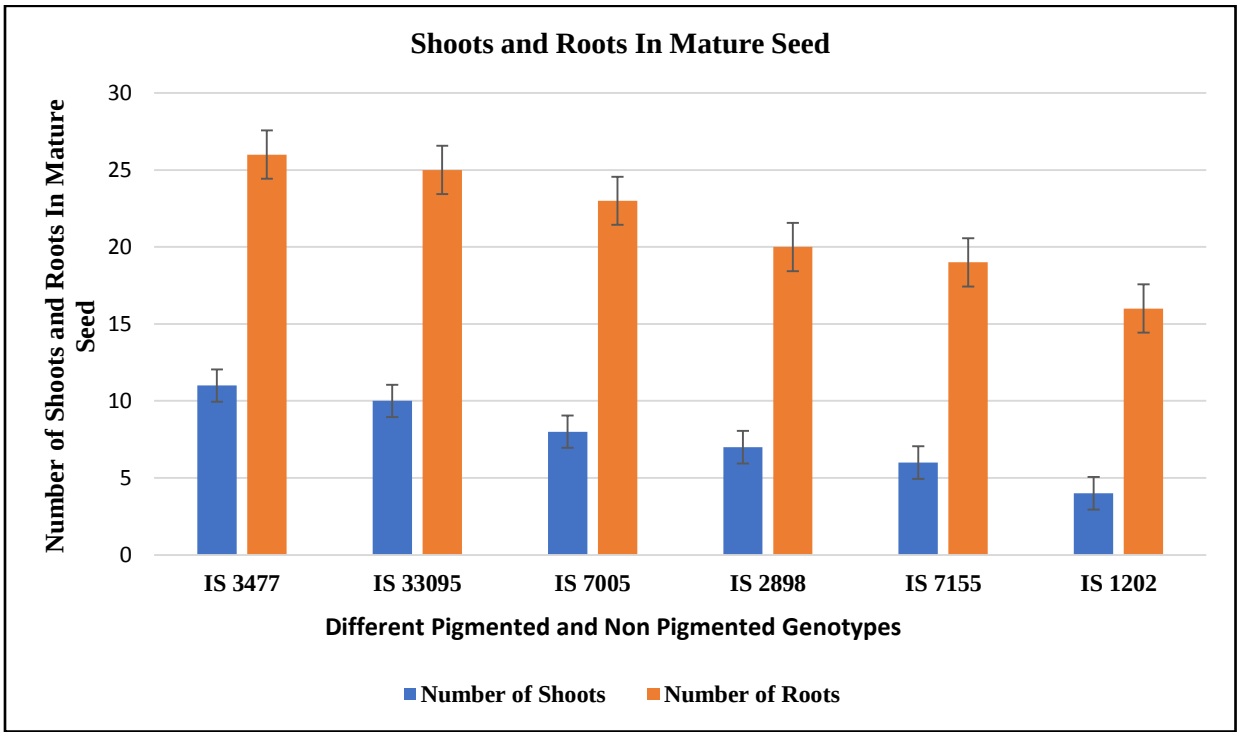


Figure 2. Number of shoots and roots in Mature seed

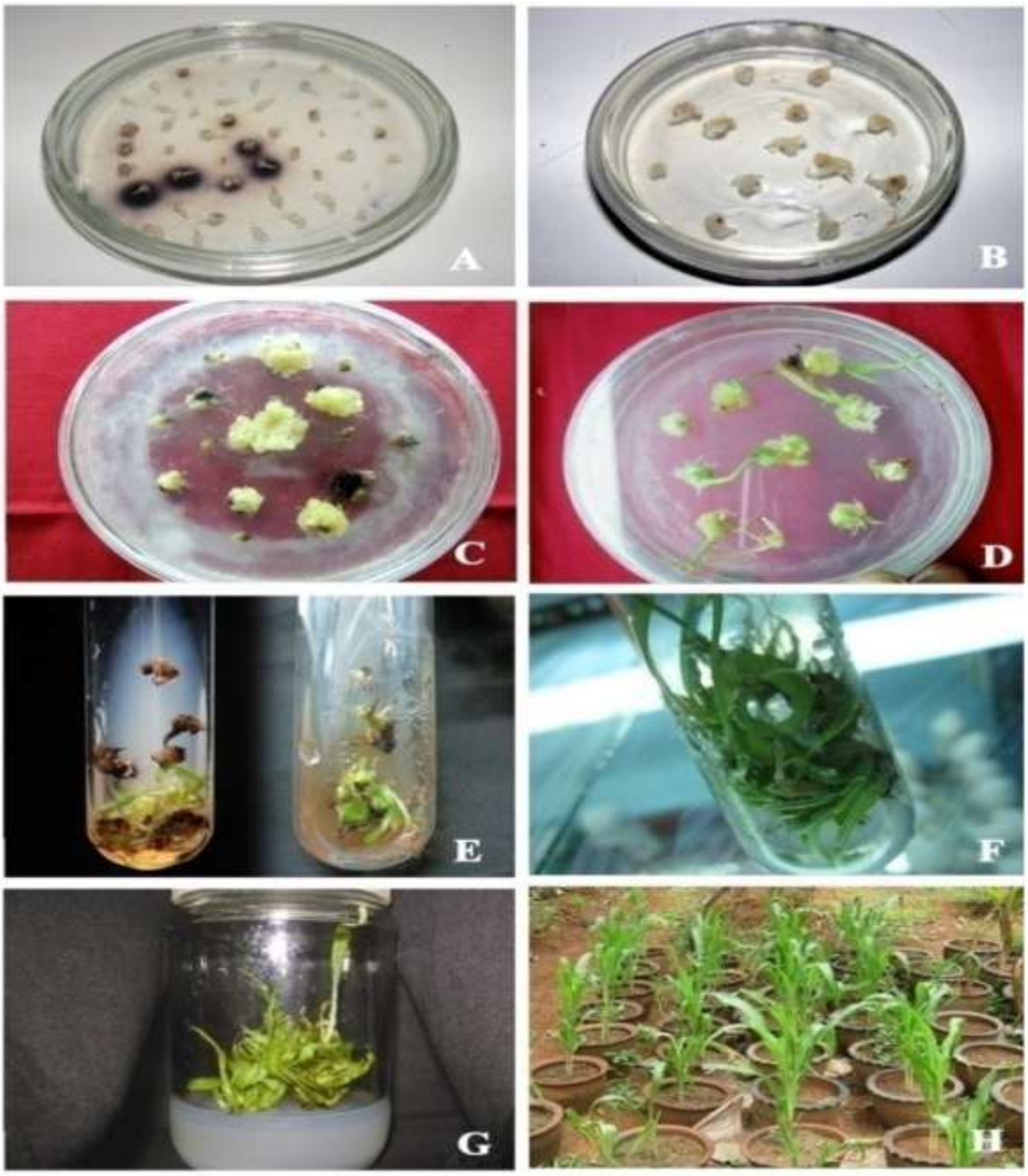
Table 9. Comparative effect of 2, 4-D, and BAP and their combination in callus induction frequency from mature seed explants

Variety	PGR concentration mg/l	Total no of explants inoculated	No. of explants responded with E. Calli	E. Calli frequency (%)
IS 3477	2,4-D 2.5 mg/l	25	18±1	72
	BAP 0.5mg/l	25	12±2	48
	2,4-D 2.5 mg/l + BAP 0.5 mg/l	**25**	**19**±1	**76**
IS 33095	2,4-D 2.5 mg/l	25	13±1	52
	BAP 0.5mg/l	25	15±1	60
	2,4-D 2.5mg/l + BAP 0.5mg/l	**25**	**17**±1	**68**
IS 7005	2,4-D 2.5 mg/l	25	14±2	56
	BAP 0.5 mg/l	25	12±1	48
	2,4-D 2.5 mg/l + BAP 0.5mg/l	25	16±2	64
IS 2898	2,4-D 2.5 mg/l	25	11±1	44
	BAP 0.5 mg/l	25	10±1	40
	2,4-D 2.5 mg/l + BAP 0.5 mg/l	25	13±1	52
IS 7155	2,4-D 2.5 mg/l	25	8±2	32
	BAP 0.5mg/l	25	5±1	20
	2,4-D 2.5 mg/l + BAP 0.5 mg/l	25	10±2	40
IS 1202	2,4-D 2.5 mg/l	25	8±2	32
	BAP 0.5 mg/l	25	6±1	24
	2,4-D 2.5 mg/l+ BAP 0.5 mg/l	25	9±1	36

Table 10. Effect of different PGR concentrations on regeneration from Mature seed

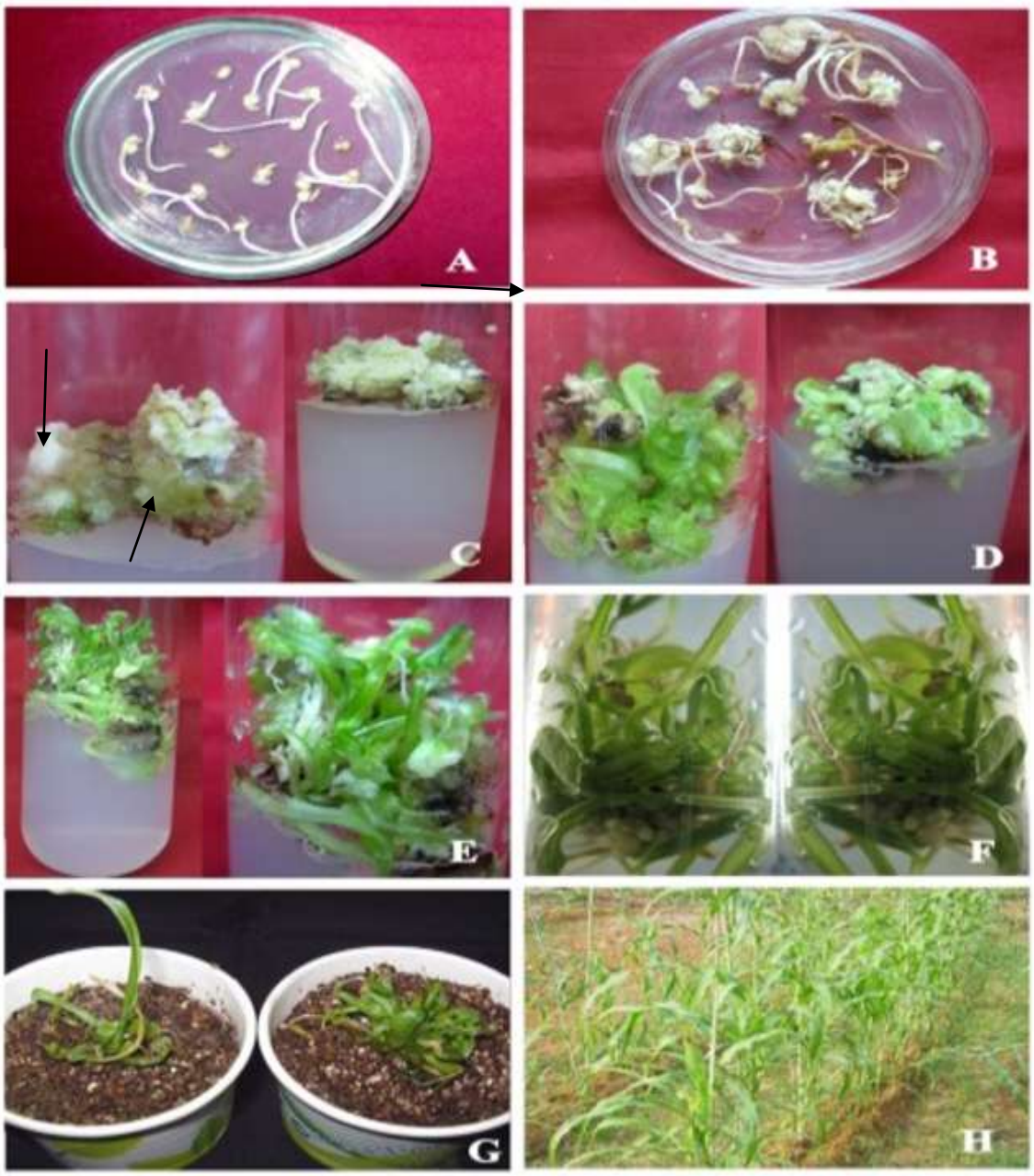
BAP	KN	**ZN**	TAD	GA3	No. of Shoots and Roots	
					Shoots	Roots
1.0					10±1	20±2
1.5					8±1	17±2
2.0					9±1	16±2
2.5					8±1	15±2
3.0					5±1	21±1
	1.0				12±1	24±3
	1.5				8±1	15±1
	2.0				6±1	12±2
	2.5				6±1	10±3
	3.0				5±1	15±2
		1.0			10±2	23±3
		1.5			**11**±1	**26**±1
		2.0			10±1	24±1
		2.5			9±2	21±2
		3.0			10±1	20±3
			1.0		8±1	16±3
			1.5		6±1	15±1
			2.0		9±2	16±2
			2.5		8±1	15±1
			3.0		8±2	15±2
				1.0	5±1	9±1
				1.5	6±1	10±2
				2.0	4±1	9±1
				2.5	3±0	6±1
				3.0	2±0	5±2

Picture 1. Stepwise depiction of callus induction and subsequent regeneration process from immature embryo explants in six distinct *Sorghum bicolor* (L.) Moench cultivars. The images illustrate the progression from initial explant inoculation to the development of embryogenic calli and their subsequent regeneration. (A) Immature embryos from different genotypes used as explants for callus initiation. (B) Explants exhibiting initial signs of callus formation after three weeks of culture. (C) Calli showing the morphological differences between embryogenic (white) and non-embryogenic (black arrow) types. (D) Calli undergoing subculture, with embryogenic calli becoming more compact and organized, while non-embryogenic calli become less coherent (black). (E) Embryogenic calli transferred to regeneration medium, showing the emergence of embryoids within nine days. (F) Embryoids progressing into well-defined shoot buds under light conditions. (G) Shoot buds growing into elongated shoots. (H) Fully developed shoots with potential for further growth and acclimatization.



Picture 2. Sequential depiction of the callus induction and subsequent regeneration process using mature seed explants from six distinct *Sorghum bicolor* (L.) Moench cultivars. The images illustrate the progression from initial explant preparation to the development of embryogenic calli and their subsequent regeneration. (A) Mature seeds from various cultivars used as explants for callus initiation. (B) Explants displaying the initiation of callus formation after ten days of culture. (C) Calli exhibiting the distinction between embryogenic (white arrow) and non-embryogenic (black arrow) types. (D) Subcultured calli, with embryogenic calli becoming more nodular and compact (white arrow), and non-embryogenic calli becoming less structured (E) Embryogenic calli transferred to regeneration medium, displaying the emergence of embryoids within a specific timeframe. (F) Embryoids maturing into green shoot buds under suitable light conditions. (G) Shoot buds progressing into well-

developed elongated shoots. (H) Fully matured shoots with potential for further cultivation and adaptation.



4. DISCUSSION

The current study represents a significant advancement in enhancing the tissue culture response of Sorghum, achieved through an extensive exploration of both pigmented and non-pigmented genotypes , as well as varied growth conditions and explant sources. Sorghum's well-known genotype-specific responses in tissue culture pose a formidable challenge, necessitating the search for consistent and robust tissue culture protocols across diverse Sorghum genotypes. Consequently, the strategy of systematically screening multiple genotypes to identify elite genotypes with the highest embryogenic callus establishment and regeneration frequencies has

been adopted, aligning with previous research [21-23]. In the realm of tissue culture media, previous studies investigating the *In vitro* characteristics of cereals identified MS (Murashige and Skoog) [24], B5 [25], LS [26], and I6 medium [27] as the most effective formulations. Notably, MS medium has been the predominant choice for Sorghum tissue culture, consistently yielding favorable results. The current study leveraged the well-established efficacy of MS medium in Sorghum tissue culture and incorporated it as the basal medium, thus ensuring a solid foundation for experimentation [28]. Among the explants evaluated across the six genotypes, the immature embryo explant emerged as the superior choice for producing embryogenic callus, surpassing the mature seed explant. The non-pigmented genotypes (IS 3477, IS 33095, and IS 7005) exhibited higher growth rates, greater regeneration frequencies, increased numbers of regenerated plantlets per explant, and a higher number of roots per culture. Conversely, the pigmented lines (IS 2898, IS 7155, and IS 1202) demonstrated comparatively lower performance across most characteristics. This variability in response based on genotype aligns with existing findings in the realm of sorghum research [29-31].

4.1 Significance of Explant Size and Developmental Stage in Tissue Culture Success: Numerous studies underscore the critical role of explant size in initiating callus formation. Research has demonstrated that embryos within the range of 0.8 to 1 mm in length exhibit optimal responsiveness, with smaller immature embryos (ranging from 0.5 mm to 1.5 mm) showing higher rates of callus development and subsequent plant regeneration compared to larger embryos [32-33]. In light of these findings, the present study emphasized the significance of selecting the appropriate size and developmental stage of explants. Specifically, immature embryos ranging from 0.5 to 2.0 mm and mature seeds harvested at the appropriate time were identified as the most conducive explant sources for generating highly embryogenic callus across all six examined genotypes. This strategic choice aligns to maximize embryogenic callus production and subsequent plant regeneration [34-36]. Within the Graminaceae family, the current investigation revealed two distinct callus types and six unique callus variations. This observation coincides with prior studies that have recognized the importance of these callus types in terms of genetic stability, plant regeneration potential, and overall callus characteristics [37-39]. Notably, the embryogenic callus identified in this study appears to align with the aforementioned observations, underscoring the consistency in these callus traits across diverse studies.

4.2 Managing Phenolic Production: During the regeneration phase, specifically around the sixth and ninth weeks, the emergence of shoots and roots prompted a notable release of phenolics in callus derived from both immature embryos and mature seeds, particularly in cultivars IS 2898, IS 7155, and IS 1202. Given that phenolics can hinder plant development, strategies to mitigate their adverse effects were explored. Various chemical agents, including PVP, ascorbic acid, and activated charcoal, were introduced into the culture media to counteract phenolic synthesis. While activated charcoal and ascorbic acid treatments successfully reduced phenolic production, they did not substantially impact necrosis frequency, the percentage of regenerating explants, or the number of regenerating plantlets. These results closely echo previous

observations in Sorghum tissue cultures, highlighting the intricate challenge of managing phenolics' influence on plant development [40].

4.3 Effect of Chemicals on Phenolic Suppression: In the context of this study, the implementation of different chemicals, such as PVP, ascorbic acid, and activated charcoal, aimed to curb the secretion of phenolic compounds in the culture media. Prior reports have illuminated the difficulty of employing chemicals to modulate phenolic production in Sorghum cultures. For instance, the use of ascorbic acid, glutathione, cysteine, activated charcoal, and Polyclar AT (insoluble polyvinylpyrrolidone) has been explored to counteract the accumulation of detrimental phenolics in Sorghum tissue cultures [41-43]. Notably, the addition of 200 mg/L Polyclar AT was observed to marginally inhibit pigments. The implementation of these approaches has underscored the multifaceted nature of phenolic suppression, with various compounds targeting factors such as basal callusing, vitrification caused by ascorbic acid, or adsorption of non-phenolic medium components via PVP and activated charcoal.

4.4 Impact of Genotype and Auxin Concentrations on Callus Development: The assessment of cumulative and embryogenic weights over 12 weeks, indicative of callus development rate, underscored significant variations attributed to both genotypes and auxin concentrations. Notably, higher auxin concentrations in the callus induction medium appeared to impede the rapid development of embryogenic callus. This observation is in line with findings that highlight the delicate balance between auxin levels and embryogenic callus formation. The influence of genotypes on culture response was consistently evident across all explant types, aligning with previous reports of genotype-specific effects on sorghum callusing ability [44]. Genetic disparities in pigment synthesis, embryogenic callus establishment, and plant regeneration were likewise noted in seed-derived sorghum explants [45].

4.5 Optimizing Regeneration Protocols: Regeneration protocols for sorghum were systematically optimized through the current study. A combination of 2.0 mg/L IAA and 0.1 mg/L BAP was identified as highly suitable for effective shoot induction, while 0.1 mg/L BAP and 1 mg/L NAA facilitated root initiation, leading to efficient regeneration. Similar optimal regeneration strategies have been documented in sweet sorghum genotypes [46]. Additionally, the potential of TDZ in enhancing regeneration was demonstrated, as evidenced by the successful application of 0.2 mg/L TDZ for promoting regeneration in barley and wheat-derived callus cultures [47]. The combination of 2 mg/L BAP and 0.5 mg/L 2, 4-D emerged as a favorable regimen for achieving maximum regeneration percentage in sorghum, while hormone-free mediums were conducive to shoot initiation [48]. These findings underscore the multifaceted nature of regeneration enhancement strategies, considering factors ranging from hormone concentrations to specific culture conditions.

4.6 Importance of Mature Seed for Callus Induction: The benefits of using mature seed encompass ease of isolation, simplified sterilization processes, heightened resilience to physical manipulation, and reduced time required for material preparation post-seed sowing. Notably, mature seed eliminates the necessity for bagging panicles to prevent cross-pollination and

provide greater flexibility in harvest timing, as mature seeds of varying sizes exhibit uniform responsiveness. This insight aligns with previous studies that have emphasized the practical advantages of mature seed utilization [49].

4.7 Enhancing Regeneration Efficiency: Furthermore, the study underscores the achievement of enhanced callus induction and regeneration efficiency in sorghum by meticulous attention to various factors, including genotype selection, explant size, culture medium composition, plant growth regulator type, and concentration, as well as environmental conditions like temperature and light intensity. These findings echo the efforts of other researchers who have optimized regeneration protocols using diverse cytokinin concentrations, such as BAP, KN, and ZN, to foster efficient regeneration [50-56].

5. In Conclusion: This study delves into Sorghum tissue culture, shedding light on key processes like explant initiation, callus induction, and regeneration. The insights gained have practical implications for agriculture, especially in tailoring protocols to specific Sorghum genotypes . The research identified optimal concentrations of plant growth regulators (PGRs), emphasizing the potential for fine-tuning conditions to boost callus formation and regeneration. Understanding the distinct characteristics of embryogenic and non-embryogenic calli is crucial, providing a foundation for efficient tissue culture strategies. The study meticulously explores the regeneration phase, detailing embryoid formation, shoot development, and rooting, offering a roadmap for protocol improvement. The successful transition of plantlets from tissue culture to natural environments showcases Sorghum's adaptability, holding promise for agricultural productivity.

In conclusion, this research significantly contributes to the understanding of Sorghum tissue culture, addressing key stages such as explant initiation, callus induction, regeneration, and successful acclimatization. The findings provide a foundation for further optimization of tissue culture protocols, considering genotype-specific responses and variations in PGR concentrations. The successful transfer of plantlets to soil underscores the potential applicability of tissue culture-derived Sorghum plants in large-scale agricultural practices, offering opportunities for crop improvement through genetic transformation and enhanced agricultural productivity.

Author Contributions: The conception of the study's topic and the initial drafting of the manuscript were carried out by **Bangaru Naidu Thaddi**. Experimental procedures were carried out by **Bangaru Naidu Thaddi** and **Nageswar Rao Seeram**. All authors participated in reviewing and approving the final version of the manuscript.

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Ethics Approval: The study is classified as "Not Applicable" or "NA" for ethics approval.

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Data Availability Statement: All data supporting the findings of this study are included in the manuscript. The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request. We adhere to the data-sharing policy of Springer Nature, and we encourage transparency and accessibility in research. The minimal dataset required to interpret, replicate, and build upon the findings reported in the article is provided. For inquiries regarding specific data or access conditions, please contact the corresponding author.

REFERENCES

1. Rao PS, Prakasham RS, Rao PP, Chopra S. Sorghum as a sustainable feedstock for biofuels. In: Jose S, Bhaskar T (eds) Biomass and biofuels. CRC Press Taylor & Francis Group, Boca Raton. 2015;2–48.
2. Naidu BT, Mani NS, Varaprasad B. Antioxidant and Free Radical Scavenging Activity of *Sorghum bicolor* (L.) Moench. Journal of Pharmacy Research. 2009;2(10):1659–1662.
3. Thaddi BN, Nallamilli MN. Estimation of Total Bioactive Compounds in Pigmented and Non-Pigmented Genotypes of Sorghum (*Sorghum bicolor* (L.) Moench). International Journal of Advanced Research in Science and Technology. 2014a;3:86–92.
4. Thaddi BN, Nallamilli MN. Assay of *In vitro* Free Radical Scavengers and Their Antioxidant Activity of Sorghum (*Sorghum bicolor* (L.) Moench). International Journal of Bioassays. 2014b;3(10):3350–3357.
5. Laopaiboon L, Thanonkeo P, Jaisil P, Laopaiboon P. Ethanol Production from Sweet Sorghum Juice in Batch and Fed-batch Fermentations by Saccharomyces cerevisiae. World Journal of Microbiology and Biotechnology. 2007;23:1497–1501.
6. Rooney WL, Blumenthal J, Bean B, Mullet JE. Designing Sorghum as a Dedicated Bioenergy Feedstock. Biofuels, Bioproducts, and Biorefining. 2007;1(2):147–157.
7. Thaddi BN, Nallamalli MN. Two-Dimensional Gel Electrophoresis Seed Proteome Map of Pigmented and Non-Pigmented Sorghum Genotypes (*Sorghum bicolor* L. Moench). European Journal of Experimental Biology. 2018;8(6):34.
8. Thaddi BN, Nallamalli MN. Seed Proteomics Approach for Identifying Sorghum Genotypes (*Sorghum bicolor* (L.) Moench) Using 2-Dimensional Gel Electrophoresis and MALDI–TOF–Mass Spectrometry. Current Proteomics Journal. 2015;12(4):264–272.
9. Elkonin LA, Lopushanskaya RF, Pakhomova NV. Initiation and maintenance of friable, embryogenic callus of sorghum (*Sorghum bicolor* (L.) Moench) by amino acids. Maydica. 1995; 40:153–157.

10. Raghavendra Rao RV, Pavan Kumar G, Subba Rao MV, Manga V. Differential in vitro response of genotypes of Sorghum. Phytomorphology. 2000; 50:253–226.
11. Hagio T. Adventitious shoot regeneration from immature embryos of sorghum. Plant Cell Tissue Organ Cult. 2002; 68:65–72.
12. Grootboom AW, Mkhonza NL, O'Kennedy MM, Chakauya E, Kunert K, Chikwamba RK. In vitro culture and plant regeneration of sorghum genotypes using immature zygotic embryos as plant source. Int J Bot. 2008; 4:450–455.
13. Seetharama N, Sairam RV, Rani TS. Regeneration of sorghum from shoot tip cultures and field performance of the progeny. Plant Cell Tissue Organ Cult. 2000;61:169–173.
14. Zhong H, Wang W, Sticklen M. In vitro regeneration of *Sorghum bicolor* (L.) Moench: efficient plant regeneration from shoot apices. J Plant Physiol. 1998; 153:719–726.
15. Maheshwari M, Lakshmi NJ, Yadav SK, Varalaxmi Y, Lakshmi AW, Vanaja M, Venkateswarlu B. Efficient plant regeneration from shoot apices of sorghum. Biol Plant. 2006; 50:741–744.
16. Raghuwanshi A, Birch RG. Genetic transformation of sweet sorghum. Plant Cell Rep. 2010; 29:997–1005.
17. Howe A, Sato S, Dweikat I, Fromm M, Clemente T. Rapid and reproducible Agrobacterium-mediated transformation of sorghum. Plant Cell Rep. 2006; 25:784–791.
18. Gurel S, Ekrem G, Rajvinder K, Joshua W, Ling M, Han Q, Peggy GL. Efficient reproducible Agrobacterium-mediated transformation of sorghum using heat treatment of immature embryos. Plant Cell Rep. 2009; 28:429–444.
19. Liu GQ, Godwin ID. Highly efficient sorghum transformation. Plant Cell Rep. 2012; 31:999–1007.
20. Liu GQ, Gilding EK, Godwin ID. Additive effects of three auxins and copper on sorghum in vitro root induction. In Vitro Cell Dev Biol Plant. 2013;49:191–197.
21. Bhaskaran S, Neumann AJ, Smith RH. Origin of somatic embryos from cultured shoot tips of *Sorghum bicolor* (L.) Moench. In Vitro Cell Dev Biol. 1988;24:947–950.
22. Rao AM, Padmasree K, Kishor PBK. Enhanced plant regeneration in grain and sweet sorghum by asparagine, proline, and cefotaxime. Plant Cell Rep. 1995;15:72–75.
23. Pola S, Mani NS, Ramana T. Plant tissue culture studies in *Sorghum bicolor*: Immature embryo explants as the source material. Int J Plant Prod. 2008;2:1–14.
24. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962;15:473–497.
25. Gamborg A, Shyluk JP, Brar DS, Constable F. Morphogenesis and plant regeneration from callus of immature embryos of sorghum. Plant Sci Lett. 1977;10:67–74.
26. Ketchum JLF, Gamborg OL, Hanning GE, Nabors MW. Millet tissue culture for crops project progress report. Department of Botany, Colorado State University, Fort Collins, Colorado. 1987;38–39.
27. Cai T, Butler L. Plant regeneration from embryogenic callus initiated from immature inflorescence of several high tannin sorghums. Plant Cell Tissue Organ Cult. 1987; 9:245–252.
28. Hagio T. Varietals difference of plant regeneration from callus of sorghum mature seed. Breed Sci. 1994; 44:121–126.
29. Rao RVR, Pavankumar G, Subba Rao MV, Manga V. Differential in vitro response of genotypes of sorghum. Phytomorphology. 2000;50:253–260.

30. Sairam RV, Seetharama N, Shymala T, Devi PS. Plant regeneration from scutella of immature embryos of diverse sorghum genotypes. Cereal Res Commun. 2000; 28:279–285.
31. Pola S, Mani NS. Somatic embryogenesis and plantlet regeneration in *Sorghum bicolor* (L.) Moench from leaf segments. J Cell Mol Biol. 2006; 5:99–107.
32. Rathus C, Nguyen T, Able JA, Gray SJ, Godwin ID. Optimizing sorghum transformation technology via somatic embryogenesis. In Sorghum T.C and Transformation, edited by N. Seetharama and I. Godwin. 2004;25–34.
33. Serhantova V, Ehrenbergerova J, Ohnoutkova L. Callus induction and regeneration efficiency of spring barley cultivars registered in the Czech Republic. Plant Soil Environ. 2004; 50:456–462.
34. Morrish F, Vasil V, Vasil IK. Development morphogenesis and genetic manipulation in tissue and cell cultures of the gramineae. Adv Genet. 1987; 24:431–482.
35. Pola S, Mani NS, Ramana T. Mature embryo as a source material for efficient regeneration response in sorghum (*Sorghum bicolor* L. Moench). Seed Sci. 2009; 26:93–104.
36. Lusardi MC, Lupotto E. Somatic embryogenesis and plant regeneration in sorghum species. Maydica. 1990; 35:59–66.
37. Sai Kishore N, Visarada KBRS, Lakshmi YA, Pashupatinath E, Rao SV, Seetharama N. In vitro culture methods in sorghum with immature embryo as an explant material. Cell Biol Morphogenesis. Plant Cell Rep. 2006; 25:174–182.
38. Kaeppler HF, Pedersen JF. Media effects on phenotype of callus cultures initiated from photoperiod-insensitive, elite inbred sorghum lines. Maydica. 1996; 41:83–89.
39. Carvalho CHS, Zehr UB, Gunaratna N, Anderson J, Kononowicz HH, Hodges TKD, Axtell JD. Agrobacterium-mediated transformation of sorghum: Factors that affect transformation efficiency. Genet Mol Biol. 2004; 27:259–269.
40. Wernicke W, Brettell R. Morphogenesis from cultured leaf tissue of *Sorghum bicolor* (L.) Culture initiation. Protoplasma. 1982; 111:19–27.
41. Walter WM, Purcell AE. Evaluation of several methods for analysis of sweet potato phenolics. J Agric Food Chem. 1979; 27:942–946.
42. Sharma N, Chandal KPS. Effects of ascorbic acid on axillary shoot induction in Tylophora Indica (Burm. f) Merrill. Plant Cell Tissue Organ Cult. 1992; 29:109–113.
43. Durt P, Wulf O. Activated charcoal catalyses sucrose hydrolysis during autoclaving. Plant Cell Tissue Organ Cult. 1993;32:97–99.
44. Cai T, Daly B, Butler L. Callus induction and plant regeneration shoot positions of mature embryos of high-tannin sorghum. Plant Cell Tissue Organ Cult. 1987; 9:245–252.
45. Bhaskaran P, Jayabalan N. In vitro plant regeneration and mass propagation system for *Sorghum bicolor* – A valuable major cereal crop. J Agric Technol. 2005; 1:345–363.
46. Murthy UR, Visarada AA, Bharathi M. Developing tissue culture system for sorghum. Cereal Res Commun. 1990; 18:355–358.
47. Shan XD, Li RQU. Thidiazuron promotes in vitro regeneration of wheat and barley. In Vitro Cell Dev Biol Plant. 2000; 36:207–210.
48. Syamala D, Devi P. Efficient regeneration of sorghum (*Sorghum bicolor* (L.) Moench) from shoot-tip explant. Indian J Exp Biol. 2003; 41:1482–1486.
49. Manjula SM, Kuruvinashetti MS, Harti CC. Regeneration, establishment and evaluation of somaclonal in *Sorghum bicolor* (L.) Moench. Euphytica. 2000; 115:173–180.

50. Eudes F, Acharya S, Selinger LB, Cheng KJ. A novel method to induce direct somatic embryogenesis, secondary embryogenesis and regeneration of fertile green cereal plants. Plant Cell Tissue Organ Cult. 2003;73:147–157.
51. Gao ZJ, Jayaraj S, Muthukrishnan, Larry Claflin, Liang GH. Efficient genetic transformation of sorghum using a visual screening marker. Genome. 2005;48:321–333.
52. Visarada KBRS, Sai Kishore N, Balakrishna D, Rao SV. Transient gus expression studies in sorghum to develop a simple protocol for Agrobacterium mediated genetic transformation. J Genet Breed. 2003;57:147–154.
53. Prathibha B, Devi, Sticklen MB. Culturing shoot tip clumps of *Sorghum bicolor* and optimal microprojectile bombardment parameters for transient expression. J Cytol Genet. 2001;2:89–96.
54. Maheswari M, Jyothi Lakshmi N, Yadav SK, Varalaxmi Y, Vijayalakshmi A, Vanaja M, Venkatachalam B. Efficient plant regeneration from shoot apices of sorghum. Biol Plant. 2006;50:741–744.
55. Jogeswar G, Ranadheer D, Anjaiah V, Kavi Kishor PB. High-frequency somatic embryogenesis and regeneration in different genotypes of *Sorghum bicolor* (L.) Moench from immature inflorescence explants. In Vitro Cell Dev Biol Plant. 2007;43:159–166.
56. Gupta S, Khanna VK, Rameshwar S, Garg GK. Strategies for overcoming genotypic limitations of in vitro regeneration and determination of genetic components of the variability of plant regeneration traits in sorghum. Plant Cell Tissue Organ Cult. 2006;86:379–388.