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Research Article
Tissue Culture of Sweet Sorghum via Mature Embryos

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Abstract

Background and Objective: Several factors affect the optimization of tissue culture protocols of cereals including hormones and media composition. This the first study in East and Central Africa to assess the effect of the hormone 2,4-D on callus initiation and regeneration of sweet sorghum via mature embryos. **Materials and Methods:** Callus induction and regeneration of 5 sweet sorghum varieties IESV92008DL, IESV92001DL, IESV92021DL, ICSV700 and ICSV93048 were evaluated using mature embryos as a source of explants and MS medium supplemented with five levels of the hormone 2,4-D (0, 1, 2, 4 and 6 mg L^{-1}). **Results:** The highest callus induction frequency was observed in 6 mg L^{-1} of 2,4-D level for all the genotypes while the lowest callus induction frequency was observed in 0 and 1mg L^{-1} of 2,4-D level. The highest embryogenic callus induction frequency was observed in 4 and 6 mg L^{-1} of 2,4-D for all the genotypes while the lowest embryogenic callus induction frequency was observed in 0 and 1 mg L^{-1} of 2,4-D. Regeneration efficiency was observed higher for the 2 genotypes ICSV93046 and ICSV700. **Conclusion:** This study revealed that the auxin 2,4-D level 4 and 4 mg L^{-1} are very important for callus initiation and regeneration of sweet sorghum and helpful for researcher to set up other protocols for improvement of sweet sorghum crop through tissue culture and transformation techniques.

Key words: Kinetin, 2, 4, D, sweet sorghum, mature embryos, callus, tissue culture, transformation techniques

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sweet sorghum *(Sorghum bicolour* (L.) Moench) is the type of sorghum that has high sugar in the stalk just like sugarcane, Sweet sorghum used as the staple food for over 500 M people in the world, especially in African and Asian countries. Sorghum is rich in antioxidants and it is grain-free of gluten and for this reason, provides an attractive grain replacement for people suffering from celiac disease. Sorghum is a drought-resistant crop addresses global climate change and limited water resources issues, sweet sorghum used as the source of food, beer, biofuel, feed and fiber[1-4]. Several reports are indicating the tolerance of sorghum to drought and high temperature than corn, wheat and other cereal crops, sorghum has the unique ability to grow under a wide range of harsh environmental conditions[5].

New cultivars of sweet sorghum have been developed with high sugar and the development of a new fermentation process has been important for the production of ethanol from sweet sorghum juice. Research on ethanol production from sweet sorghum juice has been carried out using yeast in batch processes[6,7].

Sorghum is a recalcitrant genus for tissue culture and genetic transformation, There are several reports of transformation of sorghum using various explants types including immature embryo or inflorescence and shoot apical meristem, using particle bombardment, *Agrobacterium* and free selectable marker methods[8-11]. Low embryogenic callus frequency, production phenolic and poor plant regenerability are major constrain in grain sorghum tissue culture[12,13] and the same applies to sweet sorghum[14-16].

Transformation of sorghum via *Agrobacterium*-mediated method was reported unsuccessful due to it is recalcitrant for tissue culture and genetic engineering[17]. Regeneration of transgenic sorghum was successfully reported using *Agrobacterium*-mediated transformation[18]. This was followed by reports from other researchers using the *Agrobacterium*-mediated transformation method[19]. Several factors affect transformation efficiency, including the sensitivity of sorghum explants to *Agrobacterium* infection, type of explants and composition of the culture media. Addition of coconut water to the co-cultivation medium together with the use of vigorous and actively growing immature embryos as explants for infection and the removal of excess *Agrobacterium* significantly improved the survival rate of explants which are critical for the success of transformation[20]. The purpose of this study was to provide optimized protocols for the transformation of sorghum by determining the best level of the hormone 2,4-D for callus formation and regeneration for each genotype, success in plant transformation is dependent on the ability to regenerate a whole plant from transformed tissues or cells.

MATERIALS AND METHODS

Study area: This study was carried out at Bioscience for eastern and central Africa (BecA-ILRI Hub) in Kenya from August-December, 2014.

Plant materials: Seeds from 5 sweet sorghum varieties obtained from ICRISAT (IESV92008DL, IESV92001DL, IESV92021DL, ICSV700 and ICSV93048) were sterilized in a conical flask containing 70% ethanol for 30 sec then the seeds were soaked in 2.5% sodium hypochlorite for 1 hr before washing with sterile distilled water three times. The sterile seeds were used as sources of mature embryos as explants for callus induction and regeneration of sweet sorghum.

Callus induction and culture conditions: All callus induction experiments were performed on callus induction medium contained MS salts and vitamins supplemented with 0.6 g L^{-1} L-Proline, 0.5 g L^{-1} casein hydrolysate, 0.2 mg L^{-1} Kinetin and 45 g L^{-1} sucrose. The pH of the medium was adjusted to 5.8 with 1 M NaOH or 0.1M HCl 2.5 g L^{-1}, gelrite was added to solidify the medium. Callus induction medium was sterilized by autoclaving. Five levels of 2, 4-D (0, 1, 2, 4 and 6 mg L^{-1}) were used to establish their efficacy in establishing callus and regeneration from five different varieties using mature embryos.

The Mature embryo explants were obtained from sterile seeds washed in distilled and autoclaved water. Mature embryos were cultured using sterile forceps on a callus induction medium containing different 2,4-D levels in doubled filter clean bench. The embryos were orientated with the embryo axis in contact with the medium. Twenty embryos were cultured in a 90×15 mm Petri dish for each 2,4-D level. The cultures were all covered with aluminium foil and incubated in the dark at 25±1°C for 2 weeks. Callus induction frequency was recorded after 4 weeks of culture on callus induction medium and embryogenic callus induction frequency also calculated after transfer to maturation medium while regeneration efficiency was calculated after 4-week incubation on regeneration medium.

Regeneration of plants: After 6 weeks of callus induction all embryogenic callus were transferred to baby jars containing shoot induction medium comprising MS basal salts and supplemented with 30 g L^{-1} sucrose, 500 mg L^{-1} casein

hydrolysate, 600 mg L^{-1} proline, 2 mg L^{-1} 6-BA and 2.5 g L^{-1} activated charcoal for 14 days to initiate shoots. The number of regenerated plantlets per calli was evaluated.

Acclimatization of regenerated plants was accomplished in the soil in the glasshouse. The number of regenerated plants was counted to compute the regeneration efficiency, it was calculated as the percentage of the number of regenerated shoots compared to the number of calli regenerating at least one shoot. Plantlets were maintained in the glasshouse till they matured.

Statistical analysis: The experiments were designed in RCBD four replication per treatment. Callus induction frequency was calculated on the number callus induced from the total number of explants were cultured, these data were used to compute callus induction frequency. Analyses of variance (ANOVA) were done by using stat view statistical software to test the statistical significance of differences among explants source and 2,4-D levels. Mean separation was done using the least significance difference (LSD) test at a 5% probability level.

RESULTS

The hormone 2,4-D at 6 mg L^{-1} gave the highest callus induction frequencies CIF among all 2,4-D level used in this study. While 0 and 1 mg L^{-1} of 2,4-D gave the lowest CIF among all the levels. The Callus Induction Frequencies (CIF) gave clear differences among the five sweet sorghum genotypes used in this study, the genotype IESV92001DL gave the highest callus induction frequency among all genotypes and it was observed to be 32.50 at 6 mg L^{-1}, however, 4 mg L^{-1} of 2,4-D gave an average of CIF at 27.50 expressed in Fig. 1. The genotype IESV92008DL was observed to give the lowest CIF among all genotypes in this study, the level of 2,4-D 4 and 6 mg L^{-1} was observed to give the average of 3.75 and 13.75 at respectively (Fig. 1). The genotype IESV92021 gave callus which was producing a phenolic compound and it was affecting callus induction frequency, it gave the highest CIF at 6 mg L^{-1} with an average of 20.00 while the lowest CIF for this genotype was observed to be 1.25 at 1 mg L^{-1} (Fig. 1), while 2 and 4 mg L^{-1} levels of 2,4-D gave averages of 8.75 and 15.00, respectively.

The genotype ICSV700 gave the highest callus induction frequencies at 4 and 6 mg L^{-1} of 2,4-D with averages of 17.50 and 20.00, respectively, while the lowest CIF was observed at 1 and 2 mg L^{-1} and it was observed to be 2.50 and 8.75, respectively (Fig. 1). The genotype ICSV93046 gave the highest CIF at 6 mg L^{-1} (31.25), the levels of 2,4-D 1, 2 and 4 mg L^{-1} produced CIF with averages of 3.75, 12.50 and 23.75 respectively (Fig. 1).

Mature embryos from the genotype IESV92008 gave the lowest callus induction frequencies among all the varieties while the highest callus induction was observed from the mature embryos of the varieties (ICSV93046 and IESV92001) (Fig. 1).

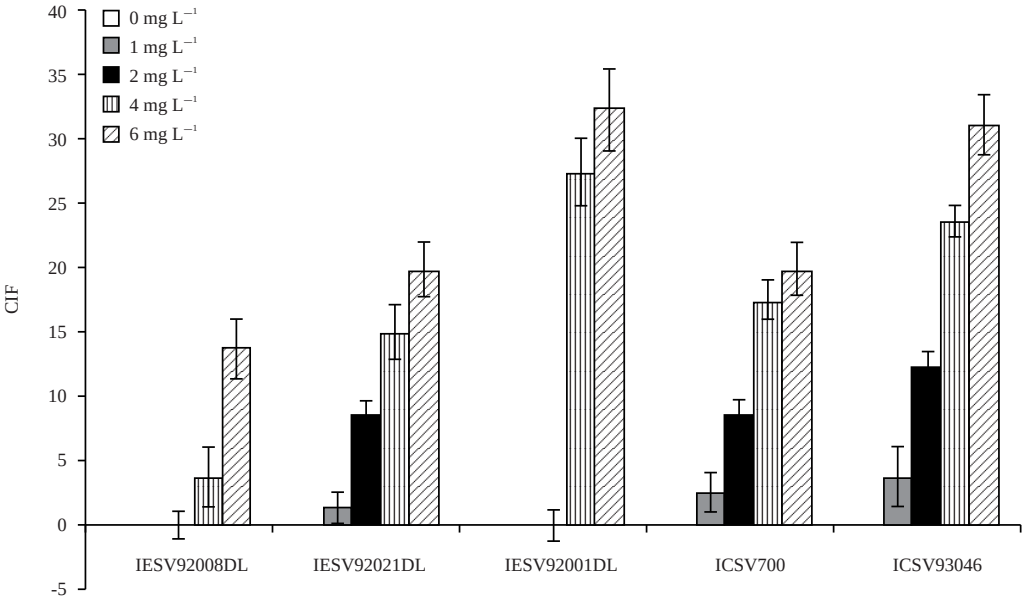

Fig. 1: Callus induction frequency for mature embryos of sweet sorghum using different levels of 2,4, D (0, 1, 2, 4 and 6 mg L^{-1})
Values are means of four replications and vertical bars are standard errors

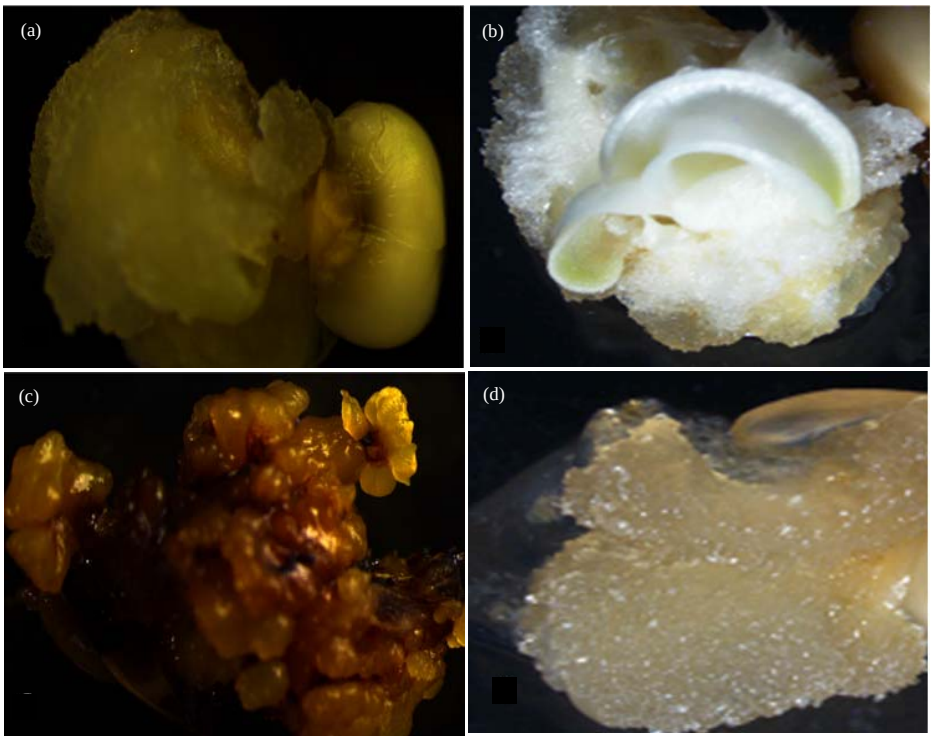

Fig. 2(a-d): Types of callus induced from sweet sorghum via mature embryos using different levels of 2,4, D. (a) Embryogenic callus from ICSV700, (b) White friable embryogenic callus of IESV93046, (c) Brown callus from IESV92021 produced phenolic compound and (d) Non-embryogenic watery callus from IESV92008

Table 1: Embryogenic callus induction frequency from mature embryos

2,4-D levels (mg L^{-1})	IESV92001	Genotype IESV92021	ICSV700	ICSV93046
0	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
1	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
2	0.00±0.00^a	6.25±2.39^b	6.25±1.25^b	8.75±1.25^b
4	18.75±1.25^b	10.00±4.08^b	10.00±2.04^b	11.25±1.25^b
6	15.00±2.04^c	11.25±1.25^b	10.00±2.04^b	16.25±2.39^c
LSD	3.30	6.17	4.77	4.28
p-value	<0.0001	0.0035	0.0007	<0.0001

Values are Mean±SE of four replicates. Mean followed by the same letter in the same column are not significantly different at $p \leq 0.05$. SE: Standard errors

The genotype ICSV700 observed to produce embryogenic and regenerable callus from mature embryos in Fig. 2a while the genotype ICSV93046 induced friable and white regenerable embryogenic callus in Fig. 2b, also the genotype IESV92021 observed to initiate brown callus which produced phenolic compound on callus induction medium and it has affected tissue culture response in Fig. 2c and the genotype IESV92008 produced non-embryogenic watery callus Fig. 2d.

Differences in Embryogenic Callus Induction Frequencies (ECIF) were observed among all the 5 genotypes used in this research, the genotype IESV92001 gave the highest embryogenic callus induction frequency among all the varieties at 4 mg L^{-1} of 2,4-D and it was observed to be 18.75±1.25 it was significantly higher than that of all other levels of 2,4-D used, while 6 mg L^{-1} gave ECIF of 15.00±2.0 and it was observed to be significantly higher than that of all other levels of 2,4-D in Table 1. While the genotype ICSV93046 gave the highest callus induction frequency among all the genotypes at 6 mg L^{-1} of 2,4-D (16.25±2.39) and it was significantly higher than that of ECIF of all other genotypes but there were no significant differences between the levels 2 and 4 mg L^{-1} of 2,4-D and were observed to be 8.75±1.2 and 11.25±1.2 ,respectively (Table 1). The genotype ICSV700 gave the lowest embryogenic callus induction frequency at 4 and 6 mg L^{-1} of 2,4-D concentrations with ECIF of 10.00±2.04, while 2 mg L^{-1} of 2,4-D level produced ECIF of 6.25±1.2, there were no significant differences observed between the three levels of 2,4-D for this genotype (Table 1).

Fig 3(a-d): Regeneration of plants from sweet sorghum via immature embryos. (a) The multiple shoots induced on regeneration medium for the genotype ICSV93046, (b) Developments of roots on the plantlets, (c) Hardening process of the plantlets at the screen house and (d) Regeneration of mature plants at the screen house

The genotype IESV92021 produced the highest ECIF at 6 mg L^{-1} of 2,4-D and it was observed to be 11.25±1.2 but it was not significantly higher than that of 2 and 4 mg L^{-1} of 2,4-D, the two-level of 2,4-D 2 and 4 mg L^{-1} gave ECIF at 6.25±2.39 and 10.00±4.08 and there were no significant differences observed between the two levels (Table 1).

The highest regeneration efficiency was observed from callus induced from mature embryos of the genotypes ICSV93046 and IESV92021 at a concentration of 2,4-D at 6 mg L^{-1} in Table 2 and it was observed to be 46 and 27, respectively, it gave shoots on shoot induction medium in Fig. 3a. The genotypes ICSV93046 and ICSV700 are the most regenerable genotypes among all the varieties and they are the most promising genotypes for tissue culture among all the genotypes used in this study, they were able to produce shoots on 2,4-D and 6 mg L^{-1} of 2,4-D in Fig. 3a, the shoots were able to produce very strong roots and there was no need for transferring the plantlets to root induction medium in Fig. 3b. The plantlets from most of the genotypes were able to survive during the hardening stage can be seen in Fig. 3c and they observed to produced mature plants in the screen house in Fig. 3d.

This project focuses on developing protocols for callus induction from mature embryos of sweet sorghum few reports

Table 2: Regeneration efficiency from mature embryos

2,4-D levels (mg L^{-1})	IESV92001	Genotype IESV92021	ICSV700	ICSV93046
2	0	13	25	13
4	0	0	25	21
6	27	46	8	29

SE: Standard errors

is available for callus regeneration of sorghum from mature embryos and this study indicates that it is possible to regenerate sweet sorghum via immature embryos.

DISCUSSION

This research focuses on developing protocols for callus induction and regeneration of sorghum through tissue culture techniques. There are many factors that affect the response of crops to tissue culture, including the source of explants, the composition of the culture media and the size of explants were critical for the success of tissue culture techniques. Optimized protocols for tissue culture of temperate sweet sorghum were developed, many studies have emerged reporting different responses of maize to tissue culture depending on the concentrations of auxin used. In the other study, the different concentrations of 2,4-D used in the culture medium caused variation on callus induction responses by the explants used.

Several reports are indicating that the responses of sweet sorghum to callus induction depending on the concentrations of Auxin used in the callus induction medium[21]. These experiments indicated that callus formation was highly stimulated by the addition of 4 or 6 mg L^{-1} of 2,4-D for all four verities. The auxin 2,4-D is important for callus induction and embryogenic callus induction from mature and immature embryos in monocotyledon plants[22,23]. The output of this study has indicated that the presence of 2,4-D could induce callus formation from seeds. However, high concentrations of 2,4-D made callus subculture easy and reduce regeneration frequency for the other three varieties[24].

Mature embryos are ready and available all the season and for these reasons can be used as an effective and alternative source of explants for tissue culture of crops[25]. The variety IESV92021 produced phenolic compound on callus induction medium which affects it is the response to tissue culture. The genotype ICSV700 induced callus which becomes watery and non-embryogenic it is reported that callus induction and regeneration of cereal crops is genotypic dependant. The addition of proline promoted the embryogenic callus production and enhancement of plant regeneration during the culture of maize and sorghum and for this reason, used proline in tissue culture medium. Induction of callus from mature embryos increased with increasing the concentrations of 2,4-D in the range of 4 and 6 mg L^{-1} with the addition of 0.2 mg L^{-1} Kinetin, the induction percentage of embryogenic callus has the negative effect of Kinetin on callus culture was noted in sorghum and wheat.

It can be reported that callus induction from mature embryos of sorghum is dependent on the concentration of the 2,4-D used. It can be recommended from this study the use of the reported CIM supplemented with 4 and 6 mg L^{-1} 2,4-D for efficient induction of callus and regeneration.

Finally, in this study, a protocol for tissue culture and regeneration from mature embryos of sweet sorghum was observed. This report showed that it is possible to improve tissue culture conditions by optimizing the compositions of callus induction and plant regeneration media for specific varieties, because of reproducibility and the easy accessibility of mature seeds, the optimization of tissue culture protocols of sorghum provides the foundation for genetic transformation of for improving important traits.

CONCLUSION

The finding of this study revealed that the level of Auxin 2,4-D is very important for callus induction and regeneration of sweet sorghum, also it was found that response of tissue culture dependant on the genotype, regeneration efficiency of sweet sorghum is also genotypic dependant.

SIGNIFICANCE STATEMENT

This study discovers the importance of the hormones on callus induction and regeneration of sweet sorghum. This study will help researchers to uncover the critical areas of transformation and in vitro mutation for sweet sorghum for enhancements of this important crop.

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