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Original Article

Optimization of *in vitro* responses of various explants sources in sorghum (*Sorghum bicolor*)

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Abstract

Sorghum usually showed obstinate behavior toward induced genetic modulation. Recently, success has been achieved in media optimization for callus induction and regeneration. A complex *in-vitro* tissue culture and regeneration system comprises of many interlinked factors such as different explant sources, optimized growth media containing effective anti-phenolic compounds, and explant response to callus induction media. In the present study we have optimized the regeneration media by utilizing the immature inflorescence, immature embryos and mature embryos. We have tested the five local genotypes i.e. JR-105, Ji-2731, Keller, Mn-3025 and Juti'an from field as well as green house sources and compares the callus induction and regeneration frequency. Our results depicted that immature inflorescence produced more callus induction in contrast with immature embryos and mature embryos. Whereas, the production of the phenolic compounds are a serious concern throughout regeneration phase. The above cited factors actually prevails and hinders the regeneration process in real sense. Furthermore, our results lead us to speculate that sorghum is highly reliant on genotype for *in-vitro* tissue culture.

Keywords: Callus induction, Immature inflorescence, Immature embryos, Phenolic compounds, Sorghum

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Introduction

Sorghum (*Sorghum bicolor* L.) is a well-adopted drought tolerant cereal crop. Owing to its diverse efficacy sorghum placed at 5th position among cereal crops (Belton and Taylor, 2004). It provide grain food for humans and biomass for the production of biofuels as it is deliberately known as the first C4 high cellulosic monocot biofuel feedstock crop (Chibani et al., 2009; Raghuwanshi and Birch, 2010). While, biofuels originated from biomass have been designated as a possible itinerary to avoid the geo political concerns, energy requirements and environmental problems (Chum and Overend, 2003;

Ragauskas et al., 2006). In this current scenario, the second-generation biofuel originated from sorghum industry gain the interest and has been developed as the leading value-added market. Because, second-generation biofuels which can produce from different feedstock crops i.e. sorghum, can grow on peripheral land such as marginal and low-cost land (Ruth, 2008; Sticklen, 2008; Lee and Lavoie, 2013). Moreover, drastic energy consumption by relentless global population pressure has been evolved. Therefore, exploring of renewable alternative energy resources are the dire need of time. Additionally, the high antioxidant content and low cholesterol level of sorghum grains can improve human health (Taylor et al., 2006; Raghuwanshi and Birch, 2010). Regardless of its distinctive features, still there is a huge vacuum exist to modify the substantial traits in an improved manner for successive exploitation (Paterson et al., 2009; Dahlberg et al., 2011). Recently, the sorghum genome has been sequenced successfully and most of the genes have been identified in their chromosomal context which provide great genetic potential to improve the sorghum (Paterson et al., 2009). Therefore, efforts have been made to enhance the performance of sorghum varieties and cut short the impacts of limiting factors through traditional breeding. Nevertheless, conventional breeding for plant development has confined to several restrictions such as inability to sustainable production (Vasil, 1994). In recent years, plant transformation comprising gene induction, modulation and expression has provided a powerful tool for varietal improvement and thus, support traditional breeding in a considerable way to achieve genetic modulation for several traits (Ahmed et al., 2018; Gurel et al., 2009). These modern approaches will subsidize to biofuel and food industries. Interestingly, sorghum has been categorized as one of the toughest plant species to perform tissue culture and genetic modulation (Zhu et al., 1998). Therefore, more efforts have been made primarily on callus induction and regeneration processes (Flinn et al., 2020).

Based on several studies it is shown that an ideal and standardize *in-vitro* tissue culture system comprises on sophisticated growth media and explant sources (Abel et al., 1986; Carvalho et al., 2004; Elkonin and Pakhomova, 2000; Jeoung et al., 2002; Hill-Ambroz and Weeks, 2001). Additionally, sorghum is comprised of phenolic compounds and its derivatives along with tannins of varying levels (Dykes and Rooney, 2006; Pola et al., 2007). Phenolic metabolites are produced during *in-vitro* culturing that considerably lower down the regeneration process. Different antioxidants and absorbing agents are utilized in the culture medium to lessen the phenolic effect (Silva et al., 2020). Earlier studies confirmed that immature explant embryos were the most capable for sorghum tissue culture. It is being suggested to estimate the essential elements and conditions prior to start the *in-vitro* tissue trials which include selective genotypes with suitable types of explant and optimized media culture. Among monocotyledonous species, sorghum is strongly genotype dependent to *in-vitro* tissue culture and regeneration process. This genotypic dependency deviates from cultivar to cultivar. Genotypes which produces less tannin during *in-vitro* tissue culture are deliberately considered the most appropriate. Freshly immature embryos collected from the field source after two weeks of post-pollination have a consequential effect to callus induction (Jeoung et al., 2002; Gao et al., 2005; Howe et al., 2006; Do et al., 2016). Along with immature embryos, immature inflorescence, shoot apices and mature embryos for *in-vitro* culture had been exploited as an alternative sources of explant (Pandey et al., 2010; Pola et al., 2009). It is evident from the published reports that immature inflorescence and embryos were considered as the best available explant sources for induction of tissue callus.

Genetic transformation followed by tissue culture in sorghum is considered one of the toughest task to do. In the current study, we optimized the *in-vitro* tissue culture medium with several modifications in growth media and subjected them to various explant sources of sorghum available for tissue culturing. Whereas, explant sources included immature embryos, immature inflorescence and mature embryos in this experiment.

Material and Methods

Plant material

The sorghum genotypes i.e. Keller, Mn-3025, JR105, Ji-2731 and Juti'an were utilized in the experiment were Chinese local and collected from Tobacco Germplasm Research Institute, Chinese Academy of Agricultural Sciences, China. Five different sorghum genotypes Keller, Mn-3025, JR105, Ji-2731 and Juti'an were utilized for the regeneration tests. Sorghum plants were raised in glasshouse at Tobacco Research Institute, Chinese Academy of Agriculture Sciences with day/night temperatures of 28/21^{o}C, with

16 h light and 8 h dark photoperiod, in plastic pots supplemented with Promix soil and liquid MS media. Three different sets of sorghum with different time of sowing were also grown in the field of Jim'mo, Qingdao, China. Required dose of water and fertilizer were subjected to sorghum plants. We covered the sorghum head with a tassel bag before pollination. After 11-14 days of pollination, immature embryos were collected from sorghum panicles for further *in-vitro* tissue culture experiments.

Table-1: Media composition for *in-vitro* culture for sorghum

Medium components	Unit per liter	Callus Induction Medium (CIM)	Shooting Medium (SM)	Rooting Medium (RM)
MS salts	g	4.3	4.2	4.2
MES	g	0.5	0.5	0.5
Proline	g	1.0	–	–
2,4-D	mg	1.5	–	–
Sucrose	g	30	30	30
BAP	mg	–	1	–
IAA	mg	–	1	–
IBA	mg	–	–	1
Agar	g	7-8	7-8	7-8
PVPP	g	10	10	10
$CuSO_4$	mg	0.16	0.16	0.16
pH		5.8	5.8	5.8
Temperature	ºC	26ºC	26ºC	26ºC
Timeframe		10–20 days	6–9 weeks	2–4 weeks

MS; Murashige and Skoog (1962), **ABA;** Abscisic acid, **MES;** 2-(*N*-morpholino) ethane sulfonic acid, **2, 4-D;** 2, 4 Dichlorophenoxyacetic acid, **BAP;** 6-Benzylaminopurine, **IBA;** Indole-3-butyric acid, **IAA;** Indole-3-acetic acid, **PVPP;** Polyvinylpolypyrrolidone, **$CuSO_4$;** Copper sulfate

Collection of explant and optimization of callus induction and regeneration

We have utilized the immature embryos, mature embryos and immature inflorescence and tested the regeneration of shoot induction and root induction. About 50- 60 immature and mature embryos from each five sorghum genotypes were disinfected with 50:50 (*v: v*) sodium hypochlorite and sterilized water for 10 min with gentle agitation on a shaker. Rinsed with sterile water for 3-4 times. Immature embryos of sorghum with 1.0-1.5 mm in length were taken out by pinching and squeezing the seeds with the help of tweezer. Then placed them on growth medium of callus induction with scutellum face up. The data of callus induction frequency and morphology were recorded after every three weeks. While immature inflorescence were taken out from the main shoot and cut out into small pieces aseptically as shown in Figure 3.3. A modified growth medium compositions were followed from Liu and Godwin, 2012 (Table. 1). The callus induction media and regeneration media are subsequently changed after every seven days to avoid the production of the phenolic compounds. For callus induction, explant plant tissues were subjected on Callus Induction Medium (CIM) for 10-20 days. While, for shoot induction, embryogenic calli were subjected on Shooting Medium (SM) m for 6-9 weeks by exposing to light of 150 mol $m^{-2}s^{-1}$ and 18:6 h photoperiod at 26ºC. Regenerated shoots were shifted to rooting medium (RM) for 2-4 weeks. Then plants with elongated shoots having 2-3 leaves with healthy roots were moved to Promix soil trays before transferring to greenhouse.

Results

***In-vitro* callus induction through immature embryos**

Freshly immature embryos of five varieties e.i. JR-105, Ji-2731, Keller, Mn-3025 and Juti'an were collected after 11-14 days of pollination from the field source as well as from greenhouse source as shown in (Figure. 1). Total 393 immature embryos of JR-105 were taken and subjected to callus induction. Out of 393 only 9.9% callus were formed. The embryogenic calli were incubated onto SM for shoot induction at 25°C under 16 h light /8 h dark photoperiod, while 28% plants were regenerated. While Ji-273, Keller and Mn-3027 gave 6.9%, 9.1% and 8.3% callus induction respectively. After 6-9 weeks of incubation, multiple shoot points were observed. The shoots with 2-3 cm were transferred onto rooting media for 2-4 weeks with same controlled conditions. However, the regenertion percentage of Ji-273, Keller and Mn-3027 remained 36%, 64% and 11% respectively (Table. 2). While, Juti'an did not produce any callus. Our results showed that callus induction from the immature embryos is quite difficult aand tidious, as a lot of labour is required. We need to change the callus media after every five to seven days, as it produce deadly phenolic compounds during the callus induction.

Table-2: *In-vitro* callus induction through immature embryo

Cultivars	No. of Embryos Inoculated	No. of Callus induced	Callus Induction%	No. of Regenerates	Regeneration %
JR-105	393	39	9.92	11	28
Ji-2731	273	19	6.96	7	36
Keller	370	14	9.19	9	64
Mn-3027	325	27	8.38	3	11
Jutian	65	0	0	0	0

Table shows the number of regenerated plants by utilizing immature embryo as an explant.

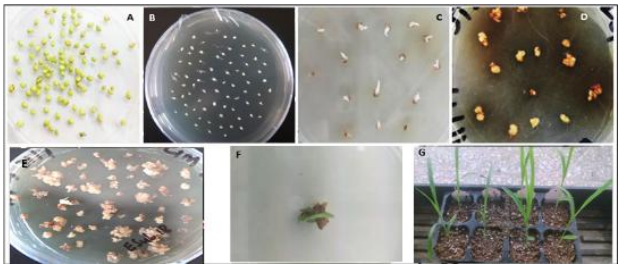

Figure-1: Different stages of sorghum *in-vitro* tissue culture by using immature embryos:
(A) 11-14 days old immature embryos after pollination; (B) immature explant embryos; (C,D,E) different stages of callus induction; (F) shoot initiation; (G) Shifting of plants in soil.

***In-vitro* callus induction through mature embryos**

Almost 50 seeds of each five genotypes were grown for 2 weeks on the callus-induction medium. The mesocotyl and radicle of sorghum seedlings were eventually developed into primary callus as shown in (Figure. 2A, 2B and 2C). In the meanwhile, a few non-embryogenic calli (rhizogenic) were also observed in our experiments. After 6 weeks of incubation under controlled conditions, poor quality of calli were detected. Further 4 weeks of subculture in callus induction media, the embryogenic and non-embryogenic calli were identified. Embryogenic calli were further subjected to plant regeneration medium. While, non-embryogenic calli were died later on. Interestingly, none of the five genotypes gave regenerates.

Figure-2: (A,B and C) Different stages of callus initiation from Juti'an mature seeds

***In-vitro* callus induction and regeneration from immature inflorescence**

The immature inflorescence of the five understudy sorghum varities i.e. JR-105, Ji-2731, Keller, Mn-3025 and Jutian were taken from greenhouse and field source. After cutting the shoot comprising of immature inflorescene were subjected to sterilization as shown in (Figure.3). The average length of the immature inflorescence was taken in cm which was in the range of 1.1cm to 1.7cm. The immature inflorescene was subjected to callus induction media for two weeks at control temperature of 24 °C by cutting them into the small pieces. Out of 85 calli in JR-105 only 8.2% were regenerated into the plants as demonstrated in (Table.3), while the ratio of the regeneration percentage of the Ji-2731, Keller, Mn-3025 and Jutian remained 7.4%, 5.2%, 12.6% and 0%, respectively.

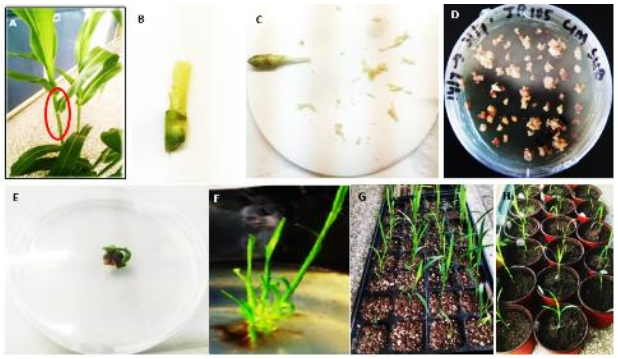

Figure-3: Different stages of sorghum *in-vitro* tissue culture by using immature inflorescence:
(A) part of the plant containing immature inflorescence; (B) expose the immature inflorescence before pollination; (C) cutting the immature inflorescence and placed on callus induction media; (D) callus induction from immature inflorescence; (E) regeneration of shoot; (F) regeneration of root; (G,H) transfer of plantlets in soil.

Table-3: *In-vitro* tissue culture through immature inflorescence

Cultivars	Inflorescence Length (cm) Ave.	No. of Callus induced	No.of Regenerates	Regeneration %
JR-105	1.5	85	7	8.2
Ji-2731	1.7	27	2	7.4
Keller	1.4	95	5	5.2
Mn-3027	1.7	71	9	12.6
Jutian	1.1	5	0	0

Table shows the number of regenerated plants using immature inflorescence.

Production of phenolic compounds

Most common issue during *in-vitro* culturing of sorghum is the production of substential quantity of phenolic compounds which are secreated from the immature embryo and immature inflorescence during callus induction. That showed a negative effect on the tissue growth and differentiation process (Dreger et al., 2019). To reduce this impact we have to change the subculture media repeatedily over course of time as it covers the whole petri dish within 7-10 days as shown in (Figure. 4). Several reports have also confirmed this drastic issue, by lowering down the frequency of tissue growth and differentiation (Gao et al., 2005; Zhao et al., 2000). While shortening the subculturing result in high labor cost and time. We utilized the PVPP in the experiment to lower down the effect but did not observe positive impact in this situation. Each five genotypes produces phenolic compounds during callus induction.

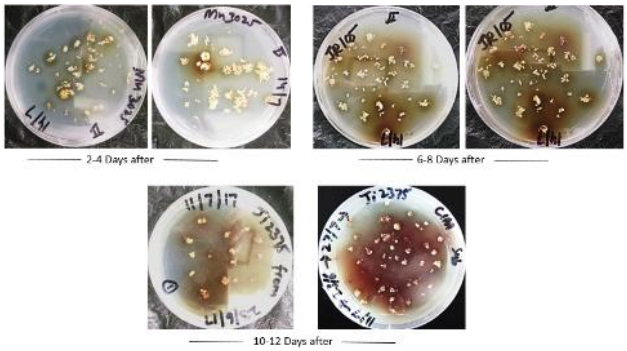

Figure-4: The production of the phenolic compounds, with different time frames in Mn-3025 (2-4 days after), JR-105 (6-8 days after) and Ji-2731 (10-12 days after) during callus formation in immature embryos and immature inflorescence which reduced the callus growth successively.

Discussion

Sorghum has tremendous potential for the production of food and biofuel. Which can fulfil the food scarcity and energy crises issues for the industries, especially under developed countries. For this instance, modern biotechnology is capable of improving sorghum along with traditional breeding approaches. Genetic modulation is a powerful tool for broaden the improved genetic sorghum germplasm (Mookkan et al., 2017). It is observed as the toughest crop for genetic modulation and several key factors are being involved during *in-vitro* culture (Emani et al., 2002). Since the initial breakthrough in *in-vitro* callus induction, improvement has been observed in media optimization and *in-vitro* explant selection, but still the callus induction rate is very low. These improvements may also include reduced timeframe of *in-vitro* culture, the feasible solution to avoid phenolic compounds as well as the availability of wide range of compatible genotypes (Hahn et al., 1983). As described earlier, the utilization of immature embryo of sorghum *in-vitro* culture system is highly time dependent, which should be broaden to non-immature embryo-based system (Chateau et al., 2000; Pola and Mani, 2006). Moreover, ventilated vessels provide good culture growth by releasing the excessive ethylene (Ezeogu et al., 2005). Furthermore, only those genotypes of sorghum had been subjected to *in-vitro* tissue culture which were already considered as model genotypes (Tadesse et al., 2003). Therefore, it should be extended to other elite genotypes.

As describe earlier, sorghum is highly genotype dependent, in this study five different cultivars i.e. JR-105,Ji-2731, Keller, Mn-3025 and Juti'an were subjected to *in-vitro* tissue culture. Different sources of explant such as immature embryos, immature inflorescence, and mature seeds have been exploited (O'Kennedy et al., 2006). These explants were taken from both field as well as greenhouse source. Our results depicted that these genotypes showed intensive recalcitrancy to the *in-vitro* tissue culture (Pola and Mani, 2006). Other reports also depicted that sorghum is also obstinate to the *in-vitro* culturing. Alongwith genotype dependency, the availibility of the explant source all around the time is not possible. Explant source is only available for the short period of time.

Another phenomenon is the release of phenolic compounds when subjected to stress that are toxic for self-destruction. We examined that after the production of phenolic compounds, callus induction and plant regeneration also decreases dramatically (Pola and Mani, 2006; Lu et al., 2009). In some cases, the production of phenolic compounds could be reduced by using a low concentration of growth agents along with rapid shifting of selection media (Lu et al., 2009). In order to attain the stable *in-vitro* tissue culture system, the callus growth media may be optimized by enhancing the competency of plant target cells. A series of chemicals in a systemic manner may be utilized to achieve the stable *in-vitro* tissue culture system (Tari et al., 2013). The above cited whole process can be split into four major steps for the ease, i.e. collection of explant material, selection of genotypes, callus induction medium and regeneration process. This study provides a comparison of various explant sources for *in-vitro* culture of sorghum, but still a detail study is needed by utilizing different factors.

Conclusion

The genetic transformation of the sorghum is considered one of the toughest task to do, because of its strong defense system against the pathogen attack. Although researchers have been succeeded in achieving the optimization of callus induction and regeneration of one of the most recalcitrant sorghum crop, the callus induction and regeneration rate is still very low and needs improvement, e.g., the utilization of various explants for tissue culture, simple optimized media, short duration of *in-vitro* culture, the possible control of phenolic compounds and compatible genotypes for tissue culture. As mentioned above, the utilization of immature embryos for callus induction and regeneration is available only for short time so there is need to explore broad spectrum compatible explants. In our experiments we utilized the five local strains such as Keller, Mn-3025, JR105, Ji-2731 and Jutian for callus induction and regeneration in order to get success. We found immature inflorescence as the best explant source for *in-vitro* tissue culture, but they are available for only a short period of time just like immature embryos. Furthermore, the collection of immature inflorescence is also time consuming process. Hence, there is a dire need to explore more cultivars which are capable of smooth genetic modulation through *Agrobacterium* transformation.

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Contribution of Authors

Ahmed RI & Rehman SU: Conceptualized part of the research, performed the experiment, data collection and manuscript write up
Akhtar LH, Mahmood K & Ahmad RT: Performed data analysis and helped in writing
Khan AM & Anum W: Helped in editing of manuscript