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Identification of *in vitro* responsive immature embryo size for plant regeneration in Sudan grass (*Sorghum sudanenses* Piper)

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Immature embryos of various sizes from six genotypes of sudan grass (*Sorghum sudanenses* Piper) were cultured to identify the best *in vitro* responsive embryo size. The immature embryos size influenced callus formation (days to callus initiation, callus induction frequency, callus growth) and plant regeneration (shoot induction frequency, shoots per callus) parameters. Immature embryos (0.7-1.5 mm) were quicker to initiate callus, induced fastly growing callus in higher frequency and regenerated shoots more frequently and intensely than immature embryos (1.6-2.5 mm) because of the initiation of callus from rapidly dividing scuttelar cells. Frequent germination and initiation of non-embryogenic callus from plumule or radicle were the main reasons for poor callus induction and plant regeneration responses from 1.6-2.5 mm size immature embryos. Thus immature embryos of 0.7-1.5 mm size may be used for embryogenic culture initiation and plant regeneration.

Keywords: sorghum, *Sorghum sudanenses*, immature embryos, immature embryo size, callus induction, plant regeneration

Introduction

The gems *Sorghum* includes many species which are source of grain, fibre, fuel and secondary products[1]. *S sudanenses*, commonly known as Sudan grass, is one of the important species of sorghum, which is primarily used as feed and fodder for animals. It crosses freely with *S. bicolor* and represents as an important source of germplasm for introgression of useful genes into *S. bicolor*. Immature embryo explant, used in maize[2], has become the most widely used explant in cereal tissue culture. In *S. bicolor*, there are a number of reports describing plant regeneration from immature embryo explant, the size of which influences plant regeneration[3-8]. Plant regeneration from the tissue cultures of *S. sudanenses* has been reported[7, 9], but the influence of the size of immature embryos on *in vitro* plant regeneration in this species has not been assessed so far. Culturing the immature embryos after certain specific days after pollination[6], measuring the length of immature embryos[7] and judging the endosperm consistency by squeezing the grain[8] are the three ways which have been used by various workers to standardize the appropriate *in vitro* responsive stage of immature embryos of *S. bicolor*. In the present investigation, the immature embryos were aseptically measured prior to their inoculation on to the culture media to determine their appropriate size.

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Materials and Methods

Plant Material and Culture Conditions

Immature caryopses of six genotypes (SDSL92101, SDSL92102, SDSL92111, SDSL92112, SDSL92115, SDSL92140) from field grown plants of sudan grass were cleansed with 5% Teepol (v/v) detergent solution and surface sterilized in 0.1% solution of mercuric chloride for 2 min followed by 3-4 rinses in sterile distilled water. Immature embryos were aseptically measured, classified into 4 embryo size classes (0.7-1.0, 1.1-1.5, 1.6-2.0 and 2.1-2.5 mm) and cultured onto MS medium supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l kinetin for callus induction. The cultures were incubated in dark at 25 ± 2 °C. The first sub culturing was made after 4 weeks to the same medium on which callus was induced. The number of days from the date of inoculation of explant on the callusing medium to the date of callus visibility was recorded. The callus induction frequency was recorded after 21 days of inoculation as percentage of total number of inoculated explants producing callus. The callus growth was recorded on a visual scale from 1 (very poor callus growth) to 4 (profuse callus

growth) before transferring callus on the regeneration medium. For plant regeneration the callus was transferred to the 2 mg/l IAA and 1 mg/l kinetin supplemented MS medium. The cultures were incubated at 25 ± 2°C in light (16 hrs photoperiod, 2500-3000 lux) under fluorescent lamp. Shoot induction frequency was calculated as percentage of total number of transferred calli producing shoot. The number of shoots/callus was recorded as the ratio of total number of shoots regenerated to the total number of calli transferred to the regeneration medium.

Statistical Analyses

The experimental design (two factor completely randomized design with four replications) had six levels of the first factor (genotype) and four levels of the second factor (embryo size). Mean responses from 5-7 immature embryos constituted one replication. All the cultured immature embryos were serially numbered and the responses were recorded on individual explant. The visual scores given for callus growth were analyzed using a non-parametric statistics[10], since these data were recorded on an ordinal scale. The percent data on callus induction and shoot induction frequencies was transformed using angular transformation procedure.

Results and Discussion

Callus Induction and Growth

During initial experiments, immature embryos of too small size (less than 0.5 mm) did not respond at all and, therefore, the longer embryo sizes (0.7-1.0, 1.1-1.5, 1.6-2.0 and 2.1-2.5 mm) were prepared. Swelling in the cultured immature embryos was observed within 4-5 days of culture followed by initiation of callus. The number of days to initiate callus was influenced by genotype, embryo size class and their interaction (Table 1). The SDSL 92102 took minimum (12.93) and SDSL 92101 the maximum (14.06) number of days to initiate callus (Table 2). As the embryo size increased, the number of days required to initiate callus also increased. In different embryo sizes, the callus initiation response of different genotypes was not uniform. Immature embryos (0.7-1.0 mm) did not exhibit any sign of germination and initiated callus from scutellum. The scutellar callus was cream to yellow in colour and compact embryogenic type (Figs 1a & b). Friable embryogenic callus and non-embryogenic callus appeared very rarely. In immature embryos of (1.1-2.0 mm) plumule and/or radicle just came out but stopped growth and callus initiation started mostly from scutellum and rarely from plumule and/or radicle. The callus from scutellum was embryogenic and that from plumule and radicle was mostly non-embryogenic (Fig. 1c). Immatue embryos (2.1-2.5 mm) germinated frequently and callus was almost always induced from plumule and radicle. The callus was dull white and gray in colour and non-embryogenic type (Fig. 1d).

Callus induction frequency was influenced by only embryo size class and different genotypes and their

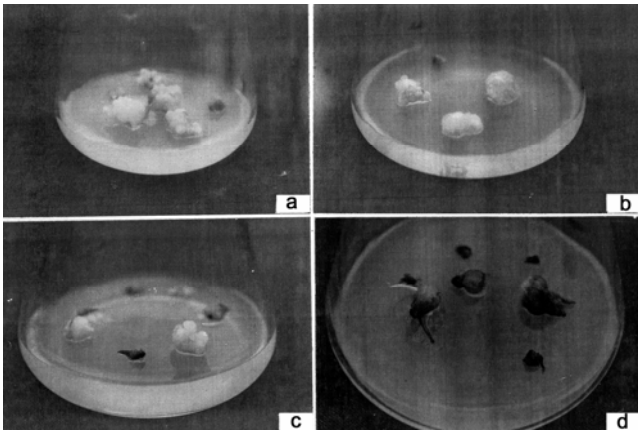

Fig. 1—Callus induction response from four embryo size classes. Highly embryogenic callus from scuttellar cells of 0.7-1.0 (a) and 1.1-1.5 mm (b) size immature embryos. Embryogenic callus from scuttellum and non-embryogenic callus from plumule and/or radicle of 1.6-2.0mm (c) and 2.1-2.5mm (d) size immature embryos.

Table 1—Analysis of variance for days to callus initiation, frequency of callus induction, frequency of shoot induction and number of shoots/callus from 4 embryo size classes of 6 genotypes

Source of variation	d.f.	MS			
		Days to callus initiation	Callus induction frequency	Shoot induction frequency	No. of shoots/ callus
Genotype	5	2.31*	330.42	49.56	1.14
Embryo size	3	151.43*	1504.03*	3330.80**	11.58**
Genotype × Embryo size	15	3.62*	137.27	62.34	0.24
Error	72	0.85	368.08	184.33	0.50

*Significant at 5% level of significance ** Significant at 1% level of significance

interactions with seed size classes did not differ significantly for this trait (Table 1). Maximum callus induction frequency was observed from 0.7-1.0 mm embryo size class followed by 1.1-1.5, 1.6-2.0 and 2.1-2.5 mm embryo size classes (Table 3). Genotypic differences with respect to callus growth were significant (Table 2). Maximum (2.72 mm) and minimum (2.03 mm) callus growths were observed in SDSL 92101 and SDSL 92140, respectively. Callus growth (2.1-2.5 mm) from immature embryo was inferior to all other embryo size classes. Embryo size classes 0.7-1.0, 1.1-1.5 and 1.6-2.0 mm did not differ from each other for callus growth. This overall trend of callus growth of embryo size classes was also exhibited by the individual genotypes.

Requirement of more number of days to callus initiation by larger embryo size classes is probably related to physiological maturity, which the embryos slowly attain during their passage through a number of developmental stages. During the attainment of maturity, such embryos are genetically programmed for germination. So larger the size of embryo, more physiologically mature and determined to germination it would be, and its initial culture response would be germination and not callus initiation. Poor callus induction response from larger embryo size class in cereal and grass tissue culture has been conclusively established that dedifferentiation of explant tissue (callus formation) can only be achieved from cells which have not passed critical stage of differentiation and are still meristematic. This is because during normal development grasses unlike dicots, do not form secondary meristems in mature cells nor do they show merestematic activity in mature cells as a wound response[11]. The plumule and radicle of germinated larger embryo size class have confined regions with meristematic activity. For proper callus induction response this region should be excised and cultured[12]. Since in the present investigation, meristematic region of plumule and radicle was not excised and cultured, the poor callus induction response was observed. Another reason for rather poor callus induction response from larger embryo

Table 2 — Effect of genotype and immature embryo size classes on days to callus initiation and callus growth

Genotype		Days to callus initiation (I) and callus growth (II) for the following embryo size classes				
		0.7-1.0	1.1-1.5	1.6-2.0	2.1-2.5	Mean
SDSL	I	9.60(g)	10.70(fg)	14.65(d)	16.80(a)	12.93{a}
92102	II	3.15(a)	2.70(ab)	2.60(ab)	1.90(b)	2.58{a}
SDSL	I	11.70(ef)	12.50(e)	14.75(cd)	17.30(a)	14.06{c}
92101	II	2.55(ab)	2.65(ab)	3.25(a)	2.45(b)	2.72{a}
SDSL	I	11.75(ef)	12.35(e)	12.70(e)	16.60(a)	13.35{bc}
92111	II	2.30(ab)	2.70(ab)	3.10(a)	2.15(b)	2.56{a}
SDSL	I	11.70(ef)	11.93(ef)	14.75(cd)	15.22(bcd)	13.40{bc}
92112	II	2.40(a)	2.20(ab)	1.95(ab)	1.75(b)	2.07{b}
SDSL	I	10.25(g)	10.70(fg)	16.0(abc)	16.90(a)	13.46{abc}
92115	II	2.50(ab)	2.70(a)	2.30(ab)	2.00(b)	2.37{ab}
SDSL	I	12.10(e)	12.05(e)	14.35(d)	16.45(ab)	13.73{ab}
92140	II	2.55(a)	1.95(ab)	1.90(ab)	1.75(b)	2.03{b}
Mean	I	11.18[c]	11.70[c]	14.53[b]	16.54[a]	13.48
	II	2.57[a]	2.48[ab]	2.51[ab]	1.99[b]	2.39

Mean days to callus initiation and callus growth values in the body of table ( ), row [ ] and column { } with different alphabets are significantly different

Table 3 — Effect of immature embryo size classes on frequency of callus & shoot induction and number of shoots/callus

Embryo size classes (mm)	Callus induction frequency (%)	Shoot induction frequency (%)	Number of shoots/callus
0.7-1.0	85.04 (a)	45.72 [a]	2.21{a}
1.1-1.5	78.45 (ab)	40.34 [ab]	1.94 {ab}
1.6-2.0	70.42 (b)	33.66 [b]	1.41{b}
2.1-2.5	67.59 (b)	14.55[c]	0.39{c}

Mean callus induction ( ), shoot induction [ ] and number of shoots/callus { } values with different alphabets are significantly different at 5% level of significance

size class might be dead or used up scutellum tissue, which initiates callus in younger embryos[2].

Shoot Induction and Shoots/Callus

In 7 to 8-week old callus, transferred to plant regeneration medium and kept in light, greening of the embryogenic calli was observed within 6-7 days of transfer and shoot regeneration could be observed in 10-15 days. Like callus induction, the shoot induction frequency and number of shoots/callus were found to be influenced by embryo size classes only. Shoot induction frequency and number of shoots/ callus from 0.7-1.0 mm (Fig 2a) and 1.1-1.5 mm (Fig. 2b) immature embryo size classes were comparable (Table 3) and maximum since most of the callus induced in these embryo size classes was embryogenic. Shoot induction and number of shoots / callus responses from 1.6-2.0 mm (Fig. 2c) embryo size class was significantly better than that of 2.1-2.5 mm (Fig. 2d) embryo size class. Shoot induction frequency and number of shoots/callus reflect the frequency and intensity of embryogenic callus in total callus mass transferred to plant regeneration media[13]. A better shoot induction frequency from the younger embryos is attributed to the source of callus (scutellum) and a poor shoot induction response from the larger embryo size[6]. But in contrast to comparable genotypic responses observed in this study, significant genotypic differences had been observed in another study[6]. The non-significant genotypic effects in present study might be the separation of variation due to the embryo size from genotypic variance. In the experiments where a study of the effect of the embryo size is not the objective, effort is made so that embryos of different sizes (0.7-2.0 mm embryos usually cultured) are represented equally in different genotypes. Since the embryo size is not measured in these experiments and randomization of embryos of different sizes in different genotypes is usually not perfect, an inevitable error due to embryo size gets included in genotypic responses. The practical implication of this observation is that while screening of genotypes conscious efforts should be made to use immature embryos of uniform size to avoid pitfalls in highlighting genotypic differences that may arise due to the size of the explant.

In conclusion, the present investigation emphasizes on the importance of immature embryo size and suggests for the use of immature embryos of 0.7-1.5 mm size for embryogenic culture initiation and plant regeneration in *S. sudanenses.*

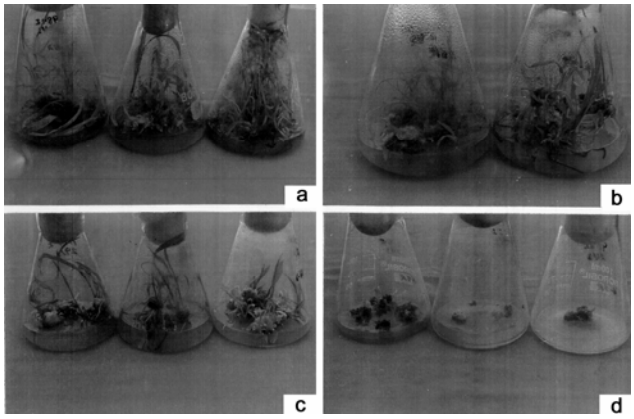

Fig. 2—Plant regeneration response from (a) 0,7-1.0 mm (b) 1.1-1.5 mm (c) 1.6-2.0 mm and (d) 2.1-2.5 mm immature embryo size classes

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