Plant Cell Tiss Organ Cult (2006) 86:379–388
DOI 10.1007/s11240-006-9140-0

ORIGINAL PAPER

Strategies for overcoming genotypic limitations of in vitro regeneration and determination of genetic components of variability of plant regeneration traits in sorghum

Sanjay Gupta · V. K. Khanna · Rameshwar Singh · G. K. Garg

Received: 28 July 2005 / Accepted: 27 June 2006 / Published online: 17 August 2006

Abstract Development of suitable strategy to overcome genotypic limitations of in vitro regeneration in sorghum would help utilize high yielding but poor tissue culture responsive genotypes in genetic manipulation programmes. A factorial experiment was conducted with two explants (immature embryos and inflorescences), eight genotypes (five *Sorghum sudanense* and three *Sorghum bicolor* genotypes), three levels of 2,4-D (1 mg l^{-1}, 3 mg l^{-1}, and 5 mg l^{-1}), and two levels of kinetin (0.0 mg l^{-1} and 0.5 mg l^{-1}). The induced callus was transferred to the regeneration media with factorial combinations of IAA (1.0 mg l^{-1} and 2.0 mg l^{-1}) and kinetin (0.5 mg l^{-1} and 1.0 mg l^{-1}). *S. sudanense* regenerated at significantly higher frequency (38.91%) and produced shoots more intensely (2.2 shoots/callus) than *S. bicolor* (26.93%, 1.26 shoots/callus). Immature inflorescences regenerated at a much higher frequency (46.48%) and produced significantly more number of shoots (2.71 shoots/callus) than immature embryos (22.35%, 0.99 shoots/callus). Moreover, differences for plant regeneration between genotypes of the same species were minimal when using immature inflorescences. Increase in the 2,4-D concentration in callus induction media exhibited inhibitory effect on callus induction, growth, shoot induction and number of shoots/callus but inclusion of kinetin in callus induction media improved these responses. Use of immature inflorescence explant and inclusion of kinetin in callus induction media could overcome genotypic limitations of plant regeneration to a large extent. The extent of variability, heritability and expected genetic advance was more in plant regeneration traits than in callus induction traits. This indicated that the variability in respect of these attributes in the genotypes may be due to the additive gene action and selection of genotypes for these characters would be rewarding.

Keywords 2,4-D · Callus · Coefficient of variation · Genetic advance · Heritability · IAA · Kinetin · Shoot induction · Shoot/callus · *Sorghum bicolor* · *Sorghum sudanense*

S. Gupta (✉)
Division of Plant Breeding, ICAR Research Complex for North East Hill Region, Umroi Road, Barapani (Umiam), Barapani, Meghalaya 793103, India
e-mail: sanitaishu@yahoo.co.in

V. K. Khanna · Rameshwar Singh
Department of Genetics and Plant Breeding, G. B. Pant University of Agriculture and Technology, Pantnagar, Udham Singh Nagar, Uttaranchal 263145, India

G. K. Garg
Molecular Biology and Genetic Engineering, G. B. Pant University of Agriculture and Technology, Pantnagar, Udham Singh Nagar, Uttaranchal 263145, India

Introduction

The genus sorghum includes many species which are source of grain, fibre, fuel, and secondary products. Sorghum [*Sorghum bicolor* L. (Moench.)] is an important grain and forage crop and is staple food for over 300 million people in semi-arid environments of Africa and Asia. *Sorghum sudanense*, 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 is an important source of germplasm for introgression of useful genes into *S. bicolor*. In both of these species, plant regenerations have been described from a range of tissues, such as immature embryos (Bhat and Kuruvinashetti 1995; Bai et al. 1995; Elkonin and Pakhomova 1996), immature inflorescences (Bai et al. 1995; Bhat et al. 1994; Kaeppler and Pedersen 1997), young leaves (Han et al. 1997), shoot tips (Nahadi and de-Wet 1995; Patil and Kuruvinashetti 1998; Shyamala and Devi 2003). In previous studies, genotypic differences for plant regeneration have been reported. However, the effective comparison of plant regeneration responses of these two species and ways to reduce genotypic limitation of plant regeneration are lacking. Plant regeneration in sorghum is achieved with a two step procedure, including induction of embryogenic calli on a 2,4-D containing medium followed by plant regeneration on a medium without growth regulator or supplemented with cytokinin and/or auxin. It is well known that the hormonal milieu of callus induction medium affects regeneration of plants in plant regeneration medium. However, this residual effect of callus induction medium has not been quantified. In order to address the above issues we conducted this large scale experiment with two species, eight genotypes, two explants, six hormonal combinations for callus induction (2,4-D and kinetin), and four hormonal combinations for plant regeneration (IAA and Kinetin) and analyzed their main and interaction effects on various plant regeneration parameters. Further, consistent genotypic differences for plant regeneration reported earlier suggest that there is a genetic basis for this and the selection of genotypes based on their in vitro performance would be rewarding if the nature of variation is heritable. In the present investigation, attempt was also made to analyze the nature of variation present for various plant regeneration parameters.

Materials and methods

Callus induction and plant regeneration

Immature embryos and immature inflorescences of five genotypes of *S. sudanense* (SDSL 98984, SDSL 98988, SDSL 981125, SDSL 981142 and SDSL 981144) and three genotypes of *S. bicolor* (2219B, GD 68727 and 981013) were used in the present study. Healthy and vigorously growing plants were bagged to prevent out crossing and caryopses of these plants were used for the isolation of immature embryos. Immature caryopses and stem portions bearing inflorescences were cleansed with 5% teepol (v/v) 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 (0.7–1.5 mm) and immature inflorescences (1–5 cm size, cut into 2–3 mm randomly shaped pieces) were aseptically dissected and placed onto six callus induction MS media (Murashige and Skoog 1962) containing factorial combinations of three levels (1 mg l^{-1}, 3 mg l^{-1}, and 5 mg l^{-1}) of 2,4-D and two levels (0 mg l^{-1} and 0.5 mg l^{-1}) of kinetin. The cultures were incubated in dark at 25 ± 2°C. 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 5 (profuse callus growth) before putting callus on the regeneration media. The first sub-culturing was made after 4 weeks to the same media on which callus was induced and the next sub-culturing was made after 3 weeks to different plant regeneration media. For regeneration of plantlets, 7–8-week-old calli, from each of the six callus induction media, were cut into small pieces of almost equal size and were transferred onto the four MS regeneration media containing factorial combinations of two levels of IAA (1.0 mg l^{-1} and 2.0 mg l^{-1}) and two levels of kinetin (0.5 mg l^{-1} and 1.0 mg l^{-1}). The cultures

were incubated at 25 ± 2°C in light (16 h 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 induced to the total number of calli transferred to the regeneration medium.

Statistical analyses

The experimental design for callus induction frequency was three factorial completely randomized design with four replications. There were eight levels of the first factor (genotype), three levels of the second factor (2,4-D), and two levels of the third factor (kinetin). Five to six explants were cultured in one flask. All the explants were serially numbered and responses were recorded on individual explant. Mean responses from 10–12 explants (two flasks) constituted one replication. Visual scores for callus growth were analyzed using a non-parametric Kruskal–Wallis statistics (Hollander and Wolfe 1973) since these data were recorded on an ordinal scale.

To test the significance of the main and the interaction effects of 2,4-D and kinetin in callusing media, on the frequency of shoot induction and number of shoots/callus, four regeneration media were considered as blocks. The design of experiment was three factors complete randomized block design with eight levels of the first factor (genotype), three levels of the second factor (2,4-D), and two levels of the third factor (kinetin).

Estimation of variances, heritability, and genetic advance

Coefficients of phenotypic and genotypic variation were computed according to the method suggested by Burton and Devane (1953), heritability and genetic advance as per Johnson et al. (1955). Correlation coefficients were calculated as according to Dewey and Lu (1959).

Result

Swelling in the scutellar tissue of immature embryos and floral primordia, rachi or rachillae of immature inflorescences was observed 7–10 days of culture and callus initiated in 13–15 days culture. Mean callus induction frequencies and callus growth of two species were comparable (Fig. 1). Both, embryogenic and non-embryogenic calli were observed in the cultures of both the explants. Embryogenic calli were either compact or friable (Fig. 2A–F). In contrast to all other genotypes which frequently produced compact callus and only rarely secreted phenols, genotype 981013 frequently produced friable callus that was full of somatic embryos (Fig. 2F) and secreted phenols. At 3 mg l^{-1} and 5 mg l^{-1} 2,4-D concentrations

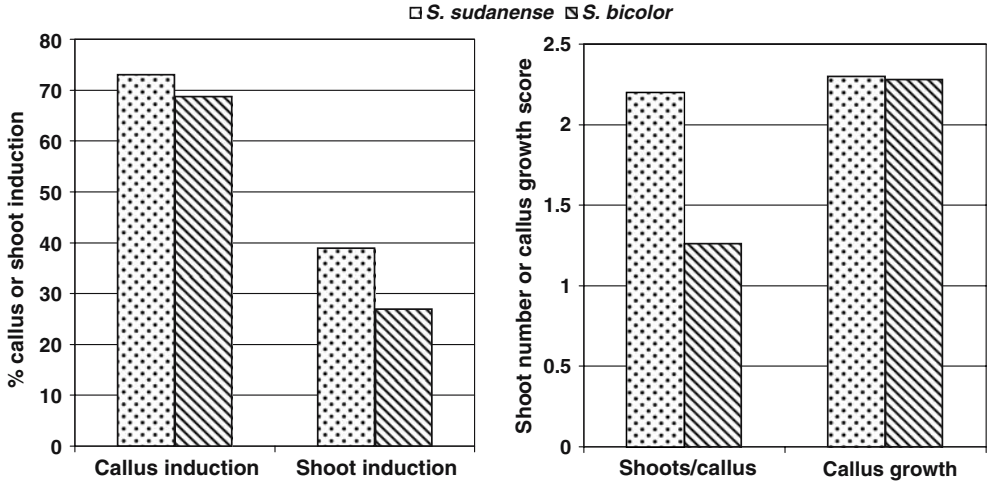

Fig. 1 Comparison of mean in vitro responses of two sorghum species

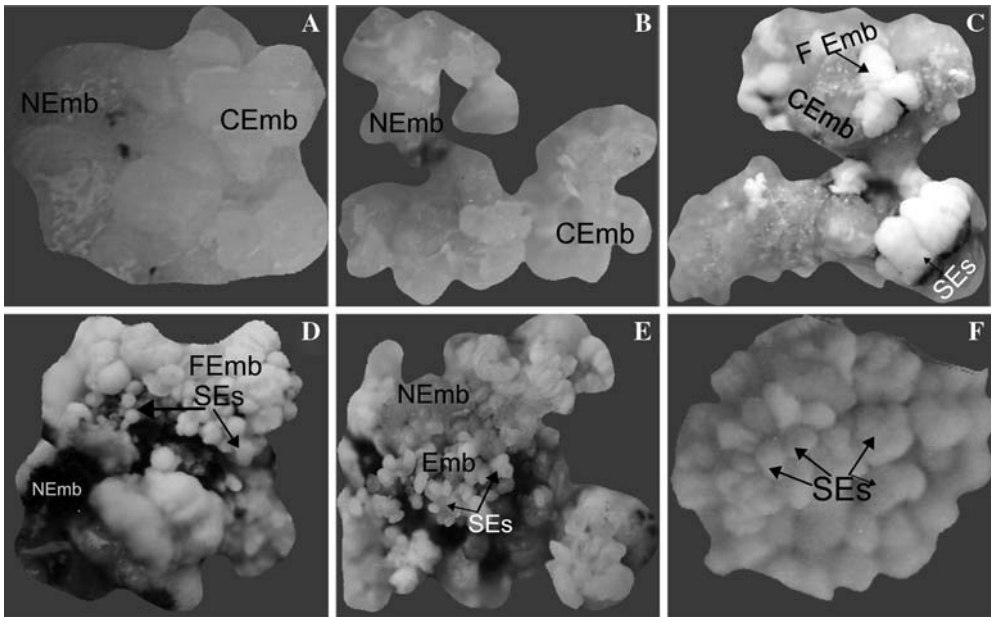

Fig. 2 (**A**) and (**B**) Compact embryogenic calli; (**C**) compact embryogenic calli with friable embryogenic calli; (**D**–**F**) white friable embryogenic calli with somatic embryos. NEmb = non-embryogenic; CEmb = compact embryogenic; FEmb = friable embryogenic; SEs = somatic embryos

many of the explants turned brown and did not produce any callus when kinetin was not present but in the presence of kinetin the callus induction response at these 2,4-D concentrations could be significantly improved (Fig. 3).

When the cultures were transferred from callusing media to different plant regeneration media and kept in light, greening of compact embryogenic calli was observed within 6–7 days of transfer. This green compact callus slowly started regenerating shoots and multiple shoots (Fig. 4A) were also frequently observed. Friable, white embryogenic callus did not become green but after 10–15 days of its transfer to plant regeneration media, it started producing shoots. Various types of organogenic responses like induction of shoot only (Fig. 4B), induction of roots only (Fig. 4C), and induction of shoot and root both (Fig. 4D) were observed.

Effect of species, genotype, and explant on plant regeneration

S. sudanense genotypes regenerated shoots more frequently and produced more shoots/callus than *S. bicolor* genotypes (Fig. 1). Genotypic differences constituted a significant source of variation for the shoot induction frequency and the shoots/callus in both of the explants (Table 1). Genotype SDSL 981142 was identified as the best genotype and the mean shoot induction response of this genotype was 10.6–25.5% higher and it produced 1.42–3.08 more shoots/callus than other genotypes. All of the genotypes exhibited significant improvement for plant regeneration responses and an overall increase of 24.13% in shoot induction frequency and 1.72 shoots/callus was recorded when immature inflorescence explant was used in place of immature embryo explant. Depending on the genotypes this improvement was 1.5–3.1 times in shoot induction frequency and 2–5.2 times in shoots/callus. Shoot induction and shoots/callus responses from immature inflorescences of even those genotypes which had performed rather poorly through their immature embryo explant, could almost equate with that of the best performing genotypes and within the species the genotypes became almost comparable for these responses.

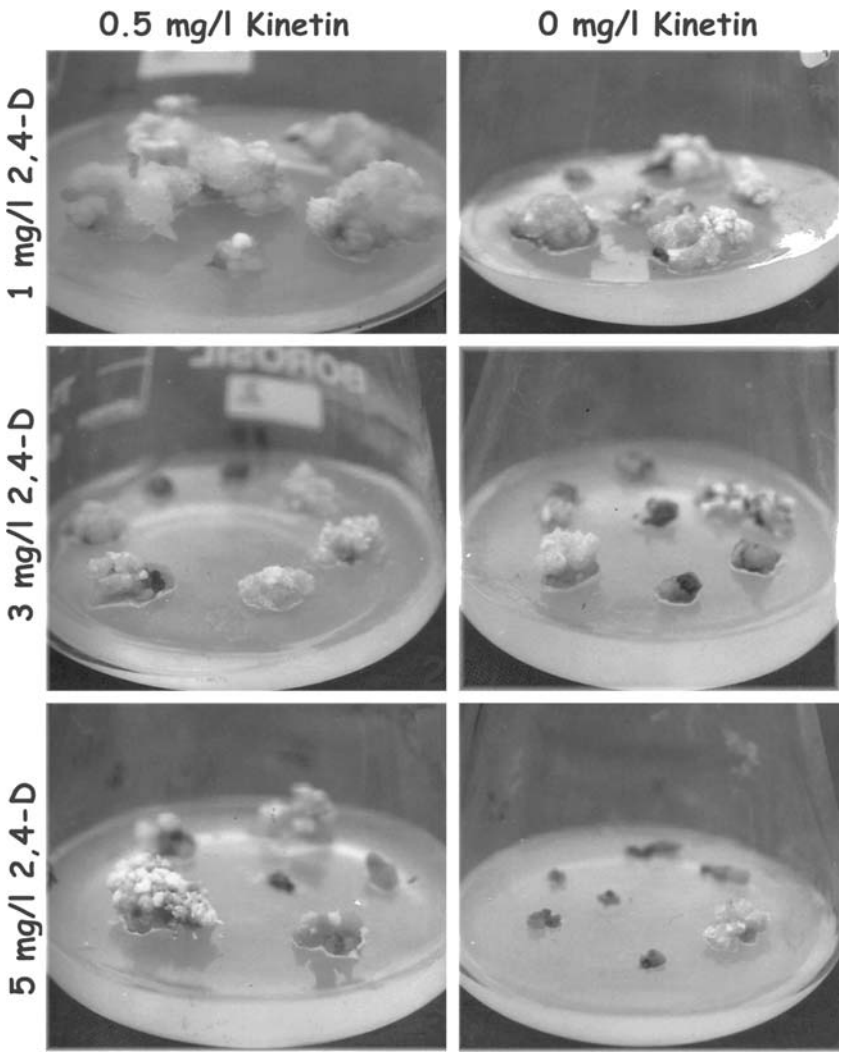

Fig. 3 Effect of 2,4-D and kinetin on callus growth

Residual effect of hormonal milieu of callus induction medium on plant regeneration

Addition of kinetin to the callus induction media resulted in the significant improvement in shoot induction frequency (19.4%) and shoot/callus (1.4). Individual genotypes registered 1.2–2.8 times increase in shoot induction frequency and 1.7–3.9 times increase in number of shoots/callus (Fig. 5). Immature inflorescences were more responsive to kinetin supplementation in callus induction medium than immature embryos. The improvement in shoot induction frequency and number of shoots/callus was more in the calli derived from *S. bicolor* genotypes than those from *S. sudanense* genotypes (Fig. 6). Significant decrease in the shoot induction and shoots/callus was observed on increasing the 2,4-D concentrations from 1 mg l^{-1} to 3 mg l^{-1} to 5 mg l^{-1}. While immature embryos were very sensitive to higher 2,4-D concentrations and exhibited significant reduction in shoot induction response at 3 mg l^{-1} and 5 mg/l 2,4-D concentrations the immature inflorescences exhibited this response only when kinetin was not present in the callusing media. On the kinetin supplemented media the immature inflorescence derived calli exhibited comparative response at all the 2,4-D levels (Fig. 7).

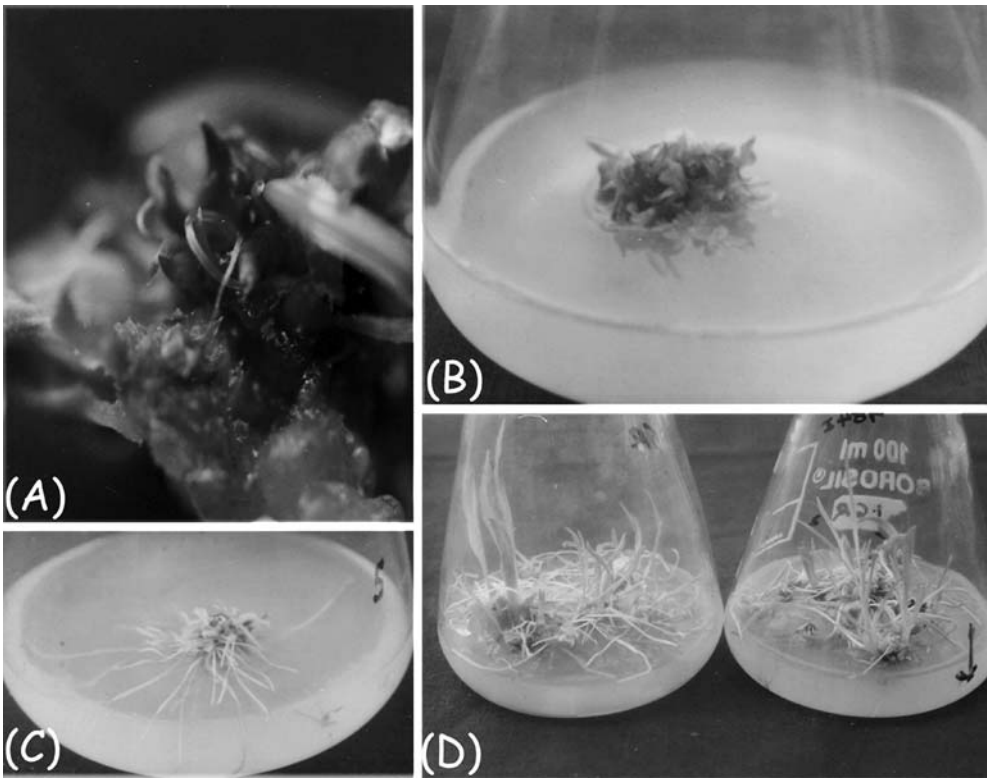

Fig. 4 Multiple shoot formation (**A**); induction of shoot only (**B**), root only (**C**) and both root and shoot (**D**)

Table 1 Effect of genotype and explant on shoot induction (%) and shoots/callus

Species/genotype	Shoot induction		Shoots/callus		Mean	
	Immature embryo	Immature inflorescence	Immature embryo	Immature inflorescence	Shoot induction	Shoots/callus
S. sudanense						
SDSL 98984	16.39 (h)	51.81 (bc)	0.64 (fg)	3.14 (bc)	34.10 {b}	1.89 {bc}
SDSL 98988	18.83 (gh)	55.53 (ab)	0.46 (g)	2.40 (bcde)	37.18 {b}	1.43 {cd}
SDSL 981125	25.40 (f)	50.03 (c)	0.99 (efg)	2.08 (bcde)	37.71 {b}	1.53 {bcd}
SDSL 981142	38.58 (d)	58.20 (a)	2.53 (bcd)	5.07 (a)	48.39 {a}	3.80 {a}
SDSL 981144	24.78 (f)	49.59 (c)	1.36 (defg)	3.39 (h)	37.18 {b}	2.38 {b}
S. bicolor						
981013	18.90 (gh)	39.03 (d)	0.73 (fg)	2.70 (bcd)	28.96 {c}	1.73 {bc}
2219B	21.80 (fg)	36.13 (de)	0.78 (fg)	1.86 (defg)	28.99 {c}	1.32 {cd}
GD 68727	14.13 (h)	31.53 (e)	0.41(g)	1.04 (efg)	22.84 {d}	0.72 {d}
Mean	22.35 [b]	46.48 [a]	0.99 [b]	2.71 [a]	34.42	1.85

Mean shoot induction and shoot /callus values in the body of table ( ), columns { } and row [ ] with different alphabets are significantly different at 5% level of significance

Genotypic basis of variation for plant regeneration traits

Phenotypic and genotypic coefficients of variations were higher for the plant regeneration traits than for the callus induction traits in both the explants (Table 2). The heritable portion of total variation could be determined with the help of heritability. Higher heritability value in both of the explants was observed for number of

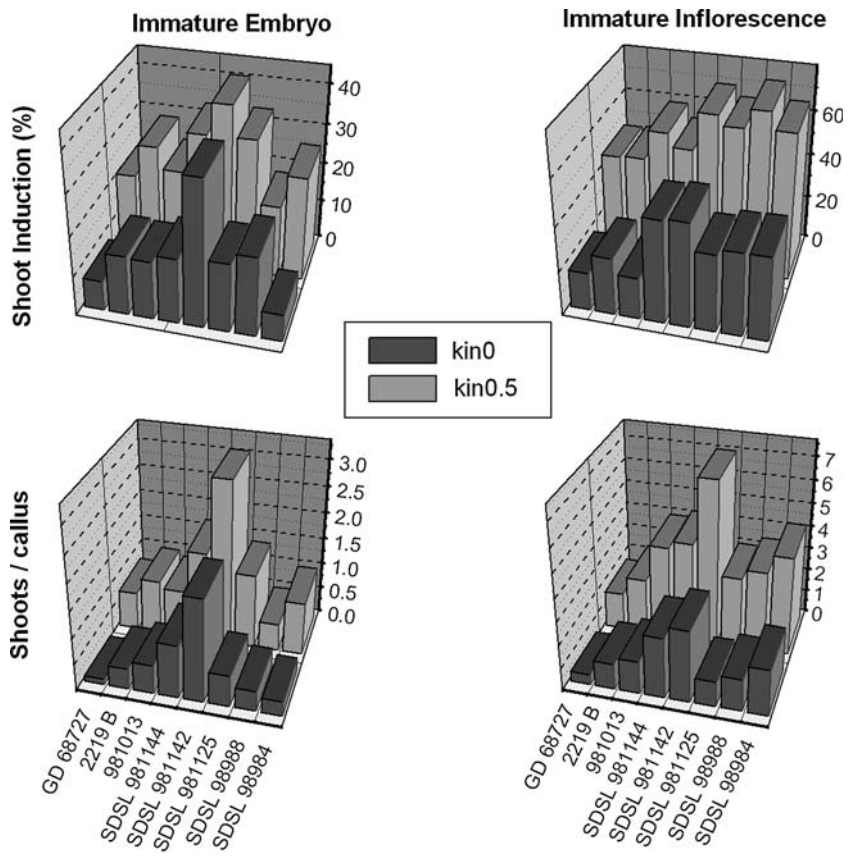

Fig. 5 Response of genotypes and explants to kinetin supplementation in callus induction media

shoots/callus followed by the shoot induction frequency and callus induction frequency. High heritability estimates of shoots/callus and shoot induction frequencies were accompanied by a high genetic advance. In both of the explants shoot induction frequency was positively correlated with the number of shoots/callus (Table 3).

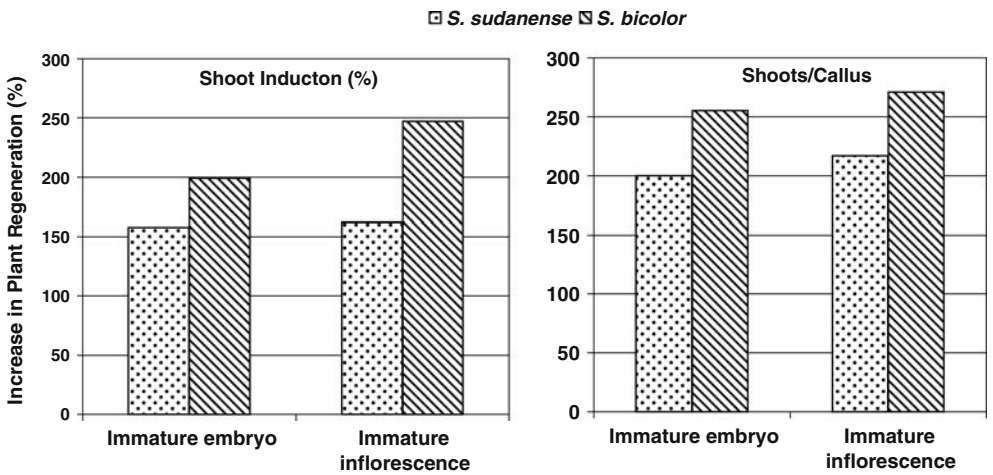

Fig. 6 Species differences for increase (%) in plant regeneration responses on kinetin supplementation in callus induction medium

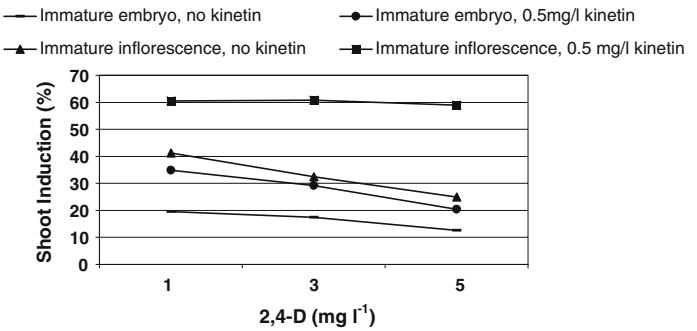

Fig. 7 Inhibitory effect of 2,4-D in callus media on shoot induction (%) from immature embryos and inflorescences in the presence and absence of kinetin

Discussion

In our effort to find out the strategy to overcome genotypic limitations of plant regeneration we could find that immature inflorescences of our genotypes were much superior to immature embryos and their performance was almost at par across the genotypes of the same species. These results suggest that the genotypic limitation, which is one of the major constraints in tissue culture, can be successfully overcome by the use of immature inflorescence explant. Comparison of meristematic status of the two explants provides an insight into the better regenerative potential of immature inflorescence explant. The relative proportion of meristematic tissues is higher in immature inflorescence as this explant has a number of meristematic tissues in the form of floral meristems, rachis, rachillae, and primordial of various floral organs while mainly the scutellum of immature embryos is the source of meristematic cells that produce embryogenic callus. Although the superiority of immature inflorescence explant over immature embryo explant for plant regeneration has been demonstrated by Bai et al. (1995), Bhat et al. (1995) the potentiality of this explant to overcome genotypic differences was not explored. Still, the use of immature inflorescences, for in vitro regeneration in sorghum is not as prevalent as the use

Table 2 Estimates of phenotypic and genotypic coefficient of variation along with heritability and genetic advance for four callus induction and plant regeneration traits in sorghum

Character	Immature embryo				Immature inflorescence			
	PCV	GCV	Heritability (%)	Genetic advance as % of mean	PCV	GCV	Heritability (%)	Genetic advance as % of mean
Callus induction frequency	9.89	6.33	47.66	9.71	12.47	6.09	23.83	6.12
Shoot induction frequency	42.63	32.11	56.76	49.84	29.59	18.49	39.03	23.80
Number of shoots/callus	74.88	70.52	88.70	137.11	56.30	43.93	60.88	70.63

PCV = phenotypic coefficient of variation; GCV = genotypic coefficient of variation

Table 3 Estimates of genotypic and phenotypic correlation coefficients plant regeneration traits

Characters	Shoot induction frequency		Number of shoots/callus	
	Immature embryo	Immature inflorescence	Immature embryo	Immature inflorescence
Callus induction frequency				
rp	0.261	0.041	0.273	0.007
rg	0.460	0.311	0.484	0.064
Shoot induction frequency				
rp			0.805*	0.640*
rg			1.00	0.818

rp = phenotypic correlation; rg = genotypic correlation

* Significant at 1% level of significance

of immature embryos. Results of the present investigation emphatically suggest for the use of immature inflorescence explant for sorghum tissue culture. Use of immature inflorescence further offers some practical advantages over immature embryos. Bagging of the panicle required to prevent change in the genotype of immature embryos through cross-pollination is not needed for immature inflorescence explant. Aseptic isolation and inoculation of immature embryos at their most in vitro responsive stage, i.e., 10–12 DAP and 0.7–1.5 mm size (Gupta et al. 2004) is tedious and time consuming as compared to immature inflorescences which exhibit morphogenetic competence over a wider size range (1–5 cm). Cai and Butler (1990) have reported that all inflorescences over 2 mm to meiosis are potentially morphogenic. The only problem with immature inflorescence is the visual identification of plants in field/glasshouse with appropriate explant size. Immature inflorescences of appropriate size remain completely covered with the leaves and flag leaf does not come out at this stage. Genotypic differences were further reduced when callus from immature inflorescence explant was induced on kinetin supplemented media. *S. bicolor* genotypes which were very poor tissue culture responsive through immature embryo explant could come very close to the *S. sudanense* genotypes when their immature inflorescence explant was used and callus induction media was supplemented with kinetin (Fig. 5). We found that 2,4-D exhibits inhibitory effect on callus induction, callus growth, shoot induction frequency and number of shoots/callus but immature inflorescence explant can produce embryogenic callus over a wide range of 2,4-D concentrations if kinetin is present in callus induction media. This observation again suggests that immature inflorescences are highly competent and may give very high embryogenic response over a wide range of 2,4-D supplementation in the presence of kinetin. Earlier Cai and Butler (1990) have reported the inhibitory effect of higher 2,4-D concentration on embryogenic callus development in *S. bicolor*. Boyes and Vasil (1984) in *S. arundinaceum* and Wang and Vasil (1982) in *Pennisetum purpureum* have also reported the inhibitory effect of higher concentrations of 2,4-D on embryogenic callus development. So, another important finding to overcome the genotypic limitations of plant regeneration is that callus induction medium must be supplemented with strong cytokinin like kinetin and 2,4-D level should be kept at minimum. *S. sudanense* regenerated more frequently and intensely than *S. bicolor*. Since two species exhibited differential response to kinetin supplementation in callus induction media it is probable that these species have differential endogenous kinetin levels. Morphologically these two species differ in tiller number and *S. sudanense* produces very high number of tillers. It is probable that *S. sudanense* contains more cytokinin than *S. bicolor* since cytokinins have been shown to be involved in tiller bud elongation in cereals (Isbell and Morgan 1982; Langer et al. 1973). This also explains the more responsiveness of *S. bicolor* to kinetin supplementation in callus induction media (Fig. 6). Further studies are required to establish differential cytokinin concentration in these two species and to correlate it with better plant regeneration response of *S. sudanense*.

Our results of genetic analysis of plant regeneration traits suggest that the extent of variability and heritability is more in shoots/callus and shoot induction frequency than in callus induction frequency. Although heritability in the present investigation has been calculated in the broad sense, its estimates can reliably be followed when the high heritability values for the trait is accompanied by high genetic advance. Shoot induction frequency and number of shoots/callus exhibited high genetic advance values along with high heritability values. This indicates that the variability in respect of these attributes in the population may be due to the additive gene action and genotypes selected for these characters would perform constantly. A significantly positive correlation between shoot induction frequency and number of shoots/callus suggests that selection of genotypes with high shoot induction frequency would also result in selection of genotypes with more number of shoots/callus and so the need of counting of shoots in a large selection programme may be obviated. Such studies are missing in sorghum tissue culture. High heritability estimates for plant

regeneration have been reported by Lazar et al. (1984) in wheat; Wu and Chen (1987) in rice. Relatively lower heritability for callus growth as compared to shoot-forming capacity has been reported by Beckert and Qing (1984).

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