Plant regeneration from immature inflorescence derived callus cultures of salt tolerant kallar grass (*Leptochloa fusca* L.)

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Abstract Efficient plant regeneration has been achieved from immature inflorescence derived callus cultures of salt tolerant grass *Leptochloa fusca* (L.). Young inflorescence explants displayed wide-ranging responses for callus induction and plant regeneration when subjected to different cold treatment durations and without cold treatment exposure (control) prior to its inoculation to MS medium supplemented with different concentrations/combinations of plant growth regulators (PGRs). The PGRs included auxins: 2, 4-dichlorophenoxy acetic acid (2, 4-D), picloram (Pic), 3, 6-dichloro-2-methoxy benzoic acid (dicamba) and cytokinins: Kinetin (KN), N6-benzyl adenine (BA). These treatments promoted different callus induction frequencies as well as various callus types such as type 1, type 2 and type 3. Induction of type 2 callus (white and compact) with potential for regeneration was obtained from cold treated (3 days at 10 °C) immature inflorescence cultured on MS medium containing 2.0 mg/l dicamba and 0.25 mg/l BA. The study demonstrated that 2.0 mg/l dicamba and 0.25 mg/l BA induced callus promoted improved frequency compared to zilch shoot regeneration response with other combinations involving 2, 4-D, picloram, KN and BA. Full strength MS supplemented with 2.0 mg/l NAA and 0.5 mg/l BA was found to be optimal for plant regeneration. The regeneration frequencies ranged from 13.8 ± 1.366 to 55.5 ± 2.766 with highest number of shoots (19.1 ± 0.560) per 50–60 mg of callus as explants after 28 days of inoculation. Plant regeneration was also obtained on the dicamba callus induction medium itself within 21 days inoculation of immature inflorescence explants. Half strength MS medium both semisolid and liquid devoid of plant growth regulators promoted

highest frequency (92.8 ± 4.099 and 100 ± 0.00) of rooting in regenerated shoots. Plants with well developed roots were successfully transferred to pots and grown to maturity with normal flowering and seed set. This is the first report on induction of callus and subsequent plant regeneration in kallar grass using immature inflorescence explants.

Keywords *Leptochloa fusca* · Immature inflorescence · Callus induction · Plant regeneration

Abbreviations

BA	N6-benzyl adenine
2, 4-D	2, 4-dichlorophenoxy acetic acid
Dicamba	3, 6-dichloro-methoxy benzoic acid
KN	Kinetin
MS	Murashige & Skoog
NAA	Naphthalene acetic acid
Pic	Picloram

Introduction

Leptochloa fusca L. of the family Poaceae (Graminae) commonly known as Kallar grass is a C-4 plant that grows under stressful conditions in low soil fertility, high salinity and pH 11.0. It is a valuable forage grass and used for saline soil reclamation (Akhter et al. 2003). Keeping in view the importance of grasses in general and kallar grass in particular, development of a in vitro plant regeneration protocol is a prerequisite for propagation of these precious genera, manipulation of genome through genetic transformation and somatic hybridization (Wang and Ge 2006; Han et al. 2009; Li et al. 2010; Zhang et al. 2010; Seo et al. 2011; Song et al. 2012). Plant regeneration in cereal and grasses is commonly influenced by a number of factors such as explants origin, genotype, media supplements, concentrations and

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types of plant growth regulators (Giri and Laxmi 2000; Gonzalez et al. 2001; Shyamkumar et al. 2009; Li et al. 2011; Zhang et al. 2012). Plant regeneration from callus cultures has been reported in many grass species. In vitro cultures have been initiated from a wide range of explants such as nodes, immature embryos, immature inflorescence, mature seeds, and shoot meristems. To mention a few, embryogenic callus induction and plant regeneration was obtained from nodal explants in grasses (Goldman et al. 2004; Murugesan et al. 2008). Plant regeneration from seed derived tissues in bahia grass (Shatters et al. 1994), *Cenchrus ciliaris* L (Shweta and Suresh 2002), perennial ryegrass (Liu et al. 2008), Italian rye grass (Lee et al. 2009) and *Eremochloa ophiuroides* Munro (Shyamkumar et al. 2009) was also reported. Further, induction of embryogenic callus and subsequent of plant regeneration from young shoot meristems (Sairam et al. 2002; Dalton et al. 2003; Murugesan et al. 2008) immature embryos (Christiansen et al. 2005) and pistils (Gugsa et al. 2006) has also been demonstrated. Immature inflorescences have been recognized as an important explants source for regeneration in many cereals and grasses (Neibaur et al. 2008; Murugesan et al. 2008; Yadav et al. 2009). The present work is envisaged aiming at transferring salt tolerant trait from kallar grass to rice through somatic hybridization. The present finding is an initial attempt in this direction to develop efficient in vitro regeneration protocol in kallar grass. Till date, there is no report on efficient plant regeneration using immature inflorescence explants in *L. fusca*. In this communication, we are reporting for the first time an efficient method for induction of callus and plant regeneration in kallar grass using immature inflorescence explants.

Materials and methods

Plant material

Leptochloa fusca (2n=20) plant material was kindly provided by Central Salinity and Soil Research Institute (CSSRI), Karnal, India and CSSRI Regional Centre Lucknow, India. Plants were established in soil and propagated under net house conditions at CPMB, Osmania University, Hyderabad, India.

Preparation of explants

Young immature inflorescences still wrapped inside the sheaths of kallar grass were collected from net house grown plants during blooming season (April to December each year). Immature inflorescence explants were

given cold treatment for 1, 2, 3, 4, 5, 6, and 7 days at 10 °C. The spikelets were excised and cut into 2.0 cm length segments. The segments were surface sterilized with 0.1 % (w/v) mercuric chloride for 5 min and rinsed five times with sterile distilled water giving each rinsing treatment of 5 min duration.

Basal medium consisted of MS (Murashige and Skoog 1962) salts supplemented with 30 g/l sucrose. Media was solidified with 0.9 % (w/v) agar (Fisher Scientific Qualigens, Mumbai, India). The pH of the media was adjusted to 5.8 before autoclaving.

Influence of cold treatment (days), callus induction from immature inflorescence, maintenance and culture incubation conditions in *L. fusca* (Kallar grass)

Approximately 2.0 cm length inflorescence segments were dissected out from main spike and cultured on callus induction medium. Influence of cold treatment ranging from 1, 2, 3, 4, 5, 6 and 7 days and plant growth regulators (PGRs) with a specific concentrations such as 2, 4-D: 2.0 mg/l; KN: 0.5 mg/l; Picloram: 2.0 mg/l; KN: 0.5 mg/l; Dicamba: 2.0 mg/l; KN: 0.5 mg/l was studied to evaluate callusing frequency.

Influence of different plant growth regulators on induction of callus from immature inflorescence of *L. fusca*

Plant growth regulator combinations involving auxins: 2, 4-dichlorophenoxyacetic acid (2, 4-D), 3, 6-dichloromethoxy benzoic acid (dicamba) and 4-amino-3, 5, 6-trichloropicolinic acid (picloram), cytokinins: N⁶-benzyl adenine (BA), kinetin (KN) were evaluated for callus induction. Different concentrations and combinations of plant growth regulators employed for callus induction are as follows:

- i. 2, 4-D at concentrations of 1.0, 2.0, 3.0 and 4.0 mg/l in combination with KN or BA (0.25, 0.5 and 1.0 mg/l)
- ii. Dicamba (1.0, 2.0, 3.0 and 4.0 mg/l) in combination with KN or BA (0.25, 0.5 and 1.0 mg/l)
- iii. Various concentrations of picloram (1.0, 2.0, 3.0 and 4.0 mg/l) in combination with KN or BA (0.25, 0.5 and 1.0 mg/l)

The cultures were incubated in dark at 25±1 °C in a culture room. Callus induction frequency was recorded as the percentage of explants produced callus tissue by the end of culture period (4 weeks). Cultures were maintained by sub-culturing at an interval of 4 weeks on callus induction medium.

The experiments were performed thrice maintaining a minimum of 10 replicates each time. Each replicate consists of 4 spikelets (23 individual flowers) of

Table 1 Influence of cold treatment (days) and PGRS (2, 4-D, Dicamba, Picloram, KN) on callus frequency from immature inflorescence of *L. fusca* (Kallar grass)

Days to cold treatment	^a Culture response					
	Days to callus induction			Frequency of callus induction (Mean% ± SE)		
	D + K ^a	Pic + K ^b	Di + K ^c	D + K	Pic + K	Di + K
C	10	14	12	41.2±1.951	33.7±1.177	25.4±1.915
1	11	12	–	61.8±2.371	41.2±1.104	NS
2	11	11	–	53.3±2.260	38.1±1.422	NS
3	10	13	12	49.9±2.737	47.3±0.750	41.6±1.919
4	11	14	12	38.9±3.435	38.5±1.530	30.2±0.75
5	13	12	13	58.3±2.290	46.0±1.501	22.7±1.146
6	11	13	14	39.8±1.177	44.6±1.126	17.5±1.166
7	14	14	12	49.0±1.177	45.1±1.562	19.7±0.75

^a observations were recorded after 4 weeks of culture

D = 2, 4-D; Pic Picloram; Di Dicamba; K Kinetin

¥ a 2, 4-D: 2.0 mg/l; KN: 0.5 mg/l; b Picloram: 2.0 mg/l; KN: 0.5 mg/l; c Dicamba: 2.0 mg/l; KN: 0.5 mg/l; NS: Not studied

C Control

The experiment was performed thrice maintaining a minimum of 10 replicates each time. Each replicate consists of 4 spikelets (23 individual flowers) of immature inflorescence

Mean is the average response of 10 replicates and the mean % value in the table shows the average frequency of 3 repeated experiments

immature inflorescence. The mean value mentioned in the Tables accounts for the average culture response of 10 replicates. Further, the mean percentage (%) value is the average frequency of 3 repeated experiments.

Effect of plant growth regulators and strength of MS nutrient on plant regeneration from immature inflorescence derived callus culture of *L. fusca* (kallar grass)

Callus pieces (explants) weighing approximately 50–60 mg were transferred to hormone-free, half-strength MS medium, or full-strength MS medium supplemented with a combination of auxin; 2, 4-D (1.0, 2.0, 3.0, 4.0 mg/l) or NAA (0.25, 0.5, 1.0 mg/l) and cytokinin; KN (0.5, 1.0, 2.0 mg/l), BA (0.25, 0.5, 1.0, 2.0), ZN (0.0625, 0.125 mg/l) or TDZ (0.125, 0.25 mg/l). The cultures were incubated in the light at 25±2 °C with a 16 h-light/8 h dark photoperiod. The number of shoots per callus explants was counted at 1 week interval up to 4 weeks and the data was recorded at the end of 4th week.

Influence of Plant growth regulators and strengths MS nutrient on rooting of regenerated shoots of *L. fusca* (kallar grass)

The regenerated shoots were transferred to ½ MS medium supplemented with NAA (0.5, 1.0 mg/l) and IBA (0.5, 1.0 mg/l). Regenerated shoots were also inoculated on to

½ MS medium devoid of any plant growth regulators. Rooting response was studied using both semisolid and liquid medium. After rooting, shoots were transferred to pots and acclimatized in the growth chamber and grown to maturity.

Statistical analysis

Latin square experimental design was followed for the experiments and statistical analysis. Experiment was performed thrice maintaining a minimum of 10 replicates each time. Each replicate consists of 4 spikelets (23 individual flowers) of immature inflorescence for callus induction, proliferation; plant regeneration and rooting were used in each experiment and treatments given. Mean value in all the statistical analysis tables shows the average response of 10 replicates. Statistical analysis of data such as mean, SE, two way, one way ANOVA and multiple comparisons was done using Matlab Version 5.3, and SPSS version 10.0 Math Works Inc. (USA) statistical packages.

Results

Callus induction

The influence of cold treatment duration (1, 2, 3, 4, 5, 6 and 7 days) and PGRs viz., 2, 4-D, dicamba, Pic, KN and BA on induction of callus using immature inflorescence explants

Table 2 Influence of different plant growth regulators on induction of callus from immature inflorescence of *L. fusca*

Plant growth regulators (mg/l)				Days to callus induction (Without cold treatment)	Days to callus induction (With cold treatment)	^a Frequency of callus induction (Mean % ± SE) Without cold treatment	^a Frequency of callus induction (Mean % ± SE) With cold treatment
Dicamba ^b				KN	BA		
1.0	0.25	–	11		11	27.5±1.530	39.8±1.585
	0.5	–	13		14	24.9±2.163	36.3±1.919
2.0	0.25	–	14		14	28.9±1.299	52.1±2.318
	0.5	–	12		12	22.3±1.501	41.6±1.919
3.0	1.0	–	11		12	19.7±0.750	32.0±3.010
	0.25	–	13		13	23.6±1.299	44.6±2.614
4.0	0.5	–	14		14	18.4±0.750	39.4±2.020
	1.0	–	13		14	21.0±1.299	46.4±1.888
1.0	0.25	–	11		12	30.2±1.985	47.3±2.018
	0.5	–	14		15	26.3±1.333	31.5±1.530
2.0	1.0	–	11		12	31.5±1.530	45.5±1.562
	–	0.25	11		11	28.9±1.501	40.3±2.607
3.0	–	0.5	12		13	23.6±0.750	34.2±2.546
	–	0.25	14		12	24.4±0.433	43.3±2.007
4.0	–	0.5	13		13	24.0±0.866	38.0±1.732
	–	1.0	13		14	26.2±0.779	31.0±1.146
1.0	–	0.25	11		12	22.3±0.750	39.4±1.530
	–	0.5	12		13	28.0±1.146	35.5±0.750
2.0	–	1.0	12		12	27.5±1.530	40.7±1.452
	–	0.25	11		13	24.9±1.333	32.8±1.333
3.0	–	0.5	13		14	25.3±1.177	30.2±0.750
	–	1.0	12		13	30.6±0.433	43.4±2.538
Picloram ^b							
1.0	0.25	–	12		12	31.9±2.665	41.6±4.056
	0.5	–	12		13	30.2±1.299	35.0±1.919
2.0	0.25	–	13		14	31.5±2.007	40.2±3.433
	0.5	–	14		13	34.6±1.594	45.5±2.706
3.0	1.0	–	11		12	32.8±3.031	42.7±2.655
	0.25	–	13		14	26.2±1.530	33.5±3.994
4.0	0.5	–	13		11	39.0±2.663	74.9±2.280
	1.0	–	15		14	31.0±1.146	34.6±1.171
1.0	0.25	–	14		14	28.9±0.750	35.0±1.585
	0.5	–	12		12	33.2±1.919	46.9±2.874
2.0	1.0	–	13		12	34.1±2.018	41.6±3.579
	–	0.25	14		13	30.6±1.562	46.4±2.445
3.0	–	0.5	13		12	34.1±2.018	42.9±2.665
	–	0.25	14		14	31.5±2.737	48.2±2.178
4.0	–	0.5	13		12	30.2±2.018	41.6±2.874
	–	1.0	12		13	29.7±0.433	36.7±2.280
1.0	–	0.25	12		11	22.3±1.501	31.9±3.059
	–	0.5	13		14	32.8±1.530	44.2±3.382
2.0	–	1.0	14		15	24.5±1.171	29.3±1.562
	–	0.25	12		13	33.2±1.177	54.3±2.693
3.0	–	0.5	12		12	28.0±2.433	42.5±2.324
	–	1.0	13		12	31.5±0.750	41.1±4.584

Table 2 (continued)

Plant growth regulators (mg/l)				Days to callus induction (Without cold treatment)	Days to callus induction (With cold treatment)	^a Frequency of callus induction (Mean % $\pm$ SE) Without cold treatment	^a Frequency of callus induction (Mean % $\pm$ SE) With cold treatment
Dicamba ^b KN BA							
2, 4-D ^c							
1.0	0.25	–	12		13	31.5 $\pm$ 2.729	41.9 $\pm$ 2.945
	0.5	–	13		14	27.6 $\pm$ 1.501	34.1 $\pm$ 2.018
2.0	0.25	–	11		11	28.9 $\pm$ 1.985	37.2 $\pm$ 4.191
	0.5	–	12		13	35.9 $\pm$ 2.324	61.8 $\pm$ 2.371
	1.0	–	14		12	20.1 $\pm$ 1.888	21.8 $\pm$ 1.146
3.0	0.25	–	13		14	34.1 $\pm$ 2.280	49.0 $\pm$ 3.804
	0.5	–	13		13	36.7 $\pm$ 2.018	52.5 $\pm$ 2.007
	1.0	–	14		15	30.2 $\pm$ 1.985	38.5 $\pm$ 2.894
4.0	0.25	–	14		13	29.4 $\pm$ 1.530	52.6 $\pm$ 0.866
	0.5	–	12		11	32.8 $\pm$ 2.737	53.0 $\pm$ 1.594
	1.0	–	12		15	33.7 $\pm$ 2.324	42.0 $\pm$ 2.453
1.0	–	0.25	13		12	31.5 $\pm$ 2.007	39.4 $\pm$ 4.036
	–	0.5	11		13	24.9 $\pm$ 0.779	32.4 $\pm$ 2.874
2.0	–	0.25	13		12	29.7 $\pm$ 0.433	35.9 $\pm$ 2.663
	–	0.5	13		12	30.6 $\pm$ 1.146	50.3 $\pm$ 1.177
	–	1.0	13		14	30.2 $\pm$ 2.280	37.2 $\pm$ 1.950
3.0	–	0.25	12		14	27.6 $\pm$ 0.750	24.1 $\pm$ 2.746
	–	0.5	13		12	29.7 $\pm$ 0.433	48.2 $\pm$ 2.412
	–	1.0	15		17	23.6 $\pm$ 0.779	30.2 $\pm$ 0.750
4.0	–	0.25	14		12	30.6 $\pm$ 0.433	51.3 $\pm$ 3.950
	–	0.5	13		12	32.4 $\pm$ 1.171	48.6 $\pm$ 3.410
	–	1.0	13		14	26.2 $\pm$ 2.280	38.1 $\pm$ 3.309

^a Observations were recorded after 4 weeks of culture

The experiment was performed thrice maintaining a minimum of 10 replicates each time. Each replicate consists of 4 spikelets (23 individual flowers) of immature inflorescence

Mean is the average response of 10 replicates and the mean % value in the table shows the average frequency of 3 repeated experiments

^b Three days cold treated immature inflorescence; ^c One day cold treated immature inflorescence

showed variable responses compared to explants without cold treatment. The explants without cold treatment showed callus induction frequency ranging from 41.2 $\pm$ 1.951 to 25.4 $\pm$ 1.915 across different PGRs combinations. Callus induction frequency was higher in 2, 4-D containing media than on media containing dicamba or Pic during the initial phases of experimentation, while assessing the influence of cold treatment duration. The percentage of callus initiation (61.8 $\pm$ 2.371) was obtained on media containing 2.0 mg/l 2, 4-D and 0.5 mg/l KN from 1 day cold treated immature inflorescence (Table 1). On the other hand, 3 day cold treatment generated callus induction frequencies of 41.6 $\pm$ 1.919 and 47.3 $\pm$ 0.750, respectively on media containing 2.0 mg/l either dicamba or Pic and 0.5 mg/l KN (Table 1). Two day cold treatment has shown least effect (38.1 $\pm$ 1.422) on induction of callus in the presence of PGRs Pic 2.0 mg/l and

KN 0.5 mg/l (Table 1). Similarly, media supplemented with 2.0 mg/l dicamba and 0.5 mg/l KN showed lowest frequency (17.5 $\pm$ 1.166) of callus initiation with 7 days cold treated inflorescence (Table 1). The callus induction frequency obtained with immature inflorescence without cold treatment ranged from a lowest response of 18.4 $\pm$ 0.750 to highest of 39.0 $\pm$ 2.663 across different PGRs evaluated (Table 2). On the other hand, the callus induction response from cold treated (3 days) explants was with a highest frequency of 74.9 $\pm$ 2.280 to a lowest of 21.8 $\pm$ 1.146 using different PGRs (Table 2). Callus induction frequency of 52.1 $\pm$ 2.318 was obtained on media supplemented with 2.0 mg/l dicamba and 0.25 mg/l KN and the lowest frequency of callus induction (31.5 $\pm$ 1.530) on media containing 4.0 mg/l dicamba and 0.5 mg/l KN (Table 2). The effect of various combinations of dicamba (1.0, 2.0, 3.0, 4.0 mg/l);

Fig. 1 High frequency plant regeneration from immature inflorescence derived callus cultures of kallar grass (*Leptochloa fusca*) (a) Induction and growth of callus from immature inflorescence (ImInf) explants of *L. fusca* after 4 weeks in dicamba/BA medium Type 1 callus: yellow and compact (one arrow); Type 2 callus: white and compact (two arrows); Type 3 callus: white, translucent, watery and soft (three arrows) (b) Further growth and proliferation of callus on ImInf explants after first subculture in 6 weeks duration (c) Profuse growth and proliferation of callus after second subculture with single white and compact Type 2 callus mass (arrow) with regeneration potential (d) Profuse growth and proliferation of Type 2 callus (e) High frequency plant regeneration from immature inflorescence derived callus (f) Rooting of regenerated shoots (g) Growth of plant to maturity in net house conditions (Bar= 1.0 cm)

BA (0.25, 0.5, 1.0 mg/l) and 3 days cold treated immature inflorescence promoted better callus induction frequency (43.3 ± 2.007) when cultured on 2.0 mg/l dicamba; 0.25 mg/l BA. Similar response of callus induction frequency (43.4 ± 2.538) was obtained on media supplemented 4.0 mg/l dicamba and 1.0 mg/l BA. The lowest callus initiation frequency was obtained on media supplemented with 4.0 mg/l dicamba and 0.5 mg/l BA. The immature inflorescence given a cold treatment for 3 days and inoculated on media supplemented with different combinations of Pic (1.0, 2.0, 3.0, and 4.0) and KN or BA (0.25, 0.5, and

1.0) has shown varying callus induction frequencies. The peak callus induction frequency (74.9 ± 2.280) was obtained from a combination of 3.0 mg/l Pic and 0.5 mg/l KN whereas the lowest frequency (33.5 ± 3.994) from 3.0 mg/l Pic and 0.25 mg/l KN (Table 2). Likewise, various combinations and concentrations of Pic and BA also illustrated wide-ranging response to callus induction. A combination of Pic: BA (4.0: 0.25 mg/l) has shown callus induction frequency (54.3 ± 2.693). Immature inflorescence cold treated for 1 day showed significantly enhanced callus induction frequency (61.8 ± 2.371). Lowest callus induction

Table 3 Effect of plant growth regulators and MS nutrient on plant regeneration from immature inflorescence derived callus culture of *L. fusca* (kallar grass)

MS + PGRS mg/l									^a Culture response	^d Regene ration%	No.of shoots/callus
	2, 4-D	KN	BA	ZN	TDZ	NAA	IAA	Di			
1.0	0.5	–	–	–	–	–	–	–	CG	NR	–
0.5	0.5	–	–	–	–	–	–	–	C	NR	–
0.5	1.0	–	–	–	–	–	–	–	C	NR	–
1.0	1.0	–	–	–	–	–	–	–	C	NR	–
1.0	0.5	0.25	–	–	–	–	–	–	C	NR	–
1.0	0.5	0.5	–	–	–	–	–	–	C	NR	–
0.5	0.5	0.5	–	–	–	–	–	–	CY	NR	–
0.5	1.0	0.5	–	–	–	–	–	–	CW	NR	–
1.0	–	0.25	–	–	–	–	–	–	CY	NR	–
1.0	–	0.5	–	–	–	–	–	–	CW	NR	–
0.5	–	0.5	–	–	–	–	–	–	CW	NR	–
1.0	0.5	0.5	–	–	–	–	–	–	CW	NR	–
2.0	0.5	0.5	–	–	–	–	–	–	GY, B	NR	–
3.0	0.5	0.5	–	–	–	–	–	–	GY, B	NR	–
4.0	0.5	0.5	–	–	–	–	–	–	GY, F,B	NR	–
1.0	–	–	0.0625	–	–	–	–	–	G	NR	–
1.0	–	–	0.125	–	–	–	–	–	G, B	NR	–
1.0	–	–	–	0.125	–	–	–	–	G	NR	–
1.0	–	–	–	0.25	–	–	–	–	CY	NR	–
–	0.5	0.5	–	–	–	–	–	–	B	NR	–
–	–	–	0.0625	–	–	–	–	–	Bl	NR	–
–	–	–	0.125	–	–	–	–	–	Bl	NR	–
–	–	–	–	0.125	–	–	–	–	Bl	NR	–
–	–	–	–	0.25	–	–	–	–	Bl	NR	–
–	2.0	–	–	–	0.5	–	–	–	G, B	NR	–
–	1.0	–	–	–	0.5	–	–	–	Bl	NR	–
–	0.5	–	–	–	0.5	–	–	–	B	NR	–
–	–	2.0	–	–	0.5	–	–	–	GR	24.9±2.56	19.1±0.560
–	2.0	–	–	–	0.25	–	–	–	B	NR	–
–	1.0	1.0	–	–	0.25	–	–	–	G, Bl	NR	–
–	–	2.0	–	–	0.25	–	–	–	G, Bl	NR	–
–	–	2.0	–	–	0.25 ^b	–	–	–	Bl	NR	–
–	–	2.0	–	–	0.5 ^b	–	–	–	GR	13.8±1.366	13.0±0.418
–	0.6	2.0	–	–	1.0	–	–	–	G, Bl	NR	–
–	1.0	2.0	–	–	1.0	–	–	–	B	NR	–
–	1.0	2.0	–	–	1.0 ^d	–	–	–	B	NR	–
–	–	–	–	–	– ^b	–	–	–	G, Bl	NR	–
–	–	–	–	–	– ^c	–	–	–	Bl	NR	–
–	–	0.25	–	–	–	–	–	2.0	GR	55.5±2.766	8.08±0.260

^aobservations were recorded after 4 weeks of culture^b½ MS; ^cMS devoid of hormones; ^dSucrose 6 %

C Compact callus; CG Compact callus with some greenness; CY Compact white to yellow callus; CW White compact callus; GY Yellowish callus with some greenness; B Browning of the callus; G callus with prominent green callus; F fragile callus; Bl blackening of callus; GR complete green callus with regeneration; NR No regeneration

^dThe experiment was performed 3 times maintaining a minimum of 10 replicates each time. Each replicate consists of four immature inflorescence derived calli weighing approximately 0.05 g

percentage (38.9±3.435) was observed in cold treated (4 days) inflorescence and inoculated on media supplemented with 2.0 mg/l 2, 4-D and 0.5 mg/l KN. The culture media supplemented with 4.0 mg/l 2, 4-D and 0.25 mg/l KN or BA has shown better callus induction frequency (62.6±0.866 or 51.3±3.950) and was significant (Table 2).

Immature inflorescence gave rise to 3 different types of callus. They were yellow and compact (type 1), white and compact (type 2) and white, translucent, watery and soft (type 3). The watery callus eventually turned brown on further subculturing (Fig. 1a). Culture of callus along with the mother explants in their first subculture improved callus

Table 4 Influence of plant growth regulators and MS nutrient on rooting of regenerated shoots of *L. fusca* (kallar grass)

$\frac{1}{2}$ MS + PGRS (mg/l) + PVP		^a Culture response					
		No of days to rooting		Frequency of rooting (Mean% $\pm$ SE)		No of roots (Mean $\pm$ SE)	
NAA	IBA	Semi solid	Liquid	Semi solid	Liquid	Semi solid	Liquid
0.5	–	15	17	84.6 $\pm$ 3.149	83.8 $\pm$ 1.899	3.6 $\pm$ 0.432	13.6 $\pm$ 1.115
1.0	–	17	18	64.2 $\pm$ 2.064	100 $\pm$ 0.00	2.7 $\pm$ 0.237	15.1 $\pm$ 1.351
–	0.5	10	11	100 $\pm$ 0.00	100 $\pm$ 0.00	3.7 $\pm$ 0.468	12.8 $\pm$ 0.748
–	1.0	10	10	100 $\pm$ 0.00	83.3 $\pm$ 2.399	3.3 $\pm$ 1.120	14.3 $\pm$ 1.520
C	C	9	7	92.8 $\pm$ 4.099	100 $\pm$ 0.00	5.2 $\pm$ 0.688	35.8 $\pm$ 1.276
^b –	–	11	B	92.8 $\pm$ 4.099	B	5.3 $\pm$ 0.704	B

^a observations were recorded after 4 weeks of culture^b Without PVP

C Control B Browning

growth pattern and subsequent maintenance (Fig. 1b). White and compact (type 2) callus was found and identified as highly responsive callus source for plant regeneration compared to type 1 and type 3 callus (Fig. 1c). At times type 2 callus with high regeneration potential could be selected out from type 1 callus and maintained separately for their use in regeneration (Fig. 1d).

Plant regeneration from callus cultures

Callus (white and compact: type 2) induced particularly on dicamba media developed large number of shoot primordia (with visible green sectors) during 14 to 18 days of culture on regeneration media. In contrast, callus initiated on 2, 4-D and Pic media remained undifferentiated after transfer to regeneration media even up to a prolonged duration (Table 3). Regeneration frequency of 24.9 ± 2.56 was obtained on full strength MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BA with an average of 19.1 ± 0.560 shoots per 50–60 mg of callus as individual explants after 4 weeks of culture (Fig. 1e). However, regeneration frequency was 13.8 ± 1.366 on $\frac{1}{2}$ strength MS media compared to MS full strength media and the average number of shoots of 13.0 ± 0.418 per explants was obtained. Plant regeneration was also observed on the callus induction media (dicamba) itself with a regeneration frequency of 55.5 ± 2.766 and an average number of shoots 8.08 ± 0.260 per callus explants (Table 3). The immature inflorescence derived callus regeneration study involved the auxins (2, 4-D, Di, NAA and IAA) and cytokinins (KN, BA, Zeatin and TDZ). Media supplemented with 2, 4-D singly with KN and BA mainly promoted callusing (Table 3). But media containing 2, 4-D along with both BA and KN together induced yellowish callus with green sectors (Table 3). Media supplemented with 2, 4-D and Zeatin promoted callusing with green sectors. However, with only zeatin blackening of callus was obtained. Media supplemented with only TDZ induced callus which turned brown after 1 week of culture, whereas with 2, 4-D and TDZ together, blackening of callus was obtained (Table 3). Media supplemented with NAA and KN promoted callusing which turned

Fig. 2 Schematic representation of the protocol for callus induction and plant regeneration in *L. fusca* using immature inflorescence explants

Table 5 Univariate ANOVA analysis illustrating the influence of cold treatment and PGRS (2, 4-D, Dicamba, Picloram, and KN) on callus induction from immature inflorescence of *L. fusca*

Source	Type III sum of squares	df	Mean square	F	Sig
Corrected model	347.530 ^a	16	19.307	16.085	0.000
Intercept	3,967.563	1	3,967.563	3,305.481	0.000
hormone	192.295	2	96.147	80.103	0.000
day	47.908	6	7.985	6.652	0.000
hormone3* day	73.963	10	7.394	6.160	0.000
Error	866.615	722	1.200		
Total	5,543.000	741			
Corrected Total	1,214.146	740			

^aR Squared=0.286 (Adjusted R Squared=0.268)

black after 1 weeks of culture. However, media supplemented with NAA and BA initially promoted callusing with green sectors and blackening of callus after 4 weeks of culture (Table 3). In the plant regeneration study using MS basal media (1/2 strength (a) and full strength (b) MS nutrients) without any plant growth regulators is considered as control (Table 3). The first visible culture response of callus in 1/2 strength MS include appearance of green sectors after 1 weeks of culture. However, the callus turned brown at the end of 4 weeks (Table 3). The callus totally turned brown without any sign of regeneration when cultured on MS full strength medium (Table 3).

Induction of rooting in regenerated shoots and transfer of plants to soil

Root induction could not be achieved with NAA or IBA supplemented individually either in full or half strength MS medium due to browning of the shoots. Addition of antioxidant PVP (0.5 %w/v) enhanced the response where shoots produced roots at all concentrations. The maximum number of 3.6 ± 0.432 roots per shoot bunch was obtained in 1/2 MS semisolid medium and PVP supplemented with 0.5 mg/l NAA. Induction of rooting was observed after 15 days with rooting frequency of (84.6 ± 3.149) (Table 4). The increased number of roots (3.7 ± 0.468 /shoot bunch) was observed in medium containing 0.5 mg/l IBA and PVP compared to medium supplemented with NAA. Maximum number of (100 ± 0.00) shoots produced roots

showing root initiation after 10 days of incubation (Table 4). Maximum rooting response was observed in 1/2 MS semi-solid medium without any plant growth regulators with a maximum of 5.2 ± 0.688 roots/shoot bunch at the end of 4 weeks of culture. Whereas 1/2 MS semisolid medium and PVP showed an average number of 5.3 ± 0.704 roots/shoot bunch. However, the average number of roots was significantly high in liquid half strength MS medium compared to roots developed on semisolid medium. The maximum number of (15.1 ± 1.351) roots per shoot bunch was obtained in 1/2 MS liquid medium and PVP supplemented with 1.0 mg/l NAA (Fig. 1f). Initiation of rooting was observed after 18 days with 100 ± 0.00 response. A similar response was obtained with 1.0 mg/l IBA and PVP with maximum shoots (83.3 ± 2.399) promoting root induction after 10 days of incubation (Table 4). Maximum response was observed on 1/2 strength MS+PVP devoid of hormones with 100 ± 0.00 shoots producing roots after 7 days of incubation and with an average number of 35.8 ± 1.276 roots per shoot bunch (Table 4). Plants were successfully transferred with survival percentage of 50 %, and established in soil under net house conditions at CPMB (Fig. 1g), Osmania University, Hyderabad, India. Growth of plants to maturity, flowering and seed set was observed. Starting from the inoculation of immature inflorescence explants through callus induction, plant regeneration and transfer of in vitro rooted plants to soil took a time duration of 84 days. The schematic representation of the protocol is depicted in Fig. 2. However, quite occasionally plant regeneration also occurred in

Table 6 Multiple comparisons of PGRS (2, 4-D, Picloram, Dicamba, KN) for induction of callus from immature inflorescence of *L. fusca*

(I)hormone	(J)hormone	Mean difference (I-J)	Std. Error	Sig
2, 4-D/KN	Picloram/KN	0.4176 ^a	0.09377	0.000
	Dicamba/KN	1.3941 ^a	0.10272	0.000
Picloram/KN	2, 4-D/KN	-0.4176 ^a	0.09377	0.000
	Dicamba/KN	0.9766 ^a	0.10272	0.000
Dicamba/KN	2, 4-D/KN	-1.3941 ^a	0.10272	0.000
	Picloram/KN	-0.9766 ^a	0.10272	0.000

Based on observed means ^aThe mean difference is significant at the 0.05 level

Table 7 Univariate ANOVA analysis showing the effect of concentrations of Dicamba and BA for the induction of callus from immature inflorescence of *L. fusca*

Source	Type III sum of squares	df	Mean square	F	Sig
Corrected model	29.510 ^a	10	2.951	2.564	0.005
Intercept	1,987.146	1	1,987.146	1,726.783	0.000
concentration	2.041	3	0.680	0.591	0.621
mg	6.278	2	3.139	2.728	0.067
Concentration* mg	21.277	5	4.255	3.698	0.003
Error	481.026	418	1.151		
Total	2,544.000	429			
Corrected Total	510.536	428			

^aR Squared=0.058 (Adjusted R Squared=0.035)

dicamba callus induction itself within 21 days, curtailing the overall protocol duration by 35 days and finally bringing the total protocol period to 49 days (Fig. 2).

Statistical analysis

The analysis was carried out through two way analysis of variance (ANOVA) for the overall data under the effect of PGRs on induction of callus from immature inflorescence explants. In addition we have also tested individually the treatment used for their significance in Post-Hoc analysis through multiple comparison tests of different PGRs, their concentrations/combinations and cold treatment durations (Tables 5, 6, 7 and 8). The data was evaluated through two-way ANOVA for the overall data under the influence of different PGRS on induction of roots using liquid media and found that different PGRs and their concentrations used did not significantly affect the induction of roots. The effect on media without PGR was found significant at LSD (Least significance difference) ≤ 0.05 in term of frequency of rooting and roots per regenerated shoots (Tables 9 and 10).

Discussion

Present investigation revealed the potential of immature inflorescence as explants for callus induction and plant regeneration in *L. fusca*. This study described an

efficient and reproducible plant regeneration protocol from immature inflorescence derived callus culture. The number of days of cold treatment and type of auxin and cytokinin employed showed significant effect on callus induction and subsequent plant regeneration. The ultimate objective of the study was to optimize induction of callus from immature inflorescence segments of *L. fusca*, subsequent plant regeneration, rooting, acclimatization and growth of plants to maturity.

Observations from experiments have shown that treatment of inflorescence at low temperature was necessary for high frequency callus induction irrespective of plant growth regulators evaluated. Percentage callus induction varied significantly depending on the plant growth regulator, number of days of cold treatment and media composition. Callus initiation frequency also varied depending on days of cold treatment and different plant growth regulators employed. However, callus could be induced from cold treated immature inflorescence cutting across 1 to 7 days and the PGRs concentrations used. Initiation of callus was observed after 10–15 days of inoculation on the induction media in majority of the cases involving different PGRs combinations. The plant growth regulator concentrations/combinations had shown synergistic effect with duration of cold treatments.

Callus induction and plant regeneration from young inflorescence was previously studied and improved by

Table 8 Univariate ANOVA analysis showing the influence of concentrations of Dicamba and KN for the induction of callus from immature inflorescence of *L. fusca*

Source	Type III sum of squares	df	Mean square	F	Sig
Corrected model	53.100 ^a	10	5.310	5.278	0.000
Intercept	2,4550.044	1	2,450.044	2,435.403	0.000
concentration	5.219	3	1.740	1.729	0.160
mg	20.792	2	10.396	10.334	0.000
Concentration* mg	27.362	5	5.472	5.440	0.000
Error	420.513	418	1.006		
Total	3,024.000	429			
Corrected Total	473.613	428			

^aR Squared=0.112 (Adjusted R Squared=0.091)

Table 9 Univariate ANOVA analysis of the influence of PGRS (NAA, IBA), antioxidant PVP and semisolid media for the induction of roots from regenerated shoots of *L. fusca*

Source	Type III sum of squares	df	Mean square	F	Sig
Corrected model	62.803 ^a	5	12.561	4.428	0.002
Intercept	966.823	1	966.823	340.867	0.000
mg 2	4.455	1	4.455	1.571	0.215
hormone2	30.955	3	10.318	3.638	0.018
mg2* hormone2	0.818	1	0.818	0.288	0.593
Error	170.182	60	2.836		
Total	1,297.000	66			
Corrected Total	232.985	65			

^a.R Squared=0.270 (Adjusted R Squared=0.209)

a combination of plant growth regulators in *Tripsacum dactyloides* (Salon and Earle 1998), zoysia grass (Murugesan et al. 2008). Induction of embryogenic callus was usually promoted by auxin especially 2, 4-D. The auxin 2, 4-D alone was used for regeneration of embryogenic callus in *Phragmites australis* (Lauzer et al. 2000). A combination of 2, 4-D and BA was used in zoysia grass for the generation of callus with regeneration potential (Murugesan et al. 2008). It was reported in seashore paspalum, that the use of dicamba significantly increased the plant regeneration frequency from immature inflorescence derived callus (Neibaur et al. 2008). The use of dicamba was also identified as the most important factor in optimizing the tissue culture and stable genetic transformation response in barley (Castillo et al. 1998; Trifonova et al. 2001). In rye, dicamba was found to be superior to 2, 4-D in generation of regenerable callus (Jimmy and Lörz 1989). For the induction of embryogenic callus in bermuda grass and millets, 2, 4-D was superior to dicamba (Rao et al. 1995; Jain et al. 2005; Jogeswar et al. 2007). In the present study, dicamba was found to be the very valuable auxin for callus induction

and most importantly subsequent plant regeneration. The dicamba containing callus induction medium promoted all three types of callus (type 1, type 2 and type 3) compared to type 1 and type 2 in media with 2, 4-D + KN and only type 1 in Pic + KN and 2, 4-D + BA fortified medium. Although type 2 callus was obtained in callus induction medium containing 2, 4-D + KN; however, plant regeneration could not be achieved. The yellow type 1 callus was induced in all callus induction medium containing 2, 4-D, Pic and dicamba but plant regeneration could not be obtained.

Conclusion

The present study develops an efficient, reproducible plant regeneration procedure using immature inflorescence explants for the first time in *L. fusca*. The protocol developed can be used for propagation and somatic hybridization study for the transfer of salinity from this vital salt tolerant forage grass to cultivated rice. The findings obtained in this study will be helpful for in vitro manipulation studies in *L. fusca*.

Table 10 Univariate ANOVA analysis showing the influence of MS media (1/2 strength) with and without PGRS (NAA, IBA), antioxidant PVP in liquid media for the induction of roots from regenerated shoots of *L. fusca*

Source	Type III sum of squares	df	Mean square	F	Sig
Corrected model	7,222.803 ^a	5	1,444.561	262.503	0.000
Intercept	14,719.005	1	14,719.005	2,674.709	0.000
mg 2	26.273	1	26.273	4.774	0.033
hormone2	7,211.864	3	2,403.955	436.842	0.000
mg2* hormone2	0.000	1	0.000	0.000	1.000
Error	330.182	60	5.503		
Total	23,101.000	66			
Corrected Total	7,552.985	65			

^a.R Squared=0.956 (Adjusted R Squared=0.953)

Experiment was performed thrice maintaining a minimum of 10 replicates each time. Each replicate consists of 4 spikelets (23 individual flowers) of immature inflorescence

Mean value in all the statistical analysis tables shows the average response of 10 replicates

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