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Rapid propagation of lemongrass (*Cymbopogon flexuosus* (Nees) Wats.) through somatic embryogenesis *in vitro*

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Abstract. Somatic embryos induced from callus cultures of lemongrass *[Cymbopogon flexuosus* (Nees) Wats.] on Murashige and Skoog medium supplemented with 5 mg/l of 2,4-D, 0.1 mg/l of NAA and 0.5 mg/l of Kn developed into plantlets when plated on a medium supplemented with 3 mg/l of BA, 1 mg/l of GA_3 and 0.1 mg/l of NAA. The regeneration potential of callus was retained for more than 2 years on the nutrient medium supplemented with comparatively lower levels of growth regulators (2,4-D at 2 mg/l, NAA at 0.1 mg/l and Kn at 0.25 mg/l). Approximately 30-35 plantlets were produced after two months of culture per 100 mg of callus inoculated. Regenerants were transplanted into soil and transferred to the field for assessment of various morphological and biochemical characteristics. The results of 1 year of field trials showed that plants derived from somatic embryoids were more uniform in all the characteristics examined when compared with the field performance of plants raised through slips by standard propagation procedures. Thus, a procedure has been developed for high frequency long term plant production of lemongrass through *in vitro* methods.

Abbreviations. 2,4-D : 2,4 - dichlorophenoxyacetic acid; NAA : α - naphthalene acetic acid; Kn: kinetin; BA: benzyladenine; GA_3: gibberllic acid; MS: Murashige and Skoog (1962) basal medium

Introduction

Cymbopogon flexuosus (Nees) Wats., commonly known as lemongrass, yields a valuable essential oil which is widely used in perfumery, cosmetics and pharmaceuticals (Verma et al. 1985). Citral, the major constituent of lemongrass oil is used in the synthesis of vitamins A & E, β - ionone, methyl ionone, synthetic violets and non-toxic adhesives besides its use in perfumery and flavour industries (Guenther 1950, Pillay 1961, Robbins 1983). This constituent varies greatly in quantity from plant to plant in wild populations, because species of *Cymbopogon* are naturally cross pollinated and highly heterozygous (Sreenath and Jagadishchandra 1991). Intra-clonal variations have also been reported in field grown populations (Patra et al. 1990). In India, natural growth of lemongrass has dwindled because of indiscriminate collection and deforestation (Husain 1991). Different lemongrass strains have shown a steady decline in the yield of oil over a period of time (Rao et al. 1991). Hence, necessity arises for a reliable *in vitro* propagation method for developing superior producing lines.

Tissue culture work done so far on *Cymbopogon* species (Sreenath and Jagadishchandra 1991) does not include detailed studies on the development of embryogenic callus from somatic tissue and subsequent regeneration for the production of somaclones in *C. flexuosus*. However, detailed work on somatic embryogenesis has been reported in *C. martinii* (Baruah and Bordoloi 1989, Nayak et al. 1992). Somatic embryogenesis has been emphasised as an efficient method for rapid *in vitro* multiplication of plants as well as a tool for crop improvement (Ammirato 1989, Sharp et al. 1982). Embryogenic cell lines retain their regenerating potential for a long period in culture and often give rise to uniform and normal plant population (Vasil 1982). The present paper reports callus culture and plant regeneration through somatic embryogenesis from nodal explants of lemongrass. Morphological and chemical characteristics of regenerated plants are also discussed.

Materials and Methods

Callus culture. Nodal segments from basal region of mature tillering plants of *C. flexuosus* growing in the experimental gardens of the Aromatic & Medicinal Plants Division of the Institute were used as explants. Segments measuring ca 1 cm were cleansed with 3% (v/v) Teepol detergent solution and surface sterilised in 0.1% (w/v) mercuric chloride solution for 15 min followed by 3-4 rinses in sterile distilled water, prior to inoculation onto nutrient agar medium. MS (Murashige and Skoog 1962) and N_6 (Chu et al. 1975) basal media supplemented with 2,4-D and Kn with or without NAA at varying concentrations were used for induction of callus. One explant was placed per culture tube and replicated 15 times for each treatment. The medium was supplemented with 30 g/l of sucrose, gelled with 0.8% agar and pH was adjusted to 5.7 before autoclaving. Cultures were raised in 25 mm X 15 mm culture tubes or 150 ml Erlenmeyer's flasks under cool white fluorescent light emitting 50 μ mole m^{-2} s^{-1} for 16 h photoperiod

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in a growth room maintained at 25 ± 2 °C. Callus was subcultured every 30-35 d. A comparison of callus growth on MS and N_6 basal media with different modifications was made after 30 d. of callus culture by weighing fresh callus in each set of experiment from 15 different culture tubes.

Plant regeneration. For plant regeneration 2 months old embryogenic callus weighing approximately 100 mg per tube was transferred to MS basal medium supplemented with varying combinations and concentrations of BA, NAA and GA_3. After 8 weeks of growth, average number of plantlets per culture and length of shoots were calculated from 25 different culture tubes for each treatment. This was followed at 4 month intervals up to 30 months, to assess embryogenic and regeneration potential in long term callus cultures. Regenerants were transferred to pots, hardened for about 30 d and subsequently planted in the field.

Plant characteristics. Data on characters like plant height, tiller number per clump, total herb weight and percentage of essential oil content were recorded from field-propagated as well as culture-derived plantations. Comparison was made between 45 plants from each group in the first year of cultivation. Essential oil samples from the freshly harvested lemongrass leaves were obtained through steam distillation (Vogel 1986) using a Clevenger's apparatus. Coefficient of variation (C.V.) was calculated as

$$C.V. = \frac{\text{Standard Deviation (SD)}}{\text{Mean}} \times 100$$

Level of significance was calculated by students 't' test.

Results and Discussion

Callus initiation and growth

Swellings in the meristematic region of the nodal segment was observed after 10-15 d of culture, followed by initiation of callus in the subsequent 30-40 d, when cultured on both MS and N_6 basal media supplemented with different combinations and concentrations of auxin and kinetin (Table 1). 2,4-D (0.5-5 mg/l), NAA (0.1-2 mg/l) or Kn (0.1-0.5 mg/l) used singly failed to stimulate callusing in explants on either of the basal media tested. A combination of 2,4-D and Kn was necessary for callus initiation and growth in *C. flexuosus*, in accordance with reports on many graminaceous species (Vasil 1986) including some other species of *Cymbopogon* (Mathur et al. 1988, Baruah and Bordoloi 1989). After 30 d of culture callus growth was better when the above combinations were used in MS medium compared to N_6 medium. However, optimal callusing response was obtained on MS medium supplemented with 5 mg/l of 2,4-D, 0.5 mg/l of Kn and 0.1 mg/l of NAA, where 62% of explants responded positively (Table 1). As in *C. flexuosus* in the present study, higher levels of 2,4-D (3-5 mg/l) were also very effective in callus induction in *C. martinii* (Baruah and Bordoloi 1989) but failed to initiate callus in *C. winterianus* (Mathur et al. 1989). During subculture, callus proliferation was much faster on MS medium with reduced levels of 2,4-D (2 mg/l) and Kn (0.5 mg/l) along with 0.1 mg/l of NAA. Use of comparatively lower levels of 2,4-D for better callus growth in *C. flexuosus* is in conformity with reports in *C. martinii* (Baruah and Bordoloi 1989). In general, callus was embryogenic in nature and small somatic embroys were visible on the surface of the callus after 15-20 d of growth.

Plant regeneration through somatic embryogenesis

Embryogenic callus (Fig. 1) was grown for 2 months in callus maintenance medium with 2,4-D supplements. Some green spots were observed in the callus but subsequent development of roots and shoots did not occur until transfer to regeneration medium. Somatic embryos (Fig. 2) developed readily into green plantlets with distinct shoots and roots on MS basal medium supplemented with BA (1-3 mg/l), GA_3 (0.5-2 mg/l) and NAA (0.1-0.5 mg/l) in different combinations. However, the frequency of embryo germination was best on MS medium containing 3 mg/l of BA, 1 mg/l of GA_3 and 0.1 mg/l of NAA (Table 2). Approximately 100 mg of inoculum produced 30-35 plantlets, which attained height of 6-8 cm within 8 weeks of growth. This observation is in agreement with report in *C. martinii* (Baruah and Bordoloi 1989), but is in contrast to reports in other species of *Cymbopogon* (Sreenath and Jagadishchandra 1991), where precocious germination of somatic embryos into green plantlets occurred on 2,4-D supplemented callus induction medium.

Table 1. Effect of 2, 4-D, Kn and NAA on induction and proliferation of lemongrass callus in different basal media.

Growth regulators (mg/l)	Basal medium	% Explants showing callus induction mean ± SE *	Average callus proliferation fresh weight (mg) ± SE *
2,4-D (0.5-5.0)	MS	-	-
	N_6	-	-
NAA (0.1-2.0)	MS	-	-
	N_6	-	-
Kn (0.1-0.5)	MS	-	-
	N_6	-	-
2,4-D (1.0) + Kn (0.1)	MS	12.3 ± 0.2	22.3 ± 0.3
	N_6	9.1 ± 0.3	20.6 ± 0.1
2,4-D (3.0) + Kn (0.1)	MS	15.6 ± 0.1	26.8 ± 0.2
	N_6	13.2 ± 0.4	23.4 ± 0.6
2,4-D (3.0) + Kn (0.5)	MS	13.2 ± 0.2	25.1 ± 0.4
	N_6	13.1 ± 0.3	20.3 ± 0.2
2,4-D (5.0) + Kn (0.5)	MS	30.6 ± 0.4	85.1 ± 0.2
	N_6	25.3 ± 0.1	61.7 ± 0.1
2,4-D (5.0) + Kn (0.5)	MS	62.7 ± 0.4	121.8 ± 0.3
+ NAA (0.1)	N_6	56.2 ± 0.2	75.5 ± 0.6
2,4-D (5.0) + Kn (0.5)	MS	58.3 ± 0.3	80.1 ± 0.5
+ NAA (0.5)	N_6	50.4 ± 0.3	46.3 ± 0.1

* Standard Error of means.

Table 2. Effect of NAA, BA and GA_3 on plantlet regeneration from embryogenic callus of lemongrass.

Growth regulators in MS basal medium (mg/l)	No. of germinated somatic embryos/ culture Mean ± SE	Length (cm) of shoots Mean ± SE
NAA (0.1) + BA (1.0)	18.5 ± 0.4	5.8 ± 0.2
NAA (0.5) + BA (1.0)	12.1 ± 0.3	5.0 ± 0.4
NAA (0.1) + BA (2.0)	22.3 ± 0.1	5.6 ± 0.1
NAA (0.5) + BA (2.0)	18.1 ± 0.3	5.2 ± 0.3
NAA (0.1) + BA (3.0)	25.3 ± 0.2	5.2 ± 0.5
NAA (0.1) + BA (3.0) + GA_3 (0.5)	33.1 ± 0.3	6.5 ± 0.5
NAA (0.1) + BA (3.0) + GA_3 (1.0)	33.7 ± 0.2	7.5 ± 0.3
NAA (0.1) + BA (3.0) + GA_3 (2.0)	30.5 ± 0.5	7.2 ± 0.1

Addition of GA_3 to regeneration medium (Table 2) was not essential for germination of somatic embryos, but enhanced the frequency of germination and growth of shoots. Embryo germination was delayed up to 15 d in medium devoid of GA_3. Addition of low levels of NAA (0.1-0.5 mg/l) to BA supplemented media was found to be essential for germination of embryos; but when NAA was used at higher levels, shoot formation was inhibited and only roots were formed. Because germinated embryos had very slender roots, for development of good root system they were transferred to semisolid MS basal medium supplemented with different auxins (1 mg/l of NAA, IAA, IBA) separately. Optimal response was obtained using 1 mg/l of IBA. The morphogenic potential of embryogenic callus was retained for more than 2 years (Table 3) when grown on callus maintenance medium with comparatively lower levels of 2,4-D (2 mg/l), whereas at higher concentration (2,4-D, 5 mg/l) it was lost after 6 months.

Table 3. The regeneration potential of lemongrass callus in long-term culture.

Number of months in culture	Number of plantlets(Mean ± SE)per approx. 100 mg fresh weight of callus grown in MS medium with	
	2mg/l 2,4-D +0.5mg/l Kn +0.1mg/l NAA	5mg/l 2,4-D +0.5mg/l Kn +0.1mg/l NAA *
2	33.7 ± 0.2	23.3 ± 0.5
6	33.5 ± 0.6	8.7 ± 0.8
10	33.5 ± 0.1	-
14	33.7 ± 0.2	-
18	32.2 ± 0.3	-
22	31.5 ± 0.4	-
26	30.7 ± 0.2	-
30	30.1 ± 0.3	-

* Regeneration potential of callus was lost after six months.

Table 4. Comparison of different morphological and chemical characteristics between normally propagated plants and culture-regenerated plants. (Data from 45 plants from each group)

Character	Normally propagated plants				Culture-regenerated plants			
	Mean	SD	Variance	CV(%)	Mean	SD	Variance	CV(%)
Plant height (cm)	190.11	48.12	2315.12	25.31	193.4	23.36	545.68 **	12.07
Tiller no. per clump.	24.08	12.44	154.75	51.66	28.67	8.81	77.61 *	30.72
Herb wt. per clump (kg)	0.524	0.508	0.258	96.94	0.714	0.315	0.099 *	44.11
Total essential oil(%) +	0.35	0.11	0.01	31.42	0.36	0.03	0.0009 **	8.33

+ : Oil yield per 100 g fresh biomass ; * : Significantly different from the normally propagated clone ($P < 0.05$) by Student's 't' test ; ** : Not Significant

Regenerants (Fig. 3) were maintained in half strength liquid MS medium prior to transfer to pots filled with a mixture of soil and sand in the ratio of 2:1 (V/V). Following transfer to pots, plants were maintained at room temperature for 3-4 weeks and subsequently transferred to the field (Fig. 4) for further evaluation of propagated plants.

Analysis of morphological and chemical characteristics

Various characteristics of normal field-cultivated and culture-derived clonal plants are summarized in Table 4. No significant difference was found either in mean plant height or in content of essential oil at the harvesting stage between the two groups of plants. However, the variances as well as the co-efficients of variation (C.V.) for plant height and essential oil content were much smaller in the culture-derived plants than in the field-cultivated plants, indicating that culture-regenerated plants were more uniform. The variance and C.V. values for tiller number and herb weight per clump were significantly smaller in case of the culture-derived plants, revealing more homogeneity. However, the high SD values obtained (Table 4) may be due to the small number of samples taken. Hatano et al. (1988) observed high uniformity in alkaloid content of culture-derived clonal plants of *Aconitum carmicheli* compared to the field-cultivated clones. It was also suggested that *in vitro* plant propagation through somatic embryogenesis may result in promoting more homogeneity in quality of secondary metabolites (Hiraoka et al. 1986, Shoyama et al. 1988).

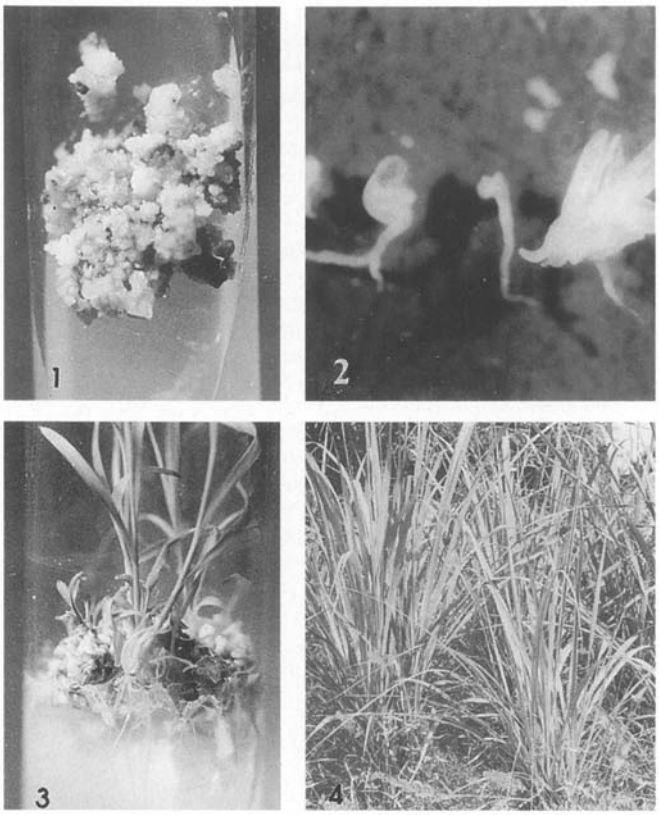

Fig. 1. Embryogenic callus of lemongrass after 8 weeks of culture.
Fig. 2. Germinating somatic embryos.
Fig. 3. Somatic embryo-derived regenerated plantlets.
Fig. 4. Regenerants established in the field.

Thus, the procedure outlined above should be useful not only for efficient propagation of lemongrass species for stable supply of high quality plants but also for the long term conservation of the genotypes for future improvement programmes.

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