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# Somatic embryogenesis and regeneration of *Cenchrus ciliaris* genotypes from immature inflorescence explants

C.B. YADAV, P. JHA, C. MAHALAKSHMI, V. ANJAIAH and V. BHAT*

*Department of Botany, University of Delhi, Delhi-110007, India*

## Abstract

An efficient, highly reproducible system for plant regeneration *via* somatic embryogenesis was developed for *Cenchrus ciliaris* genotypes IG-3108 and IG-74. Explants such as seeds, shoot tip segments and immature inflorescences were cultured on Murashige and Skoog (MS) medium supplemented with 2.0 - 5.0 mg $dm^{-3}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg $dm^{-3}$ $N^6$-benzyladenine (BA) for induction of callus. Callus could be successfully induced from all the three explants of both the genotypes. But the high frequency of embryogenic callus could be induced only from immature inflorescence explants. Somatic embryos were formed from nodular, hard and compact embryogenic calli when 2,4-D concentration was gradually reduced and BA concentration increased. Histological studies of somatic embryos indicated the presence of shoot apical meristem with leaf primordia. Ultrastructural details of globular and scutellar somatic embryos further validated successful induction and progression of somatic embryogenesis. Shoots were differentiated upon germination of somatic embryos on MS medium containing 2,4-D (0.25 mg $dm^{-3}$) and BA or kinetin (1 - 5 mg $dm^{-3}$). Roots were induced on ½ MS medium containing charcoal (0.8 %), and the regenerated plants transferred to pots and established in the soil showed normal growth and fertility.

*Additional key words*: buffel grass, embryogenic callus, histological studies, tissue culture.

## Introduction

Buffel grass (*Cenchrus ciliaris* L.) is a perennial, polyploid and warm-season forage grass with an extensive native range from tropics of Africa to India (Bhat *et al*. 2001). It is highly drought-tolerant having high biomass productivity (Martin *et al*. 1995, Rao *et al*. 1996). Owing to its apomictic mode of reproduction, the genetic improvement of buffel grass was restricted to selection methodologies (Echenique *et al*. 1996). A proper comparison of the developmental events during somatic embryogenesis (SE) of apomictic and sexual genotypes will help in determining the role of phytohormones and in greater understanding of the process of induction of somatic embryogenesis. Several candidate genes have been isolated from apomictic *Cenchrus* and their functional validation required an efficient protocol on genetic transformation which is not available yet. Plant regeneration through somatic embryogenesis has been successfully accomplished in many plant species (recently *e.g*., Liu *et al*. 2007, Junaid *et al*. 2007, Ganesh Kumari *et al*. 2008, Sanatombi *et al*. 2008). Previous studies on tissue culture in buffel grass have demonstrated plant regeneration from embryogenic callus, induced from immature inflorescences (Kackar and Shekhawat 1991) as well as from immature and mature embryos (Ross *et al*. 1995). But, a detailed understanding of somatic embryogenesis through histological and ultrastructural studies is not available. Moreover, in order to overcome the limitation of the availability of explants round the year, seed derived shoot tip explants were also tested in the present study for optimizing regeneration. Studies on the interaction between exogenously supplied hormones with endogenous levels in the explants will help in determining the optimum concentrations for efficient plant regeneration. Hence, the present study was conducted to optimize somatic embryogenesis and plant regeneration from different explants of sexual and apomictic genotypes.

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*Abbreviations*: BA - $N^6$-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; SE - somatic embryogenesis; IBA - indole-3-butyric acid; NAA - α-napthaleneacetic acid.

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* Author for correspondence; fax: (+91) 27662091, e-mail: bhatv64@rediffmail.com

## Materials and methods

Seeds of two *Cenchrus ciliaris* genotypes IG-3108 (apomictically reproducing) and IG-74 (sexually reproducing) were obtained from Indian Grassland and Fodder Research Institute, Jhansi, India.

Three explant types of these two genotypes (mature seeds, shoot tips from 3- to 4-d-old seedlings and immature inflorescences about to emerge out of the boot leaf) were sterilized in 70 % ethanol for 1 min, by 0.1 % $HgCl_2$ solution for 5 min, thoroughly washed 4 - 5 times with sterile distilled water and then were placed on the callus induction media consisting of MS medium (Murashige and Skoog 1962) with 0.50 mg $dm^{-3}$ BA and varying concentrations of 2,4-D (2, 3, 5 mg $dm^{-3}$). The pH of media was adjusted to 5.8 and agar (0.8 %) was added before autoclaving it for 15 min at 121 °C. For obtaining shoot tips, seeds of *Cenchrus ciliaris* were surface-sterilized as mentioned above and germinated on a moist blotting paper or on basal MS medium in dark at 25 °C for 3 - 4 d. Emerging shoot apices (3 - 5 mm) consisting of shoot apical meristem and a part of the mesocotyl were excised from 3 to 4-d-old seedlings and were used for callus induction. More than six segments were placed in each Petri plate with three replications and they were incubated in dark at 25 °C. The morphogenic potential of induced callus was assessed by sub-culturing the callus at two-weeks intervals on to fresh callus induction medium.

The callus induction frequency was determined after 20 d of inoculation and the callus mass at an interval of two weeks up to four subcultures before transferring the callus to regeneration medium. Callus colour was recorded as ice white and brown, yellow and light brown, white (creamy), milky white or egg white callus. Callus texture was recorded as vitreous, fragile, friable, compact, nodular and hard.

After 4 - 6 weeks of culture initiation, hard nodular and milky white calli induced on each of the induction media was transferred to media with low 2,4-D concentration (from 5 to 0.25 mg $dm^{-3}$) to induce somatic embryos. For shoot regeneration, cultures containing somatic embryos were incubated at 25 ± 2 °C under continuous irradiance (35 μmol $m^{-2}$ $s^{-1}$) provided by fluorescent tubes. MS media containing various concentrations of BA (1, 2, 3 and 5 mg $dm^{-3}$) or kinetin (1, 2, 3 and 5 mg $dm^{-3}$) in combination with 2,4-D (0.25 mg $dm^{-3}$) were used for germination of somatic embryos and shoot regeneration. The embryogenic callus induction frequency, the shoot regeneration frequency, and the number of shoots regenerated per calli 20 d after their subculture on the regeneration medium were recorded.

Well-formed shoots from somatic embryos (2 - 3 cm long) were transferred to different rooting media (½ MS, ½ MS + charcoal, MS + 2 % sucrose, MS + 2 % sucrose + charcoal, MS + 0.5 mg $dm^{-3}$ IBA, MS + IBA + charcoal). Prior to hardening, the *in vitro* regenerated plantlets were maintained on semi-solid agar medium containing very low amount of sucrose (0.4 %). After 20 - 21 d, the plantlets were taken out of the culture flasks and washed properly to remove the adhering agar from the roots and placed in test tubes containing sterile tap water for 2 - 3 d and subsequently they were transferred to pots containing soil. The root induction frequency and the mean number of roots formed per shoot were also recorded.

For histological studies fresh, regenerating and non-regenerating calli of various textures were fixed in Carnoy's solution (acetic acid and alcohol; 1:3) for 24 h and stored in 70 % ethanol. The selected samples were passed through tertiary butyl alcohol dehydration-infiltration series and embedded in *Petrowax* according to Johnsen (1940). Sections were cut at 10 μm thickness and Mayer's adhesive (Johnsen 1940) was used for floating the adhesive smeared slides to facilitate spreading on the sections. The sections were dewaxed using xylene and brought to dehydration through graded alcohol series. Northen's variations of fosters tannic acid, ferric chloride saffranine and fast green FCF (Johnsen 1940) stain series was used for staining of sections of calli samples. The sections were cleared with xylene and mounted in DPX mount. The sections were examined and photographed in upright binocular microscope (*Nikon*, Tokyo, Japan).

For scanning electron microscopy (SEM) studies samples of somatic embryos were fixed in 2 % glutaraldehyde in phosphate buffer (1 M) for 24 h at 4 °C. After dehydration through graded acetone series followed by storing in 70 % acetone, the samples were dried to their critical point and were mounted on aluminium stubs using epoxy resin based *Araldite* glue. Finally, the mounted specimens were coated with gold, examined and photographed in a *LEO 435VP* scanning electron microscope (*LEO 435 VP*, Cambridge, UK).

Statistical analysis was carried out using statistical package for social sciences (*SPSS*) software to analyze the influence of explants, genotypes and media combinations on callus induction. Three factorial completely randomized block design with three replications was used for the calculation of callus induction frequency of different explants. Two factorial completely randomized design with four replications was used for calculating the shoot regeneration frequency and the number of shoots/calli of two genotypes at various concentrations of cytokinins for immature inflorescence explants.

## Results and discussion

**Callus induction, growth and quality:** All the three explants (seeds, shoot tips and immature inflorescences) exhibited successful induction of callus at 2 to 5 mg $dm^{-3}$ 2,4-D along with 0.5 mg $dm^{-3}$ BA. The period of callus

induction varied from 6 d for immature inflorescences to 8 - 10 d for mature seeds, and 12 d for shoot tips. High callus induction frequencies were observed from seeds (79.4 %) and immature inflorescence explants (67.6 %) of IG-3108 (Table 1). Significant differences were observed among genotypes used, concentrations of 2,4-D and various explants, as well as interactions between explants and genotypes, and genotypes and 2,4-D concentrations. But there was no significant interaction between explants and 2,4-D.

The calli induced from all the explants were of non-embryogenic and non-regenerative type. These were multiplied in MS media containing 2 - 5 mg $dm^{-3}$ 2,4-D and 0.5 mg $dm^{-3}$ BA. Maximum callus growth was observed from explants of genotype IG-3108. While seeds showed highest callus growth at 2 mg $dm^{-3}$ 2,4-D, shoot tips and immature inflorescences recorded highest callus growth at 3 mg $dm^{-3}$ 2,4-D (Table 1). Our results are consistent with the results of Colomba *et al*. (2006) who demonstrated significant differences between various concentrations of 2,4-D on callus induction, embryogenic callus and somatic embryo formation. The morphogenic potential of the induced callus was assessed on the same induction medium. After three sub-cultures, embryogenic and nonembryogenic calli were observed. Nodular, hard, compact and milky white calli were obtained from immature inflorescences, which had high regeneration potential (Fig. 1*A*,*B*). Non-embryogenic calli induced from seeds were highly non-friable and brown or yellow (Fig. 1*C*,*D*). The calli, induced from shoot tips were loose and watery and turned black after first sub-culture. A maximum of 33.6 % embryogenic calli were observed from immature inflorescences of IG-74 (Fig. 2). There was a significant influence of genotype and the media used for sub-culturing on the embryogenic calli formed. However, interaction between media and genotype was not significant showing that these two variables had independent effect on embryogenesis.

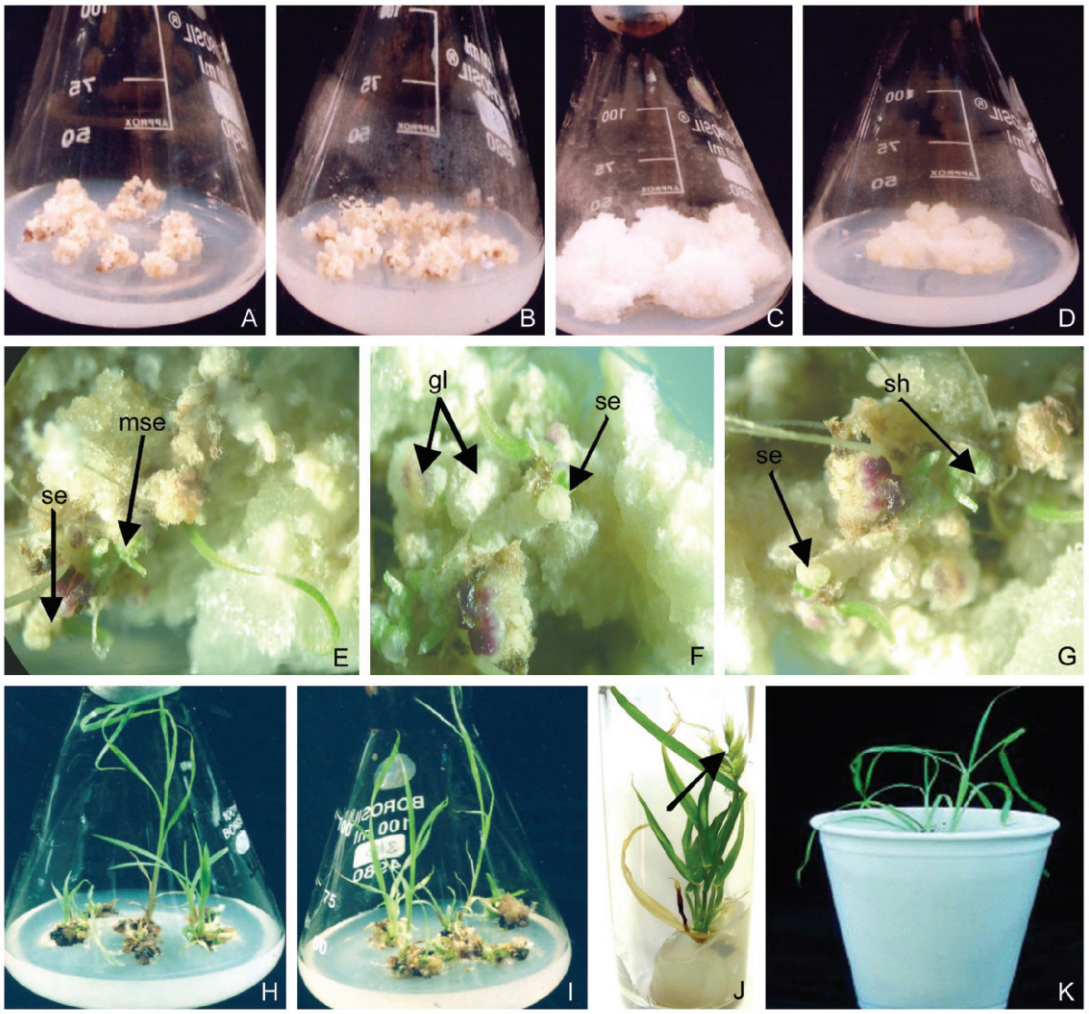


Fig. 1. Calli induced from two explants of IG-3108 and IG-74: *A*,*B* - compact, nodular and white calli from immature inflorescence *C*,*D* - loose, watery calli from seeds. Stereomicroscopic photographs showing developmental stages of somatic embryos and shoot primordia from calli induced from immature inflorescence (*E*,*F*,*G*; gl - globular embryo; se - scutellar embryo; mse - mature somatic embryo; sh - shoot primordium). Complete plantlet (*H*,*I*), fertile plant showing inflorescence (*J*; *arrow*) and regenerated plants from immature inflorescence derived calli in potting mixture (*K*).

Table 1. Frequency of callus induction and callus mass from three explants (seed, shoot tip and immature inflorescence) of genotypes IG-3108 and IG-74 on MS medium with 0.5 mg $dm^{-3}$ BA and varying concentrations of 2,4-D (2, 3 and 5 mg $dm^{-3}$) measured 14 d after inoculation. Mean ± SE, $n$ = 3.

| Explant | 2,4-D [mg $dm^{-3}$] | IG-3108 frequency [%] | mass [g] | IG-74 frequency [%] | mass [g] |
|---|---|---|---|---|---|
| Seed | 2 | 65.5 ± 1.4 | 14.0 ± 0.6 | 50.4 ± 0.9 | 11.0 ± 0.7 |
| | 3 | 79.4 ± 1.5 | 12.0 ± 0.6 | 63.4 ± 1.7 | 12.4 ± 0.2 |
| | 5 | 61.5 ± 2.6 | 5.8 ± 0.4 | 41.0 ± 1.3 | 3.8 ± 0.8 |
| Shoot tip | 2 | 23.6 ± 0.2 | 2.4 ± 0.1 | 26.8 ± 1.3 | 1.8 ± 0.6 |
| | 3 | 31.5 ± 1.9 | 3.0 ± 0.5 | 38.8 ± 0.7 | 2.0 ± 0.1 |
| | 5 | 17.8 ± 1.6 | 1.5 ± 0.2 | 20.7 ± 0.2 | 0.5 ± 0.1 |
| Inflorescence | 2 | 51.3 ± 2.5 | 8.5 ± 0.2 | 44.4 ± 1.9 | 5.4 ± 0.8 |
| | 3 | 67.6 ± 1.4 | 10.0 ± 1.0 | 51.6 ± 2.1 | 6.0 ± 1.1 |
| | 5 | 50.4 ± 1.5 | 6.0 ± 0.5 | 28.3 ± 1.2 | 3.0 ± 0.3 |

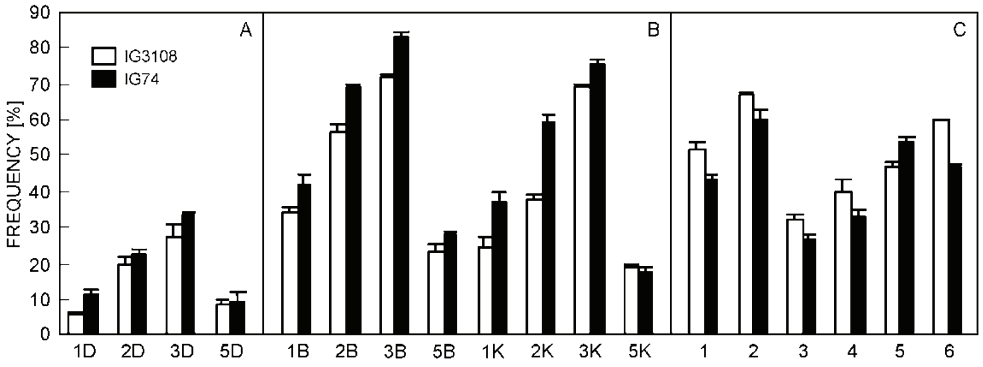


Fig. 2. Frequency of embryogenic callus induction on MS media with 1, 2, 3 and 5 mg $dm^{-3}$ 2,4-D (1D, 2D, 3D and 5D) + 0.5 mg $dm^{-3}$ BA (*A*); shoot regeneration frequency on MS media with 1, 2, 3 and 5 mg $dm^{-3}$ BA (1B, 2B, 3B, 5B) or 1, 2, 3 and 5 mg $dm^{-3}$ kinetin (1K, 2K, 3K, 5K) + 0.25 mg $dm^{-3}$ 2,4-D (*B*) and frequency of root induction on ½ MS (1), ½ MS + charcoal (2), MS + 2 % sucrose (3), MS + 2 % sucrose + charcoal (4), MS + 0.5 mg $dm^{-3}$ IBA (5) and MS + 0.5 mg $dm^{-3}$ IBA + charcoal (6) from immature inflorescence of two genotypes (*C*).

*C. ciliaris* reproduces predominantly through apomixis, which necessitated *in vitro* genetic manipulation for its genetic improvement. Higher frequency of callus induction and rate of growth was observed for all the explants except shoot tips, in various types of media in the apomictic genotype (Table 1), whereas sexual genotype exhibited better rate of embryogenic callus induction, shoot regeneration (Fig. 2) and number of shoots/callus (Table 2) from immature inflorescences. Differential response of genotypes was observed for callus growth where two kinds of calli (embryogenic and nonembryogenic) were observed. Similar observation was made by Vikrant and Rashid (2003) in *Paspalum scrobiculatum* who also reported two types of calli. Apomictic cultivar showed higher callus growth for all the explants. Since callus growth increases with increased level of endogenous auxins, it is probable that higher callus growth from apomictic explants recorded in our study could be due to higher endogenous auxin levels. In a comparative study between sexual and apomictic genotypes of *Paspalum simplex* for plant regeneration, Molinari *et al*. (2003) also observed a slightly higher number of regenerating calli with respect to the total number of calli in the sexual group than in the apomictic group. Two distinct types of calli (fast growing non-regenerative and slow growing regenerative) were also reported by Kackar and Shekhawat (1991) in *Cenchrus ciliaris.* In contrast, Kumar *et al*. (2005) reported fast growing embryogenic calli compared to slow growing non-embryogenic callus in *Dichanthium annulatum.*

**Somatic embryogenesis:** For induction of somatic embryogenesis**,** calli were sub-cultured fortnightly on MS medium containing 3 mg $dm^{-3}$ 2,4-D and 0.5 mg $dm^{-3}$ BA. When the 2,4-D concentrations were reduced from 2 - 5 mg $dm^{-3}$ to 0.25 mg $dm^{-3}$ and cytokinin concentrations increased from 0.5 mg $dm^{-3}$ to 1 - 5 mg $dm^{-3}$ in the media during subculture, most of the calli turned into cup-shaped (globular) embryogenic structures from nodular compact embryogenic calli. The globular shaped embryos developed into scutellar shaped embryos which later turned green in the same medium (Fig. 1*E*,*F*,*G*). Shoot primordia had emerged from the scutellar somatic embryos. Somatic embryogenesis was observed only

from embryogenic calli derived from immature inflorescences whereas calli from seeds became nonembryogenic while calli from shoot tips turned black. Failure to induce embryogenic calli and subsequent induction of somatic embryos from seeds and shoot tips indicated the requirement for more optimization of media. Presence of numerous floral primordia with meristematic potential might have increased embryogenic competence of calli derived from immature inflorescences.

**Plant regeneration:** Shoots were successfully induced on MS media containing varying concentrations of BA (1, 2 and 3 mg $dm^{-3}$) and 0.25 mg $dm^{-3}$ 2,4-D from calli derived from immature inflorescences (Fig. 1*H,I*). Maximum shoot induction frequency was observed in IG-74 (82.8 %) on MS medium containing 3 mg $dm^{-3}$ BA (Fig. 2), where higher number of shoots per callus (7.3) was also recorded (Table 2) compared to MS medium containing kinetin and 2,4-D. Media and genotypes significantly influenced the shoot regeneration frequency as well as the number of shoots per callus. *In vitro* flowering was observed in a few plants (Fig. 1*J*). Auxin was required in our study at a very low concentration for achieving higher rate of shoot regeneration, in contrast to earlier study by Kackar and Shekhawat (1991) who reported plant regeneration in an auxin free medium. But our results are partially corroborated by Colomba *et al*. (2006) who obtained shoot regeneration in 3 - 4 weeks after embryogenic cultures were transferred to regeneration medium containing NAA and BA. Significant genotypic differences were also observed in shoot differentiation and maintenance of regeneration capacity in sorghum (Cai and Butler 1990), barley (Hanzel *et al*. 1985) and wheat (Rajyalakshmi *et al*. 1991).

Table 2. Number of shoots per calli of two genotypes at 0.25 mg $dm^{-3}$ 2,4-D and varying concentrations of BA or kinetin (1 - 5 mg $dm^{-3}$) measured 14 d after subculture on shoot induction medium. Mean ± SE, $n = 3$.

| Cytokinin | [mg $dm^{-3}$] | IG-3108 | IG-74 |
|---|---|---|---|
| BA | 1 | 1.3 ± 0.4 | 3.3 ± 0.1 |
| | 2 | 3.0 ± 0.3 | 4.6 ± 0.8 |
| | 3 | 5.3 ± 0.7 | 7.3 ± 0.0 |
| | 5 | 1.0 ± 0.3 | 2.0 ± 0.2 |
| Kinetin | 1 | 2.0 ± 0.1 | 2.0 ± 0.2 |
| | 2 | 2.3 ± 0.2 | 3.0 ± 0.1 |
| | 3 | 3.6 ± 0.7 | 5.0 ± 0.2 |
| | 5 | 1.3 ± 0.1 | 2.6 ± 0.0 |

**Rhizogenesis and hardening:** 25-d-old regenerated plants on ½ MS containing charcoal (0.8 %) showed maximum root induction frequency in both the genotypes (Fig. 2). The genotypes, media and their interaction had significant impact on root induction frequency. These rooted plants were gradually acclimatized in the culture room and transferred to the pots containing soil and sand mixture and kept in the greenhouse for 2 or 3 d. The hardened plants were transferred to the field (Fig. 1*K*), where they showed normal growth and fertility. Rooting was also reported by Kackar and Shekhawat (1991) on ½ MS, where addition of 0.1 mg $dm^{-3}$ NAA induced root hairs as well, whereas Sankhla and Sankhla (1989) observed profusely growing well-developed roots on basal MS medium.

**Histological and scanning electron microscopic studies of somatic embryos:** Histological studies indicated that the potentially regenerating calli composed of compact cell masses of comparatively uniform and smaller size with densely staining cytoplasm and prominent nucleus intermingled with high starch granule containing cells and these regenerating cells were without intercellular spaces and exhibited meristematic activity. On the contrary, the vitreous and other non-regenerating types of calli had invariably composed of loose masses of cells of various dimensions and shapes ranging from vesicular to symphonous/polysymphonous type. SEM further confirmed different stages (globular and scutellar) of somatic embryogenesis in both the genotypes (Fig. 3).

This is the first report on histological studies with structural details of somatic embryogenesis and plant regeneration in *C. ciliaris*. Regenerative calli were clearly distinguishable from non-regenerative type as the former showed densely staining cytoplasm with prominent nucleus with high starch granules. Dome shaped shoot apical meristem with leaf primordia were visualized, which confirmed the induction of somatic embryogenesis. These types of cells were also observed in regenerating calli by earlier workers (Kohlenbach 1978, Vasil and Vasil 1982), however, most of the graminaceous crops showed regeneration through somatic embryogenesis as described in *Pennisetum americanum* (Vasil and Vasil 1982) and *P. purpureum* (Haydu and Vasil 1981). The somatic embryos exhibited the morphology of a typical monocot type of embryo showing bipolar organization, coleoptile and coleorhiza being represented for shoot and root apices bearing scutellum laterally. The same type of morphology of somatic embryos has been reported in *P. americanum* (Vasil and Vasil 1982) and finger millet (Sivadas *et al*. 1992). Scanned electron microscope studies in our study also clearly indicated globular and scutellar shaped embryoids.

**In conclusion**, efficient and reproducible *in vitro* plant regeneration was achieved through callus derived somatic embryogenesis from immature inflorescence explants of both sexual and apomictic genotypes of *C. ciliaris*. This is the first report on *in vitro* plant regeneration of a sexual genotype derived explant in *Cenchrus ciliaris*. This can be used for developing genetic transformation protocol as well as for studying the developmental events during apomictic process and to understand hormonal regulation. Many candidate genes can now be expressed to verify their function in the sexual genotype.

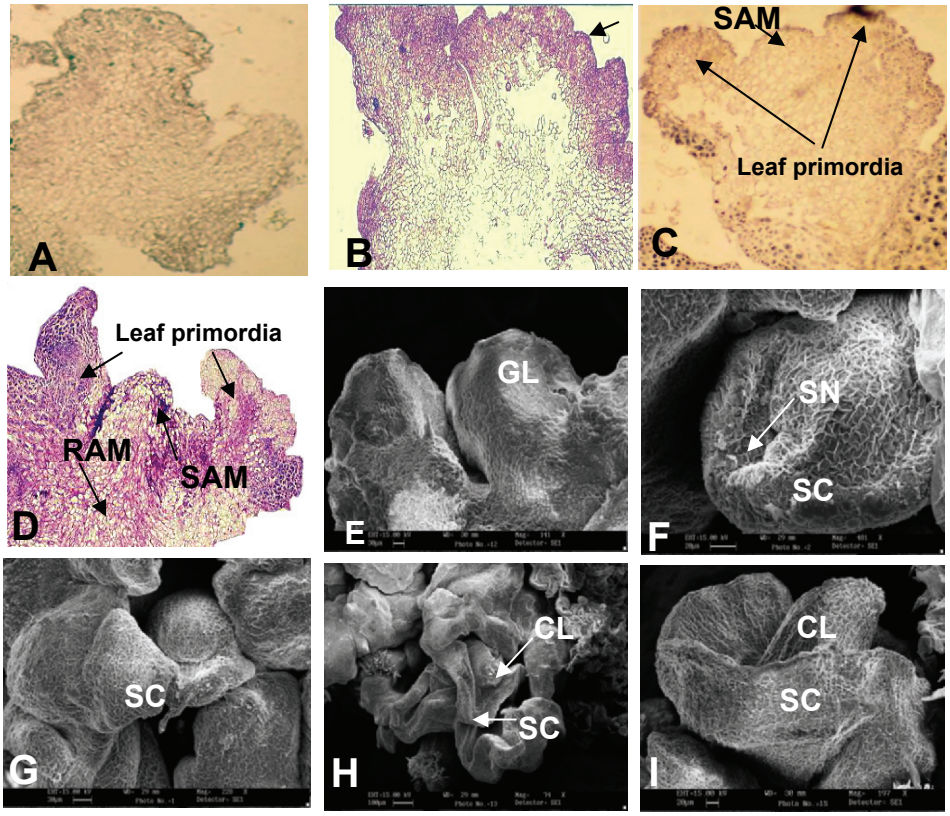


Fig. 3. Histological studies of somatic embryos induced from immature inflorescence derived callus: *A* - non regenerative type; *B* - formation of scutellar notch and establishment of polarity in a somatic embryo (*arrow*); *C*,*D* - sections of somatic embryos showing leaf primordial and dome shaped shoot apical meristem (SAM) and root apical meristem (RAM). SEM of somatic embryos of different stages: *E* - globular shaped (GL); *F* - formation of the lateral scutellar notch (SN) and scutellum (SC) in the somatic embryos; *G* - fused somatic embryos with scutellar differentiation; *H* - somatic embryos at different stages of development (CL - coleoptile); *I* - embryoid showing the scutellum and coleoptile.

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