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Optimization of callus induction and plant regeneration from germinating seeds of apomictic *Cenchrus ciliaris* L. (Poaceae)

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Abstract
A highly efficient plant regeneration system *via* somatic embryogenesis was developed using germinating seeds of *Cenchrus ciliaris* genotypes IG-3108, IG-727 and EC397537. We tested different media supplements effects on callus induction, somatic embryogenesis, shoot induction and root formation. Seeds were cultured on Murashige and Skoog (MS) medium supplemented with 3.5 - 5.5mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction. Genotype IG-727 showed highest callus induction frequency (93%) at 5.5mg/l 2,4-D as compared to other genotypes. Hard and compact embryogenic calli were transferred on MS medium supplemented with 3.5-5.5mg/l 2,4-D and 0.5 mg/l BAP for somatic embryogenesis. For plant regeneration and rooting, embryogenic calli were transferred onto MS medium supplemented with 2-3mg/l IAA and 1-2mg/l BAP with incubation at 25^{0}C under an alternative photoperiod scheme (16 h light/8h dark).The maximum shoot induction frequency (60%) and root induction frequency (48%) was observed in genotype IG-727 at MS+3mg/l IAA+2mg/l BAP and MS+3 mg/l IAA+1 mg/l BAP media combination, respectively. The plantlets were transplanted to pots, where they exhibited morphologically normal growth.

Keywords: Apomixis, *Cenchrus ciliaris*, Forage crop, Somatic embryogenesis

Buffel grass (*Cenchrus ciliaris* L.) is one of the most important forage grasses in the tropical and subtropical regions of the world. It is a perennial, polyploid and warm-season forage grass with an extensive native range from the 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). The buffel grass used as pasture grass for livestock is highly palatable to all kinds of grazing animals and also be used for silage or hay making (Khan,1970). Buffel grass reproduces asexually through seeds (apomictic mode of reproduction); the genetic improvement through conventional breeding methods is difficult, time-consuming, and restricted to selection methodologies (Echenique *et al*., 1996). On the other hand apomictic mode of reproduction may facilitate varietal improvement by genetic transformation, since no further breeding step is required to fix inheritance of the transferred trait (Vielle-Calzada *et al*., 1996). Several candidate genes for apomixis have been isolated from apomictic *Cenchrus* and transfer of apomictic trait/and or their functional validation is required an efficient protocol for genetic transformation. Limited preliminary attempts showing callus transformation in *C. ciliaris* using gene gun (Bhat *et al*., 2001) and *Agrobacterium* mediated (Batra and Kumar,2003) demonstrated the genetic transformation in this forage species. Therefore, an efficient genetic transformation protocol is required for improvement of this forage species through genetic engineering, which is not available yet. Highly efficient *in-vitro* plant regeneration is a prerequisite for successful genetic transformation. Hence, an efficient *in vitro* culture protocol for plant regeneration in this forage species would be highly valuable.

Previous studies on tissue culture in buffel grass have demonstrated plant regeneration from embryogeneic callus induced from seeds (Batra and Kumar, 2002; Yadav *et al*., 2009), immature inflorescences (Kackar and Shekhawat, 1991; Yadav *et al*., 2009) and immature and mature embryos (Ross *et al*., 1995). Compared with immature inflorescences and shoot tips, mature seeds are preferred explants as they can be stored and are available throughout the year (Ross *et al*., 1995). Batra and Kumar (2002) and Yadav *et al*. (2009) used mature seeds from *Cenchrus* as explants to investigate callus induction and plant regeneration. Although the induction frequency of callus was high, the formation of embryogenic callus was low. Hence, procedures for inducing highly regenerative embryogenic callus from mature seeds must be improved. In the present study

seeds of different *C. ciliaris* genotypes were used as explants to investigate various factors that may affect callus induction and the subsequent formation of shoots and roots. Specifically, medium supplements were optimized and the effects of different concentrations and combinations of phytohormones on plantlet regeneration were studied.

Mature and healthy seeds of two released varieties (IG-3108 and IG-727) and one exotic germplasm accession (EC397537) of *C. ciliaris* available at IGFRI Central Research Farm, Jhansi, India were used in the present study. The mature seeds were manually dehusked, sterilized in 70% ethanol for 3min and in a 0.2% $HgCl_2$ solution for 5-10min. They were then washed 3-5 times in sterilized water. Surface sterilized seeds were planted onto petri dishes (90 x 15 mm) containing 25ml MS media (Murashige and Skoog, 1962) supplemented with 500mg/l casein hydrolysate, 0.3g/l proline, 0.04g/l cystine, 30g/l sucrose and varying concentration of 2,4-D (3.5,4.5,5.5 mg/l). Media pH was adjusted to 5.8. Media were solidified with 0.8% (w/v) agar (Fisher Scientific Qualigens, Mumbai, India). For each treatment 20-25 seeds were planted onto 3 petri dishes with a total of 3 replicates per treatment. After 3-4 weeks of seed inoculation calli were formed. Callus induction frequency was recorded. Hard nodular and milky white calli induced on each of the callus induction media was transferred to same media composition with various concentration of 2,4-D (3.5,4.5, 5.5 mg/l) in combination with BAP (0.5mg/l) for embryogenesis in dark. For shoot initiation, embryogenic callus were incubated at 25^{0}C under an alternative photoperiod scheme (16 h light/8h dark) on the same media. For each treatment 15-20 embryogenic calli were planted on regeneration media in three conical flasks (100ml) as one replicate, with three replicates performed. Embryogenic callus induction frequency was recorded. After 6-7 weeks of seed inoculation, the regenerated shoots with embryogenic calli were transformed on to MS medium supplemented with various concentrations of IAA (2-3mg/l) and BAP (1-2mg/l) for shooting and rooting under the same conditions. Soot induction and root induction frequencies were recorded. Following incubation for 2 weeks, the plantlets were removed from the culture flasks and washed to remove adhering agar from the roots. The plantlets were then placed in test tubes containing sterile water for 2-3 d and subsequently transferred into pots containing soil rite. Root induction frequencies and mean numbers of roots formed per shoot were recorded.

In the present study, effect of plant growth harmones on callus induction embryogenesis and regeneration from mature seed explants were observed. The surface sterilized seeds of different genotypes were inoculated on to a basal MS medium, containing different concentrations (3.5, 4.5, 5.5 mg/l) of 2, 4-D for callus induction. After two weeks, callus formation was observed from seed explants (Fig. 1a).The callus induction frequency was observed to select the best media combination for embryogenic callus induction. The high callus induction frequencies observed were 93.20±1.86, 81.75±0.59 and 71.60±1.13 for IG-727, IG-3108 and EC397537, respectively. The highest callus induction rate (93%) occurred in IG-727 in MS media containing 5.5mg/l 2,4-D (Table 1). In grasses better response of 2, 4-D for callus induction has been observed (Batra and Kumar, 2002). Yadav *et al*. (2009) observed 79% callus induction frequency whereas, Batra and Kumar (2002) reported 80% from seeds of IG-3108. Our results are consistent with those of Batra and Kumar (2002) and Yadav *et al.* (2009) who also observed differences in callus induction due to various concentrations of 2,4-D. Our results showed significant improvement in embryogenic callus induction when compared to earlier studies, such as Yadav *et al*. (2009), which reported approximately 40% induction of embryogenic callus from immature zygotic embryos. Statistical analyses showed significant effects based on genotype and hormone treatment on the production of embryogenic callus. The significant increase in the induction of embryogenic callus may depend on endogenous hormones, which might vary from genotype to genotype in *C. ciliaris*. Somatic embryos developing from embryogenic callus turned green in the presence of light after 4–5 weeks of callus culture (Fig. 1b). The regenerated shoots from somatic embryos were transformed on to MS medium supplemented with varying concentrations of IAA (2-3mg/l) and BAP (1,2 or 3mg/l) for regeneration (Fig. 1c).The highest frequency of shoot regeneration (60%) occurred when 3mg/l IAA and 2 mg/l BAP were used. Auxin at a very low concentration was required in our study to achieve high rates of regeneration. This is in contrast to an earlier study by Kackar and Shekhewat (1991) who reported plant regeneration in auxin free media. Our results are similar to those of Batra and Kumar (2002) and Yadav *et al*. (2009) who also obtained shoot regeneration in media containing BAP and IAA. Significant genotypic differences were also observed in regeneration capacity in sorghum (Cai and Butler, 1990), barley (Hanzel *et al*., 1985) and wheat (Rajyalakshmi *et al*., 1991). Rooting of shoots was

Table 1. Influence of different concentrations of PGR on callus induction from mature seeds of *C. ciliaris* genotypes IG-727, IG-3108 and EC397537

2,4-D Concentration (mg/l)	Callus induction frequency (%)		
	IG-727	IG-3108	EC397537
3.5	81.72±1.49	81.75±0.59	71.60±1.13
4.5	70.61±1.30	66.18±0.95	56.78±1.89
5.5	93.20±1.86	59.99±1.11	48.38±1.54

successful on media containing MS + 2 mg/l IAA + 1 mg/l BAP, MS +3mg/l IAA + 1 mg/l BAP, MS +2 mg/l IAA + 2 mg/l BAP, and MS +3 mg/l IAA + 2 mg/l BAP. The maximum (48%) root induction frequency occurred using genotype IG-727 with MS + 3mg/l IAA + 1mg/l BAP. Well rooted plants were transferred to pots and grew normally (Fig. 1d) when compared to non-regenerated mother plants. Rooting was reported by Sankhla and Sankhla (1989) who reported profusely growing well developed roots on basal MS medium. Whereas Kackar and Shekhawat (1991) and Yadav *et al*. (2009) observed root hair as well on root in ½ MS with the addition of NAA. In conclusion, we have optimized the efficiency and reproducibility of *in vitro* procedures for high frequency plant regeneration of *C. ciliaris* from seed explants through callus derived somatic embryogenesis. This report also suggests the possibility

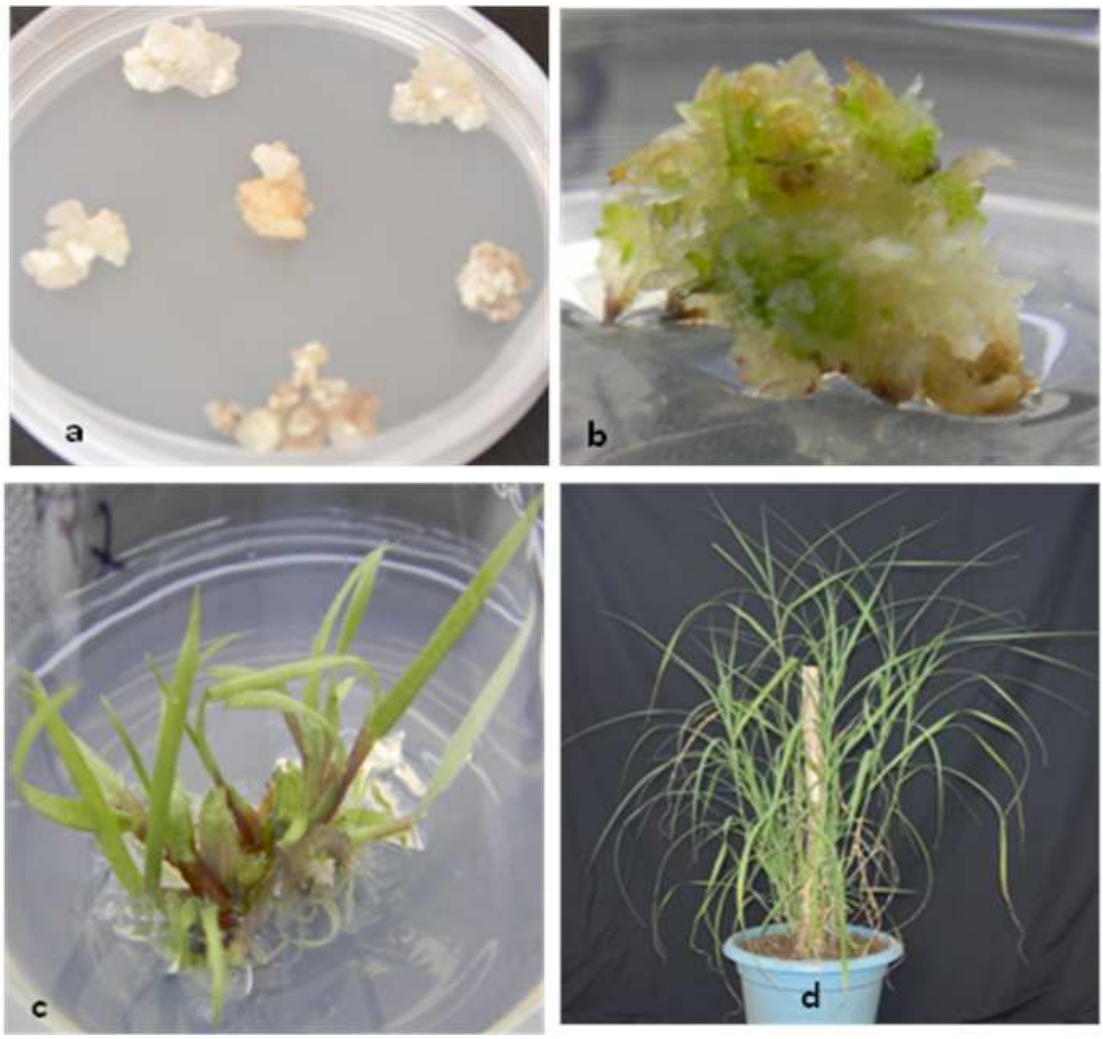

Fig 1. Plant regeneration in *C. ciliaris* genotype IG-727 (a) Callus induction (b) Shoot organogenesis (c) Root organogenesis (d) Plant transferred in pot

of improving regeneration procedures for specific genotypes. Our regeneration system provides a foundation for the genetic transformation of *C. ciliaris*, thus enabling genetic engineering improvements of this important forage grass.

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