In Vitro Plant Regeneration *via* Callus Induction in a Rare Sexual Plant of Buffelgrass (*Cenchrus ciliaris* L.)

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In vitro plant regeneration *via* callus induction in a rare sexual plant of Buffelgrass (*Cenchrus ciliaris* L.)

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Introduction

Buffelgrass (*Cenchrus ciliaris* L.) is one of the most important perennial forage grasses of the world. It is a polyploid forage grass suited to pastures, range-lands, tropics and sub-tropics of Australia, South Africa, and India. It reproduces predominantly through apomixis which provides a means of clonal propagation through seeds. In many of the plants apomixis shows dominance over sexuality, hence occurrence of obligate sexual plant in natural population is rare and over the time apomictic individuals outnumber sexuals. Being protogynous in nature, cross pollination from neighbouring apomictic plants leads to the production of either facultative or obligate apomictic genotypes. Apomictic mode of reproduction in buffelgrass makes its genetic improvement through conventional breeding difficult, time consuming and restricted to selection of elite lines from natural variants. Lack of sexual reproduction in *C. ciliaris* has severely limited the possibilities of genetic improvement of this species by hybridization (Yadav *et al*., 2012).
Sexual plant in *C. ciliaris* has occasionally been identified. A sexual plant of buffelgrass *w*as identified earlier in the germplasm collection of *C. ciliaris* (Kumar *et al*., 2010) which is perennial in nature and shows characteristic features different from normal apomictic buffelgrass. Under natural conditions the plant shows very poor growth and survival, but under intensive care, the plant survives well and flowers 3-4 times in a year. The plant is very useful for genetic improvement of this species through hybridization and identification of gene(s) for apomixis.

Since the sexual plant is self-incompatible and reproduces sexually, it can be maintained by vegetative propagation or multiplied by tissue culture. Mature seeds have been one of the preferred explants for tissue culture because of its ready availability through-out the year (Kumar and Bhat, 2012), the sexual plant does not produce seeds on self-pollination. Hence, we used different parts of the plant to standardize tissue culture protocol. Here we report, high frequency plant regeneration *via* callus induction from immature inflorescence of the sexual plant which would be very useful in maintenance, multiplication and genetic manipulation of the plant with a gene of interest.

Materials and Methods

Leaf-base, nodes and immature inflorescence of the obligate sexual buffelgrass were used as explants for callus induction. The explants were surface sterilized using 70% ethanol for 1 min followed by two washes with sterile distilled water prior to their treatment with 0.2% $HgCl_2$ for 5 min. The explants were then washed four times with sterile distilled water. The explants were cultured on MS medium supplemented with phytohormone (2,4-D, Kinetin and BAP) in dark at 25±2°C for 3 weeks for callus induction. Calli induced from the explant were sub-cultured once on the same freshly prepared medium and embryogenic Calli were transferred to regeneration medium supplemented with Kinetin and BAP, cultured in light at 25±2°C for 4 weeks under 16 h photoperiod for shoot organogenesis. Regenerated shoots were sub-cultured on regeneration medium and cultured for another 3 weeks under similar conditions. Regenerated shoot were transferred to MS medium containing IBA and activated charcoal, and cultured in light for 3 weeks for root organogenesis. Regenerated plants with shoots and roots were transplanted into pots filled with sterilized soil. The plants were hardened and grown under field conditions.
For molecular analysis, genomic DNA was isolated from 100 mg leaf of the regenerated plants. PCR was performed using the genomic DNA to screen the tissue cultured plants for mode of reproduction using a sexuality-specific SCAR marker (Kumar *et al*., 2010) and two apomixis-specific SCAR markers (Apo-C470 and Apo-C930). Embryo sac analysis of the tissue cultured plants was carried out using pistil-clearing technique (Young *et al*., 1979) to confirm the mode of reproduction.

Results and Discussion

In an attempt towards standardization of tissue culture protocol for the rare obligate sexual buffelgrass plant, different parts of the plant were used as explants. Out of the explants tested, callus induction was observed only from immature

inflorescence. Callus induction was observed after 7-8 days of culturing immature inflorescences in dark with the frequency of 27-100%. Although, MS medium supplemented with 2,4-D (6.0 mg/L) showed the highest callus induction frequency, regenerability of the calli was found to be poor (22%) probably because of the carry-over effect of 2,4-D in the calli used for shoot organogenesis. Initially the most abundant form of calli was rapidly growing, loosely packed calli but on sub-culturing the calli after 21 days, white, loosely packed, friable and globular embryogenic calli appeared.

Among the different combinations of phytohormones used for shoot organogenesis, the best response was observed on MS medium supplemented with 2.0 mg/L Kinetin. Only globular, white, friable calli were found to have regenerability. Greening of the calli within 2 weeks of culture and shoot organogenesis after 3 weeks of culture was observed on MS medium supplemented with Kinetin (2.0 mg/L) with a regeneration frequency of 42%. BAP also produced plants from embryogenic calli, but regeneration frequency as well as health of the regenerated plants was better on Kinetin supplemented medium. Only one sub-culturing to avoid any somaclonal variation might be responsible for the observed lower regeneration frequency. Root organogenesis started on regeneration medium itself, however profuse rooting was observed only after transferring the regenerated shoots to rooting medium. The best response for rooting was observed on MS medium with IBA (2.0 mg/L) and activated charcoal (2.0 g/L). We could regenerate up to 30 plants from the embryopgenic calli induced from a single inflorescence. When rooted shoots were transferred to soil in pots and hardened inside the culture room, 21 plants could establish successfully in soil (Fig. 1) with 70% survival of the tissue cultured plants. The tissue cultured plants showed normal flowering and seed setting on open-pollination.

Fig. 1: Plants regenerated from calli induced on a single piece of immature inflorescence.

Sexuality-specific SCAR marker produced a specific band of 260 bp in all the tissue cultured plants (Fig. 2a). This indicated sexual mode of reproduction in the tissue cultured plants. Apomixis-specific SCAR markers did not show amplification in any of the tissue cultured plants (Fig. 2b). However, the markers showed specific bands in the apomictic plant. This did not ruled out facultative sexual mode of reproduction in the tissue cultured plants, therefore to confirm sexual mode of reproduction embryological analysis of the tissue cultured plants was carried out. Embryo sac analysis showed presence of eight-nucleated (sexual) embryo in all the 25 pistils analyzed from the tissue cultured plants. Thus, the tissue cultured plants maintained genetic fidelity.

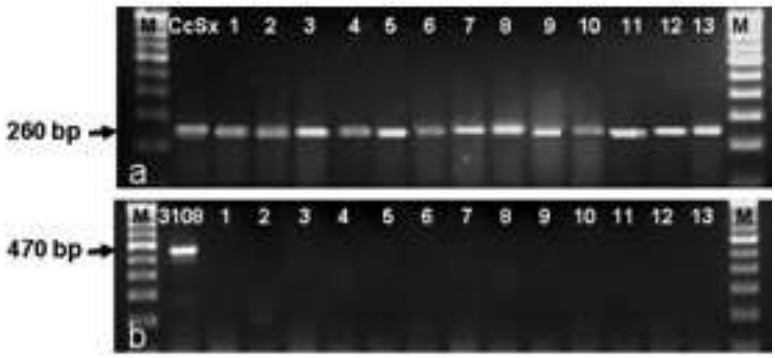

Fig. 2: Molecular analysis of tissue cultured *C. ciliaris* plants for mode of reproduction using SCAR markers. (**a**) Sexuality-specific

SCAR marker (CcSex-260) showing amplification in mother sexual plant (CcSx) and all the tissue cultured plants. (**b**) Apomixis-specific SCAR marker (Apo-C470) showing amplification (470 bp) in apomictic (3108) plant but no

amplification in the tissue cultured plants. M = 100 bp DNA ladder; CcSx = mother sexual (IGFRI-CcSx/08/1) plant; 3108 = an apomictic (IG-3108) plant; 1-13 = thirteen different tissue cultured plants *C. ciliaris*.

Conclusion

Since the obligate sexual buffelgrass is meager and does not produce seeds on self-pollination, multiplication and maintenance of the genotype through tissue culture would be a reliable strategy. Embryogenic calli were induced on MS medium with 2,4-D (3.5 mg/L) and shoot organogenesis on MS medium with Kinetin (2.0 mg/L). Rooting from the regenerated shoots was achieved on MS medium containing IBA (2.0 mg/L) and activated charcoal (2.0 g/L). Molecular analysis of the tissue cultured plants using SCAR markers and confirmation by embryo sac analysis proved reliability of the protocol in maintaining plant genotype. The tissue culture protocol would be very useful for clonal multiplication and genetic manipulation of this rare genotype of *C. ciliaris*.

References

Kumar, S. and V. Bhat. 2012. High-frequency direct plant regeneration via multiple shoot induction in the apomictic forage grass *Cenchrus ciliaris* L. *In Vitro Cell. Dev. Biol*.–Plant 48: 241-248.

Kumar, S., A. Chandra, M. G. Gupta and G. P. Shukla. 2010. Molecular and embryological analyses of rare sexual plant in buffelgrass (*Cenchrus ciliaris* L.). *Range Mgmt & Agroforestry* 31:36-40.

Kumar, S., N. Sahu and A. Singh. 2015. High-frequency *in vitro* plant regeneration via callus induction in a rare sexual plant of *Cenchrus ciliaris* L. *In Vitro Cell. Dev. Biol.-Plant* 51: 28-34.

Yadav, C. B., Anuj, S. Kumar, M. G. Gupta and V. Bhat. 2012. Genetic linkage maps of the chromosomal regions associated with apomictic and sexual modes of reproduction in *Cenchrus ciliaris*. *Mol. Breeding* 30: 239-250.

Young, B. A., R. T. Sherwood and E. C. Bashaw. 1979. Cleared pistil and thick sectioning techniques for detecting aposporous apomixis in grasses. *Can. J. Bot.* 57: 1668-1672.

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