In Vitro Cell.Dev.Biol.—Plant (2015) 51:28–34
DOI 10.1007/s11627-015-9664-2

MICROPROPAGATION

High-frequency *in vitro* plant regeneration via callus induction in a rare sexual plant of *Cenchrus ciliaris* L.

Suresh Kumar · Neha Sahu · Archana Singh

Received: 3 January 2014 / Accepted: 8 January 2015 / Published online: 29 January 2015 / Editor: Baochun Li

Abstract *Cenchrus ciliaris* L. is an important perennial forage grass that grows throughout the semi-arid tropics. It reproduces predominantly by apomixis, which provides a means of clonal propagation through seeds. The absence of sexual reproduction in *C. ciliaris* limits the possibilities of genetic improvement by hybridization. A rare obligate sexual plant of *C. ciliaris* (IGFRI-CcSx-08/1) that is self-incompatible in nature requires vegetative propagation to maintain its genotype. *In vitro* culture is one of the possible ways for clonal multiplication and a prerequisite for genetic manipulation. Here, we report high frequency *in vitro* plant regeneration via callus induction from immature inflorescences of this obligate sexual plant. Embryogenic calli were induced on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (3.5 mg L^{-1}), and shoot organogenesis was obtained on MS medium supplemented with kinetin (2.0 mg L^{-1}). Rooting from the regenerated shoots was achieved on MS medium containing indolebutyric acid (2.0 mg L^{-1}) and activated charcoal (2.0 g L^{-1}). The survival rate of the plants under *ex vitro* conditions was 70%. Molecular analysis of the tissue cultured plants using sequence characterized amplified region (SCAR) markers and by embryo sac analysis revealed obligate sexual reproduction in all the regenerated plants. The tissue cultured plants were true to the mother plant type. The high frequency of *in vitro* plant regeneration achieved by this protocol would be very useful for clonal multiplication and genetic manipulation of this rare genotype of *C. ciliaris*.

Keywords Apomixis · Forage grass · Immature inflorescence · SCAR marker · Sexual reproduction

S. Kumar · N. Sahu
Division of Crop Improvement, Indian Grassland and Fodder Research Institute, Jhansi 284003, India

S. Kumar (✉) · A. Singh
Division of Biochemistry, Indian Agricultural Research Institute, New Delhi 110012, India
e-mail: sureshkumar3_in@yahoo.co.uk

S. Kumar
e-mail: sureshkumar@iari.res.in

Introduction

Cenchrus ciliaris L. is an important perennial, polyploid (2n=4x=36) forage grass grown throughout the tropical and subtropical regions of the world. It is suitable for pastures and rangelands of Australia, South Africa, and India (Bhat *et al.* 2001). *C. ciliaris* is highly palatable to grazing animals, but its high (3–5%) lignin content reduces its digestibility. It reproduces predominantly through aposporous apomixis (see Kumar *et al.* 2010a). Apomixis provides a means of clonal propagation through seeds because the progeny are genetically identical to the mother plant. Though apomixis is prevalent in several plant species, it predominantly occurs in the Rosaceae, Compositae, and Poaceae families. In most species, apomixis shows dominance over sexuality and obligate sexual plants in natural populations are rare. Over time, apomictic individuals outnumber sexual ones. *C. ciliaris* is protogynous in nature, and cross-pollination from neighboring apomictic plants leads to the production of either facultative or obligate apomictic progenies.

Sexual plants are required for hybridization and development of mapping populations for genetic and molecular studies on apomixis. Obligate sexual *C. ciliaris* plants have occasionally been identified (Bray 1978; Kumar *et al.* 2010a). A natural variant within an Indian accession in the germplasm collections of *C. ciliaris* was identified as an obligate sexual plant (Kumar *et al.* 2010a). This plant is morphologically distinct from common apomictic *C. ciliaris* plants. It is dwarf, perennial, and produces small panicles with relatively few,

awnless spikelets. It has thick, small leaves and shorter internodes. The plant can be maintained by vegetative propagation using slips (divisions of clump with some roots and shoots). Under field conditions, the plant shows very poor growth, but with intensive care the plant grows well and flowers three times in a year under Jhansi (25° 27′ N, 78° 35′ E and 271 m.a.s.l.) conditions. The plant is tetraploid (2n=4x=36), protogynous, self-incompatible, and bears viable pollen. It shows poor seed setting on cross-pollination and produces genetically diverse progenies. The plant has been registered as a novel germplasm (INGR No. 11062) with National Bureau of Plant Genetic Resources, New Delhi (Kumar *et al.* 2013a).

Molecular markers linked to apospory have been reported in several grasses including *C. ciliaris* (Gustine *et al.* 1997; Pessino *et al.* 1998; Ozias-Akins *et al.* 1998; Martinez *et al.* 2003; Dwivedi *et al.* 2007). A sequence characterized amplified region (SCAR) marker linked to sexual reproduction in *C. ciliaris* was also identified (Kumar *et al.* 2010b). The SCAR marker (CcSex-260) produces a specific band in obligate sexual as well as facultative plants but not in obligate apomictic *C. ciliaris* plants. Recently, two apomixis-specific SCAR markers (Apo-C470 and Apo-C930) were identified and validated (Kumar *et al.* unpublished results).

Tissue culture and transformation protocols for the sexual plant (IGFRI-CcSx-08/01) would be desirable for basic studies on sexuality/apomixis. The choice of explants is an important factor that influence *in vitro* plant regeneration and success in genetic transformation (Batra and Kumar 2003; Yadav *et al.* 2009; Kumar and Bhat 2012). There are several reports on efficient and responsive plant regeneration via callus induction from different explants in *C. ciliaris* (Kackar and Shekhawat 1991; Ross *et al.* 1995; Batra and Kumar 2002; Colomba *et al.* 2006; Yadav *et al.* 2009). Mature seeds are the preferred explants because of their ready availability throughout the year (Batra and Kumar 2002, 2003; Kumar and Bhat 2012), but explants obtained from vegetative parts will be required from this self-incompatible sexual plant.

The present study was undertaken to develop an *in vitro* culture and plant regeneration protocol for this obligate sexual, self-incompatible *C. ciliaris* plant and to assess genetic fidelity of the regenerated plants using molecular markers. This standardized protocol will be very useful for maintenance and multiplication of this plant as well as for genetic manipulation and basic studies of apomixis/sexuality.

Materials and Methods

Explants and their preparation. Leaf-base, node, and immature inflorescence explants (still covered by the boot leaf) were collected in the morning from the obligate sexual *C. ciliaris* plant (IGFRI-CcSx-08/01) maintained in pots at Indian Grassland and Fodder Research Institute, Jhansi, India. The explants were surface sterilized by immersion in 70% (*v/v*) ethanol for 1 min, then washed twice with sterile distilled water, immersed in 0.2% (*w/v*) $HgCl_2$ and 0.1% (*v/v*) Tween-20 for 5 min, and then washed four times with sterile double distilled water. Immature inflorescences were cut into three pieces (1–1.5 cm in length).

In vitro culture media and conditions. Explants were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with sucrose (30 g L^{-1}) and plant growth regulators (PGRs; dependent on the treatment and stage of development), adjusted to pH to 5.8 with 1.0 N NaOH, and solidified with Gelrite (Duchefa Biochemie B.V., Haarlem, The Netherlands) (2.5 g L^{-1} for callus induction and plant regeneration and 2.0 g L^{-1} for rooting). Media were sterilized by autoclaving at 121°C for 20 min. When cooled to bearable warmth, the medium was dispensed into sterile culture vessels (30 ml per 90×15 mm Petri dishes or 50 ml per GA-7 Magenta boxes, Sigma, St. Louis MO).

Callus induction and plant regeneration. Explants (3–5 per 90 mm Petri plate and 15 per treatment) were placed horizontally on medium supplemented with one of several PGR treatments (Table 1) and cultured in darkness at 25±2°C for 3 wk. Callus induction frequency and quality (based on physical appearance) of the callus were recorded. The calli were

Table 1. Callus induction from immature inflorescences of a rare sexual plant of *Cenchrus ciliaris* (IGFRI-CcSx-08/1) on MS medium supplemented with different concentrations of growth regulators

2,4-D (mg L^{-1})	Kinetin (mg L^{-1})	BAP (mg L^{-1})	Callus induction frequency (%)[a]	Quality of callus[b]
0.0			00.0	N A
1.0			34.3±1.3b	Medium
2.0			45.7±1.7c	Poor
3.0			67.3±1.3d	Good
3.5			88.0±2.0h	Good
4.0			47.7±2.3c	Poor
4.5			86.3±2.3g	Good
5.0			46.3±1.7c	Poor
5.5			67.7±1.7d	Medium
6.0			99.7±0.3h	Good
2.0	0.5		47.7±1.3c	Medium
3.0	0.5		79.3±1.7f	Medium
4.0	0.5		75.7±1.3e	Good
2.0		0.5	29.7±1.3a	Poor
3.0		0.5	30.3±1.3a	Poor
4.0		0.5	30.0±1.7a	Poor

[a] Values represent means ± SE. Means within a *column* followed by different *lowercase letters* are significantly different ($p \leq 0.05$)

[b] Good—Friable, white embryogenic calli; Medium—Whitish, muddy calli; Poor—Muddy, translucent calli.

subcultured onto fresh medium, and after an additional 3 wk, white embryogenic calli were transferred to regeneration medium supplemented with various cytokinin treatments (Table 2) in Petri plates and cultured for 4 wk in light (40 μmol m^{-2} s^{-1}, 16 h photoperiod provided by F40/CW fluorescent tubes, Philips India Limited, Gurgaon, India) at 25±2°C. The cultures were scored for number of shoots and regeneration frequency. Regenerated shoots were subcultured onto fresh medium in Magenta boxes, cultured for another 3 wk under similar conditions, then transferred to Magenta boxes containing rooting medium with various auxin treatments (Table 3), and cultured under similar conditions for 3 wk. Regenerated plants with well-developed roots were removed and washed carefully under running tap water to remove medium adhering to roots. The plants were transplanted into sterilized soil in pots, grown inside the culture room for 1 wk, shifted to shade in the open environment for 3–4 d, and finally grown under field conditions.

Polymerase chain reaction (PCR)-based analysis of regenerated plants. Genomic DNA was isolated using the DNeasy Plant Mini Kit (QIAGEN Gmbh, Germany). About 100 mg young leaf tissue from each regenerated plant was ground into fine powder using liquid nitrogen. DNA concentrations were quantified by agarose gel (0.8%) electrophoresis using the 1 Kb DNA ladder/molecular weight marker as the standard (MBI Fermentas, Lithuania). The mode of reproduction was analyzed using a sexuality-specific SCAR marker (Kumar *et al.* 2010b) and two apomixis-specific SCAR markers (Kumar *et al.*, unpublished results). Amplifications were performed in 25 μl reaction volumes, each containing 100 ng genomic DNA, 200 μM each dNTP, 10 pmol of primers, 2 mM $MgCl_2$, 1× Taq buffer, and 3 U Taq DNA polymerase. Amplification conditions were 94°C for 5 min followed by 38 cycles of (94°C for 60 s, 60°C for 60 s, and 72°C for 30 s), followed by a final extension at 72°C for 5 min. The PCR products were visualized by agarose gel (1.4%) electrophoresis at constant voltage (2 V cm^{-1}) for 2 h.

Table 2. Effect of cytokinin treatments on shoot organogenesis from embryogenic calli induced from immature inflorescences on MS medium supplemented with 3.5 mg L^{-1} 2,4-D

Cytokinin (mg L^{-1})	No. of calli cultured	No. of shoots regenerated[a]	Regeneration frequency (%)
BAP 1.0	46	09±1.0	19.6a
BAP 2.0	46	16±1.7	34.8c
BAP 3.0	46	15±1.7	32.6c
Kinetin 1.0	38	11±1.3	29.0b
Kinetin 2.0	38	16±2.0	42.1e
Kinetin 3.0	38	15±1.3	39.5d
BAP 1.0 + Kinetin 1.0	44	15±1.3	34.1c
BAP 2.0 + Kinetin 1.0	44	16±1.7	36.4c
BAP 1.0 + Kinetin 2.0	44	17±2.0	38.6d
BAP 2.0 + Kinetin 2.0	44	15±1.7	34.1c

[a] Values represent means ± SE. Means within a *column* followed by different *lowercase letters* are significantly different ($p \leq 0.05$)

Table 3. Effect of auxin treatments on rooting of regenerated shoots derived from MS medium supplemented with 2.0 mg L^{-1} kinetin. All media contained activated charcoal (2.0 g L^{-1})

Auxin[a] (mg L^{-1})	No. of shoots cultured for rooting	Rooting frequency (%)[b]
IAA 1.0	16	48.3±1.3b
IAA 2.0	16	65.3±1.3d
IAA 3.0	16	64.7±1.7d
IBA 1.0	16	41.3±1.3a
IBA 2.0	16	70.7±1.7e
IBA 3.0	16	66.7±1.3d
NAA 1.0	14	48.0±2.0b
NAA 2.0	14	65.0±1.7d
NAA 3.0	14	62.0±1.3c

[a] *IAA* indole-3-acetic acid, *IBA* indolebutyric acid, *NAA* naphthaleneacetic acid

[b] Values represent mean of the number of shoots showed rooting. Means within a *column* followed by different *lowercase letters* are significantly different ($p \leq 0.05$)

Embryo sac analysis of tissue cultured plants. Analysis was carried out using the modified pistil-clearing technique (Young *et al.* 1979). Inflorescences were collected at the 75% stigma exertion stage and fixed in a mixture of 95% ethanol (40 ml), 40% formaldehyde (3 ml), glacial acetic acid (3 ml), and distilled water (14 ml) for 24–48 h, then transferred to 70% ethanol and stored at 4°C for up to 1 wk. Pistils were excised and passed through an ascending series (85 and 100%) of ethanol, then placed in ethanol and methyl salicylate (1:1 ratio for 2 h, then a 1:3 ratio for 4 h), and finally incubated overnight in 100% methyl salicylate. Slides were prepared by mounting the processed pistil with methyl salicylate and examined under differential interference contrast microscope. At least 25 pistils (from 3 to 4 inflorescences) were analyzed from each of the tissue cultured plants.

Statistical analysis. All experiments were analyzed using a completely randomized design with three replicates. Analysis of variance was conducted, and Duncan's multiple range test at $p \leq 0.05$ was used to compare the means of treatments.

Results and Discussion

Response of different explants to in vitro culture. The type of explants is important in determining its response to *in vitro*

culture and callus induction frequency (Gonçalves and Romano 2013; Nordine *et al.* 2014), which is likely due to their differential reactivity to medium components. None of the three types of explants produced callus on PGR-free MS medium. Callus was obtained from immature inflorescences when cultured on PGR-containing media (Fig. 1*a*, *b*), but not from leaf-base or nodal explants (Fig. 1*c*).

Callus was initiated from immature inflorescences after 7–8 d. Callus induction frequencies after 3 wk were influenced significantly by treatment and ranged from 30 to 100% (Table 1). The 6.0 mg L^{-1} 2,4-D treatment resulted in a callus induction frequency of 100%. Rapidly growing, friable calli turned white and embryogenic after 3 wk of culture (Fig. 1*d*). In a previous study, callus induction frequency of 67.6% was reported from immature inflorescences of *C. ciliaris* (IG-3108) on MS medium supplemented with 3.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} N^6-benzyladenine (Yadav *et al.* 2009) This frequency was obtained with only 2,4-D (3.0 mg L^{-1}) in this study using the IGFRI-CcSx-08/1 genotype. Genetic variability for response to tissue culture has been reported in *C. ciliaris* (Batra and Kumar 2002; Yadav *et al.* 2009).

Plantlet regeneration. Greening of the calli was observed within 1 wk of culture on regeneration medium. Shoot organogenesis started after 2 wk. After 4 wk, the highest regeneration frequency (42.10%) was observed on MS medium supplemented with 2.0 mg L^{-1} kinetin (Table 2). Shoots regenerated on kinetin-supplemented medium (Fig. 1*e*) had better leaf morphology and shoots were longer than those regenerated on BAP-containing medium. The combination of kinetin and BAP did not improve regeneration frequency compared to kinetin alone. Although the highest observed regeneration frequency was about half of that reported (82.8%) by Yadav *et al.* (2009), all the regenerated plants from this study were found to be true to the mother plant type. The lower regeneration frequency may be attributed to only one subculture, resulting in lesser amounts of embryogenic calli, compared to three repeated subcultures by Yadav *et al.* (2009). Repeated

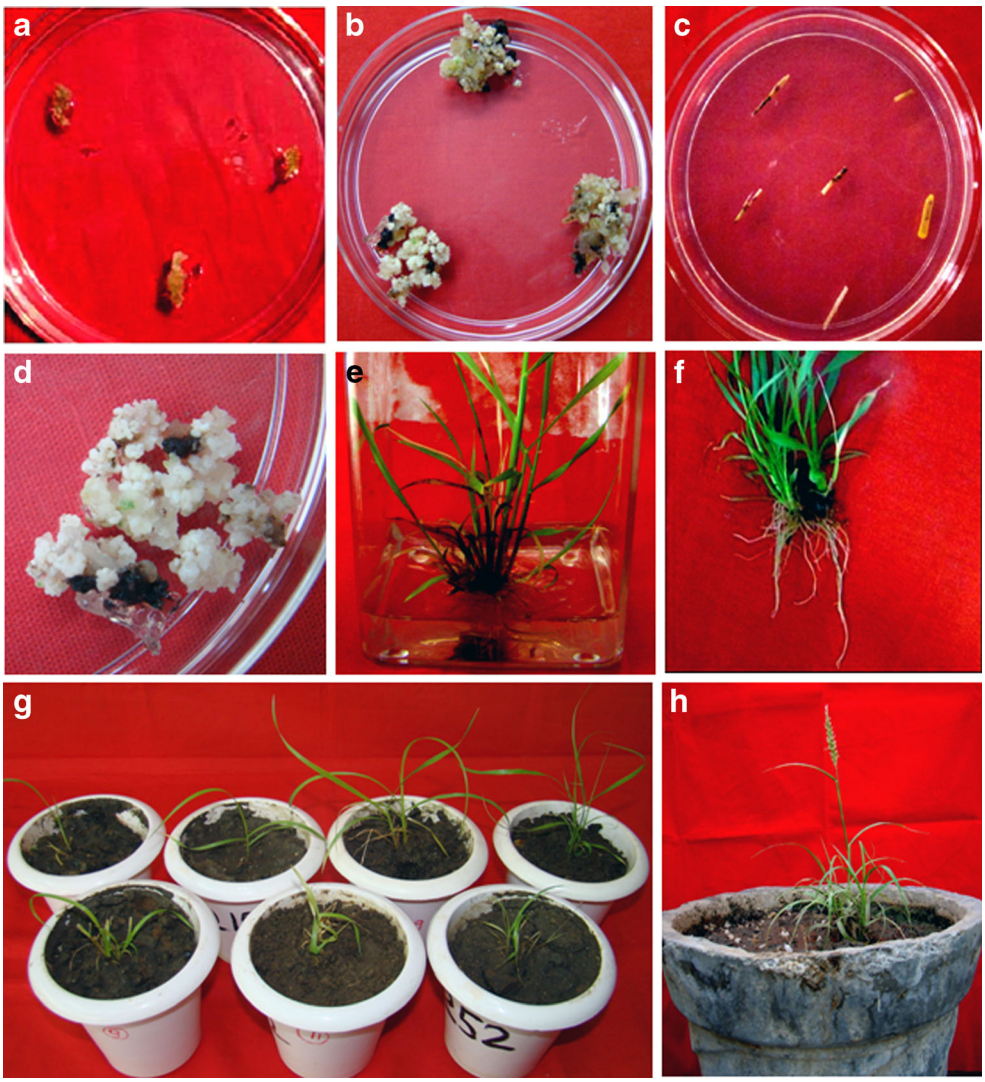

Figure 1. *In vitro* culture and clonal multiplication of a rare obligate sexual *C. ciliaris* plant. (*a*) Immature inflorescence explants; (*b*) calli induced from immature inflorescence explants; (*c*) nodal explants after 3 wk of culture on callus induction medium; (*d*) embryogenic calli after one subculture; (*e*) shoots regenerated from embryogenic calli; (*f*) rooting of regenerated shoots; (*g*) plants regenerated from the calli induced on a piece of immature inflorescence; (*h*) a tissue cultured obligate sexual *C. ciliaris* plant flowering.

subculture of calli was not practiced in this study to avoid somaclonal variation. Somaclonal variation in plants regenerated from repeatedly subcultured calli has been reported, particularly in grasses (Gupta *et al.* 1997, 1998), and repeated subculture may be used to create genetic variability in apomictic species like *C. ciliaris*.

Root organogenesis from the regenerated shoots initiated on regeneration medium, but profuse rooting could be obtained only after transferring the shoots to rooting medium. The best response for rooting (70.66%) was observed on MS medium supplemented with 2.0 mg L^{-1} indolebutyric acid and 2.0 g L^{-1} activated charcoal (Table 3). Addition of activated charcoal in the rooting medium resulted in a significant improvement in rooting (Fig. 1*f*) (data not shown). Similar observation was reported by Kumar *et al.* (2013b).

A lesser content of Gelrite (2.0 g L^{-1}) in the rooting medium minimized damage to the roots while removing the plants from the rooting medium. When the rooted shoots were transferred into soil in pots (Fig. 1*g*) and gradually acclimatized, the survival rate of the plants was 70%. Rooting in *C. ciliaris* has been reported on basal MS medium (Sankhla and Sankhla 1989), half-strength MS medium (Kackar and Shekhawat 1991), and auxin-supplemented MS media (Batra and Kumar 2002; Yadav *et al.* 2009; Kumar and Bhat 2012).

We could regenerate up to 30 plants from the embryogenic calli induced from three pieces of an immature inflorescence, and 21 plants could be established in soil under field conditions (Fig. 1*h*). This number is more than the number of seeds obtained from the inflorescence on cross-pollination. Due to the smaller size of the inflorescences (with only 9–20 healthy spikelets), we observed 5–13 well-filled mature seeds per inflorescence from a cross-pollinated sexual plant.

Molecular marker analysis. Sexuality-specific SCAR marker (CcSex-260) produced a specific band of 260 bp in all the tissue cultured plants as well as in the obligate sexual mother plant (Fig. 2*a*). This indicated the sexual mode of reproduction in the tissue cultured plants. Apomixis-specific SCAR markers (Apo-C470 and Apo-C930) did not show amplification in any of the tissue cultured plants (Fig. 2*b*, *c*). However, these SCAR markers showed specific bands (470 and 930 bp, respectively) in apomictic (positive control) plant. This clearly indicated the absence of the apomictic mode of reproduction in the tissue cultured plants. Because the CcSex-260 SCAR marker gives amplification in both the obligate sexual and facultative plants (Kumar *et al.* 2010b), these tests did not rule out the facultative sexual reproduction. To confirm the obligate sexual mode of reproduction, embryo sac analysis was carried out.

Embryo sac analysis. Microscopic examination of all cleared pistils from the tissue cultured plants and from the mother sexual *C. ciliaris* plant (Fig. 3*a*) revealed the presence of an eight-nucleated embryo sac typical for sexual reproduction with three antipodal cells (Fig. 3*b*). Pistils from IG-3108 plant (Fig. 3*c*), used for cross-pollination of the tissue cultured plants, showed four-nucleated embryo sacs (one egg cell, two synergids, and one polar nucleus) without antipodal cells typical of apomictic reproduction (Fig. 3*d*). The presence of antipodal cells in the embryo sac categorizes development as sexual, and the absence of antipodal cells categorizes it as

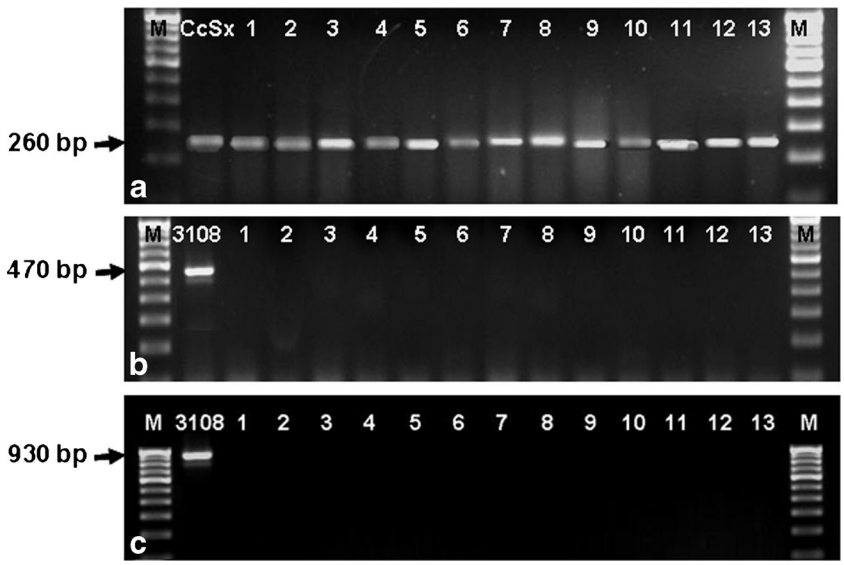

Figure 2. Molecular analysis of tissue cultured *C. ciliaris* plants for mode of reproduction using SCAR markers. (*a*) Sexuality-specific SCAR marker (CcSex-260) showing amplification in mother sexual plant (CcSx) and all tissue cultured plants; (*b*) apomixis-specific SCAR marker (Apo-C470) showing amplification (470 bp) in apomictic (3108) plant but no amplification in the tissue cultured plants; (*c*) apomixis-specific SCAR marker (Apo-C930) showing amplification (930 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*.

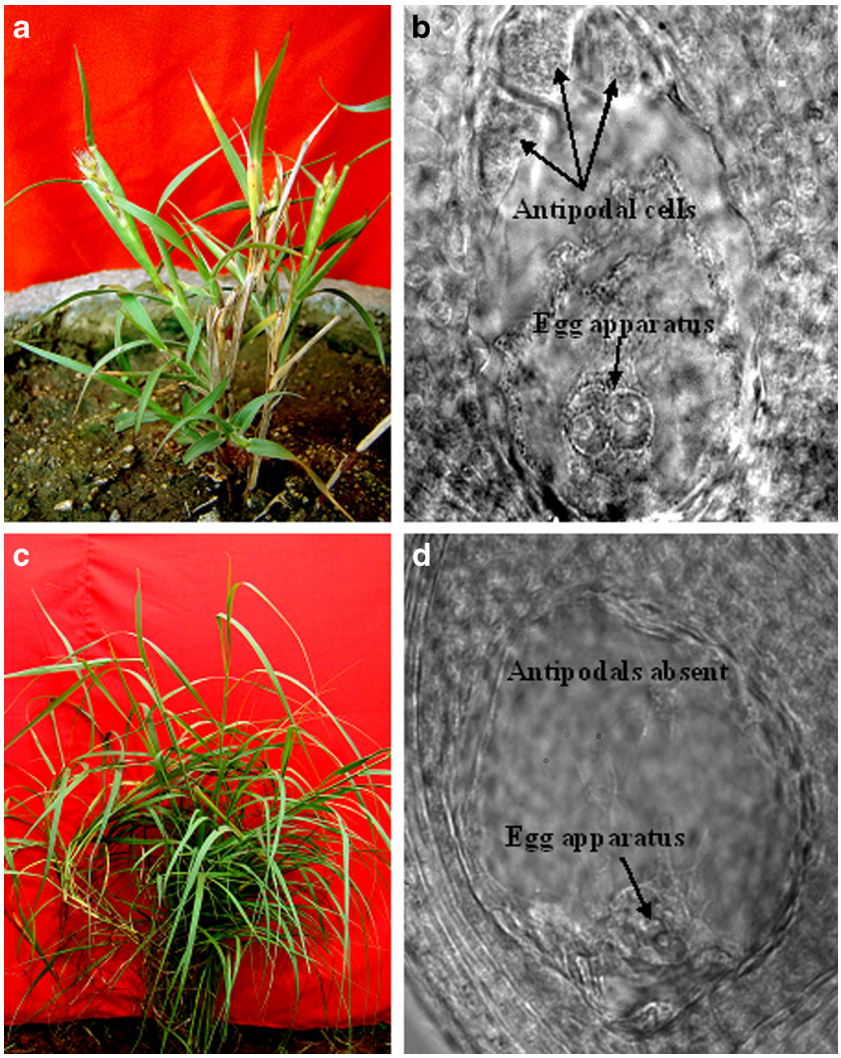

Figure 3. Two different types of *C. ciliaris* plants and embryo sacs. (*a*) The sexual *C. ciliaris* (IGFRI-CcSx/08/1) plant; (*b*) sexual embryo sac (with three antipodal cells) from a tissue cultured plant; (*c*) a common apomictic *C. ciliaris* plant (IG-3108); (*d*) a typical four-nucleated apomictic embryo sac, without antipodal cells.

apomictic (Young *et al.* 1979). When sexual and apomictic embryo sacs are observed on the same inflorescence, the plant is designated as facultative.

All of the tissue cultured plants appeared to maintain genetic fidelity with the mother sexual plant and showed the typical self-incompatibility, and meager growth, flowering, and seed setting following cross-pollination with an apomictic (IG-3108) plant. Since the sexual *C. ciliaris* (IGFRI-CcSx-08/1) plant cannot be propagated through seed, multiplication and maintenance of this rare genotype by *in vitro* culture would be more reliable.

Conclusion

The present report describes, for the first time, a high frequency *in vitro* plant regeneration procedure for a rare, obligate sexual plant of *C. ciliaris* via callus induction from immature inflorescences. The highest callus induction rate was obtained on MS medium supplemented with 3.5 mg L^{-1} 2,4-D, shoot organogenesis on MS medium supplemented with 2.0 mg L^{-1} kinetin, and rooting on MS medium containing 2.0 mg L^{-1} indolebutyric acid and 2.0 g L^{-1} activated charcoal. Regenerated plants were successfully acclimatized to *ex vitro* conditions. All the tissue cultured plants were found to be true to the mother plant type. This protocol provides a successful and rapid technique that could be useful for clonal multiplication, synthetic seed production, *ex situ* conservation, and genetic manipulation of this rare genotype for basic studies on sexuality/apomixis.

Acknowledgments We are grateful to the Director, IGFRI, Jhansi, for providing necessary facilities and encouragements to carry out the study.

References

Batra S, Kumar S (2002) *In vitro* high frequency plant regeneration in buffel grass (*Cenchrus ciliaris* L.). J Plant Biol 29:191–194

Batra S, Kumar S (2003) *Agrobacterium*-mediated transient GUS gene expression in buffel grass (*Cenchrus ciliaris* L.). J Appl Genet 44: 449–458

Bhat V, Dalton SJ, Kumar S, Bhat BV, Gupta MG, Morris P (2001) Particle-inflow gun-mediated genetic transformation of buffel grass (*Cenchrus ciliaris* L.): optimizing biological and physical parameters. J Appl Genet 42:405–412

Bray RA (1978) Evidence for facultative apomixis in *Cenchrus ciliaris*. Euphytica 27:801–804

Colomba EL, Grunberg K, Griffa S, Ribotta A, Mroginski L, Biderbost E (2006) The effect of genotype and culture medium on somatic embryogenesis and plant regeneration from mature embryos of fourteen apomictic cultivars of buffel grass (*Cenchrus ciliaris* L.). Grass Forage Sci 61:2–8

Dwivedi KK, Bhat SR, Bhat V, Baht BV, Gupta MG (2007) Identification of a SCAR marker linked to apomixis in buffel grass (*Cenchrus ciliaris* L.). Plant Sci 172:788–795

Gonçalves S, Romano A (2013) *In vitro* culture of lavenders (*Lavandula* spp.) and the production of secondary metabolites. Biotechnol Adv 31:166–174

Gupta MG, Gupta S, Bhat BV, Bhat V (1997) *In vitro* regeneration and somaclonal variation in a tropical pasture grass, *Dichanthium annulatum*. Range Manag Agrofor 18:25–30

Gupta S, Bhat BV, Bhat V, Gupta MG, Bhagmal (1998) Somaclonal variation for facultative apomixis in Marvel Grass (*Dichanthium annulatum*, Forssk. Stapf.). Forage Res 24:111–114

Gustine DL, Sherwood RT, Huff DR (1997) Apospory-linked molecular markers in buffel grass. Crop Sci 37:947–951

Kackar A, Shekhawat NS (1991) Plant regeneration from cultured immature inflorescences of *Cenchrus setigerus* and C. *ciliaris*. Ind J Exp Biol 29:62–64

Kumar S, Bhat V (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, Chandra A, Gupta MG, Shukla GP (2010a) Molecular and embryological analyses of rare sexual plant in buffel grass (*Cenchrus ciliaris* L.). Range Manag Agrofor 31:36–40

Kumar S, Chandra A, Gupta MG, Shukla GP (2010b) SCAR marker linked to sexuality in *Cenchrus ciliaris* L. Range Manag Agrofor 31:149–150

Kumar S, Chandra A, Gupta MG, Shukla GP (2013a) IGFRI-CcSx-08/1 (IC0590889; INGR11062), an Anjan grass (*Cenchrus ciliaris* L.) germplasm with a rare obligate sexual plant. Ind J Plant Genet Resour 26:99

Kumar S, Tiwari R, Chandra A, Sharma A, Bhatnagar RK (2013b) *In vitro* direct plant regeneration and *Agrobacterium*-mediated transformation of lucerne (*Medicago sativa* L.). Grass Forage Sci 68: 459–468

Martinez EJ, Hopp HE, Stein J, Ortiz JPA, Quarin CL (2003) Genetic characterization of apospory in tetraploid *Paspalum notatum* based on the identification of linked molecular markers. Mol Breeding 12: 319–327

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nordine A, Tlemcani CR, El Meskaoui A (2014) Regeneration of plants through somatic embryogenesis in *Thymus hyemalis* Lange, a potential medicinal and aromatic plant. In Vitro Cell Dev Biol - Plant 50:19–25

Ozias-Akins P, Roche D, Hanna WW (1998) Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus which may have no allelic form in sexual genotypes. Proc Natl Acad Sci U S A 95:5127–5132

Pessino SC, Evans C, Ortiz JPA, Armstead I, do Valle CB, Hayward MD (1998) A genetic map of the apospory region in *Brachiaria* hybrids: identification of two markers closely associated with the trait. Hereditas 128:153–158

Ross AH, Manners JM, Birch RG (1995) Embryogenic callus production, plant regeneration and transient gene expression following particle bombardment in the pasture grass in *Cenchrus ciliaris* (Gramineae). Aust J Bot 43:193–199

Sankhla A, Sankhla N (1989) Tissue culture studies on desert plants: *Cenchrus ciliaris*. Curr Sci 58:872–874

Yadav CB, Jha P, Mahalakshmi C, Anjaiah V, Bhat V (2009) Somatic embryogenesis and regeneration of *Cenchrus ciliaris* genotypes from immature inflorescence explants. Biol Plantarum 53:603–609

Young BA, Sherwood RT, Bashaw EC (1979) Cleared pistil and thick sectioning techniques for detecting aposporous apomixis in grasses. Can J Bot 57:1668–1672