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# High-frequency direct plant regeneration via multiple shoot induction in the apomictic forage grass *Cenchrus ciliaris* L.

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**Abstract** Genetic improvement of the apomictic forage grass species *Cenchrus ciliaris* L. based on conventional breeding methods is difficult and time-consuming. However, *in vitro* genetic manipulation of such species would provide a promising approach. A rapid and high-frequency *in vitro* plant regeneration protocol is essential for successful application of transgenic technology. This study reports on such a rapid, high-frequency and genotype-independent plant regeneration protocol for *C. ciliaris* L. Using the multiple shoot induction approach, up to 20 shoots per explant could be induced from shoot tips cultured on MS (Murashige and Skoog) medium when supplemented with 3.0 mg $L^{-1}$ TDZ. Two cultivars (IGFRI-3108 and IGFRI-727) and three exotic germplasm accessions (EC-397670, EC397496, and EC397336) showed equivalent responses to the protocol. Shoot tips from 4-d-old *in vitro* grown seedlings were used as explants for multiple shoot induction. Regenerated shoots were cultured on MS medium supplemented with gibberellic acid (2.0 mg $L^{-1}$) for shoot elongation. The regenerated shoots were rooted on MS medium supplemented with indole-3-acetic acid (3.0 mg $L^{-1}$). When transferred to soil in pots, hardened plants displayed up to 85% survival under field conditions.

**Keywords** Buffel grass · *Cenchrus ciliaris* · Apomixis · Multiple shoot induction · Thidiazuron

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## Introduction

*Cenchrus ciliaris* L. (also known as buffel grass, anjan grass, or African foxtail grass) is one of the most important forage grasses of tropical and subtropical regions of the world. It is an apomictic perennial range grass suitable for pastures in Australia, South Africa, and India (Bhat *et al*. 2001). Buffel grass can withstand heavy grazing, and it is extremely fire resistant. Being drought tolerant in nature, this species is well adapted to global arid and semiarid regions (Rao *et al*. 1996). Although highly palatable to all kinds of grazing animals, substantially high lignin content (3–5%) reduces its digestibility (Minson and Bray 1986). As an apomictic species (reproducing asexually through seeds), genetic improvement through conventional breeding methods is difficult, time-consuming, and restricted to the selection of promising lines from natural variants (Echenique *et al*. 1996). On the other hand, apomixis may facilitate varietal improvement by genetic transformation, since no further breeding step is required to fix inheritance of the transferred character trait (Vielle-Calzada *et al*. 1996). Unlike in sexual reproduction, apomixis conserves the genotype of the maternal parent. Components of apomixis include fertilization-independent embryo development (parthenogenesis), female gamete formation without meiosis (apomeiosis), and functional endosperm formation to ensure developmental adaptation (Koltunow and Grossniklaus 2003). Dissection of the molecular mechanisms underlying

apomixis will be critical for the efficient use of apomixis in crop plant improvement. Apomixis has been highly attractive to plant breeders as a means for preservation of desirable gene combinations which are otherwise disrupted by recombination and segregation during sexual reproduction. Effective exploitation of apomixis would provide tremendous opportunities to maintain hybrid vigor over successive generations, hence accelerating the breeding process through fixation of desirable trait combinations (Koltunow and Tucker 2008). In addition, artificial male sterility in an apomictic crop would have an added benefit of preventing gene flow through pollen, leading to transgene contamination (Spielman *et al*. 2003). Though apomixis is considered to be a complex trait, inheritance mechanisms have been analyzed in many species, with most studies indicating a monogenic and dominant trait with simplex inheritance (Yadav *et al*. 2011). Unfortunately, there has been no success to date in transferring apomixis to a crop species. Genes of interest have been available in our laboratories for transgene validation in *C. ciliaris* (Yadav *et al*. 2009), which require an efficient genetic transformation protocol. However, an efficient transformation protocol is not yet available, apart from earlier preliminary attempts to standardize transformation protocols for this species (Bhat *et al*. 2001; Batra and Kumar 2003). *In vitro* culture protocol is a prerequisite for successful plant genetic transformation. Hence, an efficient, genotype-independent *in vitro* culture protocol for plant regeneration in this forage species would be highly valuable.

Explant choice is one of the most important factors that influence *in vitro* plant regeneration (Trieu and Harrisson 1996) and success of transformation events. Although reports on plant regeneration via callus induction in buffel grass have been made (Ross *et al*. 1995; Batra and Kumar 2002; Colomba *et al*. 2006; Yadav *et al*. 2009), most have not been very efficient and responsive to different genotypes of *C. ciliaris*. Plant regeneration frequency and response to tissue culture procedures remain low (Ross *et al*. 1995; Colomba *et al*. 2006). The time period required for producing intact plants is considerably longer (Batra and Kumar 2002), and explants used for callus induction followed by plant regeneration are not readily available (Yadav *et al*. 2009). In addition, most of the earlier protocols required a callus phase which is associated with considerable genotypic variation. Callus culture increases the probability of somaclonal variation, especially when repeated subculture is required to produce embryonic calli. This observation requires a search for an alternate, genotype-independent path for plant regeneration which would also be compatible with genetic transformation methodology.

Shoot tips provide a preferred source of explant material as they tend to produce genetically similar plants because no intermediate callus phase is involved (Bao *et al*. 2001; Yookongkaew *et al*. 2007; Kumar and Chandra 2009). Shoot tip has been reported to be a better explant for transformation as compared to callus (Cho *et al*. 2003; Weber *et al*. 2003; Yookongkaew *et al*. 2007). Sadia *et al*. (2010) reported the use of shoot apical meristem culture for rapid micropropagation of *Sorghum bicolor*. Zhang *et al*. (2010) reported stable transformants of *Poa pratensis* using cocultivation of apical meristem derived from *in vitro* grown seedling with *Agrobacterium*.

Thidiazuron (TDZ), a phenylurea-type cytokinin, is one of the various kinds of substituted urea that has been investigated for multiple shoot induction from apical meristems in different plant species (Goldman *et al*. 2003; Gairi and Rashid 2004; Ganeshan *et al*. 2006; Kumar and Chandra 2009). Ganeshan *et al*. (2006) reported a maximum of 69 shoots per explant on culture medium containing a combination of TDZ and 6-benzylaminopurine in oat, as compared to 35 shoots per explant in case of wheat. Yookongkaew *et al*. (2007) also observed multiple shoot induction from shoot apical meristems using TDZ in rice cultivars and reported this tissue culture system to be suitable for *Agrobacterium*-mediated transformation of rice.

The present study was undertaken to optimize a rapid, high-frequency, direct plant regeneration protocol in *C. ciliaris*. The reported protocol for genotype-independent, direct plant regeneration via multiple shoot induction will also be very useful for studying the developmental events in the apomictic process and genetic improvement of this forage species.

## Materials and Methods

*Seed source and preparation.* Mature and healthy seeds of two cultivars (*viz*. IGFRI-3108 and IGFRI-727) and three exotic germplasm accessions (EC 397670, EC 397496 and EC 397336) of *C. ciliaris* available at IGFRI Central Research Farm, Jhansi, India, were used in this study. The seeds were manually dehusked and surface sterilized using 70% (*v*/*v*) ethanol for 1 min followed by two washes with sterile distilled water prior to treatment with 0.2% (*w*/*v*) $HgCl_2$ and 0.1% (*v*/*v*) Tween-20 for 5 min. The seeds were then washed four to five times with sterile double distilled water to remove traces of mercuric chloride. The seeds were soaked overnight in 10 ppm solution of gibberellic acid ($GA_3$; Sigma-Aldrich, St. Louis, MO) to break dormancy and synchronize germination.

*In vitro culture media and conditions.* The treated seeds were germinated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with TDZ (Table 1). The MS medium was prepared by dissolving 34.08 g $L^{-1}$ of dehydrated MS medium containing (30 g $L^{-1}$)

**Table 1.** Effect of TDZ on multiple shoot induction from shoot tips of buffel grass (*C. ciliaris* L.) cultured on MS medium

| TDZ (mg $L^{-1}$) | No. of shoots observed[z] | | | | | |
|---|---|---|---|---|---|---|
| | After 3 wk culture | | | After 5 wk culture | | |
| | IGFRI-3108 | IGFRI-727 | EC-397670 | IGFRI-3108 | IGFRI-727 | EC 397670[y] |
| 0 | 1.00±0.00a | 1.00±0.00a | 1.00±0.00a | 1.00±0.00a | 1.00±0.00a | 1.00±0.00a |
| 1 | 4.16±0.32b | 3.03±0.32b | 3.33±0.66b | 6.23±0.42b | 4.61±0.26b | 5.33±0.66b |
| 2 | 8.26±0.67c | 6.34±0.66c | 7.34±0.33c | 11.17±0.67c | 9.72±0.74c | 10.72±1.33c |
| 3 | 14.05±1.32d | 12.46±0.99e | 13.46±0.67e | 20.33±1.67d | 15.06±0.97d | 16.06±0.67d |
| 4 | 10.13±0.33c | 9.67±1.33d | 9.33±0.67d | 11.76±0.66c | 10.11±1.42c | 9.66±0.33c |
| 5 | 9.23±0.32c | 8.96±0.67d | 9.26±0.66d | 10.06±0.32c | 9.42±0.99c | 9.33±0.67c |

Means followed by different *lowercase letters* in a *column* are significantly different ($p \leq 0.05$) by DMRT

[z] Values represent means±SE. The number of explants in each treatment, $n=15$, and experiments were repeated three times

[y] Represents the exotic germplasm collections tested in this study—no significant variation among the germplasm collections was observed

sucrose (PT010; HiMedia Laboratories, Mumbai, India) and 440 mg $L^{-1}$ $CaCl_2 \cdot H_2O$. The pH of the medium was adjusted to 5.8 with 1.0 N NaOH, and the final volume was adjusted to 1 L with double distilled water. Gelrite (Duchefa, Haarlem, Netherlands) was used at 2.5 g $L^{-1}$ to solidify regeneration media. However, in rooting medium, only 2.0 g $L^{-1}$ of Gelrite was used. Media were sterilized by autoclaving at 121°C for 18 min. The autoclaved media were cooled down to 50–60°C before adding the required amount (0–5 mg $L^{-1}$) of TDZ from a filter-sterilized stock solution (1.0 mg $mL^{-1}$ TDZ). The stock solution of TDZ was prepared by dissolving 50 mg of thidiazuron (Duchefa) in 10 mL of dimethyl sulfoxide and making up the final volume to 50 mL with double distilled water. The solution was filter sterilized using a 0.2-μm filter disk (Sartorius Minisart) and stored at −20°C in an amber-colored bottle that was wrapped with aluminum foil to protect from light.

*Multiple shoot induction and plant regeneration.* Shoot tips (2–3 mm), along with the primordial leaf and scutellum (Fig. 1*a*), were excised from 4-d-old *in vitro* grown seedlings using a sterile scalpel blade. The shoot tips (five explants per petri plate) were placed horizontally on solid MS medium supplemented with the same concentration of TDZ as was previously used for seedling germination. The explants were cultured in the plate for 3 wk at 25±2°C under 16 h photoperiod (40 μmol $m^{-2}$ $s^{-1}$), followed by subculture onto freshly prepared TDZ-containing medium in a 500-mL wide-mouth bottle with 75 mL medium, and cultured for another 2 wk under similar conditions. Observations were recorded on multiple shoot induction after 3 and 5 wk of culture. Regenerated shoot clumps (three clumps per bottle) were then transferred to MS medium supplemented with $GA_3$ (0–3 mg $L^{-1}$) in a wide-mouth bottle and cultured in light for 2 wk to allow shoot elongation (Fig. 1*b*). Elongated shoots were divided into two to three clumps of shoots, and were transferred to MS medium containing indole-3-acetic acid (IAA; 0–3 mg $L^{-1}$, Table 2) in a wide-mouth bottle and cultured in light for 3 wk to allow root organogenesis. Regenerated plants with shoots and roots were separated from each other, and individually transplanted into pots filled with sterilized soil (Fig. 1*g*). The potted plants were grown inside the culture room for a week, and then shifted to shade in an open environment for 3–4 d. Finally, the plants were grown in sunlight under field conditions until maturity (Fig. 1*h*).

*Statistical analysis.* Experiments were repeated three times with 3 replications in a completely randomized design. Statistical analysis was performed by analysis of variance. Duncan's multiple range test (DMRT) at $p \leq 0.05$ was used to compare the means of treatments. All the five genotypes were used in different experiments. However, EC-397670 was taken as representative of the germplasm collection accessions (Table 1, Fig. 2) as no significant differences were observed between these genotypes (Fig. 3).

## Results and Discussion

An efficient and reproducible plant regeneration protocol is a prerequisite for genetic transformation. In an attempt to standardize the direct plant regeneration protocol for *C. ciliaris*, 20 shoots per explant were induced on MS medium supplemented with 3.0 mg $L^{-1}$ TDZ. Shoot tips containing the primordial leaf together with the scutellum were used as explant material for multiple shoot induction. In order to ensure year-round availability of explant and to avoid microbial contamination in the cultures, shoot tips from *in vitro* grown seedlings were used as the explant source. The scutellar region was included in the explant in order to provide a supply of endogenous growth regulators to the explant during the initial period of establishment (Smith and Murashige

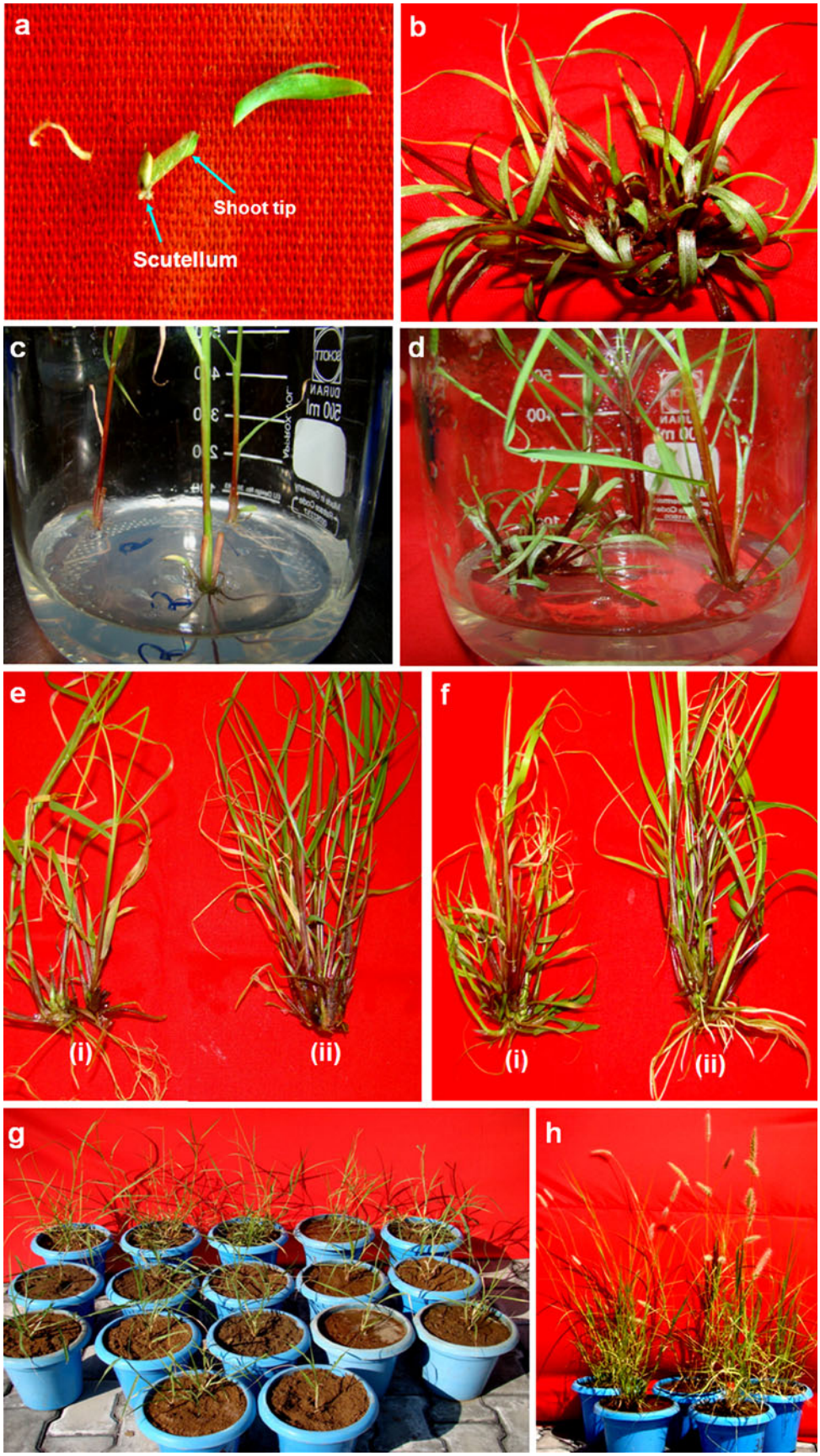


**Figure 1.** Plant regeneration via multiple shoot induction in buffel grass (cv. IGFRI-3108). *a*. Four-d-old seedling used to excise the explant containing shoot tip and scutellum. *b*. Multiple shoots induced from the explant after 5 wk of culture on MS medium with 3.0 mg $L^{-1}$ TDZ. *c*. Shoot induced from the explant on phytohormone-free MS medium. *d*. Shoots elongated on MS medium containing $GA_3$ (2.0 mg $L^{-1}$). *e*. Root organogenesis from multiple shoots regenerated on MS medium with 2.0 mg $L^{-1}$ TDZ and 2.0 mg $L^{-1}$ IAA in rooting medium(*i*); no rooting in absence of IAA in rooting medium (*ii*). *f*. Effect of TDZ concentration on subsequent root organogenesis. More shoots induced with 3 mg $L^{-1}$ TDA with fewer roots on MS medium containing 2.0 mg $L^{-1}$ IAA (*i*); lesser shoots with 2.0 mg $L^{-1}$ TDZ with more roots induced on MS medium containing 3.0 mg $L^{-1}$ IAA (*ii*). *g*. Plants regenerated via multiple shoot induction from a single shoot tip. *h*. Normal growth and flowering on tissue-cultured *C. ciliaris* plants.

**Table 2.** Effect of IAA on root organogenesis from the shoots regenerated on TDZ-containing MS medium

| TDZ used in regeneration medium (mg $L^{-1}$) | IAA in rooting medium (mg $L^{-1}$) | Rooting after 3 wk of culture[z] | | | | |
|---|---|---|---|---|---|---|
| | | IGFRI-3108 | IGFRI-727 | EC 397670 | EC-397496 | EC 397336 |
| 1 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1 | 1 | 1.13 | 1.09 | 1.03 | 1.06 | 1.03 |
| 1 | 2 | 2.36 | 2.31 | 1.61 | 1.65 | 1.39 |
| 1 | 3 | 2.67 | 2.39 | 2.33 | 2.36 | 2.13 |
| 2 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 2 | 1 | 0.45 | 0.50 | 0.19 | 0.17 | 0.17 |
| 2 | 2 | 0.81 | 0.80 | 0.79 | 0.81 | 0.76 |
| 2 | 3 | 1.12 | 1.09 | 1.10 | 1.06 | 1.07 |
| 3 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 3 | 1 | 0.10 | 0.09 | 0.00 | 0.00 | 0.00 |
| 3 | 2 | 0.50 | 0.48 | 0.43 | 0.46 | 0.44 |
| 3 | 3 | 0.97 | 0.96 | 0.96 | 0.94 | 0.93 |
| 4 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 4 | 1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 4 | 2 | 0.11 | 0.09 | 0.00 | 0.00 | 0.00 |
| 4 | 3 | 0.13 | 0.12 | 0.10 | 0.13 | 0.11 |
| 5 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 5 | 1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 5 | 2 | 0.09 | 0.08 | 0.00 | 0.00 | 0.00 |
| 5 | 3 | 0.11 | 0.10 | 0.00 | 0.00 | 0.00 |

[z]Values represent mean of the number of root(s) per shoot. The number of explant (clump of shoots) used in each treatment was 3, and treatments were replicated three times

1982). Seeds were germinated on MS medium containing TDZ in order to induce high concentrations of endogenous cytokinin. A similar strategy was earlier adopted for rice by Yookongkaew *et al.* (2007). Direct plant regeneration through multiple shoot induction from the explant was adopted, as it does not involve an intermediate callus phase, and tends to produce genetically similar plants (Bao *et al.* 2001). In most of the earlier reports on plant regeneration in buffel grass, a callus phase has been involved (Ross *et al.* 1995; Batra and Kumar 2003; Colomba *et al.* 2006; Yadav *et al.* 2009). Plant regeneration using this system requires a longer period to establish cultures and to obtain embryogenic calli, along with the risk of somaclonal variation in the plants regenerated from callus (Bregitzer and Tonks 2003; Kishore *et al.* 2006; Yookongkaew *et al.* 2007). Shoot tips provided preferred explants for *in vitro* culture

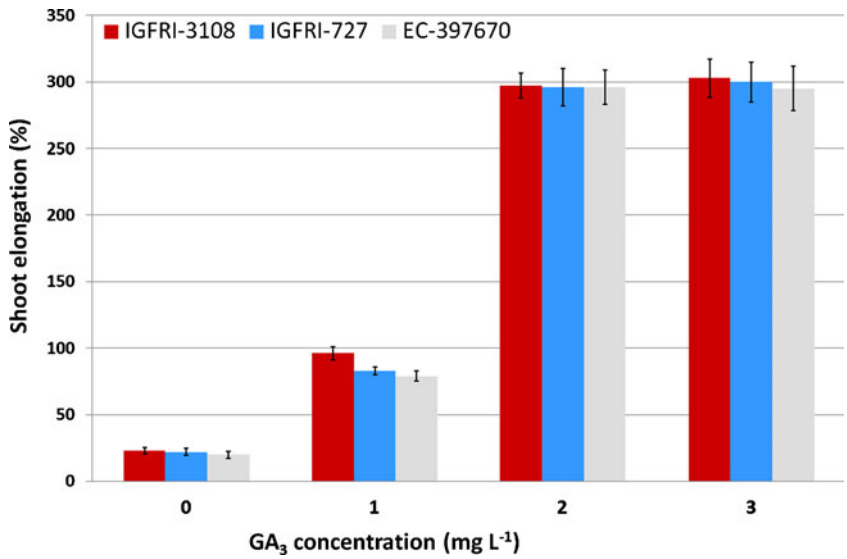


**Figure 2.** Effect of gibberellic acid on elongation of regenerated shoot in *C. ciliaris*. Regenerated shoots with 2–3 cm height were cultured on MS medium containing different concentration of $GA_3$. Observations were taken after 2 wk of culture. *Vertical bars* represent ±SE.

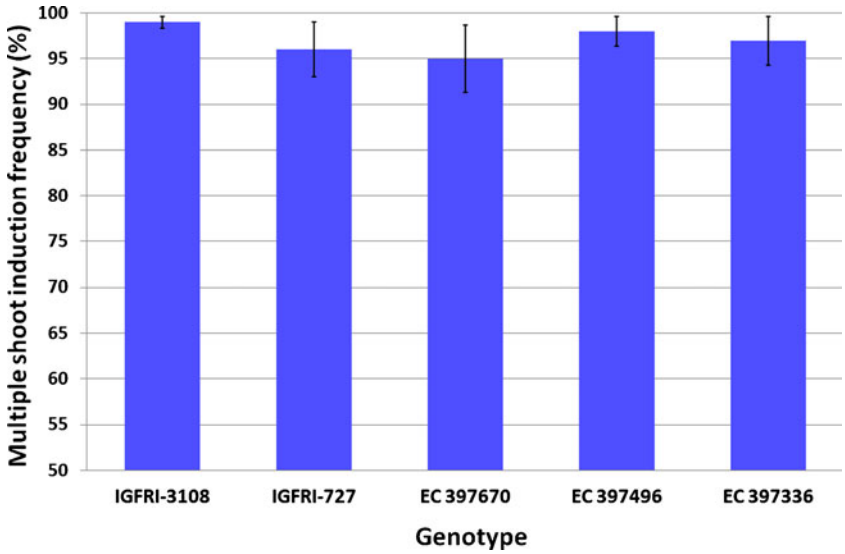


**Figure 3.** Multiple shoot induction frequency in different genotypes of *C. ciliaris*. Observations were taken after 5 wk of culture. *Vertical bars* represent ±SE.

due to ease of handling as compared to material from other sources. Previous reports have suggested that grass meristems are morphogenetically plastic, and hence can be manipulated to produce multiple shoots (Bhalla and Singh 2006).

When shoot tips were cultured on regeneration medium, swelling of the explant was observed within 4 d of culture on TDZ-containing medium. Single-shoot growth was initially observed, but multiple shoot induction was subsequently observed on medium supplemented with TDZ. Single-shoot growth continued on the phytohormone-free medium (Fig. 1*c*), whereas multiple shoots ranging from 5 to 14 were observed after 3 wk of culture of the explants on MS medium with different concentrations of TDZ (Table 1). To a certain extent, with increasing concentration of TDZ, an increase in number of multiple shoots was observed. The maximum number of shoots observed was 20 per explant on MS medium with 3.0 mg $L^{-1}$ of TDZ in cv. IGFRI-3108. Higher concentrations of TDZ in the medium resulted in a reduced number of shoots. TDZ is involved in reprogramming and differentiation of the competent cells necessary for adventitious bud development, and has been reported to induce synthesis or accumulation of endogenous cytokinins because of an increase in synthesis, a decrease in catabolism, or conversion of storage forms to biologically active cytokinins (Hutchinson and Saxena 1996). At lower concentrations of TDZ (1–2 mg $L^{-1}$), although lower numbers of shoots were induced, shoot growth was observed to be normal. In contrast, at higher concentrations of TDZ (3–5 mg $L^{-1}$), although the number of shoots induced was considerably higher, stunted growth was observed. Yookongkaew *et al*. (2007) also reported similar observations in rice, including a continuous increase in the number of multiple shoots per explant with increasing concentrations of TDZ. The rice shoots regenerated on medium containing ≥6.0 mg $L^{-1}$ TDZ were not healthy. TDZ has been reported to cause stunted growth of the regenerating shoots (Brassard *et al*. 1996; Kumar and Chandra 2009). In the present case, shoots regenerated on medium containing ≥3.0 mg $L^{-1}$ TDZ were found to be stunted, but on transfer to medium containing $GA_3$ within 2 wk, normal growth was observed. Gibberellic acid is known to promote shoot elongation in grass species (Tan and Qian 2003). To overcome the problem of stunted growth, $GA_3$ was used. Although repeated subculture of regenerated shoots on hormone-free medium may diminish the residual effects of TDZ, serial subculturing adds to the time required for regeneration of plant. $GA_3$ concentration at 2.0 mg $L^{-1}$ in the shoot elongation medium was found to be optimal, as higher concentrations of $GA_3$ did not result in a significant improvement in shoot elongation (Fig. 2).

The concentration of TDZ in the regeneration medium exerted significant effects on the number of shoots induced and subsequent rooting from the regenerated shoots. In the absence of TDZ in the regeneration medium, growth of a single shoot, along with rooting, was observed. Shoots regenerated on TDZ-containing medium failed to root on medium without IAA (Table 2, Fig. 1*e*). When the shoots regenerated on MS medium containing 3.0 mg $L^{-1}$ TDZ were transferred to rooting medium with 3.0 mg $L^{-1}$ IAA, rooting was observed within 3 wk of culture. MS medium with 2.0 mg $L^{-1}$ TDZ, however, produced lower numbers of shoots, and rooting from the shoots on medium containing 3.0 mg $L^{-1}$ IAA was superior (Fig. 1*f*, Table 2). With increasing concentration of TDZ in the regeneration medium, the concentration of IAA required for normal rooting also increased. However, shoots regenerated on medium containing very high concentrations of TDZ (>5.0 mg $L^{-1}$) failed to root even after 6 wk of culture on medium containing >5.0 mg $L^{-1}$ IAA in rooting medium (data not shown). Generally, shoots are separated from a clump before placement on rooting medium in order to reduce cytokinin accumulation and promote root formation. But when individual

shoots were separated from clumps and placed on rooting medium, death of a number of shoots was observed (data not shown). Therefore, a larger clump of shoots was divided into two to three smaller clumps and placed on the rooting medium. This effect may be a contributory factor to poor rooting even at a higher concentration of IAA (3.0 mg $L^{-1}$). A higher concentration (3.0 mg $L^{-1}$) of TDZ in the regeneration medium necessitated the use of $GA_3$ for shoot elongation, and subsequently a higher concentration of IAA was required for rooting, which might have affected overall plant health *in vitro* (Fig. 1*f*).

The rooted plants were hardened by transplanting into soil followed by growth inside the culture room for a wk, followed by transfer to shade in an open environment for 3–4 d. Gradual exposure of the plants to the open environment was necessary to avoid plant death due to desiccation. The plants were then shifted to sunlight in field conditions. Survival rate of the regenerated plants was 85%, with up to 17 tissue-cultured plants being established under field conditions from a single shoot tip explant (Fig. 1*g*). About 15% of the plants died during hardening because of insufficient rooting from the regenerated shoots. The tissue-cultured plants showed normal growth, flowering, and seed setting (Fig. 1*h*). Direct regeneration of plants required relatively short period (10–11 wk) compared to the regeneration via callus phase (20–22 wk) reported earlier by Batra and Kumar (2002). With a minor variation in the number of shoots induced from an explant, all the genotypes tested in the present study responded equally well for multiple shoot induction (Fig. 3). Thus, the direct plant regeneration protocol for *C. ciliaris* is highly efficient, rapid, and genotype independent. It does not involve a callus phase, and so a reduced time period is required for regeneration of complete plant, reducing the likelihood of somaclonal variation in the tissue-cultured plants. The value of this protocol for subsequent *Agrobacterium*-mediated genetic transformation activities in buffel grass has hence been demonstrated.

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