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Research Article

Meristem tip culture in *Phragmites australis* and genetic fidelity study using SRAP markers

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Abstract

The improved sugarcane varieties grown in and around the world are majorly evolved through interspecific hybridization of *Saccharum* species. Only few promising hybrids have been developed from allied genera such as *Erianthus* and *Sclerostachya*. Several other genera, including *Phragmites*, *Vetivera*, *Narenga* and *Neyurida*, deserve further research. Successful exploitation of these allied genera resources require careful characterization, evaluation and conservation using different methods including tissue culture techniques. Among them, *Phragmites australis* is used to remediate waste water and remove heavy metals. In this study, meristem tip culture was standardized for five clones of *Phragmites* to evaluate their response and growth. Among the five clones of *Phragmites* studied, two clones (*IND 89-711* and *IND04-1326*) showed better regeneration and growth. The *in vitro* derived plants were compared with the mother plants using four SRAP markers to check their genetic fidelity. Monomorphic bands were obtained in both the *in-vitro* and mother plants. No polymorphism was detected, thus proving the genetic uniformity of the micropropagated plants.

Keywords: Allied genera, *Phragmites*, *Saccharum spontaneum*, Apical meristem, Genetic fidelity

INTRODUCTION

Sugarcane has been grown widely in the world as an industrial crop for sugar and bio energy production. The sugarcane cultivars grown throughout the world are predominantly derived from the interspecific hybridization of *S. spontaneum* and its other related species (Legendre and Breaux, 1983; Burner and Legendre, 1993). The importance of extending the genetic basis has grown in order to exploit useful traits from related genera of sugarcane. *Erianthus*, *Phragmites*, *Vetivera*, *Narenga*, *Sclerotachya* and *Neyurida* could be used for intergeneric hybridization to widen the genetic base of sugarcane. Among them, the genus *Phragmites* has the ability to tolerate soil pollution and water logging conditions (Knight *et al.*, 2000; Srivastava *et al.*, 2011). The genus *Phragmites* of family Poaceae comprises perennial, rhizomatous, and stoloniferous and tall (2.0–6.0 m) grass

species, viz., *Phragmites australis*, *P. karka*, *P. communis*, *P. longivalvis*, *P. maxima* and *P. prostrate* (Poonawala *et al.*, 1999). *P. australis* (common reed) and *P. karka* (flute grass) are two commonly occurring species on the Indian sub-continent. *Phragmites australis* a polyploid perennial grass with clonal architecture is found all over the world and is particularly invasive in Kashmir's Himalayan aquatic ecosystems (Shah and Reshi, 2012; Saltonstall, 2002; Lambertini *et al.*, 2006)). *P. australis* (hereinafter referred to as reed), is one of the most promising natural wetland bioenergy crops with significant socioeconomic value (Leiblein-Wild, and Tackenberg, 2014). It's wide intraspecific diversity makes it ideal for studying adaptation and response to environmental change. Several studies have been published on this species (Maron *et al.*, 2004; Monty *et al.*, 2013, Amsellem *et al.*, 2000, Lavergne, and

Molofsky, 2007; Tsutsui *et al.*, 2000). Reed is abundant and often the dominant species where it occurs.

An adaptive amphibious species *Phragmites australis* is a transitional species of two closely attached ecosystems, viz., aquatic and terrestrial and commonly occurs in marshy wetlands (Mal and Narine, 2004). The growing period is long in most of the subtropical and mild temperate zones, facilitating reed plants to adapt the variations in temperature, soil nutrients and available oxygen (wetlands) together with certain physiological features such as transpiration rates in connection to the seasonal variations (Haslam, 1972). This crop is suitable for cultivation in barren land such as lands polluted with heavy metals.

Such useful traits of *Phragmites* could be used for further improvement in sugarcane breeding programme. Exploitation of these genetic resources would require characterization and assessment of the genetic stocks, using different experimental methods including classical breeding techniques and tissue culture techniques (Berding and Roach, 1987). Hence, it is necessary to conserve these potential germplasm resources. *Phragmites australis* collections are maintained in the germplasm field at ICAR- Sugarcane Breeding Institute, Coimbatore. In this study an attempt has been made to regenerate the micro propagated plantlets from *Phragmites australis* for *in vitro* conservation studies. In addition, a genetic fidelity test was performed using SARP primers, confirming the uniformity of the regenerated plants with the mother plant.

MATERIALS AND METHODS

The present investigation was carried out in the micropropagation laboratory, ICAR-Sugarcane Breeding Institute, Coimbatore during Dec 2020-Mar 2021. The clones used for this study are given in **Table 1**. The samples were collected from 4-5 months old healthy plants (**Fig. 1**).

Table 1. List of *Phragmites australis* clones used in the study

S. No.	<i>Phragmites australis</i> clones
1	IND 04-1326
2	IND 05-1423
3	IND 03-1315
4	IND 04-1334
5	IND 89-711

The standardized micropropagation protocol published by Neelamathi *et al.* (2022) was followed for regeneration of *Phragmites* clones. The basal MS media supplemented with different components are listed in **Table 2**. The excised apical meristem was transferred to the initiation medium. The explants were transferred to medium containing activated charcoal and the culture tubes were incubated in the dark room for 15 days at 25 °C. After 15 days, the explants were transferred to the solid medium without charcoal in the test tubes and allowed for the shoot initiation. The developing shoots were transferred to fresh bottle with liquid MS media containing 0.05 mg/l 6-BAP, 0.5 mg/l NAA, 0.015 mg kinetin, along with 100 ml/l coconut water. The cultures were maintained by sub-culturing in fresh media every 20-30 days. After it was transferred to rooting media for 15 to 25 days containing half MS media. The well developed rooted plants are transferred to poly bags and kept under shade net for 45 days. Then the grown plants are transferred to pots for hardening.

To assess the genetic fidelity of *in vitro* raised plantlets genomic DNA was extracted from the young leaf tissues of both *in vitro* derived plantlets and parent *Phragmites* clones. Young leaves from both *in vitro* derived plantlets and parent clones were collected and frozen immediately in liquid nitrogen for further DNA isolation. The leaf samples were ground to a powder in liquid nitrogen and genomic DNA was extracted using CTAB method

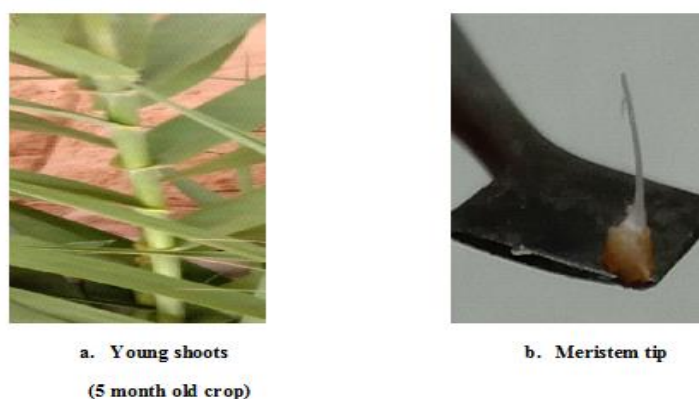


Fig. 1. Meristem tip taken from young shoot of *Phragmites australis*

Table 2. Media composition (Per litre) used for micropropagation techniques in *Phragmites australis*

Shoot initiation media	Shoot elongation media	Shoot multiplication media	Rooting media
Glycine-2 mg	Glycine-2 mg	Glycine-2 mg	Glycine-1mg
Pyridoxine-0.5 mg	Pyridoxine-0.5 mg	Pyridoxine-0.5mg	Pyridoxine-0.25 mg
Nicotinic acid-0.5 mg	Nicotinic acid-0.5 mg	Nicotinic acid-0.5 mg	Nicotinic acid-0.5 mg
Thiamine-0.1mg	Thiamine-0.1mg	Thiamine-0.1mg	Thiamine-0.1mg
Mesoinositol -100 mg	Mesoinositol -100 mg	Mesoinositol -100 mg	Mesoinositol -100 mg
Sucrose-20 g	Sucrose-20 g	Sucrose-20 g	Sucrose-30 g
Phytigel -2.6 g	Phytigel -2.6 g	6-BAP-0.05 mg	NAA-5 mg
Activated charcoal -3.5 g		NAA-0.5 mg	
		Kinetin-0.015 mg	
		Coconut water-100 ml	

Table 3. Details of SRAP Primer sequence used in the study

Primer Name	Primer Sequence
E6	GACTGC GTACGAATTGCA
M5	TGA GTCCAAACCGGAAG
E3	GACTGCGTACGAATTGAC
M14	TGAGTCCAAACCGGACC
M6	TGAGTCCAAACCGGACA

(Zhang *et al.*, 2008). Purity and quantity of extracted genomic DNA were estimated by Nanodrop and resolving in 1% (w/v) agarose gel.

PCR was carried out using four SRAP primer pair combinations which consisted of four forward primers and four reverse primers. The marker sequences are referred from (Suman *et al.*, 2008) and combinations of SRAP markers used for PCR amplification are E6M5, E3M5, E3M14 and E2M6 (**Table 3**). PCR amplification reactions were performed in a Mastercycler Pro (Eppendorf, Germany) using the following conditions: 5 min at 94°C; 5 cycles of 94°C, 1 min; 35°C, 1 min; and 72°C, 2 min; additional 35 cycles of 94°C, 1 min; 50°C, 1 min; and 72°C, 1 min; extension of 5 min at 72°C; and a final storage at 4°C (Li and Quiros, 2001). PCR products were resolved using 3% (w/v) agarose gel and documented.

RESULTS AND DISCUSSION

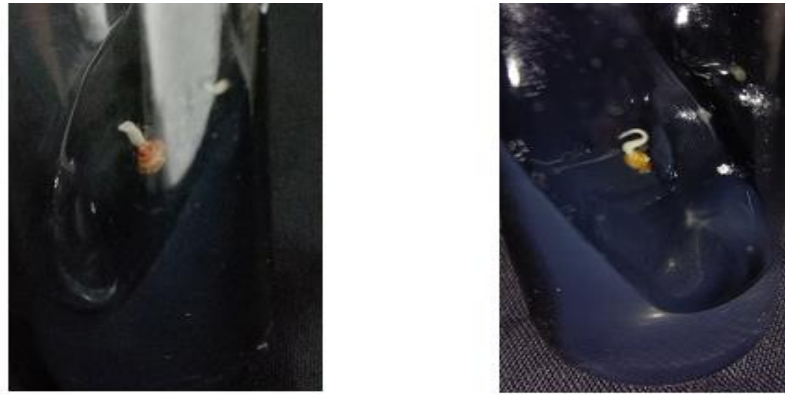
Micropropagation (clonal propagation through the apical meristem) is the most viable and successful way for producing pathogen-free stock material. Micropropagation has several advantages, including fast multiplication of new varieties, enhanced plant vitality, and germplasm storage. It is the ideal method for propagation since it generates offspring that are phenotypically similar to the mother plant (true to type) and has a considerably faster multiplication rate. Meristem culture can also be useful in eliminating pathogens (Hendre *et al.*, 1983). The protocol of apical meristem culture with two or three leaf primordia was standardized by Sreenivasan

and Jalaja (1981). In this study, the shoot apex from the each five clones of *Phragmites australis* were collected and excised to the size of 2 mm for virus free apical meristem culture. Since viruses can be eliminated using optimal meristem size as reported by Victoria *et al.* (1999), Chatenet *et al.* (2001), Parmessur *et al.* 2002 and Balamuralikrishnan *et al.* (2002).

The excised shoot apex of 2 mm size from each five clones of *Phragmites australis* (IND 04-1326, IND 05-1423, IND 89-711, IND 03-1315 and IND 04-1334) were inoculated in shoot initiation medium containing basal MS media. The activated charcoal is used to absorb the phenolic residues from the explants produced during the growth. Since the inclusion of activated charcoal in initiation medium would promote development of shoot by preventing phenol secretion (Zhang *et al.*, 2000). Visible single shoots were observed after fifteen days of inoculation (**Fig. 2**).

Single shoots were transferred to the shoot multiplication medium without the use of activated charcoal. Shoot elongation occurred in 35 days to 90 days and only two clones *viz.*, IND 89-711 and IND04-1326 showed better response (**Fig. 3**).

The elongated shoot was transferred to the multiplication MS medium with 0.05 mg/l 6-BAP, 0.5 mg/l NAA, 0.015 mg kinetin, and 100 ml/l coconut water. IND 89-711 and IND04-1326 showed better growth and development.



Initiation of apical meristematic tip in the medium after 15th day of inoculation
Fig. 2. Shoot tip initiation in shoot initiation medium (Activated charcoal)



Elongation of shoot in 35 days and above
Fig. 3. Shoot tip elongation in shoot elongation medium



Multiplication of shoot after repeated sub culturing of about 90 days
Fig. 4. Transfer of elongated shoot in to multiplication medium

Hence, these two clones were transferred to rooting media containing half MS composition (**Fig. 4**). The rooted plants were hardened in green house condition. Plantlets formed secondary and tertiary roots during hardening (**Fig. 5**). After 45 days the hardened plants were

transferred to pots (**Fig. 6**). Previous findings on tissue culture on *Phragmites australis* only commenced with mature seeds or other plant parts through embryogenic calli regeneration (Straub *et al.*, 1988; Guo *et al.*, 2014; Lee *et al.*, 2012).



Fig. 5. *Phragmites australis* clones transfer in to rooting medium



Fig. 6. Hardening of *Phragmites australis* clone IND 89-711 and IND 04-1326

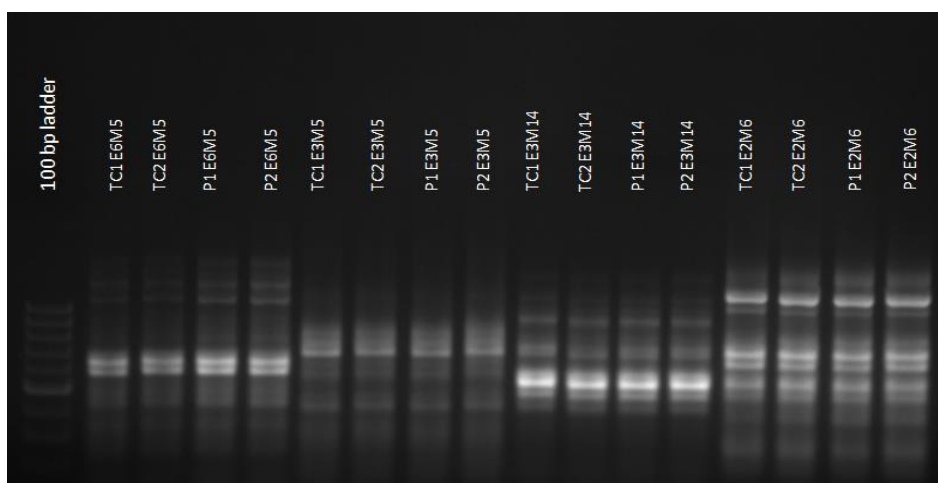


Fig. 7. Genetic fidelity analysis in *Phragmites australis* clones IND 89-711 and IND 04-1326 using SRAP markers

Primers used: E6M5, E3M5, E3M14, E2M6

Tissue culture plantlet 1 (TC1) - IND 89-711; Tissue culture plantlet 2 (TC2) -IND 04-1326; Phragmites parent plant 1 (P1)-IND 89-711; Phragmites parent plant 2 (P2) - IND 04-1326

Genetic fidelity of *in vitro* derived *Phragmites australis* clones IND 89-711 and IND04-1326 were studied using SRAP markers. A total of 25 distinct and scorable bands were produced by four combinations of SRAP (Sequence related amplified polymorphism) primers. The number of bands in each primer combination ranged from 5-8, with maximum bands observed in the combination E2M6. All the combinations of SRAP marker showed monomorphic bands across all the samples (Fig.7). There were no variants observed in the banding pattern of *in vitro* derived plantlets and the source parent clone. These results suggest that the micropropagation protocol followed in this study is appropriate and applicable for mass clonal propagation of true-to-type *Phragmites* clones. SRAP markers were already used for assessing the genetic fidelity of tissue culture plants (Clapa *et al.*, 2020; Borsai *et al.*, 2020).

This meristem tip culture on allied genera of sugarcane germplasm will form the basis for micropropagation of *Phragmites australis*. It could be used in future for *in vitro* conservation and regeneration studies of sugarcane germplasm. The study also proves that the micropropagation can generate true to type *in vitro* plantlets for further application.

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