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Factors influencing bud break and rooting and mass-scale micropropagation of three *Phragmites* species: *P. karka*, *P. communis* and *P. australis*

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Abstract A procedure has been described for the large-scale micropropagation of three *Phragmites* species, *P. karka*, *P. communis* and *P. australis*, from axillary buds excised from the main and side branches. Position of the buds on the branches had an effect on the bud break and establishment of the cultures under *in vitro* conditions. Lower buds of *P. australis* and middle buds of *P. karka* and *P. communis* were the most suitable. The presence of yeast extract as one of the ingredients of the sprouting medium helped in the early detection of systemic contamination. Multiple shoot formation and root initiation were obtained on Murashige and Skoog's basal medium supplemented with different concentrations of BA – 0.5 mg/l for *P. karka*, 0.25 mg/l for *P. communis* and 0.1 mg/l for *P. australis* – 0.5 mg/l Kn and 2% sucrose (w/v). Shoots and roots elongated on half-strength MS basal medium with 2% sucrose but without any plant growth regulators. A zone of root hair was observed in the case of *P. australis*. Hardening occurred on 95% of the plantlets within 30 days of transfer to the polyhouse. Over 10,000 plants were produced from three buds of each species within 9 months. The plants were supplied to a private company for their industrial waste treatment.

Key words *P. karka* · *P. communis* · *P. australis* · Micropropagation · Bud position · Rooting

Abbreviations *BA* 6-Benzylaminopurine · *Kn* Kinetin · *YE* Yeast extract · *MS* Murashige and Skoog (1962) basal medium

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Introduction

Phragmites Trin. is a genus of tall perennial grasses widely distributed in the temperate and tropical regions. These grasses belong to the family Graminae. They are long-rhizomatous and stoloniferous. Culms are usually 60–400 cm in height but do not exceed 1,000 cm, woody or herbaceous to persistent and generally unbranched above or branched above especially when the main culm is damaged. The genus comprises six species, *P. australis*, *P. communis*, *P. karka*, *P. longivalvis*, *P. maxima* and *P. prostratus*. Of these, *P. communis* and *P. karka* occur in India and are used for the manufacture of rayon and paper pulp, thatching, the making of mats, baskets, chairs etc. They are also excellent plants for binding loose soils. The reed, when young, is used as fodder for cattle and is medicinally important (Anonymous 1969). *P. australis*, besides the uses mentioned above, is also being used for the treatment of many industrial wastes in constructed wetlands. The plants have a system of rhizomes containing thick hollow air passages. The "root zone" supports a very large population of aerobic microorganisms which ultimately help in reducing the chemical oxygen demand (COD) and biological oxygen demand (BOD) of the waste. Reed beds are efficient in handling waste waters with BOD levels up to 500 mg/l (Kenneth and Biddlestone 1995) and have also been used for processing polluted waters ranging from domestic waste water to industrial discharge (Stomp et al. 1994) and distillery wastes (Thermax, India, personal communication). These treatment systems are environmentally friendly, low-cost and low-energy-demanding solutions to today's advanced waste water treatment systems which are generally energy intensive, expensive and relatively ineffective in the removal of many trace contaminants (Bishop and DeWaters 1988; Negri 1996).

Our investigations have developed from the need for a large number of plants of these species. In India, *P. communis* and *P. karka* grow naturally on the banks of lakes and streams in Himalayas and other surrounding areas. They spread by creeping rhizomes (Anonymous 1969).

P. australis is not native to India, and the European population is believed to be in decline due to unknown causes (Straub et al. 1988). This species has almost lost the capacity for sexual reproduction (Tzvelev 1989). Conventionally, the crop is not under cultivation because the importance of the species as a wetland plant has only been realized comparatively recently. Micropropagation is one solution to meet the immediate demand for the large number of plants required for the effluent treatment of ever-expanding industries. With this method, the required species can be propagated in a small area in as short a time as needed, even in countries where the natural conditions are not favorable.

There are a very few reports on plant regeneration of these species by tissue culture and none for large-scale micropropagation. Sangwan and Gorenflot (1975) studied callus formation and cell suspension of *P. communis*. Guoliang et al. (1987) obtained embryogenic callus and plantlet regeneration of *P. communis*. Straub et al. (1988) initiated embryogenic callus from mature seeds of *P. australis* and established over 250 plants in the greenhouse.

In this paper we report for the first time a rapid micropropagation system for three *Phragmites* species, *P. australis*, *P. karka* and *P. communis*, using axillary buds from the main and side branches of the plants, which would be ideal for their large-scale production.

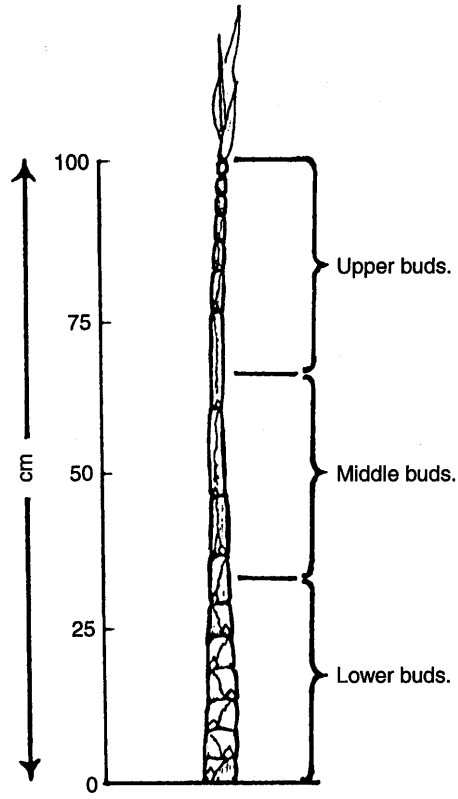

Fig. 1 Categorization of the buds with respect to their position on the branch

Materials and methods

The plant material

Ten genetically identical plants of each *Phragmites* species, *P. karka*, *P. communis* (Indian varieties) and *P. australis* (Danish variety) were collected from Thermax Private, Pune, India, and transferred to pots in a polyhouse for further growth.

Explant collection and preparation

The main and side branches of the plants were cut, thin leaf sheaths removed, and 1.5- to 2.5-cm-long explants were excised so as to have at least one axillary bud per explant. Nodal segments were collected from the base to the top of the branches. Three types of the buds were categorized from the top to the bottom as upper, middle and lower buds (Fig. 1). Each category comprised one-third of the total height of an approximately 100-cm-long branch.

Surface sterilization

The nodal segments of the explants were cleaned first with 0.5% (v/v) detergent (Labolene, Qualigens Fine Chemicals, Mumbai, India) for 8–10 min followed by 0.05–0.1% (v/v) antiseptic (Savlon, Imperial Chemical Industries, Calcutta, India) for 8–10 min. After three washings with distilled water to remove any traces of detergent and/or antiseptic, the buds were disinfected with 0.08–0.1 g % (w/v) $HgCl_2$ for 8–10 min followed by a sterile, distilled water wash in a laminar air-flow cabinet.

Sprouting

MS supplemented with BA (0.5 mg/l), Kn (0.5 mg/l), YE (1,000 mg/l), sucrose (2 g % w/v) and agar agar (0.7 g % w/v, Hi-Media, India) (SM medium) was used for the initial sprouting of the buds. Buds from all three species were cultured in test tubes (2.5×15 cm), containing 20 ml of the medium.

Multiplication

After 30 days of incubation, entire sprouted buds were transferred into 40 ml of liquid medium in 250-ml Erlenmeyer flasks.

The following media were used for the large-scale multiplication of different species: (1) M1. *P. karka*: MS supplemented with (0.5 mg/l) BA, (0.5 mg/l) Kn and 2% sucrose; (2) M2. *P. communis*: MS supplemented with (0.25 mg/l) BA, (0.5 mg/l) Kn and 2% sucrose; (3) M3. *P. australis*: MS supplemented with (0.1 mg/l) BA, (0.5 mg/l) Kn and 2% sucrose.

Sprouted buds gave rise to a clump of 8–12 shoots in 25 days following transfer to the multiplication media. The clumps were cut into smaller clumps having 3–4 shoots and then transferred into the respective fresh medium to establish a large number of shoot cultures.

Rooting

Root initiation was obtained on all three multiplication media for the respective species. Half-strength liquid MS basal medium with 2% sucrose and without any growth regulators (M4 medium) was used for the elongation of roots and shoots before transfer to the soil.

Culture conditions and observations

All the cultures were incubated at 25°±1°C with a photoperiod of 16 h at a light intensity of 11.7 μE m^{-2} s^{-1} supplied by cool-white fluorescent bulbs. The pH of all the media was between 5.6 and 5.8

before autoclaving. Autoclaving was carried out at 121 °C at 15 lb pressure for 20 min.

In all experiments, at least 100 nodal segments were inoculated in culture media and the experiment was repeated three times. Significance of differences between pairs of means of bud categories (upper, middle, lower) was determined by Student's '*t*' test (Snedecor and Cochran 1967).

Sprouting, appearance of contamination and the number of clumps obtained per flask (multiplication rate) were determined after 30 days in culture.

Transplantation

The rooted plantlets were removed from the culture vessels and washed off, and each plantlet was separated and transplanted in a potting mixture of autoclaved soil : sand (1 : 1) in polybags (10×5 cm). The plants were acclimatized in greenhouse conditions.

Results and discussion

The explants of all three species showed a high degree of contamination (65–75%). Contaminants appeared mainly as white and yellow pigment producing bacteria that were detectable after 8–10 days of incubation in the medium. Increasing the concentration and/or time of Savalon treatment from 0.05% to 0.1% and from 8 min to 10 min and the $HgCl_2$ treatment from 0.08% to 0.1% and from 8 min to 10 min did not help in reducing the contamination, indicating that the contamination was systemic in nature. This could be due to the fibrous nature of the root system, the presence of air pockets in root cortex tissue and/or the marshy plant habitat (Anonymous 1969).

The position of the nodal buds on branches considerably affected bud break in all three species (Table 1). No buds of *P. australis* sprouted from the upper buds of the stem. All turned brown after 10 days of incubation. In the case of *P. communis* and *P. karka*, however, 10% and 15% of the upper buds sprouted, respectively. The rest all either turned brown or became contaminated. The browning phenomenon was attributed to the fact that the buds were too tender to withstand the surface sterilization treatment, especially in the case of *P. australis*. Reducing the concentrations and or time of treatment did not help due to the contamination problem. There were no significant differences in the sprouting of the middle and lower buds of *P. karka* and *P. communis*. Lower buds of *P. australis* however showed higher sprouting than the middle buds of that species. Within the three species studied, bud break (Fig. 2a) was maximum in *P. karka*, followed by *P. communis*, and was minimum for *P. australis*.

During further experiments, it was observed that cultures of lower buds of *P. australis* could be established with two passages as opposed to three to four passages for middle buds. Only 30% of the middle buds could be established in culture. For *P. communis* and *P. karka*, however, middle buds could be established with two passages compared to four to five passages for the lower buds. Establishment, therefore, appeared to be strongly influenced by the maturity of the lower buds of *P. communis* and *P. karka*.

Table 1 Sprouting response of different bud categories of *Phragmites species*

Phragmites species	Percentage of buds sprouted (mean[a] ± SE)[b]		
	Upper	Middle	Lower
P. karka	15.2 ± 2.4[c]	80.0 ± 6.1[d]	75.2 ± 5.2[d]
P. communis	10.3 ± 3.2[c]	65.3 ± 5.2[d]	66.1 ± 6.2[d]
P. australis	0.0[c]	35.6 ± 4.0[d]	55.7 ± 3.6[e]

[a] Category means followed by different letters are highly significant at $P \leq 0.01$

[b] Mean of three independent experiments, each with at least 100 samples after 30 days of incubation on SM. Sprouting includes sterile and contaminated buds together

Guoliang et al. (1987) showed YE to be inhibitory to callus induction and callus growth from *P. communis* seeds. The inhibitory effect increased with increasing concentration of YE. During the present investigation, the inhibitory effect of YE was not observed in any of the three species studied. Presence and/or absence of YE in the medium did not affect sprouting and/or multiplication. However, it helped us in the early detection of contaminants. Similar results have been obtained in three species of bamboo, *Dendrocalamus strictus*, *Bambusa arundinacea*, *B. vulgaris*, *Casuarina* and cotton in our laboratory. We could detect contamination at a very early stage when the respective initiation media were supplemented with YE. Incorporation of YE in an initiation medium, therefore, can be an important ingredient for the species when contamination is a constraint.

After sprouting, multiplication of the sprouts was attempted in liquid medium as, in nature, *Phragmites* is a wetland plant (Kenneth and Biddlestone 1995). Initially, M1 medium (SM without YE) was used for all three species. Upon transfer to this medium the sprouted buds gave rise to 8–12 shoots within 25 days of incubation (Fig. 2b). A multiplication ratio of 1 : 3 was obtained in all species on the same medium. During shoot multiplication, 100% of the shoots of *P. karka* showed root initiation on the same medium.

Root initiation in the other two species was obtained in media containing lower concentrations of BA, 0.25 mg/l for *P. communis* and 0.1 mg/l for *P. australis*. This result suggests that higher concentrations of BA may have a deterimental effect on root primordia and that this effect varies from species to species. Joshi and Nadgauda (1997) have also shown BA to be inhibitory for root induction in *B. arundinacea*. We obtained similar results with another species of bamboo, *Dendrocalamus strictus*, which also belongs to the family Graminae: increasing BA concentration in bamboo increased the multiplication ratio with decreasing rooting percentage of the shoot multiples in vitro. Sangwan and Gorenflot (1975) reported root formation from *P. communis* callus occurring on all media used except those lacking growth regulators or those supplemented with high auxin concentrations.

The shoots and the roots obtained on the respective media were too small to be transferred to soil. In order to in-

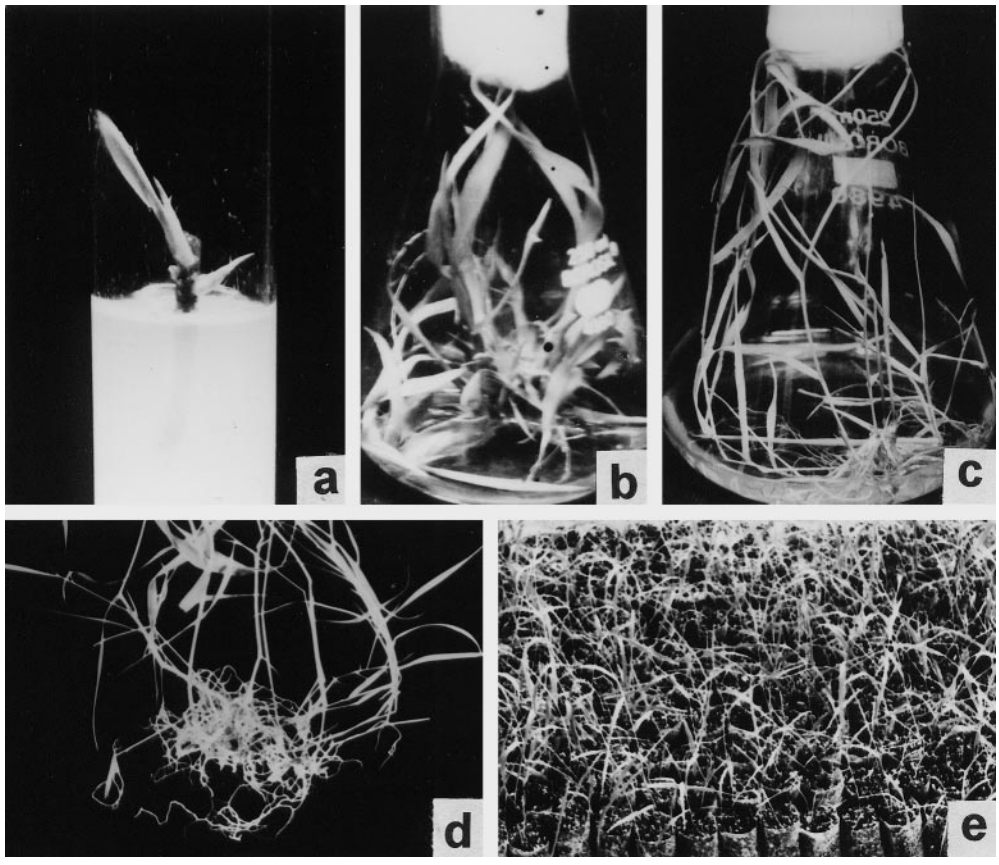

Fig. 2 **a** Sprouted bud on SM, **b** multiple shoot formation on M1 medium, **c** shoot and root elongation on M4 medium, **d** root hair zone on M4 medium, **e** hardened plants in polyhouse

Table 2 Survival of in vitro-propagated *Phragmites* species grown under polyhouse conditions for 30 days

Phragmites	Total no. of plants[a]		Percentage survival	Efficiency of the procedure[b]
	Transferred	Survived		
P. karka	10,303	10,000	97.06	3333
P. communis	11,205	10,644	94.99	3548
P. australis	12,505	12,029	96.19	4010

[a] Total plants produced from three buds of each species within 9 months; transferred in three batches containing 3,500–4,500 plantlets

[b] $\text{Efficiency} = \frac{\text{No. of plants produced}}{\text{No. of explants} = 3}$

crease the size of plantlets, we transferred the clumps onto M4 medium. Plants 10- to 12-cm long and showing a zone of root hair (Fig. 2c, d) were obtained within 15 days of incubation. Upon transfer to autoclaved soil : sand (1 : 1), 95% of the plantlets survived (Table 2) in a polyhouse maintained at 25° ± 1 °C and 80 ± 5% humidity for 30 days. The morphological and growth characteristics of the plants were free of visible abnormalities (Fig. 2e).

The efficiency of the procedure was calculated as "The number of complete plants which are obtained from an explant in a given time period". Over 3,000 plants were obtained from one bud in nine months for each species (Table 2). These plants were successfully established in the greenhouse and in the field and were supplied to a private company for waste treatment. Straub et al. (1988) reported the production of over 250 individual plants of *P. australis* from embryogenic callus.

To our knowledge, this is the first report of a large-scale micropropagation of *Phragmites* species using axillary buds. The method described here is invaluable for the treatment of large volumes of waste water in wetlands where large numbers of plants are needed. This technology is also very important for those areas where the required species of *Phragmites* are not available or are limited in numbers. One of the major requirementes when selecting candidate plants for the waste treatment is the ability to grow on contaminated sites taking into consideration the climate or tolerance of the contaminant (Stomp et al. 1994). The method described here has an added advantage that if the explant is collected from a plant adapted to a particular industrial waste, the propagules and the plants obtained thereof may not need any further adaptation to treat similar waste. This would save the time required for the adaptation.

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