Micropropagation of Indian pandan (*Pandanus amaryllifolius* Roxb.), a rich source of 2-acetyl-1-pyrroline

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A protocol for clonal propagation of *Pandanus amaryllifolius*, a rich source of 2-acetyl-1-pyrroline (2AP), an aromatic compound, has been established. Genetic fidelity of the tissue-culture-raised plantlets was ascertained through identical isozymic and RAPD profiles. The 2AP content of *in vitro* plantlets was analysed by gas chromatography in the backdrop of the mother plant.

THE most important aroma component of scented rice, 2-acetyl-1-pyrroline (2AP)[1] was also detected in pandan leaves (*Pandanus amaryllifolius* Roxb.) from Thailand[2–6]. While comparing the 2AP content of the plants from Thailand[7] with the same from West Bengal, India, higher content was observed in the latter. A micropropagation programme of this native Indian *P. amaryllifolius* Roxb. (http://www.scictr.edu.sg/ssc/publication/veg/pandanac.html) was, hence undertaken.

Micropropagation has been advocated as one of the most viable biotechnological tools for *ex situ* conservation of germplasms (http://www.rbgkew.org.uk/conservation). Similar reports are plenty and the present group itself has been successful in the micropropagation of a number of plants, maintaining genetic stability of the tissue-cultured clones[8–10]. The tissue-cultured *Pandanus* plantlets were, thus, subjected to isozyme and RAPD analysis to ascertain their genetic fidelity. Finally, 2AP content of the mother population and tissue-cultured clones was analysed by gas chromatography (GC).

P. amaryllifolius Roxb., of family Pandanaceae[11], has been located from different wild populations of district south 24 Parganas, West Bengal, India and grown in the Experimental Farm at Bose Institute since last five years. Meristems with sheathing leaf bases from newly emerging tillers, used as explants, were thoroughly washed with few drops of Tween 20 and surface sterilized with 0.25% (w/v) mercuric chloride for 10 min followed by rinsing with sterile double-distilled water four times. Explants were established in MS basal medium[12] supplemented with 0.55 mM myo inositol, 88 mM sucrose, 0.022 mM BA (N^6-benzyladenine) and 0.003 mM IAA (indole-3-acetic acid). Media were gelled with 0.75% agar (Difco Bacto). Multiplication was attained in the same media. The concentration of BA was decreased passage-wise (0.022 mM, 0.011 mM, 0.005 mM, 0.002 mM) before transferring to rooting medium. For root induction, both agar-gelled and liquid media with coir[13] were supplemented with 0.01 mM IBA (indole-3-butyric acid) and 0.002 mM kinetin. Cultures were kept under 16 h photoperiod (40–80 μ mol m^{-2} s^{-1}) at 25 ± 1°C and 78% relative humidity.

Isozymic analyses of four enzymes – Esterase (EST, E.C.3.1.1.1), peroxidase (PRX, E.C.1.11.1.7), acid phosphatase (ACP, E.C.3.1.3.2) and glutamate oxaloacetate transaminase (GOT, E.C.2.6.1.1) were done following standard protocol[14–16] after extraction of 2 g of leaves of both *in vivo* and *in vitro* plants. The protein content was estimated by the folin-phenol method[17]. Densitometric scan of the gels was done using Biorad Gel Documentation System (Gel Doc 1000, version 1.5).

For RAPD analysis, leaf sample from mother plants and randomly selected tissue culture clones of *P. amaryllifolius* were used. DNA was extracted by CTAB method[18] with minor modification[10]. DNA concentration in the samples was adjusted to 25 mg dm^{-3} for PCR reaction in each sample. PCR amplifications were performed according to the method of Williams *et al.*[19] using twenty oligonucleotide (decamer) primers, OPB01–OPB20 (Operon Tech., Alameda, USA). Amplifications were carried out in a Thermal Cycler (Perkin Elmer System – 2400, Norwalk, USA) with an initial denaturation of 120 s at 94°C and temperature profile of each cycle was: 60 s denaturation at 94°C, 60 s annealing at 35°C and 120 s for extension at 72°C. Reaction continued for 45 cycles followed by 300 s hold at 72°C to ensure that primer extension was completed. PCR reaction mixture of 0.025 cm^3 consisted 1× buffer, 0.2 mM dATP, dCTP, dGTP, dTTP, 2 mM $MgCl_2$, 0.2 μM of primer, 100 ng template DNA and 1 unit Taq DNA polymerase. Amplified products were electrophoresed in 1.8% agarose gel with λ DNA/*Eco*R1–*Hin*dIII double digest and $\phi \times 174$ *Hae*III digested DNA as size markers.

Finely chopped, fresh *P. amaryllifolius* leaves (5 g fresh weight) of both *in vivo* and *in vitro* plants directly from glass vessels with three replications in each case were considered as the source of 2AP. Samples were added to 50 ml of 0.25 mg/l TMP (2,4,6-trimethylpyridine), the internal standard solution. The mixture was stirred for 30 min, filtered, the filtrate transferred to a separating funnel, followed by addition of 5M NaOH and then 50 ml dichloromethane. Extraction was done twice and the dichloromethane solution was pooled, dried with anhydrous sodium sulphate, concentrated to 1 ml using a rotary evaporator under reduced pressure and at a temperature of 26°C. The concentrated extract was transferred to a V-shaped vial and left open to the air at room temperature until its volume decreased to 0.1 ml before it was subjected to quantitative analysis by capillary GC, with an FID. The whole experimental process was repeated for extraction and analysis of standard 2AP of exactly known amounts

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Table 1. Per cent vitrified and normal plantlets of *Pandanus amaryllifolius* with decreasing concentration of BA for four successive cultural passages

Concentration of BA (mM)							
0.022 I subculture		0.011 II subculture		0.005 III subculture		0.002 IV subculture	
Vitrified	Normal	Vitrified	Normal	Vitrified	Normal	Vitrified	Normal
76.67	23.33	56.67	43.33	40.00	60.00	13.33	86.67

$n = 30$.

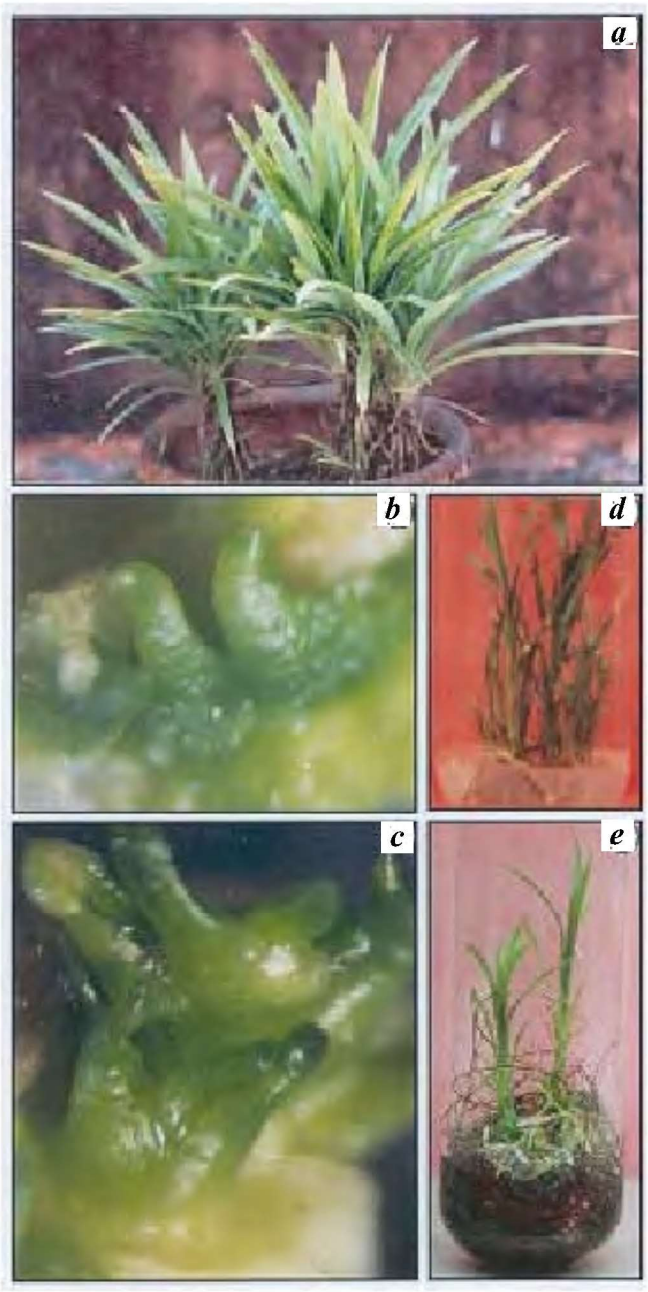

Figure 1. Micropropagation of *Pandanus amaryllifolius*. ***a***, Mother plant; ***b***, ***c***, Photomicrographs of adventitious shoot proliferation; ***d***, Full grown *in vitro* plantlet in agar-gelled medium; ***e***, Rooting in liquid medium with coir as matrix.

in a dilution series in order to obtain a standard calibration curve. GC analyses were performed on an Agilent 6890 GC equipped with Agilent 7683 injector and an FID (Agilent Technologies). A fused silica capillary column HP-5MS, biphenyldimethylpolysiloxane, with dimension 60 m × 0.25 mm i.d. and 1.0 µm film thickness (Agilent Technologies) was programmed starting at 60°C. The temperature was ramped to 120°C at a rate of 5°C per minute, and up to 180°C at a rate of 7°C per minute, resulting in an overall separation time of 21 min. The GC injector was in a split mode with a split ratio of 20 : 1. The injector temperature was set at 250°C. Purified helium gas at a flow rate of 1 ml per minute was used as the GC carrier gas. Quantification of 2AP of the leaf samples was carried out using the method of Mahatheeranont *et al.*[6], with TMP as the internal standard.

The explant excised from the tillers of *P. amaryllifolius* mother plant (Figure 1 *a*) resulted in multiple shoot bud proliferation (Figure 1 *b* and *c*). The shoot buds were then multiplied, but prolonged stay in culture containing 0.022 mM BA resulted in vitrified plantlets. Higher percentage of unvitrified plantlets (Table 1) was recorded with decreasing concentration of BA for four consecutive subcultural passages (Figure 1 *d*). A stepwise scaling down of the concentration of BA with subcultural passage has also been found beneficial in other monocots[10]. Rooting was induced in liquid medium with coir (Figure 1 *e*) and the number of roots developed per plant was always more ($P < 0.001$) in comparison to agar-gelled medium (Table 2). The technique of using coir, a biodegradable matrix for tissue culture, was developed earlier by the present group and employed in a number of plants[13].

After securing a large number of *in vitro* plantlets, samples were selected randomly after an interval of five cultural passages (one passage of twenty-eight days) from the fifth to twentieth passage for isozyme and RAPD analysis, since prolonged stay in cultural condition may jeopardize the total endeavour[20].

The mother and tissue cultured plants of *P. amaryllifolius* revealed identical isozymic profiles, though the relative activity of all the isozymes was more in the case of the mother plant in comparison to tissue-cultured plants. Among the four isozymic profiles, esterase showed maximum polymorphism (five bands, Rmf 0.16, 0.27, 0.4,

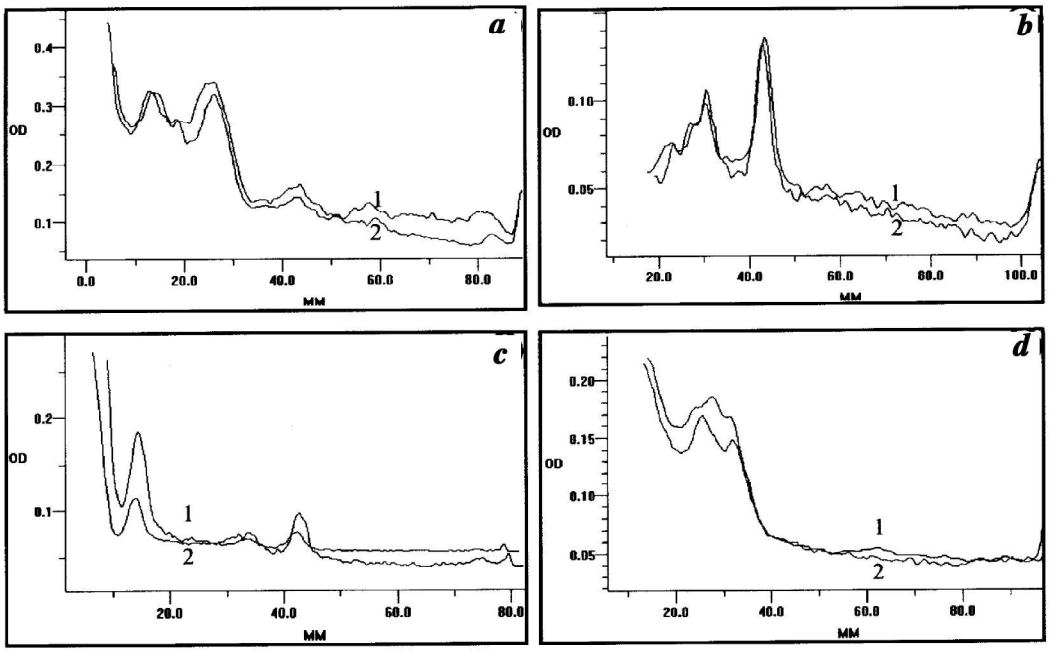

Figure 2. Isozymic profiles of mother (1) and *in vitro* (2) plants of *P. amaryllifolius*. ***a***, Esterase; ***b***, GOT; ***c***, Acid phosphatase; ***d***, Peroxidase.

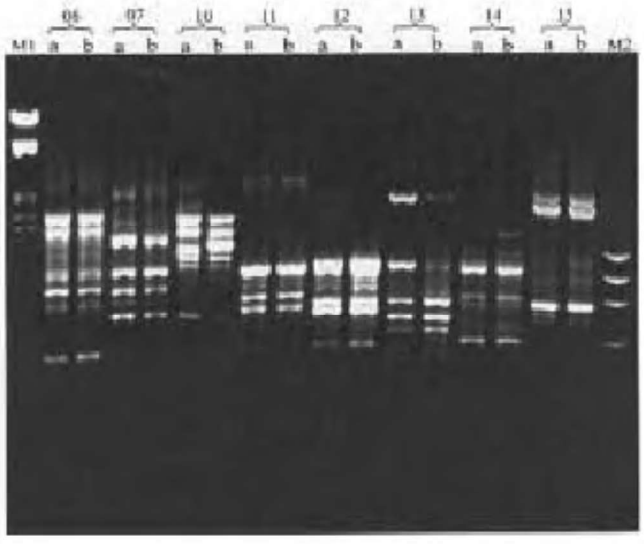

Figure 3. RAPD profiles of mother (***a***) and *in vitro* (***b***) plants of *P. amaryllifolius* with OPB primers (06, 07, 10–15). M1, λ DNA/*Eco*R1–*Hin*dIII double digest; M2, ϕ × 174 *Hae*III-digested DNA as size markers.

Table 2. Comparative response of rooting of *P. amaryllifolius* grown *in vitro* on agar-gelled medium (A) and liquid medium with coir (C)

Root induction (mean number of days taken for root initiation)		Number of roots (per plant)	
A	C	A	C
18.6 ± 0.7	5.0 ± 0.4	1.6 ± 0.2	5.0 ± 0.5

Mean ± SE (n = 10).

0.49 and 0.67; Figure 2 *a*) followed by GOT (four bands, Rmf 0.06, 0.13, 0.16 and 0.33; Figure 2 *b*), acid phosphatase (three bands, Rmf 0.18, 0.43 and 0.56; Figure 2 *c*) and peroxidase (three bands, Rmf 0.21, 0.25 and 0.29; Figure 2 *d*).

Twenty primers were screened for RAPD analysis. For the sake of brevity, profiles with maximum polymorphism (generated by primers 06, 07, 10–15) are presented (Figure 3). As there was no difference in RAPD profiles between mother and tissue cultured plants, only one profile of a randomly selected tissue cultured plant is being presented in comparison with that of the mother plant.

The comparative study between the mother and tissue-cultured plants with respect to 2AP manifested identical retention time of both 2AP and TMP (Figure 4 *a* and *b*) though relative peak area differed. The area ratio of 2AP/TMP was 5.7602 in case of mother plant and 1.6049 in case of tissue-cultured plant. 2AP was calculated by means of standard calibration curve plotted as a correlation of the amount and peak area ratios between the standard (2AP) and internal standard (TMP) in the dilution series, ranging between 0.1 and 10 mg/l, which resulted in a linear calibration curve with regression value of 0.9995 (Figure 5). The mean quantity of 2AP (μg/g of dry weight) was 527.7 and 368.8 in case of mother and tissue-cultured plant respectively. Since the detection of 2AP was the major focus of the present work, other volatile compounds as manifested in the chromatograms were not taken into consideration.

Pandanus is a vegetatively propagated plant. The *in vivo* plants of the present study were essentially derived from a wild plant of unknown age, which resulted in the present

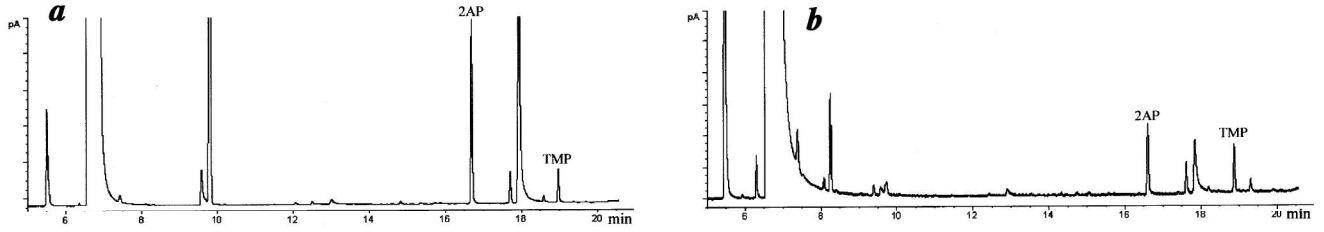

Figure 4. Reconstructed chromatograms of GC analysis of an extract of leaves by solvent of mother (***a***) and *in vitro* (***b***) plants of *P. amaryllifolius* using a fused silica capillary column HP-5MS, biphenyldimethylpolysiloxane, with dimension 60 m × 0.25 mm i.d. and 1.0 μm film thickness.

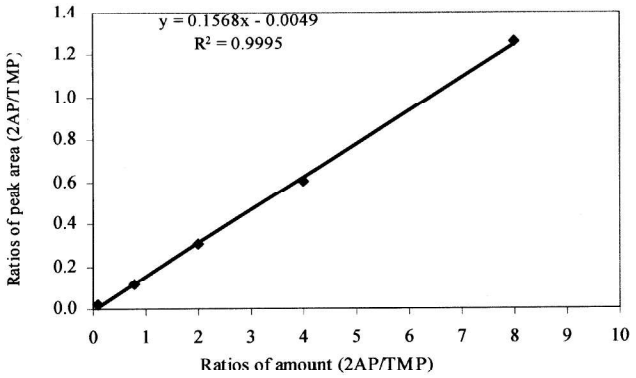

Figure 5. Relationship between ratios of concentration and ratios of peak area of 2AP and TMP showing linear response of the FID used.

population in the experimental farm by repeated cuttings during the past five years. Perhaps, therefore, 2AP content was high in the *in vivo* plants. The tissue-culture-derived plants grown in glass vessels would require time to attain the 2AP content up to the level of the mother plant after field transfer since genetically though alike, the expression of a qualitative trait is dependent on maturity.

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