In vitro flower induction in callus from a juvenile explant of sugarcane, *Saccharum officinarum* L., Var. CoC 671

Under natural growth conditions, flower formation usually commences when a plant attains maturity. The age of a plant is genetically controlled and is species-specific. The plant flowers only when genetic factors, including photoperiod response are congenial. These conditions can often be altered so that the plant can be induced to undergo an early reproductive phase. Such an attempt to induce flowering *in vitro* from juvenile explants of sugarcane is reported here.

Sugarcane is a polyploid, clonally propagated, highly heterozygous mosaic responsible for about 60% sugar production in the world. Sugarcane callus culture and regeneration was demonstrated long ago[1–3]. These pioneering attempts resulted in the introduction of tissue culture methods in sugarcane breeding programme[4–6].

Sugarcane has evolved under tropical conditions with corresponding shorter days and longer nights. The proper day length and the number of days vary both in the tropics and sub-tropics, which results in non-synchronous flowering under natural conditions. In commercial sugarcane production, flowering is not desirable. The accumulation of sucrose in the internodes will be minimized and the hydrolysis of sucrose takes place upon maturity of the cane[7]. Non-flowering or shy-flowering varieties should be used in areas where flowering is a serious problem. In India, flower initiation usually takes place during August and flowering takes almost 240–270 days after planting in early-maturing sugarcane varieties. Hence, *in vitro* studies are most suitable to understand the mechanism of flowering in sugarcane. The present correspondence describes the response of sugarcane callus on modified MS medium[1], which directly induced inflorescence *in vitro*.

The pioneering work on *in vitro* flowering was reported in cassava, without hormones in the culture medium[8]. *In vitro* inflorescence was developed from auxiliary buds of *Morus alba*[9] and *Hibiscus cannabinus* through inflorescence culture[10]. Precocious flowering in bamboos through inflorescence culture was also reported[11]. Other researchers have also reported *in vitro* flowering from cotyledon cultures of groundnut[12], callus-derived plantlets of *Papaver somniferа*[13], and somatic embryogenesis and *in vitro* flowering in *Brassica nigra*[14]. However, there are no reports available to date on the development of inflorescence directly from callus cultures.

Young leaf rolls from 3–4-month-old sugarcane plants were cultured on modified MS medium[1] supplemented with 3% sucrose, 3 mg/l 2,4-D, 100 mg/l polyvenylpyrrolidone, 10% (v/v) coconut water (liquid endosperm of 6–7-month-old coconuts) and the amino acid proline with varying concentrations, ranging from 0 to 100 mg/l (Table 1). The flasks lacking proline in the culture medium served as control. The callus was initiated after 15 days in dark. The callus obtained was kept in a photoperiod of 6 h light (40 μEinstein $m^{-2} s^{-1}$) and at a temperature of 25 ± 2°C. Flasks containing the callus were maintained for periods up to 180 days without subculturing. The flasks incorporated with 40 mg/l proline in the culture medium induced *in vitro* flowers. The floral part, mainly anther, was subjected to sectioning using cryostat (Bright Instrument Co, Huntingdon, England). A section of about 6 μ thickness was obtained and stained with safranin.

Flowering from vegetative tissue was observed *in vitro* on long-term callus culture of sugarcane meristematic tissue. The flowering was observed after 180 days of callus culture without subculturing (Figure 1 *a*). Callus cultures with 40 mg/l proline in the culture medium induced inflorescence. The inflorescence had a cluster of flowers consisting of few stamens with filament topped by anther (Figure 1 *b*). The transverse section of the anther showed two anther lobes with epidermis, tapetum, vascular bundle and pollen sacs (photograph not shown). The peduncle had two branches (Figure 1 *b*), an abnormal phenomenon compared to the inflorescence *in vivo*. The culture medium without proline induced friable callus (Figure 1 *c*). The callus cultures responded differentially upon incorporation of proline at increased concentrations in the culture medium (Table 1).

The development of inflorescence in sugarcane Var. CoC 671 may be due to the photoperiod response or the use of proline in the culture medium, or it may be due to the cumulative effect of the above. The incorporation of proline in the culture medium might have repressed the synthesis of proline in the cells. Proline is generally synthesized in plants as an osmotolerant under stress condition[15–17]. In earlier reports, 500 mg/l proline was used to initiate embryogenic callus from immature inflorescence on MS medium[18]. The proline concentration seemed to be higher when compared to our study, where 100 mg/l proline in the culture medium induced callus but inhibited further proliferation (Figure 1 *d*). Maintenance of callus for 180 days without subculturing in fresh medium might have created osmotic and nutritional stress to the growing tissue, resulting in induction of inflorescence. The long-term callus cultures in sugarcane increased the fresh weight of the callus by incorporation of 10 mg/l proline in the culture medium[19]. But long-term culture of tissues resulted in non-synchronous plantlet production and loss of organ-forming capacity[20]. Thus, the physiological conditions of *in vitro*-grown tissue and the role of proline during post-stress conditions seem to be the

Table 1. Callus culture with varying proline concentration and response of callus

Proline concentration, mg/l	Response of long-term callus culture
Control*	Normal, friable callus after 180 days of culture
20	Slightly brownish callus with retarded growth after 180 days of culture
40	*In vitro* flowering was observed after 180 days of culture
80	Brownish-black callus with retarded growth after 180 days of culture
100	Further growth of the callus ceased and callus turned completely black in colour after 180 days of culture

*Without proline.

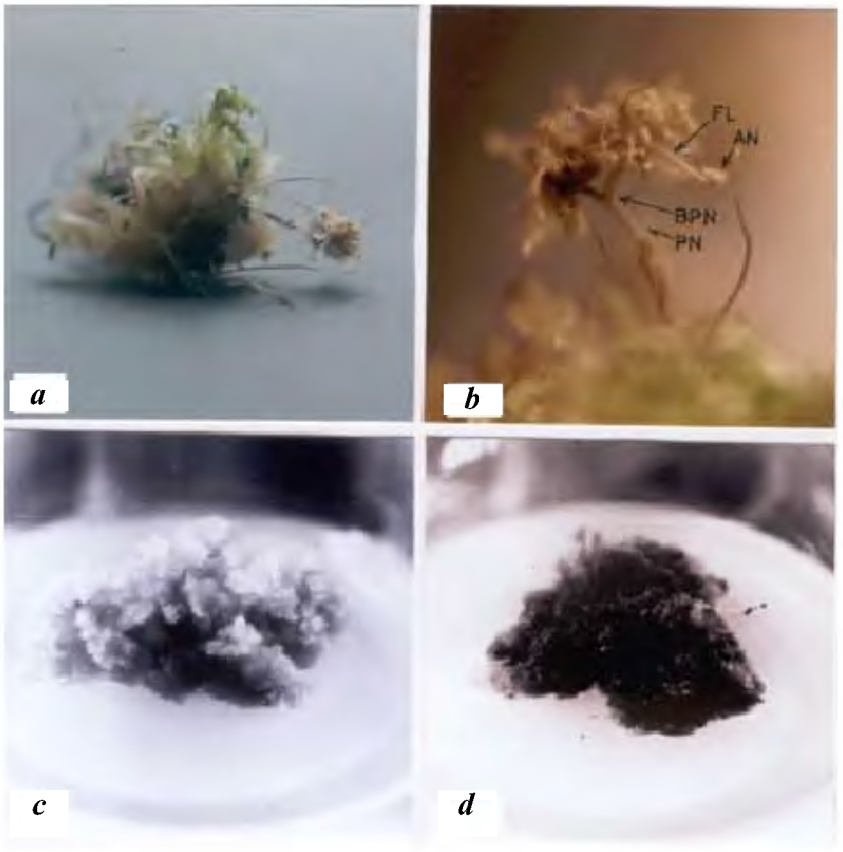

Figure 1. ***a***, *In vitro* flowering in sugarcane Var. CoC 671 on modified MS medium showing spikelet inflorescence raised on a distinct stalk; ***b***, Primary inflorescence raised on the peduncle (PN) which is branched (BPN) giving rise to two distinct inflorescence consisting of many stamens having anther filaments (FL) topped by anthers (AN); ***c***, Normal, friable callus without proline in culture medium after 180 days of culture; and ***d***, Retarded callus growth which turned black in colour with 100 mg/l proline in the culture medium after 180 days of culture.

factors for induction of precocious flowering in sugarcane.

Phytohormones influence many diverse developmental processes ranging from seed germination to root, shoot and flower formation[21]. The initiation of flowering has been explained by the presence of flower bud initials in the region of embryonic axis, and the addition of cytokinin might have triggered the mechanism promoting the precocious flowering in groundnut[22]. Precocious flowering was also observed in bamboos, where the plant flowers only once in its lifetime[11,23]. In cassava, *in vitro* flowering was induced without the use of hormones in the culture medium[8]. *In vitro* flowering in sunflower was also reported with low concentration of sucrose and 1 mg/l each of NAA and GA_3, and 0.5 mg/l activated charcoal[24]. However, increase in sucrose concentration (30 g/l) in the culture medium did not result in flowering in groundnut[12]. But, in our investigation *in vitro* flowering was observed with higher sucrose concentrations (30 g/l). The addition of inhibitors of flowering like CCC (2, chloroethy trimethyl ammonium chloride) to inhibit endogenous GA synthesis, ACC (1-amino cyclopropane-1-carboxylic acid) and AVG (aminoethoxyl vinyl glycine) to promote and inhibit ethylene production respectively, did not inhibit *in vitro* flowering in sunflower[24,25]. Perhaps, *in vitro* flowering is not linked to hormonal regulations only.

Transgenic plants with altered fertility in *Arabidopsis* were generated by manipulating genes that control flowering and pollen formation, where the expression of *LEAFY* or *APETALA 1* genes has been shown to result in precocious flowering[26,27]. Flowering from callus of a juvenile explant of sugarcane might be the combinatorial effect of 2,4-D, proline and sucrose concentrations, which might have triggered the ectopic expression of genes or the signal molecules or proteins as in *Arabidopsis*, which resulted in precocious development of inflorescence.

The present work emphasizes the development of inflorescence directly from callus of juvenile explants. The flower induction in sugarcane callus *in vitro* is bound to be a precocious phenomenon, as *in vivo* flowering in sugarcane Var. CoC 671 takes about 240–270 days after planting with low flowering. Our study can add to the previous understanding of hormonal induction of flowering and also the biosynthetic pathway leading to the formation of inducers or signal molecules, which might have triggered the flowering genes in the presence of proline in the culture medium. *In vitro* flowering in sugarcane can be taken as an advantage in terms of understanding the phenomenon of shy-flowering/low-flowering and also the correlation between the sucrose metabolism and flowering in sugarcane as the accumulation and concentration of sucrose in the internode tissues vary with cane maturity. However, the mechanism of *in vitro* flowering of juvenile explants of sugarcane deserves a thorough biological analysis going far beyond the present study.

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ACKNOWLEDGEMENTS. We thank the Department of Biotechnology, Government of India for financial support and Gulbarga University, Gulbarga for providing facilities.

Received 14 June 2002; revised accepted 11 September 2002

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Design and development of the Scanning Force Microscope for imaging and force measurement with sub-nanonewton resolution

The Scanning Force Microscope (SFM)[1], which started as a derivative of the Scanning Tunnelling Microscope (STM)[2], has emerged as one of the most powerful tools in the field of surface sciences, study of nano-mechanical properties and nanolithography. The operating principle of the SFM is based on measurement of forces of the order of 10^{-13} to 10^{-4} N between the sharp tip and the surface of the sample. The probing tip is attached to a cantilever and the force acting on the tip causes a small deflection of the cantilever. This deflection is detected and mapped as the tip scans the surface to obtain the image of the surface. Though SFMs are available commercially, it is difficult to improvize on them for experiments other than their basic function, i.e. imaging. In this communication we present the design and fabrication of a SFM with optical deflection detection system and a piezoelectric inertial drive mechanism for coarse approach. This design is easy to machine, assemble and can be modified for different physico-chemical experiments using SFM.

The major components of a force microscope are: a sharp tip mounted on a soft cantilever, the cantilever deflection detection system, feedback to monitor and control the deflection, mechanical scanning system (piezoelectric tube to raster scan the sample with respect to the tip), tip–sample approach mechanism, and an image display and measurement system via interface. Out of the various deflection detection options available, ranging from tunnelling detection method[1], piezoresistive detection[3], optical deflection detection (ODD)[4] to interferometric detection[5] using optical fibre[6], we have implemented the ODD method using a laser diode[7]. This method is used in most of the commercial SFMs, since it offers very high force resolution (~10^{-12} N), compactness and operational simplicity. The choice of ODD system for cantilever deflection detection makes the optical stage the most crucial part of the SFM design and fabrication. The optical stage consists of a laser diode (L; λ = 670 nm, power = 3 mW, RS components), position-sensitive detector (quadrant photo detector (QPD); RS components, UK), reflecting cantilever ($k \sim 0.2$–20 N/m, Olympus Optical Co, Japan) and collimating lenses. The position-sensitive detector is a quadrant photodiode, which gives a signal proportional to the position of the spot of light. For maximum sensitivity, the laser spot on the QPD should move as much as possible for a given cantilever deflection. To maximize the spot movement, we have used the concept of the optical lever. This has been achieved by adding a reflecting mirror (M; polished silicon wafer) in the optical path from the cantilever to QPD (Figure 1 *a*). Optical lever ratio = (Length of the laser path/ Length of the cantilever). For a cantilever of length 100 µm, we have achieved an optical lever ratio of ~ 500. Cantilevers have been chosen using two broad guidelines; the resonant frequency of the cantilever should be high (~ kHz), the minimum requirement for the cantilever resonant frequency is that it should be greater than the data acquisition rate, building and acoustic frequencies of the instrument), the spring constant is chosen depending on contact or non-contact mode.

We have chosen a piezoelectric tube-based inertial drive (ID) mechanism[8] for the coarse approach. The advantages of using ID are low cost, simplicity in design, controllability via interface with computer and UHV compatibility[8]. The working principle behind the ID is that the driver (D) moves only when the inertial force due to movement of the supporting glass tube (driven by a piezoelectric tube) is larger than the static friction between the driver and the walls of the glass tube (Figure 1 *a*). When a saw-tooth waveform is applied to the