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Technologies for cost reduction in sugarcane micropropagation

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High cost involved in micro propagation is a major constraint to its popular use in sugarcane (*Saccharum* spp. hybrid). This study describes two technologies for cost reduction in sugarcane micropropagation, that is, direct regeneration of complete plantlets on the same medium and substituting *in vitro* rooting by *ex vitro* rooting in conventional micro propagation. A protocol for one step regeneration of complete plantlets was developed for sugarcane cultivar CoS96268. Complete plantlets were regenerated in 42 days on regeneration medium using leaf disc explants, pretreated on MS medium supplemented with 3 mg/l 2,4-D for eight days. More than 95% explants exhibited regeneration with an average of 23 shoots per explants. After 42 days on regeneration medium, each explants produced 6 to 8 healthy plantlets which could be successfully hardened. A protocol for *ex vitro* rooting of micro shoots, raised from axillary bud culture was also standardized. *In vitro* shoots, 5 to 6 cm long, treated overnight with 20 mg/l NAA, led to formation of complete plantlets with more than 90% root induction. These plantlets possessed more than 6 roots of 4 cm average length per plantlet and exhibited 95% survival when transferred to polybags containing soil. Thus, direct adventitious regeneration and *ex vitro* rooting can be applied to sugarcane micro propagation to reduce cost of plant production.

Key words: *Ex vitro* rooting, direct organogenesis, sugarcane.

INTRODUCTION

Commercial sugarcane is a major field crop accounting for about 75% of sugar production worldwide. It is one of the most efficient biomass crops with adaptability to both tropical and subtropical conditions, and is now being targeted as a biofactory with potential for the integrated production of sugar and other industrial and high-value products. Sugarcane is vegetatively propagated for commercial planting by stem cuttings.

Production of disease-free seedlings in such large numbers during the planting season is laborious and time consuming. In addition, it requires a substantial quantity of crushable cane that otherwise could be used for sugar production. Tissue culture offers an opportunity to mass produce disease-free planting material and is now used to supplement commercial sugarcane propagation in many countries including Brazil, the United States, India and Cuba.

However, high costs involved in micro propagation are a major constraint to its popular use (Sluis, 2006). Several alternatives such as automation, shifting of production activities to countries with low wage regime (Chu and Kurtz 1989), substitution of costly media components (Ghosh, 1996) and enhanced multiplication rates (Sluis, 2006) have been suggested to reduce costs of tissue culture plants. Cost reduction in tissue culture can also be achieved through adding liquid nutrient medium to exhausted agar media (Maene and Debergh, 1985), computerized changing of nutrient solutions at different stages of growth (Tissart and Vendercook, 1985), use of industrial robots (De Bry, 1986) and nutrient mist generation in special culture enclosure (Weather and Giles, 1988). Most of these options are difficult to implement, either due to technological and biological limitations of the plant species or due to economic aspects of off-shoring production activities. Improving the efficiency of existing micro propagation procedures

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through labor friendly means and efficient use of resources is the first step for any kind of cost reduction in tissue culture plant production.

The number of plants produced by micro propagation is proportional to the manpower (Rowe, 1986; Smith, 1986) and space available for propagation. This space is highly defined in terms of temperature and hygiene, which requires great deal of expenses on electricity for cooling and lighting. In this case, the electricity consumption for cooling is approximately 50% of the electricity consumption for the lighting (Kozai 1991). Devising practical strategies which reduce manpower, space and power requirements would mean significant reduction in cost of micro propagation. Direct adventitious regeneration of complete plantlets offers efficient strategy as it reduces time in culture and need for separate rooting and multiplication medium thus, reducing manpower required for repetitive culture transfers and while, rooting of *in vitro* shoots *in vivo,* can lead to reduced per plant costs as it waves off the need for asepsis and specialized handling and leads to simultaneous hardening of plantlets (De bergh and Maene, 1981). This leads to reduced space and power requirements besides adding to labor efficiency.

Direct adventitious plant regeneration in sugarcane has been achieved from immature inflorescence tissues (Desai et al., 2004), immature leaf thin cell layers (Lakshmanan et al., 2006), and immature leaf disc explants with or without pre-emergent inflorescence (Snyman et al., 2006), leaf segments (Gill et al., 2006) and leaf midrib explants (Franklin et al., 2006), while *ex vitro* rooting in sugarcane has been reported only for sugarcane cultivar Q165 (Laxmanan et al., 2006). This study envisaged development of a system for direct regeneration of complete plantlets in CoS96268 through direct adventitious organogenesis and standardize a protocol for *ex vitro* rooting of micro shoots, raised from axillary bud culture; to reduce time, space and manpower requirement in sugarcane micro propagation.

MATERIALS AND METHODS

Direct adventitious regeneration

To develop a system for direct regeneration of complete plantlets in CoS96268 through direct adventitious organogenesis, leaf roll disc explants from shoot tops of field grown sugarcane (CoS96268) plants were prepared as described by Snyman et al. (2006). Healthy shoot tops with intact apical meristem and young leaves were collected from the field-grown plants of elite early maturing cultivar CoS96268. Cylinders of young leaf rolls (5 to 8 cm height) were cut-removed just above the shoot tip. These cylinders were wiped with 70% ethanol for 1 h and surface-sterilized with 0.1% (w/v) mercuric chloride for 10 min. After the treatment, they were washed thoroughly in sterilized distilled water. The outer leaf sheaths were removed aseptically one by one until the cylinder reached approximately 0.5 cm in diameter. The cylinders were chopped transversely into thin slices (0.5 to 1.0 mm thick), these thin slices of leaf tissues were cultured on MS medium containing 0, 1, 2, 3, 4 and 5.0 mg/l 2,4-D under continuous dark for 5, 8 and 10 days (Franklin et al., 2006). After pretreatment for the respective number of days, these were transferred to regeneration medium (0.2mg/l BAP and NAA 0.1mg/l) and incubated under a 16 h photoperiod. All the chemicals used (sucrose, agar and plant growth regulators) in the study were of Duchefa Biochemie, Netherlands. The experiment was performed with three replicates in completely randomized design and repeated thrice to ensure reproducibility. Data were recorded on percent explants regenerating and number of shoots.

Ex vitro rooting

To standardize a protocol for *ex vitro* rooting of micro shoots, axillary bud cultures were initiated from mature nodal segments using a protocol standardized in our laboratory (data not shown). Nodal segments of sugarcane CoS96268 (3 to 4 cm long with axillary buds) were collected from 6 to 12 months old mother plants. The nodal segments were wiped with 70% alcohol and washed with Tween twenty (detergent and surfactant) for 30 min followed by surface sterilization with 0.1% mercuric chloride ($HgCl_2$) solution (20 min) followed by rinsing with distilled water and cultured aseptically on MS medium supplemented with 3 mg/l BAP. One nodal segment was implanted in each culture bottle. The pH of the medium was adjusted to 5.8 before autoclaving at 1.4 kg cm^{-2} for 20 min. The induced shoots were multiplied on MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA for five passages. Effect of auxin (NAA) pretreatment on *ex vitro* rooting was studied. Clumps of *in vitro* micro shoots were separated to obtain single micro shoots. These micro shoots were kept in distilled water containing 0, 10, 20, 30 and 40 mg/l NAA such that only the base of the micro shoots remained in the solution. These shoots were then transferred to moist sand bed in a green house for twenty one days. Humidity was maintained at 60 to 70% and temperature was maintained at 35 to 40°C. The shoots were sprayed with ½ strength MS medium (Murashige and Skoog, 1962) once every week. After three weeks, the plantlets were carefully removed from the sand, data were recorded and the plantlets were then transferred to poly-bags containing soil, sand and farmyard manure (1:1:1). The experiment was performed with ten replicates in completely randomized design and repeated thrice to ensure reproducibility. Data on percent root induction, average number of roots, average root length and post rooting survival of plantlets were collected. Mean values over the responses in various treatments and standard error were calculated.

RESULTS AND DISCUSSION

The direct organogenesis pathway can be very useful for large scale applications as it eliminates/minimizes callus formation in culture and minimizes culture duration (Lakshmanan et al., 2006) leading to fast multiplication from widely available leaf explants, while retaining clonal identity. Direct adventitious regeneration potential of immature leaf disc explants is less as compared to inflorescence tissues (Snyman et al., 2006). However, the availability of floral materials is restricted in time and space. This study successfully utilized pretreatment of immature leaf disc explants on 2,4-D supplemented MS medium to enhance direct adventitious regeneration in these explants. Franklin et al. (2006) have reported that pre-treatment of leaf midrib segments can lead to

Table 1. Influence of pretreatment period with 2,4-D on direct adventitious regeneration of shoots from leaf disc explants of sugarcane cultivar CoS96268.

Pretreatment period with 2,4-D (day)	Leaf disc explant regenerating (%)	Mean number of shoot ± SD per explant after various culture interval on shoot initiation medium (Franklin et al., 2006)		
		21 day	28 day	42 day
0	2.00	3.0 ± 0.5[a]	6.0 ± 0.7[a]	8.0 ± 1.8[a]
5	40.00	5.8 ± 0.8[a]	7.2 ± 1.0[ab]	11.4 ± 2.0[ab]
8	95.00	8.2 ± 0.9[b]	12.3 ± 1.8[b]	19.0 ± 2.4[b]
10	70.00	3.4 ± 0.2[a]	5.6 ± 1.0[a]	8.2 ± 2.0[a]

Values (mean ± standard deviation) in a column followed by same letter are not significantly different.

enhanced shoot organogenesis in sugarcane, however, the same has not been described for leaf disc explants which have been widely recommended for direct adventitious organogenesis/embryogenesis (Laxmanan et al., 2006, Snyman et al., 2006). Moreover, there are no reports of direct adventitious regeneration in CoS 96268.

In this study, it was observed that leaf disc explants pretreated on MS medium containing 3.0 mg/l 2,4-D for 8 days under continuous dark when transferred to MS medium containing 0.1 mg/l NAA and 0.2 mg/l BAP under light conditions led to the development of up to 23 shoots directly on the leaf disc (Figure 1a). These shoots attained 2 to 5 cm height within 30 days of culture initiation and also developed roots on the same medium (Figure 1b). The rooted plantlets could be separated in propagules of 2 to 3 shoots (Figure 1c) and 6 to 8 such plantlets could be obtained from each explant. The plantlets were successfully transferred to the soil with 93% establishment rates. The regeneration of shoots and subsequently, plantlets were dependent on duration of pretreatment. Of the various durations of pretreatment tested (Table 1), eight days pretreatment of leaf disc explants on 2,4-D supplemented MS medium led to significantly higher shoot organogenesis on shoot initiation medium (Franklin et al., 2006) when compared to other treatments. On this treatment, 19 to 20 shoots were observed at the end of 42 days. Pretreatment of plant material with growth regulators is known to increase regeneration rates in several plant species (Yancheva et al., 2003). Our results are in line with those of Franklin et al. (2006) for sugarcane.

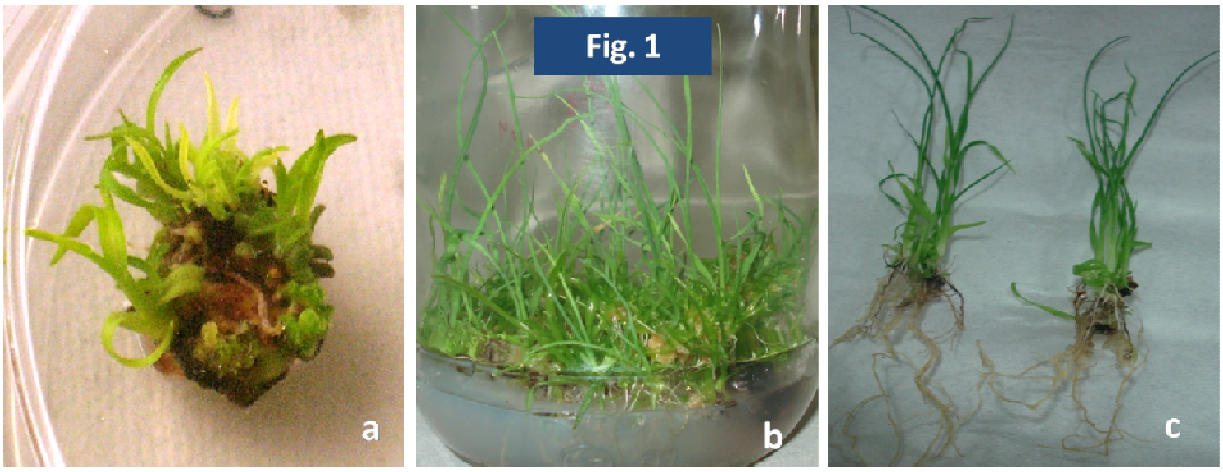
Continuous culture of explants on auxin medium results in the formation of embryogenic callus. Therefore, effect of plant growth regulators (BAP and NAA) on direct adventitious regeneration of shoots from pretreated leaf disc explants was also investigated (Table 2). It was found that MS medium supplemented with 0.2 mg/l BAP and 0.1 mg/l NAA had best results for shoot regeneration (Table 2). More than 95% explants respond with 24 shoots regenerating per explant (Figure 1a), on this medium. These results are different from those of Franklin et al. (2006) who found best shoot regeneration in MS medium supplemented with 0.1 mg/l BAP and 0.1 mg/l NAA for sugarcane varieties.

The hormonal combination used in this study also favored simultaneous rooting of regenerated shoots on the same medium (Figure 1b). Thus, complete plantlets were obtained within 42 days inoculation incubation of pretreated leaf discs on regeneration medium. These plantlets were separated in propagules of 2 to 3 shoots (Figure 1c) and successfully hardened prior to planting in soil.

Ex vitro rooting

The time required for *in vitro* rooting in sugarcane ranges from 4 to 6 weeks (Pawar et al., 2002; Ramanand et al., 2007). *Ex vitro* rooting as a measure to reduce cost and induce efficiency of sugarcane in micro propagation by coupling the rooting and hardening phase was successfully attempted. For some plants, it has been possible to treat the shoots formed in cultures as mini-cuttings and to root them *in vivo*. Usually, the shoots are treated with a high concentration of auxins (either by saturating the potting mix with auxin solution or dipping the basal cut end in the rooting powder (Debergh and Maene, 1981). Sharma et al. (1999) reported direct rooting and hardening of tea micro shoots in the field. *Ex vitro* rooted sugarcane plantlets showed excellent growth when transferred to poly-bags containing soil, sand and farmyard manure in 1:1:1 ratio (Figure 5). Even though, *in vitro* has been effective in sugarcane (Ramanand et al., 2007) propagation, its rooting has the potential to reduce production cost as most of the space in culture room is occupied by rooting cultures. Successful *ex vitro* rooting will mean availability of more space in culture room reducing power consumption and need for aseptic operations and also reduce the time for hardening, as the *ex vitro* rooting and primary hardening can take place simultaneously.

In this study, efficient rooting of sugarcane micro shoots was obtained in 3 weeks treatment of these shoots overnight with auxin (NAA). A positive influence of auxin treatment was observed on *ex vitro* adventitious rooting in micro shoots of sugarcane. This is in line with observations on *in vitro* rooting of sugarcane cultures (Pawar et al., 2002; Ramanand et al., 2007). Less or no



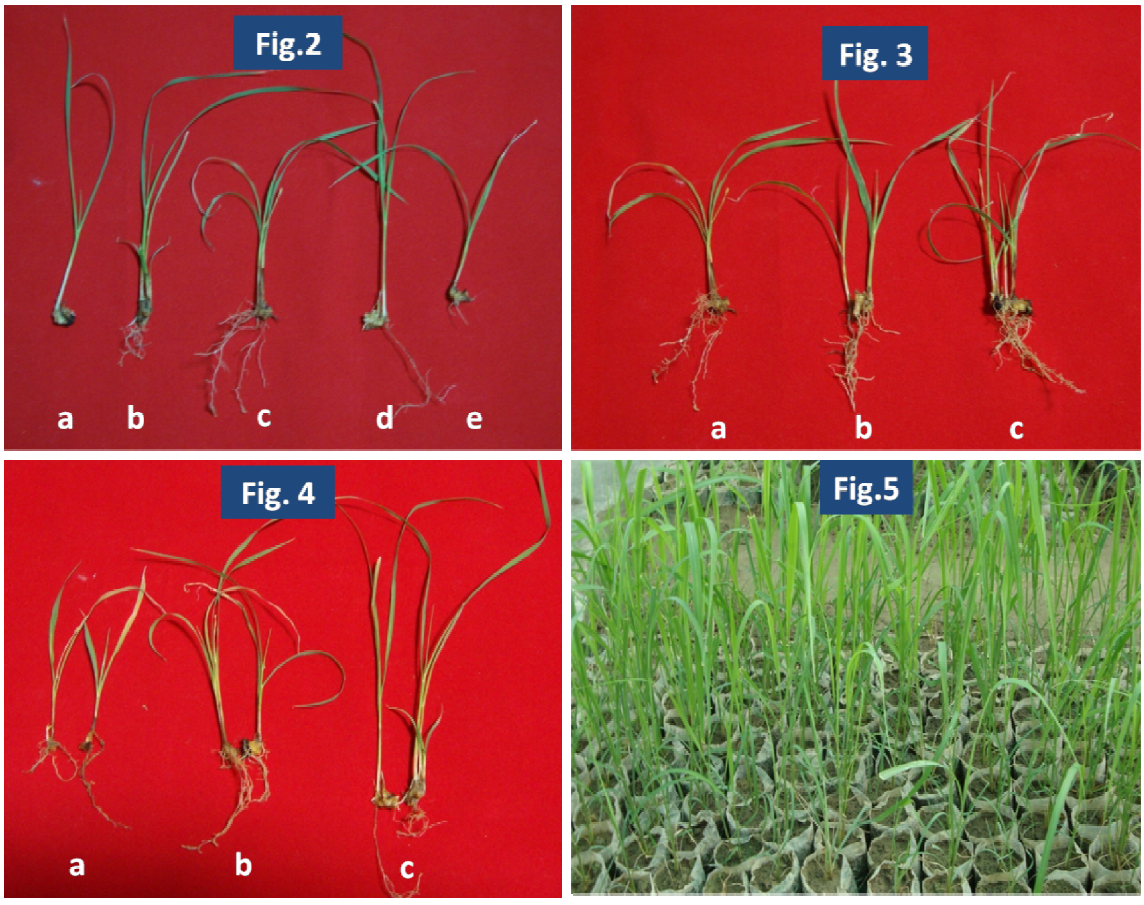


Plate – 1: Two technologies for cost reduction in sugarcane micro propagation: Direct adventitious regeneration and *Ex vitro* rooting. Test variety CoS96268.
Fig.1.a Direct shoot organogenesis in leaf disc explants on regeneration medium, b. Formation of complete plantlets on regeneration medium, **c.** complete plantlets with well developed root-shoot system.
Fig.2, Effect of Auxin concentration on *ex vitro* rooting, a, b, c, d, e, are plantlets developed after overnight treatment of *in vitro* shoots with 0,10, 20, 30, 40 mg/l NAA respectively.
Fig.3, Effect of propagule size on *ex vitro* rooting, a, b, c are rooted plantlets developed from one, two and three shoot propagules respectively.
Fig.4, Effect of shoot size on *ex vitro* rooting, a, b and c are plantlets formed by using 2-3cm, 5-6 cm and more than 7 cm long shoots respectively.
Fig.5, *ex vitro* rooted plants showing excellent growth when transferred to soil.

Table 2. Effect of plant growth regulators on direct adventitious regeneration of shoots from leaf disc explants of sugarcane cultivar CoS96268 pretreated for 8 days on MS medium supplemented with 3 mg/l 2,4-D.

PGR (mg/l)		Leaf disc explant regenerating (%)	Mean number of shoot ± SD per explant after various culture interval		
BA	NAA		21 day	28 day	42 day
0.0	0.0	0	0	0	0
0.2	0.0	20	6.0 ± 1.7^{b}	10.3 ± 1.0^{e}	20.0 ± 1.6^{d}
0.5	0.0	30	5.8 ± 0.8^{a}	7.2 ± 1.2^{abc}	10.2 ± 1.2^{b}
1.0	0.0	50	3.0 ± 0.8^{a}	5.6 ± 1.0^{abc}	8.0 ± 1.0^{b}
2.0	0.0	75	3.0 ± 0.5^{a}	5.8 ± 0.8^{ab}	7.2 ± 0.8^{a}
5.0	0.0	00	0.0	0.0	0.0
0.0	0.1	00	0.0	0.0	0.0
0.2	0.1	95	8.2 ± 0.9^{b}	12.3 ± 1.8^{d}	24.0 ± 1.4^{e}
0.5	0.1	80	6.4 ± 1.2^{b}	11.4 ± 2.0^{bc}	18.0 ± 3.3^{cd}
1.0	0.1	50	3.4 ± 0.2^{a}	7.0 ± 1.6^{f}	11.0 ± 1.2^{b}
2.0	0.1	90	1.4 ± 0.8^{a}	7.5 ± 2.6^{c}	8.0 ± 2.0^{b}
5.0	0.1	10	1.6 ± 0.2^{a}	3.3 ± 2.0^{a}	4.0 ± 1.6^{a}

Values (mean ± standard deviation) in a column followed by same letter are not significantly different ($P > 0.05$), n = 20.

Table 3. Effect of NAA on *ex vitro* rooting of sugarcane micro shoots. Shoots were harvested from *in vitro* cultures of CoS96268 raised from axillary buds and multiplied for 5 passages on 0.8 mg/l BAP + 0.1 mg/l NAA. Shootlets were treated overnight with 0 to 40 mg/l NAA in 25 ml water. Observation was taken after 21 days of planting in sand, under green house conditions.

Parameter	Root induction (%)	Mean number of root	Mean root length (cm)
Control	10.00	2.2 ± 0.4^{a}	2.6 ± 0.3^{a}
10 mg/l NAA	60.00	4.5 ± 0.7^{a}	3.1 ± 0.4^{a}
20 mg/l NAA	90.00	6.7 ± 0.8^{b}	4.2 ± 0.5^{b}
30 mg/l NAA	70.00	4.0 ± 0.4^{a}	2.8 ± 0.4^{a}
40 mg/l NAA	20.00	$4 .2 \pm 0.6^{a}$	1.6 ± 0.3^{a}

Values (mean ± standard deviation) in a column followed by same letter are not significantly different ($P > 0.05$), n = 20.

root formation was observed in control and only 60% rooting was observed in micro shoots treated with 10 mg/l NAA (Table 3), this is due to the fact that low levels of auxins often result in failure of adventitious rooting (Cooper, 1935; Smith and Wareing, 1972). Ninety percent rooting was obtained in shoots treated with 20 mg/l NAA with an average of more than 6 roots per plantlet. Figure 2 shows representative plantlets formed after *ex vitro* rooting of *in vitro* shoots of CoS96268: a, b, c, d and e, are respectively, the plantlets developed after *ex vitro* rooting of *in vitro* shoots treated with 0,10, 20, 30 and 40 mg/l NAA

The effect of length of *in vitro* shoot and the size of propagule used was also investigated (Table 4). It was found that *in vitro* shoots of 5 to 6 cm are best for *ex vitro* rooting (Figure 4). 90 to 95% rooting was observed in micro shoots of 5 to 6cm. The percent root induction in other two sizes of micro shoots was decisively less. The size of propagule also had significant effect on *ex vitro* rooting of sugarcane micro shoots (Figure 3). The percent root induction in all the size categories increased with increase in number of shoots per propagule. However, for 5 to 6 cm shoots, the increase in percent root induction (90 to 95%) was very less. Mean number of roots was significantly better in shoots of 5 to 6 cm when used singly or in a propagule. The variation inmean root length among different treatments was insignificant. This study utilized sand as a medium and misting chamber to maintain temperature and humidity regimes for *ex vitro* rooting. These strategies have been successfully utilized for different species (Rajbhandary, 1991).

A cost analysis of micro propagation using *ex vitro* rooting (data not shown), revealed more than 50% cost reduction of the plantlets raised through conventional micro propagation. Direct adventitious regeneration from leaf explants showed great potential to reduce cost per plant. More than 60 plants per leaf spindle (each with 2 to 3 shoots) were produced within 50 days of culture initiation. This technology is already being applied

Table 4. Effect of propagule and shoot size on *ex vitro* rooting. Observation was taken after 21 days of planting in sand, under green house conditions.

Propagule size	Shoot size (cm)	Root induction (%)	Mean number of root	Mean root length
One shoot	2-3	30.00	3.8 ± 0.6^a	2.2 ± 0.5^a
	5-6	90.00	6.7 ± 1.2^b	4.2 ± 0.4^a
	> 7	80.00	4.0 ± 0.8^a	4.8 ± 0.4^a
Two shoots	2-3	45.00	4.8 ± 0.6^a	5.0 ± 0.6^a
	5-6	93.00	8.5 ± 1.0^b	4.4 ± 0.6^a
	> 7	85.00	5.0 ± 0.7^a	6.4 ± 0.4^a
Three shoots	2-3	65.00	5.8 ± 0.6^a	4.2 ± 0.8^a
	5-6	95.00	8.6 ± 1.2^b	5.4 ± 0.8^a
	> 7	80.00	6.2 ± 0.8^b	6.6 ± 0.8^a

Values (mean ± standard deviation) in a column followed by same letter are not significantly different ($P > 0.05$), n = 20.

commercially through a patented technology in Australia as "Smartsett^{TM}" (Laxmanan et al., 2006). Our technology is different as it uses more convenient leaf rolls over thin cell layer besides pretreatment of explants to enhance regeneration efficiency. However, further improvement in the technology is underway to make it applicable at commercial level. Both technologies need to be applied at commercial level for real time test and application.

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