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SHORT COMMUNICATION

Shoot induction and regeneration using internodal transverse thin cell layer culture in *Sesamum indicum* L.

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Abstract An efficient protocol for shoot regeneration was developed for sesame (*Sesamum indicum* L.) internodes using the transverse thin cell layer (tTCL) culture method. The frequency of shoot regeneration and the number of adventitious buds produced from regenerated shoots depend significantly on explant age, thickness of the tTCL sections, and the phytohormones supplemented to the culture medium. A combination of 6-benzyladenine (2.0 mg l^{-1}) and α-naphthaleneacetic acid (0.5 mg l^{-1}) was found to be the best phytohormone combination for shoot bud induction, with the maximum number of shoots obtained when the tTCL sections were 0.5–1.0 mm thick and derived from 4- to 6-week-old seedlings of sesame. Well-developed shoots were rooted on MS medium without phytohormones, and 80% of the regenerated plantlets were successfully established in soil.

Keywords Internodes · Organogenesis · Sesame · Shoot regeneration · Transverse thin cell layers

Abbreviations

BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
IAA	Indole-3-acetic acid
NAA	α-Naphthaleneacetic acid
GA_3	Gibberellic acid
tTCL	Transverse thin cell layer

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Introduction

Sesame, *Sesamum indicum* L. (family Pedaliaceae), is an annual herb native to the tropics. It is considered to be a highly valuable oilseed crop throughout the world, and sesame seed oil is used for both dietary and therapeutic applications. Sesame seed oil is unique among the commercially available edible oils obtained from oil-seed crops in being very stable at room temperature as well as having a high percentage of desirable mono- and poly-unsaturated fatty acids (C18:1 MUFA and C18:2 PUFA, respectively) and natural antioxidants such as sesamin, sesamolin, and sesamol. Similar to numerous other crops, biotechnological techniques coupled with classical breeding methodology have a great potential for effecting genetic improvement of *S. indicum*. However, genetic transformation in this crop remains difficult, essentially due to the lack of an efficient plant regeneration protocol.

There have been a number of reports on the micropropagation of sesame, namely, from shoot-tip culture (Rao and Vaidyanath 1997b), nodal culture (Gangopadhyay et al. 1998), and leaf disc culture (Sharma and Pareek 1998). There have also been reports of somatic embryos in sesame being obtained from zygotic embryos (Ram et al. 1990) and of seedling-derived callus (Mary and Jayabalan 1997; Xu et al. 1997). A few attempts have also been made to regenerate this plant species in vitro from hypocotyls

and cotyledonary explants (Rao and Vaidyanath 1997b; Younghee 2001; Were et al. 2006), but the success of regeneration was low in most cases. The aim of the study reported here was therefore to develop a successful shoot regeneration strategy in a sesame (*S. indicum*) cultivar based on a transverse thin cell layer (tTCL) culture system.

The thin cell layer culture method was originally developed by Tran Thanh Van for 'programming different patterns of morphogenesis' in tobacco (Tran Thanh Van 1981). The underlying concept has subsequently been successfully applied for somatic embryogenesis and shoot regeneration in many dicotyledonous and monocotyledonous plants, including a few orchid species and other horticultural crops, such as *Dendrobium* (Wang et al. 2007) and *Lilium* (Nhut et al. 2001) and in the micropropagation of leguminous and medicinal plants, including *Panax ginseng* (ginseng) and *Phaseolus vulgaris* (common bean) (Nhut et al. 2003a). It has also been used in in vitro regeneration systems for cereals and grasses, including *Digitaria sanguinalis* (large crabgrass), *Oryza sativa* (rice), *Sorghum bicolor* (sorghum), and *Zea mays* (corn) (Nhut et al. 2003b), fruit crops, including *Musa* sp. (banana), *Pancinus trifoliate* (trifoliate orange), *Cocos nucifera* (coconut palm), *Garcinia mangostana* (mangosteen), and *Lycopersicon esculentum* (tomato) (Nhut et al. 2003c), and woody plants, including *Dendrocalamus* spp. (bamboo), *Pinus radiate* (Montery pine), *Populus* spp. (poplar), and *Sequidendron* spp. (conifers) (Texeira da Silva 2003). However, to the best of our knowledge, there has been no report of a successful plant regeneration system for sesame using tTCL culture.

We report here our investigation on the various factors that influence shoot regeneration in *S. indicum* in the tTCL culture system. The effect of explant source and concentration of plant growth regulators was studied to establish an efficient regeneration protocol for this species that can be used in genetic transformation, cell line selection, and other practical applications.

Materials and methods

Seeds of *S. indicum* L. (cv. Dhavari) were surface sterilized in 0.2% $HgCl_2$ for 2 min. After repeated washings with sterilized distilled water, the seeds were germinated on hormone-free MS medium (Murashige and Skoog 1962) with 3% sucrose and 0.8% agar (w/v) in the dark at 25°C. About 4- to 6-week-old seedlings derived from these seeds were used as the explant source for the regeneration experiments.

For shoot organogenesis, root and stem segments with nodes and internodes were transversely sliced into TCL sections approximately 0.5–2.5 mm thick and inoculated onto MS medium with 3% sucrose (w/v) supplemented with different phytohormones either alone or in combination and at various concentrations—6-benzyladenine (BA); kinetin; 2,4-dichlorophenoxyacetic acid (2,4-D) + BA; 2,4-D + kinetin; indole-3-acetic acid (IAA) + kinetin; IAA + BA; α-naphthaleneacetic acid (NAA) + BA. The medium was solidified with 1% agar (w/v). The pH was adjusted to 5.7–5.8 with 1 mol l^{-1} KOH or HCL prior to autoclaving at 121°C for 15 min. The cultures were placed in a culture chamber maintained at 28°C and cultured under a 16/8-h light/day photoperiod (illumination (50 μmol m^{-2} s^{-1}). After 14 days in culture, the percentage of explants giving shoots (shoot induction frequency) and the number of shoots regenerated per responsive explant (shoot regeneration efficiency) were recorded. The experiments were repeated at least three times, and the data of three experiments were pooled for statistical analysis.

After 2 weeks, the adventitious shoots were transferred into MS medium supplemented with 1 mg l^{-1} NAA + 5 mg l^{-1} BA + 3 mg l^{-1} gibberellic acid (GA_3) for shoot elongation (optimization data not shown). Rooting of the shoots was carried out on MS medium without phytohormones. All cultures were incubated in a growth cabinet under a 16/8-h light/dark photoperiod at 28°C. Those plantlets that developed a few roots were transplanted into a sand–soil mixture and kept in the greenhouse conditions (28°C) for 2–3 months for acclimatization and their eventual development into mature plants.

Results

The tTCL explants from the root, internode, and node of *S. indicum* were evaluated for the induction of shoot morphogenesis on medium containing different concentrations/combinations of phytohormones. Among the explants tested, only internodal tTCLs showed shoot induction; no shoot regeneration occurred in root and nodal explants. Internodal tTCL explants cultured on MS basal medium devoid of plant growth regulators did not initiate adventitious bud formation. A wide range of hormonal combinations, namely, IAA + kinetin, IAA + BA, 2,4-D + BA, 2,4-D + kinetin, NAA + BA, and BA and kinetin alone, were tested for their efficiency in inducing plant regeneration from internodal tTCL explants. No shooting response was observed in any phytohormone combination other than the NAA + BA combination; when cultured with the other hormone combinations, the explants absorbed water, swelled up, and formed dark-green hardened structures. Among the combinations of NAA and BA tested, the best result (15 shoots/explant) was observed on MS medium containing 2 mg l^{-1} BA and 0.5 mg l^{-1} NAA. The frequency of shoot induction (percentage of explants

producing shoots) varied between 5.0 and 29.8%, and the shoot regeneration efficiency (number of shoots per explant) varied from two to 15 (Table 1). Shoot organogenesis occurred directly without the formation of a distinct intermediate callus, and well-defined shoot buds were visible within 3 weeks of culture.

The age of the explants and the thickness of the explants' transverse sections are known to profoundly affect shoot induction frequency and efficiency. Stem nodes were numbered from the base to the shoot tip, with the first node from the base being numbered internode1. When internodal tTCL explants were placed on shoot regeneration medium containing 2 mg l^{-1} BA and 0.5 mg l^{-1} NAA, the highest induction frequency was obtained from explants derived from internode3 of 4- to 6-week-old seedlings. The percentage of explants responsive to developing shoots, i.e., the shoot induction frequency, was 29.8%, and the average number of adventitious buds per responsive explant, i.e., the shoot regeneration efficiency, was 15.3 (Fig. 1). Nevertheless, shoot regeneration efficiency was low in very young explants (derived from internode4) or old explants (derived from internode1). The thickness of the tTCL sections also affected the shoot regeneration frequency as very thin internodal sections (0.2–0.5 mm) turned brown and nonviable in regeneration medium during the culture period. The tTCL sections with a thickness of 1.0 mm gave rise to 12–15 shoots per responding explant, whereas the shooting efficiency decreased to five to seven shoots in the explants that were 1.5 mm thick. The shoots that developed from 2.0-mm-thick internodal sections did not grow normally into plantlets in shoot elongation medium, with the shoot buds remaining stunted for nearly 1 month on the medium, after which they died. Shoot regeneration was not observed at all from tTCL sections $\geq$2.5 mm thick (Table 2).

The regenerated shoots derived from the tTCLs were normal and responded well to the shoot elongation medium containing 3 mg l^{-1} GA_3 in addition to 5 mg l^{-1} BA and 1 mg l^{-1} NAA. Well-defined roots were formed from these regenerated shoots when cultured in MS medium without phytohormones. After 2 months of culture under in vitro conditions, the young plantlets were transferred to greenhouse conditions where they slowly were allowed to acclimatize. In terms of phenotype, about 80% of the regenerated plantlets were similar to plantlets derived from the seeds of the same sesame cultivar (Fig. 2) and subsequently grew into healthy mature plants.

Discussion

The aim of the study reported here was to develop a suitable regeneration protocol for sesame. In preliminary experiments using conventional explants, such as the shoot tip, hypocotyl, cotyledon, and leaf disc, based on previous reports on sesame, we did not obtain the successful regeneration of plantlets from *S. indicum* L. (cultivar *Dhavari*) (data not shown). This may be due to cultivar differences, as has been mentioned in previous regeneration studies on sesame (Rao and Vaidyanath 1997a, b; Gangopadhyay et al. 1998). Numerous plant species for which tissue culture is known to be difficult have now been

Table 1 Effect of different combinations of plant growth regulators on in vitro shoot regeneration of *Sesamum indicum* L. cv. Dhavari

Concentration of plant growth regulators (mg l^{-1})		Percentage of explants forming shoots	Average no. of shoots per explant
α-Naphthaleneacetic acid	6-Benzyladenine		
0	0.5	0	0
0	1	0	0
0	2	0	0
0.5	0.5	5.0 ± 0.283 a	2.74 ± 0.15 a
0.5	1	18.2 ± 1.038 b	3.04 ± 0.11 a
0.5	1.5	25.2 ± 0.335 c	6.96 ± 0.31 b
0.5	2	29.8 ± 0.695 d	15.0 ± 0.14 c
0.5	2.5	24 ± 0.283 e	8.42 ± 0.16 d
0.5	3	14 ± 0.40 a	5.18 ± 0.08 e
1	1	0	0
1	2	8.8 ± 0.522 a	2.68 ± 0.09 a
1	3	6.8 ± 0.335 a	3.06 ± 0.12 a

Values are given as the mean ± standard error of three experiments, each using 100 explants (20 explants/plate). Means followed by different lower-case letters are significantly different (Fisher test, $p < 0.05$)

Data were scored after 21 days of culture

Transverse thin cell layer (tTCL) sections from internodes one to four were randomly inoculated onto the culture medium

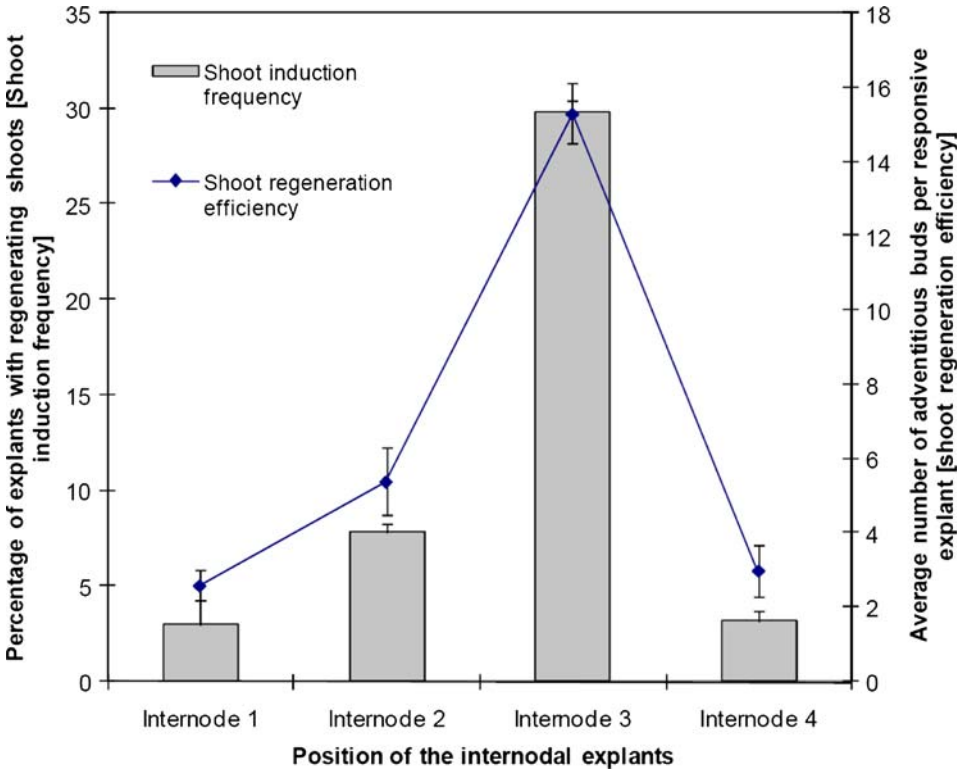

Fig. 1 Influence of position of the explant (internodal transverse thin cell layer, tTCL) on the plant on adventitious bud formation. Shoot induction frequency and shoot regeneration frequency are given as the percentage of explants with shoots and the average number of adventitious buds per responsive explant, respectively

Table 2 Effect of tTCL thickness on shoot regeneration efficiency

Thickness of the tTCL (mm)	Shooting efficiency (no. of shoots/explant)
0.5	0
1	15.3 a
1.5	5.8 a
2	1.8 b
2.5	0

Values are given as the mean ± standard error of three experiments each using 100 explants (20 explants/plate). Means followed by different lower-case letters are significantly different (Fisher test, $p < 0.05$)

Data were scored after 21 days of culture

The tTCL explants from internodes one to four were randomly inoculated on the culture medium

made amenable to in vitro culture methods by capitalizing on TCL technology. To the best of our knowledge, this is the first report of shoot regeneration in sesame using the TCL culture method.

Cytokinins along with auxins are routinely employed in in vitro plant culture systems, either singly or in combination, for shoot induction and development. Using internodal tTCLs as explants, we tested different combinations of phytohormones and obtained the best shooting response in MS medium with 2.5 mg l^{-1} BA and 0.5 mg l^{-1} NAA: shoot regeneration was rapid, with 10–15 shoot buds originating per explant within 14 days of culture (Fig. 2). These results compare favorably with those reported on shoot regeneration in this plant species using conventional explants.

The choice of the right explant is very important for regeneration using TCL technology. In addition to the culture media and environmental conditions, the source of the explant, i.e., the internodal region, appears to be a critical determining factor, as no significant results were obtained using tTCLs from nodes and roots. Similar results were obtained in different species of tobacco, where explants from only day-neutral plants produced floral buds in vitro; in similar culture medium and environmental conditions, explants from long-day and short-day tobacco plants gave rise to vegetative buds (Bridgen and Veilleux 1985). We found that the thickness of the explant influenced cell proliferation in our sesame culture system since 90% of the ≤0.5-mm-thick TCL sections turned necrotic in the culture medium. The frequency of shoot regeneration decreased when sections >1 mm were used as explants (Fig. 1). It is to be noted that explant thickness has also been shown to be an important factor in other plants, such as *Lilium longiflorum* and *Sorghum bicolor* (Gendy et al. 1996; Nhut et al. 2001).

Among the other determining factors, the age of the mother plant is significant, as mother plants younger or older than 4–6 weeks had a reduced frequency of shoot bud initiation and development into mature plantlets. The position of the explants on the mother plant was found to

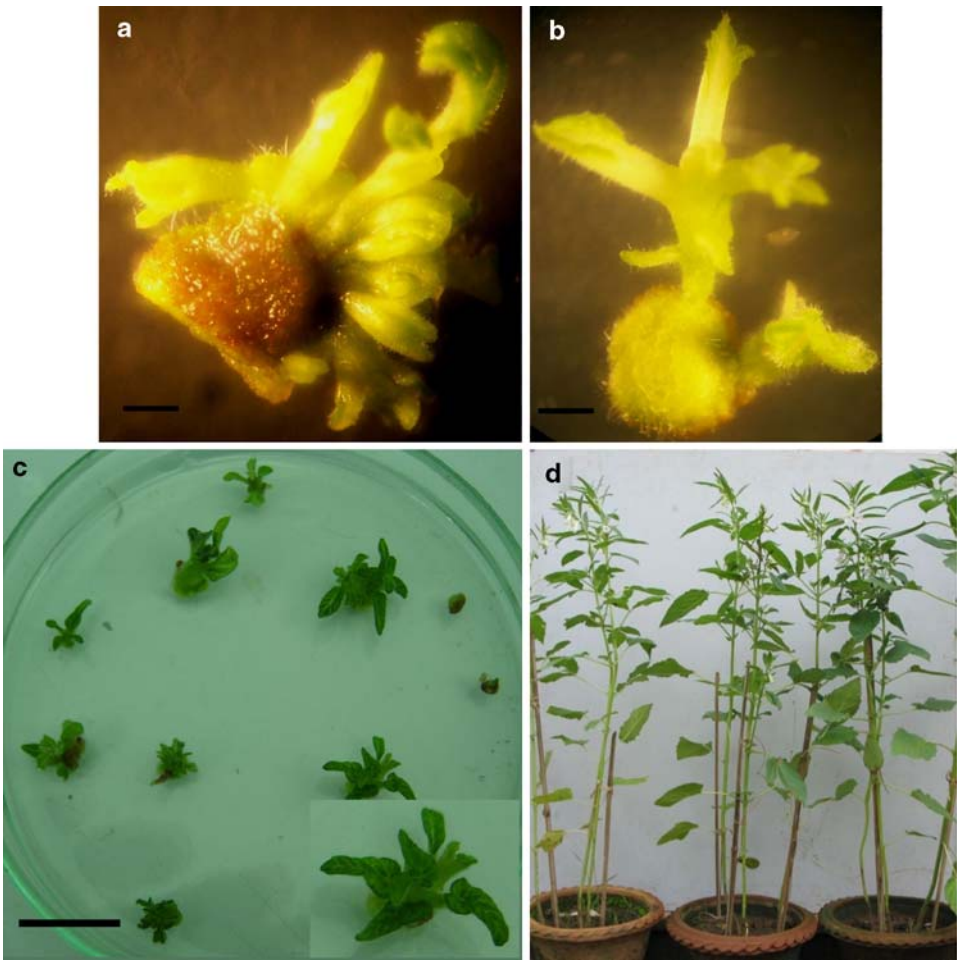

Fig. 2 Shoot organogenesis from tTCL sections of *S. indicum*. **a** Shoot buds originating from the tTCL explants after 3 weeks of culture on shoot induction media with 2 mg l^{-1} 6-benzyladenine (BA) and 0.5 mg l^{-1} α-naphthaleneacetic acid (NAA). *Bar*: 0.25 mm. **b** A reduced number of shoot buds are produced from a thick section. *Bar*: 0.25 mm. **c** Shoot buds grow into well-developed shoots in MS basal medium with 1 mg l^{-1} NAA + 5 mg l^{-1} BA + 3 mg l^{-1} gibberellic acid (GA_3). *Bar*: 2 cm. *Inset* Enlarged view of one of the TCL explants with developing shoots. **d** Greenhouse-acclimatized regenerated plantlets grown ex vitro as potted plants

affect their morphogenetic capacity, as internodes from the basal part of the main axis of the mother plant (denoted by internode3) (TableI2) gave the highest frequency of shoot regeneration. In addition to the position of explants, the orientation of explants on media have been known to affect shooting, with young explants inoculated in upright orientation exhibiting a high frequency of shoot regeneration in *Dendrobium candidum* (Wang et al. 2007). However, the orientation of the explants on the medium did not seem to be a crucial factor in our sesame plantlet regeneration protocol (data not shown).

Based on our results, we conclude that the tTCL culture system reported here using internodal sections as explants is a rapid and direct shoot regeneration method for sesame (*S. indicum*). This in vitro plantlet formation strategy can be utilized for genetic transformation experiments so as to develop improved cultivars of this important oil-seed crop.

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