Plant Cell, Tissue and Organ Culture **29**: 71–74. 1992.

In vitro plant regeneration from leaf-derived callus in ginger (*Zingiber officinale* Rosc.)

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Received 10 September 1990; accepted in revised form 31 October 1991

Key words: leaf, organogenesis

Abstract

Excised tissues from young leaves of ginger cv. Maran were cultured on revised Murashige and Skoog medium supplemented with various concentrations of growth regulators. The presence of 2, 4-D in the culture medium at 9.0–22.6 μM resulted in callus growth. Organogenesis and plantlet formation occurred when the concentration of 2,4-D is reduced to 0.9 μM and with the addition of 44.4 μM BA into the medium. The rate of plant regeneration increased when the growth regulators are completely removed from the culture medium in the subsequent subcultures. The plantlets developed extensive root systems when they were put in MS liquid medium with 5.4 μM of NAA. The establishment of these plantlets in soil is about 80%.

Abbreviations: BA – N^6-benzyladenine, NAA – α-naphthaleneacetic acid, 2,4-D – 2,4-dichlorophenoxyacetic acid

Introduction

Zingiber officinale Rosc. is a herbaceous perennial, usually grown as an annual, the rhizomes of which are used as the spice ginger. India is the largest producer and exporter of dry ginger contributing about 50% of the world's production. Other important ginger producing countries are China, Taiwan, Nigeria, Jamaica and Australia. 'Rhizome rot' caused by *Pythium aphanidermatum* and bacterial wilt caused by *Pseudomonas solanacearum* are the major constraints in increasing ginger production. The absence of seed set in ginger makes conventional breeding methods inapplicable. *In vitro* culture offers a method for creating variation and exploiting the resultant variation for crop improvement.

Contribution No. 135 from National Research centre for Spices, Calicut-673 012, Kerala, India.

Rapid clonal multiplication of ginger by shoot-tip culture was reported by earlier workers (Hosoki & Sagawa 1977; Pillai & Kumar 1982; Ilahi & Jabeen 1987; Bhagyalakshmi & Singh 1988; Noguchi & Yamakawa 1988). The potential use of tissue culture in ginger was demonstrated by De Lange et al. (1987), who reported complete elimination of nematodes by tissue culture method. Kulkarni et al. (1984) reported isolation of *Pythium*-tolerant ginger by using culture filtrate as the selecting agent. This is the first report of organogenesis and plant regeneration from leaf tissue-derived callus in ginger.

Materials and methods

The aerial shoots, comprised of sheathing leaf bases enclosing the youngest leaf inside, from 2-month-old plants of ginger cv. Maran grown in

a greenhouse, were collected during July-August and made into 4–5 cm long sections. They were first washed in running water and then were surface disinfected in 0.1% mercuric chloride solution for 5–10 min. They were then washed 3–4 times in sterile water. The outer leaf sheaths were removed aseptically and small segments (0.5–1.0 cm) of innermost leaf tissue were placed in modified MS (Murashige & Skoog 1962) medium supplemented with various concentrations of 2,4-D and NAA (Table 1) for production of callus.

Two-month-old calluses were subcultured on a series of media with 2,4-D (0 and 0.9 μM), BA (0–44.4 μM), kinetin (0–46.5 μM) and media free of growth regulators for organogenesis and plantlet formation (Table 2). The calluses were subcultured at monthly intervals on fresh medium of the same composition. Regenerated plants were placed in MS liquid medium with 5.4 μM NAA using filter paper rafts for improved rooting. Well-rooted plants were transferred to poly bags filled with 1:1 mixture of garden soil and coarse sand and kept in humid chamber for 15 days.

Borosil culture tubes, 25 × 150 mm or 32 × 200 mm, were used with one explant in each tube. For solidifying the media 0.65% 'Qualigens' bacteriological grade agar was used. The pH was adjusted to 5.8 prior to autoclaving for 20 min at 120°C and 98 kPa. All the cultures were incubated at 25 ± 2°C with a 14-h photoperiod at an irradiance of 30 $\mu molm^{-2}s^{-1}$ provided by 'Philips' cool white fluorescent tubes.

Results and discussion

Explants taken from the innermost leaf tissues of 2-month-old ginger plants were cultured on revised MS medium with various levels of NAA and 2,4-D for induction and proliferation of callus (Table 1). 2,4-D at 9.0–22.6 μM concentrations resulted in callus growth after 4 weeks of culture, the optimum level being 13.6 μM of 2,4-D (Fig. 1). NAA had no effect on callus induction when used in concentrations below 16 μM but induced callus when used in concentrations above 16 μM. However, the amount of callus produced was negligible.

Table 1. Effect of 2,4-D and NAA on leaf tissue explants of ginger.

2,4-D μM	NAA μM	Production of callus	
		Amount of callus[a]	No. of cultures callusing (%)[b]
2.3	0	0	0
4.5	0	+	60
9.0	0	+++	70
13.6	0	+++	87
18.1	0	++	67
22.6	0	++	47
0	2.7	0	0
0	5.4	0	0
0	10.7	0	0
0	16.1	+	13
0	21.5	+	33
0	26.9	+	40

[a] 0 = No response
+ = < 0.5 g ++ = 0.5–2.0 g +++ = > 2.0 g
[b] Number of replications = 15

Two-month-old callus derived from leaf tissues was further subcultured on a series of revised MS media supplemented with varying levels of auxins and cytokinins for proliferation of callus and organogenesis (Table 2). When the callus was cultured on MS medium without growth regulators immediately after its induction, it resulted in rhizogenesis. Both kinetin and BA when used alone did not result in organogenesis even after 4–5 cycles of subculture. When MS medium with 0.9 μM of 2,4-D and either 23.2 μM of kinetin or 22.2 μM BA was used, organogenesis was noticed in the form of small shoot primordia. When the concentration of BA or kinetin was further increased to 44.4 μM and 46.5 μM respectively, well-developed shoots were obtained. The number of shoots per culture were higher in BA (15–35) than in kinetin (10–25). The rate of shoot production and plantlet formation increased considerably to 30–65 shoots per culture when growth regulators were completely removed from the medium in later subcultures. It takes about 6–8 months for the callus to differentiate and develop into a full-fledged plantlet (Fig. 1).

Shoots developed from the callus were placed on the revised MS medium with 5.4 μM of NAA for rooting. An extensive root system developed within 5 weeks. Rooting was better when liquid medium was used instead of solid medium. The

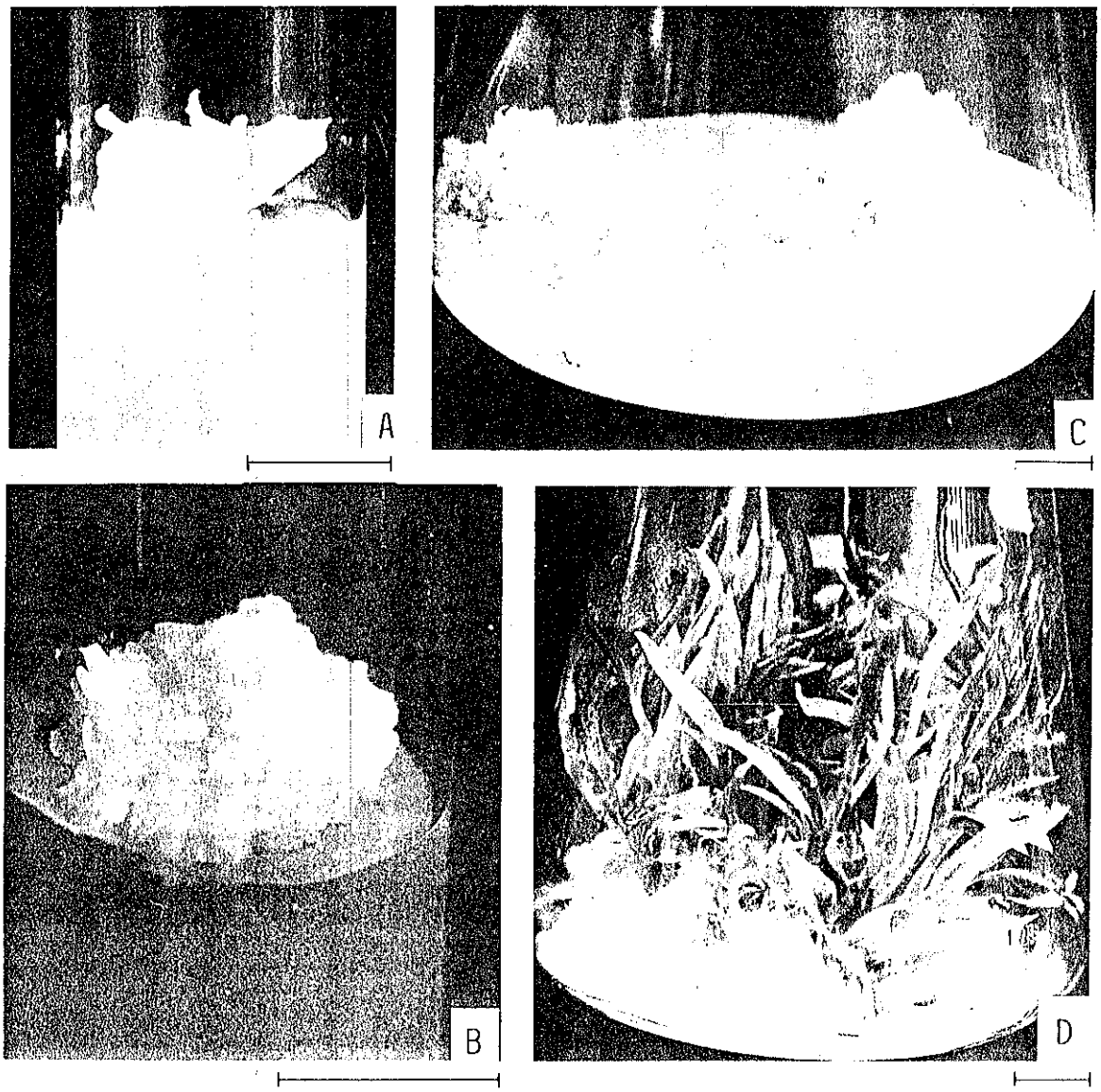

Fig. 1. In vitro organogenesis and plantlet formation from leaf-derived callus of ginger. (*A*) Production of callus from leaf tissue, (*B*) Organogenesis from leaf derived callus, (*C*) Shoot formation, and (*D*) Plantlet development.

establishment of the rooted plantlets in the soil (1 garden soil:1 sand) was above 80% when high humidity was maintained for the first two weeks after transfer to soil by placing them in a humid chamber. Most of the plants were morphologically similar at the nursery stage with 2% of the plants showing some variation in having leaves with white chlorotic patches and wavy margins. This protocol is being used routinely in this laboratory for the production of somaclones.

High contamination of cultures was reported when rhizomes or vegetative buds are used for micropropagation (Hosoki & Sagawa 1977). By using the leaf tissues this problem was eliminated almost completely. It is believed that only clones derived from meristem, shoot-tip and bud cultures are generally phenotypically homogeneous, thereby indicating genetic stability (Hu & Wang 1983). If an intermediary callus phase is involved, as is the case in most regeneration via

Table 2. Effect of 2,4-D, kinetin and BA on organogenesis and plantlet formation in callus of ginger*.

2,4-D (μM)	Kinetin (μM)	BA (μM)	Morphogenic response	Percentage of cultures showing response	Mean no. of shoots/culture tube [± SD]	Range of shoots/culture tube
0	0	0	Only roots developed	93	–	–
0	4.7	0	Slow callus growth	40		
0	13.9	0	Slow callus growth	53		
0	27.9	0	Slow culture growth	60		
0	0	4.4	Slow callus growth	47		
0	0	13.3	Slow callus growth	60	–	–
0	0	26.6	Slow callus growth	33		
0.9	4.7	0	Slow callus growth	67		
0.9	13.9	0	Slow callus growth & root initiation	60	–	–
0.9	23.2	0	Good callus growth with shoot bud primordia	80	–	–
0.9	46.5	0	Well developed shoot formation	73	18.6 ± 6.2	10–25
0.9	0	4.4	Slow callus growth	67	–	–
0.9	0	13.3	Good callus growth	87	–	–
0.9	0	22.2	Shoot bud primordia developed	67	–	–
0.9	0	44.4	Well developed shoot formation	73	24.4 ± 6.2	15–35

*Mean of 15 replications SD = Standard deviation

organogenesis, the frequency of genetic changes is increased (Hu & Wang 1983). When already differentiated tissues like leaf tissues were used for plant regeneration, through an intermediary callus phase there may be a possibility of increased rate of somaclonal variation that can be exploited for crop improvement in ginger, especially since other conventional methods of creating variation are ineffective.

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