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Plant regeneration from anther derived callus cultures of ginger (*Zingiber officinale* Rosc.)

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SUMMARY

Ginger anthers collected at the uninucleate microspore stage were subjected to a cold treatment (0°) for 7 d and induced to develop profuse callus on MS medium supplemented with 2–3 mg l^{-1} 2, 4-D. Plantlets could be regenerated from these calli on MS medium supplemented with 5–10 mg l^{-1} BAP and 0.2 mg l^{-1} 2,4-D. The regenerated plantlets could be established in soil with 85% success, when they were planted in potting mixture of garden soil, sand and vermiculite in equal proportions and kept in a humid chamber initially for 22–30 d. This is the first report of successful regeneration of plants from ginger anther cultures and the protocol can be used for possible development of androgenic haploids and dihaploids in ginger.

Ginger, one of the oldest and most important spices, has been cultivated in tropical Asia for over 3000 years (Purseglove, 1975). Ginger is the underground rhizome of *Zingiber officinale* Rosc., of the family Zingiberaceae. In India, ginger is cultivated on 67,200 hectares and about 28,310 tonnes are exported from the country earning a foreign exchange of Rs. 7,26,272 (Spices Board, 1998).

Ginger cultivation is affected by various diseases of fungal, bacterial, viral and mycoplasmal origin. Of these, soft rot caused by *Pythium aphanidermatum*, yellows caused by *Fusarium oxysporum*, bacterial wilt caused by *Pseudomonas solanacearum* and leaf spot caused by *Phyllosticta zingiberi* result in considerable crop losses. Though crop improvement work on ginger started in 1950, it was restricted to the evaluation of germplasm and the selection of a few high yielding clones from them (Rattan, 1994). Studies on mutation breeding (Datta and Biswas, 1985) using ethyl methyl sulfonate (0.05–1.0%) revealed cytological irregularities like fragment and ring configurations coupled with retarded growth with increase in concentration of treatment, but were otherwise inconclusive. The exploration of possibilities of polyploidy breeding in obtaining flowering and seed set met with conflicting results. Ramachandran and Nair (1992) found induced tetraploids to be more vigorous and flowered in the second year whereas Ratnambal and Nair (1982) observed no flowering in the induced tetraploids. Thus, lack of variability for resistance to major diseases and pests and extremely rare seed set make conventional breeding programmes ineffective in ginger. Evolving high yielding, high quality lines with resistance or tolerance to diseases and pests will substantially increase production and productivity.

It is under these circumstances that biotechnological tools can suitably be used for crop improvement in vegetatively propagated crops like ginger, especially when the production of haploids can be used for inducing mutations, for transformation, for cell line selection and in the production of homozygous lines for further breeding programmes.

Earlier reports on ginger concentrated on micropropagation and aspects like plant regeneration from callus cultures, *in vitro* conservation, synthetic seeds, the production of flavour and flavour components using cell culture and exploiting somaclonal variation for disease resistance (Nirmal Babu *et al.*, 1992, 1996). Attempts to develop haploids in ginger (Ramachandran and Chandrasekharan Nair, 1992; Samsudeen *et al.*, 1997) yielded only callus tissue from anthers. The present study was undertaken against this background and plantlets were regenerated from callus tissue induced from anthers.

MATERIALS AND METHODS

Source of explant

Ginger, *Zingiber officinale* Rosc. cv. Maran, (diploid and tetraploid) one of the most popular varieties suitable for dry ginger was used for this study. Inflorescences were collected during August/September, from field-grown plants.

Preparation of explant

Inflorescences were washed in running tap water and kept at 0°C for cold treatment for 1–9 d. After cold treatment, individual flowers were separated from inflorescences and surface sterilized with 0.1% mercuric chloride for 10 min. They were then washed three times in sterile water to remove all traces of $HgCl_2$. Selected flowers were dissected and anthers (with uninucleate microspores, Figures 1:1, 2 and 3) were inoculated in different combinations of medium. Calli developed from anthers were subcultured for further proliferation and plant regeneration.

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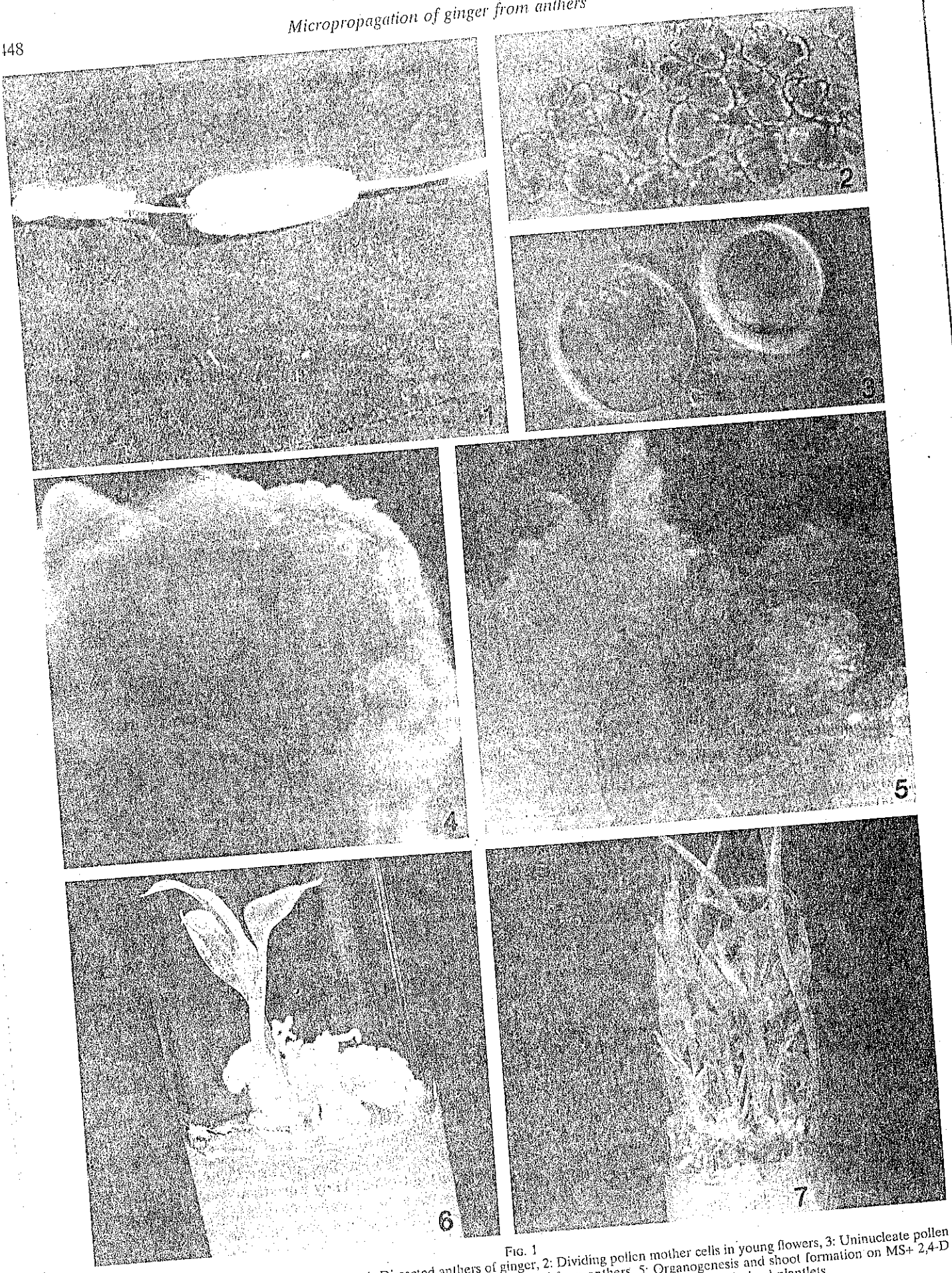

FIG. 1

Plant regeneration from anther derived callus. 1: Dissected anthers of ginger, 2: Dividing pollen mother cells in young flowers, 3: Uninucleate pollen as seen in interference contrast microscope, 4: Morphogenetic calli developed from anthers, 5: Organogenesis and shoot formation on MS+ 2,4-D (0.2 mg l^{-1}) + BAP (10 mg l^{-1}), 6: Well-developed plantlet, 7: Multiplication of anther derived plantlets.

Culture medium and conditions

MS (Murashige and Skoog, 1962) basal medium was used in the present study. Sucrose was used as the carbon source at the rate of 20 g l^{-1} in all the experiments. Two major auxins, namely α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) and two cytokinins namely 6-benzylaminopurine (BAP) and 6-furfurylamino purine (kinetin) were used at concentrations ranging from 0–10 mg l^{-1}. For solidifying the culture medium, 'Qualigens' bacteriological grade agar was used at a concentration of 7 g l^{-1}.

The medium was sterilized by autoclaving at 121°C for 20 min at 1.08 kg cm^{-2} pressure. The cultures were incubated at 22 ± 2°C and were given a photoperiod of 14 h with a light intensity of 3000 lux, provided by Philips cool white fluorescent tubular lamps. Cultures were incubated in dark for an initial 2–3 d for callus induction.

The separated anthers were cultured on MS medium supplemented with 0.2–3.0 mg l^{-1} of 2,4-D for callus initiation. The calli developed from these anthers were transferred to MS basal medium supplemented with various combinations of growth regulators for regeneration. For plant regeneration the calli were subcultured for 2–4 cycles at monthly intervals onto fresh medium of same combination.

Hardening and planting out

Healthy *in vitro* plants with good roots were taken out and washed carefully to remove all traces of medium sticking to the roots. They were then transplanted into polybags containing a mixture of garden soil, sand and vermiculite in equal proportions. The transplanted plantlets were kept in a humid chamber for 3–4 weeks for hardening and establishment. They were then kept in the nursery for the first year. In subsequent years they were transferred to earthen pots (30 cm diameter) for evaluation.

RESULTS AND DISCUSSION

Callus induction

Callus developed from anthers cultured in media containing 2,4-D at a concentration of more than 1.0 mg l^{-1} and at 3 mg l^{-1} both callus induction and proliferation was observed, giving about 3 g of callus in about 30 d of culture when incubated in light (Figure 1:4). Of the cold treatments tried, callus was induced only from anthers that had been subjected to 7 d cold treatment. Attempts to culture anthers in liquid medium led to the formation of friable non-regenerating callus in six weeks.

Influence of cold pre-treatment of immature flower buds for at least 3 d prior to culture, on enhanced callus production had been observed by Prasad *et al.* (1990). Custers *et al.* (1994) discussed the role of temperature and they observed a switch from gametophytic to sporophytic development at 17°C in *Brassica napus*, and increased frequency of androgenesis, when subjected to 4°C, was reported by Bajaj (1983).

Plant regeneration

Plant regeneration from anther derived calli occurred in the presence of high concentration of BAP, either alone or in combination with a low concentration of 2,4-D. MS medium with 2,4-D at 0.2 mg l^{-1} and BAP at 10 mg l^{-1} was the best for organogenesis and plant regeneration (Table I). The plant regeneration was by organogenesis and shoot development (Figure 1:5). Seventy per cent of the cultures gave morphogenetic response with a range of 1–20 shoots (Figure 1:6) and an average of 12.6 shoots per culture tube. These shoots were multiplied and rooted on MS basal medium with 1 mg l^{-1} NAA (Figure 1:7) before hardening and field establishment. Plant regeneration from callus from anthers of diploid and tetraploid ginger were similar. Among the cytokinins tried, BAP was better in inducing the callus to regenerate. Similar studies by Zhang *et al.* (1992) indicated that the regenerative capability of rice anther callus was lowered by the presence of kinetin in the medium.

Hardening and field establishment

The plantlets were hardened by maintaining the humidity around 90% by coverings with polybags for 20–30 d.

Ever since the first report of androgenesis in *Datura* (Guha and Maheshwari, 1964), anther culture has gained considerable importance in efforts to produce haploids and dihaploids and has been reported in many crop species (Bajaj, 1983). Various factors like genotype, physiological condition and age of the plant, stage of pollen, thermal trauma, chemical treatment and composition of the medium influence androgenesis (Sopory, 1979; Maheswari *et al.*, 1980). Cold treatment improves the viability of cultured pollen and represses gametophytic differentiation, which causes higher frequency of androgenesis.

TABLE I

Effect of 2,4-D and BAP on morphogenesis and plantlet development from anther derived callus of ginger in MS basal medium

2,4-D (mg l^{-1})	BAP (mg l^{-1})	Morphogenesis	Response (%)	Shoots/culture tube	
				Mean* (±SD)	Range
0.0	0.0	Only roots	90	–	–
0.0	1.0	Only roots	70	–	–
0.0	2.0	Slow callus growth	80	–	–
0.0	5.0	Plantlet development	30	1.6 ± 1.3	1–3
0.0	10.0	Plantlet development	50	2.3 ± 2.1	1–6
0.2	0.0	Callus	90	–	–
0.2	1.0	Callus	80	–	–
0.2	2.0	Slow growth	50	–	–
0.2	5.0	Plantlet development	40	7.3 ± 2.4	1–10
0.2	10.0	Plantlet development	70	12.3 ± 6.4	1–20

*Mean of 20 replications; SD: Standard Deviation

Haploid cells are in general unstable in culture and have a tendency to undergo endomitosis to form diploid cells. This property of cell culture has been exploited for obtaining homozygous tobacco plants (Nitsch, 1969; Kochhar *et al.*, 1971).

In the present study, excised anthers with uninucleate pollen mother cells were cultured on revised MS medium after an initial cold treatment of 7 d at 0°C. These anthers developed callus and subsequently regenerated into plantlets. MS medium was found to be suitable for both anther callus induction and regeneration in ginger, although many basal media like those of White (1963) and Nitsch and Nitsch (1969) with growth regulators, have been used for culturing excised anthers. The presence of haploids or dihaploids among the regenerated plants could not be indexed in the present study. Since there is high possibility of obtaining haploids or dihaploids in anther cultures, further studies are needed for indexing and isolating them from the regenerated progenies. Ramchandran and Nair (1992), working with the same cultivars, observed no seed set in the artificially induced tetraploid, despite its high pollen fertility (85%). The diploid, in contrast, exhibited a high degree of sterility (13% viability). This confirms the sporophytic nature of the incompatibility system in ginger, since gametophytic incompatibility breaks down in the induced polypoids as shown by Pandey (1983). Induction of androgenesis in ginger from tetraploid anthers can thus give rise directly to diploid plants showing large genetic variation, as reported for *Pelargonium roseum* (Tokumasu and Kato, 1979). This attempt at plant regeneration from anther derived callus cultures, in diploid and tetraploid ginger, has been made in this direction.

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