4 Tissue Culture and Biotechnology of Ginger

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Recent advances in plant tissue, cell, and protoplast culture combined with genetic engineering have opened up new and exciting possibilities in propagation, gene manipulation, crop improvement, and germplasm conservation in many plant species. Micropropagation has been established as a sound commercial proposition, especially in ornamentals and plantation crops to produce nuclear stock free from pathogens and viruses. Technologies like anther, pollen, and protoplast culture will speed up the process of producing better varieties. All these developments have contributed to the acceptance of in vitro culture techniques as viable and valuable tools. In addition, the potential of in vitro conservation and cryopreservation to conserve genetic resources, plant varieties, cell lines, and pollen through the establishment of in vitro gene banks is becoming a reality. It is now possible to manipulate genetically cultured cells and tissues to produce improved plants and high-value substances.

The cell- and tissue-culture techniques have a tremendous advantage in horticultural crops, especially those that are propagated vegetatively. Ginger is no exception, more so because the conventional breeding programs are hampered due to lack of fertility and natural seed set. Rhizome rot caused by *Pythium* spp. and bacterial wilt caused by *Ralstonia solanacearum* are major diseases affecting ginger that are spread primarily through infected rhizomes. Tissue-culture techniques would help in the production of pathogen-free planting material of high-yielding varieties. Since no source of resistance is available in the germplasm, somaclonal variation could be an important source of variability to evolve high-yielding, high-quality disease-resistant lines. Tissue-culture techniques could also be used for in vitro pollination and embryo rescue in ginger. However, the applicability of such techniques depends upon the ability to regenerate plants effectively within in vitro cultures. Protocols for micropropagation, plant regeneration, in vitro pollination, protoplast culture, the development of synseeds, and cryopreservation are available. These can be effectively used in ginger crop–improvement programs. All these aspects are discussed, reviewed, and updated in this chapter.

Micropropagation

Many workers reported micropropagation of ginger using shoot meristems (Hosoki and Sagawa, 1977; Nadgauda et al., 1980; Pillai and Kumar, 1982; Ilahi and Jabeen, 1987; Bhagyalakshmi and Singh, 1988; Saradha and Padmanabhan, 1989; Balachandran et al., 1990; Choi, 1991a; Choi and Kim, 1991; Samsudeen, 1996; Nirmal Babu, 1997), base of the pseudostem (Ikeda and Tanabe, 1989; Choi, 1991b; Nirmal Babu, 1997; Nirmal Babu et al., 1998), and roots (Nel, 1985). The most commonly used medium was the MS basal medium (Murashige and Skoog, 1962) supplemented with IAA, IBA, NAA,

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Table 4.1 In vitro responses of ginger

Explant used	*Media composition*	*In vitro response*	*Reference*
Vegetative buds and rhizome bits with axillary buds	MS + 2 mgl^{-1} NAA (liquid medium)	Multiple shoots and in vitro rooting	Nirmal Babu et al. (1996e)
Vegetative buds	MS major elements +Ringe-Nitsch minor elements, vitamins, 1 ppm BA, 2% sucrose	Multiple shoots with roots	Hosoki and Sagawa, 1977
Vegetative buds, ovary, rhizome, and leaf sheath	MS + 0.5 ppm NAA MS + 2 mgl^{-1} 2,4-D	Callus	Choi (1991), Nirmal Babu et al. (1997)
Rhizome	MS + 0.5 mgl^{-1} 2,4-D + 0.5 mgl^{-1}BA	Plantlets	Ilahi and Jabeen (1987)
Flower, inflorescence	MS + 10 mgl^{-1} BA + 0.2 mgl^{-1} 2,4-D	Conversion of flowers to plants	Nirmal Babu et al. (1992b)
Anther	MS + 1.5 mgl^{-1} 2,4-D + 200 ml/l coconut milk	Callus, roots	Ramachandran and Nair (1992)
Callus derived from bud, ovary, leaf	MS + 10 mgl^{-1} BA, 0.2 mgl^{-1} 2,4-D	Callus induction and plant regeneration	Nirmal Babu (1997)
		Organogenesis and plantlet formation	Nirmal Babu et al. (1992a, 1996b)
Callus derived from vegetative buds	MS + 0.1 mgl^{-1} Kin, 0.2 mgl^{-1} BA, 10% coconut milk	Shoot regeneration	Nadgauda et al. (1980)
In vitro plantlets	MS +9–12% sucrose		
	MS +75 g/l of sucrose	In vitro rhizomes	Bhat et al. (1994)
	MS + 2 g/l sucrose and 1g/l of mannitol		Sharma and Singh (1995)
			Peter et al. 2002

[a]IISR, Unpublished information from author's laboratory.
MS, Murashige and Skoog (1962) medium;
SH, Schenk and Hildebrandt (1972) medium;
WPM, Woody Plant Medium (McCown et al., 1979) medium.
IAA: Indole-3-acetic acid
IBA: Indole-3-butyric acid
NAA: α-naphlhalene acetic acid
2, 4-D: 2,4-Dichloropenoxy acetic acid
BAP: 6-Benzylaminopurine

2,4-D, kinetin, and BAP in different concentrations and combinations (Table 4.1). Only Pillai and Kumar (1982) used SH (Schenk and Hildebrandt, 1972) medium.

Nirmal Babu (1997) tested various explants—vegetative bud, immature inflorescence, leaf (pseudostem), ovary, and anther—for their morphogenetic responses (see Figure 4.1A–L) on MS basal medium supplemented with cytokinins (BAP and kinetin) and auxins (NAA and 2,4-D). MS basal medium supplemented with auxin (NAA 0–4 mgl^{-1}) and cytokinin (BAP 0–4 mgl^{-1}) gave positive response in inducing multiple shoots and roots. The presence of NAA at low concentrations (1 mgl^{-1}) resulted in good growth of culture, root induction, and shoot multiplication and addition of BAP at 4 mgl^{-1} increased the multiple shoot induction, but reduced root induction. NAA alone at high concentration (2–4 mgl^{-1}) inhibits shoot growth as well as root induction, whereas BAP alone at higher concentration (4 mgl^{-1}) induced only multiple shoots and rarely roots. Earlier workers also reported that BAP and NAA combinations were best for shoot

Figure 4.1 Stages in ginger tissue culture: (a) ginger plant; (b) vegetative bud explant and culture initiation; (c) multiple shoots; (d) regenerating callus; (e) organogenesis and embryogenesis from ovary derived callus; (f) conversion of floral buds into vegetative buds; (g) in vitro fruit formation; (h, i) plant regeneration from anther culture; (j) in vitro *rhizome* formation; (k, l) stages in hardening.

multiplication in ginger (Sakamura et al., 1986; Charlwood et al., 1988; Sakamura and Suga, 1989).

The induction of both roots and shoots in the same medium reduces the time taken for cloning considerably, and in ginger it takes about 60 days for an explant to develop

into a well-developed plantlet in the first cycle using a primary explant from the greenhouse-grown plant. When the subcultures are initiated from the in vitro developed axillary shoots, the rate of proliferation was further increased and the time taken for plantlet development was reduced to 45 days per cycle (Nirmal Babu, 1997).

Vegetative Bud Culture

Vegetative bud explants containing both shoot tip and axillary buds were used for clonal multiplication. Growth regulators, NAA (1 to 4 mgl^{-1}) and BAP (1 to 4 mgl^{-1}) were tested in various combinations. Only 50 percent of the explants could be established, whereas the rest were lost due to contamination. The explants took nearly 20 days for exhibiting first signs of growth and bud break and subsequently produced both multiple shoots as well as roots in all the growth-regulator concentrations tried. The number of shoots ranged from 1 to 9 and the number of roots ranged from 1 to 10 after 60 days of culture (see Figure 4.1a–c). Both NAA and BAP induced multiple shoots and roots in ginger vegetative bud explants. However, the cultures responded differently in different treatments. MS medium supplemented with 4 mgl^{-1} NAA and 4 mgl^{-1} BAP gave the lowest response (40 percent) for production of multiple shoots or roots, whereas that supplemented with 1 mgl^{-1} NAA and 4 mgl^{-1} BAP or NAA alone (1 mgl^{-1}) gave highest culture response (90 percent). The mean number of multiple shoots ranged from 1.2 to 5.2 in different treatments. MS medium supplemented with 3 mgl^{-1} each of NAA and BAP gave the lowest number (1.2) of multiple shoots, whereas while that supplemented with 1 mgl^{-1} NAA and 4 mgl^{-1} BAP gave the highest number (5.2).

The mean number of roots ranged from 0.7 to 5.4 in different treatments. MS media supplemented with 1 mgl^{-1} NAA gave the highest number (5.4) of roots and MS media supplemented with 4 mgl^{-1} BAP gave the least number (0.7) of roots.

Considering that both induction of multiple shoots and roots are equally important, development of both in a single medium reduces the time taken for plant development. In such cases, MS medium with 1 mgl^{-1} NAA alone or 1 mgl^{-1} NAA and 4 mgl^{-1} BAP was ideal for multiplication of ginger from vegetative bud explants (see Table 4.1). These treatments were significantly superior to the rest with respect to multiple shoot production and root induction. The plantlets from the vegetative bud cultures are healthy, robust, and 8 to 12 cm tall with three to four roots. These plantlets were hardened in a humid chamber for 20 to 25 days with 85 percent establishment.

Inflorescence Culture and Development of Shoots from Floral Meristem

Floral meristems from young inflorescences, when the determination of an individual floral meristem was not canalized, can be induced to grow vegetative shoots in vitro. In rhizomatous crops like ginger, the use of floral meristems reduces the problem of culture contamination that is so common when rhizome explants are used.

In ginger, vegetative shoots were produced in 70 percent of the explants when 1-week-old inflorescences were cultured on MS medium supplemented with 10 mgl^{-1} BAP and 0.2 mgl^{-1} 2,4-D. One or rarely two plantlets developed per axil of the bract in the majority of the cases (see Figure 4.1f). In about 26 percent of the cultures multiple shoots ranging from 5 to 25 were induced. These shoots grew into complete plantlets in 7 to 8 weeks' time. However, in 20 percent of the cultures the older flower buds developed into flowers and opened while in culture (Table 4.2). These are the meristems that were already differentiated into flower primordia (Nirmal Babu et al., 1992b).

The development and maturity of flower in the aseptic cultures of ginger inflorescence will also result in the exciting possibility of aseptic pollen, which can directly be used for conservation in a cryopollen gene bank and for in vitro pollination.

Table 4.2 Morphogenetic response of immature inflorescence explants on MS basal medium

Sl. No.	*Explant*	*Culture medium*	*Cultures responded (%)*[a]	*Morphogenetic response*
1.	One-week-old whole inflorescence	MS + 10 mgl^{-1} BAP + 0.2 mgl^{-1} 2,4-D	20	Opening of flower buds and development of normal flowers
			44	Conversion of floral buds to vegetative buds with 1–2 shoots
			26	Conversion of floral buds to vegetative buds and multiple (5–25) shoots
			2	Flower bracts developed into leaf-like structures
2.	Single flower culture	MS + 10 mgl^{-1} BAP + 0.2 mgl^{-1} 2,4-D	40	Development of fruit but ovary splits open and no further growth
			20	Development of fruit and subsequent development of plantlets from fruits

[a]Mean of 20 replications

Single Flower Cultures and Development of Fruit

Sexual reproduction is an important mechanism for the introduction of variability through recombination in a population. In nature, ginger produces many flowers but fails to set fruit. New biotechnological methods like in vitro pollination and embryo rescue are employed in many plant species for obtaining viable seeds and subsequently the plants when normal mechanisms fail to develop seeds due to incompatibility and failure in embryo development. By culturing young 1-week-old immature inflorescences on MS medium supplemented with 10 mgl^{-1} BAP and 0.2 mgl^{-1} 2,4-D, it was possible to effect in vitro pollination and the ovary developed into trilocular fruits by 60 to 90 days in 20 percent of the cultures (see Figure 4.1g), and subsequently plants could be recovered from the fruits. In 20 percent of the cultures single plantlets were seen emerging directly from the ovaries by the seventh week (Nirmal Babu et al., 1992b). These plantlets developed roots simultaneously and later produced tillers. Development of seeds by in vitro pollination in ginger was also reported by Valsala et al. (1997). In nature also, rare fruit set was reported (Purseglove et al., 1981). The possibility of in vitro–induced seed development will open up new possibilities of sexual reproduction and development of seed-derived progenies of ginger by selfing and hybridization hitherto ineffective in ginger, thus bringing entirely new directions to ginger crop–improvement programs.

Plant Regeneration from Callus

Successful development of a plant regeneration protocol is essential in that it has a cascading effect. The success of many other advanced in vitro techniques such as in vitro mutant selection, protoplast fusion, and genetic transformation depends directly upon the ability to regenerate plants from the derived tissue. In addition, polyploidy, aneuploidy, and chromosome structural changes, which commonly occur in fast-dividing cells, along with many other factors, result in accumulating heritable variation in the regenerating plants, giving rise to new variability in the gene pool (somaclonal variation) that can be used for crop improvement.

Callus can be initiated in vitro by culturing small explants on growth-supporting medium supplemented with exogenous growth-regulating factors. During this process, cell differentiation and specialization, which might be occurring in the intact plant tissue, are reversed and the explant gives rise to new tissue that is composed of meristematic and unspecialized cells. During dedifferentiation, storage products typically found in resting cells tend to disappear. New meristems are formed in the tissue and these give rise to undifferentiated parenchymal cells without any of the structural order that was characteristic of the organ or tissue from which they were derived. Although callus remains unorganized, as growth proceeds, some kind of specialized cells may be again formed. Such differentiation can take place at random, but may be associated with centers of morphogenesis, which can give rise to organs. Thus, callus culture is usually made up of two types of tissues, differentiated and nondifferentiated.

Callus Induction

Many parts of the whole plant may have an ultimate potential to produce callus in vitro, but it is frequently found that callus cultures are more easily established from some organs than others. Young meristematic tissues are most suitable and meristematic areas in older parts of a plant can also give rise to callus. Monocotyledons react differently and are less likely to form callus than dicotyledons (Pierik, 1987). A difference in the capacity of tissue to give rise to callus is particularly apparent in monocotyledons. For example, in most cereals, callus growth can be obtained only from organs such as zygotic embryos, germinating seeds, seed endosperm, seedling mesocotyl, and very young leaves but so far never from mature leaf tissue (Green and Philips, 1975; Dunstan et al., 1978).

Nirmal Babu (1997) reports that in ginger, callus was successfully induced in vegetative bud, young leaf, ovary, and anther tissues on MS medium supplemented with various levels (0.5 to 5.0 mgl^{-1}) of NAA and 2,4-D for induction and proliferation of callus. Although auxins, in general, induce callus formation, this worker reported that only 2,4-D at concentrations ranging from 0.5 to 5.0 mgl^{-1} was effective in inducing callus in all explants tried, with the best concentration being 3 mgl^{-1}. NAA induced a slight amount of callus at higher concentrations of 3 to 5 mgl^{-1} only. The explants differed in their ability to form callus. The best is vegetative bud followed by anther explant. Leaf explant gave the least amount of callus. The amount of callus produced was 2.7 g per tube in leaf and 3.4 g per tube in vegetative bud. Callus tissue was not of one kind. Strains of callus differing in appearance, color, degree of compaction, and morphogenetic potential commonly arise from a single experiment. In ginger, the callus was loose, friable, and pale yellow in color. In the subsequent cultures, the callus contained some hard organized embryogenic "lumps" (meristemoids) within the mass

of loose cells (Nirmal Babu, 1997). The callus could be maintained and multiplied by monthly subcultures on the same media.

The earlier reports on callus induction in ginger were those of Pillai and Kumar (1982), Kulkarni et al. (1987), Sakamura and Suga (1989), Choi (1991b), Malamug et al. (1991), Kacker et al. (1993), Ilahi and Jabeen (1992), and Samsudeen (1996).

Plant Regeneration

Two-month-old calli derived from various explants—vegetative bud, young leaf, ovary and anther tissues—were cultured on a series of MS basal media supplemented with varying levels of auxin (2,4-D) and cytokinins (Kinetin and BAP) for morphogenesis and plant regeneration (Table 4.3)

When fresh calli, immediately after its induction, was cultured on MS medium without growth regulators, it resulted in rhizogenesis. The cytokinins, kinetin, and BAP when used individually did not result in organogenesis even after four to five cycles of subculture. But addition of cytokinins (kinetin and BAP) at concentrations of 5 to 10 mgl^{-1} in the presence of 0.2 mgl^{-1} 2,4-D resulted in induction of morphogenesis—both organogenesis and embryogenesis—and subsequent development of plantlets. MS medium supplemented with 10 mgl^{-1} BAP and 0.2 mgl^{-1} 2,4-D gave the best morphogenic response. Once the morphogenic pathway of the cultures was determined, they continued to show their morphogenic potential in subsequent subcultures even after 2 to 3 years.

In vegetative bud-derived callus, the number of plantlets developed per culture were higher in BAP (28 to 60) than in kinetin (8 to 26). The rate of shoot production and plantlet formation increased considerably to 36 to 62 shoots per culture when growth regulators were completely excluded from the medium in later subcultures after the induction of morphogenesis (see Figure 4.1c).

MS medium supplemented with 10 mgl^{-1} BAP and 0.2 mgl^{-1} 2,4-D was also the best for leaf-derived callus. The number of shoots per culture was higher in BAP (15 to 35). Transfer to growth regulator-free culture medium after initial morphogenesis resulted in enhancement of plant regeneration to 30 to 60 shoots. The time taken for the leaf-derived callus to differentiate and develop into a complete plantlet ranges from

Table 4.3 Effect of explant on plant regeneration through callus phase on MS basal medium[a]

Sl. No.	Explant	Callus production (g)[b]	Days taken for morphogenesis[c]	Morphogenetic pathway[b]	No. of plantlets ±SD[b]
1.	Vegetative bud	3.40	146	Organogenesis (shoot) and embryogenesis	26.2 (±6.2)
2.	Leaf	2.70	162	Organogenesis (shoot)	24.4 (±6.2)
3.	Ovary	3.10	131	Embryogenesis and organogenesis (shoot)	47.6 (±9.3)
4.	Anther	3.20	120	Organogenesis (shoot)	12.6 (±3.1)

[a]Mean of 10 replications
[b]Supplemented with 3 mgl^{-1} 2,4-D.
[c]Supplemented with 0.2 mgl^{-1} 2,4-D and 10 mgl^{-1} BAP.

6 to 8 months, whereas while it takes about 5 to 7 months for the vegetative bud-derived callus.

In callus derived from ovary tissues morphogenesis was observed on MS media supplemented with 5 to 10 mgl^{-1} BAP or kinetin and 0.1 to 0.2 mgl^{-1} 2,4-D and also with 5 mgl^{-1} BAP or kinetin and 0.2 mgl^{-1} 2,4-D after four to five subcultures. In both the media white globular heart-shaped embryo-like structures were observed in 67 to 80 percent of the cultures. In about 22 percent of the cultures both organogenesis and embryogenesis were observed in the same culture. This process continued in the subsequent cultures on the same medium. The embryogenic calli were separated and used for subsequent cultures. When growth regulators were removed from the culture medium at this stage, the callus turned green and the rate of morphogenesis increased considerably, resulting in many embryo-like structures that could be separated easily with a gentle tap. On subsequent transfer to fresh medium without growth regulators, the embryoids developed into complete plants. The somatic embryos produced secondary embryoids by adventitious embryogenesis resulting in a large number (100 to 300) of tiny embryoids (see Figure 4.1e). This type of multiplication continued on growth regulator-free medium even after 2 years of continuous culture, indicating that once the callus turns embryogenic, growth regulators are not needed for further multiplication. The embryoids produced were either compactly arranged or loosely arranged. The development of these embryoids into complete plantlets was higher when NAA (1 mgl^{-1}) was added to the culture medium, which also enhanced rooting. This protocol with a production potential of a large number of tiny propagules is ideally suited for in vitro manipulations such as in vitro mutagenesis, in vitro polyploidization, and in vitro selection against biotic and abiotic stresses. The somatic embryos are ideally suited for direct DNA transfer using a particle-delivery system and development of transgenics.

The explants differed in their morphogenetic response with regard to the morphogenetic pathway as well as the plantlets regenerated. The plant regeneration was by organogenesis in leaf- and anther-derived callus, whereas it was by both organogenesis and embryogenesis in vegetative bud-derived and ovary-derived callus. Histological studies have proved the origin of both organogenesis and embryogenesis. Embryo development followed typical monocotyledonous stages of development from globular, heart-shaped to torpedo-shaped embryos with clear-cut scutellum and coleoptile development. The embryo showed clear shoot and root poles with no vascular connection between the host tissue and the embryo. The explants differed with respect to days taken for morphogenesis and their plant regeneration potential. Anther-derived callus was quickest to respond (120 days) compared to leaf-derived callus, which is slowest (162 days). The mean number of plantlets recovered ranged from 12.6 from anther-derived calli to 47.6 in ovary-derived calli (see Table 4.2). The embryogenic calli derived from ovary were canalized and produced an increasing number of somatic embryos in the subsequent cultures. These somatic embryos also resulted in repetitive embryogenesis and budding resulting in a large number (over 100) of somatic embryos in a given culture tube. Thus, the ovary was the best among the explants tried for plant regeneration and recovery. This efficient plant-regeneration system is ideally suited for in vitro selection, in vitro mutagenesis, and genetic manipulation experiments (Nirmal Babu et al., 1996b).

Shoots developed from the callus were placed on the modified MS medium with 1 mgl^{-1} of NAA for rooting that was earlier identified as ideal for root induction. An extensive root system developed within 5 weeks in this medium. Rooting was better when liquid medium was used instead of solid medium.

Successful plant regeneration in ginger was earlier reported (Nadgauda et al., 1980; Ilahi and Jabeen, 1987; Kulkarni et al., 1984; Malamug et al., 1991; Kackar et al., 1993). The explants used were young sprouts and young leaf segments and the plant regeneration was via organogenesis and embryogenesis. Earlier studies indicate BAP at high concentration resulted in morphogenesis and plant regeneration from these explants.

Anther Culture

Since the first report of induction of androgenesis in *Datura* (Guha and Maheshwari, 1964), anther culture has gained considerable importance in our efforts to produce haploids and dihaploids. Haploid plantlets are formed in two distinct ways: by embryos originating directly from microspores without callusing (direct androgenesis) or by organogenesis from haploid callus tissue. Haploid cells, in general, are unstable in culture and have a tendency to undergo endomitosis to form diploid cells. This property of cell culture can be exploited for obtaining homozygous lines of ginger.

Callus formation and the development of roots and rhizome-like structures were reported from excised ginger anthers cultured on MS medium containing 2,4-D and coconut milk (Ramachandran and Nair, 1992).

Samsudeen et al. (2000) reported regeneration of plantlets from excised anthers. They cultured excised anthers with uninucleate pollen mother cells and pollen were inoculated on modified MS medium supplemented with 0.2 to 3.0 mgl^{-1} 2,4-D after cold treatment. Cold treatment was given for 1, 2, and 7 days at 0°C. Of the three different cold treatments tried, callus was induced only from anthers, which had 7 days of cold treatment. These anthers developed friable callus in solid as well as in liquid medium in about 6 weeks. Occasional development of roots was observed in liquid cultures. Their study has shown that solid medium is better than liquid medium for ginger anther culture. 2,4-D at 3 mgl^{-1} was good for both callus induction and callus proliferation, giving about 3 g of callus in about 30 days of culture when incubated in light. Calli obtained from ginger anthers were cultured on MS basal medium supplemented with 2,4-D (0 to 0.2 mgl^{-1}) and BAP (0 to 10 mgl^{-1}). MS medium with 2,4-D at 0.2 mgl^{-1} and BAP at 10 mgl^{-1} was the best for organogenesis and plant regeneration. The plant regeneration was by organogenesis and shoot development (see Figure 4.1h). Seventy percent of the cultures gave a morphogenetic response with a range of 1 to 20 shoots and an average of 12.6 shoots per culture tube (see Figure 4.1i). These shoots were multiplied and rooted on MS basal medium with 1 mgl^{-1} NAA before hardening and field establishment. The regenerated plants are being indexed for the selection of haploids or dihaploids.

Effect of Explant on Plant Regeneration

The various explants tried (i.e., vegetative bud, leaf, ovary, and anther) differed in callus induction, morphogenetic ability of callus, and pattern of morphogenesis through the callus phase (see Table 4.3). Anther-derived callus was quicker to turn morphogenetic followed by ovary-, vegetative bud-, and leaf-derived calli. The origin of callus determined the morphogenetic pathway it follows for development of plantlets.

Histological studies confirmed that plant regeneration in ginger callus was by organogenesis as well as embryogenesis, depending upon the origin of the explant. Calli derived from vegetative bud as well as ovary showed both organogenesis and embryogenesis, whereas those from leaf and anther followed only the organogenetic path of development. In addition, among the different explants tried, ovary was the most effective for plant regeneration through the callus phase, which gave 47.6 plantlets per tube, followed by vegetative bud (26.2), leaf (24.4), and anther (12.6). The morphogenetic calli obtained from all the explants showed their morphogenetic ability even after repeated subcultures both on the same medium or on growth-regulator–free medium, indicating that growth regulators are needed only for induction of morphogenesis. Once the callus turns morphogenetic, it retains the morphogenic ability for a long time if cultured on the same medium. These cultures remained morphogenetic and produced plantlets even after 2 years of repeated subcultures (Nirmal Babu, 1997).

Hardening and Field Establishment

A substantial number of micropropagated plants do not survive transfer from in vitro conditions to greenhouse or field environments due to the "delicate" nature of plants raised in vitro. Compared to in vitro conditions, the outside atmosphere, which has substantially lower relative humidity, higher light, and septic environment, causes stress to the micropropagated plants. Shoots and plants in culture are grown in conditions that provide little physiological stress since a carbon source is provided, reducing the need for photosynthesis. Due to the high relative humidity and low light within in vitro conditions, the anatomy and physiology of tissues are different from those of plants grown in greenhouse conditions. In addition, the aseptic environment in vitro reduces the stress of pathogens. Various factors such as development of epicuticular wax, functional stomata, root system, and increased photosynthetic ability influence the acclimatization. Most plant species grown in vitro require a gradual acclimatization and hardening for survival and growth in a natural environment (Preece and Sutter, 1991; George, 1996). As the plantlets are progressively acclimatized, the rate of water loss from their leaves decreases and the photosynthetic ability of the plant increases, especially in the leaves newly produced after transfer, resulting in a higher rate of establishment.

Nirmal Babu (1997) reported that ginger plantlets micropropagated as well as callus-regenerated plants were hardened and transplanted in a porous soil mixture of vermiculite, sand, and garden soil in equal proportions with 57 to 85 percent success depending upon the origin of explant. High humidity was maintained for the initial 20 to 30 days by keeping them in a humid chamber. The humidity was reduced gradually for hardening and establishment. The survival rate of transplanted plantlets ranged from 57 to 85 percent and the time taken for hardening ranged from 22 days in direct regenerated plants to 30 days in callus-regenerated plants. Plants regenerated from ovary-derived callus have the lowest establishment rate of 57 percent, whereas those multiplied directly from vegetative buds have the highest rate of 85 percent field establishment. The tissue-cultured plantlets obtained by direct multiplication and plantlets regenerated from vegetative bud-, leaf-, ovary-, and anther-derived calli differed in their size at the time of hardening. There is a direct correlation between plant size and survival rates; the bigger plantlets are easier to establish after hardening than the smaller plantlets. In general, plantlets multiplied directly from vegetative buds were bigger and hence have

Table 4.4 Morphological features of plantlets derived from various explants at the time of hardening[a]

	Direct regeneration		*Callus regeneration*			
Explant	*Vegetative bud*	*Inflorescence*	*Vegetative bud*	*Leaf*	*Ovary*	*Anther*
No. of plantlets/culture	5.2	2.8	26.2	24.4	47.6	12.6
Plant height (cm)	11.9	11.2	8.7	2.7	6.2	6.8
No. of roots/plant	3.2	3.2	2.3	3.1	1.3	2.0
No. of leaves/plant	7.8	7.2	5.4	4.2	6.0	8.0
Leaf length (mm)	52.0	48.0	51.0	45.0	39.0	30.0
Leaf breadth (mm)	6.0	5.0	6.0	4.0	3.0	4.0
Days taken for hardening	22.0	25.0	30.0	30.0	30.0	30.0
Establishment (%)	85.0	80.0	70.0	74.0	57.0	68.0
Rhizome wt. in 1st season (g)	3.3	2.8	1.6	1.2	0.8	0.7

[a]Mean of 10 replications

Table 4.5 Morphological characters of micropropagated plants developed through direct regeneration (MP) and callus regeneration (CR)

Sl. No.	*Plant type*	*Plant ht. (cm)*	*No. of tillers/plant*	*No. of leaves/plant*	*Width of rhizome (cm)*	*No. of nodes/finger*	*Internode distance (mm)*	*Yield/plant (g)*
1.	MP	91.0±23.8	11.4±4.4	8.8±4.6	2.8±0.5	9.2±1.1	5.1±0.8	382.0±225
2.	CR	91.5± 6.1	10.2±2.2	8.8±1.2	2.7±0.2	8.1±0.7	4.9±1.0	447.4±243
3.	C	76.4±16.6	8.4±1.1	9.2±0.7	2.7±0.5	8.0±0.7	4.9±1.7	352.0±114

± Standard deviation; C: Control

a higher rate of establishment compared to plantlets regenerated through callus, which gave lower rate of establishment (see Tables 4.4 and 4.5). At the nursery stage, most of the plants were morphologically similar (see Figure 4.1k and l) except a few having leaves with white chlorotic patches and wavy margins.

Tissue-cultured plants of ginger could be hardened and acclimatized to the field conditions with relative ease due to the genetic nature of the zingiberaceous crops that are conventionally propagated through vegetative means. Earlier studies in ginger and other zingiberaceous crops like turmeric, cardamom, and *Kaempferia* support this view (Hosoki and Sagawa, 1977; Nadgauda et al., 1980; Bhagyalakshmi and Singh, 1988; Vincent et al., 1992).

Tissue-cultured plants were maintained in polythene bags for the first season and then transferred to earthern pots. Rhizomes of tissue-cultured plantlets were too small (0.7 to 3.3 g) to harvest after the first season (see Figure 4.2b) and if harvested, the rhizomes may dry up if care is not taken. The micropropagated plantlets behaved like seedlings of similar zingiberaceous crops. The size of the rhizome increased over the years and developed into normal size comparable to that of mother plants only in third year. This indicates that tissue-cultured plantlets cannot be directly used for commercial cultivation

and need to be maintained for at least two to three crop seasons in the nursery before commercial planting (Nirmal Babu, 1997; Nirmal Babu et al., 1997, 1998, 2000).

Evaluation of Somaclones

The success of any in vitro culture technique depends either on the ability to clone the genotypes for production of uniform planting material or the ability to bring about variations that can be exploited in crop-improvement programs (see Figure 4.2a–h). The genetic uniformity of plants multiplied by tissue culture depends on a number of factors, with the two most important being the method of multiplication and the genotype.

Accumulated information now shows that plants propagated by precocious shoots show no more spontaneous mutation than those propagated by conventional means. Plants regenerated from callus or cell suspension cultures may show a varying proportion of structural or physiological abnormalities depending upon the species, origin, and the age of culture (Yeoman, 1986). Other factors such as growth regulators (D'Amato, 1978; Zakhlenyuk and Kunakh, 1987), composition of the culture medium (Bayliss, 1977; Feng and Quyang, 1988), culture conditions (Cerutti, 1985; Jackson and Dale, 1988), and culture method (Wilson et al., 1976) influence somaclonal variation. The reasons for variations in micropropagated plants can also be due to the variation that existed in the source plant (preexisting variation), epigenetic or physiological effects, and genetic changes (Swartz, 1991; George, 1996). Extensive studies conducted during the last decade have shown that the cell and callus cultures, especially on periodical subculture, undergo various morphological and genetic changes: polyploidy, aneuploidy, chromosome breakage, deletions, translocations, gene amplifications, inversions, and mutations (Nagl, 1972; Meins, 1983; D'Amato, 1985). In addition, there are changes at the molecular and biochemical levels, including changes in the DNA, rearrangement of genes, somatic crossing over, altered nucleotide methylation, perturbation of DNA replication by altered nucleotide pools, and slicing or activation of genes by mutations in associated noncoding regions and transposons (Scowcroft, 1984) and enzymes (Cullis, 1983; Day and Ellis, 1984; Ball and Seilleur, 1986; Brettel et al., 1986). Thus, in vitro technology is a powerful tool for the induction of much-needed genetic variability in ginger.

Morphological and Biochemical Characterization

Morphological and biochemical characterization of 4-year-old micropropagated as well as callus-regenerated plants was reported by Nirmal Babu (1997) in comparison with conventionally propagated plants (see Table 4.6). Direct regenerated plants and callus-regenerated plants as separate groups when compared with conventionally propagated plants revealed a good amount of variation with regard to plant height, number of tillers and of leaves per plant, girth of rhizome, number of nodes per finger, internodal distance, yield per plant (see Figure 4.2a–d), dry recovery percentage, and oleoresin and fiber contents. Micropropagated and callus-regenerated plants have higher mean values with regard to plant height, number of tillers, number of nodes per finger, and yield per plant compared to controls, whereas they have lesser mean values with regard to number of leaves per plant and oleoresin and fiber contents. With regard to the width of rhizomes,

Figure 4.2 Somaclonal variation and microrhizomes in ginger: (a) bold rhizomes harvested from ginger somaclone (CR 1222); (b) comparison of rhizomes from TC plants with that of conventionally propagated plants; (c) tissue-cultured plants after second year of nursery in comparison with conventionally propagated plants; (d) variations in rhizome size, shape, and internodal length in micropropagated plants; (e) growth recovery of microrhizome-derived plants in comparison with conventionally propagated plants; (f) initial growth of microrhizome-derived plants in comparison with conventionally propagated plants; (g) microrhizomes from ginger tissue cultures; (h) rhizomes harvested from microrhizomes as planting material.

Table 4.6 Morphological characters, yield, and quality attributes of promising somaclones in ginger

Sl No.	Somaclone	Plant ht. (cm)	No of tillers/ plant	Rhizome size	Yield (g)	Dry recovery (%)	Oleoresin (%)	Fiber (%)
1.	MP 61-9	81	13	Medium	870	26.0	2.5	4.4
2.	MP 74-15	78	7	Bold	780	23.5	3.8	4.3
3.	MP 76-1	96	10	Medium	474	27.5	4.5	4.2
4.	MP 76-3	93	9	Medium	398	25.7	2.8	3.8
5.	CR 10-1	79	9	Medium	367	23.5	4.2	3.8
6.	CR 816	79	10	Medium	390	24.0	3.0	5.0
7.	CR 818	83	9	Bold	300	22.0	1.6	3.6
8.	CR 822	76	11	Medium	398	24.7	3.8	3.9
9.	CR 855	69	8	Medium	472	25.0	2.0	4.3
10.	CR 1222	87	8	Bold	600	21.0	3.9	3.9
11.	Control	101	12	Medium	513	28.0	3.8	4.9

MP, micropropagated plants; CR, callus-regenerated plants

internodal distance, and dry recovery percentage, all the three groups are on par. When we consider the range observed within the group, micropropagated plants showed the highest range with regard to plant height, number of tillers and leaves per plant, width of rhizome, and number of nodes per finger, whereas callus-regenerated plants showed the highest range with regard to internodal distance and yield.

Variations were also observed among tissue-cultured and callus-regenerated plants in their oleoresin content, fiber content, and dry recovery. The oleoresin content ranged from 1.6 to 7.6 percent, crude fiber percentage ranged from 3.6 to 6.7, and dry recovery from 21.2 to 31.8 percent among the tissue-cultured plants, whereas the control had 5.2 percent oleoresin, 5.9 percent fiber, and 28 percent dry recovery.

Nirmal Babu (1997) and Nirmal Babu et al. (1996b) observed variations in both micropropagated and callus-regenerated plants with respect to their tolerance to *Pythium aphanidermatum* and *Ralstonia solanacearum (Pseudomonas solanacearum)* when the plants were inoculated twice with the organism. Eight somaclones showed a relatively low percentage of disease incidence (*P. aphanidermatum* infection). However, these lines succumbed to infection in field trials and pot culture experiments (Kumar et al., unpublished data), indicating insufficient levels of disease resistance. Isolation of *Pythium*-tolerant lines was earlier reported in ginger (Kulkarni et al., 1987). Intraclonal variations are known in ginger and turmeric, and many promising lines and varieties were identified after clonal selection (Rajeevan and Mohanakumaran, 1993; Khader et al., 1994; Rattan et al., 1994). This accounts for variations observed in conventionally propagated plants. These preexisting variations are also reflected at higher level in micropropagated and callus-regenerated plants. Thus, somaclonal variation is an important source of variability and can be exploited for crop-improvement programs in ginger.

A few promising lines having important yield attributes and other useful characters could be selected from both micropropagated and callus-regenerated lines (Nirmal Babu, 1997).

In Vitro Selection

Kulkarni et al. (1984) reported isolation of *Pythium*-tolerant ginger by using culture filtrate as the selecting agent. In vitro selection for resistant types to *Pythium* and *Pseudomonas* is in progress at IISR using culture filtrates of the pathogen (see Figure 4.3), or pathotoxin as the selecting agent (Nirmal Babu et al., 1996b, Dake et al., 1997).

Identification of Promising Lines

A few promising lines with regard to yield, disease resistance, and other quality attributes could be identified from the tissue-cultured plantlets (see Table 4.4). Somaclones MP 61-9, CR 10-1, CR 816, and CR 822 showed comparatively lower disease incidence (*Pythium* infection) and also high per plant fresh rhizome yield of 870, 367, 373, and 358 g, respectively. Somaclone 855 with a fresh rhizome yield of 472 g per plant showed comparatively lower disease incidence against both *P. aphanidermatum* and *R. solanacearum*. Somaclones MP 74-15 and CR 1222 were also high yielders with 780 and 600 g of fresh rhizomes per plant, respectively. Somaclone, MP 74-15 with a fresh rhizome yield of 780 g and CR 818 with a fresh rhizome yield of 398 g also have bold rhizomes. In addition, the rhizomes of CR 1222 were attractive with extrabold fingers (Figure 4.2D), which was latter found to be a polyploid. Based on biochemical assays, somaclones CR 818 (3.6 percent) and MP 49-7, MP 70-4, MP 97, and CR 10-1 (all with 3.8 percent) were identified as low-fiber types, which is a preferred character.

In Vitro Micro Rhizome Induction

Species that normally produce such organs as bulbs, tubers, and corms can be induced to form these miniature propagules within in vitro cultures under appropriate environmental conditions. Plants that naturally produce tubers can be induced to produce miniature versions of the storage organs in a medium containing high cytokinin levels (George, 1993). Miniature storage organs have a great advantage as they can be readily removed from a culture flask in a dormant condition and stored ex vitro without precautions against sepsis. If they are produced in vitro from disease-free stocks, microtubers provide an ideal method for propagating and distributing disease-free planting material. When planted in soil, they behave as normal tubers.

In vitro induction of rhizomes and their germination in ginger has been reported by various workers (Sakamura et al., 1986; Sakamura and Suga, 1989; Bhat et al., 1994; Sharma and Singh, 1995; Nirmal Babu, 1997; Nirmal Babu et al., 2003). Bhat et al. (1994) reported in vitro induction of rhizomes in ginger at higher sucrose concentrations (9 to 12 percent). Quality analysis of in vitro–developed rhizomes indicated that they contain the same constituents as the original rhizome but with quantitative differences. The composition of basal medium seems to affect the composition of oil (Sakamura et al., 1986; Sakamura and Suga, 1989; Charlwood et al., 1988). Sharma and Singh (1995) reported microrhizomes with four to five buds weighing 73 to 459 mg that were induced on MS medium with 75 g/l sucrose. After storage in moist sand at room temperature for 2 months, 80 percent of the microrhizomes sprouted into plants.

Geetha (2002) and Peter et al. (2002) tried various combinations of sucrose and mannitol in different concentrations to induce microrhizomes in ginger (see Table 4.7).

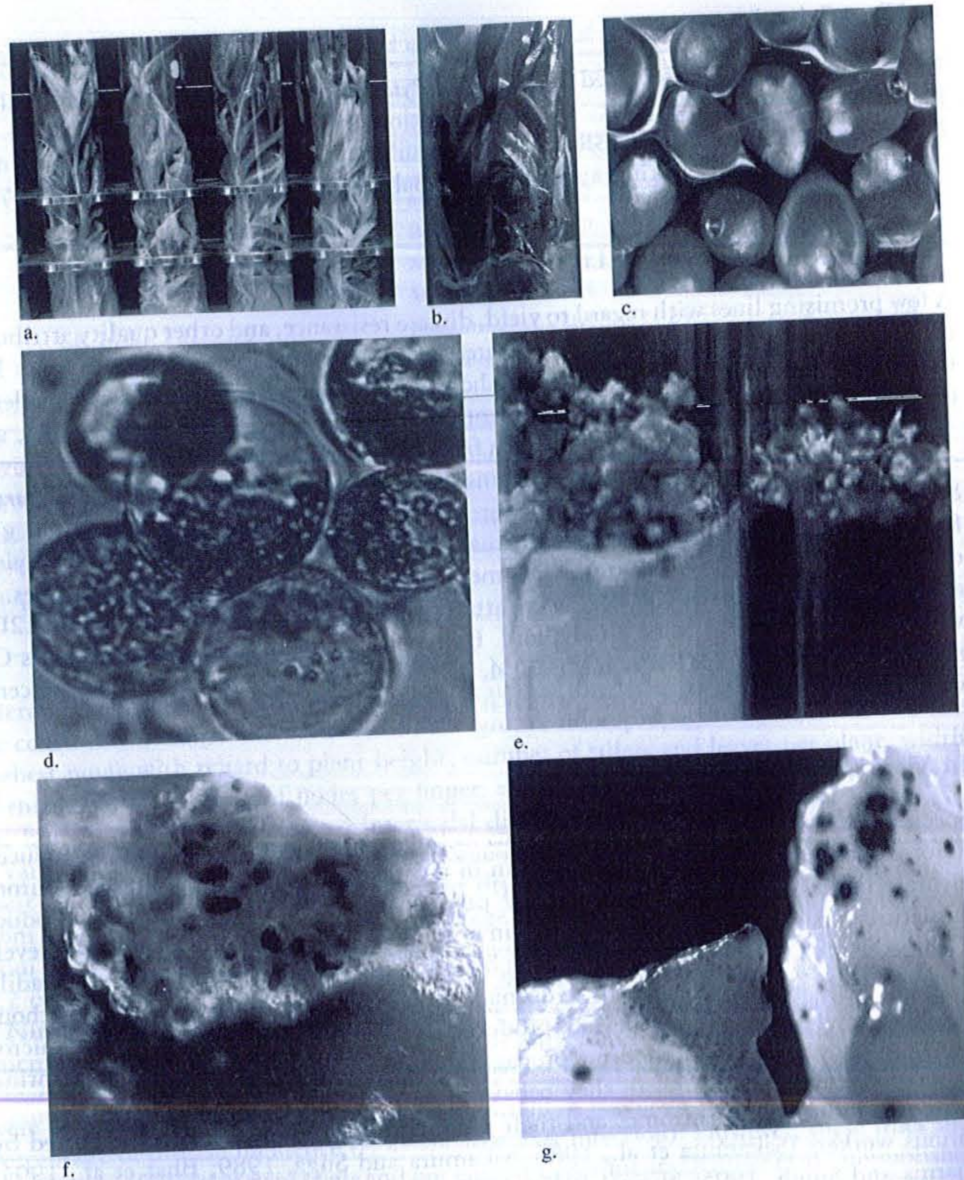

Figure 4.3 In vitro conservation and other biotechnological approaches in ginger: (a) cultures under medium-term conservation in minimal growth medium; (b) one-year-old culture under minimal growth; (c) synthetic seeds; (d) isolated protoplasts; (e) in vitro selection of embryoids against disease-causing organisms; (f) oil cells in cell suspension cultures; (g) transient expression of GUS in embryogenic calli.

Microrhizomes of 0.05 to 15 g fresh weight per explant were induced in ginger tissue cultures in 1 to 12 months on MS basal medium supplemented with higher levels of carbon source. In 3 percent sucrose, microrhizome formation was observed in 30 to 40 percent cultures only after 12 months in culture. In the medium with 1 or 1.5 percent

Table 4.7 Effect of sucrose and mannitol on induction of microrhizomes in ginger on ms basal medium

Sucrose (%)	*Mannitol (%)*	*Time period for induction of microrhizome (months)*	*Percentage response*[a]	*Fresh weight (g)*[a]
3.0	—	12	30–40	0.05–1.0
9.0	—	1	80–100	5–15
10.0	—	1	80–100	5–10
12.0	—	1	80–100	3–6
1.0	1.0	8	50–60	0.06–1.2
1.5	1.5	8	50–60	0.1–1.2
2.0	1.0	8	30–40	0.02–0.5
3.0	3.0	No induction	—	—
3.0	6.0	No induction	—	—
5.0	5.0	No induction	—	—
6.0	6.0	No induction	—	—

MS, Murashige and Skoog (1962) medium.
[a]Mean of 20 replicates

each of sucrose and mannitol, time taken for microrhizome formation was reduced to 8 months with 50 to 60 percent of the cultures responding. In this combination comparatively smaller microrhizomes, weighing up to 0.05 to 1.2 g, were produced. Microrhizomes were produced in 80 to 100 percent of cultures when sucrose concentrations were increased to 9, 10, and 12 percent. The microrhizomes from these cultures were larger with a fresh weight of 3 to 15 g in 1 to 6 months. The other combinations of sucrose and mannitol did not induce microrhizomes (see Table 4.7). Microrhizomes resembled the normal rhizomes in all respects, except for their smaller size. They consisted of two to four nodes and one to six buds. They also have the aromatic flavor of ginger and resembled the normal rhizome in anatomical features. The presence of well-developed oil cells, fibers, starch grains, and curcumin cells was also observed. These microrhizomes were directly planted in the field without any hardening with 80 percent success. Care has to be taken to ensure soil moisture in the first 20 days after planting. The plants derived from the microrhizomes although shorter in size (see Table 4.8), have more tillers per plant. The microrhizomes weighing 2–15g, gave a fresh rhizome yield of 100 to 800 g per plant, and an average yield of 10.5 kg per 3 m^2 bed, whereas the control gave a per bed yield of 15 kg (Figure 4.2e–h). The fresh rhizome yield of conventionally propagated plants when 20 to 30 g of seed rhizome was used, was 400 to 1,100 g per plant. In comparison with conventional propagation, the per bed yield was lower in microrhizome-derived plants, but the seed material used was also lesser in the latter. Microrhizomes gave lesser yield per unit area, but very high recovery vis-à-vis the weight of seed material used, and coupled with freedom from disease will make microrhizomes an ideal source of planting material. Microrhizome-derived plants, although slow in initial field establishment, were able to pick up later and grew on par with the control within 4 to 5 months. Microrhizome-derived plants produced more tillers per plant than the micropropagated and the control plants. Field data analysis also indicated that microrhizomes are more stable than micropropagated plants (Nirmal Babu et al., 2003).

Table 4.8 Morphological data of microrhizhome-derived plants compared with control (var: Jamaica)

Days after	*Plant Height*	*Tiller/ plant*	*Leaf/ tiller*	*Leaf Length*	*Leaf Breadth*	*No. of nodes*	*Petiole Length*	*Inter-nodal Dist.*
30 mr	24.92	4.60	9.30	21.03	2.80	8.07	0.30	5.77
c	31.88	4.04	8.08	20.31	2.82	7.41	0.39	3.33
60 mr	29.72	14.2	16.0	25.76	3.10	13.01	0.20	3.74
c	32.02	5.01	9.03	22.1	2.81	8.20	0.30	2.29
90 mr	53.12	16.6	19.6	23.24	2.20	18.2	0.30	1.60
c	53.90	5.70	9.50	23.20	2.90	9.10	0.30	2.82

mr, microrhizomes; c, control mother plant

In Vitro Conservation and Other Biotechnological Approaches

Synthetic Seeds

Synthetic seeds or artificial seeds in ginger were made by encapsulating in vitro–regenerated shoot buds, somatic embryos, and calli in 5 percent sodium alginate gel. The beads were round, uniform in size, and also sufficiently strong for easy handling (see Figure 4.3c). The encapsulated synthetic seeds were stored up to 9 months when maintained aseptically in MS basal medium at 22±2°C. Such synthetic seeds germinated on MS medium supplemented with 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} IBA into normal plants with 80 percent success (Sajina et al., 1997; Geetha, 2002; Swapna, 2002; and Peter et al., 2002).

Synthetic seeds form ideal source material for germplasm conservation and exchange. In addition, disease-free planting material can be moved from one place to another by using encapsulated propagules, especially in ginger, where major diseases are transmitted through infected rhizomes (see Figure 4.3c). Disease-free encapsulated shoot buds were produced in ginger by Sharma et al. (1994).

In Vitro Conservation and Cryopreservation of Germplasm

As in vitro techniques are becoming more important in crop improvement through somatic cell genetics, genetic stocks are assuming more variable forms from in vitro plantlets to protoplasts and DNA (Withers, 1985). With due precautions, the genotypes of plants propagated by node or shoot culture can be preserved without change. This type of in vitro culture can therefore be used to maintain genotypes over long periods. Fortunately, several ways have been found to reduce the rate of growth of cultured material so that it can be kept unattended for moderate lengths of time.

In Vitro Conservation

Modifying the constituents of culture medium by decreasing the carbohydrate/nutrient supply, changing the osmotic potential using the combinations of sucrose and mannitol and withdrawal of growth regulators from the culture medium, storage in reduced light, desiccation combined with cold treatment, reduced oxygen tension, and the use of growth regulators such as absisic acid induces slow growth in many crop species.

At the IISR, ginger plantlets could be successfully conserved for extended periods of over 12 months on half-strength MS basal medium supplemented with 15 gl^{-1} each of sucrose and mannitol. The cultures were sealed with aluminum foil and maintained at a temperature of 22±2°C. Thus, in ginger, minimal growth was induced by minimizing the evaporation loss using aluminum foil to seal the culture vessel, reduction of both the carbon source and the nutrients to half strength, and addition of mannitol. Ginger cultures grew well at 22±2°C but deteriorated when kept under lower temperatures of 5°C and 10°C. The rate of growth was higher when full-strength MS medium was used. High concentration of sucrose (30 gl^{-1}) increased culture growth substantially, resulting in exhaustion of culture medium. When the concentration of sucrose was reduced to 20 gl^{-1} and nutrient concentration to half, the cultures could be maintained for a much longer period of 200 to 240 days with a survival percentage of 75 to 81 depending upon the closure used. Addition of mannitol (10 to 15 gl^{-1}) and reduction of sucrose to lower levels (15 to 10 gl^{-1}) induced slow growth, and subsequently 73 to 80 percent of the cultures could be maintained for a period of 360 days when the culture vessels were closed with aluminum foil (see Figure 4.3a and 4.3b). Full- or half-strength MS medium supplemented with 10 or 15 gl^{-1} each of sucrose and mannitol and 1/2 MS with 20 gl^{-1} sucrose and 10 gl^{-1} mannitol allowed the cultures to be maintained for 360 days (see Table 4.9) (Nirmal Babu, 1997; Nirmal Babu et al., 1999, 2000; Geetha, 2002; and Peter et al., 2002).

According to Balachandran et al. (1990), ginger cultures could be maintained up to 7 months without subculture by using polypropylene caps as culture vessel enclosures. Dekkers et al. (1991) reported that ginger shoots could be maintained for over 1 year at ambient temperatures (24 to 29°C) in a medium containing mannitol (25 gL^{-1}) with an overlay of mineral oil.

At present, over 100 core collections of ginger are maintained at the IISR in vitro gene bank with yearly subculture. The small-sized plantlets kept in the conservation medium for over 5 years with yearly subculture when transferred to the multiplication medium (MS + 30 gl^{-1} sucrose and 1 mgl^{-1} NAA) gave normal growth with good multiplication rate. These plantlets were established easily with >80 percent success and developed into normal plants similar to the mother plants. Thus, the in vitro conservation technique is a safe alternative for a vegetativley propagated crop such as ginger for conservation and exchange of disease-free planting material.

Cryopreservation

Cryopreservation offers a better alternative when the base germplasm of any crop can be preserved for long durations with minimum effort. However, protocols for viable cryopreservation and post-thaw recovery of cryopreserved material are not available for ginger.

Geetha (2002) and Peter et al. (2002) reported that ginger in vitro-derived shoot tips could be successfully cryopreserved with limited success by pretreating the shoot tips with 0.75 M sucrose, desiccating for 1 hour, and plunging into liquid nitrogen (−185°C). Only 20 percent of the cultures developed into plantlets after cryopreservation. They also reported that encapsulated shoot buds of ginger (Synseeds) could be successfully cryopreserved after preculture on 0.75 M sucrose for 3 days and desiccated for 4 hours on laminar airflow. Plantlets could be regenerated from 20 percent of the cultures after cryopreservation.

Table 4.9 Effect of media components and culture vessel closures on induction of minimal growth in ginger cultures

Treatments			Growth rate				Duration of storage with 80% survival (months)
Basal medium Concentration (MS)	Sucrose (S) + Mannitol (M) (g/l)	Closure types	Increase in height (cm)[a] Mean	SD	Average no. of shoots/culture Mean	SD	
Full strength	30 S + 0 M	CP	7.2	0.56	4.6	0.70	3–4
Full strength	30 S + 0 M	SC	10.4	0.88	4.9	0.88	4–5
Full strength	20 S + 0 M	CP	5.6	0.98	2.6	0.84	3–4
Full strength	20 S + 0 M	SC	8.6	0.52	3.3	1.06	4–5
Full strength	20 S + 10 M	CP	4.7	0.73	2.8	0.92	4–5
Full strength	20 S + 10 M	SC	6.8	0.63	2.6	0.97	6–7
Full strength	15 S + 15 M	CP	6.3	0.60	2.7	0.95	4–5
Full strength	15 S + 15 M	SC	6.6	0.41	3	0.82	7–8
Full strength	10 S + 10 M	CP	3.7	0.60	1.5	0.71	5–6
Full strength	10 S + 10 M	SC	4.8	0.72	2	0.82	7–8
Half strength	30 S + 0 M	CP	6.4	0.81	3.4	0.97	3–4
Half strength	30 S + 0 M	SC	6.9	0.57	4	0.82	5–6
Half strength	20 S + 0 M	CP	5.4	0.40	1.9	0.88	4–5
Half strength	20 S + 0 M	SC	6.9	0.37	1.7	0.82	5–6
Half strength	20 S + 10 M	CP	5.1	0.42	1.7	0.67	4–5
Half strength	20 S + 10 M	SC	6.3	0.37	1.9	0.74	6–7
Half strength	15 S + 15 M	CP	4.8	0.49	2.3	0.82	5–6
Half strength	15 S + 15 M	SC	5.7	0.54	3.4	0.84	10–12
Half strength	10 S + 10 M	CP	5.4	0.77	2.2	0.63	5–6
Half strength	10 S + 10 M	SC	5.9	0.32	2.8	0.79	10–12

MS, Murashige and Skoog medium; S, sucrose; M, mannitol; SC, screw cap; CP, cotton plug.
[a]Mean of 10 replications.

Cell Suspension Culture

Plant cells accumulate secondary metabolites at specific cell sites at specific developmental stages under specific conditions of cell culture; hence, plant tissue culture can be used as an alternative to whole plants as a biological source of potentially useful metabolites and biologically active compounds (Yeoman, 1987). The genetic makeup, the regulation of gene expression, the cellular physiology, and the regulation of metabolism must be considered in order to develop highly productive and practical plant cell lines.

Production of volatile constituents in ginger cell cultures were reported earlier by Sakamura and Suga (1989). Ilahi and Jabeen (1992) also reported preliminary studies on alkaloid biosynthesis in callus cultures of ginger, and Charlwood et al. (1988) have reported the accumulation of flavor compounds by cultures of ginger. Nirmal Babu (1997) reported successful establishment of cell suspension cultures in ginger. These cultures are maintained by weekly transfers to the fresh nutrient medium containing MS basal salts and 1 mgl^{-1} of 2,4-D for over 2 years and are in continuous growth and multiplication. The cells were heterogeneous, and during the process of subcultures,

some of the cells differentiated into oil-producing cells (see Figure 4.3f), although the number of oil-producing cells were less for commercial exploitation. These reports are very preliminary and much more work needs to be done before ginger cell cultures can be used for commercial production of flavor components in vitro.

Protoplast Isolation and Culture

Successful isolation, culture, and fusion of protoplasts are important because of their role in studies of plant improvement by cell modification and somatic hybridization. Another aspect of considerable interest is the storage of protoplast through immobilization and cryopreservation, which are of great importance, especially in the pharmaceutical industry (Bajaj, 1989a, 1989b).

Protoplasts were successfully isolated from young in vitro–derived leaves (see Figure 4.3f) using an enzyme mixture containing 0.5 percent macerozyme R10, 3 percent hemicellulase, and 5 percent cellulase Onozuka R10 and mechanically macerating the plamolysed leaf tissue after incubating at 15°C for 10 hours and at 30°C for 6 hours. The protoplast yield was 2.5 × 10^5 per gram of leaf tissue with 55 percent viability. The isolated protoplasts were round and were filled with chloroplasts. Cell suspension cultures required a slightly different concentration of enzyme mixture with 1 percent macerozyme R10, 3 percent hemicellulase and 6 percent Onozuka cellulase R10. The incubation conditions were 15°C for 10 hours and 30°C for 8 hours. The yield of protoplasts was lesser from a callus/cell suspension with 1 × 10^5 protoplasts per gram weight of callus in an isolation solution containing cell protoplast washing (CPW) salts, 7 percent mannitol, 1 percent macerozyme, 3 percent hemicellulase, and 6 percent cellulase Onozuka R10. The protoplasts isolated from cell suspension cultures were round, with little or no chloroplasts inside, with 72 percent viability (see Table 4.10). The protoplasts derived from leaf tissue were heterogeneous and comprised of protoplasts of different sizes (0.15 to 0.21 mm). The protoplasts derived from cell suspension cultures were mostly of uniform size (0.39 mm). They were cultured up to 20 days as droplet cultures in MS liquid medium with 0.5 mgl^{-1} BAP, 0.5 mgl^{-1} NAA, and 0.5 mgl^{-1} 2,4-D supplemented with 3 percent sucrose and 7 percent mannitol. The cell contents became dense by 1 week and fresh medium needed to be added at 7-day intervals. The protoplasts started regenerating the cell wall within 2 to 3 days. Within 3 to 4 weeks of culture, cells started dividing. Protoplasts plated on liquid as well as solidified medium

Table 4.10 Effect of source tissue, enzyme concentration, and incubation conditions on yield of protoplasts

Source	Enzyme solution	Incubation conditions		Protoplast yield	Viability (%)	Size (mm)
Leaf	0.5% Macerozyme R 10 +3% Hemicellulase +5% Onozuka cellulase R10	16 h	15°C for 10 h, 30°C for 6 h, 53 rpm in dark	2.5 × 10^5/g of leaf	55 with chloroplasts	0.21
Callus	1% Macerozyme R10 + 3% Hemicellulase + 6% Onozuka cellulase R10	18 h	15°C for 10 h, 30°C for 8 h, 53 rpm in dark	1 × 10^5/g of callus	72 no chloroplasts	0.39

after 20 days gave similar results. The dividing cells developed into microcalli in 50 to 70 days in MS medium with 1 mgl^{-1} NAA and 1 mgl^{-1} BAP. The protoplasts started cell division within 2 to 3 days and developed into microcalli in 50 to 70 days. So far, no reports are available on plant regeneration from protoplast-derived microcalli (Geetha et al., 2000).

Genetic Transformation

The tremendous progress made during the last decade has demonstrated that refinement of routine in vitro techniques coupled with recombinant DNA technology and genetic engineering have opened up new vistas for plant improvement (Potrykus et al., 1985; Potrykus and Spangenberg, 1995). The *Agrobacterium*-mediated gene transfer is most successful in plants, but has limited applications in monocots due to host range limitations. Bombardment of intact plant cells with high-velocity, DNA-coated microprojectiles is another very effective method for production of transgenic plants (Sanford et al., 1987; Franks and Birch, 1991).

Nirmal Babu (1997) reported transient expression of GUS in ginger embryogenic calli (see Figure 4.3g) when it was bombarded with microprojectiles (1.6 μm gold particles) using a BioRad PDS-1000/He gene gun at 900 and 1,100 psi helium pressure with the target distance of either 6 or 9 cm. The vector used was pAHC 25 containing GUS (β-glucuronidase) and BAR (phosphinothricin–acetyl transferase) as reporter and selectable marker genes respectively and carrying Ubi-1 (ubiquitin) promoter (Christensen and Quail, 1996). The best GUS score was obtained when the target distance was 9 cm with 900 psi helium pressure. The GUS score of 133 blue spots per square centimeter indicates not only the optimization and efficiency of the biolistic process, but also the ability of the ubiquitin promoter to drive the expression of the reporter gene (see Figure 4.3g).

Molecular Characterization

With the advent of molecular biology techniques, DNA-based markers very efficiently augment morphological, cytological, and biochemical characters in germplasm characterization, varietal identification, clonal fidelity testing, assessment of genetic diversity, validation of genetic relationship, phylogenetic and evolutionary studies, marker-assisted selection, and gene tagging. Owing to plasticity, ubiquity, and stability, DNA markers are easier, efficient, and less time consuming, especially in perennials where morphological markers are few. The relatively easy to use, low-cost, and highly accurate nature of the polymerase chain reaction (PCR)–based technologies such as RAPD, AFLP, and microsatellites are widely appreciated.

RAPD Profiling of Ginger Cultivars

Genomic DNA was successfully isolated in the authors' laboratory from young and fresh leaves of ginger plants using the modified cetyl amino butane (CTAB) method of Ausubel et al. (1995). Development of RAPD profiles for various ginger cultivars (see Figure 4.4a) and related species is in progress at the Indian Institute of Spices Research to study the interrelationships and to identify the core collections in the germplasm.

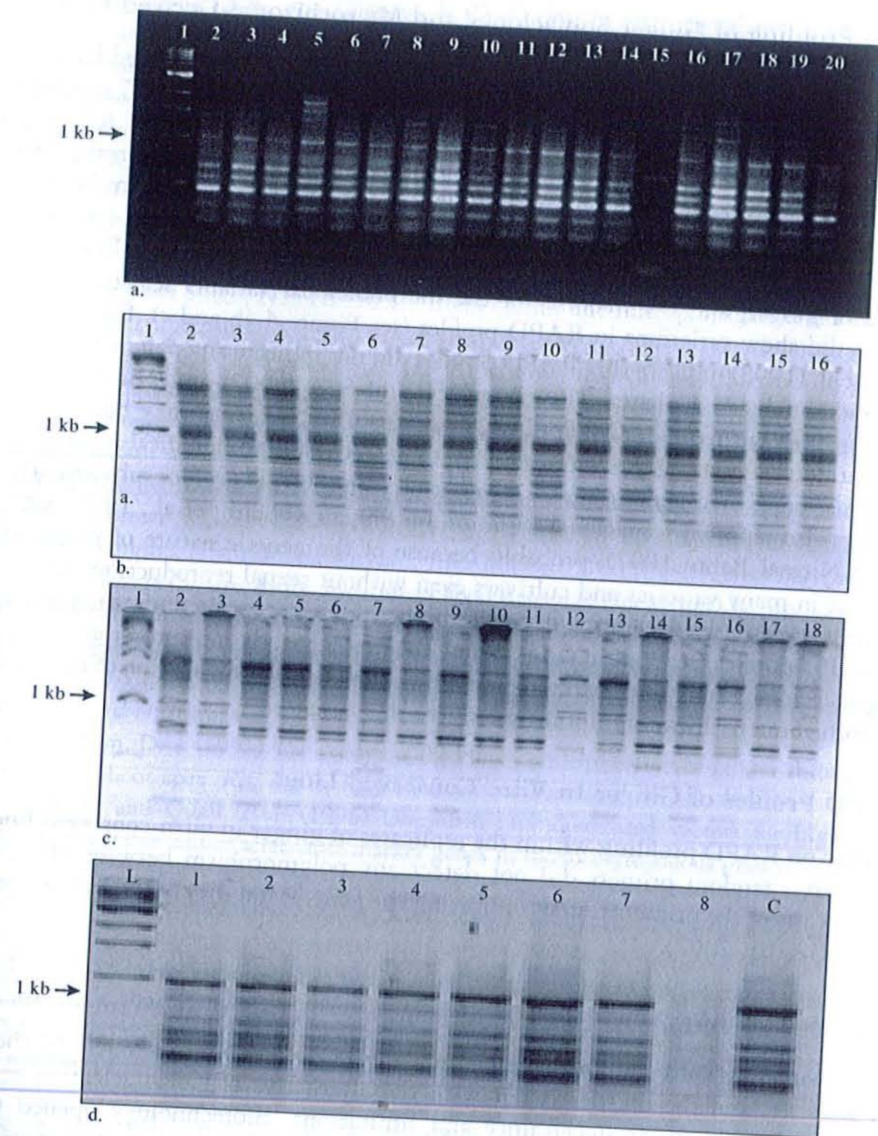

Figure 4.4 Molecular characterization of ginger: (a) RAPD profiles as expressed by OPERON primer OPD 11 among different cultivars of ginger collected from different geographical regions. lanes: 1 1 kb ladder, 2 Maran, 3 Australia, 4 Jamaica, 5 Gurubathani, 6 Bhainse, 7 RRE, 8 Hawaiian, 9 Sabarimala, 10 Mananthody, 11 Anamica, 12 Bisonvally, 13 China, 14 Jugijan, 15 IISR Rajitha, 16 IISR Varada, 17 Wyanad local, 18 Nadan, 19 PGS 43, 20 V2-E5-3. (b) RAPD profiles as expressed by OPERON primer OPE 01 in micropropagated plants of ginger: lanes: 1. 1 Kb Ladder, 2 RRE 1, 3 M Control, 4 MP 49-7, 5 MP 50-3, 6 MP 54-2, 7 MP 54-4, 8 MP 61-4, 9 MP 61-9, 10 MP 61-10, 11 MP 64-3, 12 MP 68-4, 13 MP 70-3, 14 MP 70-5, 15 MP 74-4, 16 MP 76-3. (c) RAPD profiles as expressed by OPERON primer OPD 10 in callus regenerated plants of ginger: lanes: 1 1 Kb Ladder, 2 RRE 1, 3 M Control, 4 CR 3, 5 CR 4, 6 CR 7, 7 CR 9, 8 CR 10-1, 9 CR 13, 10 CR 20, 11 CR 42, 12 CR 64, 13 CR 67, 14 CR 816, 15 CR 817, 16 CR 1222, 17 CR 1441, 18 AC-1. (d) RAPD profiles of microrhizome derived plants of ginger, as expressed by OPERON primer OPB 10: L—1kb ladder, lanes 1, 2, 3, 4, 5, 6, 7, 8: microrhizome derived plants, C—control.

RAPD Profiling of Ginger Somaclones and Microrhizome-Derived Plants

Suja (2002) and Nirmal Babu et al. (2003) used RAPD profiles amplified by 11 operon primers as an index for estimating genetic fidelity of selected "variants" among micropropagated and callus-regenerated plants. They observed differences in RAPD profiles observed in some of the micropropagated plants that indicated micropropagation even without the callus phase induced variations in 9 of the 13 plants tested. Similar differences were noticed among 12 out of 15 callus-regenerated plants. In general, this indicates a high amount of variability among the selected micropropagated and callus-regenerated plants of ginger, and the majority of the morphological variants selected from earlier studies did show variations in RAPD profiles (see Figure 4.4b and c). Earlier studies by Rout et al. (1998) indicate that RAPD profiles did not indicate any polymorphism among the micropropagated plants. The observations of Suja (2002) and Nirmal Babu et al. (2003) differ with the earlier finding. The variability observed may be due to bigger population size used by the latter workers to detect the morphological variants first and confirmation of their genetic nature of variation using RAPD profiles subsequently. Other workers also reported somaclonal variation in ginger (Kulkarni et al., 1987; Samsudeen, 1996; Nirmal Babu, 1997), probably because of the genetic nature of ginger that has resulted in many varieties and cultivars even without sexual reproduction. However, the microrhizome-derived plants did show a high degree of genetic uniformity as expressed by RAPD profiles (see Figure 4.4d). Thus, the present study indicated that direct micropropagation of ginger results in somclonal variation; hence, propagation of ginger through microrhizome pathway significantly reduces this variation.

RAPD Profiles of Ginger In Vitro Conserved Lines

Studies on RAPD profiling within the replicates of ginger in vitro-conserved lines using 10 operon random primers did not detect any polymorphism between the conserved lines in any of the primers tested, indicating the genetic stability of the in vitro-conserved lines (Geetha, 2002).

Conclusion

This marvelous spice and medicinal plant, ginger, is constrained severely by the absence of seed set, and the breeder is left with the alternative of clonal selection or induced mutations with all its uncertainty and limitations. Biotechnology opened up many potential avenues such as tissue culture, somaclonal variation, in vitro mutagenesis and selection, molecular fingerprinting, recombinant DNA technology, and genetic modification through transgenics for creating disease-resistant lines. Concerted efforts are needed to solve the serious problems besetting this "great medicine" and "universal cure" as described in the Indian systems of medicine, which is a great spice unparalleled in the range of applications and uses.

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