INDUCTION OF GENETIC VARIABILITY IN GINGER (*Zingiber officinale* Rosc.) THROUGH *IN VITRO* FERTILIZATION

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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(Plant Biotechnology)

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KERALA, INDIA

2005

DECLARATION

I hereby declare that this thesis entitled **"Induction of genetic variability in ginger (*Zingiber officinale* Rosc.) through *in vitro* fertilization"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara,
19-12-05

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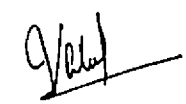
Certified that this thesis, entitled **"Induction of genetic variability in ginger (*Zingiber officinale* Rosc.) through *in vitro* fertilization"**is a record of research work done independently by **Mrs. Rethidevi A.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.



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Rethidevi A.

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ABBREVATIONS

2,4 – D	:	2,4 dichloro phenoxy acetic acid
BA	:	Benzyle Adenine
BAP	:	Benzyl Amino Purine
cm	:	Centimeter
CTAB	:	Cetyl trimethyl ammonium bromide
d NTP	:	deoxy Nucleotide Tri Phosphate
DAP	:	Days after pollination
DNA	:	Deoxy ribonucleic acid
EDTA	:	Ethylene Diamine Tetracetic acid
gl^{-1}	:	gram per litre
GA_3	:	Gibberellic Acid
h	:	hour
IAA	:	Indole Acetic Acid
IBA	:	Indole Butyric Acid
Kin	:	Kinetin
mA	:	Milli Ampere
mgl^{-1}	:	Milligram per litre
min	:	Minute
mm	:	Millimeter
µM	:	Micro Molar
M	:	Molar
MS	:	Murashige and Skoog
NAA	:	Naphthalene Acetic Acid
NOA	:	Naphthoxy Acetic Acid
PCR	:	Polymerase Chain Reaction
ppm	:	Parts per million
psi	:	Pounds per square inch
RAPD	:	Random amplified Polymorphic DNA


Dedicated to My
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ABBREVATIONS

2,4 – D	:	2,4 dichloro phenoxy acetic acid
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DAP	:	Days after pollination
DNA	:	Deoxy ribonucleic acid
EDTA	:	Ethylene Diamine Tetracetic acid
gl^{-1}	:	gram per litre
GA_3	:	Gibberellic Acid
h	:	hour
IAA	:	Indole Acetic Acid
IBA	:	Indole Butyric Acid
Kin	:	Kinetin
mA	:	Milli Ampere
mgl^{-1}	:	Milligram per litre
min	:	Minute
mm	:	Millimeter
µM	:	Micro Molar
M	:	Molar
MS	:	Murashige and Skoog
NAA	:	Naphthalene Acetic Acid
NOA	:	Naphthoxy Acetic Acid
PCR	:	Polymerase Chain Reaction
ppm	:	Parts per million
psi	:	Pounds per square inch
RAPD	:	Random amplified Polymorphic DNA

rpm	:	revolutions per minute
TAE	:	Tris Acetic acid EDTA
UPGMA	:	Unweighted Pair Group Method of Arithmetic Averages
UV	:	Ultraviolet
µl	:	Micro litre
µM	:	Micro Molar


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Introduction

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) is an important commercial spice, valued throughout the world for its aroma, flavour and pungency. The aromatic rhizomes of ginger find application both as a spice and in medicine.

At present ginger is ranked as fourth important spice crop of India standing next to chillies, black pepper and turmeric. India is the largest producer, consumer and exporter of the same, producing about 2,30,000 million tonnes from an area of 70,000 ha and has earned foreign exchange to the tune of Rs. 5950 lakhs through the export of 13,000 million tonnes in the year 2004-2005 (FAO, 2005). Although ginger is grown in almost all parts of India, Kerala makes significant contribution through production of world-renowned Cochin and Calicut ginger and contributes a major share to Indian production. In Kerala, ginger is cultivated in an area of 8418 ha with a production of 2086 tonnes in the year 2002-2003 (Spices board, 2005).

The increasing demand of ginger and its suitability for varied preparations have put forth the need for promising ginger types having special quality attributes. But at present, ginger crop in India is facing severe threat due to the incidence of soft rot and bacterial wilt diseases. Low quality of Indian ginger due to high fibre content of our popular cultivars coupled with high cost of production are other bottlenecks of ginger cultivation in the country. Hence there is an imperative need for developing high yielding cultivars with low fibre content and tolerance or resistance to devastating diseases.

The genetic variability in ginger is locked up due to exclusive vegetative propagation. Conventional generative breeding methods to develop resistant and high yielding genotypes cannot be employed in ginger mainly due to lack of natural seed set. Investigations carried out by Sathiabhama (1988) revealed that pollen tube growth in ginger is not sufficient to fertilize the deeply seated ovules. Zenkteler

(1969) and Zenkteler and Melchers (1978) have shown that germinable seeds could be produced in extremely wide crosses through placental pollination.

Valsala (1994) proved that germinable seeds could be produced in ginger through *in vitro* pollination and fertilization techniques. But the germination of the *in vitro* produced seeds was found to be highly erratic. Attempts were done to standardize the conditions for successful regeneration from the *in vitro* produced ginger seeds in subsequent years by Bindu (1997) and Shankar (2003). But they also came up with the same result of erratic germination. So the conditions for getting regeneration of plantlets from the *in vitro* produced seeds of ginger are yet to be standardized.

Sheeba (1996) have generated two autotetraploids through colchiploidy. The high pollen fertility observed in the autotetraploids could be used with advantage, in heterosis breeding. Generation of progenies with altered ploidy levels is possible by crossing autotetraploids and diploids, which can be exploited for targeted characters.

Since it is difficult to get successful germination of *in vitro* produced seeds of ginger, one better option is to go for embryo or endosperm culture to get direct or indirect organogenesis or somatic embryogenesis from the hybrid tissues. The success of the present study will open up new vistas in crop improvement of ginger.

Therefore, the present investigation was undertaken with the following objectives:

- To assess genetic variability among selected ginger cultivars including induced autotetraploids through molecular characterization.
- To refine *in vitro* pollination technique in ginger with respect to seed germination or embryo regeneration.
- To create genetic variability in ginger by crossing diploids with autotetraploids and diploids through *in vitro* fertilization.

Review of literature

2. REVIEW OF LITERATURE

The available literature on various aspects relevant to the present investigation "Induction of genetic variability in ginger (*Zingiber officinale* Rosc.) through *in vitro* fertilization" is reviewed here under.

2.1 ORIGIN AND DISTRIBUTION

Zingiber officinale Rosc. is not seen in wild state, hence the country of origin is not known with certainty. The Sanskrit name, 'Singabera' gave rise to the Greek name 'Zingiberi' from which the Latin name 'Zingiber' was derived. It might have originated in south East Asia, especially in India and China and later introduced to countries like Japan, Sierra Leone, Nigeria, Australia and West Indies. Spaniards introduced ginger to Jamaica which is now world famous for the quality (Purseglove, 1978).

2.2 SPECIES AND VARIETIES

Zingiber officinale Rosc. is a monocot belonging to the family zingiberaceae of the order zingiberales. The family zingiberaceae consists of two subfamilies; aromatic zingiberoideae and non-aromatic costoideae. The sub family zingiberoideae includes important genera like *Aframomum, Amomum, Curcuma, Eletteria, Hedychium, Kaempferia* and *Zingiber*. The principal genus of costoideae is *Costus* (Purseglove, 1978).

The genus *Zingiber* consists of about 80 to 90 species of perennial rhizomatous herbs distributed throughout South East Asia and extending to Queensland and Japan (Purseglove *et al.*, 1981). *Z. mioga,* a condimental vegetable rarely set seeds as reported by Adaniya (1991). Sirat (1994) used *Z. ottensii* rhizome as a poultice in postnatal treatments and as an appetizer.

2.3 VARIABILITY STUDIES

In ginger there is no natural seed set and it is always propagated vegetatively and the number of clones available is limited. Each center of production produces a distinctive type and this may be due to soil, climatic, and cultural differences (Purseglove, 1978).

Very little variability has been reported among the genotypes grown in the same area, but with a good amount of variability among the varieties grown in different states. Improved ginger cultivars such as Suprabha, Suruchi, and Surabi perform well for a number of yield and quality related traits as reported by Sreekumar *et al* (1980). Varada, Mahima, and Rejatha are released varieties from Kerala. Varada exhibits wide adaptability while Mahima is notable for the high rhizome yield and Rejatha for the volatile oil content (Naidu *et al.*, 2000 and Sarma and Sasikumar, 2002).

2.3.1 Vegetative and economic attributes

Nybe (1978) investigated morphological variations in twenty five types of ginger and found that height of plant, number of leaves per tiller and number of roots per plant were maximum in Valluvanad and the number of primary fingers per plant and the secondary fingers per plant was more in Wynad local and Bajpai respectively.

Mohanty etal. (1981) studied twenty eight cultivars of ginger for variations in number of tillers, number of leaves, plant height, leaf width, weight of straw, number of adventitious roots, number of root tubers, total number of rhizome fingers, girth of secondary fingers and rhizome yield. The varietal differences for all the characters studied were significant.

Ravindran *et al.* (1994) evaluated accessions for various morphological, yield and quality characters and revealed considerable variability for most of these traits.

Sujatha *et al.* (1994) reported plant height to be highest in cultivar Calcutta, more leaves and tillers in PGS –10 and maximum fresh yield in Kuruppampady and dry yield in PGS-667.

Himagiri was found to perform well for tillers per plant, leaves per plant, leaf length and breadth, rhizome length and breadth and yield and the performance was consistent under both rain fed and irrigated conditions (Korla, 1999).

The yield was positively and significantly correlated with tillers per clump, internodal distance of rhizome and plant height and was negatively correlated with fibre content as reported by Chandra and Govind (1999).

Nybe *et al.* (1980) revealed that morphological characters and economic characters such as length and girth of primary and secondary fingers were found positively correlated with yield. Weight of mother rhizome and internodal distance of rhizome were the most variable characters.

2.3.2 Quality attributes

Nybe *et al.* (1980) reported that Rio-de-Janeiro was the best in oleoresin recovery where as Karakal recorded maximum oil content. However, the yield of aromatic oil and oleoresin when worked out on per hectare basis were found maximum in Maran. They also observed minimum fibre content in China and maximum in Kuruppampady closely followed by Maran.

Sreekumar *et al.* (1980) evaluated 30 ginger cultivars and the results indicated that cultivars Thingpuri, Rio-de-Janeiro and China were suitable for fresh ginger production and Tura, Nadia and Maran suitable for dry ginger. Maran was found suitable for extraction of oleoresin and volatile oil content, but it had high fibre content (Saikia and Shadeque, 1992).

Considerable variations were observed by Shamina *et al.* (1997) for total free amino acids, protein and total phenols. Nizam and Jayachandran (1997) observed maximum non-volatile ether extract in Kuruppampady. Korla (1999) observed that though SG 61 had high volatile oil and oleoresin content, it had high crude fibre content and poor yield. More fibre content in Khasi local and less in Nadia was reported by Chandra and Govind (1999).

Gingerol content, antioxidant activity and pungency intensity were higher in tetraploid types than in diploid types as investigated by Nakasone *et al.* (1999). Singh *et al.* (2000) observed highest rhizome yield and oil content in Thinladium and Nadia respectively.

2.3.3 Incidence of pests and diseases

Pests and diseases are important production constraints. The common pathogens associated were *Ralstonia* sp, *Pythium* spp and *Fusarium oxysporum*. *Pratylenchus coffeae* increased the severity of infection by these pathogens along with *Fusarium* as reported by Rajan *et al.* (2002).

None of the accessions screened by Raveendran *et al.* (1994) showed tolerance or resistance to leaf spot disease, rhizome rot and bacterial wilt. Maximum variation within cultivated ginger occurs in northern India. This is suggested to be due to geographical spread from its centre of origin in South East Asia accompanied by genetic differentiation into locally adapted populations caused by mutations.

All the twenty five types in ginger studied were susceptible to soft rot, leaf spot and shoot borer incidence and among them, Maran was relatively tolerant to soft rot whereas Rio-de-Janeiro was the most susceptible type as reported by Nybe (1978). Ali *et al.* (1994) observed SG-600 to be the most resistant cultivar against *Pythium* spp and heavy losses occurred in local cultivars.

Setty *et al.* (1995) observed that though none of the cultivars were resistant against leaf spot Narasapatom and Tura were moderately resistant with a disease index of less than 5 per cent. Singh and Edison (2000) reported that accessions SG-554 and RGS-5 were resistant to *Phyllosticta* leaf spot disease of under Chattisgarh conditions.

Pruthi (1998) reported *Conogethes punctiferalis*, as a serious pest that damages by boring central shoots of the plants, which turn yellow and finally dry the crop. Dohroo (2001) found *Pythium*, *Fusarium* and *Verticillium* to be associated with storage rot of ginger and these reached its maximum intensity in April at 18.5^{0}C and 67.5 per cent relative humidity.

2.3.4 Flowering behaviour

Ginger is a shy flowering crop, when raised from seed bits of around 15g and maintained as an annual crop (Hooker, 1894 and Holttum, 1950). Pillai *et al.* (1978) reported that, of the 35 germplasm collections maintained AT CPCRI, Kasaragod all but six flowered and the flowering started in the last week of October and lasted till early December, The peak being in November. Nybe (1978) reported 0.5 to 12 per cent flowering in various ginger types under Vellanikkara conditions.

Valsala (1994) reported that biennial plants flowered as early as first July and it extended to last week of October whereas plants maintained as annuals flowered only by middle of September and the season extended to third week of November. The seven cultivars studied registered cent percent flowering when they were maintained as biennials and in annuals, percentage of flowering was only 25 per cent. Soo *et al.* (2000) observed that flowering occurred between 18 and 25 August irregularly, at 16.00-17.00 h.

According to Shankar (2003), flowering season in ginger as annuals at central Kerala extended from September to November. The cultivars flowered within a

period of 133.6 to 164.8 days after planting. The duration of flowering was maximum of 54 days (Rio-de-Janeiro) and minimum of 25 days (autotetraploid Z-0-86).

Flowering was not observed in any of the tetraploid ginger plant by Ratnambal and Nair (1982). But Ramachandran (1982) reported that induced tetraploids of ginger flowered during the second year of planting. Sheeba (1996) observed flowering in autotetraploid ginger derived from the variety Rio-de-Janeiro during August in second year of planting. The autotetraploids derived from Himachal Pradesh flowered in subsequent years.

2.3.5 Molecular markers to assess the variability

In ginger, twenty-eight cultivars were compared for peroxidase isozyme patterns by fuzzy cluster analysis in Fujan. The cultivars differed in isozyme pattern activity and intensity. They were divided into three types viz., da-fei-jiang,huang and zhu-zi-zang (He *et al.*, 1995).

Seedling progenies of turmeric (*Curcuma longa*) showed maximum similarity and differed distinctly from the clonally propagated accessions through isozyme analysis. Shamina *et al.* (1998) collected fifteen accessions of *Curcuma longa* from different geographical areas in India along with a few seedling progenies and studied them for variation based on polymorphism of isozyme.

RAPD markers were used to evaluate the genetic stability of micropropagated plants of *Zingiber officinale* cv. V3S18. Fifteen arbitrary decamers were used to amplify DNA from *in vivo* and *in vitro* plant material to assess the genetic fidelity (Rout et al., 1998). All RAPD profiles from micropropagated plants were monomorphic and similar to those of field grown control plants.

RAPD analysis of 10 turmeric plants each propagated conventionally, regenerated from shoot tips, leaf base callus and immature inflorescence was carried out using 15 primers to detect the polymorphism if any and was found that all the plants were alike (Neeta et al., 2003).

Twelve cultivars of *Kaempferia parviflora* were studied using random amplified polymorphic DNA (RAPD) markers and the results were analysed for a similarity among the cultivars, and an unweighted pair group method cluster analysis was performed. The dendrogram showed good relationship between the banding patterns of cultivars and the morphological traits of leaves, petioles and rhizomes; especially the internal skin colour of rhizomes (Pojanagaroon et al., 2004). The dendrogram showed good relationship between the banding patterns of the cultivars and the morphological traits of leaves, petioles and rhizomes; especially the internal skin colour of rhizomes. In addition, the relationship between the banding patterns and their quantities of chemical components in essential oil of Krachai-Dam rhizomes was obvious.

According to the AFLP plymorphism study conducted among various species of *Zingiber* by Kavitha *et al.* (2005), no polymorphism was detected at more than 500 AFLP loci screened in ginger cultivars and it is the most extreme case of genetic narrowness in a species. Analysis revealed high variability in *Zingiber cernuum* and relatively low variation in other species.

The relationships among 19 accessions of Zingiberaceae belonging to 11 species of *Boesenbergia,* six species of *Kaempferia,* and two species of *Scaphochlamys* from Southern Thailand were studied using random amplified polymorphic DNA (RAPD) profiles from leaf tissue samples. Data obtained from the RAPD fingerprints from the samples clarified some doubts in morphological classification. The dendrogram resulting from cluster analysis, UPGMA and a principal component analysis of the RAPD result confirms a higher degree of

relationship between *Boesenbergia* and *Scaphochlamys* than between *Boesenbergia* and *Kaempferia* (Vanijajiva *et al.*, 2005)

2.4 INFLORESCENCE

Inflorescence in ginger is a bracteate spike or raceme directly springing from the rhizome, rarely emerging from the aerial shoot. The inflorescence has a slender scape of 10 to 20 cm and a cylindrical cone like spike 4 to 7 cm in length and 1.50 to 2.50 cm in diameter with appressed ovate or elliptic green bracts. Each bract subtends a single flower with a lateral or obliquely posterior bracteole. Occasionally in a bract more than one flower is seen. The bracts are spirally arranged (Nybe, 1978, Pillai *et al.*, 1978 and Jayachandran *et al.*, 1979).

2.5 FLORAL MORPHOLOGY

The flowers are small as compared to those of other related genera and are born in the axil of a bract. They are trimerous, zygomorphic, bisexual and epigynous. The calyx is tubular or bell shaped dividing above into three short teeth and split on one side. The corolla is tubular below with three yellowish lobes of which the dorsal lobe is curved over the anther. Androecium consists of six stamens of which the outer three are reduced to staminodes and are seen at the base of the corolla tube. The inner lateral stamens are united to form a deep purple coloured labellum. The posterior stamen of the inner whorl is the only fertile stamen and is enclosed by the labellum. The stamen has short, broad filament and two prominent anther lobes. The style is long and slender and passes through the groove formed by the anther lobes and ends in a capitate stigma. Stigma has a circular aperture surrounded by stiff hairs and it protrudes above the anthers (Nybe, 1978, Jayachandran *et al.*, 1979 and Soo *et al.*, 2000). The ovary is inferior, trilocular with several ovules per ovary (Pillai *et al.*, 1978). The ovary measured a mean length of 2.71 mm and diameter of 2.59 mm and with a mean ovule number of 24.43. The ovules measured a mean length of 539.13 μm and breadth of 324.41 μm at the middle (Valsala, 1994).

2.6 FLORAL BIOLOGY

Ginger inflorescence takes approximately 29 to 32 days from the spike initiation to first blooming. In a spike, one or two flowers opened daily and blooming of flowers in a spike was completed with in a period of 9 to 22 days. The blooming of individual flowers takes place in an acropetal succession and the flower falls on the next day of blooming (Usha, 1984 and Valsala, 1994).

Valsala (1994) and Shankar (2003) reported that anthesis started by 15 h and continued up to 16 h. In contrast Das (1999) found that anthesis under green house occurred around 13.00-14.00 h and under field conditions around 9.00 – 10.00 h.

According to Pillai *et al.* (1978) and Jayachandran *et al.* (1979), anther dehiscence almost coincided with the flower opening. In contrast, Usha (1984) and Valsala (1994) observed anther dehiscence 10 min to 2 h after opening. Time of stigma receptivity remains still unravelled owing to the failure of hand pollination to set seeds.

2.7 POLLEN STUDIES

2.7.1 Pollen morphology

The pollen grains of ginger flowers are round, ovoid to ellipsoidal, highly heterogenous in size and filling and are limited by a very thick exine. Exine is striate or tuberculate to areolate in *Zingiber officinale* (Mangaly and Nayar, 1990). A well-defined pore is absent in pollen grains. The anther lobes are filled with plenty of pollen grains (Pillai *et al.*, 1978; Jayachandran *et al.*, 1979 and Usha, 1984).

Seasonal influence was noticed on diameter of pollen grains, which ranged from 43.08 µm to 95.43 µm in different ginger cultivars (Shankar, 2003).

2.7.2 Pollen fertility and germination

Pollen fertility in ginger reported by various workers ranged from 0.9 to 40 per cent (Pillai *et al.*, 1978; Usha, 1984; Sathiabhama, 1988; Bindu, 1997 and Adaniya, 1998). Pollen fertility in the ginger cultivar Nadia was 14.15 per cent and 35.28 per cent in SG-66.The larger pollen grains of diameter 68.78 to 94.64 µm were well stained with acetocarmine while smaller grains of diameter 53.32 to 65.58 µm did not get stained (Valsala, 1994).

Pillai *et al.* (1978) obtained 14.50 percent pollen germination in a medium with 8 per cent sucrose, 3 percent gelatin and 60 ppm boric acid in moist chamber operated at 26.5^{0}C. With the same medium, Usha (1984) got only 6 per cent pollen germination. Sathiabhama (1988) reported 17.81 per cent pollen germination in the same medium except for the reduction of gelatin to one per cent. Maximum pollen tube length observed was 108 µm and coiling of pollen tube was also noticed. Valsala (1994) got only 7.51 per cent pollen germination in ME_3 medium. The pollen tube growth was significantly higher in the ME_3 medium and maximum tube length obtained was 1042.17 µm. Coiling was absent when ME_3 medium was used for germination. Bindu (1997) got a pollen germination ranging from 2.80 to 6.13 per cent and mean pollen tube length ranging from 51.9 to 72.16 µm in ME_3 medium.

Sheeba (1996) reported that high pollen fertility (64%) was recorded for the autotetraploid derived from Rio-de-Janeiro. Shirai (2001) reported that induced tetraploid strains had a higher pollen fertility and germination (27.4 to 74.2% and 4.8 to 12.9% respectively) than the diploid counterparts (0.3 to 6.2 % and 0.0 to 0.1%) respectively.

Shankar (2003) reported that the fertility, viability and tube length of the pollen grains was found to be influenced by the season and position of flowers in the inflorescence. The mean pollen fertility was highest (46.01 %) during the mid period

of the flowering season and in flowers emerging from the upper portions of the inflorescence (36.74 %). Pollen viability using ME_3 medium was highest in early season (8.36 %) and the flowers from the lower portion of the inflorescence (9.18%). The mean tube length of the germinated pollen grains was maximum in mid season (240.68 µm) and for flowers from middle portion of inflorescence (226.05 µm). He also noticed that the genotype of the cultivar influenced the pollen size, fertility, viability and tube length. The autotetraploids showed more fertility percentage and viability percentage when compared to corresponding diploids.

2.8 STIGMA RECEPTIVITY, STYLE LENGTH, OVULE AND OVARY SIZE

Observations under hand lens by Jayachandran *et al.* (1979) indicated that stigma receptivity coincided with anther dehiscence. Valsala (1994) reported variations among cultivars in style length (3.0 to 4.2 cm), ovary length (2.0 to 3.0 mm) and diameter (2.0 to 3.0 mm), ovule length (469.87 to 617.62 µm) and ovule breadth (303.26 to 373.13 µm) of different ginger cultivars.

According to Shankar (2003) the ovary measured a mean length of 2.25 mm and a diameter of 2.23 mm and recorded a mean ovule number of 21.56 per ovary. The ovary of Maran was the largest followed by that of Z-0-78 (autotetrapolid derived from Himachal Pradesh).

2.9 *IN VITRO* HYBRIDIZATION

In vitro crossing between selected cultivars showed that Rio-de-Janeiro as female parent can be crossed with Kuruppampady, SG-66, Nadia and as male parent with Kuruppampady, Nadia, SG-66 and Bajpai. Selfing studies showed that the cultivars Rio-de-Janeiro, Kuruppampady, Bajpai, SG-66 and Nadia could be selfed by *in vitro* pollination and fertilization techniques (Bindu, 1997). According to Shankar (2003) selfing of five ginger cultivars *in vitro* was successful. He got successful development of seeds by crossing autotetraploids with diploids and vice versa, but the seeds were not germinated into plantlets.

2.9.1 Reasons for lack of seed set in ginger

Several reasons were put forward for lack of seed set in ginger. Chromosomal aberrations during meiosis and defects in micro and megasporogenesis, which lead to defective gametes and sterility, are reasons suggested by Pillai *et al.* (1978). Even though high sterility exists in ginger, some amount of pollen germination occurs. Since anther lobes of ginger contain a lot of pollen grains, low pollen fertility may not be the reason for lack of seed set in ginger.

Another school of thought was that failure of seed set is due to self-incompatibility existing in stigma and style (Pillai *et al.*, 1978). Usha (1984) observed that incompatibility may not be the factor for failure of seed set, as it failed to set seed following bud pollination or after removing the stigma and style.

Pollen pistil interaction under *in vivo* conditions using fluorescence microscopy was studied by Sathiabhama (1988). It was observed that pollen tube growth was only 95µm whereas the style length was up to 39000µm and the possibility of pollen tube reaching the ovule at 39000µm is quite remote and suggested that insufficient pollen tube growth is the main reason for lack of seed set. She further opinioned that presence of spiny stigma prevent germination of pollen grains either by injuring the pollen grains or by preventing the adherence of pollen grains to the stigmatic surface. The pollen tube growth was very slow and it advanced, got coiled. Besides, the opened flowers are retained in the plant for less than 12h after anthesis and by the time the pollen germinate and grow, abscission layer is formed in the stylar region and flowers are shed.

Das (1999) reported that lack of seed set despite selfing and cross-pollination might be due to homology of bivalents, with irregular segregation of genomic complements leading to sterile gamete formation.

2.9.2 *In vitro* pollination and fertilization

The technique of *in vitro* pollination and fertilization is an effective tool for getting seed set in species where pre fertilization barriers block seed set. Some of the barriers to fertilization are (a) inability of pollen to germinate on foreign stigma, (b) failure of pollen tube to reach the ovule due to excessive length of the style, or slow growth of the pollen tube, which fails to reach the stigma before the ovary abscises, (c) bursting of the pollen tube in the style. The most important application of the technique is for the production of rare hybrids (Bhojwani and Razdan, 1983).

2.9.3 Technique of *in vitro* pollination and fertilization

The prerequisites and procedures to be adopted for successful *in vitro* pollination has been described by Kanta and Maheswari (1963).

The technique consists of

1. A detailed study of floral biology of the crop
2. Studies on pollen germination and tube growth
3. Standardisation of a surface sterilization technique for flower and flower buds without affecting viability of pollen grains and receptivity of gynoecium.
4. Identification of a suitable medium that support development of ovule or ovary to mature seed or fruit.
5. Standardisation of appropriate pollination technique so that fertilization and growth of ovule takes place.
6. Identification of suitable culture conditions, which promote growth of ovule to viable seeds.
7. Histological examination of ovule or ovary at various stages to know the fertilization and development of embryo and endosperm.
8. Standardisation of culture conditions for germination of seeds.

2.9.4 *In vitro* fertilization studies in zingiberaceous crops

A) Ginger

Valsala (1994) reported seed set in ginger for the first time by *in vitro* pollination. Shankar (2003) also got *in vitro* seed set in ginger later.

The flower buds have to be collected on the day of anthesis or the next day of anthesis as reported by Valsala (1994). She perfected a three-step surface sterilization procedure in ginger. The flower buds on the day of anthesis, prior to flower opening were surface sterilized by dipping in 500 mgl^{-1} streptocycline for 1h followed by wiping with 70 per cent alcohol and rinsing with 0.1 per cent mercuric chloride for three minutes. Shankar (2003) followed the same procedure for his *in vitro* pollination studies in ginger.

Both Valsala (1994) and Shankar (2003) reported successful development of ovule after placental pollination in ginger. In placental pollination, the placenta bearing the ovules was exposed by completely peeling the ovary wall and pollination was done on it with pollen grains suspended in pollen germination medium.

Valsala (1994) found half MS, SH and Nitsch's medium good for ovary development of ginger after *in vitro* pollination. Shankar (2003) found semi solid half strength MS to be superior for the culture establishment of ginger ovary and ovule. Vitamin supplementation by adding the vitamin stock of MS medium in double the normal quantity was beneficial for ovule development.

Valsala (1994) found 6-8 per cent sucrose optimum for the development of ovaries and ovules of ginger while Shankar (2003) obtained maximum percentage of ovule development in 3 per cent sucrose.

Valsala (1994) and Shankar (2003) obtained maximum seed development when pollinated ovaries of ginger were cultured in half MS + BAP 2.5mgl^{-1} +

NAA0.5 mgl^{-1} supplemented with coconut water 15 per cent (v/v). The auxins and cytokinins alone, induced ovule development but combinations proved to be better. Valsala (1994) found that the combinations of NAA 0.5 mgl^{-1} with varying concentrations of BAP from 2 to 10 mgl^{-1} showed positive effect. The effect of BAP could be replaced by kinetin (2 and 5 mgl^{-1}) or 2 iP (2.5 mgl^{-1}). The effect of NAA (0.5 to 2 mgl^{-1}) could be replaced by 2,4-D (0.5 to 1 mgl^{-1}) or IAA (0.05 to 0.2 mgl^{-1}). GA did not favour ovary and ovule development. Supplements like coconut water (10 to 12 per cent v/v) and casein hydrolysate (200 to 500 mgl^{-1}) enhanced ovule development. Similarly, inflorescence extrtact of ginger (0.3 to 3 per cent) promoted ovule development.

The cultures were incubated at 26 + 2^0 C in an air-conditioned culture room under diffused light as reported by Valsala (1994) in ginger.

Valsala (1994) reported ginger seeds incubated in a medium of half MS with 2,4-D 8.0 mg l^{-1} for two months and then in a plant growth regulator combination of BAP 9.0 mg l^{-1} and 2,4-D 0.1 mg l^{-1} in half MS showed germination. Valsala et al. (2001) observed radicle emergence in a media combination of half MS + 6 per cent sucrose + NAA (0.2 mg l^{-1})+ BA (1.0 mg l^{-1}) + GA_3 (2.0 mg l^{-1})+ coconut water 15 % v/v.

Shankar (2003) subjected the seeds of ginger raised through *in vitro* pollination to various *in vitro* and in vivo germination studies. Though the in vivo treatments failed to induce germination, 80 days old mature seed obtained from the cross diploid (Rio-de-Janeiro) X autotetraploid (Z-0-78) germinated under *in vitro* conditions after seed priming treatment (50 ppm KNO_3 for 8 h) and also the cross of autotetraploid (Z-0-86) X diploid (Maran) after seed imbibition in water for one day when cultured in half MS +2,4-D (0.5 mg l^{-1}), BAP (6.0 mg l^{-1}) and GA_3 (5.0 mg l^{-1}) along with 3 per cent sucrose showing radicle emergence. Seed of the cross between diploid (Rio-de-Janeiro) X diploid (Maran) and diploid (Rio-de-Janeiro) X

autotetraploid (Z-0-86) germinated after culturing in solid medium of half MS +2,4 D (0.25 mg l^{-1}) + BAP (9.0 mg l^{-1}) by producing radicle.

B) Turmeric

Renjith (1999) and Vijayasree (2001) obtained seed set in turmeric by *in vitro* placental pollination. They utilized similar procedure for surface sterilization of turmeric flower buds as that reported by Valsala (1994) in ginger but with a lower concentration of streptocycline (350 mgl^{-1})

Renjith (1999) observed that *in vitro* pollinated gynoeciums of turmeric recorded maximum ovule swelling when cultured in half MS with NAA 0.5 mgl^{-1} and BAP and kinetin both at 1 mgl^{-1} and 3 per cent sucrose. Organic supplements like coconut water 15 per cent v/v and casein hydrolysate 200 mgl^{-1} enhanced ovule development.

The cultures were incubated at 26 + 2^0 C in an air-conditioned culture room under diffused light as reported by in turmeric Renjith (1999) and Vijayasree (2001). Both of the workers could not get germination of *in vitro* produced seeds of turmeric even though they got *in vitro* germination of in vivo produced seeds.

C) Kacholam

Bhurke (2002) achieved seed set in kacholam by *in vitro* placental pollination. For surface sterilization he used the same procedure as reported in ginger (Valsala, 1994).

Bhurke (2002) reported the following four combinations favourable for ovule development of kacholam. Maximum ovule swelling was observed in cultures with hormone combinations viz.,

Half MS + 2,4-D 0.2 mgl^{-1}

Half MS + BA 1.0 mgl^{-1} + kinetin 1.0 mgl^{-1}

Half MS + BA 0.5 mgl^{-1}+ NAA 3.0 mgl^{-1}

Half MS + BA 1.0 mgl^{-1}+ kinetin 3.0 mgl^{-1}+ 2,4-D 0.2 mgl^{-1}

Organic supplements did not favour ovule development. The cultures were incubated at 26 + 2^0 C in an air-conditioned culture room under diffused light. The seeds obtained did not germinate.

2.9.5 *In vitro* fertilization studies in other crops

Generally flower buds on the day of anthesis or one or two days of anthesis respond to *in vitro* pollination. The seed set was reported to be higher when the ovules were excised 1-2 days after anthesis than on the day of anthesis (Kanta *et al.*, 1962; Kanta and Maheswari, 1963 and Rangaswamy and Shivanna, 1967). On the contrary, in *Lilium*, while making interspecific crosses, seed set was observed when the flowers were pollinated three days prior to anthesis (Tuyl *et al.*, 1991).

Zenketeler (1984) and Razdan (1993) reported that the ovaries large in size and containing many ovules are the best experimental material for *in vitro* pollination. Most of the initial successful attempts of *in vitro* pollination are reported in members of families Solanaceae (*Nicotiana tabacum, N. alata, N. rustica, Petunia hybrida*), Papaveraceae (*Papaver somniferum, Argemone mexicana, Eschscholtzia californica*) and Caryophyllaceae (*Melandrium album, M. rubrum, Dianthus caryophyllus, Agrostemma githago*) where in all the above cases, the placentae are covered with several hundred ovules. Abundant supply of viable pollen and abundant growth of pollen tubes on ovules and placentae are other important factors for successful *in vitro* pollination.

Castano and Proft (2000) noted that success has been obtained mainly with the species of the same families Brassicaceae, Caryophyllaceae, Lilliaceae, Papaveraceae, Primulaceae and Solanaceae. The direct *in vitro* pollination of ovules allows them to overcome pre and post zygotic barriers of incompatibility resulting in

subsequent development of embryos in 57 species representing 14 families. The best results were obtained in species in which the ovaries are large and contain many ovules.

In the initial years of *in vitro* pollination and fertilization technique, the Nitsch medium identified for ovary development was used (Kanta and Maheswari, 1963 and Usha, 1965). Modified Nitsch's medium in some angiosperms was suggested by Kanta and Maheswari (1963)

Tuyl *et al.* (1991) reported that MS medium was useful for the production of interspecific hybrids of *Gossypium*, *Nicotiana* and *Lilium* following *in vitro* pollination. Castano and Proft (2000) identified both MS and Gamborg's B_5 medium for the culture of isolated ovules of chicory after *in vitro* ovular pollination.

The sucrose concentration influences ovary and ovule development and seed germination after *in vitro* pollination. Generally sucrose has been used at a concentration of 4-5 per cent (Kanta and Maheswari, 1963 and Usha, 1965). For the swelling of capsules of *Lilium* hybrids, MS medium supplemented with 7 per cent sucrose gave best results (Tuyl *et al.*, 1991).

Castano and Proft used MS medium and Gamborg's medium with 4 and 6 percent sucrose for culturing chicory ovules. In *Lilium*, a high concentration of 10 per cent sucrose gave successful results when cultured in Brewbaker and Kwack's agar plate medium (Chi, 2000).

Casein hydrolysate at 500 ppm was found to enhance ovule development in some angiosperms (Kanta and Maheswasri, 19630. Usha (1965) observed seed set in *Antirrhinum majus* through *in vitro* pollination on addition of 25 per cent coconut water to Nitsch's medium. In *Lilium*, auxin is essential at the initial stages of ovule developmwnt (Tuyl *et al.*, 1991).

In *Petunia diffusa*, day light (10-12 ft candle at 25 + 2^{0}C) was found optimum for *in vitro* seed development (Rangaswamy and Shivanna, 1967). Tuyl *et al.* (1991) incubated cultures of *Lilium* in a climate room of light intensity 12 Wm-2, photoperiod 16 h and temperature 24^{0}C for the production of interspecific hybrids.

2.9.6 Embryo and endosperm development following *in vitro* fertilization

In *Papaver rhoeas*, the embryos after intra-ovarian pollination grew faster than in nature. The cotyledons differentiated within 14 days after pollination and by the third week the plumule and the root tip were well-differentiated and one month old seeds had fully developed embryo (Kanta, 1960).

Kanta and Maheswari (1963) reported that in test tube fertilization of *Eschscholzia californica*, the embryo showed normal development during its early growth. However the endosperm was poorly developed and degenerated before becoming cellular. In test tube fertilization of *Nicotiana rustica* and *Nicotiana tabacum* fertilized ovules enlarged considerably but 25 per cent of them aborted showing only an irregular embryonic mass and a degenerating endosperm.

In the test tube fertilization of *Papaver somniferum*, the embryo developed concomitantly with the endosperm. Normally developing seeds from three days old culture showed a two-celled embryo. Dissections from nine days old seed showed a globular pro embryo. In fifteen days old seed, a differentiated embryo and twenty-two days old seed a fully formed normal dicotyledonous embryo and cellular endosperm (Kanta and Maheswari, 1963).

2.10 SEED GERMINATION STUDIES

2.10.1 Germination of seeds produced *in vivo*

Cardamom seeds have highest germination (39.33%) after acid scarification for 10 minutes and soaking in GA_3 for 24 h combined with sowing in the open site (Raja, 1993). Germination of cardamom seeds improved significantly after acid

treatment (nitric acid 25 % for 10 min) and continuous washing in water for 24 days giving 85 percent germination as observed by Chaudhury and Chandel (1995). Korikanthimath and Mulge (1998) found acid treatment (nitric acid 20 % for 10 min), GA_3 100 mg l^{-1} for 12 h and. Planofix (NAA 75 mg l^{-1} for 12 h) increased germination of cardamom seeds. Higher doses of growth regulators decreased germination.

Spiegel-Roy *et al.* (1985) reported Nitsh's medium with IAA 10^{-5} M and GA_3 10^{-6} M as the best for germination of seeds of seedless grape cultivars. Deemir and Gunay (1996) observed the increased gertmination percentage in seeds of cucumber after priming with 3 per cent KNO_3 for five days.

Acacia nilotica seeds scarified with H_2SO_4 followed by washing and drying and then treated with KNO_3 at 2 per cent for 24 h showed higher germination percentage as reported by Palani *et al* (1996). Treatment with KCl and KH_2PO_4 for 24 h had no germination effect.

Cantliffe and Abebe (1996) reported priming the tomato seeds with KNO_3 or PEG 8000 solutions for6-8 days consistently improved germination at 35^0 C. Khandelwal and Sen (1996) observed one-year-old seeds of *Eragrostis* soaked in 50 ppm KNO_3 for 24 h showed higher germination.

Buyukalaca *et al.* (1997) reported that embryos of pistachio seeds were germinated on solid growth regulator free MS medium and half strength MS without sucrose and liquid growth regulator free half strength MS medium without sucrose.

The seeds of *Coriandrum sativum* L. germinated on MS basal medium with three per cent sucrose (Stephan and Jayabalan, 2001). Kitsaki *et al.* (2004) observed *in vitro* germination of seeds from *Ophrys* species (Orchidaceae) with Malmgren's

medium as a basal sowing and growing medium supplemented with pineapple juice or coconut milk.

Half strength MS basal medium devoid of plant growth regulators found to be the best for asymbiotic germination of immature seeds of *Dendrobium tosaense* (Lo *et al.*, 2004). Sreelatha *et al.* (2005) succeeded in getting positive response of Kannanthali (*Exacum bicolor*) seeds to *in vitro* seed culture. The seeds of this plant are not reported to germinate in natural condition. By culturing the seeds in the medium half MS + 2.0 mg l^{-1} IBA they could get the best response.

2.10.2 Germination of seeds produced *in vitro*

The *in vitro* raised seeds may require special treatments, media and culture conditions for germination. *In vitro* seeds of *Papaver rhoeas* soaked in tap water for 24 h germinated in modified White's medium (Kanta, 1960).

Usha (1965) observed seeds of *Antirhinum majus* produced through *in vitro* pollination germinated in Nitsh medium with 4 per cent sucrose. *In vitro* produced seeds of Petunia germinated on moist filter paper and on nutrient agar medium on which it was developed. The germination was more on moist filter paper (Rangaswamy and Shivanna, 1967).

In vitro produced seeds of *Nicotiana tabacum* varied in their time taken for germination and sometimes the seeds dried out and passed to dormancy (Balatkova *et al.*, 1976). The caryopses of maize produced *in vitro* dried at room temperature for several days and then kept for germination on moist filter paper at 28^0C germinated within 7 days after incubation (Gengenbach, 1977).

Castano and Proft (2000) cultured ovules from *in vitro* pollinated flowers of chicory on various media combinations. The ovules developed into seedlings in three

combinations viz.,

- MS + IAA 4 mgl^{-1} + kinetin 0.5 mgl^{-1} + GA_3 5 mgl^{-1} with 4 per cent sucrose and supplemented with glycine 9.5 mgl^{-1} + casein hydrolysate 500 mgl^{-1}.

- B_5 + IAA 4 mgl^{-1} + kinetin 0.5 mgl^{-1} + GA 5 mgl^{-1} with 4 per cent sucrose and supplemented with glycine 7.5 mgl^{-1} + casein hydrolysate 500 mgl^{-1}.

- MS + IAA 0.5 mgl^{-1} + kinetin 0.5 mgl^{-1} + GA_3 5 mgl^{-1} with 4 per cent sucrose and supplemented with glycine 7.5 mgl^{-1} + casein hydrolysate 500 mgl^{-1}

2.11 EMBRYO RESCUE TECHNOLOGY

In crop improvement programme, post fertilization barriers can be overcome by Embryo Rescue Technology (Bhojwani and Razdan, 1983).

When embryos cannot be easily excised, whole ovule can be cultured and similarly when ovule is too small to be removed, whole ovary can be cultured. Thus embryo rescue technique can be done by embryo culture, ovule culture and ovary culture. This technique is utilized for the production of certain rare hybrids, which are otherwise impossible in nature due to embryo abortion.

Inomata (1996) described methods of overcoming interspecific and intergeneric barriers in *Brassica* and its wild relatives, through embryo, ovule and ovary culture. The method is successfully utilized for interspecific hybridization in crops like tomato and brinjal (Blestos *et al.*, 1998).

2.11.1 Ovule culture

The first successful culture of ovules was reported by Maheswari (1958) in *Papaver rhoeas*. The ovules were cultured on Niitsh medium containing vitamins,

kinetin (0.4 mg l^{-1}) and IAA (5 mg l^{-1}). Raising mature seeds by culturing ovules of *Allium cepa* containing globular or older embryos is comparatively easier as reported by Guha and Johri (1966). Spiegel-Roy *et al.* (1985) obtained normal embryos and seedlings by culturing abortive ovules and seeds of rape on Nitsh medium with IAA 10^{-5} M and GA_3 10^{-6} M. Kristiansen and Vainstein (1995) obtained viable hybrids ovule culture in genus *Alstroemeria.*

Ishikawa *et al.* (2001) efficiently produced interspecific hybrids by culturing immature ovules with placenta 7-14 days after pollination on 2 g l^{-1} gelrite solidified MS medium containing 3 percent sucrose. Ovule culture 60 days after pollination was recommended for producing interspecific hybrids in large scale crosses of lily (Jin *et al.*, 2001). Michishita *et al.* (2001) obtained hybrid seedlings from *Rhododendron kaempferia* ovules by exposing ovules 4 months after crossing to 50 mg l^{-1} GA3. The best medium for culturing apricot ovules was modified SH medium with 4 per cent sugar or 4 percent sorbitol as reported by Qing *et al.* (2002).

Caryopsis cultures of Paspalum scrobiculatum produced embryogenic callus in N_6 medium with 4.5 µm 2,4 D either alone or with 4.5 µm BA. The combination of TDZ (11.25 µm to 22.5 µm) and 2,4 D (4.5 µm) stimulated differentiation of shoot buds in the embryogenic callus cultures (Rashid, 2002a).

2.11.2 Embryo culture

Hannig (1904) for the first time cultured embryos of the cruciferous genera- *Raphanus* and *Cochleria*. Since then, *in vitro* zygotic embryo culture technique has been tried in plant breeding and horticulture as well as in basic studies on embryo physiology and biochemistry.

In monocots a well-studied plant for embryo rescue is barley (*Hordeum vulgare*). Norstog (1965) has described the following procedure for the isolation of immature barley embryos of smaller than 0.2 mm size. In barley, the region of the

ovule that contains the embryo is beak like. The beak was excised and transferred to a drop of sterile paraffin oil. Tissues of the beak were carefully teased apart to release the embryo. The isolated embryo was lifted out in the oil film using a micro spatula and transferred to the nutrient medium. The presence of oil prevents drying up of the embryo.

Shankar (2003) could not get plant regeneration in ginger through embryo culture after *in vitro* pollination.

2.11.3 Embryo nurse endosperm transplant

The nutritional requirements of young embryos are complex. The technique of embryo-nurse endosperm transplant can supplement these requirements.

Zeibur and Brink (1951) showed that *in vitro* growth of excised immature embryos (300-1100μm long) of barley was considerably promoted by surrounding the embryo with the endosperm of another seed of the same species. A modified endosperm transplant technique for younger embryo culture has been described by Lautour *et al.* (1978) and Williams and Lautour (1980). They inserted an excised hybrid embryo into a cellular endosperm dissected out from a normally developing ovule of one of the parents or a third species and cultured the nurse endosperm with the transplanted embryo on an artificial medium. Using this technique, many interspecific hybrids could be produced in *Trifolium* genus, which could not be reared by growing embryos directly on the medium.

2.11.4 Precocious germination

Precocious germination is the phenomenon of seedling formation without complex normal embryonic development. Excised immature plant embryo on nutrient medium sometimes tend to bypass the stage of dormancy and cease to undergo linear embryogenic mode of development and develop into weak seedlings (Bhojwani and Razdan, 1983).

According to Kent and Brink (1947) incorporating casein hydrolysate in the culture medium could prevent precocious germination of excised immature barley embryos. Norstog (1972) reported that exogenous factors like reduced oxygen tension, elevated temperature and high light intensity prevented precocious germination. Studies conducted further showed that the presence of some inhibitors like ABA might be preventing precocious germination.

2.11.5 Culture requirements

2.11.5.1 Media requirements for developing embryo

The culture medium should support progressive and orderly development of embryos. Hannig (1904) reported that mineral salt-sucrose solution was sufficient to culture mature embryos (2mm long) of crucifers.

Monnier (1978) observed that though MS medium supported maximum growth of embryos of *Capsella bursa-pastoris*, the survival frequency of the embryos were very low, whereas in the Knop's medium which was least toxic, the growth of the embryo was very poor. So he developed Monnier's medium, which was a modification of MS medium, containing high concentrations of K^+ and Ca^{2+} and a reduced level of NH_4^+ ions supported both growth and survival of embryos.

Rashid (2002) found N_6 medium to be superior to MS medium for culturing zygotic embryo explants of *Paspalum scrobiculatum*. Solid SH medium was used for embryo culture in black pepper as reported by Nair and Gupta (2003).

2.11.5.2 Growth regulators

An auxin or cytokinin, or both are required for the growth of embryo and for callusing embryos.

Raghavan and Torrey (1964) reported that for culturing globular embryos of *Capsella* on mineral salt medium with only 2 percent sucrose, it was necessary to

supplement with IAA (0.1 mg l^{-1}), kinetin (0.001 mg l^{-1}) and adenine sulphate (0.001 mg l^{-1}). The plantlets were obtained from zygotic embryos of *Taxus brevifolia* when cultured on half strength Gamborge's B_5 medium supplemented with BA 10 µM (Chee, 1995) while the plantlets were obtained from 40 days old embryos of *Juniperus cedrus* when cultured on Quoirin and Le Poivere half strength medium supplemented with BA 5 µM (Harry *et al.*, 1995).

2.11.5.3 Carbohydrate and osmotic pressure of culture medium

Sucrose is the commonly used carbohydrate for embryo culture (Beek *et al.*, 1944). Sucrose is also essential in the medium to maintain suitable osmolarity, which is extremely important for immature embryos. Eight to twelve percent of sucrose was required for the culture of pro-embryos (Norstog, 1961 and Monnier, 1978).

The stimulatory effect of high concentrations (12-18%) of sucrose on *in vitro* development of excised globular embryos (smaller than 80 µm) of *Capsella* can be obtained by combinations of IAA, kinetin and adenine sulphate added to the basal medium with only 2 per cent sucrose (Raghavan and Torrey, 1964).

Pancholi *et al.* (1995) reported that immature embryos excised from *Musa velutine* seeds when cultured on half strength MS medium with 2 per cent sucrose formed plantlets.

2.11.5.4 Amino acids and vitamins

The addition of amino acids, singly or in combination to the culture medium may stimulate embryo growth. Hanning (1904) reported that asparagine was very effective in enhancing embryo growth of crucifers.

Casein hydrolysate (CH) an amino acid complex was used widely as an additive to the embryo culture. Kent and Brink (1947) observed that CH inhibited precocious germination and promoted pre-germinal embryo development in

Hordeum vulgare. According to them, the optimum level of CH for *Cucumis melo* embryos to form plants (Beharav and Coheu, 1995).

Maheswari and Lal (1961) found that vitamins such as B_1 and B_6 or even a mixture of vitamins stimulated ovary growth of *Iberis amara*. Vitamin E increased seed fertility in *Dendrobium nobile* (Ito, 1966).

2.11.5.5 Natural plant extracts

Beek *et al.* (1942) observed that normal seedlings from *Datura* embryos as small as 150-200 μm long and as young as 10 DAP could be developed by incorporating coconut in the culture medium containing mineral salts, vitamins and amino acids. Later, it has enabled the successful culture of young embryos of a number of species.

2.11.5.6 pH of the medium

Pancholi *et al.* (1995) reported that the optimum pH for the growth and morphogenesis of immature embryos of *Musa velutina* under *in vitro* conditions was 5.8 at 23 to 28 ^{0}C.

2.11.5.7 Culture maintenance

Embryos of most plants grew at temperature between 25 and 30 ^{0}C (Narayanaswamy and Norstog, 1964). According to them, light was not critical for embryo growth. However, in barley, light was known to suppress precocious germination of immature embryos (Norstog, 1972).

Behrav and Coheu (1995) reported that the hybrid embryo of the cross between *Cucumis melo* and *Cucumis metuliferus* was grown best at 25 ^{0}C with a photoperiod of 16 h. The zygotic embryo of *Taxus brevifolia* produced organs like shoot and root under 16 h photoperiod (Chee, 1995).

2.11.6 Routes of hybrid plant production through embryo culture

2.11.6.1 Direct organogenesis

For the culture of immature embryos of *Actinidia*, the best medium was MS basal containing GA_3 0.01- 0.05 mg l^{-1} + mannitol 8 percent as suggested by Kin *et al.* (1995). Lekshmy (1989) cultured embryos of mature hybrid seeds of banana on modified Knudson's medium to develop plantlets.

Sudhadevi *et al.*, (1993) reported production of multiple shoots from hybrid embryo between Kew X Ripley Queen varieties of pineapple on MS medium containing NAA 3 mg $l^{-1}$and BAP 2 mg l^{-1}. Shoot elongation was observed on MS with BAP 4 mg l^{-1} and IBA 1mg l^{-1} and they were rooted by giving a pulse treatment with IBA 2 mg l^{-1}.

Muniswamy and Sreenath (1996) reported that zygotic embryos of *Coffea bengalensis* were matured when incubated on MS medium with ABA 1 mg l^{-1} + BAP or kinetin 1 mg l^{-1} by 30 days. They germinated into plantlets when cultured on half strength MS with BAP 0.1 mg l^{-1}.

Asif *et al.* (2001) observed that germination was more in *in vitro* zygotic embryo culture of wild banana compared to green house grown seeds. Burun and Poyrazoglu (2002) observed the highest plantlet development from barley embryos in Randolph and Cox medium. But Qing *et al.* (2002) reported that the best medium for culturing young embryos of apricot was modified SH medium.

Ondrej *et al.* (2002) observed better development of *Cucumis* embryos was achieved on medium with lower concentration of GA_3 but high concentration positively stimulated embryogenesis of zygote and pro-embryo culture.Excised zygotic embryos of bottle palm germinated when cultured in MS medium with 3 per cent sucrose and 3 g l^{-1} activated charcoal (Sarasan *et al.*, 2002).

2.11.6.2 Indirect organogenesis

Callus growth of ginger seeds obtained through ***in vitro*** pollination was observed in the medium containing half MS + 2,4 D (0.5 mg l^{-1}), BAP (6.0 mg l^{-1}) and GA_3 (5.0 mg l^{-1}) along with 3 per cent sucrose (Shankar, 2003).

Wang (1987) obtained callus induction and plant regeneration from mature embryos of maize. Callus was obtained on MS medium with 1-2 mg l^{-1} 2, 4-D and plant regeneration was obtained on MS medium without hormones.

Roy and De (1989) reported tissue culture and plant regeneration from immature embryo explants of *Calotropis gigantea.* Callus was obtained from immature embryos when cultured on MS medium with NAA 0.1 mg l^{-1} and BAP 0.1 to 0.5 mg l^{-1}. Rooting was reduced when shoots were transferred to auxin supplemented Bonner's solution or half strength MS basal salt solutions.

Callus initiation in loblolly pine was improved by the addition of abscissic acid (3.7μM), silver nitrate (20 μM) and guanosine (10 μM) to the medium and by optimizing the cytokinin levels (BA 2.8 μM and kinetin 2.83μM) in the presence of 50 mgl^{-1} activated carbon (Pullman *et al.*, 2003). Callus induction from mature embryos of wheat was noticed in a medium containing thiamine 1.0 mgl^{-1}, casein hydrolysate 1.0 mgl^{-1}, dicamba 2.5 mgl^{-1} and L- proline 0.69 mgl^{-1} (Zale *et al.*, 2004).

Ovular callus culture studies in *Carica papaya* by Kavitha and Chezhiyan (2005) revealed that maximum percentage callusing was obtained on MS medium supplemented with kinetin 0.5 mg l^{-1}+ IBA 0.1 mg l^{-1}. Percent greening of the callus was also high in the same medium.

MS medium containing 2,4 D (2 mg l^{-1}) plus B_5 vitamins, MS medium containing 2,4 D (1 mg l^{-1}) with no vitamins or MS medium containing picloram

(2.2 mg l^{-1}) and 2,4 D (0.5 mg l^{-1}) plus MS vitamins were used for callus initiation from immature embryos of wheat. The calli regenerated in Full MS basal medium (Haliloglu and Baenziger, 2005).

2.11.6.3 Somatic embryogenesis

Chuang and Chang (1987) observed somatic embryogenesis and plant regeneration in callus culture derived from mature zygotic embryos of *Dysosma pleiantha*. Callus was obtained in MS medium supplemented with 2,4-D 1 mg l^{-1}. Embryoids were formed on media containing 2,4-D 0.1 to 0.5 mg l^{-1} and they germinated on MS or B_5 with BA 1 mg l^{-1} and GA_3 1 mg l^{-1}.

Induction of embryogenic callus and subsequent somatic embryogenesis was possible at a lower concentration of 2,4 D (1.0-2.0 mg l^{-1}) when immature zygotic embryos of *Paspalum scrobiculatum* were used as explants. Addition of amino acids like L-proline or L- tryptophan to the medium significantly enhanced embryogenesis. These embryos readily germinated in hormone free medium (Rashid, 2002b).

Kaun *et al.* (2002) reported that highest rate of embryogenesis was obtained when immature epicotyle and cotyledons of groundnut were cultured in MS medium supplemented with 4 mgl^{-1} of 2,4 D. Somatic embryos thus obtained germinated when plated on a medium with 50 mgl^{-1} ascorbic acid and 0.5 mgl^{-1} NAA.

Direct somatic embryogenesis was obtained from mature zygotic embryos of sandalwood when cultured on MS medium containing thidiazuron 2 mg l^{-1} or benzyl amino purine 2 mg l^{-1}. Somatic embryos germinated in half strength MS supplemented with 1.4 μM GA_3 (Rai and McComb, 2002).

When longitudinal sections of three weeks old zygotic embryos of bottle palm were cultured on MS + 2,4 D 3 mg l^{-1}, somatic embryos were formed directly

on the cut surface without callusing. But the germination of somatic embryos was problematic (Sarasan et al., 2002).

Somatic embryoids were developed from seeds of ginger obtained through *in vitro* pollination when cultured on half strength MS + 2,4 D (0.5 mg l^{-1}), BAP (6.0 mg l^{-1}) and GA_3 (5.0 mg l^{-1}) along with 3 per cent sucrose and also in half strength MS + 2,4 D (0.1 mg l^{-1}), BAP (8 mg l^{-1}) along with 3 per cent sucrose (Shankar, 2003). But he could not get plant regeneration from these somatic embryoids.

According to Samaj *et al.* (2003), a developmental switch from nonpolar pre embryogenic units to polarized transition units in maize embryogenic callus was effected by auxin deprivation from the culture medium.

As per the reports of Tokuji and Kuriyama (2003) gibberellin inhibited the early stage of embryonic cell differentiation and uniconazole, an inhibitor of GA synthesis promoted the secondary embryogenesis from the primary embryo in carrot. Somatic embryos were obtained from zygotic embryos of *Areca catechu* when incubated in MS medium containing dicamba 9.05 to 36.2 µM. They developed into plantlets in basal medium free of growth regulators (Wang *et al.*, 2003).

Simultaneous callus formation and regeneration from embryo explants of cumin was reported on B_5 medium supplemented with 1 ppm BA, 0.2 ppm NAA and 0.4 ppm IAA (Ebrahimie *et al.*, 2003). Somatic embryogenesis and plant regeneration was obtained when the zygotic embryo explants of *Cryptomeria japonica* were cultured on a medium containing 3.2 µM 2,4 D and 1.8 µM BA in dark (Igasaki *et al.*, 2003).

Embryogenic callus could be produced from immature zygotic embryos of *Commiphora wightii* (Arnott) Bhandari, after reciprocal transfer on media containing 2,4,5 tri chloro phenoxy acetic acid (0.1mg l^{-1}) and kinetin (0.1mg l^{-1}) or media

devoid of growth regulators. Maximum growth of calli was got in modified MS (MS with low level of nitrogen [50mg N l^{-1} of $(NH_4)_2$ SO_4 and 200 mg N l^{-1} of KNO_3] and calcium (220 mg l^{-1} Ca Cl_2) supplemented with BA (0.25 mg l^{-1}) and IBA (0.1mg l^{-1}). Embryoids germinated in hormone free MS medium (Kumar *et al.*, 2003).

According to Pullman *et al* (2003), the weight of embryogenic tissue from megagametophytic explants of loblolly pine was increased by brassinolide (0.005 to 0.25 μM) by 66 per cent. It also stimulated callus initiation in recalcitrant families of the crop. The same concentration of brassinolide was used to improve embryogenic callus growth in rice also.

Callusing was induced mature embryos, immature embryos and inflorescence explants of wheat grass by incubating in MS medium with 22.6 μM 2,4 D under dark. After 6-8 weeks, calli were transferred to liquid AA medium with 6.8 μM 2,4 D , 2 per cent sucrose and 3 per cent (w/v) sorbitol and incubated in a gyratory shaker at 80 rpm in dark. Somatic embryoids were induced from the callus on solid MS + 9.1 μM 2,4 D and 3 per cent sucrose. Plantlet regeneration was obtained in solid MS medium + 0.9 μM kinetin (Wang *et al.*, 2003).

Gairi and Rashid (2004) reported that thidiazuron at 10 μM concentration induced somatic embryogenesis in non-responsive caryopses of rice after a short treatment with 20 μM 2,4 D for three days. The addition of 2,3,5 tri iodo benzoic acid (0.1mgl^{-1}) and/or colchicine (0.02%) into the medium promoted somatic embryogenesis in maize (Obert and Barnabas, 2004).

Immature zygotic embryos of papaya when cultured on half MS + 2,4 D (2.0 mg l^{-1})under dark for a period of 2 months, embryogenic callus was produced. After 2 months, calli were incubated on ½ MS basal medium for 3 months to develop somatic embryos. Shoots were developed in ½ MS + BAP 0.4 mg l^{-1} and NAA 0.02

mg l^{-1}. Rooting was noticed in MS basal liquid medium + IBA 1.0 mg l^{-1} (Anandan *et al.*, 2005).

2.11.6.4 Embryo suspension culture

Kageyama *et al* (1991) studied somatic embryogenesis of explants from mature seeds of melon through suspension culture. The culture period in the initial culture medium (MS supplemented with 1 mg 2,4-D, 1 mg NAA, 0.1 mg BA and 30 g sucrose/litre) influenced the structural characteristics of the somatic embryoids. When the suspension was cultured for 7 days in the initial medium, most of the somatic embryoids which developed had normal morphogenesis. A larger number of normal somatic embryoids were obtained when the 7-day-old cultures were washed with MS medium containing 0.5% activated charcoal.

Rapidly growing finely dispersed embryogenic cell suspension cultures were established from embryogenic callus in *Eschscholzia californica* and maintained in B5 liquid media supplemented with 0.5 mg l^{-1} (2.26 µM) 2,4-dichlorophenoxyacetic acid [2,4-D]. Somatic embryo production was substantially reduced at shaker speeds above 40 rpm. Glucose and sucrose were the most effective carbon sources. The development of somatic embryos was promoted by $AgNO_3$ at concentrations below 10 mg l^{-1} (58.8 µM). A semi-solid medium containing 1.5 g l^{-1} Gel-rite produced the highest frequency of somatic embryo conversion, and promoted the efficient growth of plantlets (Park and Facchini, 2001).

2.12 MICROPROPAGATION OF GINGER

2.12.1 Enhanced release of axillary buds

Hosoki and Sagawa (1977) reported clonal propagation of ginger through enhanced release of axillary buds. Buds from rhizome in storage were cultured in a medium consisting of MS major elements, Ringe-Nitsh minor elements and vitamins, 2.0 per cent sucrose and BAP 1 mg l^{-1}. De Lange *et al.* (1987) got

germination of shoot buds encapsulated in 4 per cent sodium alginate gel *in vitro* to form roots and shoots.

Dogra *et al* (1994) achieved *in vitro* propagation of ginger using rhizome buds and these buds produced multiple shoots when culured aseptically on MS medium supplemented with 2.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA.

Sharma and Singh (1997) reported high frequency *in vitro* multiplication of disease free clones of ginger by culturing small, active buds on MS medium supplemented with 2 mg l^{-1} kinetin and 20 g l^{-1} sucrose. An average of 7.7 shoots per bud was obtained after 4 weeks of culture.

Babu *et al.* (1992) reported plantlet formation from immature inflorescence (1 to 10 days old flower buds) in modified MS medium supplemented with BA 10 mg l^{-1} and 2,4-D 0.2 mg l^{-1}.

2.12.2 Callus mediated organogenesis

Choi (1991) reported callus-mediated organogenesis in ginger from explants of pseudostem containing one leaf blade. Callusing was best on medium containing NAA 0.5 ppm while shoot and root formation were best on medium containing NAA 0.1 to 1 ppm and BA 1 ppm.

Rout and Das (1997) reported efficient plant regeneration in *Z. officinale* achieved using callus derived from shoot primordia grown on MS media. Organogenesis was best on media supplemented with BA (5 mg l^{-1}), IAA (1 mg l^{-1}), adenine sulphate (100 mg l^{-1}) and sucrose 3 percent. Shoots were rooted on half MS supplemented with IBA or IAA (1 mg l^{-1}) and 2 per cent sucrose.

Palai *et al* (2000) reported plant regeneration from callus culture on MS basal medium supplemented with BAP (5 mg l^{-1}), adenine sulphate (100 mg l^{-1}), IAA (1.5

mg l^{-1}) and 3 per cent sucrose. Regenerated shoots were rooted on half strength MS medium supplemented with IAA (0.25-0.5 mg l^{-1}) within 7-8 days of culture. These *in vitro* produced plantlets were successfully established in soil.

Paul *et al.* (2005) developed an indirect method of plantlet regeneration in ginger using various explants like sprouted bud, shoot tip, leaf and pseudostem. Callusing was obtained in all the explants except mature leaf in MS medium supplemented with 2,4 D alone (1.0-2.0 mg l^{-1}) and in combination with BAP (0.5-3.0 mg l^{-1}). Shoot morphogenesis was obtained from one month old calli in MS + BAP 3.0 mg l^{-1}. Regenerated shoots were subcultured to medium of same composition for further shoot proliferation and rooting.

2.12.3 Somatic embryogenesis

In monocots, immature inflorescence, immature embryos, or mature seeds are the choice explants for initiating embryogenic cultures (Bhaskaran and Smith, 1990). Somatic embryogenesis in ginger was reported by Kackar *et al.* (1993). The leaf segments cultured on MS medium with sucrose 8 per cent and dicamba 2.7 µM produced embryoids. IAA and NAA were not effective in producing embryogenic cultures. Plant regeneration was achieved when embryogenic cultures were transferred to MS medium containing BAP 8.9 µM. The plantlets produced were successfully established in soil.

Suma and Kesavachandran (2005) reported a protocol for plant regeneration via somatic embryogenesis obtained from callus derived from young buds of *Zingiber oficinale* Rosc. MS medium with 2,4 D 1 mg l^{-1} and BA 0.5 mg l^{-1} was most effective in inducing and maintaining embryogenic cultures. The somatic embryos germinated on half strength MS medium supplemented with 3 per cent sucrose and 3 mg l^{-1} BA.

Materials and Methods

3. MATERIALS AND METHODS

The details regarding the experimental material and methodology adopted for conducting various aspects of the present study 'Induction of genetic variability in ginger (*Zingiber officinale* Rosc.) through *in vitro* fertilization' are presented in this chapter.

3.1 EXPERIMENTAL MATERIAL

The cultivars of ginger maintained at the College of Horticulture as listed in Table 1, formed the parent material for the *in vitro* fertilization programme.

Table 1. List of ginger cultivars used as the parent material

Cultivars	Identity	Code No.
Z-0-78	Autotetraploid from Himachal Pradesh evolved from the 0.25% colchicine treatment at Department of Plantation crops and Spices	V_1
Z-0-86	Autotetraploid from Rio-de-Janeiro evolved from the 0.1% colchicine treatment at Department of Plantation crops and Spices	V_2
Kodakara local	Local cultivar from Thrissur district	V_3
Kuruppampady	Local cultivar from Ernakulam district	V_4
Mahima	Released variety from IISR	V_5
Maran	Cultivar from Assam	V_6
Rejatha	Released variety from IISR	V_7
Rio-de-Janeiro	Genotype from Rio-de-Janeiro of Brazil	V_8
Varadha	Released variety from IISR	V_9

3.1.1 Production and management of the crop

The planting of the two consecutive crops was done in April 2004 and April 2005. The potting mixture was prepared (1 sand: 1 soil: 1 farm yard manure) and subjected to solarization as per the Package of Practices Recommendations of Kerala Agricultural University (KAU, 1996) before filling in pots. The rhizome bits of 15 to 20g and 200g of the selected cultivars were planted in pots. Five potted plants of each cultivar were maintained. The plants, which were raised from 15 to 20g rhizome bits, were maintained as biennials. The crop was maintained following cultural, manurial and various plant protection operations as per the Package of Practices Recommendations of Kerala Agricultural University (KAU, 1996).

3.2 MOLECULAR CHARACTERIZATION OF PARENT PLANTS

Random Amplified Polymorphic DNA analysis was done to assess the polymorphism among the parents at the genetic level.

3.2.1 DNA isolation

A modified CTAB extraction procedure reported by Doyle and Doyle (1987) was followed for the extraction of genomic DNA in ginger.

The third and fourth leaves from top were taken from the selected cultivars using sterile blades. The leaf samples were collected on ice, washed with tap water and immediately used for extraction. Details are given below.

A. Reagents:

1. 4X Extraction buffer

Tris HCl	: 4.8g
EDTA di sodium salt	: 0.74g
Sorbitol	: 2.56g
Sterile milliQ water	: 100ml

Sorbitol, Tris HCl and EDTA were dissolved in about 80ml sterile milliQ water and the pH was adjusted to 7.5 using concentrated HCl. The volume was

then made up to 100ml, a pinch of Sodium metabisulphate (0.38 percent) was added prior to extraction.

2. Lysis buffer

1M Tris HCl, pH 8	: 20.0ml
0.25M EDTA	: 20.0ml
5M NaCl	: 40.0ml
Sterile milliQ water	: 20.0ml
Cetyl Trimethyl Ammonium Bromide (CTAB)	: 2.0g

3. TE buffer

10mM Tris HCl, pH 7.6	: 0.3028g
1mM EDTA	: 0.0930g
Distilled water	: 200ml

4. Sarcosin (5%)

Sarcosin	: 5.0g
Distilled water	: 100ml

All the above four reagents were prepared and autoclaved before use.

5. Chilled isopropanol
6. Chloroform: isoamyl alcohol mixture (24:1 v/v)
7. β mercapto ethanol
8. Ethanol (100% and 70%)

B. Procedure:

The leaf sample (0.5g) was ground in sterile mortar, with 3ml of extraction buffer and 50 µl β mercapto ethanol and a pinch of sodium meta bisulphate. The homogenate was poured into a centrifuge tube (50ml) containing 4ml pre-warmed lysis buffer and 1ml (5%) sarcosin. The tube was kept for 15 minutes at 65^0C and was occasionally mixed by inversion. Equal volumes of chloroform: iso amyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10,000 rpm for 10 minutes at 4^0C. The upper aqueous phase was pipetted out and saved in a centrifuge tube. To the centrifuge tube containing the

aqueous phase, 0.6 volume pre chilled isopropanol was added. The tube was kept at -20^0C for half an hour for precipitation. The contents were mixed gently by inversion until the DNA was precipitated. The DNA was pelleted by centrifuging at 10,000 rpm for 10 minutes at 4^0C. The isopropanol was poured off, drained well and the pelleted DNA was washed with 70% alcohol and centrifuged at 10,000 rpm for 3 minutes. The pellet was air dried and resuspended in 200µl TE buffer.

3.2.2 Assesssment of quality of DNA

The quality of isolated DNA was evaluated through electrophoresis.

Electrophoresis of DNA samples.

A. Reagents

1. Agarose
2. 50 X TAE buffer

Tris base	:242g
0.5M EDTA, pH 8	:100ml
Glacial acetic acid	: 57.1ml

The contents were mixed well, autoclaved and stored at room temperature.

3. 6 X Gel loading dye

Bromophenol blue	: 0.25%
Xylene cyanol FF	: 0.25%
Glycerol in water	: 30%

The components were mixed well, autoclaved and stored at 4^0C

4. Ethidium bromide

B. Procedure

Gel buffer 1X TAE was prepared from the 50X TAE stock solution. Gel buffer 1X TAE was taken in a conical flask (100ml for large gel and 30 ml for small gel). Agarose (1.0% for DNA samples and 1.4% for RAPD samples) was weighed, added to the flask, stirred and boiled till the agarose dissolved completely. Ehidium

bromide (2µl) was added into the flask, mixed well and it was allowed to cool to 40^{0}C. The open ends of the gel-casting tray was sealed with cello tape and placed on a leveled horizontal surface and the comb was placed properly on the tray. The dissolved agarose was poured gently into the tray. The gel was allowed to solidify for 30 minutes and then the comb was removed carefully. The gel was then placed in electrophoresis unit with the wells directed towards the cathode. 1X TAE buffer was added to cover the gel with 2 to 3 millimeter of buffer. The DNA sample (5 µl) was mixed with gel loading dye. The samples were then loaded carefully into the well using micropipette. Standard DNA molecular weight markers were loaded in one well and a negative control was also loaded in another well in the case of electrophoresis for RAPD assay. The cathode and anode of the electrophoresis unit were then connected to the power supply and the gel was run at constant voltage (45mA). The power supply was turned off when the loading dye moved to the required distance.

3.2.3 Gel documentation

The gel was taken from electrophoresis unit and viewed under UV light of 320nm. The image of the gel was monitored and stored in a gel documentation system Alpha Imager-2000, Alpha InnoTech, USA.

3.2.4 Random Amplified Polymorphic DNA (RAPD) analysis

3.2.4.1 Screening of random primers for RAPD

A total of 19 decamer primers (Operon Technologies, USA) under two different series viz., OPE and OPF (Table 2) were screened for amplification of genomic DNA extracted from one of the ginger cultivars. The procedure of Demeke *et al.* (1992) was modified and was used for the amplification of genomic DNA.

In polymerase chain reaction, the following programme was followed.

Cycle 1

i) DNA denaturation at 94^{0}C for 3 min.

ii) Annealing of the primer to template DNA at 37^{0}C for 1 min.

iii) Primer extension at 72^{0}C for 1 min.

Number of cycle repeats: 1

Cycle 2

i) DNA denaturation at 94^{0}C for 1 min.

ii) Annealing of the primer to template DNA at 37^{0}C for 1 min.

iii) Primer extension at 72^{0}C for 1 min.

Number of cycle repeats: 31

The reaction mixture (25µl) consisted of

- 10 X assay buffer
- 1mM dNTP mix
- Taq DNA polymerase
- Primer
- Template DNA
- Milli Q water

A master mix without the template DNA and primer was prepared using the reaction mixture for the required number of reactions. From this master mix, 22µl was pipetted into each PCR tube. 1µl of primer and 2.0µl of template DNA were added. Heated lid was used while running PCR. The PCR tubes were loaded in the DNA Engine (MJ research) and the aforesaid programme was run. The amplified products (20 µl) were resolved on 1.4 per cent agarose gel using 1X TAE buffer. The gel was viewed under UV light in the transilluminator and then documented using alpha imager.

The quality of amplification was scored as good (>5 amplicons), average (4-5 amplicons) and poor (<4 amplicons).

Table. 2. List of primers used for screening

Sl. No.	Primer code	Primer sequence
1	OPE-3	CCAGATGCAC
2	OPE-5	GCAGGGAGGT
3	OPE-6	AAGACCCCTC
4	OPE-7	AGATGCAGCC
5	OPE-11	GAGTCTCAGG
6	OPE-12	TTATCGCCCC
7	OPF-1	ACGGATCCTG
8	OPF-2	GAGGATCCCT
9	OPF-3	CCTGATCACC
10	OPF-5	CCGAATTCCC
11	OPF-6	GGGAATTCGG
12	OPF-7	CCGATATCCC
13	OPF-8	GGGATATCGG
14	OPF-9	CCAAGCTTCC
15	OPF-10	GGAAGCTTGG

3.2.4.2 Screening of ginger cultivars by RAPD

Nine primers that gave 4-9 amplicons in the initial screening were selected. They were OPE3, OPE7, OPE11, OPE12, OPF1, OPF3, OPF5, OPF6 and OPF7 Genomic DNA (20-50ng) of the cultivars Kodakara local (V_3), Kuruppampady (V_4), Mahima (V_5), Maran (V_6), Rejatha (V_7). Rio-de-Janeiro (V_8), Varada (V_9) and Z-0-78 (V_1), was subjected to amplification using the selected random primers following the thermal cycles used for primer screening.

3.2.5 Cluster analysis

The pattern of DNA amplification for the 9 primers was scored as 1 or 0 for the presence or absence of bands respectively and the data were analyzed using NTSYS pc 2.0 software package. A dendrogram was constructed for the 8 cultivars based on the clustering using unweighted Pair Group Method of Arithmetic Averages (UPGMA) using NTSYS package.

3.3 FLOWERING BEHAVIOUR

The observations on flowering behaviour of the nine ginger cultivars were taken. The duration from planting to the onset of flowering, date of appearance of first flower, last flower and the flowering duration were computed.

3.4 POLLEN STUDIES

3.4.1 Pollen fertility and viability studies

The fertility and viability of pollen from all the cultivars for early, mid and late flowering season were recorded. The pollen fertility and viability of flowers at lower, middle and upper portions of inflorescence were examined.

3.4.2 Estimation of pollen fertility

Pollen grains scooped out from flower buds at the time of anthesis were stained in 1% acetocarmine and viewed at 40X magnification. All the pollen grains that were well stained were counted as fertile and unstained as sterile. The observations were recorded for three flowers of each cultivar from nine microscopic fields. The fertility percentage was calculated using the formula.

$$\frac{\text{No.: of well stained pollen grains in a field X 100}}{\text{Total no: of pollen grains in a field}}$$

3.4.3 Pollen viability studies

The pollen grains were collected at anthesis and were incubated in a moisture chamber along with a drop of modified ME_3 medium (Leduc *et al.*, 1990). The composition of the ME_3 medium is given in Table 3. The observations were taken 24h after incubation. The total no: of pollen grains as well as the number of germinated grains were counted for three flowers of each cultivar from nine microscopic fields and the mean germination percentages were worked out. Photomicrographs of the germinated pollen grains were also taken.

3.5 REFINEMENT OF *IN VITRO* POLLINATION TECHNIQUE

The protocol for *in vitro* pollination developed by Valsala (1994) and refined by Shankar (2003) formed the basis for the present study.

3.5.1 Maintenance of asepsis

All the aseptic manipulations such as surface sterilization of flower buds, pollination, inoculation of ovules, sub culturing and various *in vitro* seed germination studies, including embryo culture were carried out under the hood of a clean sterile laminar air flow chamber. The table of laminar air flow chamber was made sterile by wiping with absolute alcohol and then exposing to ultra violet radiations for 20 to 30 min. All the inoculation aids were first steam sterilized and then flame sterilized before use.

3.5.2 Preparation of media

The chemicals used for preparing various media were of analytical grade obtained from British Drug House (BDH), Sisco Research Laboratories (SRL), Merck, Himedia and Sigma.Borosilicate glass wares of corning/borosil were used for *in vitro* studies. They were initially soaked in hot water at 100^0 C for half an hour. On cooling, they were washed with detergent solution, rinsed with potassium dichromate solution in sulphuric acid, then washed free of detergent using tap water and finally rinsed with distilled water.

Table 3. Composition of ME_3 medium (Leduc *et al.*, 1990)

Constituents	Concentration (mg l^{-1})
Macronutrients	
$MgSO_4.\ 7H_2O$	370.00
KNO_3	950.00
KH_2PO_4	85.00
$CaCl_2$	880.00
NH_4NO_3	412.50
KCl	175.00
Na_2EDTA	7.45
$FeSO_4.7H_2O$	5.55
Micronutrients	
H_3BO_3	50.00
$MnSO_4.H_2O$	16.80
$ZnSO_4.7H_2O$	10.50
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
Vitamins	
Thiamine	1.00
Pyridoxine	1.00
PEG 4000	120000.00
pH	6.0

Washed glass wares were dried in hot air oven at 60^0C and were stored away from dust and contaminants. Standard procedures were adopted (Gamborge and Shyluk, 1981) for the preparation of media. The pH of the medium was adjusted to 5.7. Semi solid medium was prepared by adding good quality agar (0.60 to 0.75%) or phytagel (0.14 to 0.20%). Sterilization of media was done by subjecting them to temperature of 121^0C at a pressure of 15psi for 20 minutes. After sterilization, the media were allowed to cool to room temperature and stored in cool, dry place.

3.5.3 Pollination technique

Flower buds were collected on the day of anthesis at 12 noon prior to flower opening. The flower buds ready to dehisce, which were protruding from the bract, were scooped out with the help of forceps and needle without injuring the ovary. The epicalyx and calyx surrounding the base of the corolla tube of the flower bud was removed, wiped with 50 per cent alcohol and then they were quickly transferred into conical flasks containing sterile water.

The flower buds were surface sterilized prior to anthesis i.e.3.00 p.m. The surface sterilization was done by wiping the flower buds with 70 per cent alcohol followed by dipping in 0.05 per cent streptocycline for two hours. In the laminar air flow they were treated with 0.1 per cent mercuric chloride for 3 minutes, then rinsed thrice with sterile water to remove traces of sterilant. Then they were air dried on sterile flamed filter paper.

Placental pollination was adopted as reported by Valsala (1994) and Shankar (2003) for the present study. In placental pollination, placenta bearing the ovules was exposed by completely peeling the ovary wall and pollination was done on it with pollen grains suspended in modified ME_3 medium. After pollination, it was transferred to inoculation medium. *In vitro* crossing between cultivars were done adopting this method. Subculturing was done at 21 days interval. The cultures were incubated in dark at 26 ± 2^0 C.

3.6 DEVELOPMENT OF OVULES AFTER *IN VITRO* POLLINATION

Development of the pollinated ovules in different media combinations was studied. The ovular development was assessed by scoring as given below:

- '++++' Very good swelling
- '+++' Good swelling
- '++' Moderate swelling
- '+' Low swelling
- '-' No swelling.

The percentage of cultures showing ovule development and percentage of ovules developed per culture were recorded. The increase in size of the ovule in the selected media combinations in terms of length and breadth at intervals of 15 days commencing from 15 DAP to 60 DAP was examined. The colour change of the ovules after *in vitro* pollination was observed. The filling of ovules was also examined at 10, 20, 30, 40 and 50 DAP in the media combination of half MS + sucrose (3%) + phytagel (0.18%) + picloram (0.2 mg l^{-1}) + BA (2.5 mg l^{-1}).

3.6.1 Experiments for refinement of media for ovule development

3.6.1.1 Basal media for ovule development

Four media combinations as modified MS and B_5 along with auxins and cytokinins as shown below were tried for culture establishment. The compositions of MS (Murashige and Skoog, 1962) and B_5 media (Gamborge *et al.*, 1968) are given in Table 4.

- Half MS + 3% sucrose + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}
- Half MS with 2X vitamins + 3% sucrose + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}
- B_5 + 3% sucrose + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}
- Half MS macro and micro nutrients + B_5 vitamins + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}

Table.4. Composition of basal media used for culture establishment, seed germination and embryo culture (Murashige and Skoog, 1962 and Gamborge *et al.*, 1968)

Constituents	Concentration (mg l^{-1})	
	MS medium	B_5 medium
Macronytrients		
KNO_3	1900.00	2500.00
NH_4NO_3	1650.00	-
KH_2PO_4	170.00	-
$MgSO_4.7H_2O$	370.00	122.09
$CaCl_2.2H_2O$	440.00	113.24
$(NH_4)_2$ SO4	-	134.00
Na $H_2PO_4.H_2O$	-	150.00
Micronutrients		
H_3BO_3	6.20	3.0
$MnSO_4.4H_2O$	22.30	10.00
$ZnSO_4.7H_2O$	8.60	2.00
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
KI	0.830	0.75
Na_2EDTA	33.60	37.30
$MnSO_4.H_2O$	-	10.00
$FeSO_4.7H_2O$	27.80	27.80
Vitamins		
Thiamine.HCl	0.10	1.00
Pyridoxine.HCl	0.50	1.00
Nicotinic acid	.05	-
Others		
Glycine	2.00	-
Myo-inositol	100.00	100.00
Sucrose	30000.00	30000.00
pH	5.8	5.8

3.6.1.2 Influence of solidifying agent on ovule development

Influence of different concentrations of agar (Sisco Research Laboratories, India) (6.0, 6.5, 7.0, 7.5 g l^{-1}) and phytagel (Himedia Laboratories, India) (1.4, 1.6, 1.8, 2.0 g l^{-1}) on ovule development were studied

3.6.1.3 Influence of organic supplements on ovule development

The effect of organic supplements added to the culture medium was studied. The organic supplements tried include milk protein (100 mg l^{-1}) and tender coconut water (10 per cent).

3.6.1.4 Influence of auxins and cytokinins on ovule development

Influence of auxins ie picloram, 2,4-D, NAA, IAA alone and in combination with different cytokinins on ovule development were studied (Table. 5).

3.6.2 Influence of parental combination on ovule development

Influence of parental combinations - autotetraploid X autotetraploid, autotetraploid X diploid, diploid X autotetraploid and diploid X diploid - on ovule development was studied

3.7 VIABILITY TEST OR TETRAZOLIUM TEST

Viability of the seeds incubated in the media combination of half MS + sucrose (3%) + phytagel (0.18%) + picloram (0.2 mg l^{-1}) + BA (2.5 mg l^{-1}) was tested at 10, 20, 30, 40 and 50 DAP with tetrazolium (2,3,5 triphenoxy tetrazolium chloride). The seeds were first soaked in distilled water for 24 h. Then they were longitudinally cut into two halves under a dissection microscope, immersed in 0.1 per cent tetrazolium solution of pH 6.5 to 7.0 and kept in dark at 38^0 C until satisfactory staining was obtained (2 to 3h). Then they were taken out from tetrazolium, immersed in water and observed under stereomicroscope and photomicrographs were taken.

Table. 5. Auxins alone and in combination with cytokinins (mg l^{-1}) tried for ovule development after *in vitro* placental pollination

Sl. no.	Growth regulators in mg l^{-1}
1	2,4 D 0.2
2	2,4 D 1.0
3	2,4 D 2.0
4	2,4 D 3.0
5	2,4 D 5.0
6	2,4 D 0.2 + kin 2.0
7	2,4 D 0.2 + BA 2.5
8	2,4 D 0.2 + BA 3.0
9	Picloram 0.1 + BA 2.5
10	Picloram 0.2 + BA 2.5
11	Picloram 0.3 + BA 2.5
12	Picloram 0.5 + BA 2.5
13	Picloram 1.0 + BA 2.5
14	NAA 0.5 + kin 2.0
15	NAA 0.5 + BA 2.5
16	NAA 1.0 + BA 3.0
17	IAA 0.1 + BA 2.5
18	IAA 0.5 + BA 2.5
19	IAA 0.2 + kin 2.0

3.8 SEED GERMINATION STUDIES

Influence of different media combinations on the germination of the seeds 60 to 80 days after *in vitro* pollination was studied. The seeds for the germination studies were initially incubated in media combination of half MS + 3 per cent sucrose + 0.65 per cent agar + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}. Observations on germination were recorded at 30, 60 and 90 days of inoculation.

3.8.1 Influence of thiourea on seed germination

Influence of thiourea alone (1.0, 2.0 mg l^{-1}) or in combination with auxins and cytokinins (BA 1.0 mg l^{-1} + NAA 0.2 mg l^{-1}) was studied. The cultures were incubated in dark at 26 $\pm$ 2^0 C and subcultured at 21 days interval.

3.8.2 Influence of IBA on seed germination

Effect of IBA alone (1.0, 2.0, 3.0, 4.0, 5.0 mg l^{-1}) was studied. The cultures were incubated in dark at 26 $\pm$ 2^0 C and subcultured at 21 days interval.

3.8.3 Influence of GA_3 on seed germination

Effect of GA_3 in combination with different auxins and cytokinins was studied. The growth regulator combinations are given below:

- GA_3 0.5 mg l^{-1}
- GA_3 2.0 mg l^{-1} + BA 1.0 mg l^{-1} + NAA 0.2 mg l^{-1}
- GA_3 5.0 mg l^{-1} + 2,4-D 0.5 mg l^{-1} + BA 6.0 mg l^{-1}
- GA_3 5.0 mg l^{-1} + 2,4-D 0.5 mg l^{-1} + NAA 0.2 mg l^{-1} + thiourea 2.0 mg l^{-1}
- GA_3 5.0 mg l^{-1} + IBA 3.0 mg l^{-1}

The cultures were incubated in dark at 26$\pm$5^0 C and subcultured at 21 days interval.

3.8.4 Influence of combinations of auxins and cytokinins on seed germination

Influence of different combinations of auxins and cytokinins on seed germination was studied (Table. 6). Subculturing was done at 21 days interval.

Table 8. Growth regulator combinations tried for inducing direct organogenesis from embryo along with endosperm

Sl. no.	Growth regulators (mg l^{-1})	Incubation condition
1	IBA 3.0	Dark
2	NAA 0.5 + BA 2.5	Light
3	NAA 1.0 + BA 1.0	Dark
4	Picloram 0.2 + BA 2.5	Dark
5	Picloram 0.2 + BA 6.0 + GA_3 5.0	Light
6	2,4-D 0.2 + BA 9.0	Light
7	2,4-D 0.2 + BA 10.0	Light
8	2,4-D 0.2 + BA 1.0 + kin 1.0	Dark
9	IAA 0.2 + BA 4.0	Light
10	IAA 0.2 + BA 1.0 + kin 1.0	Dark
11	BA 3.0	Light
12	BA 8.0	Light
13	TDZ 2.2	Light

Basal medium: Half MS +3 % sucrose

No.of replications : four

Table 6. Combinations of auxins and cytokinins tried for seed germination

Sl. no.	Basal medium	Growth regulators (mg l^{-1})	Conditions	Age of seed
1	Half MS	IBA 3.0 + BA 1.0	Dark	60-80 days
2	Half MS	NAA 0.5 + BA 2.5	Dark	60-80 days
3	Half MS	2,4 D 0.2 + BA 5.0	Light	60-80 days
4	Half MS	2,4 D 0.1 + BA 9.0	Light	60-80 days
5	Half MS	2,4 D 0.2 + BA 2.5	Dark	15-30 days
6	B_5	2,4 D 0.2 + BA 2.5	Dark	15-30 days
7	Half MS	Picloram 0.2 + BA 2.5	Dark	15-30 days

3.9 EMBRYO CULTURE

During first year, embryo culture studies were done with embryo and endosperm excised from seeds of 40 to 60 DAP and incubated in different media combinations involving auxins, cytokinins and GA_3 as given in Table 7. Observations were recorded after 90 days of inoculation.

During second year embryo was excised along with endosperm from seeds of 15 to 30 DAP and cultured in various media combinations involving different growth regulators like 2,4-D, IAA, NAA, IBA, picloram, BA, kinetin, TDZ, GA_3 and adenine to get direct or indirect organogenesis (Table 8 and 9). Observations were recorded after 40 days of inoculation.

Table 7. Growth regulator combinations tried to induce indirect organogenesis from embryo along with endosperm (40 to 60 DAP)

Sl.no.	Basal medium	Growth regulators (mg l^{-1})	Incubation condition
1	Half MS	2,4-D 1.0	Dark
2	Half MS	2,4-D 2.0	Dark
3	Half MS	2,4-D 3.0	Dark
4	Half MS	2,4-D 4.0	Dark
5	Half MS	2,4-D 5.0	Dark
6	Half MS	2,4-D 8.0	Dark
7	Half MS	2,4-D 10.0	Dark
8	Half MS	2,4-D 20.0	Dark
9	Half MS	2,4-D 1.0 + BA 0.25	Dark
10	Half MS	2,4-D 2.0 + BA 2.0	Dark
11	Half MS	2,4-D 1.0 + BA 0.5	Dark
12	Half MS	2,4-D 1.0 + BA 0.25	Dark
13	½ MS+B_5 vitamins	2,4-D 0.2 + kin0.5	Dark
14	Half MS	2,4-D 0.2 + kin 1.0 + BA1.0	Dark
15	Half MS + 6% sucrose	2,4-D 1.0 + PVP 500 + glutamine 400 + ascorbic acid 100	Dark
16	Half MS	NAA 3.0 + BA 1.0	Dark
17	Half MS	NAA 1.0 + BA 1.0	Dark
18	½ MS+B_5 vitamins	IBA 0.5 + kin 0.5	Dark
19	Half MS	IAA 0.2 + BA 4.0	Dark
20	Half MS	IAA 0.2 + BA 1.0 + kin1.0	Dark
21	Half MS	TDZ 2.0	Light

No. of replications: six

Table 8. Growth regulator combinations tried for inducing direct organogenesis from embryo along with endosperm

Sl. no.	Growth regulators (mg l^{-1})	Incubation condition
1	IBA 3.0	Dark
2	NAA 0.5 + BA 2.5	Light
3	NAA 1.0 + BA 1.0	Dark
4	Picloram 0.2 + BA 2.5	Dark
5	Picloram 0.2 + BA 6.0 + GA_3 5.0	Light
6	2,4-D 0.2 + BA 9.0	Light
7	2,4-D 0.2 + BA 10.0	Light
8	2,4-D 0.2 + BA 1.0 + kin 1.0	Dark
9	IAA 0.2 + BA 4.0	Light
10	IAA 0.2 + BA 1.0 + kin 1.0	Dark
11	BA 3.0	Light
12	BA 8.0	Light
13	TDZ 2.2	Light

Basal medium: Half MS +3 % sucrose

No.of replications : four

Table 9. Growth regulator combinations tried to induce indirect organogenesis from embryo along with endosperm (15 to 30 DAP)

Sl.no.	Basal medium	Growth regulators (mg l^{-1})	Incubation condition
1	Half MS	IAA 0.1	Dark
2	Half MS	IAA 0.2 + BA 4.0	Light
3	Half MS liquid	NAA 1.0 + BA 1.0	Dark
4	Half MS liquid	NAA 3.0 + BA 1.0	Dark
5	Half MS	NAA 1.0 + kin 1.0	Dark
6	½ MS+B_5 vitamins	2,4-D 2.0	Dark
*7	½ MS+2% sucrose	2,4-D 4.0	Dark
*8	Half MS	2,4-D 8.0	Dark
*9	Half MS	2,4-D 12.0	Dark
10	Half MS liquid	2,4 D 1.0 + BA 0.5	Light
11	Half MS	2,4-D 0.2 + BA 5.0	Light
12	Half MS	2,4 D 2.0 + kin 3.0	Light
13	Half MS	2,4-D 0.2+ kin 0.3+IBA 0.2 + NAA 0.3 + GA_3 0.5	Dark
14	Half MS	2,4-D 0.2 + kin 0.3 + IBA 0.2+ picloram 0.1+GA_3 0.5	Light
15	Half MS	2,4-D 0.5 + picloram 2.2	Dark
16	½ MS+6% sucrose	2,4-D 4.0 + TDZ 0.02	Dark
17	Half MS	kin 6.0 + adenine 200	Light
18	Half MS	TDZ 2.2	Light

No. of replications: four

* Pulse treatment for one week

Results

4. RESULTS

The results of the investigations on "Induction of genetic variability in ginger (*Zingiber officinale* Rosc.) through *in vitro* fertilization" are described in this chapter under the following headings:

1. Molecular characterization of the parent plants, used for *in vitro* crossing.
2. Flowering behaviour.
3. Pollen studies.
4. Refinement of *in vitro* pollination technique.
5. Seed viability test.
6. Seed germination studies.
7. Embryo culture.

4.1 MOLECULAR CHARACTERIZATION OF PARENT PLANTS

4.1.1 Isolation of DNA

From a composite sample of third and fourth leaf from top, DNA was isolated using Doyle and Doyle method (1987). The quality of DNA was checked by electrophoresis and the presence of an intact discrete band indicated non-degraded DNA (Plate 1).

4.1.2 Screening of random primers for RAPD

Fifteen random primers were screened with the selected PCR reaction mixture and thermal settings.

4.1.2.1 OPE series

Table 10 shows results of screening of six primers under OPE series. Number of amplicons among the primers tested varied from two to six amplicons. Out of these, OPE 11 and 12 gave good amplification with six amplicons. The performance

of OPE 3, 6 and 7 were average. From this series OPE 3, 7, 11 and 12 were selected for further studies (Plate 2).

4.1.2.2 OPF series

Table 11 shows the results of screening with nine primers in OPF series. No. of amplicons varied from zero to nine (Plate 3). Five primers viz., OPF 1, 3, 5, 6 and 7 gave good amplification with 9, 9, 9, 9 and 7 amplicons respectively. OPF 2 and 10 gave average amplification. The primers OPF 1, 3, 5, 6 and 7 were selected for further analysis.

4.1.3 Screening of ginger cutivars with selected primers

The genomic DNA extracted through Doyle and Doyle method (1987) was amplified using nine primers selected from the initial screening. The details of amplification products obtained and the number of polymorphic and monomorphic bands are given in Table 12 and 13 respectively. The ginger cultivars screened include Kodakara Local (V_3), Kuruppampady (V_4), Mahima (V_5), Maran (V_6), Rejatha (V_7), Rio-de-janerio (V_8), Varadha (V_9) and Z-0-78 (V_1). Plates 4 and 5 show the amplification pattern obtained after the gel electrophoresis of RAPD products.

In Z-0-78, certain bands were absent when amplified with OPE 12, OPE 3, OPE 5 and OPF 7, while those bands were present in other cultivars.

4.1.4 Cluster analysis

From the dendrogram constructed using the pooled data of the RAPD scores with nine different primers, the variation recorded ranged from 5 to 19 per cent. All the cultivars except Rio-de-Janeiro formed a cluster with a total variability of around 11 per cent. The cultivar Rio-de-Janeiro was more or less distinct with maximum variability (19%). The variability among the released varieties from IISR ranged from 5 to 10 per cent.

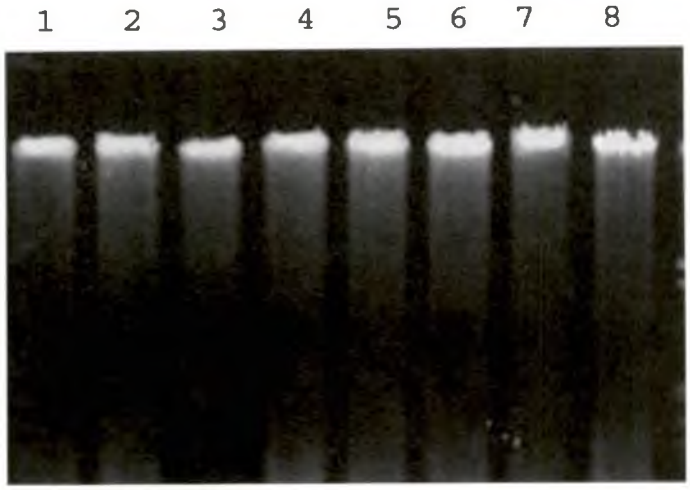


Plate 1. Genomic DNA of ginger cultivars

Lane 1. Kodakara local (V3), 2. Kuruppampady (V4), 3. Mahima (V5), 4. Maran (V6), 5. Rejatha (V7), 6. Rio-de-Janeiro (V8), 7. Varadha (V9), 8. Z-0-78 (V1).

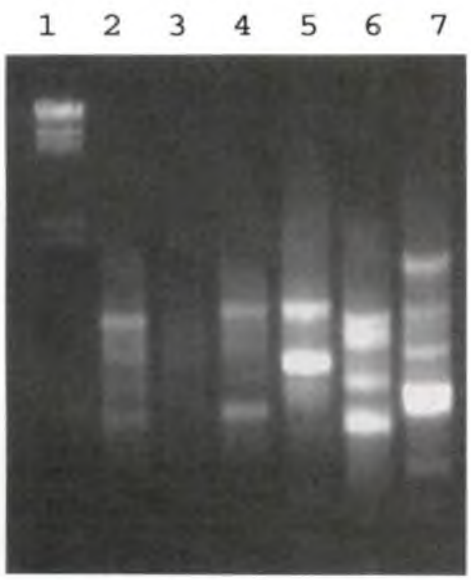


Plate 2. Screening of random primers under OPE series for RAPD

Lane 1. Molecular weight marker: λ DNA/ Eco R I / Hind III, 2. OPE 3, 3. OPE 5, 4. OPE 6, 5. OPE 7, 6. OPE 11, 7. OPE 12

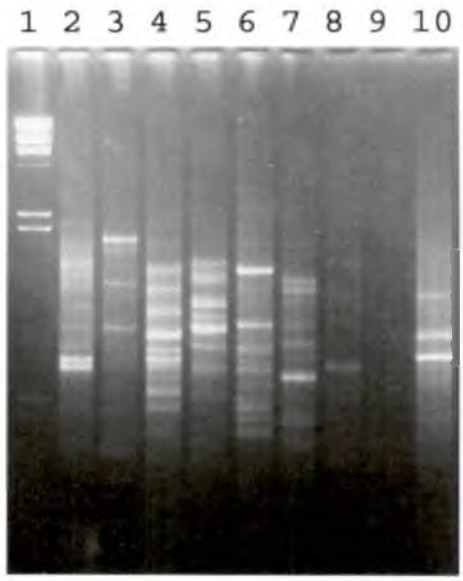


Plate 3. Screening of random primers under OPF series for RAPD

Lane 1. Molecular weight marker: λ DNA/ Eco R I / Hind III, 2. OPF 1, 3. OPF 2, 4. OPF 3, 5. OPF 5, 6. OPF 6, 7. OPF 7, 8. OPF 8, 9. OPF 9,10. OPF 10

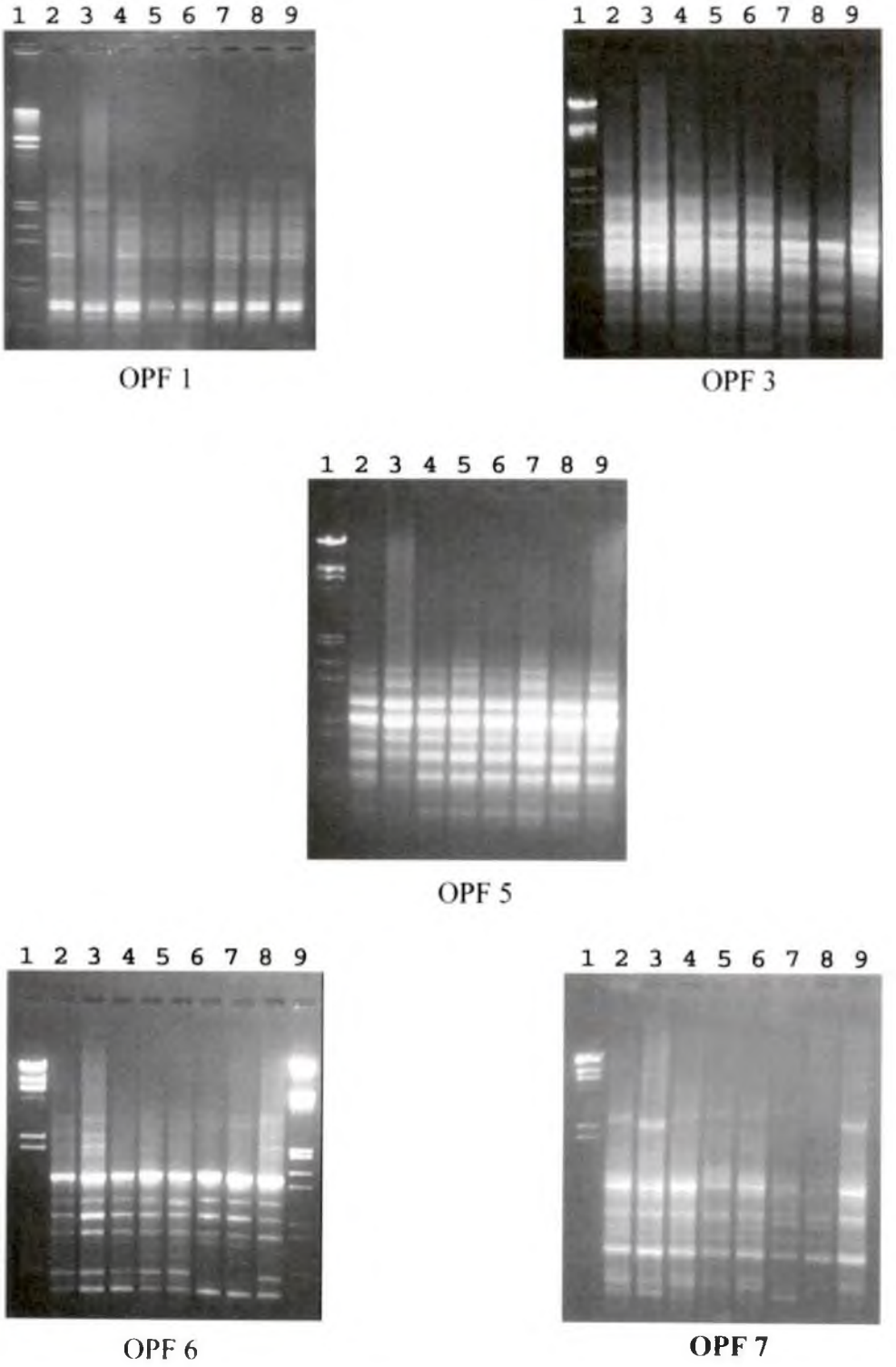
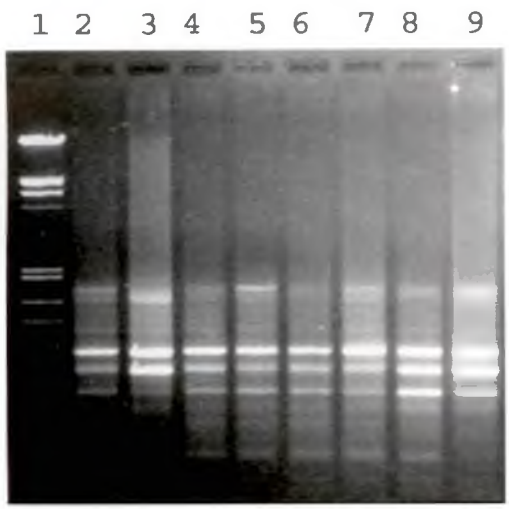
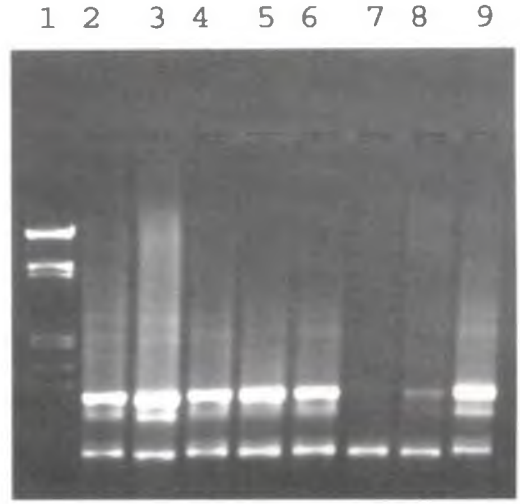


Plate 4. Screening of ginger cultivars with selected OPF primers by RAPD.

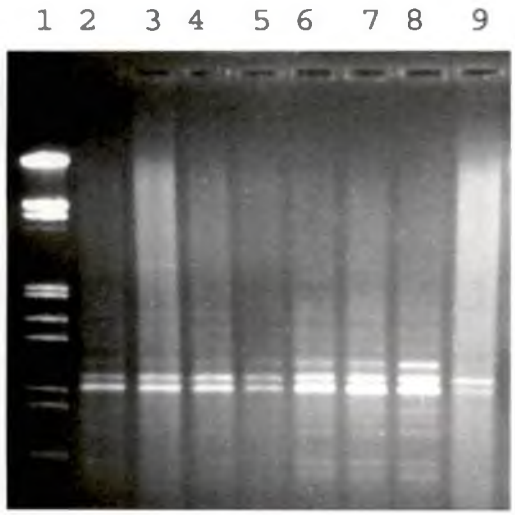
Lane 1. Molecular weight marker: λ DNA/Eco R I /Hind III,
2. Kodakara local (V3), 3. Kuruppampady (V4), 4. Mahima (V5),
5. Maran (V6),6. Rejatha (V7), 7. Rio-de-Janeiro (V8), 8. Varadha (V9),
9. Z-0-78 (V1).



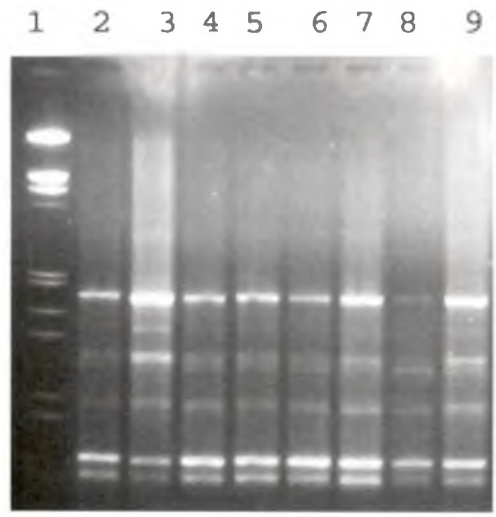
OPE 3



OPE 7



OPE 11



OPE 12

Plate 5. Screening of ginger cultivars with selected OPE primers by RAPD.

Lane 1. Molecular weight marker: λ DNA/Eco R I /Hind III,
2. Kodakara local (V3), 3. Kuruppampady (V4), 4. Mahima (V5), 5. Maran (V6),
6. Rejatha (V7), 7. Rio-de-Janeiro (V8), 8. Varadha (V9), 9. Z-0-78 (V1).

Table 10. Amplification pattern of ginger genomic DNA with different decamer primers under OPE series

Primer code	Primer sequence	No. of amplicons	Quality of amplification
OPE-3	CCAGATGCAC	4	Average
OPE-5	GCAGGGAGGT	2	Poor
OPE-6	AAGACCCCTC	4	Average
OPE-7	AGATGCAGCC	5	Average
OPE-11	GAGTCTCAGG	6	Good
OPE-12	TTATCGCCCC	6	Good

Good: > 5, Average: 4-5 and poor: < 4amplicons

Table 11. Amplification pattern of ginger genomic DNA with different decamer primers under OPF series

Primer code	Primer sequence	No. of amplicons	Quality of amplification
OPF 1	ACGGATCCTG	9	Good
OPF 2	GAGGATCCCT	4	Average
OPF 3	CCTGATCACC	9	Good
OPF 5	CCGAATTCCC	9	Good
OPF 6	GGGAATTCGG	9	Good
OPF 7	CCGATATCCC	7	Good
OPF 8	GGGATATCGG	1	Poor
OPF 9	CCAAGCTTCC	0	Poor
OPF 10	GGAAGCTTGG	4	Average

Good: > 5, Average: 4-5 and poor: < 4amplicons

Table 12. No. of amplicons obtained for each cultivar after PCR using the selected primers

Primer / Cultivar	OPE 3	OPE 7	OPE 11	OPE 12	OPF 1	OPF 3	OPF5	OPF 6	OPF 7
V_4	7	6	9	7	14	10	13	11	10
V_5	7	6	7	7	14	10	13	11	11
V_6	7	6	9	7	13	10	13	8	8
V_7	7	6	9	7	13	10	14	9	7
V_8	7	6	12	6	13	10	13	9	8
V_9	7	6	12	7	14	7	14	6	7
V_1	7	4	12	6	14	5	13	7	6
V_{10}	7	7	2	7	14	8	13	12	9

The cultivars Kodakara local and Mahima showed 95 per cent similarity in RAPD pattern. In conclusion, the cluster analysis revealed not much variability in the genetic make up of the ginger cultivars evaluated (Fig.1).

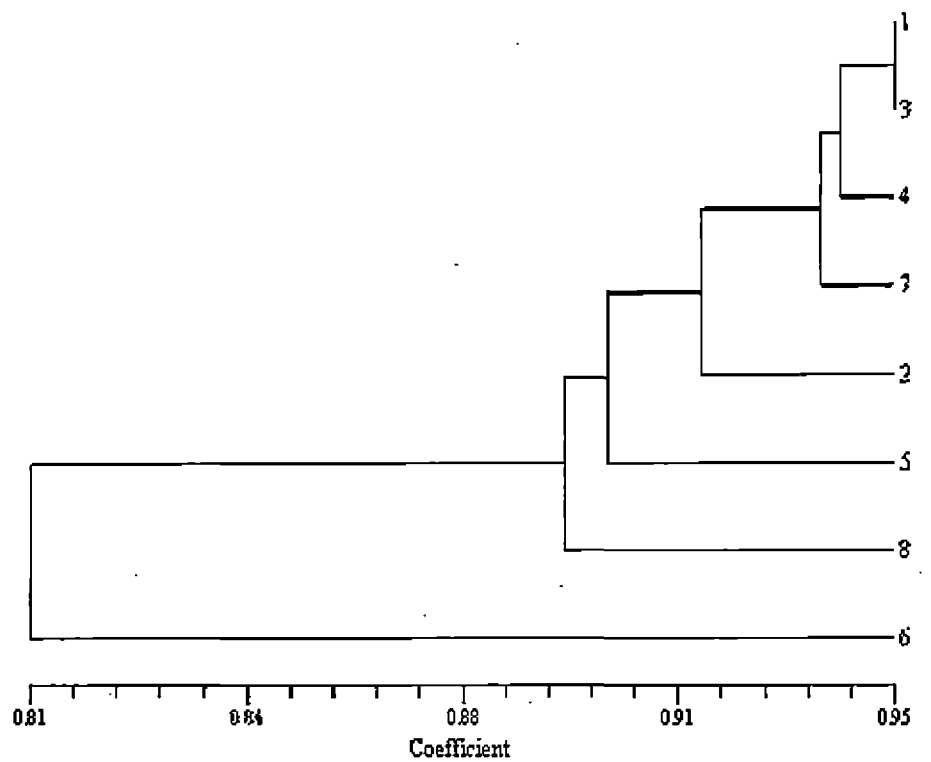


Fig 1. Dendrogram showing clusters of ginger cultivars after RAPD analysis.

1. Kodakara local (V_3), 2. Kuruppampady (V_4), 3. Mahima (V_5), 4. Maran (V_6), 5. Rejatha (V_7), 6. Rio-de-Janeiro (V_8), 7. Varadha (V_9), 8. Z-0-78 (V_1).

4.2 FLOWERING BEHAVIOUR

The observation on variability in flowering behaviour of ginger cultivars under study is presented in Table 14. The flowering season in ginger extended from August to November. Out of the nine cultivars, Kuruppampady was found to be the earliest flowering type (133 days after planting) and the cultivar Rio-de-Janeiro flowers late (169 days after planting). The flowering duration ranged from 31(Kodakara local) to 66 days (Z-0-78). The flowering duration of autotetraploids ranged from 54 (Z-0-86) to 66 days (Z-0-78). Among the diploids Rio-de-Janeiro had maximum flowering duration (56 days). Plates 6,7 and 8 show ginger plant at flowering, ginger inflorescence and ginger flower respectively.

Table 14. Variability in flowering behaviour of the selected ginger cultivars

Sl.no.	Name of the cultivar	Days for initiation of flowering (days)	Date of first flower	Date of last flower	Flowering duration (days)
1	Z-0-78	142	02-09-04	08-11-04	66
2	Z-0-86	162	20-10-04	30-11-04	54
3	Kodakara local	139	15-10-04	16-11-04	31
4	Kuruppampady	133	29-09-04	10-11-04	41
5	Mahima	142	02-09-04	11-10-04	39
6	Maran	134	30-08-04	05-10-04	36
7	Rejatha	148	08-09-04	04-11-04	37
8	Rio-de-Janeiro	169	02-10-04	09-11-04	56
9	Varadha	156	16-09-04	24-10-04	38
	Mean	149.22			44.22

Plants from 15-20g rhizome bits.

Average of five observations.

Plate 6. Ginger cultivar Varadha at flowering

Plate 7. Inflorescence of ginger

Plate 8. Opened flower on the day of anthesis

4.3 POLLEN STUDIES

Pollen fertility of nine ginger cultivars was tested by staining with acetocarmine (Plate 9). Pollen viability was examined in ME_3 medium after 24 h of incubation (Plate 10). Mean pollen fertility and viability were found to be 35.76 percent and 8.11 per cent respectively. The autotetrapolid Z-0-78 had maximum pollen fertility (52.44 %) and the lowest fertility was recorded with Rio-de-Janeiro (20.10%). With respect to pollen viability also, Z-0-78 exhibited the maximum of 15.68 per cent. The cultivar Kuruppampady showed the lowest pollen viability of 3.68 per cent (Table 15). Among the autotetraploids the pollen fertility ranged from 49.63 (Z-0-86) to 52.44 per cent (Z-0-78) and among the diploids it ranged from 20.10 (Rio-de-Janeiro) to 41.02 per cent (Varadha). The range of pollen viability was from 12.03 (Z-0-86) to 15.68 (Z-0-78) in autotetraploids and 3.68 (Kuruppampady) to 8.90 per cent (Varadha) in diploids.

Table 15. Polllen fertility and viability of ginger cultivars

Sl. no.	Name of cultivar	Pollen fertility (%)	Pollen viability (%)
1	Z-0-78	52.44	15.68
2	Z-0-86	49.63	12.03
3	Kodakara local	28.33	5.42
4	Kuruppampady	25.03	3.68
5	Mahima	40.52	8.66
6	Maran	24.07	4.74
7	Rejatha	40.72	8.13
8	Rio-de-Janeiro	20.10	5.76
9	Varadha	41.02	8.90
	Mean	35.76	8.11

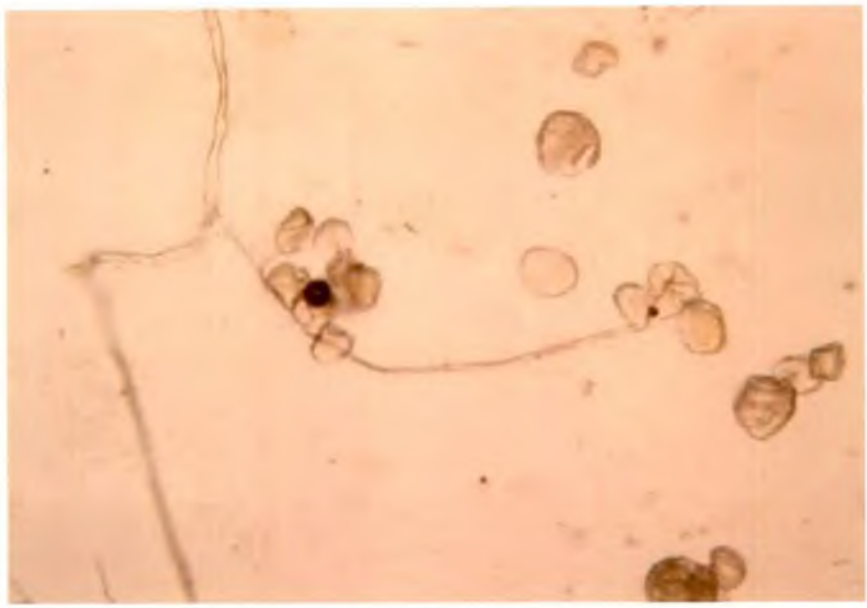


Plate 9. Pollen germination and tube growth in ginger (Varadha) (40X)

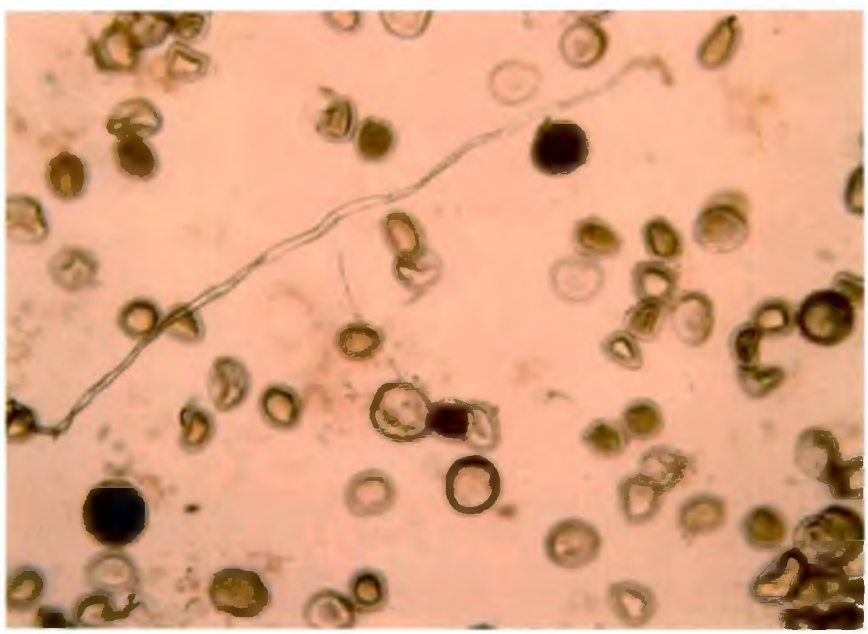


Plate 10. Pollen germination and tube growth in ginger (Z-0-78) (40X)

4.4 REFINEMENT OF *IN VITRO* POLLINATION TECHNIQUE

In vitro placental pollination was done among the autotetraploids and diploids (Plate 11). The development of the ovule after *in vitro* pollination was studied and the results obtained are as follows.

4.4.1 Experiments for refinement of media for ovule development

4.4.1.1 Basal medium for culture establishment and ovule development

Attempts were made to refine the standardized media (Valsala, 1994) with respect to salt and vitamin concentration. The parental combination selected for the study was Varadha X Z-0-78. Out of the four media combinations tried, half MS with double the quantity of vitamin stock + BA 2.5 mgl^{-1}+ NAA 0.5 mgl^{-1}, was the best recording very good ovule swelling (++++) in 88.88 per cent cultures with 79.44 per cent setting per culture. The same medium with normal quantity of vitamins produced ovules with good swelling (+++). The substitution of MS vitamins with B_5 vitamins increased the percentage of cultures with ovule swelling (+++). But in B_5 medium, ovule development was moderate (++) (Table 16).

4.4.1.2 Influence of solidifying agent in the medium on ovule development

Influence of agar and phytagel as solidifying agents in the basal medium of half MS + 3 per cent sucrose + BA 2.5 mg l^{-1} + picloram 0.2 mg l^{-1} on ovule development after *in vitro* pollination was studied (Table 17 and 18). Varying concentrations of phytagel from 0.14 to 2.0 per cent along with growth regulators were tried and it was found that phytagel at 0.18 per cent was the best with respect to ovule swelling (++++) and percentage setting per culture (88.88%).

Influence of varying concentrations of agar (0.60 to 0.75 %) as solidifying agent on ovule development was studied. The performance was best at a concentration of 0.65 per cent recording good ovule swelling (+++) and 83.33 per cent setting per culture. Phytagel (0.18 %) was found to be superior to agar as solidifying agent in the medium for ovule development.

Table 16. Basal medium for culture establishment of ginger ovules after *in vitro* pollination

Sl. No.	Media combination	Ovule swelling	Cultures with setting (%)	% Setting per culture
1	Half MS + 3% sucrose + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}	+++	77.77	80.83
2	Half MS with double quantity of vitamin stock + 3% sucrose + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}	++++	88.88	79.44
3	B_5 + 3% sucrose + BA 2.5 mg l^{-1} NAA 0.5 mg l^{-1}	++	61.11	73.33
4	Half strength MS macro and micro nutrients + B_5 vitamins + BA 2.5 mg l^{-1} NAA 0.5 mg l^{-1}	+++	83.33	80.28
	Mean		77.77	78.35

Method of *in vitro* pollination: placental

Average of 18 observations 20 DAP

Scoring: '++++' very good; '+++' good; '++' moderate; '+' low; '-' nil

Parental combination: Varadha X Z-0-78

Table 17. Influence of varying concentrations of phytagel on ovule development after *in vitro* pollination

Sl. No.	Treatment	Ovule swelling	% Setting per culture
1	0.20% phytagel	++	83.05
2	0.18% phytagel	++++	88.88
3	0.16% phytagel	+++	88.61
4	0.14% phytagel	++	82.22
	Mean		85.69

Basal medium - Half MS + 3% sucrose + BA 2.5 mg l^{-1} + picloram 0.2 mg l^{-1}
Method of *in vitro* pollination: placental
Average of 18 observations 20 DAP.
Scoring: '++++' very good; '+++' good; '++' moderate; '+' low; '-' nil
Parental combination: Varadha X Z-0-78

Table 18. Influence of varying concentrations of agar on ovule development after *in vitro* pollination

Sl. No.	Treatment	Ovule swelling	% Setting per culture
1	0.75 % agar	++	78.33
2	0.70 % agar	+++	82.22
3	0.65 % agar	+++	83.33
4	0.60 % agar	++	75.55
	Mean		79.86

Basal medium - Half MS + 3% sucrose + BA 2.5 mg l^{-1} + picloram 0.2 mg l^{-1}
Average of 18 observations 20 DAP.
Scoring: '++++' very good; '+++' good; '++' moderate; '+' low; '-' nil
Method of *in vitro* pollination: placental
Parental combination: Varadha X Z-0-78

4.4.1.3 Influence of organic supplements on ovule development

Inclusion of milk protein (100 mg l^{-1}) in the medium of half MS + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1} did not influence ovule development. Inclusion of coconut water (15%) in the aforesaid basal medium supported ovule development.

4.4.1.4 Influence of auxins and cytokinins on ovule development

4.4.1.4.1 Influence of picloram in combination with BA on ovule development

Influence of varying concentrations of picloram (0.1 to 1.0 mg l^{-1}) in combination with BA (2.5 mg l^{-1}) in the basal medium of half MS with 2X vitamins + 3 per cent sucrose + 0.18 per cent phytagel on ovule development after *in vitro* pollination was studied (Table 19). The parental combination selected for the study was Varadha X Z-0-78. Best results were obtained in the combination of picloram 0.2 mg l^{-1} + BA 2.5 mg l^{-1} recording very good ovule swelling (++++) in 97.22 per cent cultures with a setting percentage of 88.33 per culture. Picloram at 0.3 mg l^{-1} concentration also recorded good ovule swelling (+++) in 94.44 per cent cultures with 84.72 per cent setting per culture. The concentration of picloram above 0.5 mg l^{-1} negatively influenced ovule development. Reducing the concentration of picloram from 0.2 to 0.1 mg l^{-1} also reduced ovule swelling.

4.4.1.4.2 Influence of NAA on ovule development in combination with cytokinins

Influence of varying concentrations of NAA 0.5 to 1.0 mg l^{-1} along with BA 2.5 to 3.0 mg l^{-1} was studied (Table 20). The media combination of half MS with 2X vitamins + 3 per cent sucrose + 0.18 per cent phytagel + NAA 0.5 mg l^{-1} + BA 2.5 mg l^{-1} was the best, recording good ovule swelling (+++) in 88.88 per cent cultures with 80.83 per cent setting per culture. Increasing the concentration of NAA from 0.5 to 1.0 mg l^{-1} and concentration of BA from 2.5 to 3.0 mg l^{-1} reduced ovule swelling. Replacement of BA (2.5 mg l^{-1}) with kinetin (2.0 mg l^{-1}) gave good ovule swelling (+++).

Table 19. Influence of Picloram in combination with BA on ovule development after *in vitro* pollination

Sl. No.	Growth regulators in mg l^{-1}	Ovule swelling	Cultures with setting (%)	% Setting per culture
1	Picloram 0.1 + BA 2.5	++	88.88	79.72
2	Picloram 0.2 + BA 2.5	++++	97.22	88.88
3	Picloram 0.3 + BA 2.5	+++	94.44	84.72
4	Picloram 0.5 + BA 2.5	++	77.77	65.83
5	Picloram 1.0 + BA 2.5	+	66.66	65.00
	Mean		84.99	76.72

Basal medium - Half MS with 2X vitamins + 3% sucrose + 0.18% phytagel.

Method of *in vitro* pollination: placental

Average of 18 observations 20 DAP.

Scoring: '++++' very good; '+++' good; '++' moderate; '+' low; '-' nil

Parental combination: Varadha X Z-0-78

Table 20. Influence of NAA in combination with cytokinins on ovule development after *in vitro* pollination

Sl. No.	Growth regulators in mg l^{-1}	Ovule swelling	Cultures with setting (%)	% Setting per culture
1	NAA 0.5 + BA 2.5	+++	88.88	80.83
2	NAA 1.0 + BA 3.0	++	83.33	76.66
3	NAA 0.5 + Kinetin 2.0	+++	77.77	66.94
	Mean		83.33	74.81

Basal medium - Half MS with 2X vitamins + 3% sucrose + 0.18% phytagel.
Method of *in vitro* pollination: placental
Average of 18 observations 20 DAP.
Scoring: '++++' very good; '+++' good; '++' moderate; '+' low; '-' nil
Parental combination: Varadha X Z-0-78

4.4.1.4.3 Influence of 2,4 D alone or in combination with BA on ovule development

Influence of 2,4 D alone at concentrations ranging from 0.2 to 5.0 mg l^{-1} was studied in the basal medium of half MS with 2X vitamins + 3 per cent sucrose + 0.65 per cent agar (Table 21). The 2,4-D alone in the medium recorded low or no ovule development. The percentage of cultures with seed set ranged from 16.66 (2,4-D 5.0 mg l^{-1}) to 61.08 (2,4-D 0.2 mg l^{-1}) and the range of percentage setting was from 3.33 (2,4-D 5.0 mg l^{-1}) to 63.61 (2,4-D 0.2 mg l^{-1}).

Combinations of 2,4-D (0.2 mg l^{-1}) with BA 2.0 to 3.0 mg l^{-1} in the aforesaid basal medium were tried for ovule development (Table 21). The combination of 2,4-D 0.2 mg l^{-1} and BA 2.5 mg l^{-1} resulted in moderate ovule swelling (++) in 77.73 per cent cultures with a setting percentage of 77.77 per culture. Increasing or decreasing the concentration of BA from 2.5 mg l^{-1} to 3.0 and 2.0 mg l^{-1} respectively in the aforesaid combination also caused moderate ovule swelling, but the percentage setting per culture and the number of cultures showing ovule development reduced.

Table 21. Influence of 2,4 D alone or in combination with cytokinins on ovule development after *in vitro* pollination

Sl. No.	Growth regulators in mg l^{-1}	Ovule swelling	Cultures with setting (%)	% Setting per culture
1	2,4 D 0.2	+	61.08	63.61
2	2,4 D 1.0	+	49.96	61.66
3	2,4 D 2.0	+	39.99	52.22
4	2,4 D 3.0	-	16.66	5.55
5	2,4 D 5.0	-	16.66	3.33
6	2,4 D 0.2 + BA 3.0	++	72.22	67.50
7	2,4 D 0.2 + BA 2.5	++	77.73	77.77
8	2,4 D 0.2 + BA 2.0	++	51.06	52.22
	Mean		60.42	47.98

Basal medium - Half MS with 2X vitamins + 3% sucrose + 0.18% phytogel.

Method of *in vitro* pollination: placental

Average of 18 observations 20 DAP.

Scoring: '++++' very good; '+++' good; '++' moderate; '+' low; '-' nil

Parental combination: Varadha X Z-0-78

4.4.1.4.4 Influence of IAA on ovule development in combination with cytokinins

The influence of varied concentrations of IAA (0.2 and 0.5 mg l^{-1}) in combination with BA (2.5 mg l^{-1}) in the basal medium of half MS + 3 per cent sucrose + 0.18 per cent phytagel on ovule development was studied. The two combinations tried gave moderate ovule swelling (++) in 72.22 per cent cultures with 66.39 to 67.50 per cent setting per

culture (Table 22). The other combination of IAA (0.1 mg l^{-1}) with kinetin (2.0 mg l^{-1}) also caused moderate ovule swelling (++).

Table 22. Influence of IAA in combination with cytokinins on ovule development after *in vitro* pollination

Sl. No	Growth regulators in mg l^{-1}	Ovule swelling	Cultures with setting (%)	% Setting per culture
1	IAA 0.5 + BA 2.5	++	72.22	66.39
2	IAA 0.2 + Kinetin 2.0	++	72.22	67.50
3	IAA 0.1 + BA 2.5	++	83.33	61.66
	Mean		75.92	65.18

Basal medium - Half MS + 3% sucrose + 0.18% phytogel.
Method of *in vitro* pollination: placental
Average of 18 observations 20 DAP.
Scoring: '++++' very good; '+++' good; '++' moderate; '+' low; '-' nil
Parental combination: Varadha X Z-0-78

4.4.2 Influence of parental combination on ovule development

The influence of crossing between ginger cultivars on ovule development was studied (Table 23). The parental combination between autotetraploids (Z-0-78 and Z-0-86) produced seeds with very good swelling (++++). The percentage of cultures with setting (90.74 to 98.15%) and percentage setting per culture (87.03 to 94.44%) were the highest in this combination. The parental combination Z-078 X Varadha and its reciprocal also favoured very good ovule swelling (++++) in 83.33 to 88.88 per cent cultures with a setting percentage of 83.33 to 90.74 per culture. The parental combination Z-0-86 X Varadha and its reciprocal produced seeds with good swelling (+++) in 87.03 to 88.88 per cent cultures with 77.77 to 87.03 percent setting per culture. The cross between diploids Varadha and Mahima produced seeds with good development (+++) in 75.92 to 83.33 cultures with a setting percentage of 77.77 to 88.88 per culture. But the cross between the diploids Rejatha as the female parent and Kuruppampady as the male parent yielded ovules with moderate swelling (++). For this parental combination, the percentage of

cultures with setting (66.66%) and percentage setting per culture (72.22%) were the lowest.

Table 23. Influence of parental combination on ovule development after *in vitro* pollination

Sl.no.	Parental combination	Ovule swelling	Cultures with setting (%)	% Setting per culture
1	*Autotetraploid X Autotetraploid*			
	Z-0-78 X Z-0-86	++++	98.15	94.44
	Z-0-86 X Z-0-78	++++	90.74	87.03
2	*Autotetraploid X Diploid*			
	Z-0-78 X Varadha	++++	83.33	83.33
	Z-0-86 X Varadha	+++	88.88	77.77
3	*Diploid X Autotetraploid*			
	Varadha X Z-0-78	++++	88.88	90.74
	Varadha X Z-0-86	+++	87.03	87.03
4	*Diploid X Diploid*			
	Varadha X Mahima	+++	83.33	88.88
	Mahima X Varadha	+++	75.92	77.77
	Rejatha X Kuruppampady	++	66.66	72.22
	Mean		84.55	84.36

Medium: ½ MS + 3% sucrose + BA 2.5 mgl^{-1} + picloram 0.2 mgl^{-1} + 0.18% phytagel
Method of *in vitro* pollination: placental
Average of five observations
Scoring: '++++' very good; '+++' good; '++' moderate; '+' low; '-' nil

4.4.3 Post pollination changes of the ovule

4.4.3.1 Increase in size of the ovules after in vitro placental pollination

The seed development in two media combinations in terms of length and breadth of the ovule at intervals of 15 days starting from 15 DAP to 60 DAP are presented in Table 24. The parental combination selected for the study was Varadha X Z-078.

The ovules on the day of pollination measured a mean length of 0.68 mm and breadth of 0.45 mm. There was substantial increase in the size of the seeds at 15 DAP. The seeds at 15 DAP recorded a mean length of 2.0mm and breadth of 1.18 mm in the medium of half MS + 3 per cent sucrose + BA 2.5 mgl^{-1} + picloram 0.2 mgl^{-1} + 0.18 per cent phytagel while it was 1.8 mm and 0.89 mm respectively in half MS + 3 per cent sucrose + BA 2.5 mgl^{-1}+ NAA 0.5 mgl^{-1} + 0.18 per cent phytagel. At 60 DAP the ovules attained a mean length of 2.53 mm length and 1.63mm breadth. The size of the ovules attained was more in the former medium (2.68 mm length and 1.78 mm breadth).

4.4.3.2 Colour change in the ovules after in vitro placental pollination

The colour change of the developing ovules after *in vitro* pollination cultured in two media combinations were recorded as detailed in Table 25. The ovules cultured in half MS + 3 per cent sucrose + BA 2.5 mgl^{-1}+ picloram 0.2 mgl^{-1} + 0.18 per cent phytagel, turned to light pink from creamy white colour within 15 DAP, while it took 25 days for the ovules cultured in half MS + 3 per cent sucrose + BA 2.5 mgl^{-1}+ NAA 0.5 mgl^{-1}+ 0.18 per cent phytagel to become pink in colour. In the course of time seeds turned red, purple red, dark purple and finally black towards 80 DAP (Plate 12).

4.4.3.3 Endosperm filling of the seeds

Endosperm filling of the seeds cultured on two media combinations was examined at 10, 20, 30, 40 and 50 days after pollination. The culture medium was half MS with 2X vitamins + NAA 0.5 mg l^{-1}+ BA 2.5 mg l^{-1}. There was complete filling at 10 days after pollination in the former medium. The endosperm was soft and jelly like in 10 days old seeds and it became firm by 20 DAP.

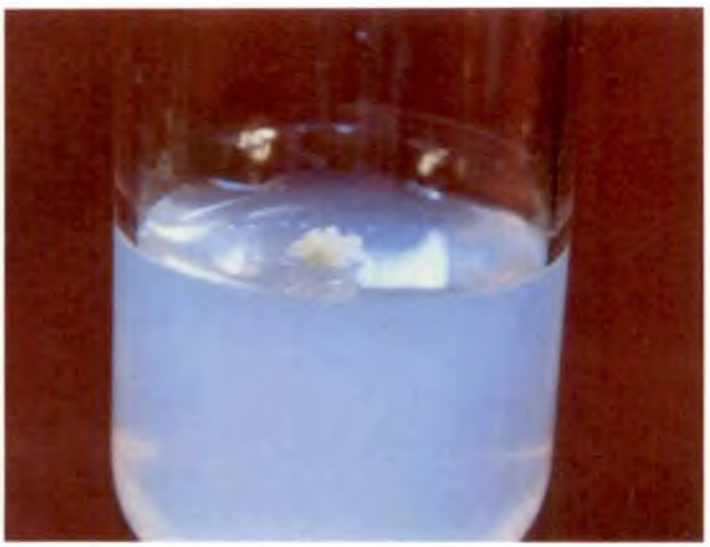


Plate 11. Developing ovules after *in vitro* placental pollen (8DAP) (40X)

15 DAP (40X)

65 DAP(40X)

Plate 12. Colour change in the ovules after *in vitro* placental pollination

Table 24. Seed development after *in vitro* placental pollination

Sl. no.	Media combination	Initial size		15 DAP		30 DAP		45 DAP		60 DAP	
		length (mm)	breadth (mm)	length (mm)	breadth (mm)	length (mm)	breadth (mm)	length (mm)	breadth (mm)	length (mm)	breadth (mm)
1	½ MS + 3% sucrose + BA 2.5 + NAA 0.5 + 0.18% phytagel	0.68	0.45	1.80	0.89	2.12	1.10	2.20	1.26	2.35	1.48
2	½ MS + 3% sucrose + BA 2.5 + picloram 0.2 + 0.18% phytagel	0.68	0.45	2.0	1.18	2.30	1.40	2.51	1.62	2.68	1.78
	Mean			1.90	1.04	2.21	1.25	2.36	1.44	2.53	1.63

Average of five observations

Method of *in vitro* pollination: placental

Parental combination: Varadha X Z-0-78

Table 25. Colour change of developing ovules after *in vitro* placental pollination

Sl. no.	Media combination	Initial colour	15 DAP	25 DAP	35 DAP	45 DAP	65 DAP	80DAP
1	½ MS + 3% sucrose + BA 2.5 + NAA 0.5 + 0.18% phytagel	Creamy white	Creamy white	Light pink	Red	Purple red	Dark purple	Black
2	½ MS + 3% sucrose + BA 2.5 + picloram 0.2 + 0.18% phytagel	Creamy white	Light pink	Red	Purple red	Dark purple	Purple black	Black

Parental combination: Varadha X Z-0-78
Method of *in vitro* pollination: placental

The hardness of the endosperm progressed upto 50 DAP. Embryo was found to be seated towards the chalazal end and the endosperm constitutes the major portion of the seed (Plate 13).

4.5 VIABILITY OF THE SEEDS

The seeds of 10, 20, 30, 40 and 50 DAP cultured in the medium of half MS + 3 per cent sucrose + 0.18 per cent phytagel + picloram 0.2 mg l^{-1} + BA 2.5 mg l^{-1} were tested for viability using tetrazolium. All the seeds tested were viable as the developing embryo and endosperm completely stained red (Plate 14). This also revealed that the embryo and endosperm were metabolically active upto 50 DAP.

4.6 GERMINATION STUDIES OF *IN VITRO* PRODUCED GINGER SEEDS

The pollinated ovules 60 to 80 DAP were cultured in media having various combinations of growth regulators for germination (Plate 15). The seeds for the germination studies were obtained after the incubation of *in vitro* pollinated ovules in the medium of half MS + 3 per cent sucrose + 0.65 per cent agar + NAA 0.5 mg l^{-1} + BA 2.5 mg l^{-1}. The results of seed germination studies are described below.

4.6.1 Influence of thiourea on germination of ginger seeds

In the basal medium of half MS + 3 per cent sucrose + 0.65 per cent agar, thiourea alone (1 to 2 mg l^{-1}) and with BA 1.0 mg l^{-1} + NAA 0.2 mg l^{-1} had no effect on the germination of seeds (Table 26)

4.6.2 Influence of IBA on germination of ginger seeds

The auxin IBA 3.0 mg l^{-1} in the basal medium of half MS + 3 per cent sucrose + 0.65 per cent agar caused radicle emergence in 60 days old seed derived from the parental combination of Kuruppampady X Z-0-78 (Plate 16). But subculturing to the same media for radicle elongation did not cause any change. Other concentrations of IBA were not effective (Table 27).

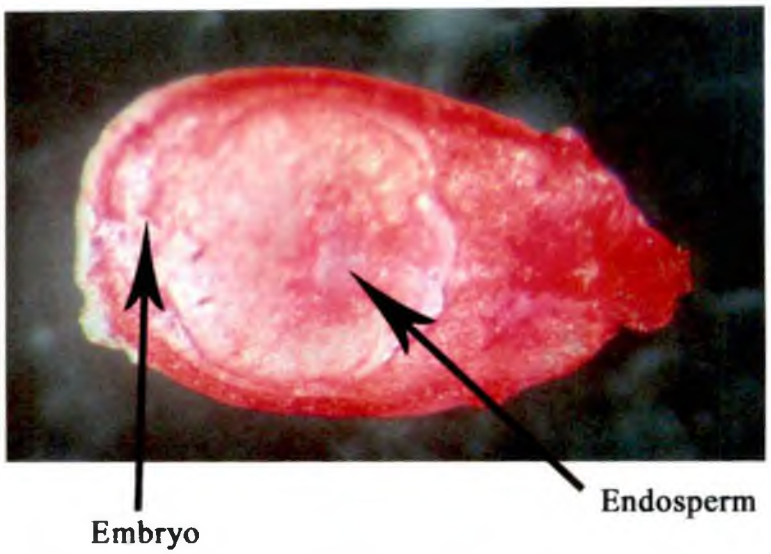


Plate 13. Endosperm filling of the seeds at 25 DAP.
Parental combination: Varadha X Z-0-78

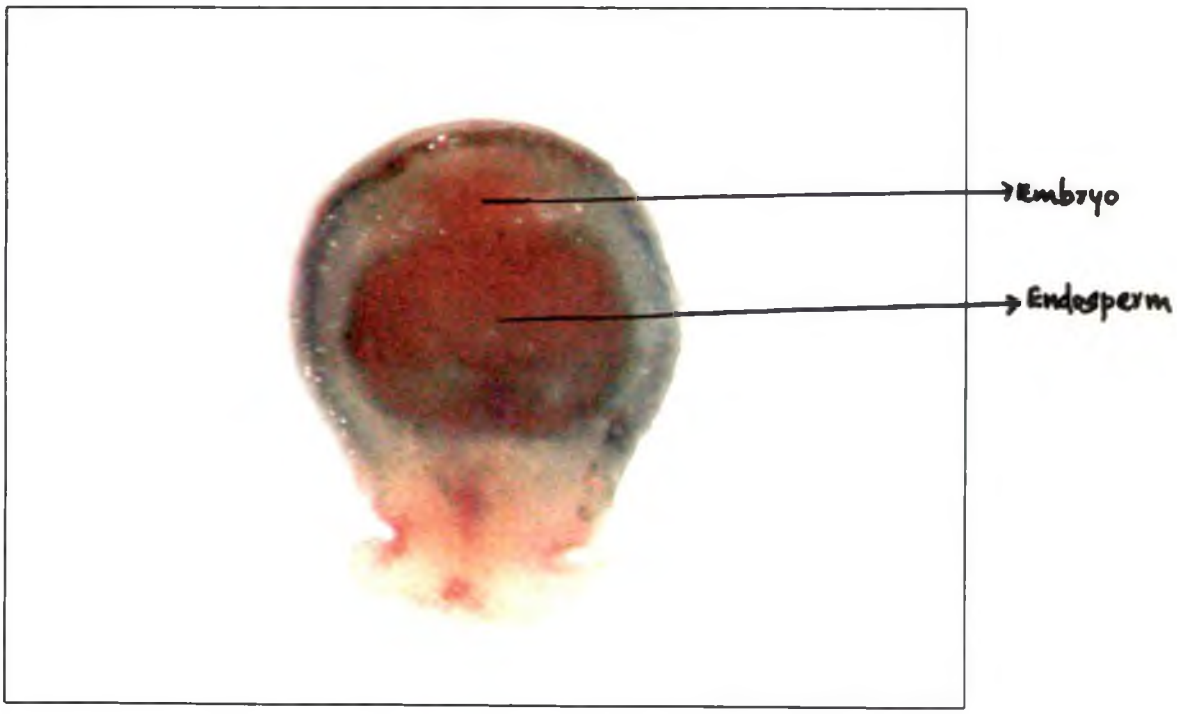


Plate 14. L.S. of the seed after tetrazolium staining
Parental combination: Z-0-78 X Z-0 86
Label embryo, endosperm

Plate 15. Ginger seeds (40-60 DAP) for germination

A close view.

Plate 16. Radicle emergence in ginger seeds0f 60 DAP

Table 26. Influence of thiourea on germination of ginger seeds

Sl. no.	Growth regulators in mg l^{-1}	Germination (%)
1	Thiourea 1.0	Nil
2	Thiourea 2.0	Nil
3	Thiourea 2.0 + BA 1.0 + NAA 0.2	Nil
	Mean	-

Basal medium - Half MS + 3% sucrose + 0.65% agar
Maturity of the seed - 60 - 80 DAP
Average of 18 observations-30, 60 and 90 DAP
Culture condition: Dark

Table 27. Influence of IBA on germination of ginger seeds

Sl. No.	Growth regulators in mg l^{-1}	Germination (%)
1	IBA 1.0	Nil
2	IBA 2.0	Nil
3	IBA 3.0	Radicle emergence
4	IBA 4.0	Nil
5	IBA 5.0	Nil

Basal medium - Half MS + 3% sucrose + 0.65% agar
Maturity of the seed - 60 - 80 DAP
Average of 18 observations-30, 60 and 90 DAP
Culture condition: Dark

4.6.3 Influence of GA_3 with other plant growth regulators on germination of ginger seeds

GA_3 0.5 mg l^{-1} alone in the basal medium of half MS + 3 per cent sucrose + 0.65 per cent agar had no influence on seed germination. Three combinations of GA_3 (5.0 mg l^{-1}) with IBA (3.0 mg l^{-1}), 2,4-D (0.5 mg l^{-1}) + BA (6.0 mg l^{-1}), and with 2,4-D (0.5 mg l^{-1}) + NAA (0.2 mg l^{-1}) + thiourea 2.0 did not induce germination of ginger seeds. GA_3 at 2.0 mg l^{-1} in combination with BA 1.0 mg l^{-1} + NAA 0.2 mg l^{-1} also was not effective in inducing germination.

Table 28. Influence of GA_3 on germination of ginger seeds

Sl. No.	Growth regulators in mg l^{-1}	Germination (%)
1	GA_3 0.5	Nil
2	GA_3 5.0 + IBA 3.0	Nil
3	GA_3 2.0 + BA 1.0 + NAA 0.2	Nil
4	GA_3 5.0 + 2,4-D 0.5 + BA 6.0	Nil
5	GA_3 5.0 + 2,4-D 0.5 + NAA 0.2 + thiourea 2.0	Nil

Basal medium - Half MS + 3% sucrose + 0.65% agar
Maturity of the seed - 60 - 80 DAP
Average of 18 observations-30, 60 and 90 DAP
Culture condition: Dark

4.6.4 Influence of combinations of auxins and cytokinins on germination of ginger seeds

The seeds cultured and maintained under dark on B_5 medium with 3 per cent sucrose and 0.18 per cent phytagel containing combinations of 2,4-D (0.2 mg l^{-1}) and BA (2.5 mg l^{-1}) germinated giving rise to somatic embryoids roots and shoot three and a half month after *in vitro* pollination (Table 29). It was found that the seed coat

was pushed apart by the callusing embryo and endosperm. The callus was embryogenic and developed globular somatic embryoids. Embryoids developed root and shoot poles, 105 days after *in vitro* pollination in the same medium (Plate 17). The parentage of the germinated seed was Z-0-78 X Varadha.

Initial incubation in auxin (2,4-D 8 mg l^{-1}) followed by exposure to combination of BA 8 mg l^{-1}+ 2, 4 – D 0.1 mg l^{-1} did not induce any seed germination. Varying combinations of BA (1.0, 2.5, 5.0, 9.0 mg l^{-1}) with other particular combinations of 2,4-D, IBA and NAA as mentioned in Table 29 did not cause seed germination.

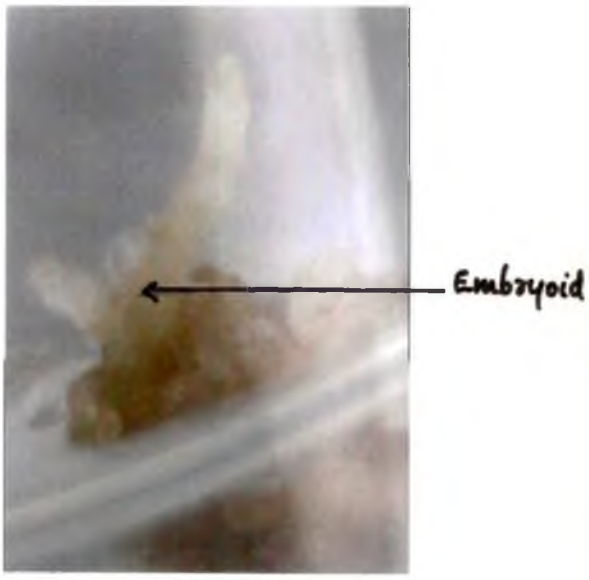
4.7 EMBRYO CULTURE

During first year, embryo culture studies were done with embryo and endosperm excised from seeds of 40 to 60 DAP and incubated in different media combinations involving auxins, cytokinins and GA_3. There was no callusing in any of the media combinations tried upto 90 days of inoculation (Table 30).

During second year embryo was excised along with endosperm from seeds of 15 to 30 DAP (Plate 18) and cultured in various media combinations involving different growth regulators like 2,4-D, IAA, NAA, IBA, picloram, BA, kinetin, TDZ, GA_3 and adenine to get direct or indirect organogenesis. Excision of the embryo and endosperm was easy compared to the mature seeds of 40 to 60 DAP. But there was no positive response observed in any of the cultures upto 40 days of inoculation (Table 31 and 32).

A close view

Callusing embryo and endosperm



A close view

Somatic embryogenesis and regeneration

Plate 17. Seed germination (via) somatic embryogenesis

Plate 18. Embryo along with endosperm (20 DAP) for culturing.

Table 29. Influence of auxin-cytokinin combinations on germination of ginger seeds

Sl. No.	Growth regulator (mg l^{-1})	Age of the seed	Germination (%)
1	Half MS + BA 1.0 + IBA 3.0	60 –80 days	Nil
2	Half MS + BA 2.5 + NAA 0.5	60 –80 days	Nil
3	Half MS + BA 5.0 + 2,4-D 0.2	60 –80 days	Nil
4	Half MS + BA 2.5 + 2,4 -D 0.2	60 –80 days	Nil
5	Half MS + BA 9.0 + 2,4-D 0.1	15-30 days	Nil
6	Half MS +Picloram 0.2 + BA 2.5	15-30 days	Nil
7	B_5 + BA 2.5 + 2,4- D 0.2	15-30 days	10 % germination

Basal medium: 3% sucrose + 0.65% agar

Average of 18 observations-30, 60 and 90 DAP

Culture condition: Dark

Table 30. Response of embryo with endosperm (40-60 DAP) to callusing in various media combinations.

Sl. no.	Basal medium	Growth regulators in mg l^{-1}	Incubation condition	Callusing %
1	Half MS	2,4-D 1.0	Dark	Nil
2	Half MS	2,4-D 2.0	Dark	Nil
3	Half MS	2,4-D 3.0	Dark	Nil
4	Half MS	2,4-D 4.0	Dark	Nil
5	Half MS	2,4-D 5.0	Dark	Nil
6	Half MS	2,4-D 8.0	Dark	Nil
7	Half MS	2,4-D 10.0	Dark	Nil
8	Half MS	2,4-D 20.0	Dark	Nil
9	Half MS	2,4-D 1.0 + BA 0.25	Dark	Nil
10	Half MS	2,4-D 2.0 + BA 2.0	Dark	Nil
11	Half MS	2,4-D 1.0 + BA 0.5	Dark	Nil
12	Half MS	2,4-D 1.0 + BA0.25	Dark	Nil
13	½ MS+B_5 vitamins	2,4-D 0.2 + kin 0.5	Dark	Nil
14	Half MS	2,4-D 0.2 + 1.0 kin + 1.0 BA	Dark	Nil
15	Half MS + 6% sucrose	2,4-D 1.0 + PVP 500 + glutamine 400 + ascorbic acid 100	Dark	Nil
16	Half MS	NAA 3.0 + BA 1.0	Dark	Nil
17	Half MS	NAA 1.0 + BA 1.0	Dark	Nil
18	½ MS+B_5 vitamins	IBA 0.5 + kin 0.5	Dark	Nil
19	Half MS	IAA 0.2 + BA 4.0	Dark	Nil
20	Half MS	IAA 0.2 + BA 1.0 +kin1.0	Dark	Nil
21	Half MS	TDZ 2.0	Light	Nil

Average of six observations – 90 days of inoculation

Table 31. Response of embryo with endosperm (15-30 DAP) to direct organogenesis in various media combinations.

Sl. no.	Growth regulators in mg l^{-1}	Incubation condition	Regeneration %
1	IBA 3.0	Dark	Nil
2	NAA 0.5 + BA 2.5	Light	Nil
3	NAA 1.0 + BA 1.0	Dark	Nil
4	Picloram 0.2 + BA 2.5	Dark	Nil
5	Picloram 0.2 + BA 6.0 + GA_3 5.0	Light	Nil
6	2,4-D 0.2 + BA 9.0	Light	Nil
7	2,4-D 0.2 + BA 10.0	Light	Nil
8	2,4-D 0.2 + BA 1.0 + kin 1.0	Dark	Nil
9	IAA 0.2 + BA 4.0	Light	Nil
10	IAA 0.2 + BA 1.0 + kin 1.0	Dark	Nil
11	BA 3.0	Light	Nil
12	BA 8.0	Light	Nil
13	TDZ 2.2	Light	Nil

Basal medium: half MS + 3% sucrose

Average of four observations – 40 days of inoculation

Table 32. Response of embryo with endosperm (15-30 DAP) to callusing in various media combinations.

Sl.no.	Basal medium	Growth regulators in mg l^{-1}	Culture condition	Callusing %
1	Half MS	IAA 0.1	Dark	Nil
2	Half MS	IAA 0.2 + BA 4.0	Light	Nil
3	Half MS liquid	NAA 1.0 + BA 1.0	Dark	Nil
4	Half MS liquid	NAA 3.0 + BA 1.0	Dark	Nil
5	Half MS	NAA 1.0 + kin 1.0	Dark	Nil
6	½ MS+B_5 vitamins	2,4-D 2.0	Dark	Nil
*7	½ MS+2% sucrose	2,4-D 4.0	Dark	Nil
*8	Half MS	2,4-D 8.0	Dark	Nil
*9	Half MS	2,4-D 12.0	Dark	Nil
10	Half MS liquid	2,4 D 1.0 + BA 0.5	Light	Nil
11	Half MS	2,4-D 0.2 + BA 5.0	Light	Nil
12	Half MS	2,4 D 2.0 + kin 3.0	Light	Nil
13	Half MS	2,4-D 0.2+ kin 0.3+IBA 0.2 + NAA 0.3 + GA_3 0.5	Dark	Nil
14	Half MS	2,4-D 0.2 + kin 0.3 + IBA 0.2+ picloram 0.1+GA_3 0.5	Light	Nil
15	Half MS	2,4-D 0.5 + picloram 2.2	Dark	Nil
16	½ MS+6% sucrose	2,4-D 4.0 + TDZ 0.02	Dark	Nil
17	Half MS	kin 6.0 + 200 adenine	Light	Nil
18	Half MS	TDZ 2.2	Light	Nil

Average of four observations – 40 days of inoculation

* Pulse treatment for one week

Discussion

5. DISCUSSION

The investigations on 'Induction of genetic variability in ginger (*Zingiber officinale* Rosc.) through *in vitro* fertilization' were carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 2004-2005.

Ginger is propagated exclusively by vegetative means due to the absence of seed set. Even then, to some extent the existing population shows variability for morphological, economic and qualitative traits including pest and disease resistance. The available variability in ginger is not fully exploited for crop improvement, as conventional hybridization is not possible in this crop.

Earlier attempts towards *in vivo* pollination by adopting artificial cross-pollination, chemical aided pollination, mentor pollination and pollination after decapitating stigma failed to induce seed set (Usha, 1984 and Sathiabhama, 1988).

In vitro pollination has been proved as a promising technique for overcoming pre fertilization barriers due to incompatibility and for raising new genotypes. Valsala (1994), Renjith (1999) and Bhurke (2002) found that *in vitro* pollination technique involving application of pollen grains in a germinating medium in close vicinity of ovules effects fertilization in ginger, turmeric and kacholam respectively. Success was also obtained in seeds set and seed development. However, the germination of *in vitro* produced seeds was highly erratic. Bindu (1997) and Shankar (2003) produced ginger seeds *in vitro* following the protocol developed by Valsala (1994). But germination of the seeds continues to be a problem and the conditions for successful germination are yet to be perfected.

5.1 MOLECULAR CHARACTERIZATION OF GINGER CULTIVARS

The genetic variability of selected ginger cultivars was assessed using RAPD. The amplification pattern of DNA of the cultivars showed that in Z-0-78, certain bands were absent when amplified with OPE 12, OPF 3, OPF 5 and OPF 7. These results could be utilized for early detection of hybrids from crosses involving Z-0-78 as one of the parents.

From the dendrogram constructed from the pooled data of the RAPD scores with nine different primers expressed very low variability in the genetic make up of the ginger cultivars evaluated (Fig 1). The variation recorded ranged from 5 per cent to 19 per cent. All the cultivars except Rio-de-Janeiro were closely related to each other. Rio-de-Janeiro was distinct from others with a maximum variability of 19 per cent. It may be due to the fact that this particular cultivar is an introduced one. The variability among the released varieties from IISR ranged from 5 to 10 per cent. The cultivars Kodakara local and Mahima are genetically more or less equal with 95 per cent similarity. These results ultimately show that the genetic variability among the cultivars of ginger under study is very narrow.

In an RAPD characterization study by Pojanagaroon *et al.* (2004) to analyze similarity among 12 cultivars of *Kaempferia parviflora* which is propagated using rhizomes like ginger and coming under Zingiberaceae, the dendrogram showed good relationship between the banding patterns and morphological and quality traits. So RAPD is effective in detecting any polymorphism in zingiberaceous crops. This technique can be used to assess the genetic fidelity of the micropropagated plants of ginger (Rout *et al.*, 1998), turmeric (Neeta *et al.*, 2003) and the relationship between different members of zingiberaceae.

Kavitha *et al.* (2005) from the variability study conducted in cultivars of *Zingiber officinale* Rosc. detected no polymorphism at more than 500 AFLP loci

screened and they reported that it was the most extreme case of genetic narrowness in a species. But the analysis revealed variability in *Zingiber cernuum.*

Low variability in ginger is due to exclusive vegetative propagation. If there is sexual reproduction in this crop, there will be chances for variability. Seedling progenies of turmeric (*Curcuma longa*-zingiberaceae) showed maximum similarity but differed distinctly from the clonally propagated accessions collected from different geographical areas in an isozyme analysis done by Shamina *et al* (1998).

5.2 FLOWERING BEHAVIOUR

The flowering season in ginger extended from August to November. The data on days to flowering (Table 14) revealed that flowering occurred within a period of 133 days (Kuruppampady) to 169 days (Rio-de-Janeiro) after planting. Cultivar difference was noticed with respect to days for initiation of flowering and flowering duration. Kuruppampady (133 days) and Maran (134 days) were the early flowering cultivars while Rio-de-Janeiro (162 days) and Z-0-86 (169 days) were the late flowering cultivars. The cultivar Z-0-78, autotetraploid from Himachal Pradesh flowers after 142 days of planting, Z-0-86, the autotetraploid from Rio-de-Janeiro flowers late (162 days) from planting. The flowering duration was maximum in Z-0-78 (66 days) and minimum in Kodakara local (31 days). The flowering duration of autotetraploids ranged from 54 (Z-0-86) to 66 days (Z-0-78). Among the diploids Rio-de-Janeiro had the maximum flowering duration (56 days).

This is in conformity with the reports of Valsala (1994) and Shankar (2003) that Maran and Rio-de-Janeiro were the early and late flowering cultivars respectively. Shankar (2003) also reported that autotetraploids flowered early compared to diploids.

The extended flowering season of autotetraploids is advantageous for crossing with other cultivars. According to Valsala (1994), raising the plants from

large sized rhizome bits (200g) induced early and cent percent flowering. Maintaining the plants as biennials made the onset of flowering early in the second year of planting. These practices will extend the period of explant availability for *in vitro* pollination and will help to make specific parental combinations.

5.3 POLLEN STUDIES

The genotype of the cultivars was found to influence the pollen fertility and viability. The autotetraploids showed more fertility and viability percentage when compared to diploids (Table 15). Among the diploids the released varieties Varadha, Mahima and Rejatha had more pollen fertility (40.52 to 41.02 %) and viability (8.13 to 8.90 %) compared to others. Among the autotetraploids the pollen fertility ranged from 49.63 (Z-0-86) to 52.44 per cent (Z-0-78) and among the diploids it ranged from 20.10 (Rio-de-Janeiro) to 41.02 per cent (Varadha). The range of pollen viability in autotetraploids was from 12.03 (Z-0-86) to 15.68 (Z-0-78) and it was from 3.68 (Kuruppampady) to 8.90 per cent (Varadha) in diploids. So the pollen viability among the nine cultivars was highest for Z-0-78 (15.68 %) and lowest for Kuruppampady (3.68 %).

Ramachandran and Nair (1992) reported that pollen fertility was higher in tetraploids (85 per cent) compared to diploids (13 per cent). Sheeba (1996) also found that autotetraploids had high pollen fertility (64%) than diploids (6%). Shankar (2003) has the same opinion that the pollen of autotetraploids Z-078 and Z-086 are more fertile and viable compared to diploids with 49.96 per cent and 56.91 per cent fertility and 10.66 and 12.83 per cent viability respectively.

The increased pollen fertility in the autotetraploids may be due to the high frequency of quadrivalent formation and the high sterility in the diploids is probably due to heterozygosity for gross structural changes of chromosomes (Ramachandran and Nair, 1992). These observations indicate that the chances of seed set will be more in crosses involving tetraploids as male parents and these cultivars will be

better choice in future generative breeding programmes. The combinations involving released varieties Varadha, Mahima and Rejatha as male parents are also good for seed set.

5.4 REFINEMENT OF *IN VITRO* POLLINATION TECHNIQUE

The ovule development was good when placental pollination was followed. Valsala (1994), Valsala *et al.* (1996), Bindu (1990) and Shankar (2003) also reported seed set and development through placental pollination, which was found to be the best in ginger.

5.4.1 Refinement of media for ovule development

5.4.1.1 Basal medium for culture establishment and ovule development

Half MS medium with 2X vitamin stock + BA 2.5 mgl^{-1}+ NAA 0.5 mgl^{-1}, was the best recording very good ovule swelling (++++) in 88.88 per cent cultures with 79.44 per cent setting per culture. The same medium with normal quantity of vitamins produced ovules with good swelling (+++). The substitution of MS vitamins with B_5 vitamins increased the percentage of cultures with ovule swelling (+++). But in B_5 medium, ovule development was moderate (++) (Table 16).

Suitability of MS medium with plant growth substances for *in vitro* culture of ginger has been reported earlier (Hosoki and Sagawa, 1977; Choi, 1991; Kackar *et al.*, 1993; Palai *et al*, 2000 and Paul *et al.*, 2005).

Shankar (2003) and Valsala (1994) found half MS good for ovary development of ginger after *in vitro* pollination. Shankar (2003) also reported that vitamin supplementation by adding the vitamin stock of MS medium in double the normal quantity was beneficial for ovule development

The comparison of vitamin stock of MS and B_5 medium (Table 4), showed that the vitamins, thiamine, HCl and pyridoxine, HCl are at higher concentration in

B_5. This may be the reason for good ovule development in MS medium having vitamins substituted with that of B_5. From the above observations it can be concluded that high amount of vitamins in the medium favours the ovule development positively.

Vitamins such as B_1 and B_6 or even a mixture of vitamins (Maheswari and Lal, 1961) stimulated ovary growth of *Iberis amara*. Vitamin E increased seed fertility in *Dendrobium nobile* (Ito, 1966). Castano and Proft (2000) identified both MS and B_5 medium for the culture of isolated ovules of chicory after *in vitro* ovular pollination. They also reported that vitamin stock of MS medium supported the ovule development in chicory.

The media standardized by Valsala (1994) could be refined by including double the quantity of MS vitamins or replacing it with B_5 vitamins.

5.4.1.2 Influence of solidifying agent in the medium on ovule development

The type and concentration of solidifying agent affects the ovule development considerably (Table 17 and 18). Phytagel (0.18%) was found to be supporting maximum ovule development (++++). The percentage of cultures with ovule development and percentage setting per ovary were also highest in the case of phytagel (0.18%). This may be due to the easier and better absorption of nutrients by the explants from the phytagel-containing medium.

The *in vitro* pollination technique standardized by Valsala (1994) could be refined in this aspect.

5.4.1.3 Influence of organic supplements on ovule development

Inclusion of milk protein (100 mg l^{-1}) in the medium of half MS + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1} did not influence ovule development. Inclusion of coconut water (15%) in the aforesaid basal medium supported ovule development.

Addition of coconut water at concentration of 15 to 25 per cent v/v to the media favoured ovule development after *in vitro* placental pollination in ginger as reported by Valsala (1994) and Shankar (2003). Usha (1965) observed seed set in *Antirrhinum majus* through *in vitro* pollination on addition of 25 per cent coconut water to Nitsch's medium.

Coconut water contains a number of components promoting cell division and a large number of free amino acids (Letham, 1974). Thus the basal media half MS supplemented with NAA (0.5 mg l^{-1}), BA (2.5 mg l^{-1}) and coconut water (15%) was found suitable for *in vitro* pollination studies in ginger.

5.4.1.4 Influence of auxins and cytokinins on ovule development

The influence of auxins and cytokinins was studied to refine the media combination for ovule development. The parental combination selected for the study was Varadha X Z-078

The combination of picloram 0.2 mg l^{-1} + BA 2.5 mg l^{-1} in the basal medium of half MS + 3 per cent sucrose + 0.18 per cent phytagel recorded very good ovule swelling (++++) in 97.22 per cent cultures with a setting percentage of 88.33 per culture (Table 19). Picloram at 0.3 mg l^{-1} concentration also recorded good ovule swelling (+++) in 94.44 per cent cultures with 84.72 per cent setting per culture. The concentration of picloram above 0.5 mg l^{-1} negatively affected ovule development. Reducing the concentration of picloram from 0.2 to 0.1 mg l^{-1} also reduced ovule swelling

Influence of varying concentrations of NAA 0.5 to 1.0 mg l^{-1} along with BA 2.5 to 3.0 mg l^{-1} was studied (Table 20). The media combination of half MS + NAA 0.5 mg l^{-1} with BA 2.5 mg l^{-1} recorded good ovule swelling. Replacement of BA (2.5 mg l^{-1}) with kinetin (2.0 mg l^{-1}) also gave good ovule swelling.

Influence of 2,4 D alone at concentrations ranging from 0.2 to 5.0 mg l^{-1} and in combination with BA in the basal medium of half MS with 2X vitamins + 3 per cent sucrose + 0.65 per cent agar was studied and found that 2,4 D alone (0.2 to 5.0 mg l^{-1}) in the medium recorded low or no ovule development (Table 21). But the combination of 2,4-D 0.2 mg l^{-1} and BA 2.5 mg l^{-1} resulted in moderate ovule swelling.

Influence of IAA in combination with BA and kinetin on ovule development was studied. Two combinations of IAA (0.2 and 0.5 mg l^{-1}) with BA (2.5 mg l^{-1}) gave moderate ovule swelling (Table 22). The other combination of IAA (0.1 mg l^{-1}) with kinetin (2.0 mg l^{-1}) also caused moderate ovule swelling (++).

Valsala (1994) and Shankar (2003) obtained maximum seed development when pollinated ovaries of ginger were cultured in the medium of half MS + coconut water (15%) + BAP 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}. The auxins and cytokinins alone, induced little ovule development but combinations were essential for maximum development. The effect of BAP could be replaced by kinetin (2 mg l^{-1}) or 2 iP (2.5 mg l^{-1}). The effect of NAA (0.5 mg l^{-1}) could be replaced by 2,4-D (0.5 mg l^{-1}) or IAA (0.05 to 0.2 mgl^{-1}).

The role of auxins in supporting ovary development into fruit is well documented. The developing seeds are a rich source of auxin (Nitsch, 1952), which is utilized for normal fruit growth in apple, pear and strawberry. Synthetic auxins such as NOA and 2, 4-D can replace the stimulus provided by pollination. Euewens and Schwale (1975) have reported that in general, high auxins (IAA) and GA_3 levels have beeen associated with active seed growth and fruit growth by cell expansion. In a number of plants, both GA_3 and auxins are highest during early to mid embryo development, at a stage when cytokinins decreased rapidly. Quartrano (1987) reported that hormones play an important role in different stages of seed development and germination. The concentration of cytokinins, auxins, gibberellins

and abscisic acid were found relatively high in extracts from seeds of different developmental stages.

The cytokinin activity at the early stage of embryogenesis is responsible for enhanced seed size by increasing cell number (Michael and Kelbitsch, 1972). Studies with isogenic mutants of barley, which vary in grain weight, demonstrated that large grain lines contain higher amounts of cytokinin at the very early stage of seeds development than small grain lines (Kelbitsch *et al.*, 1975) According to Tollennar (1977), cytokinins are found in relatively high concentration in the liquid endosperm stage of early seed growth and their presence coincides with higher rate of mitosis.

The aforesaid reports explain the requirement of growth regulators, especially auxins and cytokinins for the development of seed after fertilization.

In the present investigation to refine the medium for ovule development standardized by Valsala (1994) and refined by Shankar (2003), it was found that **the best medium for the culture of ginger ovules after *in vitro* placental pollination was half MS with 2X vitamins + 3 per cent sucrose + 0.18 per cent phytagel + picloram 0.2 mg l^{-1} + BA 2.5 mg l^{-1}.** The swelling of the ovules (++++), the percentage of cultures with seed set (97.22%) and the percentage setting per culture (88.88%) were found to be more when compared to the already standardized medium of half MS + 3 per cent sucrose + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}+ 15 per cent coconut water. Replacement of BA 2.5 mg l^{-1} with kinetin 2.0 mg l^{-1}can be explored.

5.4.2 Influence of parental combination on ovule development

Influence of parental combination on ovule development in the medium of half MS with 2X vitamins + 3 per cent sucrose + 0.18 per cent phytagel + picloram 0.2 mg l^{-1} + BA 2.5 mg l^{-1} was studied. The parental combination between autotetraploids (Z-0-78 and Z-0-86) produced seeds with very good swelling (++++). The percentage of cultures with setting (90.74 to 98.15%) and percentage setting per

culture (87.03 to 94.44%) were the highest in this combination. The parental combination Z-078 X Varadha and its reciprocal also favoured very good ovule swelling (++++) in 83.33 to 88.88 per cent cultures with a setting percentage of 83.33 to 90.74 per culture. The parental combination Z-0-86 X Varadha and its reciprocal produced seeds with good swelling (+++) in 87.03 to 88.88 per cent cultures with 77.77 to 87.03 percent setting per culture. The cross between diploids Varadha and Mahima produced seeds with good development (+++) in 75.92 to 83.33 cultures with a setting percentage of 77.77 to 88.88 per culture. But the cross between the diploids Rejatha as the female parent and Kuruppampady as the male parent yielded ovules with moderate swelling (++). For this parental combination, the percentage of cultures with setting (66.66%) and percentage setting per culture (72.22%) were the lowest (Table 23).

The aforesaid results indicate that cross incompatibility is absent for seed set in ginger through *in vitro* pollination as reported by Bindu (1997) and Shankar (2003). The autotetraploid Z-0-78 performed well both as male and female parent in crosses. This may be due to the high pollen fertility, pollen viability and ovule size.

Varadha and Mahima, the varieties released from IISR possess the highest pollen fertility and viability among the dipolids under study. Varadha as male parent, when crossed with autotetraploid Z-0-78 caused very good ovule swelling (++++). Mahima also performed well when used as male parent to cross with Varadha. The ovule size of Varadha is more when compared to other diploids. But the cross involving Kuruppampady as male parent gave only moderate development because of the poor pollen fertility and viability.

Shankar (2003) reported that crossing the autotetraploids with diploids and its reciprocal *in vitro* was successful and showed ovule development. The mean percentage of cultures showing response (100%) was maximum in the cross of

Maran X Z-0-78 but maximum percentage setting per culture (15.85) was observed in cross of Rio-de-Janeiro X Z-0-78.

So from the above discussion, it can be concluded that Z-0-78 and Varadha are the best both as male and female parents. Z-0-86 and Mahima are the next best ones which can be used as both male and female parents in crossing programmes. In these parental combinations, the probability for getting seed germination is more and the hybrids produced will have altered ploidy levels and it will be desirable.

5.4.3 Post pollination changes of the ovule

5.4.3.1 Increase in size of the ovules after in vitro placental pollination

The ovules of the cross Varadha X Z-0-78 on the day of pollination measured a mean length of 0.68 mm and breadth of 0.45 mm. There was substantial increase in the size of the seeds at 15 DAP. The seeds at 15 DAP recorded a mean length of 2.0 mm and breadth of 1.18 mm in the medium of half MS + 3 per cent sucrose + BA 2.5 mg l^{-1} + picloram 0.2 mg l^{-1} + 0.18 per cent phytagel while it was 1.8 mm and 0.89 mm respectively in half MS with 2X vitamins + 3 per cent sucrose + BA 2.5 mg l^{-1}+ NAA 0.5 mg l^{-1} + 0.18 per cent phytagel. At 60 DAP the ovules attained four-fold increase in size with a mean length of 2.53 mm length and 1.63 mm breadth. The size of the ovules attained was more in the former medium (2.68 mm length and 1.78 mm breadth) (Table24). So picloram at 0.2 mg l^{-1} favours good growth of the seeds.

Valsala (1994) reported that the ovules of ginger on the day of anthesis measured a length of 0.45 mm and a breadth of 0.32 mm and the growth was rapid in the initial 20 days after *in vitro* pollination. The length and breadth showed a linear increase with advance in age upto 80 DAP and they attained a maximum value of 2.30 mm and 1.60 mm respectively. Shankar (2003) had similar observations in his studies on *in vitro* pollination between autotetraploids and diploids in ginger.

5.4.3.2 Colour change in the ovules after in vitro placental pollination

The ovules cultured in half MS with 2X vitamins+ 3 per cent sucrose + BA 2.5 mg l^{-1} + picloram 0.2 mg l^{-1}+ 0.18 per cent phytagel, turned to light pink from creamy white colour within 15 DAP, while it took 25 days for the ovules cultured in half MS + 3 per cent sucrose + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}+ 0.18 per cent phytagel to become pink in colour. In the course of time seeds turned red, purple red, dark purple and finally black towards 80 DAP (Table 25).

According to Valsala (1994), the colour of the *in vitro* pollinated ovules of ginger during the initial stage was creamy white and in the course of development they developed purple red as if they were ripe. The mean number of days required to have purple red colour was 30.65 days. The whole ovules of the culture turned black between 60 to 90 days after pollination. The observations of Shankar (2003) are in concurrence with the above reports.

The colour of the developing ovules after *in vitro* pollination is an indication of ripeness and maturity. The current results show that the medium of half MS + 3 per cent sucrose + BA 2.5 mg l^{-1}+ picloram 0.2 mg l^{-1}+ 0.18 per cent phytagel is more supporting for the ripening and maturation of ovules after *in vitro* pollination.

5.4.3.3 Endosperm filling of the seeds

Endosperm filling of the seeds cultured on two media combinations was examined at 10, 20, 30, 40 and 50 days after pollination. The medium half MS with 2X vitamins + picloram 0.2 mg l^{-1} + BA 2.5 mg $l^{-1}$was found to be more supportive for earlier and better filling with good embryo and endosperm development than half MS with 2X vitamins + NAA 0.5 mg l^{-1}+ BA 2.5 mg l^{-1}. There was complete filling at 10 days after pollination in the former medium. The endosperm was soft and jelly like in 10 days old seeds and it became firm by 20 DAP. The hardness of the endosperm progressed upto 50 DAP. Embryo was found to be seated towards the chalazal end and the endosperm constitutes the major portion of the seed.

Filling of the ovules is an indication of active metabolism taking place inside the seed, which in turn leads to proper embryo and endosperm development. Therefore the medium of half MS + 3 per cent sucrose + 0.18 per cent phytagel + picloram 0.2 mg l^{-1} + BA 2.5 mg l^{-1} is ideal for culturing the *in vitro* pollinated ovules with respect to their endosperm filling.

5.5 VIABILITY OF THE OVULES

The seeds of 10, 20, 30, 40 and 50 DAP cultured on half MS + 3 per cent sucrose + 0.18 per cent phytagel + picloram 0.2 mg l^{-1} + BA 2.5 mg l^{-1} were tested for viability using tetrazolium. All the seeds tested were viable with developing embryo and endosperm completely stained red (Plate 13). This also revealed that the embryo and endosperm were metabolically active upto 50 DAP.

Shankar (2003), from the examination of seeds subjected to tetrazolium staining, reported that seeds from 40 DAP to 80 DAP were stained red and thus viable. As a difference from this report, the present study revealed that the immature ovules of 10 to 20 DAP were germinable giving red colour in the tetrazolium test. This may be due to the better embryo and endosperm development happening inside the ovules cultured in the aforesaid medium.

So the embryo rescue and seed germination studies could be undertaken even at 10 DAP. It also suggests that seeds developed after *in vitro* pollination are germinable if suitable conditions are arrived at.

5.6 GERMINATION STUDIES OF *IN VITRO* PRODUCED SEEDS

In the basal medium of half MS + 3 per cent sucrose + 0.65 per cent agar, thio urea alone (1 to 2 mg l^{-1}) and with BA 1.0 mg l^{-1} + NAA 0.2 mg l^{-1} had no effect on the germination of seeds (Table 26).

The 60 days old seed derived from the parental combination of Kuruppampady X Z-0-78 showed radicle emergence when cultured in dark in the medium of half MS + 3 per cent sucrose + 0.65 per cent agar + IBA 3.0mg l^{-1}. But subculturing to the same media for radicle elongation did not cause any change (Table 27).

Sreelatha *et al.* (2005) succeeded in getting positive response of Kannanthali (*Exacum bicolor*) seeds to *in vitro* seed culture. The seeds of this plant are not reported to germinate in natural condition. By culturing the seeds in the medium half MS + 2.0 mg l^{-1} IBA in dark they could get the best response. So in future, IBA should be included in germination studies.

GA_3 0.5 mg l^{-1} alone had no influence on seed germination. Combinations of GA_3 (2, 5 mg l^{-1}) with particular combinations of BA, NAA and IBA did not cause germination of ginger seeds (Table 28).

Shankar (2003) reported that 80 days old mature seed each obtained from the cross diploid (Rio-de-Janeiro) X autotetraploid (Z-0-78) and autotetraploid (Z-0-86) X diploid (Maran) germinated under *in vitro* conditions when cultured in half MS +2,4-D (0.5 mg l^{-1}), BAP (6.0 mg l^{-1}) and GA_3 (5.0 mg l^{-1}) along with 3 per cent sucrose showing radicle emergence. He found priming the 80 days old seeds in 50 ppm KNO_3 for 8 h as well as soaking in water for one day prior to inoculation favoured germination.

Castano and Proft (2000) obtained germination of the seeds from *in vitro* pollinated flowers of chicory on three media combinations containing GA_3 5 mg l^{-1} along with IAA and kinetin.

Spiegel-Roy *et al.* (1985) reported Nitsh's medium with IAA 10^{-5} M and GA_3 10^{-6} M as the best for germination of seeds of seedless grape cultivars. Cardamom

seeds have highest germination (39.33%) after acid scarification for 10 minutes and soaking in GA_3 for 24 h combined with sowing in the open site (Raja, 1993). Korikanthimath and Mulge (1998) found acid treatment (nitric acid 20 % for 10 min), GA_3 100 mg l^{-1} for 12 h and. Planofix (NAA 75 mg l^{-1} for 12 h) increased germination of cardamom seeds. Michishita *et al.* (2001) obtained hybrid seedlings from *Rhododendron kaempferia* ovules by exposing ovules 4 months after crossing to 50 mg l^{-1} GA_3. But in the present study, GA_3 along with auxins and cytokinins did not cause seed germination. Prolonged culturing of seeds in media combinations of GA_3, auxins and cytokinins may give positive results.

The seeds cultured and maintained under dark on B_5 medium with 3 per cent sucrose and 0.18 per cent phytagel containing combinations of 2,4-D (0.2 mg l^{-1}) and BA (2.5 mg l^{-1}) germinated giving rise to somatic embryoids three and a half month after *in vitro* pollination (Table 29). It was found that the seed coat was pushed apart by the callusing embryo and endosperm. The callus was embryogenic and developed globular somatic embryoids. Embryoids developed root and shoot poles in the same medium. The parentage of the germinated seed was Z-0-78 X Varadha.

Valsala (1994) reported ginger seeds incubated in a medium of half MS with 2,4-D 8.0 mg l^{-1} for two months and then in a plant growth regulator combination of BAP 9.0 mg l^{-1} and 2,4-D 0.1 mg l^{-1} in half MS showed germination. Valsala *et al.* (2001) observed radicle emergence in a media combination of half MS + 6 per cent sucrose + NAA (0.2 mg l^{-1})+ BA (1.0 mg l^{-1}) + GA_3 (2.0 mg l^{-1})+ coconut water 15 % v/v.

Shankar (2003) found that seeds produced from the cross diploid (Rio-de-Janeiro) X autotetraploid (Z-0-86) germinated after culturing in solid medium of half MS +2,4 D (0.25 mg l^{-1}) + BAP (9.0 mg l^{-1}) by producing radicle. In the present investigation seed germination could not be obtained in the media reported by Valsala (1994) and Shankar (2003).

Somatic embryoids were developed from seeds of ginger when cultured on half MS + 2,4 D (0.5 mg l^{-1}), BAP (6.0 mg l^{-1}) and GA_3 (5.0 mg l^{-1}) along with 3 per cent sucrose and also in half MS + 2,4 D (0.1 mg l^{-1}), BAP (8 mg l^{-1}) along with 3 per cent sucrose (Shankar, 2003).

Suma and Kesavachandran (2005) reported that MS medium with 2,4 D 1 mg l^{-1} and BA 0.5 mg l^{-1} was the most effective medium in inducing and maintaining embryogenic cultures derived from young buds of ginger. The somatic embryos germinated on half strength MS medium supplemented with 3 per cent sucrose and 3 mg l^{-1} BA. Paul *et al.* (2005) obtained callusing from ginger explants like sprouted bud, shoot tip, leaf and pseudostem in MS medium supplemented with 2,4 D alone (1.0-2.0 mg l^{-1}) and in combination with BAP (0.5-3.0 mg l^{-1}). Shoot morphogenesis was obtained from one month old calli in MS + BAP 3.0 mg l^{-1}.

It can be concluded from the aforesaid investigations that seed germination requires culturing of seeds in media combination of auxins and cytokinins. The present results also support this. Low concentrations of 2,4-D favours induction of callus and somatic embryoids. Since the regeneration was obtained in B_5 medium having high concentration of vitamins, it is clear that vitamins play a significant role in seed germination. It also suggests that prolonged culturing is required to get positive response.

5.7 EMBRYO CULTURE

During first year, embryo culture studies were done with embryo and endosperm excised from seeds of 40 to 60 DAP and incubated in different media combinations involving auxins, cytokinins and GA_3. There was no callusing in any of the media combinations tried upto 90 days of inoculation.

During second year embryo was excised along with endosperm from seeds of 15 to 30 DAP and cultured in various media combinations involving different growth

regulators like 2,4-D, IAA, NAA, IBA, picloram, BA, kinetin, TDZ, GA_3 and adenine to get direct or indirect organogenesis. Excision of the embryo and endosperm was easy compared to the mature seeds of 40 to 60 DAP. But there was no positive response observed in any culture upto 40 days of inoculation.

Roy and De (1989) reported plant regeneration from immature embryo explants of *Calotropis gigantean* on MS medium with NAA 0.1 mg l^{-1} and BAP 0.1 to 0.5 mg l^{-1}. Sudhadevi *et al.* (1993) reported production of multiple shoots from hybrid embryo of pineapple on MS medium containing NAA 3.0 mg $l^{-1}$and BAP 2.0 mg l^{-1}. Muniswamy and Sreenath (1996) reported that zygotic embryos of *Coffea bengalensis* germinated into plantlets when cultured on half strength MS with BAP 0.1 mg l^{-1}. For the culture of immature embryos of *Actinidia,* the best medium was MS basal containing GA_3 0.01 to 0.05 mg l^{-1} + mannitol 8 percent as suggested by Kin *et al.* (1995).

Chuang and Chang (1987) observed callusing of mature zygotic embryos of *Dysosma pleiantha* in MS medium supplemented with 2,4-D 1 mg l^{-1}. Embryoids were formed on media containing 2,4-D 0.1 to 0.5 mg l^{-1} and they germinated on MS or B_5 with BA 1 mg l^{-1} and GA_3 1 mg l^{-1}.

Induction of embryogenic callus and subsequent somatic embryogenesis was possible at a lower concentration of 2,4 D (1.0-2.0 mg l^{-1}) when immature zygotic embryos of *Paspalum scrobiculatum* were used as explants (Rashid, 2002b). MS medium containing 2,4 D (2 mg l^{-1}) plus B_5 vitamins, MS medium containing 2,4 D 1 mg l^{-1} with no vitamins or MS medium containing picloram (2.2 mg l^{-1}) and 2,4 D (0.5 mg l^{-1}) plus MS vitamins were used for callus initiation from immature embryos of wheat. The calli regenerated in Full MS basal medium (Haliloglu and Baenziger, 2005).

Kaun *et al.* (2002) reported somatic embryogenesis from immature epicotyle and cotyledons of groundnut cultured in MS medium supplemented with 4 mgl^{-1} of 2,4 D. Somatic embryos thus obtained germinated when plated on a medium with 50 mgl^{-1} ascorbic acid and 0.5 mgl^{-1} NAA. Direct somatic embryogenesis was obtained from mature zygotic embryos of sandalwood on MS medium containing TDZ 2.0 mg l^{-1} or BA 2.0 mg l^{-1}. Somatic embryos germinated in half strength MS supplemented with 1.4 µM GA_3 (Rai and McComb, 2002).

Simultaneous callus formation and regeneration from embryo explants of cumin was reported on B_5 medium supplemented with 1 ppm BA, 0.2 ppm NAA and 0.4 ppm IAA (Ebrahimie *et al.*, 2003). Somatic embryogenesis and plant regeneration was obtained when the zygotic embryo explants of *Cryptomeria japonica* were cultured on a medium containing 3.2 µM 2,4 D and 1.8 µM BA in dark (Igasaki *et al.*, 2003).

Gairi and Rashid (2004) reported that thidiazuron at 10 µM concentration induced somatic embryogenesis in non-responsive caryopses of rice after a short treatment with 20 µM 2,4 D for three days. Immature zygotic embryos of papaya callused on ½ MS + 2,4 D 2.0 mg l^{-1} under dark and somatic embryoids were developed on ½ MS basal medium. Shoots were developed in ½ MS + BAP 0.4 mg l^{-1} and NAA 0.02 mg l^{-1}. Rooting was noticed in MS basal liquid medium + IBA 1.0 mg l^{-1} (Anandan *et al.*, 2005).

Present investigations show that it is difficult to get regeneration from embryo with endosperm excised from mature seeds of 40 to 60 DAP. It also suggests that prolonged culturing in the same media with regular subculturing also may be necessary for getting positive response. Juvenility of the embryo and endosperm tisues also will be a deciding factor in regeneration. Since good filling and development were obtained even at 10 days, future embryo culture studies can be undertaken with seeds of less than 15 days.

5.8 FUTURE LINE OF WORK SUGGESTED

Molecular characterization of the nine ginger cultivars under the present study revealed that variability is very less in the ginger genome. It may be due to the exclusive vegetative propagation and absence of natural seed set in ginger. So *in vitro* fertilization is one of the best options to create variability in this crop.

In the present investigations on 'Induction of genetic variability in ginger (*Zingiber officinale* Rosc.) through *in vitro* fertilization' the technique of *in vitro* pollination in ginger was refined with respect to media combinations for ovule development. In the medium of half MS with 2 X vitamins+ 3 per cent sucrose + 2.5 BA + 0.2 picloram + 0.18 per cent phytagel viable seeds with good embryo and endosperm development were obtained. Viability test showed that embryo and endosperm were metabolically active upto 50 DAP.

In the present study seed germination and somatic embryogenesis were obtained in the medium of B_5 + 3 per cent sucrose + 0.18 per cent phytagel + 2,4-D 0.2 mg l^{-1} + BA 2.5 mg l^{-1}. This gives an indication that B_5 medium, concentration of vitamins in the culture medium and culturing in dark for prolonged period favours germination. Parental combination also has significant effect on seed development. The autotetraploids, Z-0-78 and Z-0-86 as well as the released diploid varieties Varadha, Rejatha and Mahima are ideal parents for *in vitro* fertilization.

Embryo culture studies conducted with seeds of 40 to 60 DAP did not give positive results. It appears that juvenility of the embryos is important in getting regeneration. Embryo culture studies need to be continued with the seeds of less than 15 DAP. Success in this line will complete in *vitro* fertilization protocol in ginger and the dream of heterosis breeding in this crop will be realized.

Summary

6. SUMMARY

Investigations on 'Induction of genetic variability in ginger (*Zingiber officinale* Rosc.) through *in vitro* fertilization' were carried out at the Centre for Plant Biotechnology and Molecular Biology, College of horticulture, Vellanikkara during 2004-2005.

The salient findings of the study are summarized as follows:

1. Molecular characterization studies of the eight ginger cultivars- Z-0-78 (V_1) Kodakara Local (V_3), Kuruppampady (V_4), Mahima (V_5), Maran (V_6), Rejatha (V_7), Rio-de-Janeiro (V_8) and Varadha (V_9)- by RAPD using nine random primers revealed that the genetic variability among the cultivars ranged from 5 to 19 per cent. The cultivar Rio-de-Janeiro was distinct with maximum variability. The cultivars Kodakara local and Mahima showed 95 per cent similarity in RAPD pattern. In conclusion the analysis revealed a narrow genetic base for the ginger cultivars evaluated. The variability among the released varieties from IISR ranged from 5 to 10 per cent.

2. In ginger, the flowering season extended from September to November. The cultivars showed variability with respect to crop duration for initiation of flowering and flowering duration. Flowering started within a period of 133 to 169 days after planting. Kuruppampady was the earliest flowering cultivar while Rio-de-Janeiro was the late flowering cultivar. The duration of flowering was maximum in Z-0-78 (66 days) and minimum in Kodakara local (31 days).

3. The mean pollen fertility and viability among the ginger cultivars were 35.76 per cent and 8.11 per cent respectively. The autotetraploids had high pollen fertility and viability. Among the autotetraploids the pollen fertility ranged

from 49.63 (Z-0-86) to 52.44 per cent (Z-0-78) and among the diploids it ranged from 20.10 (Rio-de-Janeiro) to 41.02 per cent (Varadha). The range of pollen viability in autotetraploids was from 12.03 (Z-0-86) to 15.68 (Z-0-78) and in diploids it was from 3.68 (Kuruppampady) to 8.90 per cent (Varadha).

4. Attempts were made to refine the *in vitro* pollination technique with respect to basal media combination, growth regulators, vitamins and solidifying agent. Studies conducted in the cross Varadha X Z-0-78 using placental pollination technique. The basal medium of half MS with 2X vitamins was superior to B_5 medium. As solidifying agent phytagel 0.18 per cent was superior to agar 0.65 per cent. Among the various plant growth regulator combinations tried the combination of picloram and BA was found to be best. The medium of half MS with 2X vitamins + 3 per cent sucrose + BA 2.5 mgl^{-1} + picloram 0.2 mgl^{-1} + 0.18 per cent phytagel was the best with respect to maximum ovule swelling, percentage of cultures with ovule development and percentage setting per culture.

5. Seed set and seed development in various crosses involving autotetraploids and diploids were assessed. The parental combinations involving autotetraploids (Z-0-78 and Z-0-86) produced seeds with very good swelling in maximum number of cultures with maximum setting per culture. The parental combination Z-078 X Varadha and its reciprocal also favoured very good ovule swelling. The parental combination Z-0-86 X Varadha as well as Varadha X Mahima and their reciprocals produced seeds with good swelling.

6. The size increase of the developing ovules after *in vitro* pollination was monitored from the day of pollination to 60 DAP. The ovules (Varadha X Z-0-78) on the day of anthesis measured a mean length of 0.68mm and breadth of 0.45mm. The seeds at 15 DAP recorded a mean length of 2.0mm and breadth of 1.18mm in the medium of half MS with 2X vitamins + 3 per cent

sucrose + BA 2.5 mg l^{-1} + picloram 0.2 mg l^{-1} + 0.18 per cent phytagel. At 60 DAP the ovules attained 2.68 mm length and 1.78 mm breadth.

7. The pollinated ovules cultured in the course of maturation turned pink and later blackened. The ovules cultured in half MS with 2X vitamins + 3 per cent sucrose + BA 2.5 mg l^{-1} + picloram 0.2 mg l^{-1} + 0.18 per cent phytagel, turned to light pink from creamy white colour within 15 DAP, while it took 25 days for the ovules cultured in half MS + 3 per cent sucrose + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1} + 0.18 per cent phytagel to turn pink. In the course of development, seeds turned red, purple red, dark purple and finally black towards 80 DAP.

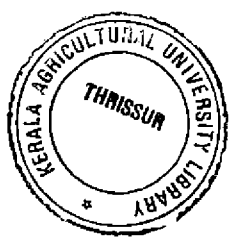
8. The endosperm filling was monitored in two media combinations from 10 to 50 DAP at an interval of 10 days. The medium of half MS with 2X vitamins + 3 per cent sucrose + 0.18 per cent phytagel + picloram 0.2 mg l^{-1} + BA 2.5 mg l^{-1} was found to be more supporting for early and good endosperm filling with good embryo development. In this medium there was complete filling at 10 days after pollination in the former medium. The endosperm was soft and jelly like in 10 days old seeds and it became firm by 20 DAP. The hardness of the endosperm progressed upto 50 DAP. Embryo was found to be seated towards the chalazal end and the endosperm constitutes the major portion of the seed.

9. The viability of the seeds developed was monitored from 10 to 50 DAP by tetrazolium test. The embryo and endosperm were stained completely red indicating that they were metabolically active upto 50 DAP.

10. Embryo culture studies conducted with seeds of 40 to 60 days maturity in various hormonal combinations did not give positive results. Hence juvenility of the embryos may be important in getting regeneration. Embryo culture in

various hormonal combinations from seeds of 15 to 30 DAP did not give response after 40 days of culturing. Prolonged culturing may give positive results.

11. Germination studies were conducted in a number of plant growth regulator combinations of auxins, cytokinins, GA_3 and thiourea. The 60 days old seed derived from the parental combination of Kuruppampady X Z-0-78 showed radicle emergence when cultured in dark in the medium of half MS + 3 per cent sucrose + 0.65 per cent agar + IBA 3.0 mg l^{-1}. It failed to grow further. The seeds of parental combination Z-0-78 X Varadha when cultured and maintained in dark on the medium of B_5 + 3 per cent sucrose + 0.18 per cent phytagel + 2,4-D 0.2 mg l^{-1} + BA 2.5 mg l^{-1} germinated by embryo callusing and somatic embryogenesis 105 days after *in vitro* pollination. It was found that the seed coat was pushed apart by the callusing embryo and it developed globular somatic embryoids. Embryoids developed root and shoot poles in the same medium. The somatic embryoid is in the initial stage of development.



References

REFERENCES

Adaniya, S. 1991. Effects of pollination season and relative humidity on seed setting potential in *Zingiber mioga* Rosc. and variations in the number of chromosomes of self pollinated progenies. *J. Jpn Soc. Hort. Sci.* 60: 361-367.

Adaniya, S. 1998. Variation in pollen fertility and germinability in ginger (*Zingiber officinale* Rosc.). *J. Jpn Soc. Hort. Sci.* 67: 872-874.

Ali, S.A., Mishra, A.K. and Mourya, K.N. 1994. Genetic variability in ginger (*Zingiber officinale* Rosc.) *Int. J. trop. Agric.* 12: 282-283.

Anandan, R., Pranathi, S., Phan, D.P., Sooriyanadhasundaram, K., Kumar, N., Sudhakar, D. and Balasubramaniam, P. 2005. Somatic embryogenesis and plant regeneration from immature embryos of papaya (*Carica papaya* L.), Co 7. *Proccedings of the National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and strategies.* 10-12 Jan., 2005, Kerala Agricultural University, Thrissur, Kerala. pp 102-104.

Asif, M.J., Mak, C. and othman, R.Y. 2001. *In vitro* zygotic embryo culture of wild *Musa accuminata* ssp. Malaccensis and factors affecting germination and seedling groewth. *Plant Cell Tissue Organ Cult.* 67: 287-270.

Babu, K.N., Samsudeen, K. and Ravindran, P.N. 1992. Direct regeneration of plantlets from immature inflorescence of ginger (*Zingiber officinale* Rosc.). *J. Spice Aromatic Crops* 1: 43-48.

Balatkova, V., Hrabetova, E. and Tupy, J. 1976. the effect of sugar nuitrition of *in vitro* pollinated placentae on seed set and dormancy in *Nicotiana tabacum* L. *Experientia* 32(10): 1255-1256.

Beek, V.O., Sin, R. and Smit, H.A.J. 1944. Factors affecting the growth of Datura embryos *in vitro*. *Am. J. Bot.* 31: 219-224.

Chee, P.P. 1995. Organogenesis in *Taxus brevifolia* tissue cultures – zygotic embryo culture and propagation via shoot organogenesis. *Plant Cell Rep.* 14: 560-565.

Chi, H.S. 2000. Interspecific crosses of lily by *in vitro* pollinated ovules. *Bot. Bull. Acad. Sci.* 41: 143-149.

Choi, S.K. 1991. Studies on the clonal multiplication of ginger through *in vitro* cuttings. *Res. Rep. Rural Development Administration* 33: 33-39.

Chuang, M. and Chang, W. 1987. Somatic embryogebnesis and plant regeneration in callus culture derived from immature seeds and mature zygotic embryos of *Dysosma pleiantha* (Hance) Woodson. *Plant Cell Rep.* 6: 484-485.

Das, P. 1999. Genetic advance heritability and path analysis in ginger (*Zingiber officinale* Rosc.) *J. Plantation Crops* 27: 27-30.

De-Lange, J.H., Willers, P. and Magdanel, H. 1987. Elimination of nematodes from ginger (*Zingiber officinale* Rosc). *J. hort. Sci.* 62: 249-252.

Demeke, T., Adams, R.P. and Chibbar, R. 1992. Potential taxonomic use of Random Amplified Polymorphic DNA (RAPD): a case study in *Brassica. Theor. Appl. Genet.* 84: 990-994.

Demir, I. And Gunay, A. 1996. The effects of seed quality on germination, emergence and subsequent seedling development in cucumber (*Cucumis sativus* L.) *Seed Abst.* 19: 20-21.

Dogra, S.P., Korla, B.N. and Sharma, P.P. 1994. *In vitro* clonal propagation of ginger (*Zingiber officinale* Rosc). Hort. J. 7: 45-50.

Dohroo, N.P. 2001. Etilogy and Management of storage rot of ginger in Himachal Pradesh. *Indian phytopathology* 54: 49-54.

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11-15.

Ebrahimie, E., Habashi, A.A., Ghareyazie, B., Ghannadha, M. and Mohammedie, M. 2003. A rapid and efficient method for regeneration of plantlets from embryo explants of cumin (*Cuminum cyminum*). *Plant Cell Tissue Organ Cult.* 75: 19-25.

Behrav, A., and Coheu, Y. 1995. Effect of kinetin and gibberellin on melon embryo culture and subsequent propagation for hybrid embryo rescue. *Plant Gr. Regul.* 16: 267-269.

Bhaskaran, S. and Smith, R.H. 1990. Regeneration in cereal tissue cultures: A review. *Crop Sci.* 30: 1328-1336.

Bhojwani, S.S. and Razdan, M.K. 1983. *Plant Tissue Culture: Theory and Practice.* Elsevier Science Publishers, New York. 368p.

Bhurke, V.V. 2002. *In vitro* pollination in kacholam (*Kaempferia galan*ga L.) for seed set. MSc. (Hort.) thesis, Kerala Agricultural University, Trichur. 95 p.

Bindu, R. 1997. *In vitro* pollination, embryo rescue and germination studies in ginger (*Zingiber officinal*e Rosc). MSc. (Hort.) thesis, Kerala Agricultural University, Trichur. 95 p.

Bletsos, F.A., Roupakias, D.G. and Thanassopulopaulos, C.C. 1998. Morphological traits of the interspecific egg plant hybrid *Solanum melongena* X *S. torvum. Acta Hort.* 467: 155-161..

Bridgen, M.P. 1994. A review of plant embryo culture. *Hort. Sci.* 29: 1243-1246

Burun, b. and Poyrazoglu, E.C. 2002. Embryo culture in barley (*Hordeum vulgare* l.). *Turkish J. Biol.* 26: 175-180.

Buyukalaca, S., Gulen, M. and Kaska, N. 1997. Embryo rescue on abortive pistachio seeds. *Acta Hort.* 441: 307-308.

Cantliffe, D.J. and Abebe, Y. 1996. Priming 'Solarset' tomato seeds to improve germination at high temperature. *Seed Abst.* 19: 25-26.

Castano, C.i. and Proft, M.P. 2000. *In vitro* pollination of isolated ovules of *Cichorium intybus* L. *Plant Cell Rep.* 19: 25-26.

Chandra, R. and Govind, S. 1999. Genetic variability and performance of ginger genotypes under mid-hills of Meghalaya. *Indian J. Hort.* 56: 274-278.

Chaudhury, R. and Chandel, K.P.S. 1995. Studies on germination and cryopreservation of cardamom (*Elettaria caedamomum* Maton) seeds. *Seed Sci. Technol.* 23: 235-240.

Chee, P.P. 1995. Organogenesis in *Taxus brevifolia* tissue cultures – zygotic embryo culture and propagation via shoot organogenesis. *Plant Cell Rep.* 14: 560-565.

Chi, H.S. 2000. Interspecific crosses of lily by *in vitro* pollinated ovules. *Bot. Bull. Acad. Sci.* 41: 143-149.

Choi, S.K. 1991. Studies on the clonal multiplication of ginger through *in vitro* cuttings. *Res. Rep. Rural Development Administration* 33: 33-39.

Chuang, M. and Chang, W. 1987. Somatic embryogebnesis and plant regeneration in callus culture derived from immature seeds and mature zygotic embryos of *Dysosma pleiantha* (Hance) Woodson. *Plant Cell Rep.* 6: 484-485.

Das, P. 1999. Genetic advance heritability and path analysis in ginger (*Zingiber officinale* Rosc.) *J. Plantation Crops* 27: 27-30.

De-Lange, J.H., Willers, P. and Magdanel, H. 1987. Elimination of nematodes from ginger (*Zingiber officinale* Rosc). *J. hort. Sci.* 62: 249-252.

Demeke, T., Adams, R.P. and Chibbar, R. 1992. Potential taxonomic use of Random Amplified Polymorphic DNA (RAPD): a case study in *Brassica. Theor. Appl. Genet.* 84: 990-994.

Demir, I. And Gunay, A. 1996. The effects of seed quality on germination, emergence and subsequent seedling development in cucumber (*Cucumis sativus* L.) *Seed Abst.* 19: 20-21.

Dogra, S.P., Korla, B.N. and Sharma, P.P. 1994. *In vitro* clonal propagation of ginger (*Zingiber officinale* Rosc). Hort. J. 7: 45-50.

Dohroo, N.P. 2001. Etilogy and Management of storage rot of ginger in Himachal Pradesh. *Indian phytopathology* 54: 49-54.

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11-15.

Ebrahimie, E., Habashi, A.A., Ghareyazie, B., Ghannadha, M. and Mohammedie, M. 2003. A rapid and efficient method for regeneration of plantlets from embryo explants of cumin (*Cuminum cyminum*). *Plant Cell Tissue Organ Cult.* 75: 19-25.

Euewens, C.J. and Schwale, W.W. 1975. Seed and pod development in Pisum sativum L. in relation to extracted and applied hormones. *J. Exp. Bot.* 26: 1-14.

FAO (Food and Agriculture organisation). 2005. Agricultural data: FAOSTAT. [On-line]. Available: http://www.fao.org. [16 Nov 2005].

Gairi, A. and Rashid, A. 2004. TDZ induced somatic embryogenesis in non responsive caryopses of rice using a short treatment with 2,4 D. *Plant Cell Tissue Organ Cult.* 76: 29-33.

Gamborg, O.L. and Shyluk, J.P. 1981. Nuitrition medoia and characteristic of plant tissue cultures. *Plant Tissue Culture: Methods and Application in Agriculture.* (ed. Thorpe, T.A.). Academic Press, New York, pp 21-44.

Gamborg, O.L., Miller, R.A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158.

Gengenbach, B.G. 1977. Development of maize caryopses resulting from *in vitro* pollination. *Planta* 134: 91-93.

Guha, S. and Johri, B.M. 1996. *In vitro* development of ovary and ovule of *Allium cepa* L. *Phytomorphology* 16: 353-364.

Haliloglu, K. and Baenziger. 2005. Screening wheat genotypes for callus induction and regeneration capacity from immature embryo cultures. *J. Plant Biochem. Biotech.* 14: 155-160.

Hannig, E. 1904. Zur Physiologie Pflanzlicher Embryoneu. I. Uber die cultur von Crucifereu – embryoneu ausserhalb des Embryosacks. *Bot. Ztg.* 62: 45- 80.

Harry, I.S. Martinez, P.C. and Thorpe, T.A. 1995. Plantlet regeneration from mature embryos of Juniperus cedrus- embryogenic explant plantlet propagation. *Plant Cell Tissue Organ Cult.* 41: 75-78.

He, C.K., Li, J.S., Guo, S.Z., Zheng, M. and Cheng, W.S.1995. The relationship between geographic distribution and the genetic difference of peroxidase enzyme of ginger germplasm in Fujia. *Acta Hort.* 402: 125-132.

Holttum, R.E. 1950. The Zingiberaceae of Malay Peninsula. *Gardens Bull.* 13: 241-249.

Hooker, J.D. 1894. *The Flora of British India.* Reeve and Co. Ltd., London, 792p.

Hosoki, T. and Sagawa, Y. 1977. Clonal propagation of ginger (*Zingiber officinale* Rosc) through tissue culture. *Hort. Sci.* 12: 451-452.

Igasaki, T., Sato,T., Akashi, N., Mohri, T., Maruyama, E., Kinoshita, I. Walter, C. and Shinohara, K. 2003. Somatic embryogenesis and plant regeneration from immature zygotic embryos of *Cryptomeria japonica* D. Don. *Plant Cell. Rep.* 22: 239-243.

Inomata, N. 1996. Overcoming the cross incompatability through embryo rescue and the transfer of characters within the genus *Brassica* crops. *Scient. Rept. Fac. Agric.* Okayama Univ. 85: 79-88.

Ishikawa, T., Takayama, T. and Ishizaka, H. 2001. Production of interspecific hybrids between *Astroemeria pelegrina* L. and *A. magenta* Bayer by ovule culture. *Euphytica* 118: 19-27.

Ito, I. 1966. *In vitro* culture of ovary in orchids (1). Effects of sugar, peptone and coconut milk upon the growth of ovary of *Dendrobium nobile. Sci. rep. Kyotoperfect Univ. Agric.* 18: 38-50.

Jayachandran, B.K. Vijayagopal, D. and. Sethumadhavan, P. 1979. Floral biology of ginger (*Zingiber officinale* Rosc.) *Agric. Res. J. Kerala.* 17: 93-94.

Jin, K.Y. Min, S.P. and Hwa, J.K. 2001. Application of *in vitro* culture methods for overcoming cross-incompatibility in interspecific crosses between *L. longiflorum* and *L. cernuum. Korean J. hort. Sci. Technol.* 19: 3787-383.

Kackar, A., Bhat, S.R., Chandal, K.P.S. and Malik, S.K. 1993. Plant regeneration (via) somatic embryogenesis in ginger. *Plant Cell Tissue Organ Cult.* 32: 289-292.

Kageyama, K, Yabe, K. and Miyajima, S.1991. Somatic embryogenesis in suspension culture of mature seed of *Cucumis melo* L. *Jpn J. Breeding* 41: 273-278.

Kanta, K. 1960. Intra-ovarian pollination in *Papaver rhoeas* L. *Nature* 188: 683-684.

Kanta, K. and Maheswari, P. 1963. Test tube fertilization in some angiosperms. *Phytomorphology* 13: 230-237.

Kanta, K., Rangaswamy. N.S. and Maheswari, P. 1962. Test tube fertilization in a flowering plant. *Nature* 194: 1214-1217.

KAU, 1996. *Package of Practices Recommendations: Crops 96.* Directorate of Extension, Kerala Agricultural University, Trichur. 215p.

Kaun, H.T., Chou, H.M., Hu, T.K. and Hsu, M.C. 2002. Somatic embryogenesis and plant regeneration from immature embryos of peanut (*Arachis hypogaea*). *J. Agric. For.* 51: 43-55.

Kavitha, P.G., Nair, P., Nair, A., Jayachandran, B.K., Sabu, M. and Thomas, G. 2005. AFLP polymorphism and *Pythium* response in *Zingiber* species. *Proccedings of the ICAR National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies.* 10-12 Jan., 2005, Kerala Agricultural University, Thrissur, Kerala. pp 232-234.

Kavitha, P.S. and Chezhiyan, N. 2005. Ovular callus culture studies in *Carica papaya* L. *Proccedings of the ICAR National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies.* 10-12 Jan., 2005, Kerala Agricultural University, Thrissur, Kerala. pp 95-99.

Kelbitsch, S.H., Michael, G. Hauser, H. and Fischbeck, G. 1975. Cytokiningehalt and konetwicklureg von Gerstenmutanen mit utershiedlicher korngrosse. *Z. pifanerzuchtun* 75: 311-316.

Kent, N. E. and Brink, R.A. 1947. Growth *in vitro* of immature *Hordeum* embryos. *Science* 106: 547-548.

Khandelwal, V.K. and Sen, D.N. 1996. Effect of potassium nitrate on seed germination of Eragrostis species. *Seed Abstr.* 19: 38-39.

Kin, M.S., Ziang, H.A.and Rong, D.C. 1995. The culture regime suitable for the rescue of hybrid embryos in *Actinidia. Acta Hort.* 40: 63.

Kitsaki, C.K., Zygouraki, S., Ziolora, M. and Kintzios, S. 2004. *In vitro* germination, protocorm formation and plantlet development of mature versus immature seeds from several *Ophrys* species (Orchidaceae). *Plant Cell Rep.* 23: 284-290.

Korikanthimath, V.S. and Mulge, R. 1998. Presowing seed treatment to enhance germination of cardamom. *Karanataka J. agric. Sci.* 11: 540-542.

Korla, B.N. 1999. Evaluation of ginger clones for quality attributes under rainfed and irrigated conditions. Hort. J. 12: 39-44.Nybe, E.V., Nair, S. and Mohankumaran, N. 1980. Assessment of yield and quality components of ginger. *Proceedings of National Seminar on Ginger and Turmeric*, (ed. Indian Institute of Spices Research) Calicut, 8-9 April, 1980. pp. 24-29.

Kristiansen, K. and Vainstein, A. 1995. Interspecific hybridization in *Alstroemeria. Acta Hort.* 420: 85-88.

Kumar, S., Suri, S.S., Sonie, K.C. and Ramawat, K.G. 2003. Establishment of embryogenic cultures and somatic embryogenesis in callus culture of guggul *Commiphora wightii* (Arnott.) Bhandari. *Indian J. Exp. Biol.* 41: 69-77.

Lautour, G., Jones, W,t. and Ross, M.D. 1978. Production of interspecific hybrids in Lotus aided by endosperm transplants. *N. Z. J. Bot.* 16: 61-68.

Leduc, N., Monnier, M. and Douglas, G.C. 1990. Germination of trinucleated pollen: formulation of a new medium for *Capsella bursa pastoris. Sex. Plant Reproduction* 3: 228-235.

Lekshmy, M.L. 1889. Cytomorphological evaluation of hybrids and embryo culture studies in banana. M. Sc. (Hort.) thesis, Kerala Agricultural University, Trichur, 157 p.

Letham, D. S. 1974. Regulators of cell division in plant tissues XX. The cytokinins of coconut milk. *Physiol. Pl.* 32: 347.

Lo, S.F., Nalawade, S.M., Kuo, C.L., Chen, C.L. and Tsay, H.S. 2004. Asymbiotic germination of immature seeds, plantlet development and *ex vitro* establishment of plants of *Dendrobium tosaense* Makino - a medicinally important orchid. *In vitro Cell. Dev. Biol. Plant* 40: 528-535.

Maheswari, N. 1958. *In vitro* culture of excised ovules of *Papaver somniferum* L. *Science* 127: 342-343.

Maheswari, N. and Lal, M. 1961. *In vitro* culture of excised ovules of *Papaver somniferum* L. *Phytomorphology.* 11: 307-314.

Mangaly, J.K. and Nayar, J. 1990. Polynolgy of South Indian Zingiberaceae. *Bot. J. Linnaean Soc.* 103: 351-366.

Michel, G. and Kelbitsch, H. 1972. Cytokinin content and kernel size of barley grains as affected by environmental and genetic factors. *Crop Sci.* 12: 162-165.

Michishita, A., Ureshino, K. and Miyajima, I. 2001. Shortening the period from crossing to the seedling stage through ovule culture of interspecific crosses of *Azalea*. *J. Jpn Soc. Hort. Sci.* 70: 54-59.

Mohanty, D.C., Das, R.C. and Sharma, Y.N. 1981. Variability of agronomic characters in ginger (*Zingiber officinale* Rosc.). *Orissa J. Hort.* 9: 15-17.

Monnier, M. 1978. Culture of zygotic embryos. *Frontiers of Plant Tissue Culture* (ed. Thorpe, T.A.) University of Calgary Press, Canada. pp 277-286.

Muniswamy, B. and Sreenath, H.L. 1996. *In vitro* plant development from cultured zygotic embryos of *Coffea bengalensis*. *Proceedings of the Plantation crops symposium XII*, 27-29 Nov. 1996, Kottayam, India. pp 25-26.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant* 15: 473-497.

Naidu, M.M., Padma, M., Yuvaraj, K.M. and Murty, P.S. 2000. Evaluation of ginger varieties for high altitude and tribal area of Andhra Pradesh. *J. Spices Aromatic Crops* 9: 50-51.

Nair, R.R., and Gupta, S.D. 2003. Somatic embryogenesis and plant regeneration in black pepper (*Piper nigrum* L.). *J. Hort. Sci. Biotech.* 78: 416-421.

Nakasone, Y., Wada, K. and Adaniya, S. 1999. Evaluation of pungency in the diploid and tetraploid types of ginger (*Zingiber officinale* Rosc.) and antioxidative activity of their methanol extracts. *Jpn J. Trop. Agric.* 43: 71-75.

Narayanaswamy, Ş. and Norstog, K. 1984. Plant embryo culture. *Bot. Rev.* 30: 587-628.

Neeta, S., Eapen, S. and George, L. 2003. Biotechnological studies of turmeric (*Curcuma longa* L.) and ginger (*Zingiber officinale* Rosc.). *Advances agric. biotech.* 11-32.

Nitsch, J.P. 1952. Plant hormones in the development of fruits. *Q. rev. Biol.* 27: 33-59.

Nizam, S.A. and Jayachandran, B.K. 1997. Effect of seed rhizome size and varieties on the quality of ginger (*Zingiber officinale* Rosc.) under open and shade. *S. Indian. Hort.* 45: 50-52.

Norstog, K. 1961. The growth and differentiation of cultured barley embryos. *Am. J. Bot.* 48: 876-884.

Norstog, K. 1965. Development of cultured barley embryos. I. Growth of 0.1-0.4 mm embryos. *Am. J. Bot.* 52: 538-546.

Norstog, K.1972. Factors relating to precocious germination in cultured barley embryos. *Phytomorphology.* 22: 134-139.

Nybe, E.V. 1978. Morphological studies and quality evaluation of ginger (*Zingiber officinale* Rosc.) types. M. Sc. (Hort.) thesis, Kerala Agricultural University, Trichur, 106 p.

Obert, B. and Barnabas, B. 2004. Colchicine induced embryogenesis in maize. *Plant Cell Tissue Organ Cult.* 77: 283-285.

Ondrej, V., Navratilova, B. and Lebeda, A. 2002. Influence of GA_3 on the zygotic embryogenesis of *Cucumis* species *in vitro*. *Biologica*. 57: 523-525.

Palai, S.K., Rout, G.R., Samantaray, S. and Das, P.2000. Biochemical changes during *in vitro* organogenesis in *Zingiber officinale*. *J. Plant Biol.* 27: 153-160.

Palani, 1996. Effect of pre sowing chemical treatment on germination and seedling growth in Acacia nilotica. *Seed Abst.* 19: 25-26.

Pancholi, N., Wetter, A. and Caligari, P.D.S. 1995. Germination of Musa velutina seeds: Comparison of *in vivo* and *in vitro* systems- immature seed culture medium and light conditions optimization for example - hybrid plant propagation. *In vitro Plant* 31: 127-130.

Park, S. U and Facchini, P.J. Somatic embryogenesis from embryogenic cell suspension cultures of California poppy, *Eschscholzia californica* Cham. *In vitro Cellular Developmental Biol. Plant.* 37: 35-39.

Paul, R., Shylaja, M.R., Nazeem, P.A., Valsala, P.A. and Nybe, E.V. 2005. Plantlet regeneration in ginger (Zingibwer oficinale Rosc.) through indirect organogenesis. *Proccedings of the ICAR National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies.* 10-12 Jan., 2005, Kerala Agricultural University, Thrissur, Kerala. pp 107-108.

Pillai, P.K.T., Vijayagopal, G. and Nambiar, M.C. 1978. Flowering behaviour, cytology and pollen germination in ginger (*Zingiber officinale* Rosc.) *J. Plantation crops* 6: 12-13.

Pojanagaroon, S., Praphet, R., Kaewrak, C. and Yotdi, A. 2004. Characterization of Krachai-Dam (*Kaempferia parviflora*) cultivars using RAPD markers, morphological traits and chemical components of essential oil from rhizomes. *Proceedings of the 42nd Kasetsart University Annual Conference*, Kasetsart, Thailand, 3-6 February 2004. pp 56-65.

Pruthy, J.S. 1998. *Major Spices of India Crop Management Post Harvest Technology.* Indian Council of Agricultuiral Research, New Delhi, 283p.

Pullman, G.S., Zhang, Y. and Phan, B.H. 2003. Brassinolide improves embryogenic tissue initiation in conifers and rice. *Plant Cell Rep.* 22: 96-104.

Purseglove, J.W. 1978. *Tropical Crops- Monocotyledons.* Longman, London, 540p.

Purseglove, J.W., Brown, E.G., Green,C.L. and Robbins, S.R.J. 1981. *Spices Vol. II.* Longman, London, 813p.

Qing, S.Z., Jianpo, G. and Yin, L.C. 2002. Experiment on culturing extra early apricot embryos. *China Fruits* 1: 17-18.

Quartrano, R.S. 1987. The role of hormones during seed development. *Plant Hormones and Their Role in Plant Growth and Development* (ed. Davies, P.J.). Kluwer Academic Publishers, London, pp. 513-515.

Raghavan, V. and Torrey, J.G. 1964. Effects of certain growth substances on the growth and morphogenesis of immature embryos of *Capsella* in culture. *Plant Physiol.* 39: 601-699.

Rai, V.R. and Mc Comb, J. 2002. Direct somatic embryogenesis from mature embryos of sandalwood. *Plant Cell Tissue Organ Cult.* 69: 65-70.

Raja, N.S.S. 1993. Pre sowing treatments of seeds to improve germination in cardamom. *J. Plantation Crops* 21: 116-117.

Rajan, P.P., Gupta, S.R. and Sarma, Y.R. 2002. Diseases of ginger and their control with *Trichoderma harzianum. Indian phytopathology.* 55: 173-177.

Ramachandran, K. 1982. Polypoidy induced in ginger by colchicines treatment. *Curr. Sci.* 51: 288-289.

Ramachandran, K. and Nair, P.N..C. 1992. Induced tetraploids in ginger. *J. Spices Aromatic Crops* 1: 39-42.

Rangaswamy, N.S. and Shivanna, K.R. 1967. Induction of gametic compatibility and seed formation in axenic cultures of a diploid self-incompatible species of Petunia. *Nature.* 216: 937-939.

Rashid, V.A. 2002a. Induction of multiple shoots by thidiazuron from caryopsis cultures of minor millet (*Paspalum scrobiculatum* L.)and its effect on the regeneration of embryogenic callus cultures. *Plant Cell Rep.* 21: 9-13.

Rashid, V.A. 2002b. Somatic embryogenesis from immature and mature embryos of a minor millet *Paspalum scrobiculatum* L. *Plant Cell Tissue Organ Cult.* 69: 71-77.

Ratnambal, M.J. and Nair, M.K. 1982. Colchicine induced tetraploids in ginger. *J. Plantation Crops* 10: 57-61.

Ravindran, P.N., Sasikumar, B. and George, J.K. 1994. Genetic resources of ginger (*Zingiber officinale* Rosc.) and its conservation in India. *Pl. Genet. Resour. Newsl.* 98: 1-4.

Razdan, M.K. 1993. *An Introduction to Plant Tissue Culture.* Oxford and IBH Publishing Co. Pvt. Ltd. New Delhi, 143p.

Renjith, D. 1999. Response of turmeric *Curcuma domestica* Vál. to *in vivo* and *in vitro* pollination. M. Sc. (Hort.) thesis, Kerala Agricultural University, Trichur, 87 p.

Rout, G. R., Das, P., Goel, S. and Raina, S. N. 1998. Determination of genetic stability of micropropagated plants of ginger using random amplified polymorphic DNA (RAPD) markers. *Botanical Bull. Academia Sinica* 39: 23-27.

Rout, G.R. and Das, P. 1997. *In vitro* organogenesis in ginger (*Zingiber officinale* Rosc). *J. Herbs Spices Medicinal Plants.* 4: 41-51.

Roy, T. and De, D.N. 1990. tissue culture and plant regeneration from immature embryo explants of *Calotropis gigantea* (Linn.). *Plant Cell. Tissue Organ Cult.* 20: 229-233.

Saikia, L. and Shadeque, A. 1992. Yield and quality of ginger (*Zingiber officinale* Rosc.) varieties grown in Assam. *J. Spices Aromatic Crops* 1: 131-135.

Samaj, J., Baluska, F., Pretova. And Volkmann, D. 2003. Auxin deprivation induces a developmental switch in maize somatic embryogenesis involving redistribution of microtubules and actin filaments from endoplasmic to cortical cytoskeletal arrays. *Plant Cell Rep.* 21: 940-945.

Sarasan, V., Ramsay, M.M. and Roberts, A.V. 2002. *In vitro* germination and induction of direct somatic embryogenesis in bottle palm [*Hyophorbe lagenicaulis* (L. Bailey) H.E. Moore], a critically endangered Mauritian palm. *Plant Cell Rep.* 20: 1107-1111.

Sarma, Y.R. and Sasikumar, B. 2002. Indian spices: Accelerating Production and Export through proactive programmes. *Souvenir on National Consultative Meeting for Accelerated production and Export of Spices*, (ed. Indian Institute of Spice Research) Calicut, 29-30 May, 2002, pp.9-21.

Sathiabhama, K.U. 1988. Investigations on cytogenetics, flowering and seed set in ginger (*Zingiber officinale* Rosc.). M. Sc. (Hort.) thesis, Kerala Agricultural University, Trichur. 108p.

Setty, T.A.S., Guruprasad, T.R. and Reddy, M.N. 1995. Susceptibility of ginger cultivars to phyllosticta leaf spot at West Coast conditions. *Environment Ecol.* 13: 443-444.

Shamina, A., Zachariah, T. J. and Sasikumar, B. 1997. Biochemical variability in selected ginger (*Zingiber officinale* Rosc.) germplasm accessions. *J. Spices Aromatic Crops* 6: 119-127.

Shamina, A., Zachariah, T.J., Sasikumar, B. and George, J.K. 1998. Biochemical variation in turmeric (*Curcuma longa* L.) accessions based on isozyme polymorphism. *J. Hort. Sci. Biotech.* 73: 479-483.

Shankar, G. 2003. Expolitation of induced variability for crop improvement in ginger (*Zingiber officinale* Rosc.). M. Sc. (Hort.) thesis, Kerala Agricultural University, Trichur. 175p.

Sharma, T.R. and Singh, B.M. 1997. High frequency *in vitro* multiplication of disease free *Zingiber officinale* Rosc. *Plant Cell Rep.* 77: 68-72.

Sheeba, P.T. 1996. Induction of autotetraploidy in ginger. M. Sc. (Hort.) thesis, Kerala Agricultural University, Trichur. 71p.

Shirai, D. 2001. *In vitro* induction of tetraploid ginger (Zingiber officinale Rosc.) and its pollen fertility and germinability. *Scientia Horticulturae* 88: 277-287.

Singh, A.K. and Edison, S. 2000. Reaction of ginger germplasm to Phyllosticta zingiberi under field conditions. *Indian phytopathology* 53: 210-212.

Singh, D.K., Sinha, S.K. and Singh, V.N. 2000. Control of bacterial wilt of ginger (*Zingiber officinale* Rosc.) with antibiotics. *J. Res.* 12: 41-43.

Sirat, H.m. 1994. Study on the terpenoides of *Zingiber offensii. Planta Medica* 60: 497-498.

Soo, K., Choic, I. And Dong, K.S. 2000. Investigation of floral structure and plant regeneration through anther culture in ginger. *Korean J. Crop Sci.* 45: 207-210.

Spices Board of India. 2005. State wise area and production of spices in India. [on-line]. Available: http://www.indianspices.com/. [16 Nov. 2005].

Spiegel-Roy, P., Sahar, N., Baron,J. and Lavi, U. 1885. *In vitro* culture and plant formation from grape cutivars with abortive ovules and seed. *J. Am. Soc. Hort. Sci.* 110: 109-112.

Sreekumar, V., Indrasenan, G. and Mammen, M.K. 1980. Studies on quantitative and qualitative attributes of ginger cultures. *Proceedings of National Seminar on Ginger and Turmeric,* (ed. Indian Institute of Spice Research} Calicut, 8-9 April, 1980. pp. 47-49.

Sreelatha, U., Baburaj, T. S., and Nazeem, P. 2005. Micropropagation of *Exacum biclor* (Kannanthali), a plant with excellent ornamental and medicinal potential. [Abstract]. *Mangroves and coastal ecosystem, Abstracts XV, Swadeshi Science Congress* 5-7 November 2005. p24.Govt. Brennen College, Thalassery, Absract no. 9.

Stephan, R. and Jayabalan, N. 2001. Propagation of *Coriandrum sativum* L. through somatic embryogenesis. *Indian J. Exp. Biol.* 39: 387-389.

Sudhadevi, P.K., Geetha, C.K. and Radhakrishnan, C. 1993. *In vitro* regeneration of plantlets from hybrid seed embryo of pineapple [*Ananas comosus* (Linn.). Merrr.]. *Proceedings of National Symposium on Horticutural research Changing Scenario.* Bangalore, p. 462.

Sujatha, K., Latha, P. and Nayar, N.K. 1994. Evaluation of ginger varieties as an intercrop in coconut gardens. *S. Indian Hort.* 42: 339.

Suma, B. and Kesavachandran, R. 2005. Somatic embryogenesis and plant regeneration from callus cultures of ginger (*Zingiber officinale* Rosc.). *Proccedings of 17th Kerala Science Congress, 29-31 Jan.,2005, Kerala Forest Research Institute, Peechi, Trichur. pp 88-89.*

Tokuji, Y. and Kuriyama, K. 2003. Involvement of gibberellin and cytokinin in the formation of embryogenic cell clumps in carrot (*Daucus carota*). *J. Plant Physiol.* 160: 133-14.

Tollenaar, M. 1977. Sink-source relationshipn during reproductoive development in maize: a review. *Maydica* 22: 49-75.

Tuyl, V.J.M., Dien, V.M.P., Creij, V.H.G.M., Klien, V.W., Franken, J. and Bino, R.J. 1991. Application of *in vitro* ovary culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. *Plant Sci.* 74: 115-126.

Usha, K. 1984. Effect of growth regulators on flowering, pollination and seed set in ginger (*Zingiber officinale* Rosc.). M. Sc. (Hort.) thesis, Kerala Agricultural University, Trichur, 125p.

Usha, S.V. 1965. *In vitro* pollination in *Antirrhinum majus* L. *Curr. Sci.* 17: 511-512.

Valsala, P.A. 1994. Standardization of *in vitro* pollination and fertilization for genetic variability in *Zingiber officinale* Rosc. Ph. D. thesis, Kerala Agricultural University, Trichur, 133p.

Valsala, P.A., Nybe, E.V., Nazeem, P.A. and Joseph, L. 2001. Hybridization in ginger *Zingiber officinale* (Rosc.) through *in vitro* pollination. Final report 1998-2001. ICAR Ad-hoc project, Kerala, India, pp1-20.

Vanijajiva, O, Sirirugsa, P and Suvachittanont, W.2005. Confirmation of relationships among *Boesenbergia* (Zingiberaceae) and related genera by RAPD. *Biochem. Systematics Ecol.* 33(2): 159-170.

Vijayasree, P.S. 2001. Refinement of in vivo and *in vitro* pollination technique in turmeric. M. Sc. (Hort.) thesis, Kerala Agricultural University, Trichur, 105p.

Wang, A.S. 1987. Callus induction and plant regeneration from maize mature embryos. *Plant Cell Rep.* 6: 360-362.

Wang, H.C., Chen, J.T., Wu, S.P., Lin, M.C. and Chang, W.C. 2003. Plant regeneration through somatic embryogenesis from zygotic-embryo-derived callus of *Areca catechu* L. (Arecaceae). *In vitro Cellular and Developmental Biol. Plant* 39: 34-36.

Wang, Z.Y., Bell, J. and Hopkins, A. 2003. Establishment of a plant regeneration system for wheat grasses (*Tinopyrum, Agropyron* and *Pascopyrum*). *Plant Cell Tissue Organ Cult.* 73: 265-273.

Williams, D. and Lautour, G. 1980. The use of embryo culture with transplanted nurse endosperm for the production of interspecific hybrids in pasture legumes. *Bot. Gaz.* 141: 251-257.

Zale, J.M., Wier, H.B., Kidwell, K.K. and Stebewr, C.M. 2004. Callus induction and plant regeneration from mature embryos of a diverse set of wheat genotypes. *Plant Cell. Tissue Organ Cult.* 76: 277-281.

Zeibur, N.K. and Brink, R.A. 1951. The stimulative effect of *Hordeum* endosperms on the growth of mature plant embryos *in vitro*. *Am. J. Bot.* 38: 253-256.

Zenkteler, M. 1969. PTPN Prace Komisji. *Biologiczney* 32: 1-71.

Zenkteler, M. 1984. *In vitro* pollination and fertilization. *Cell Culture and Somatic Cell Genomics of Plants* Vol. I (ed. Vasil, I.K.) Academic Press Inc., Florida, pp. 269-275.

Zenkteler, M. and Melchers, G. 1978. In vitro hybridization by sexual methods and by fusion of somatic protoplasts. *Theor. Appl. Genet.* 52: 81-90.

Appendices

Appendix I

Specific chemicals used	Firm
Cetyl trimethyl ammonium bromide (CTAB)	E Merck, Germany
β mercaptoethanol	E Merck, Germany
Ethidium bromide	Sigma, USA
Agarose	Genei, Bangalore
Taq DNA polymerase	Genei, Bangalore
PCR reagents (10X assay buffer, dNTPs)	Genei, Bangalore
Decamer primers	Operon, USA
Bromophenol blue	Sigma, USA
Tris base	Sigma, USA
EDTA	Sigma, USA
DNA molecular wt. marker	Genei, Bangalore
RNase	Genei, Bangalore
Phytagel	Himedia, India
Proteinase K	Genei, Bangalore
Naphthalene acetic acid	Sigma, USA
Benzyl amino purine	Sigma, USA
Indole acetic acid	Sigma, USA

Appendix II

Equipments for the study	Firm
Refrigerated high speed centrifuge	Kubota, Japan
Water purification system	Millipore, Germany
	Aquaguard
Deep freezer	Sanyo, Japan
Electronic balance	Sarturius
Laminar airflow	Kirloskar, India
Cytocentrifuge	Spinwin
Electrophoresis system	Hoefer, USA
	Biotech, Madras
	Genei, Bangalore
Thermal cycler	MJ Research, USA
	Peltire PTC 200
Transilluminator	Herolab, Germany
Documentation system	Alpha Innotech, USA
Ice flaking machine	Icematics

INDUCTION OF GENETIC VARIABILITY IN GINGER (*Zingiber officinale* Rosc.) THROUGH *IN VITRO* FERTILIZATION

By

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ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

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ABSTRACT

Investigations on 'Induction of genetic variability in ginger (*Zingiber officinale* Rosc.) through *in vitro* fertilization' were carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 2004-2005.

Molecular characterization studies of the eight ginger cultivars -Z-0-78 (V_1) Kodakara Local (V_3), Kuruppampady (V_4), Mahima (V_5), Maran (V_6), Rejatha (V_7), Rio-de-Janeiro (V_8) and Varadha (V_9)- by RAPD using nine random primers revealed that the genetic variability among the cultivars very narrow in the range of 5 to 19 per cent. The cultivar Rio-de-Janeiro was distinct with maximum variability. The variability among the released varieties from IISR ranged from 5 to 10 per cent.

The flowering season in ginger extended from August to November. The cultivars showed variability with respect to duration for initiation of flowering and flowering duration.

The mean pollen fertility and viability among the ginger cultivars were 35.76 per cent and 8.11 per cent respectively. The autotetraploids had high pollen fertility and viability compared to diploids. The range of pollen viability in autotetraploids was from 12.03 to 15.68 (Z-0-78) and in diploids it was from 3.68 to 8.90 per cent. Among the diploids the released varieties Varadha, Mahima and Rejatha had more pollen fertility and viability.

Attempts were made to refine the *in vitro* pollination technique with respect to basal media combination, growth regulators, vitamins and gelling agent. Studies conducted in the cross Varadha X Z-0-78 using placental pollination technique. The basal medium of half MS with 2X vitamins was superior to B_5 medium. As gelling agent phytagel 0.18 per cent was superior to agar 0.65 per cent. Among the various

plant growth regulator combinations tried the combination of picloram and BA was found to be best. The medium of half MS with 2X vitamins + 3 per cent sucrose + BA 2.5 mgl^{-1}+ picloram 0.2 mgl^{-1} + 0.18 per cent phytagel was the best with respect to maximum ovule swelling, percentage of cultures with ovule development and percentage setting per culture.

Seed set and seed development in various crosses involving autotetraploids and diploids were assessed. The parental combination between autotetraploids (Z-0-78 X Z-0-86) produced seeds with very good swelling in maximum number of cultures with maximum setting per culture.. The parental combination Z-078 X Varadha and its reciprocal also favoured very good ovule swelling. The parental combination Z-0-86 X Varadha as well as Varadha X Mahima and their reciprocals produced seeds with good swelling.

The size increase of the developing ovules after *in vitro* pollination was monitored from the day of pollination to 60 DAP. The size increase upto 15 DAP was rapid. At 60 DAP the ovules attained four-fold increase in size. The pollinated ovules cultured, in the course of maturation turned pink and later blackened. The seeds showed complete endosperm filling at 10 DAP. The soft and jelly like endosperm became hard by 20 DAP. Viability test with tetrazolium showed that the seeds were viable up to 50 DAP. Embryo was found to be seated towards the chalazal end and endosperm constitutes the major portion of the seed.

Germination studies were conducted in a number of plant growth regulator combinations. The 60 days old seed derived from the parental combination of Kuruppampady X Z-0-78 showed radicle emergence in the medium of half MS + 3 per cent sucrose + 0.65 per cent agar + IBA 3.0 mg l^{-1}. The seeds of parental combination Z-0-78 X Varadha germinated through somatic embryogenesis in the medium of B_5 + 3 per cent sucrose + 0.18 per cent phytagel + 2,4-D 0.2 mg l^{-1} + BA 2.5 mg l^{-1} 105 days after *in vitro* pollination. The germination was 10 per cent It was

found that the seed coat was pushed apart by the callusing embryo and it developed globular somatic embryoids. Embryoids developed root and shoot poles in the same medium. The somatic embryoid is in the initial stage of development.

Embryo culture studies with embryos excised from seeds of 15 to 60 DAP in various media combinations did not give positive response. Prolonged culturing may give positive results.