IN VITRO POLLINATION, EMBRYO RESCUE AND GERMINATION STUDIES IN GINGER, *Zingiber officinale* (Rosc.)

BY

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THESIS

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requirement for the degree of

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DECLARATION

I hereby declare that the thesis entitled "***In vitro* pollination, embryo rescue and germination studies in ginger, *Zingiber officinale* (Rosc.)**" 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, associateship or other similar title, of any other university or society.

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CERTIFICATE

Certified that the thesis entitled "***In vitro* pollination, embryo rescue and germination studies in ginger, *Zingiber officinale* (Rosc.)**" is a record of research work done independently by **Smt. Bindu, R.**, 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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BINDU,R.

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ABBREVIATIONS

ABA	- abscisic acid
BAP	- benzyl amino purine
CH	- casein hydrolysate
CW	- coconut water
DAP	- days after pollination
Na_2 EDTA	- sodium salt of ethylene diamino tetra acetic acid
GA_3	- gibberellic acid
h	- hour(s)
HNO_3	- nitric acid
H_2SO_4	- sulphuric acid
IAA	- Inode-3-acetic acid
kg cm^{-1}	- kilogram(s) per centimeter square
mg l^{-1}	- milligram(s) per litre
min	- minute(s)
MS	- Murashige and Skoog's (1962) medium
NAA	- α-Napththalene acetic acid
PEG	- poly ethylene glycol
ppm	- parts per million
s	- second(s)
SH	- Schenk and Hildebrandt (1972) medium
v/v	- volume in volume
2iP	- iso pentenyl adenine
2,4-D	- 2,4-dichlora phenoxy acetic acid
mm	- milli meter
μm	- micro meter
μM	- micro molar

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 medicines. At present ginger is ranked as the fourth important spice crop of India, standing next to chillies, black pepper and turmeric. India is the largest producer and exporter of the same, producing about 1.86 lakh tonnes from an area of 62,090 ha and has earned foreign exchange to the tune of Rs.383 million in the year 1995-96.

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 is another bottleneck 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.

Conventional generative breeding methods to develop resistant and high yielding genotypes can not be employed in ginger mainly due to lack of seed set. Investigations carried out by Sathiabhama (1988) revealed that pollen tube growth in ginger is not sufficient to fertilize the deeply seated ovules and fertilization is not taking place. 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 conditions required for germination of the seed have to be further investigated.

Achievements in this line would realize heterozygous breeding in ginger and high yielding types with specific qualities can be produced. Resistance to soft rot and bacterial wilt can be searched among wild relatives of ginger and if available can be incorporated into important cultivars. The present investigations were undertaken to standardise the conditions required for germination of *in vitro* produced seeds of ginger.

Review of Literature

2. REVIEW OF LITERATURE

Ginger, *Zingiber officinale* (Rosc.) has been described by many as a species producing flowers very scarcely and setting no seeds. Many investigators have tried to unravel the mistery of lack of seed set and fruit formation in ginger. Every efforts made so far for seed set and fruit formation under *in vivo* conditions were unsuccessful. *In vitro* pollination and fertilization techniques have been proved effective for overcoming pre- fertilization barriers encountered in selfing as well as in crossing programmes. Seed set and seed germination were obtained through *in vitro* pollination (Valsala, 1994). But the conditions required for germination need refinement. The accumulated literature on various aspects relevant to the subject matter of the present study are reviewed hereunder.

2.1 Flowering in ginger

2.1.1 Flowering behaviour

Ginger is a shy flowering crop, when raised from seed bits of around 15 g and maintained as an annual crop (Hooker, 1894; Holttum, 1950; Pillai *et al.*, 1978 and Nybe, 1978). The date of planting and size of seed material used influenced flowering. The shy flowering habit of ginger can be corrected by using large seed bits of around 200 g and by maintaining plants from 15 g seed bit as biennial. In normal annual plants, flowering occurred by middle of September and completed by last week of November. When the crop was raised from large seed bits, the flowering was earlier ie. last week of August and lasted by last week of October. In biennials, first flowering was obtained as early as Ist July and it

extended to last week of October. In annuals, the flowering season was for two months while it was extended to four months in biennials (Valsala, 1994).

2.1.2 Inflorescence

Inflorescence in ginger is a bracteate spike or raceme directly springing from the rhizome, rarely emerging terminally 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 length and 1.5 to 2.5 cm in diameter with supressed ovate or elliptic green bracts. Each bract subtends a single flower with a lateral or obliquely posterior bracteole. Occasionaly in a bract more than one flower is seen. The bracts are spirally arranged (Nybe, 1978; Pillai *et al.*, 1978; Jayachandran *et al.*, 1979 and Purseglove *et al.*, 1981).

2.1.3 Morphology of flowers

The flowers of ginger 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 6 stamens of which the outer three were reduced to staminodes and are seen at the base of the corolla tube. The inner lateral stamens are united and showing 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 passes through the grove 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; Purseglove, 1978 and

Jayachandran *et al.*, 1979). 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 midele (Valsala, 1994).

2.1.4 Floral biology

Ginger inflorescence take approximately 29 to 32 days from the spike initiation to first blooming (Usha, 1984 and Valsala, 1994).

In a spike, one or two flowers opened daily and blooming of flowers in a spike was completed within a period of 9 to 22 days (Usha, 1984 and Valsala, 1994). The blooming of individual flowers take place in an acropetal succession and the flower falls on the next day of blooming.

Anthesis in ginger cultivars were started by 3.00pm and continued upto 4.00 pm (Valsala, 1994). According to Pillai *et al.* (1978) and Jayachandran and Vijayagopal (1979), pollen shedding almost coincided with the flower opening. In contrast, Usha (1984) and Valsala (1994) observed anther dehiscence 10 mts to 2 h after opening. Stigma receptivity of ginger remains still unravelled owing to the failure of hand pollination to set seeds.

2.2 Pollen studies

2.2.1 Pollen structure

The pollen grains of ginger flowers are round, highly heterogenous in size and filling and are limited by a very thick exine. A well defined pore is absent

in the pollen grains (Pillai *et al.*, 1978; Jayachandran *et al.*, 1979 and Sathiabhama, 1988). The anther lobes are filled with pleanty of pollen grains (Jayachandran and Vijayagopal, 1979 and Usha, 1984).

2.2.2 Pollen fertility and germination

Reported pollen fertility in ginger ranged between 12 to 40 per cent (Pillai *et al.*, 1978; Usha, 1984; Sathiabhama, 1988 and Valsala, 1994).

Pillai *et al.* (1978) could obtain 14.50 per cent pollen germination in a medium with 8 per cent sucrose, 3 per cent gelatin and 60 ppm boric acid in moist chamber operating at 26.5°C. In the same medium Usha (1984) got only 6 per cent germination. Sathiabhama (1988) got 11.81 per cent pollen germination in the same medium except that gelatin was reduced to one per cent. She got a maximum pollen tube length of 108 μm and coiling of pollen tube was also observed. In the same medium Valsala (1994) got only 7.51 per cent pollen germination whereas Leduc *et al.*, 1990 reported 15.06 per cent in the ME_3 medium. The ME_3 medium contains all the major and minor nutrients and is characterized by high osmotic potential due to the presence of 12 per cent PEG-4000. The pollen tube growth was also significantly higher in the ME_3 medium and a high value of 1042.17 μm was obtained. The pH reactions ranging from 4.0 to 8.0 did not significantly influence pollen tube growth.

2.2.3 Pollination and seed set under *in vivo* condition

According to Hooker (1894) ginger is a specie never setting seeds. Pillai *et al.* (1978) reported that flower structure of ginger manifest an adaptation suitable for entemophily. Hand pollination using large quantities of pollen grains in cultivar

Rio-de-Janeiro was not effective in achieving seed set as reported by Jayachandran *et al.* (1979) and Usha (1984). Bud pollination as well as pollination after stigma and style removal were not effective (Usha, 1984).

Sathiabhama (1988) also could not obtain seed set in ginger eventhough many techniques such as bud pollination, artificial sibbing, artificial cross pollination between cultivars, chemically aided pollination, mixed pollination and mentor pollination were tried.

2.3 Reasons for lack of seed set

Several investigators have probed the reasons for lack of seed set in ginger. One school of thought was that failure of seed set in ginger is due to self incompatibility existing in stigma and style (East, 1940; Fryxell, 1957 and Pillai *et al.*, 1978).

Usha (1984) was of the opinion that incompatibility may not be the factor causing failure of seed set in ginger, as she failed to get seed by bud pollination or after removing the stigma and style.

Chromasomal abberations during meiosis and defects in micro and megasporogenesis which lead to defective gametes and sterility is another reason supported by many scientists (Ramachandran, 1969; Pillai *et al.*, 1978 and Ratnambal, 1979). Eventhough high sterility exist in ginger, some account 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.

Sathiabhama (1988) studied pollen-pistil interaction under *in vivo* conditions using fluorescence microscopy by adopting the procedure described by Kho and Baer (1968) and Kho *et al.* (1980). She found that pollen tube growth was only 95 μm whereas the style length was measured up to 3.9 cm (39000 μm). The possibility of pollen tube reaching the ovule at 39,000 μm below is quite remote. From this observation, she concluded that insufficient pollen tube growth is the main reason for lack of seed set in ginger.

Sathiabhama (1988) also added that spiny stigma of ginger flowers prevent germination of pollen grains either by injuring the pollen grains or by preventing the adherence of pollen grains in stigmatic surface. She also observed that the pollen tube growth was very slow and as it advances, get coiled. Besides, the opened flowers are retained in the plant for less than 12 h after anthesis and by the time the pollen germinate and grow, abscission layer is formed in the stylar region and flowers are shed.

2.4 *In vitro* seed set

Seed set in ginger was obtained through *in vitro* techniques (Valsala, 1994). The study can be summarised as follows:

The flower buds could be surface sterilized prior to anthesis (3.00 pm) by dipping them in streptocycline 500 mg l^{-1} for one h by wiping with 70 per cent alcohol and rinsing with mercuric chloride 0.1 per cent for 3 min. The flower buds on the day of anthesis and one day after anthesis were used for *in vitro* pollination. To standardise a method of *in vitro* pollination for selfing and crossing, nine

methods viz., stigmatic pollination, stylar pollination, intra-ovarian pollination in three ways, placental pollination in two ways, modified placental pollination and ovular or test tube fertilization (Bhojwani and Razdan, 1983) were tried. Ovules were developed in placental pollination, modified placental pollination and ovular or test tube fertilization. In placental pollination, the placenta bearing the ovules were exposed by completely peeling the ovary wall and pollination was done on the whole unit and alternatively they were cut into two pieces also.

In modified placental pollination, ovary wall of one locule was peeled away and pollination was done on the exposed ovules. In ovular pollination or test tube fertilization, the ovules were seperated and put in a cavity slide containing pollen suspension in pollen germination medium. After 24 h, it was transferred to culture medium.

In all these successful cases, pollen grains in ME_3 medium were applied over the ovules. The observation of ovules after placental pollination under fluorescence microscopy revealed that pollen germination started within 3 h of pollination and pollen tube growth was sufficient to fertilize the ovule.

Histological examination of ovules at 4 DAP showed eight celled pro-embryo and 40 DAP showed well developed embryo and endosperm rich in starch and oil grains. The aforesaid successful pollination techniques were suitable for selfing and crossing in ginger under *in vitro* condition. The pollinated ovules developed into mature seed in the medium of 1/2 MS + 6% sucrose + NAA 0.5 mg l^{-1} + BAP 2.5 mg l^{-1} + 15% CW v/v. The basal medium 1/2 MS could be replaced by SH (Schenk and Hildebrandt, 1972) or Nitsch (Nitsch, 1951). The ovules after *in vitro* pollination developed in sucrose concentration ranging from 3.0

to 12.0 per cent considering the increased seed set, 6.0 to 8.0 per cent levels of sucrose were optimum.

The effect of NAA could be replaced by 2,4-D or IAA. The effect of BAP could be replaced by kinetin or 2iP. Instead of coconut water 15 % v/v, CH 200 mg l^{-1} can also be used.

The ovules developed at a temperature of 26°C in dark, diffused light or light intensities of 500 to 1000 lux. Dark coloured mature seeds were obtained 60 to 90 DAP.

The ginger seeds recorded a mean length of 2.20 mm and breadth of 1.60 mm at 80 DAP. The arillate seeds showed two seed coats, the outer being thick and the inner one being thin. The seed coat encloses a cavity and in the cavity endosperm with embedded embryo was seen.

The *in vitro* produced seeds of ginger showed capability of germination when 80 DAP old seeds were incubated initially in the medium of 1/2 MS with 2,4-D 8 mg l^{-1} for 2 months and then in hormone combination of BAP 9 mg l^{-1} and 2,4-D 0.1 mg l^{-1}. However, the germination rate was very low and the conditions for the germination of *in vitro* produced seeds of ginger need refinement.

2.5 Germination of *in vitro* produced seeds

The *in vitro* raised seeds may often require special pre-treatments, media and culture conditions for its germination. Since such seeds are not available in ginger, works done in other crops are reviewed hereunder.

In vitro seeds of *Papaver rhoeas* soaked in tap water for 24 h germinated in modified Whites (1943) medium (Kanta, 1960).

Seeds of *Papaver somniferum* did not germinate when they were attatched to the placenta. If seperated from the placenta, they germinated within 15 days after planting in new medium. Eventhough the endosperm development was not normal, *Argemone mexicana* germinated within three months after starting the culture. The seeds of *Nicotiana rustica* and *N. tabaccum* germinated *in situ* as well as in basal medium (Kanta *et al.*, 1963).

The seeds produced by *in vitro* pollination in *Antirrhinum majus* germinated in basal medium (Nitsch, 1951) with 4 per cent sucrose (Usha, 1965).

In vitro produced seeds of Petunia were germinated in water soaked filter paper and in the same nutrient agar medium on which it was developed. The germination was more in water soaked filter paper (Rangaswamy and Shivanna, 1967).

The time taken by *in vitro* produced seeds of *Nicotiana tabaccum* for germination when sown *in vitro* was found to vary. Sometimes the seeds dried out and passed to a dormant state (Balatkova *et al.*, 1976).

The karyopses of maize produced under *in vitro* condition were dried at room temperature for several days and then made to germinate on moist filter paper at 28°C. They germinated within 7 days after incubation (Gengenbach, 1977a).

2.6 Embryo culture

Embryo culture technique is adopted in situations where the hybrid embryo fails to attain maturity due to embryo-endosperm incompatibility or poor development of the endosperm. The method can be utilised for the production of rare hybrids which are otherwise impossible in nature due to the abortion of embryo. The literature about various techniques of embryo culture, factors affecting embryo growth related to other works are reviewed hereunder:

2.6.1 Technique of embryo culture

2.6.1.1 Technique for dicots

The technique eventually consists of excision and transfer of the embryo from the abortive seed to a culture medium which will support the development of the same to full maturity (Bhojwani and Razdan, 1983). The excision of mature embryos can be done with relative ease by splitting open the seed. Seeds with a hard seed coat are dissected after soaking them in water (Raghavan, 1977). Smaller embryos require careful dissection with the aid of a microspatula and transferred to the nutrient medium. The presence of oil prevent drying up of the embryo.

Raghavan and Torrey (1963) adopted the following procedure for isolating torpedo shaped younger embryos from the ovules of *Capsella bursa pastoris*. The embryo is confined to one longitudinal half of the ovule and are clearly visible through the chalaza either because of the transparent vesicle of their suspensor. The ovule from which the embryo has to be excised was placed in the depression of a new slide with a drop of medium. With the help of a sharp mounted blade, the ovule

was split longitudinally to isolate the half containing the embryo. By carefully teasing apart the ovular tissues the entire embryo could be removed.

2.6.1.2 Technique for monocots

In monocots, a well studied plant for embryo culture 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 microspatula and transferred to the nutrient medium. The presence of oil prevent drying up of the embryo.

2.6.1.3 Embryo-nurse endosperm transplant

The nutritional requirements of younger embryos are complicated as they require more morphogenetic differentiation. The technique of embryo-nurse endosperm transplant will be helpful in such situations.

Ziebur and Brink (1951) showed that *in vitro* growth of excised immature embryos (300-1100 μm long) of Hordeum was considerably promoted by surrounding from another seed of the same species.

A modified endosperm transplant technique for young embryo culture has been described by De Lautour *et al.* (1978), Williams (1978, 1980) and Williams and De 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.

2.6.2 Role of suspensor in embryoculture

The suspensor is a ephemeral structure found at the radicular end of the pro-embryo. Usually it attains maximum development by the time of embryo reaches the globular stage. Studies suggest the active involvement of the suspensor in the development of the young embryos (Clutter *et al.*, 1977 and Bhojwani and Bhatnagar, 1978). Therefore, for the successful culture of younger embryos, suspensor may be required. There are also reports that the role of suspensor can be replaced by hormones like gibberelin and kinetin (Yeung and Sussex, 1979; Cionini *et al.*, 1976).

2.6.3 Culture requirements

2.6.3.1 Media requirements for developing embryo

The most important aspect of embryoculture is the selection of right culture medium that would support progressive and orderly development of embryos excised at different stages of development.

Hannig (1904) reported that a mineral salts-sucrose solution was sufficient to culture mature embryos (2 mm long) of crucifers. Laibach (1925) reared full plants from excised hybrid embryos (1 mm long) using only 15 per cent glucose solution.

With respect to nutrition, two phases of embryo development had been recognized by Raghavan (1966) (a) the heterotrophic phase-during this early phase, the embryo was dependent and draws up on the endosperm and the surrounding maternal tissues, (b) the autotrophic phase-during this phase, the embryo was capable of synthesising substances required for its growth. The media required changes in the two phases and even within the two phases the exogenous requirement of the cultured embryos become irrespectively simpler with the age of the embryo.

2.6.3.1.1 Growth regulators

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

Raghavan and Torrey (1964) reported that for cultivating globular embryos of *Capsella* on a mineral salts - vitamin medium with only 2 per cent sucrose, it was necessary to supplement it with IAA, kinetin and adenine sulphate.

Morgan *et al.* (1995) reported that interspecific hybrids between *Limonium perigrinum* Bergins and *Limonium purpuratum* L. were obtained on MS medium supplemented with BA 0.6 mg l^{-1} and NAA 0.3 mg l^{-1}.

Chee (1995) reported that plantlets were obtained from zygotic embryos of *Taxus brevifolia* when cultured on 1/2 strength Gamborg's B5 medium supplemented with 10 μm BA.

Harry *et al.* (1995) reported that plantlets were obtained from 40 days old embryos of *Juniperus cedrus* when cultured on Quoirin and Le Poivre 1/2 strength medium supplemented with 5 μm BA.

2.6.3.1.2 Carbohydrate and osmotic pressure of culture medium

A suitable source of carbon energy was generally required for the activation of excised mature and immature embryos. Sucrose was the best form of carbohydrate and was commonly used for embryo culture (Van over beek *et al.*, 1944, Lofland, 1950; Rijven, 1952, Burhardtova and Tupy, 1980). Sucrose is also essential in the medium to maintain a suitable osmolarity which is extremely important for immature embryos.

Mature embryos grew fairly well with 2 per cent sucrose but younger embryos required higher levels of carbohydrate (Ryczkowski, 1960, Mauney, 1961 and Smith, 1973). Eight to 12 per cent sucrose was required for the culture of proembryos (Datura-Rietsema *et al.*, 1953, Hordeum-Ziebur and Brink, 1951, Norstog, 1961, Capsella-Rijven, 1952, Veen, 1963, Monnier, 1978).

Raghavan and Torrey (1964) reported that the stimulatory effect of high concentrations (12-18 per cent) of sucrose on *in vitro* development of excised globular embryos (smaller than 80 μm) of capsella can be obtained by a combination of IAA (0.1 mg l^{-1}), kinetin (0.001 mg l^{-1}) and adenine sulphate (0.001 mg l^{-1}) added to the basal medium with only 2 per cent sucrose.

Pancholi *et al.* (1995) reported that immature embryos excised from *Musa velutine* seeds when cultured on 1/2 strength MS medium with 2 per cent sucrose and pH 5.8 at 23 to 25 degree resulted in plantlet formation.

2.6.3.1.3 Mineral salts

Monnier (1976) observed that 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, contained high concentrations of K^+ and Ca^{2+} and a reduced level of NH_4^+ ions supported both growth and survival of embryos.

Umbeck and Norstog (1979) reported that NH_4^+ ions in the medium was essential for proper growth and differentiation of immature barley embryos. With NO_3^- as the sole source of inorganic nitrogen, differentiation did not take place.

Harry *et al.* (1995) reported that excised 40 days old embryos of *Juniperus cedrus* when cultured on 1/2 strength Quoirin and Le Poivre medium without growth reulators or other supplements induced shoot buds after 2 months.

2.6.3.1.4 Amino acids and vitamins

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

Casein hydrolysate (CH), are amino acid complex, used widely as an additive to the embryo culture media. 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 *Hordeum vulgare* was 500 mg l^{-1}.

Rangaswami (1961) reported that CH was essential for culture of very young globular embryos of *Citrus microcarpa*.

Matsubara (1964) studied the effect of 18 amino acids and two amides for the culture of young *Datura tatula* embryos and observed that all except glutamine were inhibitory.

Beharav (1995) reported that the optimum level of CH for *Cucumis melo* embryos to form plants was 0.1 g l^{-1}.

Vitamins had been used in embryoculture media but their presence was not always essential. In some cases, a vitamin may even inhibit normal morphogenesis (Raghavan, 1980).

2.6.3.1.5 Natural plant extracts

Van over 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 attained by incorporating coconut water in the culture medium containing mineral salts, vitamins and aminoacids. Later, it has enabled the successful culture of young embryos of a number of species.

Zhiri *et al.* (1994) reported that when embryos of *Taxus baceata* were grown on MS medium supplemented with 1 g l^{-1} CH, 1 g l^{-1} yeast extract, 0.1 g l^{-1} ascorbic acid and 5 g l^{-1} activated charcoal resulted in plantlet formation.

2.6.3.2 pH of the medium

The pH of the ovular sap of *Capsella* was about 6.0 and its excised

embryos grow equaly well in the medium with pH 5.4 to 7.5 (Rijven, 1952). The optimum pH for early heart-shaped embryos of *Datura tatula* ranged from 5.0 to 7.5 (Matsubara, 1962). Rice embryos (8-day-old) showed best growth at two pH values viz., 5 and 9 (Sapre, 1963). Norstog and Smith (1963) had reported that for organogenic differentiation of immature barley embryos the pH of the medium was critical. The optimum value was about 4.9. At a pH above 5.2, embryo growth occurred without any appreciable differentiation.

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 8.

2.6.3.3 Culture storage

Embryos of most plants grew well at temperatures between 25 and 30°C (Narayanaswamy and Norstog, 1964). The optimum temperature for *Datura tatula* was reported to be 35°C (Matsubara and Nakahira, 1965).

According to Narayanaswamy and Norstog (1965), light was not critical for embryo growth. Matsubara and Nakahira (1965) also concluded that the growth of young Datura embryos was not influenced by light. However, in barley, light was known to suppress precocious germination of immature embryos (Norstog, 1972).

Beharav (1995) reported that the hybrid embryo of the cross between *Cucumis melo* and *Cucumis metuliferus* was grown best at 25 deg with a photoperiod of 16 h.

Chee (1995) reported that the zygotic embryos of *Taxus brevifolia* produced organs like shoot and root under a 16 h photoperiod.

2.6.4 Precocious germination

According to Quartrano (1987), the stages of seed development and germination involve rapid embryo and endosperm growth and differentiation after fertilization followed by a quiscent state of low metabolic activity and growth (dormancy) and finally a switch back to the active growth of embryo to form seedling. Excised immature plant embryos on nutrient medium sometimes bypass the stage of dormancy and develop into week seedlings (Choinski and Trelease, 1978, Dure *et al.*, 1980, Crouch and Sussex, 1981, Dure and Galau, 1981).

Kent and Brink (1947) reported that precocious germination of excised immature barley embryos can be prevented by incorporating CH in the culture medium. Ziebur *et al.* (1950) although not as effective as CH, a high sucrose level (12.8 per cent) in the medium can also bring the same effect. Precocious germination of capsella embryos are prevented by the presence of high concentration of sucrose (12-18 per cent) in the medium (Rijven, 1952). Norstog (1972) and Norstog and Klein (1972) have shown that exogenous factors like reduced oxygen tension, elevated temperature and high light intensity also prevented precocious germination. Studies conducted further showed that presence of some inhibitors like ABA may be preventing precocious germination in normal embryo development (Norstog, 1972, Dure, 1975, King, 1976 and Hsu, 1979).

2.6.5 Routes of hybrid plant production through embryo culture

2.6.5.1 Somatic embryogenesis

Somatic emrbyogenesis is most common route of plantlet production from embryos. Chuang and Chang (1987) observed somatic embryogenesis and plant regeneration in callus culture derived from mature zygotic embryos of *Dysosma pleiantha* (Hence) Woodson. The callus was obtained from wounded mature zygotic embryos of MS medium supplemented with 1 ppm 2,4-D. Embryoids were formed on media containing 2,4-D 0.1 to 0.5 mg l^{-1} and they germinated on MS or B5 with 1 ppm BAP and 1 ppm GA_3.

Luhrs and Lorz (1987) reported *in vitro* plant regeneration from embryogenic cultures of spring and winter type barley (*Hordeum vulgara* L.) varieties. Somatic embryos obtained on modified MS and CC media with CH 1 g l^{-1}. Germination of somatic embryos obtained on CC medium with 1 ppm NAA and 0.05 ppm zeatin.

Guerra and Handro (1988) reported somatic embryogenesis and plant generation in embryo cultures of *Euterpe edulis* mart. (Palmae). Direct embryogenesis was obtained on Linsmaier and Skoog medium containing 2,4-D 50 mg l^{-1}. Plantlets were obtained when somatic embryos were transferred to medium containing NAA and 2iP or without growth regulators.

2.6.5.2 Direct organogenesis

Patel and Thorpe (1983) reported *in vitro* differentiation of plantlets from embryonic explants of lodgepole pine (*Pinus contorta* Dougl. exhourd.). Shoots

were obtained on modified LP medium with 10^{-5} M BAP and roots were obtained by treating the shoots with sterilized rooting powder containing IBA.

Lekshmy (1989) cultured embryos of mature hybrid seeds from fully ripe bunches. Seeds were surface sterilized with 1 per cent silver nitrate for 10 min before extraction and excised embryos developed to plantlets when cultured on modified Knudson's medium (Knudson, 1946).

Sudhadevi (1993) reported 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}. Elongation of shoots obtained on MS with BAP 4 mg l^{-1} and IBA 1 mg l^{-1}. Rooting also obtained by giving a pulse treatment with IBA 2 mg l^{-1}.

Muniswamy *et al.* (1996) reported that zygotic embryos of *Coffea bengalensis* were matured on MS medium with ABA 1 mg l^{-1} + BAP or kinetin by 30 days. They germinated into plantlets when cultured on 1/2 strength MS with 0.1 mg l^{-1} BAP.

2.6.5.3 Indirect organogenesis

Wang (1987) obtained callus induction and plant regeneration from maize mature embryos. Callus was obtained on MS medium with 1-2 ppm 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* (Linn.) R.Br. The callus was obtained from immature embryos when cultured on MS medium with 0.1 mg l^{-1}

NAA and BAP 0.1 to 0.5 mg l^{-1}. Rooting was reduced when shoots were transferred to auxin supplemented Bonner's solution or 1/2 strength MS basal salt solutions.

2.7 Micropropagation of ginger

2.7.1 Enhanced release of axillary bud

Clonal propagation of ginger under *in vitro* conditions through enhanced release of axillary bud was first reported by Hosoki and Sagawa (1977). Buds from rhizome in storage were cultured on a medium consisting of MS major elements, Ringe-Nitsch minor elements and vitamins, 2.0 per cent sucrose and BAP 1 mg l^{-1}. Similar results were also obtained by Nadgauda *et al.* (1980). Pillai and Kumar (1982) found that the sprouted buds of the ginger rhizome grew and established well when they were cultured on SH medium also. De Lange *et al.* (1987) developed a method for elimination of nematodes from ginger by tissue culture. Later on, a number of reports on successful micropropagation of ginger were published (Bhaghyalakshmi and Singh, 1988; Ikeda and Tanabe, 1989; Inden *et al.*, 1988 and Choi, 1991a). MS medium containing 0.5 mg l^{-1} NAA and 5 mg l^{-1} BAP was best for *in vitro* regeneration of ginger (Inden *et al.*, 1988 and Choi, 1991a). Choi (1991b) found that rooting of shoots were enhanced by the addition of 2 per cent activated charcoal to the medium.

Babu *et al.* (1992a) reported plantlet formation from immature inflorescence (1 to 10 day old flower buds) in modified MS medium supplemented with 10 ppm BA and 0.2 ppm 2,4-D. In the same medium individual flowers produced plantlets as well as fruits.

Illg and Faria (1995) reported micropropagation of *Zingiber spectabile* Griff. (ornamental ginger). Multiple buds were obtained from axillary buds on MS medium with 10 μM IAA. Shoots were obtained on 1/2 MS with 10 μM BA and roots were obtained on 1/2 MS with 5 μM NAA or IAA.

2.7.2 Callus mediated organogenesis

Ilahi and Jabeen (1987) have successfully produced plantlets through callus mediated organogenesis. They reported that 1/2 strength of MS inorganic was more favourable for micropropagation of ginger than full strength. Choi (1991c) also reported callus mediated organogenesis in ginger from explants of pseudostem containing one leaf blade. Callus was produced in MS medium containing 0.5 ppm NAA. Shoot and root regeneration was obtained in a medium containing 0.1 to 1.0 ppm NAA and 1.0 ppm BA. Babu *et al.* (1992b) have also reported callus mediated regeneration in ginger. The presence of 2,4-D in the medium 9 to 22.5 μM resulted in callus growth. Organogenesis and plantlet formation occurred when the concentration of 2,4-D was reduced to 0.9 μM and with the addition of 44.4 μM BA into the medium.

2.7.3 Somatic organogenesis

Kackar *et al.* (1993) reported somatic embryogenesis in ginger. The leaf segments in MS medium with 8 per cent sucrose and 2.7 μM Dicamba produced embryogenesis. IAA and NAA were not effective in producing embryogeneic cultures. Plant regeneration was achieved when embryogenic cultures were transferred to MS medium containing 8.9 μM BA. Histological studies revealed various stages of somatic embryogenesis characteristic of monocot system. The *in vitro* raised plants were successfully established in soil.

Material and Methods

3. MATERIALS AND METHODS

The present study, "*in vitro* pollination, embryo rescue and germination studies in ginger, *Zingiber officinale* (Rosc.)" was carried out at the tissue culture laboratory, Department of Plantation Crops and Spices, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur during March 1994 to February 1996. The details regarding the experimental materials and methodology adopted for conducting various aspects of the study are described in this chapter.

3.1 Materials

3.1.1 Source of explants

Eight cultivars/clones of ginger namely, Maran, Rio-de-Janeiro, Nadia, Bajpai, SG-66, Kuruppampady, SG-603 and SG-543 having a wide geographical base were selected for the study and planted in the field for sufficient supply of flowers (Plate 1 and 2). But Maran was not included in the study as flowers were not available during the course of investigation.

3.1.2 Chemicals

Chemicals used for the preparation of various media were procured from British Drug House (BDH), SISCO Research Laboratory (SRL), Merck or Sigma.

3.1.3 Glasswares

Borosilicate glasswares of corning/borosil brand were used for the experiment. They were cleaned initially by boiling in tap water for half an h. On cooling, they were thoroughly washed with the detergent solution, rinsed with

Plate 1. Ginger cultivar SG-66 on flowering

Plate 2. Inflorescence of ginger showing flower bud on the day of anthesis

potassium dichromate solution in sulphuric acid, then washed free of detergents using tap water and finally rinsed with distilled water. Washed glasswares were dried in hot air oven at 60°C and were stored away from dust and contaminants.

3.1.4 Culture medium

3.1.4.1 Composition of media

Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962), Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) were used as basal media in the present study. Composition of these media are given in Table 1. The basal media were supplemented with different levels of cytokinins, auxins, GA_3, ethylene, casein hydrolysate, coconut water, etc. in different experiments.

3.1.4.2 Preparation of medium

Standard procedures (Gamborge and Shyluk, 1981) were followed for the preparation of the medium. Stock solutions of major and minor nutrients were prepared first by dissolving the required quantity of chemicals in distilled water and stored under refrigerated conditions in amber coloured bottles. Stock solutions for the major and minor nutrients were prepared fresh in every three months, vitamin stock solutions prepared fresh in every two months and that of phyto-hormones prepared fresh at monthly intervals.

Specific quantities of stock solutions were pipetted out into a beaker. Sucrose and inositol were added fresh and dissolved, and the volume was made upto the required level using distilled water. The pH of the solution was adjusted between 5.6 and 5.8 using 0.1 N NaOH or 0.1 N HCl. For preparing semi solid medium, good quality agar was added at 0.75 per cent level and the medium was boiled till a

Table 1. Composition of various culture media used for culture establishment

Constituents	Quantity mg l^{-1}	
	MS	SH
Macro nutrients		
KNO_3	1900.0	2500.0
NH_4NO_3	1650.0	-
$(NH_4)_2SO_4$	-	-
KH_2PO_4	170.0	1250.0
$NaH_2PO_4.H_2O$	-	-
$NH_4H_2PO_4$	-	300.0
$MgSO_4.7H_2O$	370.0	400.0
$CaCl_2.2H_2O$	440.0	200.0
$Ca(NO_3)_2.4H_2O$	440.0	200.0
Micro nutrients		
H_3BO_3	6.200	5.0
$MnSO_4.H_2O$	-	10.0
$MnSO_4.4H_2O$	22.300	-
$ZnSO_4.7H_2O$	8.600	1.0
$NaMoo_4.2H_2O$	0.250	0.10
$CaSO_4.5H_2O$	0.025	0.20
$CoCl_2.6H_2O$	0.025	0.10
KI	0.830	1.00
$FeSO_4.7H_2O$	27.800	15.00
Na_2 EDTA	33.600	20.00
Vitamins		
Thiamine, HCl	0.10	5.00
Pyridoxine, HCl	0.50	0.50
Nicotinic acid	0.05	5.00
Niacin	-	-
Others		
Glycine	2.00	-
Myo-inositol	100.00	1000.00
Sucrose	30000.00	30000.00
pH	5.8	5.8

clean solution was obtained. In case of liquid medium, agar was avoided and filter paper bridges were provided to support the explants. About 15 ml medium was poured into the culture tubes of size 15 x 2.5 cm or 10 x 2.5 cm. The tubes were then plugged with non absorbant cotton and autoclaved at 121°C and 15 Psi (1.06 kg cm^{-2}) for 20 min (Dodds and Roberts, 1982). The medium was allowed to cool to room temperature and stored in cool, dry place.

3.2 Methods

3.2.1 Maintenance of ginger in the field

The eight cultivars viz., Maran, Rio-de-Janeiro, Nadia, Bajpai, SG-66, Kuruppampady, SG-603 and SG-543 having a wide geographical base were selected for the study and planted in pots. The first crop was planted in 1994 May and the second was planted in 1995 April. A potting mixture of soil, sand and cowdung at 1:2:2 ratio was used for filling the pots. Seed bits of 15 g and 200 g weight were used for planting. The seeds were planted after treating with 0.3 per cent Dithane-M-45, 0.025 per cent Ekalux and 0.05 per cent streptocycline. Pots were irrigated during the dry spell. The manures and fertilizers were given as per the Package of Practices Recommendations, KAU, 1993.

Plant protection measures were taken against rhizome rot of ginger and also shoot borer attack at vegetative and flowering phases. Bordeaux mixture 0.1 per cent spraying was given once in a month as a prophylactic measure. Ekalux 0.025 per cent spraying was given once in three weeks to control shoot borer. Streptocycline 0.01 per cent spraying was given once in a month to reduce bacterial inoculum. Dithane-M-45 (0.3%) and Bavistin (0.1%) drenching were given according to the intensity of rhizome rot.

3.2.2 Pollen studies

3.2.2.1 Pollen fertility and viability studies

Studies were carried out to understand the fertility and viability of various cultivars at early (mid to last October), mid (early to mid November) and late (mid to last November) flowering season. Fertility and viability of the pollen grains of the flowers at lower, middle and upper portions of the inflorescences were also studied. The observations recorded were statistically analysed for interpretations.

3.2.2.2 Estimation of pollen fertility

Flower buds at the time of anthesis were used for the study. The pollen grains were stained using 1 per cent acetocarmine and viewed at 100 x magnification. All the pollengrains that were well filled and stained were counted as fertile and others as sterile. The means of a sample from 10 microscopic field served as a replication. The fertility per cent was calculated using the formula.

$$\frac{\text{Number of well stained pollen grains}}{\text{Total number of pollen grains in the field}} \times 100$$

3.2.2.3 Pollen viability studies

The pollen grains were collected at the time of flower opening and were incubated in a moisture chamber along with a drop of ME_3 medium. ME_3 medium developed by Leduc *et al.*, 1990 was earlier identified for improved pollen germination and tube growth of ginger (Valsala, 1994). The composition of the medium is

given in Table 2. The observations were taken 24 h after incubation. The total number of pollen grains as well as the number of grains were counted on 10 microscopic fields and the mean germination percentage was worked out. Mean pollen tube growth attained also measured using a calibrated occular micrometer. Photomicrographs of the germinated pollen grains were also taken.

3.2.3 Production of ginger seeds by *in vitro* pollination

3.2.3.1 Collection of explant

The flowering in ginger commenced from September and the time of anthesis observed was 3 pm. The flower buds on the day of anthesis as well as flowers one day after anthesis were used for *in vitro* pollination. They were collected from the spike with the help of a needle and forceps.

3.2.3.2 Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilization of flower buds, pollination and inoculation of ovules, subsequent sub culturings and embryo rescue were carried out under the hood of a clean laminar air flow chamber. The working table of the laminar air flow chamber was first wiped with absolute alcohol and then exposed to ultra violet light for 20 to 30 min. The petridishes as well as the inoculation aids were first steam sterilized and then flame sterilized before use.

3.2.3.3 Surface sterilization

Flower buds were surface sterilized prior to anthesis ie. 3.00 pm. Since they were directly collected from the field, first wiped with 70 per cent alcohol and given 0.05 per cent streptocycline dip for 2 h to avoid bacterial interference. Then

Table 2. Composition of ME_3 medium (Leduc *et al.*, 1990)

Constituents	Concentration (mg l^{-1})
Macro nutrients	
$MgSO_4.7H_2O$	370.00
KNO_3	950.00
H_2PO_4K	85.00
$CaCl_2.2H_2O$	880.00
NH_4NO_3	412.50
KCl	175.00
Na_2 EDTA	7.45
$FeSO_4.7H_2O$	5.55
Micro nutrients	
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
Others	
PEG-4000	120,000

they were washed and treated with mercuric chloride 0.1 per cent for 3 min under perfect aseptic conditions in the laminar air flow chamber. The buds were then rinsed thrice with sterile distilled water to remove traces of sterilant from the surface and were dried on sterile flamed filter paper.

3.2.3.4 *In vitro* pollination

The method adopted for the present study was *in vitro* placental pollination as standardised by Valsala (1994). Placenta bearing the ovules were exposed by completely peeling the ovary wall and pollination was done on it. *In vitro* crossing between cultivars as well as selfing were done by adopting this method. After pollination, ovules with pollen and a drop of pollen germination medium (ME_3) were transferred to previously prepared medium. The different media combinations as listed in Table 3 were tried to support the growth of pollinated ovules.

3.2.4 Culture conditions

The cultures were incubated at 26 $\pm$ 2°C in an air conditioned culture room under diffused light. Humidity in the culture room varied between 60 to 80 per cent according to the climate prevailed.

3.2.5 Viability test (Tetrazolium test)

The viability of the seeds were tested at 40 and 80 days after pollination (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°C until the solution was completely

Table 3. The combination of auxins and cytokinins tested for *in vitro* seed development in ginger

Sl. No.	Auxin concentration (mg l^{-1})	Cytokinin concentration (mg l^{-1})
1	NAA 0.5	BAP (7.5, 10, 15)
2	NAA 1.0	BAP (2.5, 5, 7.5)
3	NAA 2.0	BAP (5, 10, 15, 20)
4	IAA 0.1	BAP (5, 10, 15)
5	IAA 0.2	BAP (10, 15)
6	2,4-D (0.1, 0.2, 0.5, 1.0)	BAP (5, 10, 15, 20)

Basal medium: 1/2 MS + 6% sucrose and SH + 6% sucrose

evaporated out. They were observed under stereo microscope for the colour change of the embryo. Photomicrographs of the specimens were taken to interpret the results.

3.2.6 Treatments for germination of ginger seeds

3.2.6.1 Primary treatments

The seeds obtained on 80 DAP were subjected to various primary treatments like imbibition in water, keeping on moist filter paper, moist sand and basal medium (both solid and liquid) for germination.

3.2.6.2 Treatment with hormones

The seeds obtained on 80 DAP were incubated in various media combinations for germination. The combinations of growth regulators tried are furnished in Table 4.

Different levels of GA_3 (5 to 10 mg l^{-1}) and ethylene (5 to 10 mg l^{-1}) were also tested by filter sterilization as well as by autoclaving. The cultures were kept in dark and in diffused light at 26 $\pm$ 2°C and were observed for one year for germination with frequent subculturing.

3.2.6.3 Chemical scarification

Seeds were scarified with 50 per cent HCl and HNO_3 25 per cent for three to ten min and after washing they were incubated to the ½ MS medium alone and also with NAA 1.0 mg l^{-1} and BAP 10 mg l^{-1}.

Table 4. The combination of auxins and cytokinins tested for *in vitro* seed germination in ginger

Sl. No.	Auxin concentration (mg l^{-1})	Cytokinin concentration (mg l^{-1})
1	2,4-D (0.1, 0.2, 0.5, 1.0)	BAP (5, 10, 15, 20)
2	NAA 0.5	BAP (7.5, 10, 15, 20)
3	NAA (1.0, 2.0)	BAP (5, 10, 15, 20)
4	NAA 0.5	2iP (2.5, 5)
5	IAA (0.05, 0.1)	BAP (5, 10, 15, 20)
6	IAA 0.2	BAP (10, 15)
7	IAA (0.05, 0.1, 0.2)	2iP (2.5, 5)

Basal medium: 1/2 MS + 6% sucrose

3.2.6.4 Mechanical scarification

Seeds were pounded with sterile sand till the seed coat ruptured and after washing, they were incubated to the ½ MS medium alone and also with IAA 0.05 mg l^{-1} and BAP 10 mg l^{-1}.

3.2.6.5 Stratification

The seeds were kept at 4 °C for 6, 12, 18 and 24 h and were incubated to the ½ MS medium alone and also with NAA 0.5 mg l^{-1} and BAP 20 mg l^{-1}.

3.2.6.6 Washing the seeds in running water and sowing

Seeds were washed at time intervals of 2, 6, 8 and 12 h and later incubated in ½ MS medium alone and also with 2,4-D 0.2 mg l^{-1} and BAP 15 mg l^{-1}.

3.2.6.7 Hydration - dehydration - rehydration

The seeds were initially hydrated for 12 h, then dehydrated at time intervals of 5, 6, 8 and 10 h and again rehydrated for another 12 h and later incubated to ½ MS medium alone and also with IAA 0.05 mg l^{-1} and BAP 5 mg l^{-1}.

3.2.6.8 Priming of seeds with mannitol and PEG

Initially the seeds were imbibed in 12 per cent mannitol and in PEG-4000 solution for 20 days and later imbibed in water for 12 h and incubated to ½ MS medium alone and also with 2,4-D 0.2 mg l^{-1} and BAP 15 mg l^{-1}.

3.2.7 Embryo rescue studies

The seeds obtained on 20, 40, 60 and 80 DAP were subjected to embryo rescue studies. Eighty days old seeds were soaked in water for 24 h to soften the seed coat. The technique of embryo rescue was done under the hood of a clean laminar air flow chamber under a dissection microscope.

The technique followed in this study was transfer of embryo along with endosperm. First a perfect cut was given to the seed at micropylar end without injuring the embryo. Then with the help of forceps, slight pressure was applied at the chalazal end so as to easily squeeze out the embryo along with endosperm. The rescued embryo with a small bit of endosperm was incubated in ½ MS medium containing combinations of 2,4-D (0.5, 1.0), NAA (0.5, 1.0) or IAA (0.05) with BAP (5, 10, 15, 20) mg l^{-1}.

Results

4. RESULTS

The results of the experiments on "*in vitro* pollination, embryo rescue and gemination studies in ginger, *Zingiber officinale* (Rosc.)" conducted at the Plant Tissue Culture Laboratory, Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during the period 1994-1996 are presented in this chapter.

4.1 Investigations on pollen studies in ginger

4.1.1 Influence of season on pollen fertility of ginger cultivars

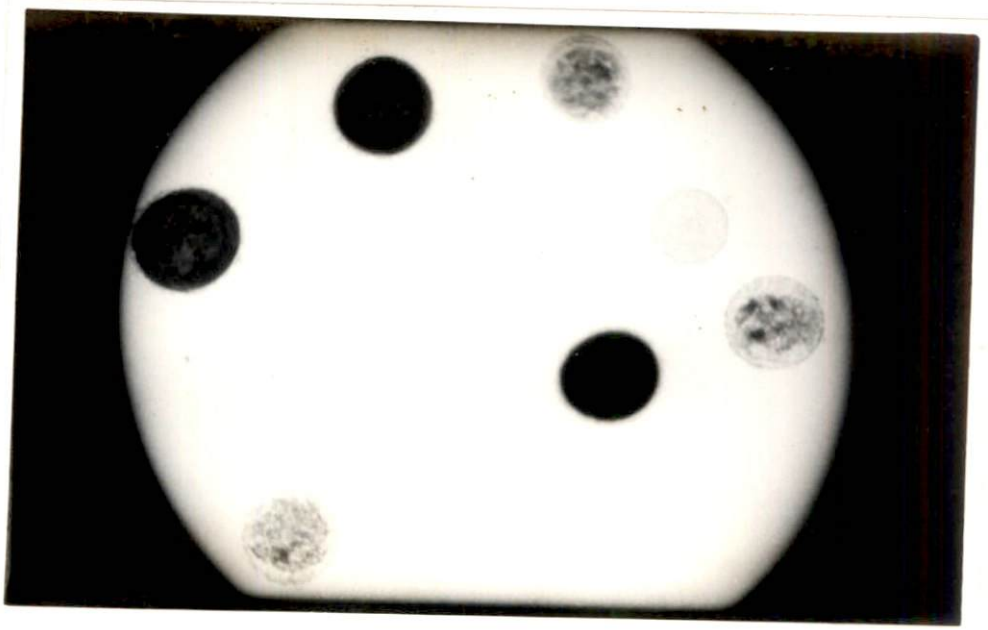
The data regarding the influence of season on fertility of different ginger cultivars are presented in Table 5 and Plate 3. The results showed that the fertility percentage of different ginger cultivars is significantly high (29.1%) during the middle of the season (early to mid October) compared to 12.05 per cent during the early period of the season (mid to last October) and 14.4 per cent during the late period of the flowering season (mid to last November). During the mid period of the season, fertility percentage in different ginger cultivars ranged from 22.4 (Bajpai) to 35.5 (Rio-de-Janeiro), while it was only 8.95 (Nadia) to 16.2 (SG-66) during the early period of the season. During the late period, pollen fertility percentage ranged from 4.8 (SG-543) to 21.7 (SG-66).

There was significant difference among cultivars with respect to pollen fertility and the cultivar SG-66 (24.36%) and Rio-de-Janeiro (21.76%) recorded high fertility among the cultivars. It was moderate in Kuruppampady (17.36%),

Table 5. Effect of season on fertility of pollen grains of ginger cultivars

Cultivars	Fertility (%)			
	Early season (Mid to last October)	Mid season (Early to mid November)	Late season (Mid to late November)	Mean
Kuruppampady	13.7	33.5	4.9	17.36
Rio-de-Janeiro	11.7	35.5	18.1	21.76
Nadia	8.95	26.6	17.9	17.82
Bajpai	10.3	22.4	18.7	17.13
SG-66	16.2	35.2	21.7	24.36
SG-603	10.7	26.2	14.9	17.26
SG-543	12.8	24.3	4.8	13.96
Mean	12.05	29.1	14.4	
CD for season	8.64			
CD for cultivars	5.66			

Plate 3. Fertile and sterile pollengrains stained with acetocarmine



SG-603 (17.26%) and Bajpai (17.13%). The cultivar SG-543 recorded the lowest fertility (13.96%).

4.1.2 Influence of season on viability and tube length of pollen grains of ginger cultivars

The data regarding the influence of season on germination and tube length of pollen grains of ginger cultivars are presented in Table 6 and Plate 4. The results showed that viability of pollen grains of different ginger cultivars are generally high in early (5.89%) and mid season (5.06%) compared to late (2.84%) flowering season. During the early season, the viability percentage ranged from 3.1 (SG-603) to 9.3 (Rio-de-Janeiro) and in mid season, it ranged from 2.9 (SG-603) to 8.4 (Rio-de-Janeiro) among cultivars. The viability percentage during late season ranged from 1.9 (SG-543) to 4.6 (Rio-de-Janeiro).

There was significant difference among ginger cultivars with respect to pollen viability and the cultivar Rio-de-Janeiro recorded significantly high viability (7.4%). Other ginger cultivars which showed comparatively better pollen viability are SG-66 (5.17%), Kuruppampady (4.67%), Bajpai (4.63%) and Nadia (4.13%). The cultivar SG-603 recorded the lowest pollen viability, (2.7%).

There was no significant difference with regard to the tube length of the germinated pollen grains collected during early (63.57 μm), mid (68.07 μm) and late (48.2 μm) seasons. Though not significant, the pollen grains of early and mid season showed better tube growth compared to late period of the season.

Table 6. Effect of season on viability and tube length of pollen grains of ginger cultivars

Cultivars	Germination %				Mean pollen tube length (µm)			
	Early season (Mid to last October)	Mid season (Early to mid November)	Late season (Mid to last November)	Mean	Early season (Mid to last October)	Mid season (Early to mid November)	Late season (Mid to last November)	Mean
Kuruppampady	6.3	5.2	2.5	4.67	55.1	65.6	43.5	54.7
Rio-de-Janeiro	9.3	8.4	4.6	7.43	54.2	66.3	69.6	63.4
Nadia	6.1	4.2	2.1	4.13	73.9	74.3	33.0	60.4
Bajpai	7.5	4.2	2.2	4.63	80.3	81.4	39.0	66.9
SG-66	5.4	6.2	3.9	5.17	64.5	78.6	62.3	68.5
SG-603	3.1	2.9	2.7	2.90	67.5	49.1	54.0	56.9
SG-543	3.5	4.3	1.9	3.23	49.5	61.2	36.0	48.9
Mean	5.89	5.06	2.84		63.57	68.07	48.2	
CD for season	1.89				CD for cultivars		14.28	
CD for cultivars	1.24							

Plate 4. Pollen germination and tube growth in ME_3 medium (x 200)

However, with regard to pollen tube length, the cultivars differed significntly. The cultivar SG-66 recorded the maximum mean pollen tube length of 68.46 μm and the cultivars Bajpai (66.9 μm), Rio-de-Janeiro (63.4 μm), Nadia (60.4 μm), SG-603 (56.9 μm) and Kuruppampady (54.7 μm) were on par with this variety. The cultivar SG-543 has produced the shortest pollen tube.

4.1.3 Influence of position of flowers in the inflorescence on pollen fertility of ginger cultivars

The data regarding the influence of position of flowers in the inflorescence on fertility of pollen grains are presented in Table 7. The results showed that the position of flowers in the inflorescence does not significantly affect the pollen fertility as the pollen from the lower, middle and upper portions recorded fertility values of 18.55 per cent, 20.66 per cent and 19.70 per cent respectively.

There was significant difference among cultivars with respect to pollen fertility and the cultivar SG-66 (24.47%) and Rio-de-Janeiro (23.7%) recorded high fertility among the cultivars studied. It was moderate in Kuruppampady (19.13%), SG-603 (18.67%), SG-543 (18.30%) and Nadia (17.53%). In this experiment the cultivar Bajpai recorded lowest fertility (15.5%).

4.1.4 Influence of position of flowers in the inflorescence on viability and tube length of pollen grains

The data regarding the influence of position of flowers in the inflorescence on viability and tube length of pollen grains of ginger cultivars are presented in Table 8. The results indicated that the germination percentage of pollen grains of flowers from lower (4.58%), middle (5.91%) and upper (3.78%) portions

Table 7. Effect of position of flowers in inflorescence on fertility of pollen grains of ginger cultivars

Cultivars	Fertility (%)			
	Position of flowers in the inflorescence			
	Lower	Middle	Upper	Mean
Kuruppampady	20.8	21.2	15.4	19.13
Rio-de-Janeiro	24.9	16.9	29.5	23.77
Nadia	8.1	26.6	17.9	17.53
Bajpai	22.4	13.8	10.3	15.5
SG-66	23.5	23.1	26.8	24.47
SG-603	10.7	24.1	21.2	18.67
SG-543	19.5	18.6	16.8	18.30
Mean	18.55	20.6	19.7	
CD for comparing cutlivars		7.11		

Table 8. Effect of position of flowers in the inflorescence on viability and tube length of pollen grains of ginger cultivars

Cultivars	Germination %				Mean pollen tube length (µm)			
	Position of flowers in the inflorescence				Position of flowers in the inflorescence			
	Lower	Middle	Upper	Mean	Lower	Middle	Upper	Mean
Kuruppampady	5.2	7.3	5.7	6.01	50.1	62.3	46.5	52.96
Rio-de-Janeiro	8.2	5.8	4.4	6.13	76.1	75.2	65.2	72.16
Nadia	3.9	8.2	3.2	5.10	69.6	76.4	32.3	59.4
Bajpai	4.2	7.5	2.8	4.83	63.2	72.3	56.2	63.9
SG-66	4.0	5.9	4.3	4.73	78.1	56.3	40.1	54.8
SG-603	3.1	2.5	2.8	2.80	63.1	56.4	36.2	51.9
SG-543	3.5	4.2	3.3	3.67	54.2	48.3	56.2	52.9
Mean	4.58	5.91	3.78		64.9	62.5	47.5	
CD for cultivars	1.59				CD for cultivars		12.95	

of the inflorescence did not differ significantly. Eventhough the data were not significantly different, the viability of the pollen grains of flowers from lower and middle portions of the inflorescence was rather high, compared to the upper portion.

The cultivars differed significantly with respect to viability of pollen grains and the cultivar Rio-de-Janeiro recorded the maximum viability of 6.13 per cent and the cultivars Kuruppampady (6.01%), Nadia (5.10%), Bajpai (4.83%) and SG-66 (4.75%) are on par with this. The viability of pollen grains of the cultivars SG-543 (3.67%) and SG-603 (2.8%) was significantly lower to others.

There was no significant difference with respect to the pollen tube length of the germinated pollen grains collected from lower (64.9 μm), middle (62.5 μm) and upper (47.5 μm) portion of the inflorescence. Though not significant, the pollen tube growth of the pollen grains collected from lower and middle portions of the inflorescence was rather high compared to the upper portion of the inflorescence.

The cultivars differed significantly with respect to pollen tube growth. The Rio-de-Janeiro (72.16 μm), Bajpai (63.9 μm) and Nadia (59.4 μm) recorded significantly longer pollen tubes compared to the rest (Table 8).

4.2 Investigations on *in vitro* pollination in ginger

4.2.1 Influence of crossing between ginger cultivars

The data on influence of crossing between ginger cultivars are presented in Table 9. The cultivar Rio-de-Janeiro as female parent could be crossed with Kuruppampady, SG-66 and Nadia and the percentage of cultures showing response ranged from 85 to 100 per cent. The percentage of ovules developed per culture ranged from 74.2 to 90 per cent. Rio-de-Janeiro as pollen parent could be crossed

Table 9. Effect of cultivars in crossing for ovule development in ginger after *in vitro* placental pollination

Sl. No.	Cross	Percentage of cultures showing response	Percentage of ovules developed per culture
1	Rio-de-Janeiro x Kuruppampady	100	74.2
2	Rio-de-Janeiro x SG-66	85	77.6
3	Rio-de-Janeiro x Nadia	100	90.0
4	Kuruppampady x Rio-de-Janeiro	100	65.8
5	Nadia x Rio-de-Janeiro	100	91.6
6	SG-66 x Rio-de-Janeiro	100	77.6
7	Bajpai x Rio-de-Janeiro	100	100.0
8	SG-603 x Rio-de-Janeiro	100	71.4
9	Kuruppampady x SG-66	100	73.6
10	Bajpai x Kuruppampady	100	40.0
11	SG-66 x Kuruppampady	75	59.6
12	SG-66 x SG-543	100	92.3
13	Nadia x SG-66	100	58.3
14	SG-543 x SG-66	80	66.9
15	SG-603 x SG-66	50	42.8
16	Nadia x SG-603	100	59.7
	Mean	93.2	71.3

Average of 18 observations, 20 DAP
Medium - 1/2 MS + 2,4-D 0.5 + BA 10 + 15% CW + 6% sucrose
Explant - Ovules after placental pollination

with Kuruppampady, Nadia, SG-66, Bajpai and SG-603 and in all crosses, percentage of cultures showing response were 100 per cent. The percentage of ovules developed per culture ranged from 65.8 to 100 per cent. The cultivar Kuruppampady as female parent could be crossed with SG-66 and as male parent is compatible with Bajpai and SG-66. The cultivar SG-66 as female parent could be crossed with SG-543 and as pollen parent with Nadia, SG-543 and SG-603. The cultivar SG-603 as male parent could be crossed with Nadia and the percentage of cultures showing response was 100 per cent.

The mean percentage of cultures showing response was 93.2 and the mean percentage of ovules developed per culture was 71.3, when all the crosses were considered together.

4.2.2 Influence of selfing in ginger cultivars

The data on influence of selfing are presented in Table 10. All the seven cultivars viz., Rio-de-Janeiro, Kuruppampady, SG-66, Nadia, Bajpai, SG-603 and SG-543 could be selfed and the percentage of cultures showing response ranged from 50 to 95.8 per cent. In selfing, maximum culture response was observed in Rio-de-Janeiro (95.8%) and minimum in Nadia and SG-543 (50%). The percentage of ovules developed per culture ranged between 62.5 to 89.8 per cent. The maximum was recorded in Rio-de-Janeiro (89.8%) and minimum in SG-543 (62.5%).

Table 10. Effect of self pollination in different ginger cultivars for ovule development after *in vitro* placental pollination

Sl. No.	Cultivar	Percentage of cultures showing ovule development	Percentage of ovules developed per culture
1	Rio-de-Janeiro	95.8	89.8
2	Kuruppampady	72.0	76.5
3	SG-66	90.0	72.5
4	Nadia	50.0	83.3
5	Bajpai	64.1	84.3
6	SG-603	61.1	79.8
7	SG-543	50.0	62.5
	Mean	69.0	78.38

Average of 18 observations, 20 DAP
Medium - 1/2 MS + IAA 0.05 + BA 15 + 15% CW + 6% sucrose
Explant - Ovules after placental pollination

4.2.3 Influence of growth regulators on ovule development in ginger after *in vitro* placental pollination

4.2.3.1 Influence of NAA and BAP

The auxin, NAA at a concentration of 0.5 to 2 mg l^{-1} along with cytokinin, BAP at 5 to 20 mg l^{-1} in the basal medium of 1/2 MS + 6 per cent sucrose supported ovule development after *in vitro* placental pollination in the cross SG-66 and Rio-de-Janeiro and this was observed in 66.40 per cent cultures (Table 11, Plate 5 and 6). The mean percentage of ovules developed per culture was 63.4. In the basal medium, the percentage of cultures showing response was 20.1 per cent and percentage of ovules developed per culture was 15 per cent.

4.2.3.2 Influence of IAA and BAP

The combination of IAA 0.1 mg l^{-1} along with BAP 5 to 20 mg l^{-1} in the basal medium of 1/2 MS + 6 per cent sucrose supported ovule development after placental pollination in the cross Rio-de-Janeiro and SG-66. IAA 0.2 mg l^{-1} along with BAP 10 to 15 mg l^{-1} also favoured ovule development. This was observed in 60.7 percentage of cultures and in control it was only 10 per cent. The mean percentage of ovules developed per culture was 69.4 and in control it was only 25 per cent (Table 12).

4.2.3.3 Influence of 2,4-D and BAP

The 2,4-D combinations ranging from 0.1 to 2.0 mg l^{-1} with BAP 5 to 20 mg l^{-1} in the basal medium of 1/2 MS + 6 per cent sucrose encouraged ovule development in the cross SG-66 and SG-543 and this was observed in 94 percentage

Table 11. Influence of NAA and BAP in ovule development in ginger after *in vitro* placental pollination

Sl. No.	Treatments (mg/l)	Percentage of cultures showing ovule development	Percentage of ovules developed per culture
1	NAA 0.5 + BAP 7.5	68.5	65.0
2	NAA 0.5 + BAP 10	62.4	69.0
3	NAA 0.5 + BAP 15	75.2	68.0
4	NAA 1.0 + BAP 2.5	70.2	80.0
5	NAA 1.0 + BAP 5.0	62.1	79.0
6	NAA 1.0 + BAP 7.5	77.7	69.0
7	NAA 2.0 + BAP 5	60.2	60.0
8	NAA 2.0 + BAP 10	72.9	72.0
9	NAA 2.0 + BAP 15	100.0	70.0
10	NAA 2.0 + BAP 20	61.2	50.0
11	Control (Basal medium)	20.1	15.0
	Mean	71.04	68.2
Average of 15 observations, 20 DAP		66.4	63.4

Basal medium - 1/2 MS + 6% sucrose
Explant - Ovules after placental pollination
Parental combination - SG-66 x Rio-de-Janeiro

Plate 5. Developing ovules of ginger after *in vitro* placental pollination

Plate 6. Developing ginger seeds after subculturing

Table 12. Influence of IAA and BAP in ovule development in ginger after *in vitro* placental pollination

Sl. No.	Treatments ($mg\,l^{-1}$)	Percentage of cultures showing ovule development	Percentage of ovules developed per culture
1	IAA 0.1 + BA 5	100	78.4
2	IAA 0.1 + BA 10	100	78.5
3	IAA 0.1 + BA 15	100	87.5
4	IAA 0.1 + BA 20	40	66.7
5	IAA 0.2 + BA 10	25	75.0
6	IAA 0.2 + BA 15	50	75.0
7	Control (Basal medium)	10	25.0
	Mean	60.7	69.4

Average of 15 observations, 20 DAP
Basal medium - 1/2 MS + 6% sucrose
Explant - Ovules after placental pollination
Parental combination - Rio-de-Janeiro x SG-66

of cultures. In the basal medium it was only 23.0 per cent. The mean percentage of ovules developed per culture was 76.8 and in control it was only 16.1 per cent (Table 13).

4.3 Seed viability test (Tetrazolium test)

The examination of tetrazolium soaked longitudinal sections of seeds of 40 and 80 DAP under stereo microscope showed that the embryos were deeply pink coloured compared to the surrounding tissues (Plate 7). The embryos of the seeds of 40 DAP took more intense pink colour than the seeds of 80 DAP.

4.4 Germination studies of *in vitro* seeds of ginger

4.4.1 Primary treatments

The seeds kept in water, moist filter paper, moist sand and basal medium (both soild and liquid) did not germinate.

4.4.2 Influence of growth regulators

4.4.2.1 Influence of 2,4-D and BAP

The results of the 2,4-D - BAP combination studies are listed in Table 14. It could be observed that 2,4-D (0.1 to 1.0 mg l^{-1}) along with BAP (5 to 20 mg l^{-1}) in the basal medium of 1/2 MS + 6 per cent sucrose had no influence on the germination of ginger seeds.

4.4.2.2 Influence of NAA with BAP and 2iP

NAA (0.5 to 2 mg l^{-1}) along with BAP (5 to 20 mg l^{-1}) in the basal medium of 1/2 MS + 6 per cent sucrose recorded no response on seed germination

Table 13. Influence of 2,4-D and BAP in ovule development in ginger after *in vitro* placental pollination

Sl. No.	Treatments ($mg\,l^{-1}$)	Percentage of cultures showing ovule development	Percentage of ovules developed per culture
1	2,4-D 0.1 + BAP 5	100	61.1
2	2,4-D 0.1 + BAP 10	100	70.5
3	2,4-D 0.1 + BAP 15	100	61.1
4	2,4-D 0.1 + BAP 20	100	92.3
5	2,4-D 0.2 + BAP 5	100	93.5
6	2,4-D 0.2 + BAP 10	98	95.2
7	2,4-D 0.2 + BAP 15	100	67.7
8	2,4-D 0.2 + BAP 20	100	92.8
9	2,4-D 0.5 + BAP 5	96	77.5
10	2,4-D 0.5 + BAP 10	98	87.5
11	2,4-D 0.5 + BAP 15	100	93.1
12	2,4-D 0.5 + BAP 20	90	78.0
13	2,4-D 1.0 + BAP 5	98	85.8
14	2,4-D 1.0 + BAP 10	100	63.1
15	2,4-D 1.0 + BAP 15	95	77.1
16	2,4-D 1.0 + BAP 20	100	94.4
17	Control (Basal medium)	23	16.1
	Mean	94	76.8

Average of 15 observations, 20 DAP
Basal medium - 1/2 MS + 6% sucrose
Explant - ovules after placental pollination
Parental combination - SG-66 x SG-543

Plate 7. L.S. of *Zingiber officinale* (Rosc.)seed 40 DAP stained with tetrazolium (x 400)

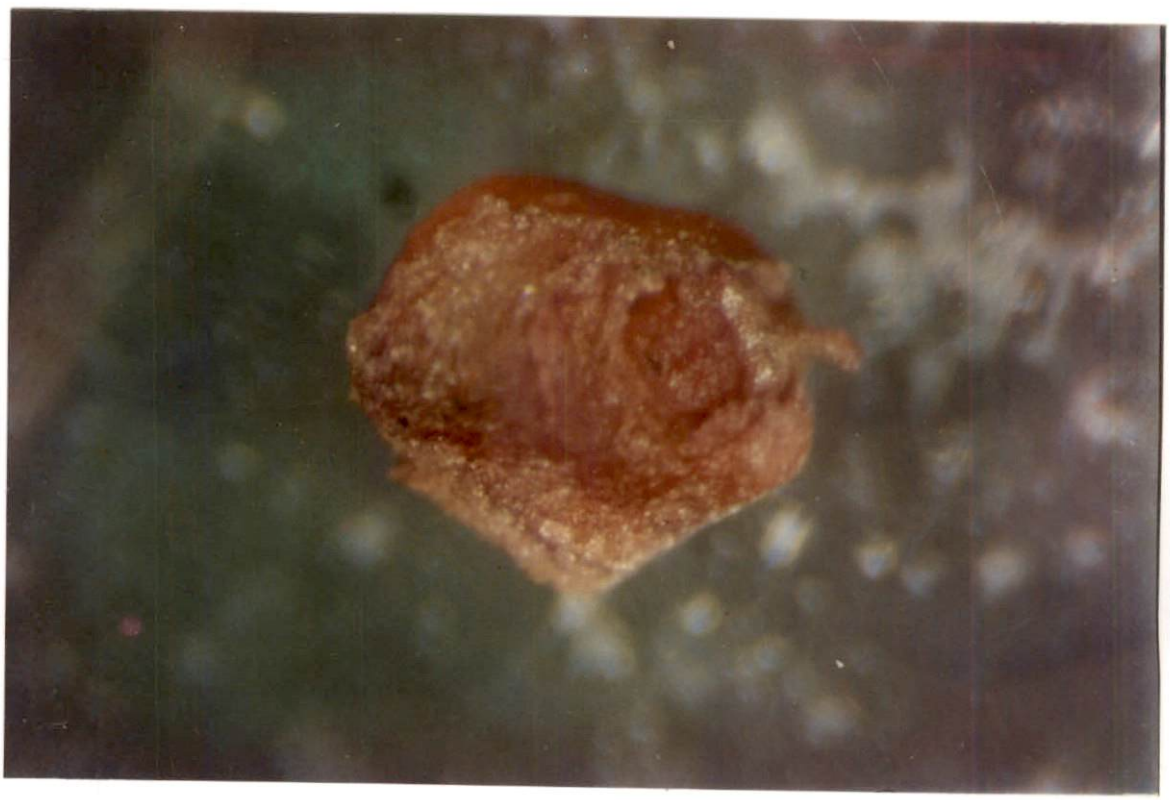


Table 14. Influence of 2,4-D and BAP in *in vitro* seed germination of ginger

Sl.No.	Treatments ($mg\ l^{-1}$)	Germination
1	1/2 MS + 2,4-D 0.1 + BA 5	Nil
2	2,4-D 0.1 + BA 10	,,
3	2,4-D 0.1 + BA 15	,,
4	2,4-D 0.1 + BA 20	,,
5	2,4-D 0.2 + BA 5	,,
6	2,4-D 0.2 + BA 10	,,
7	2,4-D 0.2 + BA 15	,,
8	2,4-D 0.2 + BA 20	,,
9	2,4-D 0.5 + BA 5	,,
10	2,4-D 0.5 + BA 10	,,
11	2,4-D 0.5 + BA 15	,,
12	2,4-D 0.5 + BA 20	,,
13	2,4-D 1.0 + BA 5	,,
14	2,4-D 1.0 + BA 10	,,
15	2,4-D 1.0 + BA 15	,,
16	2,4-D 1.0 + BA 20	,,
17	Control (Basal medium)	,,

Average of 15 observations
Medium - Basal medium + 6% sucrose
Explant - *In vitro* seeds 80 days after placental pollination

in ginger (Table 15). The combination of NAA 0.5 mg l^{-1} along with 2iP 2.5 mg l^{-1} to 5 mg l^{-1} also did not produce germination in ginger seeds.

4.4.2.3 Influence of IAA with BAP and 2iP

IAA (0.05 to 0.2 mg l^{-1}) along with BAP (5 to 20 mg l^{-1}) in the basal medium of 1/2 MS + 6 per cent sucrose recorded no response on seed germination in ginger (Table 16). IAA 0.05 to 0.2 mg l^{-1} along with 2iP 2.5 to 5 mg l^{-1} also did not positively influence seed germination.

4.4.2.4 Influence of GA_3 and ethylene

GA_3 (5 to 10 mg l^{-1}) and ethylene (5 to 10 mg l^{-1}) had no influence on seed germination in ginger (Table 17).

4.4.3 Effect of chemical scarification

The data on results of chemical scarification are furnished in Table 18. The results showed that these treatments had no response on germination of ginger seeds.

4.4.4 Effect of mechanical scarification

The results showed that this treatment also had no influence on germiantion of ginger seeds.

4.4.5 Effect of stratification

The data on influence of cold treatment for germination of ginger seeds are shown in Table 19. The results revealed that stratification had no favourable response on seed germination in ginger.

Table 15. Influence of NAA with BAP and 2iP in *in vitro* seed germination of ginger

Sl.No.	Treatments (mg l^{-1})	Germination
1	NAA 0.5 + BA 7.5	Nil
2	NAA 0.5 + BA 10	,,
3	NAA 0.5 + BA 15	,,
4	NAA 0.5 + BA 20	,,
5	NAA 1.0 + BA 5	,,
6	NAA 1.0 + BA 10	,,
7	NAA 1.0 + BA 15	,,
8	NAA 1.0 + BA 20	,,
9	NAA 2.0 + BA 5	,,
10	NAA 2.0 + BA 10	,,
11	NAA 2.0 + BA 15	,,
12	NAA 2.0 + BA 20	,,
13	NAA 0.5 + 2iP 2.5	,,
14	NAA 0.5 + 2iP 5	,,
15	Control (Basal medium)	,,

Average of 15 observations
Basal medium - 1/2 MS + 6% sucrose
Explant - *In vitro* 80 days after placental pollination

Table 16. Influence of IAA with BAP and 2iP in *in vitro* seed germination of ginger

Sl.No.	Treatments ($mg\ l^{-1}$)	Germination
1	IAA 0.05 + BA 5	Nil
2	IAA 0.05 + BA 10	,,
3	IAA 0.05 + BA 15	,,
4	IAA 0.05 + BA 20	,,
5	IAA 0.1 + BA 5	,,
6	IAA 0.1 + BA 10	,,
7	IAA 0.1 + BA 15	,,
8	IAA 0.1 + BA 20	,,
9	IAA 0.2 + BA 10	,,
10	IAA 0.2 + BA 15	,,
11	IAA 0.2 + 2iP 2.5	,,
12	IAA 0.2 + 2iP 5	,,
13	IAA 0.1 + 2iP 2.5	,,
14	IAA 0.1 + 2iP 5	,,
15	IAA 0.05 + 2iP 2.5	,,
16	IAA 0.05 + 2iP 5	,,
17	Control (Basal medium)	,,

Average of 15 observations
Basal medium - 1/2 MS + 6% sucrose
Explant - *In vitro* seeds 80 days after placental pollination

Table 17. Effect of GA_3 and ethylene on germination of *in vitro* produced seeds of ginger after *in vitro* placental pollination

Sl. No.	Treatment	Germination
1	GA_3 5 mg l^{-1}	Nil
2	GA_3 6 mg l^{-1}	,,
3	GA_3 8 mg l^{-1}	,,
4	GA_3 10 mg l^{-1}	,,
5	Ethylene 5 mg l^{-1}	,,
6	Ethylene 6 mg l^{-1}	,,
7	Ethylene 8 mg l^{-1}	,,
8	Ethylene 10 mg l^{-1}	,,

Average of 12 observations, 15 and 30 days after inoculation
Basal medium - 1/2 MS + 3% sucrose
Explant - *In vitro* seeds 80 days after placental pollination

Table 18. Effect of chemical scarification for germination of ginger seeds

Sl.No.	Treatments	Germination
1	HCl 50% for 3 mts	Nil
2	,, for 5 mts	,,
3	,, for 10 mts	,,
4	HNO_3 25% for 3 mts	,,
5	,, for 5 mts	,,
6	,, for 10 mts	,,

Average of 12 observations, 15 and 30 days after culturing
Media - 1/2 MS + 3% sucrose and 1/2 MS + sucrose 3% + NAA 1.0 mg l^{-1} + BAP 10 mg l^{-1}
Explant - *In vitro* seeds after placental pollination

Table 19. Effect of stratification for germination of *in vitro* produced ginger seeds

Sl.No.	Treatment	Germination
1	Incubating at 4°C for 6 h	Nil
2	,, 4°C for 12 h	,,
3	,, 4°C for 18 h	,,
4	,, 4°C for 24 h	,,

Average of 12 observations, 15 and 30 days after culturing
Media - 1/2 MS + 3% sucrose and 1/2 MS + 3% sucrose + NAA 0.5 mg l^{-1} + BAP 20 mg l^{-1}
Explant - *In vitro* seeds 80 days after placental pollination

4.4.6 Effect of washing the seeds in running water and sowing

The data on influence of washing the seeds in running water and sowing are given in Table 20. The results showed that this treatment had no influence on germination of ginger seeds.

4.4.7 Effect of hydration-dehydration-rehydration treatment

The results showed that this treatment had no influence on germination of ginger seeds. The data regarding this aspect are given in Table 21.

4.4.8 Effect of priming of seeds with mannitol and PEG

Priming of the seeds with mannitol and PEG-4000 each at 12 per cent concentration had no influence on germination of ginger seeds (Table 22).

4.4.9 Investigations on embryo rescue studies in ginger

Embryo rescue techniques tried had no favourable response on the germination of embryo (Table 23). Embryos along with endosperm were isolated from seeds of 20, 40, 60 and 80 DAP and were cultured in the basal medium of 1/2 MS containing auxin and cytokinin combinations.

Table 20. Effect of washing the *in vitro* produced seeds of ginger in running water and sowing for germination of seeds

Sl.No.	Treatment	Germination
1	Washing the seeds in running water for 2 h	Nil
2	,, for 6 h	,,
3	,, for 8 h	,,
4	,, for 12 h	,,

Average of 12 observations, 15 and 30 days after culturing
Media - 1/2 MS + 3% sucrose and 1/2 MS + 3% sucrose + 2,4-D 0.2 mg l^{-1} + BAP 15 mg l^{-1}
Explant - *In vitro* seeds 80 days after placental pollination

Table 21. Effect of hydration - dehydration and rehydration for germination of *in vitro* produced ginger seeds

Sl.No.	Treatment	Germination
1	Hydration for 12 h, dehydration for 5 h and rehydration for 12 h	Nil
2	Hydration for 12 h, dehydration for 6 h and rehydration for 12 h	,,
3	Hydration for 12 h, dehydration for 8 h and rehydration for 12 h	,,
4	Hydration for 12 h, dehydration for 10 h and rehydration for 12 h	,,

Average of 12 observations, 15 and 30 days after culturing
Media - 1/2 MS + 3% sucrose and 1/2 MS + IAA 0.05 + BA 1.5 mg l^{-1} + 3% scurose
Explant - *In vitro* seeds 80 days after placental pollination

Table 22. Effect of priming of seeds with mannitol and PEG

Sl.No.	Treatment	Germination
1	Initiation of seeds in 12% mannitol for 20 days and later in water for 12 h	Nil
2	Initial inhibition of seeds in 12% PEG-4000 for 20 days and later in water for 12 h	,,

Average of 12 observations, 15 and 30 days after culturing
Media - 1/2 MS + 3% sucrose and 1/2 MS + 2,4-D 0.2 mg l^{-1} + BAP 15 mg l^{-1} + 3% sucrose
Explant - *In vitro* seeds 80 days after placental pollination

Table 23. Effect of embryo rescue for producing ginger seedlings

Sl.No.	Treatment	Germination
1	2,4-D 0.5 + BA 5	Nil
2	2,4-D 0.5 + BA 10	,,
3	2,4-D 0.5 + BA 15	,,
4	2,4-D 0.5 + BA 20	,,
5	2,4-D 1.0 + BA 5	,,
6	2,4-D 1.0 + BA 10	,,
7	2,4-D 1.0 + BA 15	,,
8	2,4-D 1.0 + BA 20	,,
9	NAA 0.5 + BA 5	,,
10	NAA 0.5 + BA 10	,,
11	NAA 0.5 + BA 15	,,
12	NAA 0.5 + BA 20	,,
13	NAA 1.0 + BA 5	,,
14	NAA 1.0 + BA 10	,,
15	NAA 1.0 + BA 15	,,
16	NAA 1.0 + BA 20	,,
17	IAA 0.05 + BA 5	,,
18	IAA 0.05 + BA 10	,,
19	IAA 0.05 + BA 15	,,
20	IAA 0.05 + BA 20	,,
21	Control (Basal medium)	,,

Average of 6 observations
Basal medium - 1/2 MS + 3% sucrose
Explant - Embryo along with endosperm 20, 40, 60 and 80 DAP

Discussion

5. DISCUSSION

Studies on "*in vitro* pollination, embryo rescue and germination in ginger (*Zingiber officinale* Rosc.)" were carried out at the Plant Tissue Culture Laboratory, Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, during 1994-1996.

As ginger is exclusively propagated by vegetative means, natural variability available in this crop is very narrow. Variability could not be created among cultivars by hybridization between cultivars due to lack of seed set and fruit formation under *in vivo* conditions. Sathiabhama (1988) reported that long style coupled with inadequate pollen germination and tube growth are the main reasons for lack of seed set in ginger. Various attempts such as bud pollination, artificial cross pollination, chemical aided pollination, mentor pollination and pollination after decapitating stigma under *in vitro* failed to induce seed set (Usha, 1984 and Sathiabhama, 1988).

Valsala (1994) proved that *in vitro* pollination techniques involving application of pollen grains in a germinating medium in close vicinity of ovules bring fertilization in ginger. She could also obtain seed set, seed development and seed germination. However, the conditions required for germination of the seed have to be further investigated. Hence the present investigations were taken up.

5.1 Pollen studies in ginger

The pollen fertility was very low in the studies and it ranged between 4.8 per cent in SG-543 and 35.5 per cent in Rio-de-Janeiro (Table 5). Pollen viability

ranged between 2.5 in SG-603 to 8.2 in Rio-de-Janeiro (Table 8). This is in agreement with previous reports (Nair *et al.*, 1975; Pillai *et al.*, 1978; Jayachandran *et al.*, 1979; Sathiabhama, 1988 and Valsala, 1995). The cultivars of ginger show varying degrees of meiotic abnormalities like the formation of univalents and multivalents at metaphase-1, presence of bridges and laggards at Anaphase-1 and micronuclei formation at Telophase-1 (Ramachandran, 1969; Pillai *et al.*, 1978; Ratnambal and Nair, 1983 and Sathiabhama, 1988). Hence it can be assumed that meiotic abnormalities during microsporogenesis contribute much towards pollen sterility in ginger. Since pollen fertility and viability are very low, large quantities of pollen grains may be used for pollination.

The fertility of the pollen grains was influenced by the season and the cultivar from which it was collected. The fertility was significantly high during the mid season (29.1%) compared to early (12.05%) and late seasons (14.4%). The germination percentage was high during the early (5.89%) and mid (5.06%) seasons compared to late season (2.84%). The observation on mean pollen tube length also followed the same trend. During the early and mid seasons the pollen tube length was 63.50 μm and 68.07 μm respectively.

From the aforesaid observations, it can be suggested that pollination during the mid and early seasons can result in better seed set in ginger.

The fertility of pollen grains did not show significant relationship with position of flowers in the inflorescence. Though not significant, the results indicate that using pollen grains from lower and middle portions of the inflorescence for pollination can result in more seed set. The observations on the pollen tube length also suggests the same.

The genotype of the cultivar influenced the pollen fertility. The fertility was high in SG-66 and Rio-de-Janeiro. This was true in case of observations made on the influence of season and position of flowers in the inflorescence on pollen fertility. This trend was seen in viability studies also. This gives the indication that the chances of seed set will be more in crosses involving Rio-de-Janeiro and SG-66 as male parents and these cultivars could be successfully employed for further seed programmes.

5.2 Investigations on *in vitro* pollination in ginger

5.2.1 Effect of crossing in ginger

The exotic cultivar Rio-de-Janeiro as female parent could be crossed with Kuruppampady - a cultivar from Kerala, SG-66 - a cultivar from Himachal Pradesh and Nadia - a cultivar from Assam (Table 9). As male parent also, it could be crossed with Kuruppampady, Nadia, SG-66 and Bajpai. The cultivar Bajpai is from Assam and SG-603 is from Himachal Pradesh. The percentage of cultures showing response ranged from 85 to 100. This indicate the possibility that the high yield potential of Rio-de-Janeiro, which is recommended for green ginger production in Kerala can be transferred to Kuruppampady, SG-66, Nadia, Bajpai and SG-603, i.e., cultivars suitable for dry ginger production. Kuruppampady as female parent could be crossed with SG-66 and as male parent could be crossed with Bajpai and SG-66. Kuruppampaddy is noted for high cured yield and oleoresin content.

The mean ovule development per culture was 71.3 per cent. A ginger ovary contains approximately 24 ovules, so from a single cross, about seventeen seeds could be obtained.

5.2.2 Effect of selfing in ginger

All the seven cultivars viz., Rio-de-Janeiro, Kuruppampady, SG-66, Nadia, Bajpai, SG-603 and SG-543 could be selfed and the percentage of cultures showing response ranged from 50 to 95.8 per cent. Because of exclusive vegetative propagation, cultivars may possibly be homozygous. Hence the need for selfing does not usually arise. But if there is any heterogeneity present, *in vitro* selfing can be resorted to.

5.2.3 Effect of growth regulators for ovule development in ginger

The plant growth substances cytokinins and auxins were required for proper ovule development following *in vitro* pollination and fertilization. The combination of BAP ranging from 0.5 to 20 mg l^{-1} with 2,4-D 0.1 to 1 mg l^{-1} had favourable effect on ovule development compared to control ie. basal medium with 6 per cent sucrose. Media with growth regulators promoted ovule development in 90-100 per cent of cultures while in control it was only 23 per cent (Table 13). The effect of 2,4-D 0.1 to 1.0 mg l^{-1} could be replaced by NAA (0.5 to 2 mg l^{-1}) and IAA (0.1 to 0.2 mg l^{-1}). The results of the studies conducted by Valsala (1994) also agreed with this observation.

According to Quartrano (1987) the stages of seed development and germination involve rapid embryo and endosperm growth and differentiation, after fertilization, followed by a quiscent state of low metabolic activity and growth and finally a switch back to active growth of embryo to form seedling. Hormones play an important role in these processes. The cytokinins, auxins, gibberellins (GA_3) and

abscisic acid (ABA) were found in relatively high concentration in extracts from seeds at different development 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 amount of cytokinin at the very early stage of seed development than small grain lines (Kelbitsch *et al.*, 1975). According to Tollenaar (1977) cytokinins are found in relatively high concentrations in the liquid endosperm stage of early seed growth and their presence coincides with the higher rate of mitosis.

Euwens and Schwale (1975) have reported that in general, high auxins (IAA) and GA_3 levels have been 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 are decreasing rapidly. This explains the requirement of auxins for proper development of ovules.

5.3 Seed viability test (Tetrazolium test)

The results of tetrazolium test showed that seeds of 40 and 80 DAP were viable. The embryo portion took red colour from 40 DAP upto 80 DAP. So it can be concluded that seeds from 40 DAP itself can be subjected to germination.

The works done by Gbehounou *et al.* (1993) in the seeds of *Striga hermonthica* revealed that the supposed viability with tetrazolium colour test and germination is closely linked. They got 80 per cent germination against 92 per cent viability with tetrazolium test.

5.4 Germination studies of *in vitro* seeds of ginger

5.4.1 Effect of primary treatments on *in vitro* seed germination

The primary treatments like water soaking, incubating on moist filter paper and moist sand did not favour germination of ginger seeds. But the seeds of cardamom, another member of Zingiberaceae family germinate five to seven weeks after sowing, but germination is irregular and sprouting continue upto one year (Aiyer, 1944).

In the course of evolution, the ginger plant adaptated very much for vegetative propagation and it might have lost the capacity for survival through sexual reproduction. Besides, as the seeds were developed under *in vitro* condition, the growth substances required for proper development of the embryo may be lacking in the medium.

5.4.2 Effect of growth regulators on *in vitro* seed germination

Incubating seeds in 1/2 MS + 6 per cent sucrose along with 2,4-D 0.1 to 1.0 mg l^{-1} or NAA 0.5 to 2.0 mg l^{-1} along with BAP 5 to 20 mg l^{-1} had no influence on germination of ginger seeds. The combination of NAA 0.5 mg l^{-1} or IAA 0.05 to 0.2 mg l^{-1} with 2iP 2.5 to 5 mg l^{-1} had no influence on seed germination. GA_3 5 to 10 mg l^{-1} and ethylene 5 to 10 mg l^{-1} also had no influence on seed germination.

In the previous works, Valsala (1994) got one seed germinated out of 20 seeds. For germination, it was incubated in the medium of 1/2 MS with 2,4-D 8.0 mg l^{-1} for two months. Then they were transferred to a plant growth regulator

combination of BAP 9.0 mg l^{-1} and 2,4-D 0.1 mg l^{-1}. This revealed that plant growth substances are required for seed germination under *in vitro* conditions. The low germination can be due to various reasons. In the present study average pollen germination was only 4.75 per cent. The same degree of low viability can be expected in female gametophyte also. This demands more number of replication for the same treatment. Present study was carried out with 12 to 15 replications for each treatment.

5.4.3 Effect of seed treatments for seed germination

The seed coat of mature ginger seeds eighty days after pollination were dark and hard. Chemical and mechanical scarification to soften the seed coat did not favour seed germination. But cardamom seeds with hard seed coat when treated with concentrated H_2SO_4 or HNO_3 for two min recorded improved permeability of seed coat and there by more germination (Spices Board, 1992).

Stratification of seeds at low temperature (4°C) for 6 to 24 h had no response on seed germination. According to Outcalt (1991) stratification increases speed and percentage of germination of Ocala Pine seed in dry soil.

Seeds were washed in running water to remove if there is any inhibitors present in the seed. The seeds did not respond to this treatment also.

Subjecting embryos to stress condition also did not produce germination. Stress condition was given by dehydrating hydrated seeds for 12 h. In another set of treatments, stress condition was provided by soaking the seeds in 12 per cent each of mannitol and PEG-4000 solution for 20 days.

5.5 Embryo rescue studies

Embryos along with endosperm were rescued from seeds obtained on 20, 40, 60 and 80 DAP and were incubated in 1/2 MS medium containing auxins and cytokinins. The hormone combinations of 2,4-D (0.5, 1.0) or NAA (0.5, 1.0) or IAA (0.05) with BAP 5, 10, 15, 20 mg l^{-1} were used. Rescueing embryo along with endosperm removes the seed coat barrier, which may hasten the absorption of the medium by the embryo. This treatment also did not help for germination.

To conclude, the seeds did not germinate eventhough they were subjected to various effects of hormones, scarification, stratification, stress condition and embryo rescue. This may be due to poor embryo development, embryo-endosperm in compatibility or genetic reasons. But in the previous works (Valsala, 1994) one seed out of 20 germinated with the help of hormones i.e., 2,4-D and BAP. This shows that *in vitro* produced seeds of ginger need hormones for germination. Besides, high degree of male sterility is observed in ginger cultivars. The same degree of low viability can be expected in female gametophyte also. These factors will definitely influnece seed viability also. So for identifying conditions for the germination of *in vitro* produced seeds of ginger, the effect of plant growth substances for germination has to be studied in detail with more replications as far as possible.

Summary

6. SUMMARY

Invesgiations were carried out at the Plant Tissue Culture Laboratory, Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during 1994 to 1996 for the refinement of *in vitro* pollination technique in ginger and for identifying the conditions required for the development of embryo to seedling. The salient findings of the study can be summarised as follows.

1. Pollen fertility was influenced by season and maximum fertility was recorded during the mid flowering season i.e., early to mid November.

2. The pollen fertility in the eight cultivars studied ranged from 13.36 per cent in SG-543 to 24.36 per cent in SG-66. There was significant difference between cultivars with respect to fertility and the cultivar SG-66 recorded the maximum mean pollen fertility of 24.47 per cent followed by Rio-de-Janeiro (23.7%).

3. The pollen fertility was not influenced by position of flowers in the inflorescence.

4. The germination of pollen grains in the ME_3 medium was found to be low and varied from 2.9 to 7.4 per cent among cultivars. There was significant difference among cultivars with respect to germination of pollen grains and Rio-de-Janeiro recorded maximum pollen germination of 7.4 per cent.

5. Germination of pollen grains was influenced by season and it was high in the early and mid seasons i.e., from middle of October to middle of November. Pollen tube growth was not influenced by season of inflorescence production.

6. Pollen germination and tube growth were not influneced by position of flowers in the inflorescence.

7. The cultivar Rio-de-Janeiro as female parent can be crossed with Kuruppampady, SG-66, Nadia and as a male parent can be crossed with Kuruppampady, Nadia, SG-66 and Bajpai.

8. Kuruppampady as female parent can be crossed with SG-66 and as male parent can be crossed with Bajpai and SG-66.

9. In crossing studies the mean ovule development per culture was 71.3 per cent and mean cultures showing response was 93.2 per cent. From a single cross approximately seventeen seeds can be obtained.

10. The seven cultivars viz., Rio-de-Janeiro, Kurumppampady, SG-66, Nadia, Bajpai, SG-603, SG-543 can be selfed and the cultures showing response ranged from 50 to 95.8 per cent.

11. The combination of BAP 0.5 to 20 mg l^{-1} with 2,4-D 0.1 to 1 mg l^{-1} had favourable effect on ovule development.

12. The effect of 2,4-D 0.1 to 1.0 mg l^{-1} could be replaced by NAA (0.5 to 2 mg l^{-1}) and IAA (0.1 to 0.2 mg l^{-1}).

13. The seed viability test with tetrazolium salt at 40 and 80 DAP revealed that the seeds are viable.

14. The primary treatments like water soaking, incubating on moist filter paper, moist sand or basal medium (both solid and liquid state) did not favour germination of ginger seeds.

15. Incubating the seeds in 1/2 MS + 6 per cent sucrose along with 2,4-D 0.1 to 1.0 mg l^{-1} or NAA 0.5 to 2.0 mg l^{-1} along with BAP 5 to 20 mg l^{-1} had no influence on germination of ginger seeds.

16. The combination of NAA 0.5 mg l^{-1} or IAA 0.05 to 0.2 mg l^{-1} with 2iP 2.5 to 5 mg l^{-1} had no influence on seed germination.

17. GA_3 5 to 10 mg l^{-1} and ethylene 5 to 10 mg l^{-1} didn't influence seed germination.

18. Chemical and mechanical scarification to soften the seed coat did not influence seed germination.

19. Stratification of seeds at low temperature (4 °C) for 6 to 24 h had no response on seed germination.

20. Washing of seeds in running water to remove any inhibitors present in the seed had also no response.

21. Subjecting the embryos to stress condition by dehydrating hydrated seeds for 12 hours did not produce germination.

22. Soaking the seeds in 12 per cent each of mannitol and PEG-4000 solution for 20 days did not influence germination.

23. Embryos along with endosperm were rescued from 20, 40, 60 and 80 DAP and incubated in 1/2 MS medium with 2,4-D (0.5, 1.0 mg l^{-1}) or NAA (0.5, 1.0 mg l^{-1}) or IAA (0.05 mg l^{-1}) with BAP (5, 10, 15, 20 mg l^{-1}) did not produce germination.

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IN VITRO POLLINATION, EMBRYO RESCUE AND GERMINATION STUDIES IN GINGER, *Zingiber officinale* (Rosc.)

BY

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ABSTRACT OF A THESIS

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ABSTRACT

Investigations were carried out for the refinement of *in vitro* pollination techniques in ginger developed at the Plant Tissue Culture Laboratory, Department of Plantation Crops and Spices, College of Horticulture, mainly to identify the conditions required for the germination of *in vitro* produced seeds of ginger. The study was carried out from 1994 to 1996.

Investigations were carried to study the effect of season, cultivars and position of flowers in the inflorescence on pollen fertility and viability. The results showed that pollen fertility and viability were influenced by the season and genotype but not by the position of flowers on the inflorescence. The pollen viability was high in inflorescences produced during the early and mid period of the flowering seasons. So scheduling the pollination works for the early and mid period of the flowering seasons may lead to more seed set. The cultivars SG-66 and Rio-de-Janeiro exhibited more pollen fertility and viability. So chances of seed set will be more in crosses involving these cultivars as male parents.

Crossing studies showed that Rio-de-Janeiro as female parent can be crossed with Kuruppampady, SG-66, Nadia and as a male parent can be crossed with Kuruppampady, Nadia, SG-66 and Bajpai. So the high yield potential of Rio-de-Janeiro can be transferred to cultivars suitable for dry ginger production.

Selfing studies showed that the cultivars viz., Rio-de-Janeiro, Kuruppampady, SG-66, Nadia, Bajpai, SG-603 and SG-543 can be selfed by the *in vitro* pollination and fertilization techniques.

The *in vitro* fertilized ovules developed into mature seeds in the medium of 1/2 MS with 2,4-D 0.1 to 1.0 mg l^{-1} and BAP 5 to 20 mg l^{-1}. The effect of 2,4-D could be replaced by NAA 0.5 to 2 mg l^{-1} or IAA 0.05 to 0.2 mg l^{-1}.

The results of seed viability test with tetrazolium salt showed that seeds of 40 and 80 DAP are viable so seeds from 40 DAP onwards can be subjected to germination studies.

The studies on germination of ginger seeds showed that primary treatments like water soaking, incubating on moist filter paper, moist sand or basal medium (both solid and liquid state) did not favour germination of ginger seeds. Incubating the seeds in 1/2 MS + 6 per cent sucrose along with 2,4-D 0.1 to 1.0 mg l^{-1} or NAA 0.5 to 2.0 mg l^{-1} and BAP 5 to 20 mg l^{-1} had no influence on germination of ginger seeds. The combination of NAA 0.5 mg l^{-1} or IAA 0.05 to 0.2 mg l^{-1} with 2iP 2.5 to 5 mg l^{-1} had no influence on seed germination. GA_3 5 to 10 mg l^{-1} and ethylene 5 to 10 mg l^{-1} also did not favour seed germination.

Seed treatments like chemical and mechanical scarification, stratification, washing the seeds in running water and subjecting the embryos to stress condition by dehydrating hydrated seeds for 12 h or soaking the seeds in 12 per cent each of mannitol and PEG-4000 solution did not influence germination.

Embryo rescue involving transfer of embryo along with endosperm to culture media with varying combinations of auxins and cytokinin also did not promote development of embryo to seedling.

Appendix

APPENDIX-I

Abstract of analysis of variance for the effect of different treatments

Sl. No.	Table details and characters	Treatment MSS	Error MSS	Level of* significance
I.	Table 5. Effect of season on fertility of pollengrains of ginger cultivars			
	1) Season	596.87	23.61	1
	2) Cultivars	35.38	23.61	5
II.	Table 6. Effect of season on viability and tube length of pollen grains of ginger cultivars			
	a) Viability			
	1) Season	17.32	1.131	1
	2) Cultivars	6.66	1.131	1
	b) Tube length			
	1) Cultivars	145.77	150.5	5
III.	Table 7. Effect of position of flowers in the inflorescence on fertility of pollen grains of ginger cultivars			
	1) Cultivars	32.45	37.3	5
IV.	Table 8. Effect of position of flowers in the inflorescence on viability and tube length of pollen grains of ginger cultivars			
	a) Viability			
	1) Cultivars	4.38	1.88	5
	b) Tube length			
	1) Cultivars	167.58	123.82	5

*In percentage