IN VITRO POLLINATION IN KACHOLAM (*KAEMPFERIA GALANGA* L.) FOR SEED SET

By

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THESIS

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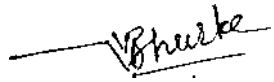
DECLARATION

I hereby declare that the thesis entitled **"*In vitro* pollination in kacholam (*Kaempferia galanga* L.) for seed set"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.



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DEDICATED TO MY MOTHER

"Start from where you are, with what you have, make something of it, never be satisfied."

- George Washington Carver

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LIST OF ABBREVIATIONS

µm - micrometer

2,4-D - 2,4-Dichlorophenoxy Acetic Acid

BA - Benzyl adenine

CH - Casein hydrolysate

cm - centimetre

CW - Coconut water

DAP - Days after pollination

FAA - Formalin acetic acid mixture

GA_3 - Gibberellic Acid

ha - hectare

HCl - Hydrochloric acid

HNO_3 - Nitric acid

IAA - Indole - 3 – Acetic Acid

IBA - Indole Butyric Acid

KC - Knudson C medium

kg - kilogram

Kin - Kinetin, N_6-furfuryl acetone

l - litre

mg - milligram

mm - millimetre

MS - Murashige and Skoog's (1962) medium

NA - Not available / Not applicable

NAA - α Naphthalene Acetic Acid

PDA - Potato Dextrose Agar medium

PEG 4000 - Polyethyleneglycol-4000

t - tonne

v/v - volume in volume

YEM - Yeast Extract Mannitol medium

INTRODUCTION

Introduction

Kaempferia galanga L., known as 'kacholam' in Kerala is an important medicinal and aromatic plant of Zingiberaceae. It is indigenous to India and is distributed in the tropics and subtropics of Asia, Africa and in Philippine islands. There are 55 species reported in the genus *Kaempferia* of which 10 are found in India, *K. galanga* L. and *K. rotunda* L. being the economically important ones.

The kacholam plant is a rhizomatous herb spreading horizontally, lying flat on the ground surface with a rosette of leaves, and the inflorescence arises directly from the rhizome. The whole plant is refreshingly aromatic and is bestowed with several medicinal properties like stimulant, expectorant, carminative and diuretic (Chopra *et al.*, 1956). It is also noted for the anti-inflammatory, larvicidal, insecticidal, anticancer and vasorelaxant activities (Sastri, 1959; Pongprayoon *et al.*, 1996; Kiuchi *et al.*, 1988; Nugroho, *et al.*, 1996 and Mustafa *et al.*, 1996). The plant is listed under the red list of medicinal plants of south India and has been stated as "critically endangered" (FRLHT, 1997).

In India *K. galanga* is cultivated throughout the plains for its rhizome. The various medicinal and aromatic properties of the rhizome make it a multipurpose plant. The economic produce of kacholam for trade is the chopped and dried rhizomes of 6 to 7 months maturity. These rhizomes are used in bulk quantities in ayurvedic medicine and in cosmetic industries. In ayurveda, kacholam is reported to be effective against diseases caused by the morbidity of 'Vatha' and 'Kapha', the major two among the 'Thridoshas'. Kacholam is an ingredient of several ayurvedic preparations such as 'Kachuradi choornam', 'Kachuradi thailam', 'Kachuradi vattu', 'Asanelaadi thailam', 'Kalyanakaghritham', 'Dasamoolarishtam' and 'Chyavanaprasam' (Sivarajan and Balachandran, 1994). The rhizomes can also be used for curing inflammatory wounds. It can also cure skin disorders, piles, oedema, fever, epilepsy, splenic disorders and asthma. Apart from rhizomes, extract of whole plant is also used in some tonics (Kirthikar and Basu, 1935; Brown, 1941; Quisumbing, 1951; Aiyer and Kolammal, 1964; Burkill, 1966).

In cosmetic industry, it is used as a perfume in hair washes, powders and other cosmetics. Its antidandruff property makes it an essential ingredient in hair washes. It is also used in the manufacture of agarbathis.

The rhizomes yield 2.4-4.0 per cent volatile oil, which is utilized in the manufacture of perfumes and curry flavourings. The constituents of the rhizome oil include para methoxy ethyl cinnamate (60.24 % of the oil), ethyl cinnamate (20.66 %), cineol, borneol, 3- carene, camphene, kaempferol, kaempferide and cinnamaldehyde.

Recently, the exploitation of rhizomes in the medicinal and perfumery industry has shot up and the demand for the same has increased. In Kerala, it has attained the status of a cash crop of homesteads. The annual consumption of the dried rhizome by various ayurvedic pharmacies in Kerala is about 16,000 kg with a unit price of Rs. 275 per kg thus valued at about Rs. 0.38 million (Joseph, 2001). The humid tropical climate and soil conditions prevailing in the state are very much suitable for cultivation of kacholam. The cultivation practices of the crop are simple consisting of propagation through rhizome bits and planting on raised beds in partially shaded conditions. Hence there is immense scope for cultivation in the households of Kerala as an intercrop in coconut gardens. Since the crop flourishes well in humid tropics, it can be cultivated successfully in various parts of south India and North-Eastern states also.

In order to meet the internal and export demand, cultivation of kacholam has to be streamlined on scientific footing. For this, high yielding and quality cultivars are imperative. No promising released varieties are available so far. At present, the cultivation is limited to local cultivars/ local selections only.

The genetic resources available in the crop exhibit limited variability for economic characters. Limited natural genetic diversity and absence of seed set are the major constraints for improvement through selection and heterosis breeding. Various *in vivo* pollination techniques previously tried for seed set did not yield

any positive result. A probe into the reasons for lack of seed set reveals that incompatibility reactions operating in the style coupled with caducous nature of the flower prevent seed set. Flowers being complete with all essential whorls and adequate pollen production, pre-fertilization barriers like spiny stigma, lengthy style and caducous nature of the flower contribute towards seedlessness (Rekha and Viswanathan, 1996).

Aforesaid type of pre-fertilization barriers observed in other crops of Zingiberaceae viz. ginger (Valsala, 1994) and turmeric (Renjith, 1999) have been overcome through *in vitro* pollination wherein the pollen fertility was low. Adequate pollen production and high pollen fertility (72.8 %) and pollen viability (68.9 %) are observed in kacholam (Rekha, 1993), which indicate a greater possibility of getting seed set in this crop through *in vitro* pollination and fertilization. In this context, an attempt for seed set through *in vitro* pollination for induction of variability was carried out.

Success in this line may open up new vistas of crop improvement in kacholam through hybridization. The hybrid plants obtained through this technique could be easily mass propagated by tissue culture techniques that are already standardized for this crop. Further exploitation of the hybrid vigour would be facilitated by vegetative propagation through rhizomes. The results obtained in this crop will be useful in certain tropical rhizomatous crops like ginger, turmeric and chittaratha (*Alpinia galanga*), where seed set is absent or less.

REVIEW OF LITERATURE

Review of Literature

The investigations on "*In vitro* pollination in kacholam (*Kaempferia galanga* L.) for seed set" aims at standardization of *in vitro* pollination technique for seed set and seed germination so that crop improvement through hybridization will be feasible in this crop. The literature on various aspects relevant to the subject matter of the investigation is reviewed here.

2.1 Origin, distribution and taxonomy

A genus of rhizomatous herbs, *Kaempferia* is supposed to have been originated in South East Asia, probably in Burma (Myanmar) and from there, it appears to have migrated across tropical Asia and Africa (Holtum, 1950). The genus is widely distributed in the tropics and subtropics of Asia and Africa (Synge, 1956). Willis (1960) suggested that family Zingiberaceae is chiefly Indo-Malayan in distribution. According to Hooker (1892) *Kaempferia galanga* L. is distributed throughout the plains of India. Gamble (1926) reported that it is distributed at low elevations along the West coast.

About 10 species occur in India with *K. galanga* L. and *K. rotunda* L. being the economically important ones. *Kaempferia galanga* L. is a monocotyledonous, rhizomatous herb belonging to the family Zingiberaceae of the order Zingiberales coming under the series Epigyne (Bentham and Hooker, 1894).

According to Hutchinson (1934), there are four tribes in the family viz. Costea, Hedychia, Globeae and Zingibera. Schumann (1904) divided the family Zingiberaceae into two subfamilies viz. Zingiberoideae and Costoideae. The Zingiberoideae is divided into three tribes viz. Globeae, Hedychieae and Zingiberaceae. The genus *Kaempferia* comes under the tribe Hedychieae.

Hooker (1892) reorganized four subgenera under the genus *Kaempferia* viz. Sincorus, Protanthium, Monolophus and Stachyanthesis. *K. galanga* L. comes under the subgenus Sincorus which also includes 11 other species viz. *K.*

marginata, K. angustifolia, K. ovalifolia, K. speciosa, K. pandurata, K. prainiana, K. roscoeana, K. parviflora, K. involucrate, K. andersoni and *K. cocinna*. The only variety reported in *K. galanga* is *K galanga var latifoloia*.

2.2 Cytogenetics

Raghavan and Venkatasubhan (1943) reported the chromosome number of *Kaempferia galanga* as 54 and ascertained six as the basic chromosome number for the genus. Raghavan and Arora (1958) also assigned a chromosome number of 2n = 54 for the same.

Sharma and Bhattacharya (1959) reported a chromosome number of 22 for *K. galanga*. Ramachandran (1969) reported a chromosome number of 2n = 54 for *K. galanga* and opined that this species is probably an aneuploid pentaploid. Rekha (1993) assigned the basic chromosome number x = 11 with somatic cell chromosome number as 55 in *K. galanga* thus concluding the species being a pentaploid.

Beltran and Kam (1984) studied the cytotaxonomy of Zingiberaceae and found that Asiatic *Kaempferia*s had a basic chromosome number of x = 11 while the African ones have x = 14.

2.3 Flowering

2.3.1 Flowering behaviour

Rajagopalan (1983) observed that in *K. galanga* flowering started in June and ended in September, the peak being during July-August. The flowers were produced directly from the rhizome and opened in succession.

Rekha (1993) reported that *K. galanga* flowered during July-August.

Biswas and Chopra (1982) reported that in *K. rotunda* flowering occurred in summer and fruiting in the early rains.

2.3.2 Inflorescence

The inflorescence in the genus *Kaempferia* is reported to be a short scape (Gamble, 1926). The floral morphology has been described by Hooker (1892) and by Kirthikar and Basu (1935). According to them, six to 12 flowers are produced from an inflorescence situated within the sheath of leaves.

Rekha and Viswanathan (1996) reported that an inflorescence of *K. galanga* contains two to 13 buds of which the outer ones open first.

2.3.3 Floral morphology and floral biology

The flowers of *Kaempferia* are fugacious and fragrant with three lanceolate bracts. They are white in colour with a purple spot in the centre. Calyx is of the same length as that of the bracts. Corolla tube is very long with filiform stigma. Both the essential whorls i.e. androecium and the gynoecium are trimerous. Out of three stamens, only one is fertile and other two are petaloid staminodes. Ovary is inferior, three celled and capsules are oblong (Hooker, 1892; Gamble, 1926; Kirthikar and Basu, 1935).

Rekha and Viswanathan (1996) reported that flowers of *K. galanga* are bisexual, complete and zygomorphic with floral parts arranged on the trimerous plan of monocotyledons. Perianth is connate at the base forming a long tube and free at the apex. The stamens are arranged in two whorls, the outer one being represented by two staminodes and the inner one being the only one fertile anther and bilobed showy labellum. The style passes through the groove formed by anther lobe. Gynoecium consists of tricarpellary syncarpous inferior ovary with 16-19 ovules in axile placentation. Style is about four cm long and ends in a spiny stigma.

The study on floral biology of kacholam showed that the flowers are well adapted for both cross as well as self pollination (Rekha and Viswanathan, 1996). The flowers produced 54,000 pollen grains per flower thus the inadequacy of pollen grains was ruled out as being a cause for lack of seed set, especially with respect to the number of ovules per flower i.e. 16-19.

The study of the gynoecium showed that the flowers had a mean style length of 4.37 cm and mean ovary length 0.34 cm.

2.3.4 Pollen studies

Rekha (1993) reported a mean pollen fertility of 72.8 per cent in *K. galanga* with acetocarmine stain test. The mean diameter of pollen grains was 114.8 µm and a maximum pollen germination percentage of 68.9 per cent with a maximum pollen tube length of 732 µm was observed in the medium of 8 % sucrose + 60 ppm boric acid + 1 % gelatin.

2.3.5 Pollination and seed set

There are no reports on seed set in *K. galanga* in spite of the flowers being complete and well adopted to cross as well as self pollination.

Rekha and Viswanathan (1996) observed that the floral characters such as white showy labellum, mild fragrance of the flower, presence of nectar and nocturnal anthesis make the flowers suitable for insect pollination. The adaptations favouring self pollination such as proximity of anther and stigma were also observed. However, there was no seed set when flowers were subjected to stigmatic and stub pollination under *in vivo* condition.

2.3.5.1 Reasons of lack of seed set

Rekha (1993) found that the cytological factors contributing to sterility were meager while the morphological features of the floral parts were responsible for seedlessness. The reasons for lack of seed set were summarised as follows:

i) Spinuous stigma preventing adherence of pollen grains

ii) Poor pollen germination under *in vivo* conditions

iii) Inability of pollen tubes to penetrate the stigmatic surface

iv) Slow growth of pollen tube through the style so that it does not reach the ovary before withering of flower

v) Coiling and bursting of the pollen tubes

vi) Caducous nature of the flower.

The seedlessness in the crop was thus mainly attributed to incompatibility factors in the style.

2.4 *In vitro* pollination and fertilization

The technique of *in vitro* pollination and fertilization is an effective tool for getting seed set in species where pre-fertilization barriers block the seed set. Some of the barriers to fertilization are

a) Inability of the pollen grains to germinate on the foreign stigma.

b) Failure of the pollen tube to reach the ovule due to excessive length of the style or due to slow growth of the pollen tube that fails to reach the base of the style before the ovary abscises.

c) Bursting of the pollen tube in the style.

The most important application of the technique is the production of rare hybrids (Bhojwani and Razdan, 1983).

2.4.1 *In vitro* pollination and fertilization technique

Kanta and Maheshwari (1963) described the prerequisites and the procedures to be adopted for the successful *in vitro* pollination.

The technique consists of

1) A detailed study of the floral biology of the crop i.e. time of anthesis, anther dehiscence, mode of pollination etc.

2) Pollen germination and pollen tube growth has to be determined.

3) Standardization of a surface sterilization technique for flowers and flower buds so that the viability of the pollen grains and the receptivity of the gynoecium are not affected.

4) Identification of a suitable medium, which can support the development of the ovule or the ovary into mature seed or fruit.

5) Standardization of appropriate pollination technique so that fertilization and growth of the ovule takes place.

6) Identification of suitable culture conditions which will promote the growth of the ovule to viable seed.

7) Histological examination of ovules or ovary at various stages of development to know the fertilization and the development of embryo and endosperm.

8) Standardization of culture medium and conditions for germination of seeds.

2.4.2 Types of *in vitro* pollination

The actual technique of *in vitro* pollination needs to be modified to suit the type of flower and the conditions/ pre-fertilization barriers present in a particular species. The different kinds of *in vitro* pollination are *in vitro* stigmatic pollination, intra ovarian pollination, *in vitro* placental pollination and *in vitro* ovular pollination (Bhojwani and Razdan, 1983). *In vitro* stylar pollination was reported by Niimi (1976). Van Tuyl *et al.* (1991) used *in vitro* grafted style pollination.

2.4.2.1 *In vitro* stigmatic pollination

The pollen grains are applied on the surface of the stigma. Usha (1965) obtained seeds in *Antirrhinum majus* by *in vitro* stigmatic pollination. In *Lilium*,

interspecific hybrids were obtained through *in vitro* stigmatic pollination (Van Tuyl *et al.*, 1991).

2.4.2.2 *In vitro* stylar pollination

The style is cut at various heights and the pollen grains are deposited on the cut surface of the style. *In vitro* seed production has been successfully reported in *Petunia hybrida* (Niimi, 1976) and in maize (Gengenbach, 1977 and Hauptli and Williams, 1988).

2.4.2.3 Grafted style pollination

Van Tuyl *et al.* (1991) devised this method in *Lilium*. Here pollen grains are applied on a compatible stigma and the style with the germinated pollen is attached to the ovary of another plant, which is incompatible. In *Lilium* crosses the style and stigma were joined together using a piece of drinking straw filled with stigmatic exudates or were stuck together only with the exudate. This technique was used to overcome the incompatibility among various groups of *Lilium*.

2.4.2.4 Intra ovarian pollination

Pollen grains are directly applied onto the ovary in intra ovarian pollination. Kanta (1960) developed seeds in *Papaver rhoeas* using modified intra ovarian pollination. In this technique, the pollen suspended in distilled water having boric acid (100 mg l^{-1}) was injected into the ovary through a hole using a hypodermic syringe. This technique was later used successfully in *Papaver somniferum* and *Eschscholtzia californica* also.

The pollen grains can also be introduced into the ovary through a slit made on the ovary wall. This technique gave positive results both in *Argemone mexicana* and in *A. ochroleuca*. This technique was used to produce interspecific hybrids between these two species (Kanta and Mahaeshwari, 1963).

2.4.2.5 *In vitro* placental pollination

Here the entire placental tissue bearing ovules attached to short pedicel is used (Rangaswamy and Shivanna, 1967 and 1971a). The placenta may be cut into two or more pieces each carrying a certain number of ovules.

Rangaswamy and Shivanna (1971b) developed a modification to the placental pollination technique. They cultured entire pistils after exposing the placenta bearing the ovules by removing the ovary wall partially and pollination was done on exposed ovules.

Valsala *et al.* (1996) reported seed development in ginger (*Zingiber officinale* Rosc.) with placental, modified placental pollination and test tube fertilization.

Renjith (1999) achieved seed development in turmeric (*Curcuma domestica* Val.) with placental pollination.

2.4.2.6 *In vitro* ovular pollination / Test tube fertilization

Pollen grains are directly applied to the excised ovules. This technique has been used in *Papaver somniferum*, *Argemone mexicana*, *Eschscholtzia californica*, *Nicotiana rustica* and *N. tabacum* (Kanta and Maheshwari, 1963).

Mature embryoids and plants were obtained after *in vitro* ovular pollination of the cruciferous species (Zenkteler, 1980).

Slusarkiewicz (1984) also reported the test tube fertilization of ovules in some species of Solanaceae. Embryos were developed in plums by this technique (Lech *et al.*, 1994).

Self-incompatibility was overcome in chicory (*Cichorium intybus* L.) by *in vitro* ovular pollination (Castano and DeProft, 2000).

Pre and post fertilization barriers in *Lilium* were overcome (Chi, 2000) using *in vitro* ovular pollination method for producing interspecific hybrids.

Fernando *et al.* (1998) achieved successful *in vitro* fertilization in conifers for the first time in Douglas fir (*Pseudotsuga menziesii*). The procedure consisted of a two- step process involving induction of pollen tubes in culture followed by introduction of isolated female gametophytes at the tips of growing pollen tubes.

2.4.3 Factors affecting seed set

2.4.3.1 Age of the flower buds

Generally, the flower buds on the day of anthesis or one or two days after anthesis respond to *in vitro* pollination. The occurrence of seed set was reported to be higher in when the ovules were excised one to two days after anthesis than on the day of anthesis (Kanta *et al.*, 1962, Kanta and Maheshwari, 1963, Rangaswamy and Shivanna, 1971a and Balatkova *et al.*, 1976).

In maize, the spike three to four days after silking was found suitable for *in vitro* pollination (Gengenbach, 1977). The flower buds one day prior to anthesis were ideal for *in vitro* pollination in *Gossypium* (Refaat *et al.*, 1984).

In *Lilium*, the seed set was observed when the flowers pollinated three days prior to anthesis. Success rate was often reduced when pollination was done two days prior to anthesis (Van Tuyl *et al.*, 1991).

2.4.3.2 Surface sterilization

An effective surface sterilization technique is an important prerequisite for successful *in vitro* culture. In the *in vitro* pollination, the technique has to be such that there is no or little harm to the pollen grains and the ovules.

In *Papaver somniferum*, the ovaries are relatively hard and can be surface sterilized by direct flaming after dipping in rectified spirit. In other species where the ovaries are more delicate, such as in *Argemone mexicana*, *Eschscholtzia californica*, *Nicotiana rustica* and *N. tabacum*, surface sterilization was done with chlorine water (Kanta and Maheshwari, 1963).

Usha (1965) reported surface sterilization of flower buds of *Antirrhinum majus*, prior to anthesis by dipping first in 70 per cent alcohol and later in strong chlorine water followed by washing with sterile distilled water.

In *Vaccinium*, satisfactory surface sterilization was achieved with one per cent sodium hypochlorite solution for 15 minutes (Munoz and Lyrene, 1985).

In *Lilium*, flower buds were treated with ethyl alcohol (70 %) for one minute followed by treatment with commercial bleach (2 % Cl) for 15 minutes for surface sterilization (Van Tuyl *et al.*, 1991).

In chicory (*Cichorium intybus* L.), surface sterilization was done by immersing the flower buds before anthesis in one per cent NaOCl for five minutes followed by rinsing with sterile distilled water (Castano and DeProft, 2000).

The surface sterilization was found unnecessary in maize since several layers of husk protect ovaries. The removal of inner husks with sterile forceps in the laminar flow chamber is sufficient to achieve successful *in vitro* culture (Gengenbach, 1977).

2.4.3.3 Nature of the explant

The factors such as the physical condition of the ovary or ovules, the extent and the type of dissection done to expose the ovules, the parts of the pistil being removed or retained constitute the nature of explant. This has an influence on the success of *in vitro* pollination.

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

Castano and DeProft (2000) noted that success has been obtained mainly with the species of the families Brassicaceae, Caryophyllaceae, Liliaceae, Papaveraceae, Primulaceae and Solanaceae. The direct *in vitro* pollination of ovules allows some pre and post zygotic barriers of incompatibility to be overcome and it has been accomplished with subsequent development of embryos in 57 species representing 14 families. The best results were obtained in species in which the ovaries are large and contain many ovules.

In the intra ovarian pollination of *Papaver rhoeas*, the retention of petals and sepals was found to accelerate the ovary development (Kanta, 1960).

In the placental or ovular pollination wetting the surface of the ovules should be avoided as it causes poor germination, bursting of pollen tube resulting in poor seed set (Balatkova and Tupy, 1968 and Zenkteler, 1980).

Rangaswamy and Shivanna (1971a) observed that in *Petunia axillaris*, *in vitro* pollination on excised ovules or a group of ovules attached to a piece of placenta did not result in seed set. Normal seed development resulted when pollination was done on intact placenta with undisturbed ovules.

In *Petunia hybrida* complete removal of the style had a deleterious effect on seed set following placental pollination. Hence the entire pistils with exposed

ovules by peeling the ovary wall alone was used for *in vitro* pollination (Wagner and Hess, 1973).

In maize, the ovaries attached to the placental tissue produced favourable development than single ovaries (Sladky and Havel, 1976; Gengenbach, 1977; Dhaliwal and King, 1978).

In vitro pollination of unfertilized ovules excised from pollinated pistils with its own pollen or that of *Malus sp.* resulted in good seed set than the ovules from unpollinated pistils (Balatkova *et al.*, 1977).

2.4.3.4 The culture medium

An appropriate culture medium is necessary for the successful development of *in vitro* pollinated ovule into a mature seed.

2.4.3.4.1 Basal medium

In the initial years of *in vitro* pollination technique, the Nitsch's medium (1951) was used (Kanta and Maheshwari, 1963; Usha, 1965; Rangaswamy and Shivanna, 1967; Jarzina and Zenkteler, 1983).

Modified Nitsch's medium was developed by Kanta and Maheshwari (1963) while Steward and Hsu (1978) developed a medium for intraspecific and inerspecific hybrids from young fertilized ovules in cotton.

In maize, Gengenbach and Green (1975) used modified LS medium to support *in vitro* seed development. In later studies, MS medium was found to be suitable for the development of maize karyopses (Dhaliwal and King, 1978; Bajaj, 1979; Havel and Novak, 1981). MS medium was also used for the production of interspecific hybrids of *Gossypium*, *Nicotiana* and *Lilium* following *in vitro* pollination (Refaat *et al.*, 1984; Slusarkiewicz, 1984; Zhou *et al.*, 1991; Van Tuyl

et al., 1991). MS medium was suitable for plum also (Lech *et al.*, 1994). Hauptli and Williams (1988) used MS medium in maize.

Valsala (1994) tried MS medium at full and half strength, SH medium and Nitsch's medium and found half MS, SH and Nitsch's medium suitable for ovary development of ginger after *in vitro* pollination.

Renjith (1999) found half MS superior to full MS for the culture establishment of turmeric ovary after *in vitro* pollination.

Castano and DeProft (2000) utilized both MS and Gamborg's B5 medium (Gamborg *et al.*, 1968) for the culture of isolated ovules of chicory (*Cichorium intybus* L.) after *in vitro* ovular pollination.

2.4.3.4.2 Sucrose concentration

The sucrose concentration influences the development of ovary, ovule and germination of seeds after *in vitro* pollination.

Generally, sucrose has been used at a concentration of four to five per cent (Kanta and Maheshwari, 1963; Usha, 1965; Rangaswamy and Shivanna, 1967).

In maize, a high concentration of 15-17 per cent sucrose gave successful results (Sladky and Havel, 1976; Gengenbach, 1977), while Dhaliwal and King (1978) could obtain viable seeds even with five per cent sucrose. Bajaj (1979) found that seven per cent sucrose is optimum for maize.

In the production of interspecific hybrids of *Lilium* by *in vitro* pollination, it was found that the sucrose concentration affects the capsule development. The highest swelling of the capsule was obtained with sucrose concentration of 10 per cent but embryos were mostly found in ovaries culture at six to eight per cent sucrose. Best results were obtained with MS medium with seven per cent sucrose (Van Tuyl *et al.*, 1991).

Lech *et al.* (1994) observed that in plum, two per cent sucrose along with other supplements could support embryo development after *in vitro* fertilization.

Valsala (1994) found six to eight per cent sucrose optimum for the development of ovaries and ovules of ginger while Renjith (1999) found three per cent sucrose superior to six per cent in case of turmeric.

Castano and DeProft (2000) used MS medium and Gamborg's B5 medium with 4 and six per cent sucrose.

Chi (2000) used agar plate containing Brewbaker and Kwack's medium (Brewbaker and Kwack, 1963) with 10 per cent sucrose for *Lilium*.

2.4.3.4.3 Effect of growth regulators and supplements

Usha (1965) could obtain seed set in *Antirrhinum majus* through *in vitro* pollination, on addition of 25 per cent coconut water in Nitsch's medium and supplements.

Casein hydrolysate at 500 mg l^{-1} was found to enhance ovule development in *Papaver spp.*, *Petunia hybrida* and maize (Kanta and Maheshwari, 1963; Wagner and Hess, 1974; Zubkova and Sladky, 1975).

Balatkova *et al.* (1977) observed the influence of growth regulators and supplements such as IAA, kinetin, tomato juice, coconut milk and yeast extract on seed development in tobacco after placental pollination. Coconut milk, tomato juice and yeast extract inhibited the seed development while the growth regulators IAA 1.0 mg l^{-1} or kinetin 0.1 mg l^{-1} improved the number of seeds per ovary. Kinetin had an inhibitory effect at higher concentration of 1.0 mg l^{-1}.

Van Tuyl *et al.* (1991) reported that in *Allium*, auxin is essential at the initial stages of ovule development.

In plum, GA_3 0.5 mg l^{-1} promoted the development of zygote after *in vitro* pollination (Lech *et al.*, 1994).

Valsala (1994) observed that hormones are essential for ovary and ovule development in ginger after *in vitro* pollination. The auxins as well as cytokinins alone, induced ovule development but combinations proved to be better. GA did not favour ovary and ovule development, organic supplements coconut water, casein hydrolysate, inflorescence extract promoted ovule development.

Renjith (1999) observed that *in vitro* pollinated gynoeciums recorded maximum ovule swelling when cultured on ½ MS medium with NAA 0.5 mg l^{-1} with BAP and kinetin both at 1.0 mg l^{-1}. Organic supplements coconut water and casein hydrolysate enhanced ovule development.

Castano and DeProft (2000) cultured ovules from *in vivo* pollinated flowers of chicory (*Cichorium intybus* L.) on various media combinations. The ovules developed into seedlings in three combinations viz.

i) MS + IAA 4.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + GA_3 5.0 mg l^{-1} with four per cent sucrose supplemented with glycine 9.5 mg l^{-1} + casein hydrolysate 500 mg l^{-1}

ii) GB_5 + IAA 4.0 mg l^{-1} + kinetin 0.5 mg l^{-1} + GA_3 5.0 mg l^{-1} with four per cent sucrose supplemented with glycine 7.5 mg l^{-1} + casein hydrolysate 500 mg l^{-1}

iii) MS + IAA 0.5 mg l^{-1} + kinetin 0.5 mg l^{-1} + GA_3 4.0 mg l^{-1} with four per cent sucrose supplemented with glycine 7.5 mg l^{-1} + casein hydrolysate 500 mg l^{-1}

2.4.3.5 Culture conditions

Cultures were usually stored in darkness or near darkness (Rangaswamy and Shivanna, 1967; Balatkova *et al.*, 1977; Dhaliwal and King, 1978). In *Petunia diffusa,* daylight (10-12 ft candle at 25 ± 2°C) was found optimum for *in vitro* seed development (Rangaswamy and Shivanna, 1967). The result of *in vitro* pollination was the same whether the cultures were incubated in light or dark in case of

cruciferous species (Zenkteler, 1969). Lech *et al.* (1994) reported that embryo development in plum after *in vitro* pollination was not influenced by light conditions.

2.4.4 *In vitro* pollination in ginger

In ginger, natural seed set is not observed due to incompatibility reaction operating in style. Valsala (1994) reported seed set and seed germination in ginger for the first time by *in vitro* pollination. The protocol standardized is summarized here.

Flower buds on the day of anthesis and one day prior to anthesis were suitable for *in vitro* pollination. Surface sterilization of flower buds was done before 3.00 pm. It consisted of dipping the flower buds in streptocycline solution (500 mg l^{-1}) for one h followed by wiping with 70 per cent alcohol and rinsing with mercuric chloride (0.1 %) for three minutes. *In vitro* pollination could be done by 5.30 pm for easy scooping out of pollen grains from anthers. Placental, modified placental and ovular pollination alongwith pollen germination medium (modified ME_3 medium) resulted in fertilization. Placental pollination was the best method of pollination as evidenced by maximum number of seeds per culture. Seed set and development could be obtained in the medium of ½ MS + NAA 0.5 mg l^{-1} + BAP 2.5 mg l^{-1} with six per cent sucrose and supplemented with coconut water (15 % v/v). The effect of BAP could be replaced by kinetin 2.0 mg l^{-1} or 2ip 2.5 mg l^{-1}. The effect of NAA could be replaced by 2,4-D 0.05 mg l^{-1} or IAA mg l^{-1}. The effect of coconut water could be replaced by casein hydrolysate 500 mg l^{-1} or inflorescence extract 0.3 per cent. The *in vitro* produced seeds of ginger germinated when 80 days old seeds were inoculated initially in the medium of ½ MS + 2,4-D 8 mg l^{-1} for two months and then in ½ MS + BAP 9.0 mg l^{-1} + 2,4-D 0.2 mg l^{-1}.

Nazeem *et al.* (1996) reported the protocol for rapid multiplication of *in vitro* germinated ginger seedlings.

2.4.5 *In vitro* pollination in turmeric

In turmeric, improvement of medium and long duration types is difficult due to lack of natural seed set. Renjith (1999) obtained seed set in turmeric by *in vitro* pollination. The medium of ½ MS + BAP 1.0 mg l^{-1} + kinetin 1.0 mg l^{-1} + NAA 0.5 mg l^{-1} with three per cent sucrose was found to be the best for ovary and ovule development. Organic supplements coconut water (15 % v/v) and casein hydrolysate (200 mg l^{-1}) enhanced the development. Intra ovarian, placental and modified placental methods of *in vitro* pollination were found to be suitable.

2.4.6 Seed germination

2.4.6.1 Germination of *in vivo* produced seeds

Germination of cardamom seeds improved significantly after acid treatment (nitric acid 25 per cent for 10 minutes) and continuous water washing for 24 days. Above 85 per cent germination could be obtained within 90 days after treatment and plating on moist filter paper (Choudhary and Chandel, 1995).

Korikanthimath and Mulge (1998) found acid treatment (nitric acid 20 % for 10 minutes), gibberellic acid (GA_3 100 mg l^{-1} for 12 h) and Planofix (NAA 75 mg l^{-1} for 12 h) increased germination of cardamom (cv malabar) seeds. Higher doses of growth regulators decreased germination.

Cantos *et al.* (1998) reported a promising method for improvement of germination of *Juniperus oxycedrus* wherein isolated embryos incubated *in vitro* on 1/3 MS medium with three per cent sucrose and GA_3 0.5 g l^{-1} reached germination levels of about 50 per cent while seeds without testa showed only 12 per cent germination *in vitro*. Intact seeds did not germinate.

Raina (1984) tackled the problem of lack of seed set in *Triticum durum* X *Secale cereale* crosses by culturing isolated hybrid embryos *in vitro*, from the

highly shriveled hybrid karyopses. The embryos placed on Taira and Larter's (1978) modified Norstog medium germinated *in vitro* into viable seedlings.

2.4.6.2 Germination of *in vitro* produced seeds

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

Seeds of *Antirrhinum majus* produced by *in vitro* pollination germinated in basal medium (Nitsch, 1951) with four per cent sucrose (Usha, 1965).

In vitro produced seeds of *Petunia* germinated on moist filter paper and on nutrient agar medium on which it was developed. The germination was more on moist filter paper (Rangaswamy and Shivanna, 1967).

In vitro produced seeds of *Nicotiana tabacum* varied in their time taken for germination and sometimes the seeds dried out and passed to dormancy (Balatkova *et al.*, 1976).

The karyopses of maize produced *in vitro* were dried at room temperature for several days and then made to germinate on moist filter paper at 28°C. Germination occurred within seven days of incubation (Gengenbach, 1977).

2.5 Ovule culture and ovary culture

The technique of *in vitro* pollination consists of culture of ovules or ovaries after pollination, for the development of seeds. Even though *in vitro* pollination would help to induce seed set, the later development of the seeds may be limited by various unknown factors. For successful *in vitro* culture of the gynoecium, the knowledge of culture of ovules and ovaries would be helpful to manipulate the conditions for its development. Hence, the available literature on ovule culture and ovary culture is reviewed here.

2.5.1 Ovule culture

The first successful culture of ovules was reported by Maheshwari (1958) in *Papaver rhoeas*. The ovules were cultured on Nitsch (1951) medium containing vitamins, kinetin (0.4 mg l^{-1}) and IAA (5.0 mg l^{-1}).

Maheshwari and Lal (1961) studied the response of ovules of *Papaver somniferum* cultured on Nitsch medium six days after pollination, to various hormones and media supplements. Casein hydrolysate promoted the growth and differentiation of the embryo. Yeast extract hastened differentiation but the mature embryo produced therein was smaller than that in casein hydrolysate. Kinetin enhanced the differentiation process, but checked the elongation of the embryo, while GA and IAA did not stimulate the embryo.

Raising mature seeds by culturing ovules containing globular or older embryo is comparatively easier and has been reported by several workers (*Gynandropsis* and *Impatiens* -- Chopra and Sabharwal, 1963; *Nicotiana tabaccum* – Duliew, 1966; *Allium cepa* – Guha and Johri, 1966).

Beasley and Ting (1974) reported that exogeneous plant growth regulators could substitute pollination, fertilization and subsequent embryo growth in cotton. Isolated unfertilized immature ovules enlarged in presence of kinetin, enlarged and produced fibres in presence of IAA or GA or both. In case of fertilized ovules of cotton, GA induced marked stimulation of fibre production while kinetin and ABA induced a marked inhibition (Beasley and Ting 1973).

Spiegel-Roy *et al.* (1985) obtained normal embryos and seedlings by culturing abortive ovules and seeds of three cultivars of grape on Nitsch medium and orchid agar medium supplemented with hormones. The best medium was Nitsch medium with IAA 10^{-5} M and GA_3 10^{-6} M.

The technique of ovule culture is useful in interspecific and intergeneric crosses, wherein the hybrid embryos abort in the developing seeds. This is of help in the crosses wherein the technique of embryo rescue fails to support the embryo development beyond heart or torpedo-shaped stages.

Subhashini *et al.* (1985) cultured ovules from the incompatible cross *Nicotiana glutinosa* x *Nicotiana megalosiphon* seven days after pollination. One seed germinated 15 days after inoculation in Nitsch medum. The other non-germinated ovules produced callus on MS medium with NAA 6.0 mg l^{-1} and BAP 2.0 mg l^{-1}. The hybridity of the callus was confirmed by cytological studies. Shooting was induced from callus by subculturing on MS medium with IAA 2.0 mg l^{-1} and kinetin 2.0 mg l^{-1}. Rooting of shoots was obtained with IAA 3.0 to 5.0 mg l^{-1} and kinetin 2.0 mg l^{-1}.

Kumlehn *et al.* (1997) produced seedlings by culturing fertilized ovules of wheat (*Triticum aestivum* L.) on modified OC medium (Kumlehn and Nitzsche, 1995) with 85.5 mg l^{-1} sucrose, 80.0 mg l^{-1} NH_4NO_3 and 0.5 mg l^{-1} kinetin. The developed embryos were isolated from the ovules three weeks after inoculation and cultured on B5 medium (Gamborg *et al.*, 1968) with organic acids of Kao and Michayluk (1975), casein hydrolysate 250 mg l^{-1}, xylose 250 mg l^{-1}, IBA 0.5 mg l^{-1} and thidiazuron 0.1 mg l^{-1} for regeneration.

Baranski (1996) reported *in vitro* gynogenesis in red beet (*Beta vulgaris* L.) by culturing ovules on MS and N_6 media supplemented with thiamine 1.0 mg l^{-1}, pyridoxine 0.5 mg l^{-1} and nicotinic acid 0.5 mg l^{-1} with hormones. Ovule response was highest in N_6 medium with IAA 0.5 mg l^{-1} and BA 0.2 mg l^{-1}.

Roh *et al.* (1996b) produced interspecific hybrids of *Lilium longiflorum* and *L. xelegans* by ovule culture. Carpels were cultured on MS medium with combinations of NAA and kinetin after pollination. MS medium with NAA 0.5 mg l^{-1} and kinetin 3.0 mg l^{-1} produced maximum number of plantlets.

Several workers have utilized the ovule culture technique to support the developing hybrids resulting from interspecific crosses.

Kristiansen and Vainstein (1995) studied methods of production of hybrids in the genus *Alstroemeria* and obtained viable hybrids using ovule culture. Early post-fertilization barriers were also overcome in various interspecific crosses in the genus *Alstroemeria* utilizing five species from Chile and two species from Brazil using ovule culture (DeJeu and Jacobsen, 1995). Lu and Bridgen (1996) reported ovule culture procedure for rescue of hybrid embryos resulting from 10 incompatible interspecific crosses of *Alstroemeria*.

Roh *et al.* (1996a) reported rescue of interspecific hybrids between *Lilium longiflorum* and *L. callosum* using ovule culture. Niimi *et al.* (1996) produced hybrids of *Lilium regale* and *L. rubellum* using ovule culture for introducing the vigorous growth and disease resistance characters from *L. regale* to *L. rubellum*.

Rhee *et al.* (1997) studied embryo development in more than 110 cross combinations of different cultivars of *Brassica campestris*, *B. campestris subsp. chinensis*, *B. juncea*, *B. oleracea* and *Raphanus sativus* using ovule culture. Inomata (1996) described methods of overcoming interspecific and intergeneric barriers in *Brassica* and its wild relatives, through embryo culture, ovule culture and ovary culture.

2.5.2 Ovary culture

The technique of ovary culture was developed by Nitsch (1951). Ovaries of *Lycopersicon esculentum* and *Cucumis angularis* were cultured two days after pollination on a simple medium containing mineral salts, trace elements and five per cent sucrose. The fruits developed *in vitro* were smaller than the natural ones, but they contained viable seeds.

Sachar and Kanta (1958) cultured ovaries of *Tropaeolum majus* two days after pollination on Nitsch basic medium containing vitamins, glycine and

thiamine. The fruit size and embryo growth were inferior as compared to that in natural condition.

Maheshwari and Lal (1958) successfully cultured the ovaries of *Iberis amara* one day after pollination on Nitsch basic medium with Whites vitamins. Additions of IAA to this medium produced fruits even larger than those formed in nature. The retention of calyx was essential for the normal development of fruits.

Guha and Johri (1966) cultured ovaries of *Allium cepa* on Nitsch medium with five per cent sucrose. The maximum ovary growth was observed when this medium was supplemented with GA, IAA and kinetin, simultaneously. The seed set was the maximum in medium containing tryptophan followed by that containing IAA. Here also, removal of perianth prior to culture retarded the ovary growth markedly.

Johri and Sehgal (1966) could produce fruits larger than the natural size by culturing ovaries of *Anethum graveolens* on Whites medium three days after pollination.

Bajaj and Collins (1968) cultured pollinated and unpollinated flowers of the cultivated strawberry (*Fragaria ananassa* D.) on White's medium (1943) and studied the effects of hormones, light and retention or removal of calyx on *in vitro* fruit development. In the pollinated flowers, the shape, size and growth of the fruits depended on the number of fertilized ovules while removal of calyx prior to culture reduced the fruit size. Ripening was hastened by GA five to 10 mg l^{-1} as well as by high light intensity. IBA or NAA slightly increased the fruit size at five to 10 mg l^{-1} but higher concentrations were inhibitory. In the unpollinated flowers, GA induced some growth but the fruit development was not complete.

Gong *et al.* (1995) produced interspecific hybrids of *Brassica campestris* and *Sinapis alba* by ovary culture. Growth regulators were not indispensable for development of ovaries but IAA 1.5 to 2.0 mg l^{-1} increased the rate of development

of ovaries and increased the yield of hybrid seeds, while BA 0.5 to 2.0 mg l^{-1} reduced it.

Bhat and Sarla (1996) produced hybrids of *Brassica nigra* and *B. campestris* using sequential ovary-ovule culture.

Ewald (1996) utilized ovary culture technique to produce interspecific hybrids from *Cyclamen persicum* and *C. purpurascens*.

2.6 Micropropagation in *Kaempferia spp.*

Micropropagation has been successfully attempted in *Kaempferia* by several workers. The media and hormonal combinations reported in micropropagation may prove to be important clues regarding culture establishment of *Kaempferia* ovaries and ovules after *in vitro* pollination. Hence, the literature on micropropagation of *Kaempferia spp.* is reviewed here.

Seeni (1990) obtained regenerated plants from *Kaempferia galanga*.

Vincent *et al.* (1991) regenerated plantlets from callus cultures of *K. galanga*.

Vincent *et al.* (1992b) reported successful micropropagation of *Kaempferia galanga* L. They reported direct shoot proliferation from axillary buds isolated from rhizomes, cultured on MS medium. Highest number of shoots was obtained 120 days after incubation on MS medium with 13.9 µM kinetin and 2.2 µM BA with three per cent sucrose. Healthy roots were induced with the multiplication of shoots without subculturing on separate rooting medium. Best rooting was observed on shoot multiplication medium containing 13.9 µM kinetin and 2.2 µM BA with three per cent sucrose.

Vincent *et al.* (1992a) successfully standardized embryogenesis in *Kaempferia galanga*. Highly embryogenic callus could be produced from

vegetative buds from rhizome explants on MS medium supplemented with 0.5 mg l^{-1} BA and 1.0 mg l^{-1} 2,4-D. The callus subcultured on MS medium supplemented with 0.1 mgl^{-1} BA and 1.0 mgl^{-1} produced globular embryoids, which developed into plantlets when transferred to hormone free medium.

Anand *et al.* (1997) standardized a protocol for the *in vitro* propagation in *Kaempferia galanga* L. MS medium supplemented with 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} IAA proved ideal for both shoot and root induction.

Joseph (1997) reported indirect organogenesis and embryogenesis in *Kaempferia galanga* L. Profuse induction of morphogenic calli was obtained from pseudostem bit explants in ½ MS medium supplemented with 2,4-D and low concentrations of BA. Shoot regeneration was obtained from calli in ½ MS medium supplemented with BA 8.0 mg l^{-1} or kinetin 6.0 mg l^{-1}. Combinations of 2,4-D with lower levels of BA and with silver nitrate (5.0 and 10.0 mgl^{-1}) and activated charcoal (0.1 %) resulted in rhizogenesis. Under dark incubation, numerous embryoids were induced in MS medium supplemented with NAA 3.0 mg l^{-1} and BA 1.0 mg l^{-1} from white embryogenic calli. MS medium with five per cent sucrose was the best medium for maturation and germination of embryoids.

Babu *et al.* (1997) reported a complete micropropagation protocol for *Kaempferia galanga* L. and *K. rotunda* L. including both micropropagation and slow growth systems.

Geetha *et al.* (1997) reported standardization of micropropagation of *Kaempferia spp.* including *K. galanga* and *K. rotunda.*

2.7 Crop improvement in kacholam

There is no promising released variety in kacholam, so far. At present, the cultivation is limited to local cultivars/local selections only. Heterosis breeding is difficult in kacholam due to lack of seed set. Some recent germplasm evaluation

studies show that the available ecotypes from Kerala show variability for morphological characters and economic characters i.e. yield and oil.

Mutation breeding undertaken by Viswanathan *et al.* (1992), Kurian *et al.* (1993) and Kanakamany (1997) revealed that exposing the rhizomes to gamma irradiation from ^{60}Co source could induce variability in kacholam for morphological and economic characters. Attempt made by Ajithmohan (1996) showed that polyploidy is not favourable for kacholam as the resultant plants were of small size and reduced vigour.

2.7.1 Germplasm evaluation and variability studies

Latha (1994) evaluated 10 types of kacholam (five collections, four selections and one irradiated population) for morphological variability and yield. There was considerable amount of variability and scope for selection for the characters as well as for economic characters such as fresh rhizome yield, dry rhizome yield and oil content. Among the types, the fresh rhizome yield ranged from 48.81 to 74.93 g/plant, dry rhizome yield from 10.00 to 19.81 g/plant and the oil content from 1.53 to 3.00 per cent.

Presannakumari *et al.* (1994) evaluated five local types of kacholam and found that they differed significantly in fresh rhizome yield, dry rhizome yield, dry weight recovery, oil content and oil yield.

Presannakumari *et al.* (1997) evaluated 12 types of kacholam and found that fresh rhizome yield ranged from 29.7 to 48.6 g/plant and the dry rhizome yield from 6.5 to 14.5 g/plant among them. The dry matter content ranged from 18.5 to 35.9 per cent while oil content ranged from 1.57 to 3.40 per cent among the types.

Even though there is no promising released variety in kacholam, cultivation of collections from Koothattukulam, Thodupuzha, Varandarapalli, Kalladikode, Ponnukkara, Perumbavoor and Vellanikkara is suggested on *ad hoc* basis (KAU, 1996).

MATERIALS AND METHODS

Materials and methods

The investigations on "*In vitro* pollination in kacholam (*Kaempferia galanga* L.) for seed set" were carried out at the Department of Plantation Crops and Spices and at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during May 2000 to October 2001.

The details regarding the experimental materials used and methodology adopted for conducting various aspects of the study are presented in this chapter.

3.1 Plant materials

Six ecotypes of kacholam collected from the cultivators' field, formed the experimental materials for the present study. The ecotypes represent various kacholam growing areas of Kerala as indicated in table 1.

Table 1. Ecotypes of kacholam under study

Ecotype	District of Kerala	Designated as
Palakkad	Palakkad	V1
Muttungal	Ernakulam	V2
Kuruppallure	Kottayam	V3
Kothamangalam	Ernakulam	V4
Kuruppanthara	Kottayam	V5
Chimmoni	Thrissur	V6

3.2 Variability studies in the kacholam ecotypes

The variability in the selected ecotypes for fresh rhizome yield, dry recovery percentage, dry rhizome yield, oil content, oil yield, oleoresin content

and oleoresin yield was estimated. The crop was raised for this purpose by adopting the package of practices of Kerala Agricultural University (KAU, 1996). The cropping field was prepared and raised beds of 1x1m size were taken at 50 cm spacing. The selected ecotypes viz. V1 to V6 were planted at a spacing of 30 x 25 cm on the raised beds, thus accommodating 12 plants on each bed. Observations were recorded from 12 plants in each ecotype as shown below.

3.2.1 Fresh rhizome yield

The rhizomes were harvested seven months after planting by uprooting individual clumps. The fresh rhizome yield was expressed as g/plant and t ha^{-1}.

3.2.2 Dry recovery percentage

The percent recovery of dry rhizome to fresh rhizome was estimated by drying 500 g of fresh rhizome immediately after harvest until a constant weight was obtained. For quick drying, the rhizomes were chopped into pieces of 2-3 mm thickness.

3.2.3 Dry rhizome yield

Dry rhizome yield per plant and per hectare were calculated for each ecotype using the corresponding dry recovery percentage and were expressed as g/plant and t ha^{-1}.

3.2.4 Estimation of volatile oil

Volatile oil was estimated by water distillation adopting Clevenger trap method as per AOAC (1980) and expressed as percentage. 50 g of powdered sample from each ecotype was used for analysis. The oil production per hectare was calculated from the oil recovery percentage and the dry rhizome yield per hectare.

3.2.5 Estimation of oleoresin

The oleoresin content was estimated by using the Soxhlet extraction apparatus as per AOAC (1980). The solvent used was hexane. Five grams of powdered sample was refluxed with 400 ml of solvent. The solvent was evaporated out from the extract until a constant weight was reached. The oleoresin content was expressed as percentage. The oleoresin production per hectare was calculated from the dry recovery percentage and the dry rhizome yield per hectare.

The quality of the oleoresin was observed by sensory evaluation and scoring technique.

3.3 Investigations for the improvement of flowering season

3.3.1 Influence of date of planting on flowering

Influence of date of planting at monthly interval from 15-05-2001 to 15-07-2001 was studied. Twelve plants were raised in each group for every ecotype, using rhizome bits of 5-10 g and observations on percentage of flowering and the flowering season were recorded. The flowering season was computed by noting the first and the last flowering in each lot.

3.3.2 Influence of seed bit size on flowering

Plants raised from three treatments of seed bit sizes, viz. T_1 (5-10 g), T_2 (20-25 g), and T_3 (biennials from 5-10 g) were compared for flowering. In each treatment, for every ecotype, 12 plants were raised. Observations on percentage of flowering, number of inflorescences per plant, duration of flowering per plant (days) and flowering season were recorded.

3.4 Floral biology and morphology

The floral biology and morphology of the selected ecotypes was studied by observing the plants raised from normal seed bit size of 5-10 g and planted in May 2001. The observations on various characters as shown below were recorded.

i) Crop duration for the initiation of flowering (days)
ii) Duration from initiation of inflorescence to blooming (days)
iii) Blooming period of an inflorescence (days)
iv) Percentage of flowering
v) Number of inflorescences per plant
vi) Number of flowers per plant
vii) Number of flowers per inflorescence
viii) Time of anthesis
ix) Time of anther dehiscence
x) Style length (cm)
xi) Ovary length (mm)
xii) Ovary diameter (mm)
xiii) Number of ovules per ovary
xiv) Length of ovule (µm)
xv) Breadth of ovule (µm)

The stage at which the inflorescence primordium was 0.5 to 1.0 cm was taken as the date of initiation of inflorescence. The length of the style was measured in cm. The length and diameter of the ovary were measured in mm. The length and breadth of the ovules was recorded in µm using a calibrated ocular micrometer.

3.5 Pollen studies

3.5.1 Estimation of pollen fertility

The pollen fertility was estimated using acetocarmine stain. Opened flowers on the day of anthesis were collected and used for the study. The pollen grains were stained with acetocarmine and viewed under a microscope at 40X magnification. All the pollen grains that were well filled and stained were counted as fertile and others as sterile. The means of a sample from five microscopic fields served as a replication. The fertility percentage was calculated using the formula

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

3.5.2 Standardization of medium for pollen germination and tube growth

The *in vitro* pollen germination and pollen tube growth of the six kacholam ecotypes were studied in the following media:

1. Modified ME_3 medium of pH 6 with 12 per cent PEG 4000 (Leduc *et al.*, 1990)
2. Brewbaker and Kwack's (1963) medium
3. Pollen germination medium reported by Rekha (1993)
4. Control (distilled water)

The detailed compositions of these media are given in tables 2, 3 and 4, respectively. The different media were prepared by dissolving the required quantity of chemicals in distilled water. The prepared media were sterilized before use by autoclaving.

The pollen grains were collected from the flowers at the time of anthesis and were incubated in a moisture chamber along with a drop of medium to be tested. The number of germinated pollen grains and the total number of pollen grains were counted on five microscopic fields and the mean germination percentage was calculated. The pollen tube growth attained was measured using a calibrated ocular micrometer.

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

Constituents	Concentration (mg l^{-1})
Macronutrients	
$MgSO_4.7H_2O$	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_2EDTA	7.45
$FeSO_4.7H_2O$	5.55
Micronutrients	
H_3BO_3	50.00
$MnSO_4.H_2O$	16.80
$ZnSO_4.7H_2O$	10.50
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Thiamine	1.00
Pyridoxine	1.00
PEG 4000	120000.00

Table 3. Composition of Brewbaker and Kwack's medium (Brewbaker and Kwack, 1963)

Constituents	Concentration (mg l^{-1})
Sucrose	100000.00
$Ca(NO_3)_2.4H_2O$	300.00
$MgSO_4.7H_2O$	200.00
KNO_3	100.00

Table 4. Composition of medium standardized by Rekha (1993)

Constituents	Concentration (mg l^{-1})
Sucrose	80000.00
H_3BO_3	60.00
Gelatin	10000.00

3.6 *In vitro* pollination

The selected ecotypes were planted in the field in the years 2000 and 2001from May onwards at 15 days interval till 15th July to get continuous supply of flowers for three months from July to September.

3.6.1 Collection of explant

Flowers were collected on the day of anthesis during early hours of the day for *in vitro* pollination. The flowers were scooped out carefully using a fine forceps and a needle-arrow without injuring the ovary.

3.6.2 Surface sterilization

The calyx and corolla of the flowers were removed using a fine forceps. The flowers were immersed in tap water and cleaned carefully to remove the soil particles adhering to the base of the ovary. The flowers were then cut into two parts i.e. upper part having the anther lobes and the lower part having the ovary along with a small portion of the style. These were then transferred to separate flasks containing the surface sterilant viz. Streptocycline 500 mg l^{-1} and kept for one hour and agitated manually at intervals for thorough soaking. Separation of the anther and ovary portions was done to ensure gentle rinsing of anther portion and to avoid loss of pollen grains during washing. This also facilitated vigorous shaking and rinsing of the ovary portion to clean the basal part of the ovary where more soil particles were found adhering.

Ovary portions were wiped with cotton soaked in 70 per cent alcohol. Both anther portions and the ovary portions were then surface sterilized inside the laminar airflow bench using 0.1 per cent mercuric chloride for three minutes. They were rinsed with sterile distilled water thrice to remove traces of the sterilant.

3.6.3 Standardization of culture medium

3.6.3.1 Preparation of media

The chemicals used for preparing the medium were of analytical grade obtained from British Drug House (BDH), Sisco Research Laboratories (SRL), Merck and Sigma.

Standard procedures were adopted (Gamborg and Shyluk, 1981) for the preparation of media. The pH of the medium was adjusted at 5.6 to 5.8 before autoclaving. Semisolid media were prepared by adding good quality agar (0.75 %). Medium was sterilized by subjecting it to a temperature of 121°C at a pressure of

1.06 kg cm^{-2} for 20 minutes in an autoclave (Dodds and Robert, 1982). After sterilization the media were stored in air-conditioned culture room in dark.

3.6.3.2 Management of the bacterial contaminations in the cultures

Heavy bacterial contamination was found to be the major problem in culture establishment. The cultures were observed for the contaminations by bacteria and the most commonly occurring bacteria were isolated on PDA and maintained on nutrient agar medium. In order to identify the most effective antibiotic for the control of bacterial interference, a sensitivity test was done on YEM medium. The antibiotics and chemicals used for the sensitivity test are given in table 6. To facilitate further selection of antibiotics, Gram staining was carried out for the four most commonly occurring bacteria (Schaad, 1992).

3.6.3.3 Culture establishment

MS medium (Murashige and Skoog, 1962) was used at full and half strength for the culture establishment (Table 5). The basal medium was supplemented with various levels of auxins, cytokinins and organic supplements as treatments. The pollinated ovaries were cultured and swelling of ovary and ovules was scored. The cultures were also observed for microbial contaminations.

3.6.3.4 Influence of auxins

The basal medium ½ MS was supplemented with IAA, NAA, IBA and 2,4-D at various concentrations as shown in table 7.

3.6.3.5 Influence of cytokinins

The basal medium ½ MS was supplemented with BA and kinetin at various concentrations, alone and in combinations as shown in table 8.

Table 5. Composition of MS culture medium used for culture establishment (Murashige and Skoog, 1962)

Constituents	Concentration (mg l^{-1})
Macronutrients	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$MgSO_4.7H_2O$	370.00
$CaCl_2.2H_2O$	440.00
$Ca(NO_3)_2.4H_2O$	440.00
Micronutrients	
H_3BO_3	6.200
$MnSO_4$	22.300
$ZnSO_4.7H_2O$	8.600
$Na_2MoO_4.2H_2O$	0.250
$CaSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.830
Na_2EDTA	33.600
Vitamins	
Thiamine.HCl	0.10
Pyridoxine.HCl	0.50
Nicotinic acid	0.05
Niacin	-
Others	
Glycine	2.00
Myo-inositol	100.00
Sucrose	30000.00
pH	5.6-5.8

Table 6. *In vitro* sensitivity of bacteria to antibiotics and other chemicals in culture establishment

Antibiotics / Chemicals	Concentrations (mg l^{-1})
Ambystryn S (streptomycin)	50, 100 and 250
Gentamicin (gentamicin)	50, 100 and 250
Kannamac (kannamycin)	50, 100 and 250
Roscicillin (ampicillin)	50, 100 and 250
Streptocycline (streptomycin + tetracycline)	250
Paraxin 250 (chloramphenicol)	500
Amoxycillin (amoxycillin)	500
Mercuric Chloride	1000
Fytolan (copper oxychloride)	1000 and 2500
Copper Sulphate	10000
Control (YEM)	-

3.6.3.6 Auxin and cytokinin combinations

The basal medium ½ MS was supplemented with auxins and cytokinins in various combinations as shown in table 9.

The seeds obtained from the cultures on the most successful media were examined for seed filling by cutting open and were stained with safranin.

3.6.3.7 Influence of sucrose concentration

The influence of different levels of sucrose (3.0, 6.0, 9.0 and 12.0 %) in ovary and ovule development was studied. The medium of ½ MS + BA 0.5 mg l^{-1} + NAA 3.0 mg l^{-1} was supplemented with different concentrations of sucrose. Placental pollination was followed.

Table 7. Auxin concentrations tried in ovule development

Treatments		
Basal medium		Auxins (mg l^{-1})
½ MS	NAA	0.5 and 1.0
½ MS	IAA	0.2
½ MS	IBA	0.2
½ MS	2,4 –D	0.2 and 1.0

Table 8. Cytokinin concentrations tried in ovule development

Treatments		
Basal medium	Cytokinins (mg l^{-1})	
	BA	Kinetin
½ MS	1.0	-
½ MS	4.0	-
½ MS	-	1.0
½ MS	-	4.0
½ MS	1.0	1.0
½ MS	1.0	3.0

Table 9. Auxin and cytokinins tried in ovule development

Cytokinins (mg l^{-1})		Auxins (mg l^{-1})
BA	Kinetin	
0.5	-	NAA 3.0
0.5	-	NAA 2.0
0.5	-	NAA 1.0
0.5	-	IAA 0.2
0.5	-	IBA 0.2
0.5	-	2, 4 –D0.2
1.0	-	NAA 3.0
1.0	1.0	NAA 0.5
1.0	3.0	NAA 0.5
1.0	3.0	IAA 0.2
1.0	3.0	IBA 0.2
1.0	3.0	2, 4 –D0.2
1.5	1.5	NAA 0.5
2.0	3.0	NAA 0.5
4.0	-	2, 4 –D (0.2) + IAA (0.2)
-	4.0	NAA 0.5
-	4.0	IAA 0.5
-	4.0	IBA 0.2
-	4.0	2, 4 –D 0.2

Basal medium: ½ MS with 3 % sucrose

3.6.3.8 Influence of organic media supplements

The influence of organic supplements such as Casein hydrolysate (CH), coconut water (CW), Yeast extract (YE) and L-glutamine (LG) in ovule development was studied (Table 10). The basal media used was ½ MS + BA 0.5 mg l^{-1}+ NAA 3.0 mg l^{-1} with 3.0 per cent sucrose. Placental pollination was followed.

Table 10. Organic media supplements tried in the ovule development

Supplements	Concentration
Casein Hydrolysate	250 mg l^{-1}
Coconut water	15% v/v
Yeast Extract	250 mg l^{-1}
L-glutamine	100 mg l^{-1}

Basal medium: ½ MS + BA 0.5 mg l^{-1} + NAA mg l^{-1} with 3% sucrose

3.6.5.4 Influence of vitamin supplementation in ovule development

The effect of doubling the vitamin stock of MS medium in ovule development was studied. Three hormone combinations in half MS basal medium were selected for this study. The ovaries after placental pollination were cultured and observed for ovule development.

3.6.6 Standardization of *in vitro* pollination techniques

Different *in vitro* pollination techniques as per Bhojwani and Razdan (1983) were tried for standardizing an appropriate *in vitro* pollination technique

for kacholam. Flowers collected on the day of anthesis were surface sterilized. The ovary or ovules were dissected / exposed using forceps and a scalpel. The pollen grains were scooped out from the anthers using a scalpel and applied on the appropriate portion of the gynoecium as per the type of pollination. A drop of pollen germination medium was applied on the pollinated gynoecium.

The pollination was completed before 2 pm to avoid shrinking of the flowers.

The ovary/ovule after pollination was placed in the culture medium and observations were recorded at intervals for swelling and development.

3.6.6.1 The different pollination techniques tried

3.6.6.1.1 Stigmatic pollination

The pollen grains were applied on the stigma, along with ME_3 pollen germination medium after removal of the corolla.

3.6.6.1.2 Stylar pollination

The style was cut at various levels and pollen grains were deposited on the cut surface of the style along with pollen germination medium.

3.6.6.1.3 Intra ovarian pollination

A cut was made just below the junction of the style and pollination was done on the opened surface of the ovary. Pollen germination medium was deposited on the surface.

3.6.6.1.4 Placental pollination

Placentas with the ovules were exposed by completely peeling the ovary wall and pollination was done on the ovules and placenta.

3.7 Post pollination changes

Observations were recorded at five, ten, 15 and 20 days intervals for the changes / development of ovary / ovules and for contaminations.

3.8 Pollen pistil interaction

Fluorescence microscopy technique (Kho and Baer, 1968, Kho *et al.* 1980) was employed to study pollen germination and tube growth through stigmatic and stylar tissues after *in vivo* and *in vitro* pollination. The pollinated pistils were fixed in FAA (Formalin 10 ml, Acetic acid 10 ml and Ethyl alcohol 8 ml) at various intervals i.e. three, six, nine, 12, 24, 36 and 48 hours after pollination. After 24 hours of fixation, the materials were transferred to glass vials containing 1 N NaOH for 8 h at room temperature in order to soften the tissue. The materials were then washed carefully with distilled water and stained with 0.1 per cent Aniline Blue in 0.1 N K_2HPO_4 for 18 h. after staining, the pistils were mounted on a microscopic slide and observed through a fluorescence microscope and photomicrographs were taken.

3.9 Histological examination of ovules

The ovules were fixed in FAA at various intervals viz. two, five, 10, 25 and 55 days after pollination. The samples were run through alcohol-xylol series. microtomed (Leica, Germany) at 10 µm and slides were prepared following standard procedures of plant microtechnique (Jenson, 1962). The slides were stained by safranin and fast green without prior removal of paraffin (Ma *et al.*, 1993). Permanent slides were prepared using DPX mountant and observed under a microscope (Leica, Germany). Photomicrographs of selected sections were taken.

3.10 Seed germination studies

Seeds developed 20 days after *in vitro* pollination were subjected to both *in vivo* and *in vitro* germination studies. Observations were recorded at ten days intervals up to 60 days.

3.10.1 *In vivo* treatments

Seeds were subjected to various pre-sowing treatments like soaking seeds in water for 24 h and acid scarification (50 % HCl for 5 minutes; 25 % HNO_3 for 10 minutes) followed by water soaking for 24 h and soaking in GA_3 solution (100 mg l^{-1}) for 12 h.

3.10.2 *In vitro* treatments

Seeds were kept on moist filter paper and were incubated on various media for germination. The compositions of the media tried for germination viz. Knudson C medium (Knudson, 1951), Taira and Larter's modified Norstog medium, Tomato embryo culture medium are given in tables 11, 12 and 13, respectively.

Table 11. Composition of Knudson C medium

Constituents	Concentration (mg l^{-1})
$Ca(NO_3)_2.4H_2O$	1000.00
$FeSO_4.H_2O$	25.00
$KI I_2PO_4$	250.00
$MgSO_4.7H_2O$	250.00
$(NH_4)_2SO_4$	250.00
$MnSO_4$	7.50
Sucrose	20000.00
Agar	9000.00

Table 12. Composition of Taira and Larters' modified Norstog medium (Taira and Larter, 1978)

Constituents	Concentration (mg l^{-1})
KH_2PO_4	900.00
KCl	750.00
$MgSO_4.7H_2O$	750.00
$CaCl_2.2H_2O$	750.00
$MnSO_4.H_2O$	3.00
H_3BO_3	0.50
$ZnSO_4.7H_2O$	0.50
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.020
$Na_2MoO_4.2H_2O$	0.030
Fe EDTA	25.00
Meso-inositol	100.00
Thiamine HCl	1.00
Pyridoxine HCl	1.00
Nicotinic acid	1.00
Casein hydrolysate	2500.00
Maleic acid *	500.00
Agar	10000.00
Sucrose	51300.00
pH (before autoclaving)	5.5

*Maleic acid dissolved in 150 ml water, pH adjusted to 5.5 with NaOH before adding to the medium

Table 13. Composition of tomato embryo culture medium (Neal and Topoleski, 1983)

Constituent	Concentration (mg l^{-1})
NH_4NO_3	1601.00
KNO_3	2022.00
$NaH_2PO_4.H_2O$	276.02
$CaCl_4.2H_2O$	441.07
$MgSO_4.7H_2O$	739.50
H_3BO_3	9.28
$MnSO_4.H_2O$	16.90
$ZnSO_4.7H_2O$	11.50
$CuSO_4.7H_2O$	0.375
$Na_2MoO_4.2H_2O$	0.242
$CoCl_2.6H_2O$	0.238
KI	0.830
$FeSO_4.7H_2O$	27.80
Na_2 EDTA	37.22
Sucrose	60000.00
Inositol	20.00
Nicotinic acid	0.50
Pyridoxine	0.50
Thiamine	1.00
Glycine	0.50

Results

The results of the experiments on "*In vitro* pollination in kacholam (*Kaempferia galanga* L.) for seed set" are described in this chapter.

4.1 Variability in the plant materials

The plant materials selected for the study were ecotypes from Palakkad (V1), Muttungal (V2), Kuruppallure (V3), Kothamangalam (V4), Kuruppanthara (V5) and Chimmoni (V6).

4.1.1 Fresh rhizome yield

Yield potential of the selected ecotypes was compared and was expressed as fresh rhizome yield, g/plant and t ha^{-1}. The mean fresh rhizome yield in the six selected ecotypes was 86.40 g/plant and it ranged from 46.87 in Chimmoni to 107.50 g/plant in Kuruppanthara. In the selected lot, Kothamangalam (103.00 g/plant) and Palakkad (98.50 g/plant) can also be considered as high yielders (Table 14). The mean fresh rhizome yield per hectare in the selected ecotypes was 6.63 t ha^{-1} and it ranged from 3.60 in Chimmoni to 8.26 t ha^{-1} in Kuruppanthara.

4.1.2 Dry recovery percentage

The ecotypes showed variation with respect to dry recovery percentage and it ranged from 24.99 in Kothamangalam to 29.15 in Palakkad with a mean of 26.99 per cent. The ecotypes Chimmoni (28.43 %) and Kuruppanthara (27.00 %) recorded higher dry recovery percentage than the mean. Even though the fresh rhizome yield was the lowest in Chimmoni, the dry recovery was high (28.43 %).

Dry rhizome yield ranged from 13.33 g/plant in Chimmoni to 29.03 g/plant in Kuruppanthara with a mean of 23.22 g/plant. Among the lot, Palakkad recorded the second highest dry rhizome yield of 28.71 g/plant. Dry rhizome yield per hectare ranged from 1.02 t ha^{-1} in Chimmoni to 2.23 t ha^{-1} in Kuruppanthara with a

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mean of 1.78 t ha^{-1} (Table 14). The ecotypes Kuruppanthara and Palakkad can be considered as high yielders.

4.1.3 Oil content and oil yield

The selected ecotypes showed variation with respect to quality parameters i.e. oil content, oleoresin and sensory perception of the oleoresin. The oil content ranged from 1.40 per cent in Kothamangalam to 2.40 per cent in Muttungal with a mean of 1.90 per cent. The oil content in Kuruppanthara, the highest fresh and dry rhizome yielder, was high (2.20 %). The oil yield per hectare among the ecotypes ranged from 20.46 kg in Chimmoni to 49.04 kg in Kuruppanthara with a mean of 33.53 kg. Palakkad and Muttungal also recorded more oil yield per hectare than the overall mean (Table 14).

4.1.4 Oleoresin content, yield and quality of oleoresin

The ecotypes showed variation with respect to oleoresin content also. It ranged from 2.66 per cent in Muttungal to 3.71 per cent in Kothamangalam with a mean of 3.21 per cent. The oleoresin recovery was slightly lower in Kuruppanthara (3.12 %), the highest fresh rhizome yielder and higher in Palakkad (3.70 %).

There was striking difference among ecotypes with respect to oleoresin yield. It ranged from 31.42 kg ha^{-1} in Chimmoni to 81.44 kg ha^{-1} in Palakkad with a mean of 58.02 kg ha^{-1}. The oleoresin yield was high in Kothamangalam followed by Kuruppanthara with 73.39 and 69.52 kg ha^{-1} respectively. The quality of oleoresin showed variation with respect to sensory evaluation and the ecotype Kuruppanthara had the most pleasing aroma (Table 14).

The genetic materials identified for high dry rhizome yield were Kuruppanthara and Palakkad. The genotype identified for high dry recovery percentage was Palakkad. Muttungal was identified as genetic material having high oil content. Kothamangalam and Palakkad were identified as materials having high oleoresin content.

Table 14. Variability in kacholam ecotypes with respect to yield and quality characters

Sl. No.	Characters	Ecotypes						Mean
		Palakkad (V1)	Muttungal (V2)	Kuruppallure (V3)	Kothamangalam (V4)	Kuruppanthara (V5)	Chimmoni (V6)	
1.	Fresh rhizome yield (g/plant)	98.50	81.25	81.25	103.00	107.50	46.87	86.40
2.	Fresh rhizome yield (t ha^{-1})	7.56	6.24	6.24	7.91	8.26	3.60	6.63
3.	Dry recovery (%)	29.15	26.59	25.78	24.99	27.00	28.43	26.99
4.	Dry rhizome yield (g/plant)	28.71	21.60	20.94	25.73	29.03	13.33	23.22
5.	Dry rhizome yield (t ha^{-1})	2.20	1.66	1.61	1.98	2.23	1.02	1.78
6.	Oil content (%)	1.60	2.40	1.80	1.40	2.20	2.00	1.90
7.	Oil yield (kg ha^{-1})	35.26	39.79	28.94	27.66	49.04	20.46	33.53
8.	Oleoresin content (%)	3.70	2.66	3.00	3.71	3.12	3.07	3.21
9.	Oleoresin yield (kg ha^{-1})	81.44	44.09	48.26	73.39	69.52	31.42	58.02
10.	Oleoresin quality based on sensory evaluation	++	+	+	++	++++	++	

Scoring: ++++: Excellent, +++: Good, ++: Fair, +: Poor

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4.2 Investigations for the improvement of flowering

4.2.1 Influence of date of planting on flowering

4.2.1.1 Percentage of flowering

The influence of three dates of planting i.e. 15th May, 15th June and 15th July on flowering was compared and the results are presented in table 15. Flowering was observed in all the six ecotypes of kacholam when planted in the month of May. The ecotypes differed in percentage of flowering and a mean 61.11 per cent flowering was observed when planting was done in May. It reduced to 48.61 and further to 23.61 when planting was done in the months of June and July, respectively. The ecotypes Muttungal, Kothamangalam and Chimmoni did not flower at all when planted in the month of July.

4.2.1.2 Flowering season

The influence of date of planting on flowering season is presented in table 16. In May planting, the flowering was from 5th July to 16th August and it got extended up to 5th October by adopting 15th June and 15th July planting. In July planting, the flowering started by 3rd August and in July planting it was by 1st October. The spread of flowering season for 15th May planting was 43 days while it was 40 days for 15th June planting. In July planting, it got reduced to 35 days. It can also be noted that in July planting, three ecotypes did not flower.

4.2.2 Influence of seed bit size and biennial habit on flowering

4.2.2.1 Percentage of flowering

In T_1 (5-10 g), the mean percentage of flowering was 62.49 and in T_2 920-25 g), it was 66.55. The mean percentage of flowering was 62.49 in T_1 while in T_2 it was 66.55. The plants in T_3 (5-10 g, biennial) showed significantly higher

percentage of flowering (87.49) as compared to annuals. In each treatment, the ecotypes varied in the percentage of flowering (Table 17).

Table 15. Influence of date of planting on percentage of flowering in kacholam ecotypes

Ecotypes	Dates of planting		
	15-05-2001	15-06-2001	15-07-2001
	Percentage of flowering		
Palakkad (V1)	83.33	66.66	50.00
Muttungal (V2)	50.00	41.66	0.00
Kuruppallure (V3)	66.66	58.33	50.00
Kothamangalam (V4)	41.66	33.33	0.00
Kuruppanthara (V5)	66.66	58.33	41.66
Chimmoni (V6)	58.33	33.33	0.00
Mean	61.11	48.61	23.61

4.2.2.2 Number of inflorescences per plant

The mean number of inflorescences per plant in T_1 was 1.29 while it was 1.34 in T_2. The plants kept as biennials (T_3) produced more number of inflorescences per plant as compared to the annual plants but the data was not statistically significant.

4.2.2.3 Duration of flowering

The mean duration of flowering was 12.74 days in T_1. In T_2, it was 10.75 days (Table 18). The plants kept as biennials (T_3) showed a significantly longer duration of flowering, with a mean value of 17.85 days.

4.2.2.4 Flowering season

In T_1, the flowering started in the last week of 24th June and ended by 9th August. In T_2, the flowering started by 22nd June and ended 2nd August.

Table 16. Influence of date of planting on flowering season

Ecotype	Dates of planting								
	15-05-2001			15-06-2001			15-07-2001		
	Date of first flowering	Date of last flowering	Duration of flowering (days)	Date of first flowering	Date of last flowering	Duration of flowering (days)	Date of first flowering	Date of last flowering	Duration of flowering (days)
Palakkad	10-07-2001	08-08-2001	29	08-08-2001	08-09-2001	32	07-09-2001	05-10-2001	29
Muttungal	11-07-2001	08-08-2001	28	09-08-2001	05-09-2001	28	-	-	-
Kuruppallure	05-07-2001	08-08-2001	34	03-08-2001	05-09-2001	34	04-09-2001	02-10-2001	29
Kothamangalam	24-07-2001	16-08-2001	24	20-08-2001	11-09-2001	23	-	-	-
Kuruppanthara	05-07-2001	08-08-2001	34	03-08-2001	05-09-2001	34	01-09-2001	30-09-2001	29
Chimmoni	25-07-2001	08-08-2001	14	22-08-2001	05-09-2001	15	-	-	-
Spread of flowering	05-07-2001	16-08-2001	43	03-08-2001	11-09-2001	40	01-09-2001	05-10-2001	35

* Seed bit size: 5-10 g

Table 17. Influence of size of seed material and biennial habit on percentage of flowering

Ecotypes	Percentage of flowering			
	Seed bit size			
	T_1	T_2	T_3	
Palakkad (V1)	100.00	66.00	83.33	
Muttungal (V2)	50.00	50.00	83.33	
Kuruppallure (V3)	66.66	91.66	91.66	
Kothamangalam (V4)	33.33	58.33	100.00	
Kuruppanthara (V5)	83.33	58.33	91.66	
Chimmoni (V6)	41.66	75.00	75.00	CD at 5% level 21.99
Mean	62.49	66.55	87.49	SEm ± 7.296

Table 18. Influence of size of seed material and biennial habit on number of inflorescences per plant

Ecotypes	Number of inflorescences per plant			
	Seed bit size			
	T_1	T_2	T_3	
Palakkad (V1)	1.33	1.33	1.77	
Muttungal (V2)	1.66	1.60	1.22	
Kuruppallure (V3)	1.25	1.27	1.87	
Kothamangalam (V4)	1.25	1.33	1.50	
Kuruppanthara (V5)	1.30	1.16	1.37	
Chimmoni (V6)	1.00	1.37	1.22	
Mean	1.29	1.34	1.49	Non significant

Table 19. Influence of size of seed material and biennial habit on duration of flowering per plant (days)

Ecotypes	Duration of flowering per plant (days)			
	Seed bit size			
	T_1	T_2	T_3	
Palakkad (V1)	13.63	16.16	17.77	
Muttungal (V2)	11.00	7.60	15.33	
Kuruppallure (V3)	13.25	8.18	17.72	
Kothamangalam (V4)	13.00	6.40	18.25	
Kuruppanthara (V5)	15.60	17.20	18.63	
Chimmoni (V6)	10.00	9.00	19.44	CD at 5% level 3.743
Mean	12.74	10.75	17.85	SEm ± 1.242

T_1: 5-10 g seed bit, T_2: 20-25 g seed bit, T_3: 5-10 g seed bit kept as biennial

Table 20. Influence of size of seed material and biennial habit on flowering season

Ecotype	Seed bit size*								
	T_1 (5-10 g)			T_2 (20-25 g)			T_3 (5-10 g; biennial)		
	Date of first flowering	Date of last flowering	Duration of flowering (days)	Date of first flowering	Date of last flowering	Duration of flowering (days)	Date of first flowering	Date of last flowering	Duration of flowering (days)
Palakkad	28-06-2000	31-07-2000	34	24-06-2000	02-08-2000	40	03-06-2001	18-07-2001	46
Muttungal	02-07-2000	27-07-2000	25	11-07-2000	25-07-2000	14	03-06-2001	16-07-2001	44
Kuruppallure	25-06-2000	28-07-2000	34	25-06-2000	31-07-2000	37	08-06-2001	18-07-2001	41
Kothamangalam	16-07-2000	09-08-2000	25	11-07-2000	28-07-2000	17	03-06-2001	19-07-2001	47
Kuruppanthara	24-06-2000	31-07-2000	38	22-06-2000	27-07-2000	36	06-06-2001	20-07-2001	45
Chimmoni	17-07-2000	02-08-2000	17	05-07-2000	02-08-2000	28	03-06-2001	16-07-2001	44
Spread of flowering	24-06-2000	09-08-2000	47	22-06-2000	02-08-2000	42	03-06-2001	20-07-2001	48

* Date of planting: 05-05-2000

In T_3, the flowering started early, in the first week of June (03-06-2001), with the receipt of summer showers and ended at the end of third week of July (20-07-2001). The spread of the flowering season in T_1 was 47 days while in T_2 it was 42 days. In T_3, it was 48 days (Table 20).

This experiment revealed that the treatment effect of T_1 and T_2 was almost the same while early flowering could be obtained in kacholam by planting normal size seed bit (5-10 g) and maintaining that crop as biennial.

4.3 Study of floral biology and floral morphology

4.3.1 Floral biology of kacholam

The crop duration from planting to the initiation of flowering ranged from 48.0 (Kuruppanthara) to 68.5 (Kothamangalam) days with a mean of 57.5 days (Table 21). Kuruppanthara, Kuruppallure and Palakkad can be considered as early flowering (48-51 days), Kuruppanthara being the earliest to flower. Kothamangalam (68.5 days) was the latest to flower. Muttungal and Chimmoni (61.5 to 66 days) can also be considered as late flowering types.

The inflorescence primordium of 0.5 to 1.0 cm length developed into a blooming inflorescence within a mean period of 13.58 days. The blooming period of an inflorescence was completed within a mean period of 11.75 days and ranged from 9.50 days (Chimmoni) to 16.40 days (Kuruppanthara).

The time of anthesis in all selected ecotypes except Palakkad was 4 to 4.30 am while in Palakkad it was 4.30 to 5.00 am. The anther dehiscence occurred shortly after anthesis. In Palakakd, it occurred between 4.45 am and 5.15 am, while in all the other ecotypes it occurred between 4.30 am and 5.00 am.

Table 21. Floral biology and morphology of kacholam ecotypes

Sl. No.	Characters	Ecotypes						Mean
		Palakkad (V1)	Muttungal (V2)	Kuruppallure (V3)	Kothamangalam (V4)	Kuruppanthara (V5)	Chimmoni (V6)	
1.	Crop duration for the initiation of flowering (days)	51.0	61.5	50.0	68.5	48.0	66.0	57.5
2.	Duration from initiation of inflorescence to blooming (days)	13.0	13.5	13.4	14.0	13.4	14.2	13.58
3.	Blooming period of an inflorescence (days)	14.9	9.3	10.7	9.7	16.4	9.5	11.75
4.	Time of anthesis (am)	4.30-5.00	4.00-4.30	4.00-4.30	4.00-4.30	4.00-4.30	4.00-4.30	-
5.	Time of anther dehiscence (am)	4.45-5.15	4.30-5.00	4.30-5.00	4.30-5.00	4.30-5.00	4.30-5.00	-
6.	Percentage of flowering	83.00	50.00	79.16	45.83	70.83	58.33	64.53
7.	No. of inflorescence per plant	1.33	1.63	1.26	1.29	1.23	1.84	1.43
8.	No. of flowers per plant	7.505	5.21	4.967	6.13	7.60	4.89	6.05
9.	No. of flowers per inflorescence	7.30	4.00	8.00	8.20	11.00	7.00	7.58
10.	Style length (cm)	4.53	4.75	4.59	4.36	4.30	4.64	4.52
11.	Ovary length (mm)	4.10	4.30	4.30	4.20	4.00	3.80	4.11
12.	Ovary diameter (mm)	2.50	2.70	3.30	2.60	2.70	2.30	2.68
13.	No. of ovules per ovary	22.00	20.40	20.80	19.60	18.60	18.60	20.00
14.	Length of ovule (µm)	86.00	98.00	114.00	102.00	82.00	96.00	96.33
15.	Breadth of ovule (µm)	61.00	62.00	58.00	61.00	65.00	60.00	61.16

The percentage of flowering (number of plants flowered) ranged from 45.83 in Kothamangalam to 83.00 in Palakkad with a mean of 64.53. The number of inflorescence per plant ranged from 1.23 in Kuruppanthara to 1.80 in Chimmoni with a mean of 1.43. Each plant produced one or two inflorescences with a mean value of 1.43 per plant. An inflorescence contained 2 to 13 flower buds with a mean value of 6.05, which opened generally one or two per day, starting from the outer ones. The number of flowers per plant ranged from 4.89 in Chimmoni to 7.51 in Palakkad with a mean of 6.05. The number of flowers per inflorescence ranged from 4.0 in Muttungal to 11.0 in Kuruppanthara with a mean of 7.58 (Table 21).

4.3.2 Floral morphology of kacholam

The inflorescence of kacholam is a scape covered by a leathery sheath, directly arising from the rhizome. Each flower is subtended by a bract and one or two bracteoles (Plate 1a, 1b). Flowers are bisexual, complete trimerous and zygomorphic (Plate 2a, 2b). Perianth is connate at the base forming a long tube and free at the apex. The stamens are arranged in two whorls. The outer whorl of stamens is represented by two staminodes, which are situated at the base of the tubular perianth. The posterior stamen of the inner whorl is the only fertile one. The other two stamens are united and form a large bilobed showy labellum, which is the most conspicuous part of the flower. The fertile stamen is bilobed and the connective tissue forms a hood above the stamen. The anther lobe forms a groove through which style passes so that the stigma comes very close to the anther. Gynoecium consists of tricarpellary syncarpous inferior ovary with 17 to 21 ovules arranged in axile placentation. The style is long, about four cm in length and ends in a spiny stigma. The mean style length in kacholam ecotypes was found to be 4.52 cm. The ovary measurement of the ecotypes had a mean length of 4.11 mm and mean diameter of 2.68 mm in the middle. The mean number of ovules per ovary recorded in the ecotypes was 20.00. Microscopic measurement of ovule with ocular micrometer showed a mean length of 96.33 µm and a mean breadth of 61.16 µm at the middle (Table 21).

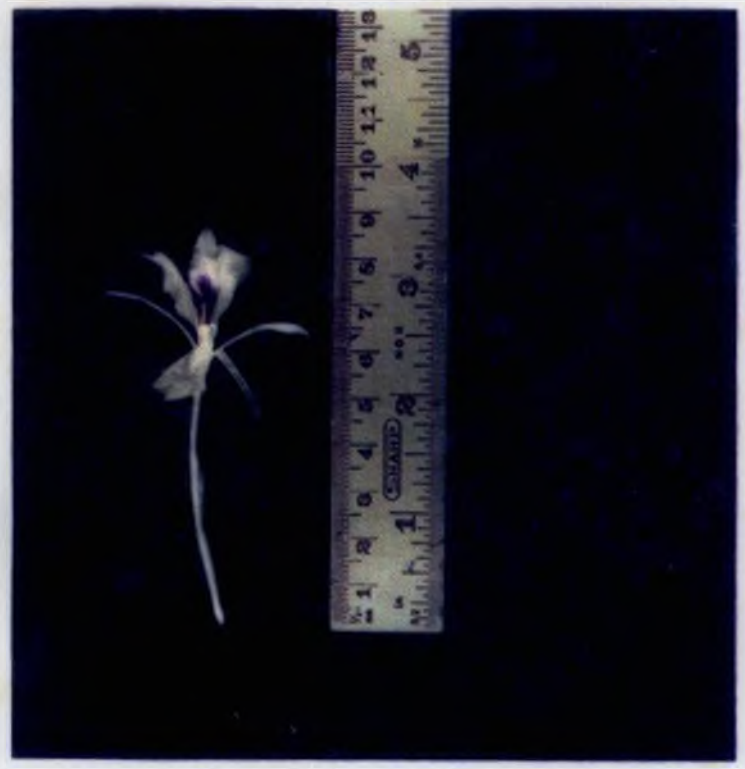
Plate 1

1a. Kacholam (*Kaempferia galanga* L.) plant

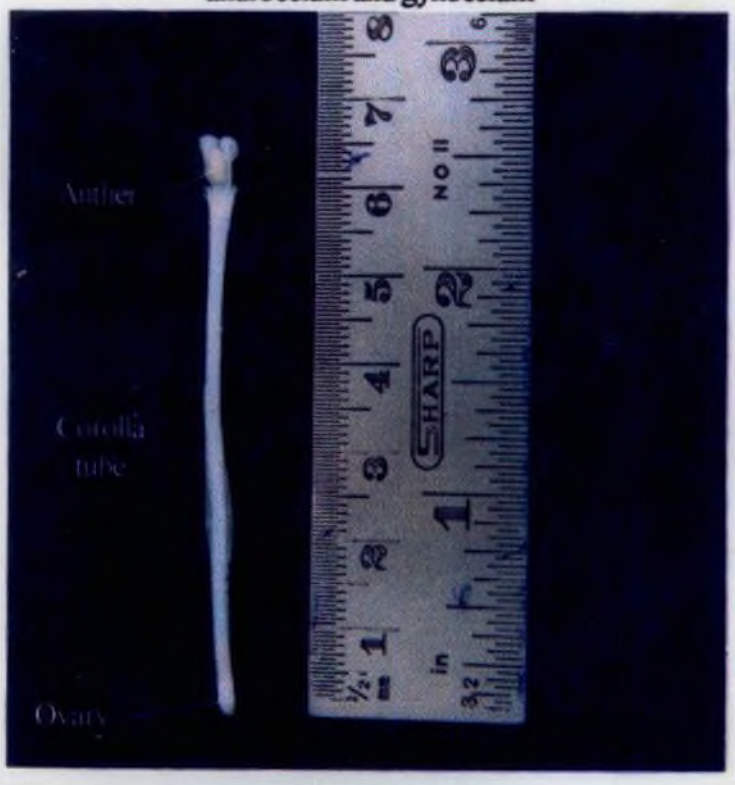
1b. Kacholam plant in flowering

Plate 2

2a. Flower of *Kaempferia galanga*



2b. Flower of *Kaempferia galanga* with androecium and gynoecium



4.4 Pollen studies

4.4.1 Pollen morphology

Kacholam flowers have sufficient pollen grains in the anthers. The pollen grains were spherical in shape, highly homogeneous in size and white in colour.

4.4.2 Pollen fertility in kacholam ecotypes

Pollen fertility was determined using acetocarmine stain. It ranged from 64.96 per cent to 86.30 per cent among the ecotypes with a mean value of 76.33 per cent (Table 22). The ecotype Chimmoni recorded maximum pollen fertility of 86.3 per cent followed by Palakkad (78.5 %) and Kuruppanthara (77.7 %). The ecotype Kothamangalam recorded the lowest fertility (64.96 %).

4.4.3 Standardization of medium for pollen germination and pollen tube growth

The pollen grains incubated in different media were observed for germination after 24 h of incubation and the data are presented in table 23. Pollen grains germinated in ME_3 medium, in Brewbaker and Kwack's medium and in the medium standardized by Rekha (1993). There was no pollen germination in distilled water. The mean germination percentage was the highest in ME_3 medium (78.79 %) followed by Brewbaker and Kwack's medium (67.57 %) and Rekha's medium (67.31 %).

The ecotypes varied in the pollen germination percentage among the media tried (Figure 1). In ME_3 medium, Palakkad recorded the highest germination percentage of 87.58 per cent while Kothamangalam the lowest (68.85 %). In Brewbaker and Kwack's medium Kuruppallure recorded the highest germination of 70.25 per cent while Kothamangalam the lowest (62.20 %). In Rekha's medium, Palakkad recorded the highest (75.03 %) germination while Kuruppallure the lowest (50.60 %).

Table 22. Pollen fertility in kacholam ecotypes

Ecotypes	Palakkad (V1)	Muttungal (V2)	Kuruppallure (V3)	Kothamangalam (V4)	Kuruppanthara (V5)	Chimmoni (V6)	Mean
Pollen fertility (%)	78.5	76.73	73.79	64.96	77.70	86.30	76.33

Table 23. Pollen germination percentage of kacholam ecotypes in various media 24 h after incubation

Sl. No.	Treatments	Pollen germination (%)						Mean
		Ecotypes						
		Palakkad (V1)	Muttungal (V2)	Kuruppallure (V3)	Kothamangalam (V4)	Kuruppanthara (V5)	Chimmoni (V6)	
1.	ME_3 medium	87.58	80.36	82.06	68.85	70.96	82.97	78.80
2.	Brewbaker and Kwack's medium	69.90	66.38	70.25	62.20	66.97	69.76	67.58
3.	Rekha's medium	75.03	68.27	50.60	68.62	71.92	69.44	67.31
4.	Distilled water	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 24. Pollen tube length of kacholam ecotypes in various media 24 h after incubation

Sl. No.	Treatments	Pollen tube length (µm)						Mean
		Ecotypes						
		Palakkad (V1)	Muttungal (V2)	Kuruppallure (V3)	Kothamangalam (V4)	Kuruppanthara (V5)	Chimmoni (V6)	
1.	ME_3 medium	826.0	819.0	802.0	766.0	794.0	848.0	809.17
2.	Brewbaker and Kwack's medium	768.0	758.0	758.0	722.0	718.0	826.0	758.33
3.	Rekha's medium	762.0	774.0	770.0	774.0	768.0	746.0	765.67
4.	Distilled water	0.0	0.0	0.0	0.0	0.0	0.0	0.0

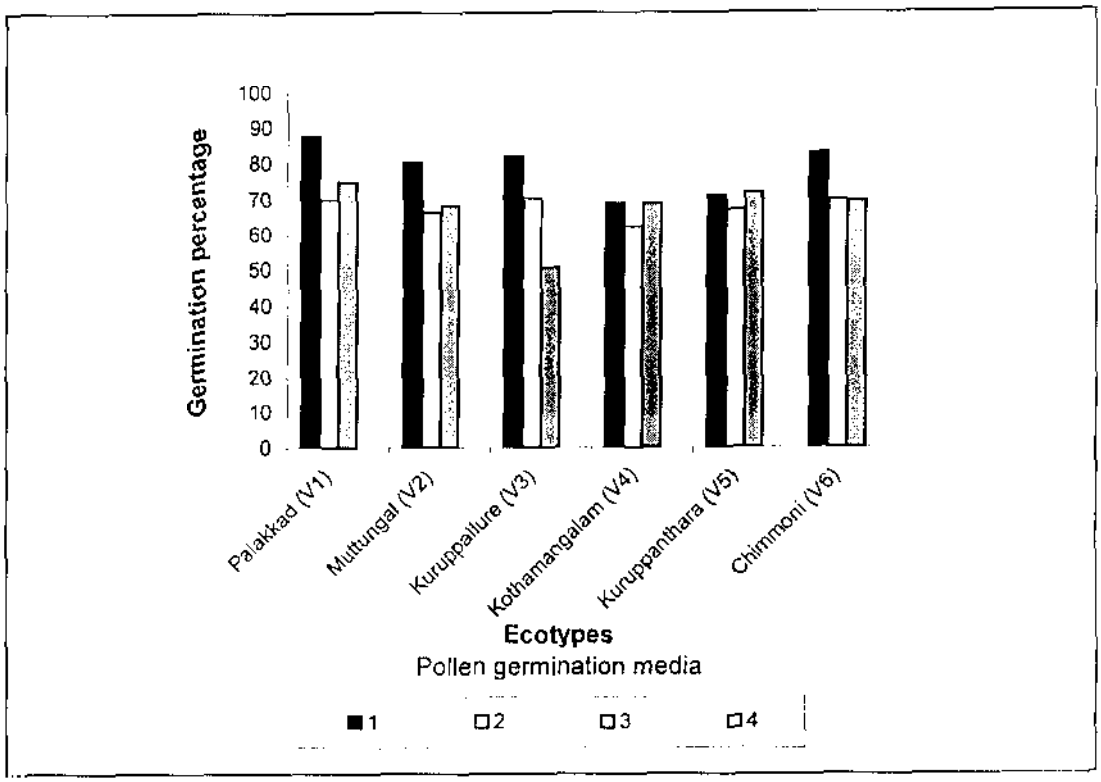


Fig.1. Pollen germination percentage of kacholam ecotypes in various media 24h after incubation

Pollen germination media:

1. ME_3 medium
2. Brewbaker and Kwack's medium
3. Rekha's medium
4. Control (Distilled water)

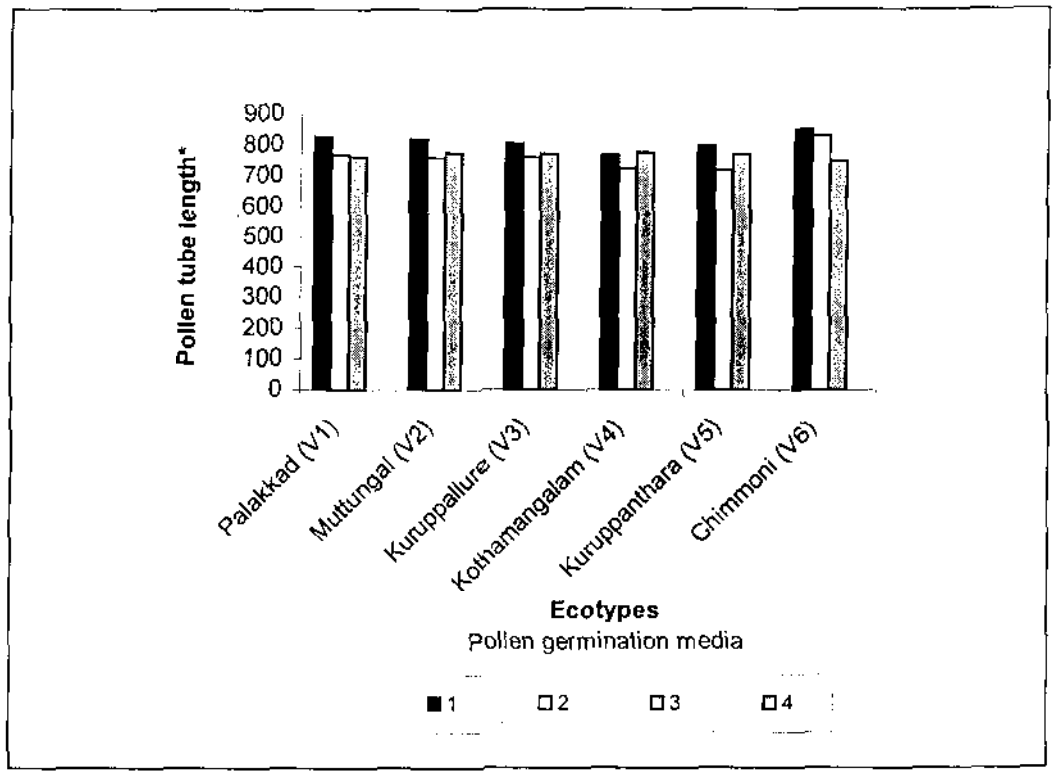


Fig.2. Pollen tube length of kacholam ecotypes in various media 24h after incubation

Pollen germination media:

1. ME_3 medium
2. Brewbaker and Kwack's medium
3. Rekha's medium
4. Control (Distilled water)

* Pollen tube length in μm

The ecotypes varied in the pollen tube growth among the media tried (Figure 2). The pollen grains germinated after 24h were observed for pollen tube growth (Table 24). The mean pollen tube growth was observed to be the maximum in ME_3 medium (809.16 µm) followed by that in the Rekha's medium (765.66 µm) and the minimum in Brewbaker and Kwack's medium (758.33 µm).

In ME_3 medium, Chimmoni recorded maximum pollen tube length of 848.0 µm and the minimum (776.0 µm) was recorded in Kothamangalam. In Brewbaker and Kwack's medium, Chimmoni recorded the maximum pollen tube length of 826.0 µm while Kuruppanthara the minimum (718.0 µm). In Rekha's medium, Muttungal recorded the maximum pollen tube length of 774.0 µm while Chimmoni recorded the minimum (746.0 µm).

4.5 *In vitro* pollination

4.5.1 Standardization of media

4.5.1.1 Basal medium for culture establishment

To decide the medium for initial *in vitro* establishment, surface sterilized ovaries with placental pollination, were incubated on two basal media i.e. half strength MS and full strength MS with three per cent sucrose and two hormone combinations BA 0.5 mg l^{-1} + NAA 3.0 mg l^{-1} and 2,4-D 0.2 mg l^{-1}. The ovules developed in both media but half MS medium was superior to full MS (Table 25).

4.5.2 Management of bacterial contamination in the cultures

Gram staining was carried out for the four most commonly occurring bacteria as per Schaad (1992). The general observations on these four types of bacteria isolated from the cultures are given in table 26. Among them, the white coloured gram negative bacterium was found most frequently and destroyed the cultures.

Table 25. Influence of basal media in the ovule development of kacholam after *in vitro* pollination

Sl. No.	Treatments*	Ovule swelling	Percentage of cultures showing ovule development **
1.	½ MS + BA 0.5 + NAA 3.0	+++	83.33
2.	MS + BA 0.5 + NAA 3.0	++	72.22
3.	½ MS + 2,4-D 0.2	+++	70.55
4.	MS + 2,4-D 0.2	++	66.66

* Hormone concentrations in mg l^{-1}
** Average of 18 observations 20 DAP; *In vitro* placental pollination
Scoring: +++: good, ++: moderate, +: low

Table 26. Observations on the most common bacteria in culture establishment

Colony	Colour	Gram test	Remarks
1	White	Negative (-)	Most common, slimy, covered entire medium surface
2	Offwhite	Positive (+)	Slimy, covered entire medium surface
3	Yellow	Positive (+)	Watery to slimy, growth nearby the explant
4	Pink	Positive (+)	Slimy, growth nearby the explant

The *in vitro* sensitivity of bacteria to seven antibiotics and three chemicals at different concentrations was studied. Seven antibiotics viz. Ambystryn S (streptomycin), Roscicillin (ampicillin), Kannamac (kannamycin), Gentamicin, Streptocycline, Paraxin 250 (chloramphenicol), amoxycillin and the fungicides viz. mercuric chloride, Fytolan (copper oxychloride) and copper sulphate were tested in this study. The results (Table 27) showed that the different types of bacteria varied in their response to the treatments. In general, the white and the pink coloured bacteria were difficult to suppress with the antibiotics and the chemicals tried, but the yellow coloured bacteria were completely suppressed by all the treatments at all the concentrations tried. In the white and the pink coloured bacteria, higher concentrations of antibiotics resulted in suppression of proliferation as compared to the lower concentrations.

Table 27. *In vitro* sensitivity of bacteria to antibiotics/chemicals in culture establishments

Treatments		Scoring for bacterial growth		
Antibiotic/chemical	Concentration (mg l^{-1})	White	Yellow	Pink
Ambystryn S (streptomycin)	50	++++	-	++++
	100	++	-	++
	250	++	-	++
Roscicillin (ampicillin)	50	+++	-	+++
	100	++	-	++
	250	++	-	+
Kannamac (kannamycin)	50	+++	-	+++
	100	++	-	++
	250	++	-	-
Gentamicin (gentamicin)	50	+++	-	+++
	100	+++	-	++
	250	++	-	++
Streptocycline (streptomycin + tetracycline)	250	+++	-	+++
Paraxin 250 (chloramphenicol)	500	++++	-	+++
Amoxycillin (amoxycillin)	500	+++	-	+++
Mercuric chloride	1000	++	-	-
Phytolan (copper oxychloride)	1000	++	-	++
	2500	-	-	-
Copper sulphate	10000	++	-	++
Control (YEM)	-	+++	+++	+++

Scoring: ++++: Full growth, +++: Growth, ++: Moderate growth,
+: Suppressed growth, -: Complete suppression

Fytolan (copper oxychloride) completely suppressed all the three types of bacteria at 2500 mg l^{-1}. The white bacteria were completely suppressed in Fytolan (2500 mg l^{-1}) whereas showed moderate (++) to full (++++) growth in the other treatments. The pink bacteria were completely suppressed in Kannamac (250 mg l^{-1}), mercuric chloride (1000 mg l^{-1}) and Fytolan (2500 mg l^{-1}) whereas showed suppressed (+) to full (++++) growth in the other treatments.

4.5.3 Influence of auxins in ovule development

The results (Table 28) showed that auxins induced ovule development in the basal medium of ½ MS with 3 per cent sucrose. 2,4-D 0.2 to 1.0 mg l^{-1} resulted in good to moderate ovule swelling in 70.55 and 55.33 per cent of cultures (Plate 3a). NAA 0.5 to 1.0 mg l^{-1} induced low ovule swelling in 27.77 to 44.44 per cent of cultures. Other auxins IAA and IBA (each at 0.2 mg l^{-1}) caused low ovule swelling in 61.11 and 55.55 per cent of cultures.

4.5.4 Influence of cytokinins in ovule development

The results of the investigations on the influence of cytokinins on ovule development (Table 29) showed that both BA and kinetin favoured ovule development individually as well as in combinations in the basal medium of ½ MS with three per cent sucrose.

BA 1.0 to 4.0 mg l^{-1} favoured low to moderate ovule development in 47.05 to 70.58 per cent of the cultures while kinetin 1.0 to 4.0 mg l^{-1} was found to favour low ovule development in 44.44 to 61.11 per cent cultures. The combination of BA and kinetin each at 1.0 mg l^{-1} resulted in ovule development with good ovule swelling in 72.22 per cent cultures (Plate 3b). Combinations of BA 1.0 mg l^{-1} with higher level of Kinetin (3.0 mg l^{-1}) resulted in moderate ovule swelling but percentage of cultures with ovule development was low (38.88).

Table 28. Influence of auxins in the ovule development of kacholam after *in vitro* pollination

Sl. No.	Treatments*	Ovule swelling	Percentage of cultures showing ovule development **
1.	NAA 0.5	+	27.77
2.	NAA 1.0	+	44.44
3.	IAA 0.2	+	61.11
4.	IBA 0.2	+	55.55
5.	2,4-D 0.2	+++	70.55
6.	2,4-D 1.0	++	53.33

* Hormone concentrations in mg l^{-1}
** Average of 18 observations 20 DAP
Basal medium: ½ MS + 3% sucrose; *In vitro* placental pollination
Scoring: +++: good, ++: moderate, +: low

4.5.5 Influence of auxins and cytokinins in ovule development

The combinations of cytokinins (BA and kinetin) and auxins (NAA, IBA, IAA and 2,4-D) supported ovule development (Table 30). BA 0.5 mg l^{-1} along with NAA 3.0 mg l^{-1} favoured maximum ovule development in 83.33 per cent of the cultures (Plate 3c). Reduction in NAA to 2.0 and then 1.0 mg l^{-1} reduced ovule swelling. Increasing BA from 0.5 to 1.0 mg l^{-1} was not favourable as it reduced ovule swelling. In this medium, replacement of NAA with IAA (0.2 mg l^{-1}), IBA (0.2 mg l^{-1}) and 2,4-D (0.2 mg l^{-1}) produced ovule development in 50.00, 83.33 and 70.58 per cent of cultures but the ovule swelling was not up to the level as that in the optimum combination of BA and NAA.

Combinations of cytokinins BA 1.0 mg l^{-1} and kinetin 1.0 to 3.0 mg l^{-1} along with NAA 0.5 mg l^{-1} showed ovule development in 66.66 and 69.23 per cent of the cultures but the ovule swelling was not optimum. To study the replacement effects of other auxins in combination with BA 1.0 mg l^{-1} and kinetin 3.0 mg l^{-1}; IAA 0.2 mg l^{-1}, IBA 0.2 mg l^{-1} and 2,4-D 0.2 mg l^{-1} were tried. Among these, the combination BA 1.0 + kinetin 3.0 + 2,4-D 0.2 mg l^{-1} was found to be the best and produced optimum ovule swelling in 83.33 per cent of the cultures (Plate 3d).

Table 29. Influence of cytokinins in the ovule development of kacholam after *in vitro* pollination

Sl. No.	Treatments*	Ovule swelling	Percentage of cultures showing ovule development **
1.	BA 1.0	+	47.05
2.	BA 4.0	++	70.58
3.	Kinetin 1.0	+	44.44
4.	Kinetin 4.0	+	61.11
5.	BA 1.0 + Kinetin 1.0	+++	72.22
6.	BA 1.0 + Kinetin 3.0	++	38.88

* Hormone concentrations in mg l^{-1}
** Average of 18 observations 20 DAP
Basal medium: ½ MS + 3% sucrose; *In vitro* placental pollination
Scoring: +++: good, ++: moderate, +: low

Table 30. Influence of auxins and cytokinins in the ovule development of kacholam after *in vitro* pollination

Sl. No.	Treatments*	Ovule swelling	Percentage of cultures showing ovule development **
1.	BA 0.5 + NAA 3.0	+++	83.33
2.	BA 0.5 + NAA 2.0	++	83.33
3.	BA 0.5 + NAA 1.0	++	66.66
4.	BA 0.5 + IAA 0.2	+	50.00
5.	BA 0.5 + IBA 0.2	+	83.33
6.	BA 0.5 + 2,4-D 0.2	++	70.58
7.	BA 1.0 + NAA 3.0	++	72.22
8.	BA 1.0 + Kin 1.0 + NAA 0.5	++	66.66
9.	BA 1.0 + Kin 3.0 + NAA 0.5	++	69.23
10.	BA 1.0 + Kin 3.0 + IAA 0.2	++	72.22
11.	BA 1.0 + Kin 3.0 + IBA 0.2	+	66.66
12.	BA 1.0 + Kin 3.0 + 2,4-D 0.2	+++	83.33
13.	BA 1.5 + Kin 1.5 + NAA 0.5	++	77.77
14.	BA 2.0 + Kin 3.0 + NAA 0.5	++	75.00
15.	BA 4.0 + 2,4-D 0.2 + IAA 0.2	+	66.66
16.	Kin 4.0 + NAA 0.5	++	83.33
17.	Kin 4.0 + IAA 0.2	++	66.66
18.	Kin 4.0 + IBA 0.2	+	66.66
19.	Kin 4.0 + 2,4-D 0.2	++	66.66

* Hormone concentrations in mg l^{-1}
** Average of 18 observations 20 DAP
Basal medium: ½ MS + 3% sucrose; *In vitro* placental pollination
Scoring: +++: good, ++: moderate, +: low

Plate 3

Developing ovules 10 days after *in vitro* placental pollination in different media combinations

3a. 1/2 MS + 2,4-D 0.2 mg l^{-1}

3b. 1/2 MS + BA 1.0 + kin 1.0 mg l^{-1}

3c. 1/2 MS + NAA 3.0 + BA 0.5 mg l^{-1}

3d. 1/2 MS + BA 1.0 + kin 3.0 + 2,4-D 0.2 mg l^{-1}

A higher concentration of BA 4.0 mg l^{-1} in combination with 2,4-D 0.2 mg l^{-1} + IAA 0.2 mg l^{-1} reduced ovule swelling. Higher concentration of kinetin (4.0 mg l^{-1}) when combined with NAA 0.5 mg l^{-1} produced ovule development in 83.33 per cent cultures but the ovule swelling was not optimum.

The replacement of NAA with other auxins , IAA 0.2, IBA 0.2 and 2,4-D 0.2 mg l^{-1} in combination with kinetin 4.0 mg l^{-1} was also not favourable.

The seeds obtained from the cultures on the most successful media were examined for seed filling by cutting open and were stained with safranin. The seeds were fully filled with endosperm along with embryo and were readily stained indicating their viability.

4.5.6 Influence of sucrose concentration in ovule development

Maximum ovule development was observed at three per cent sucrose level in basal medium of ½ MS supplemented with BA 0.5 mg l^{-1} and NAA 3.0 mg l^{-1}. At this sucrose level, good ovule swelling was observed in 83.33 per cent of cultures.

Table 31. Influence of sucrose in the ovule development of kacholam after *in vitro* pollination

Sl. No.	Treatments	Ovule swelling	Percentage of cultures showing ovule development *
1.	Sucrose 3%	+++	83.33
2.	Sucrose 6%	++	70.58
3.	Sucrose 9%	++	77.77
4.	Sucrose 12%	+	50.00

* Average of 18 observations 20 DAP
Basal medium: ½ MS + BA 0.5 mg l^{-1} + NAA 3.0 mg l^{-1}
In vitro placental pollination
Scoring: +++: good, ++: moderate, +: low

An increase in the sucrose level to 6 per cent resulted in decreased percentage of ovule development (70.58 %) and the extent of ovule swelling. A further increase in sucrose level to 9 per cent was also not favourable for ovule swelling. The percentage of ovule swelling decreased drastically to 50.00 per cent with low ovule swelling at the highest level (12 %) of sucrose tried (Table 31).

4.5.7 Influence of organic media supplements in ovule development

The results (Table 32) indicated that the organic media supplements viz. casein hydrolysate, coconut water, yeast extract and L-glutamine were not favourable for ovule swelling as the extent of ovule swelling was low. The medium containing casein hydrolysate (250 mg l^{-1}) induced low development in 70.58 per cent of cultures while the medium containing coconut water (15 % v/v), yeast extract (250 mg l^{-1}) and L-glutamine (100 mg l^{-1}) resulted in low ovule development in 41.66, 68.75 and 58.33 per cent of cultures, respectively.

4.5.8 Influence of vitamin supplementation in ovule development

The effect of doubling the vitamin stock of MS medium in ovule development was studied. For this three hormone combinations were selected from the results of other investigations (Table 33). The vitamin supplementation was found to enhance ovule development in the medium ½ MS + BA 0.5 mg l^{-1} + NAA 3.0 mg l^{-1} with 3 per cent sucrose thus achieving the maximum percentage of ovule development (88.88) with good ovule swelling. The ovule development percentage in ½ MS + BA 1.0 mg l^{-1} Kinetin 3.0 mg l^{-1} + 2,4-D 0.2 mg l^{-1} was 78.57 per cent with medium ovule swelling while that in ½ MS + 2,4-D 0.2 mg l^{-1} was only 43.75 per cent. Therefore, in these two media vitamin supplementation was not favourable.

Table 32. Influence of organic media supplements in the ovule development of kacholam after *in vitro* pollination

Sl. No.	Treatments	Ovule swelling	Percentage of cultures showing ovule development *
1.	Casein hydrolysate 250 mg l^{-1}	+	70.58
2.	Coconut water 15% v/v	+	41.66
3.	Yeast extract 250 mg l^{-1}	+	68.75
4.	L-glutamine 100 mg l^{-1}	+	58.33

* Average of 18 observations 20 DAP
Basal medium: ½ MS + BA 0.5 mg l^{-1} + NAA 3.0 mg l^{-1} + 3% sucrose
In vitro placental pollination
Scoring: +++: good, ++: moderate, +: low

Table 33. Influence of vitamin supplementation in the ovule development of kacholam after *in vitro* pollination

Sl. No.	Treatments*	Ovule swelling	Percentage of cultures showing ovule development **
1.	BA 0.5 + NAA 3.0	+++	88.88
2.	2,4-D 0.2	++	43.75
3.	BA 1.0 + Kinetin 3.0 + 2,4-D 0.2	++	78.57

* Hormone concentrations in mg l^{-1}
** Average of 18 observations 20 DAP
Basal medium: ½ MS + 3% sucrose + vitamins; *In vitro* placental pollination
Scoring: +++: good, ++: moderate, +: low

4.5.9 Effect of illumination in ovule development

To study the effect of illumination on ovule development, the *in vitro* pollinated explants were subjected to three different conditions of illumination viz. continuous diffused light, continuous dark and an initial dark condition followed by light from 10 DAP onwards. The results (Table 34) showed that continuous diffused light was the best treatment resulting in 83.33 per cent cultures with good ovule development. The other two treatments produced ovule development in 80.00 per cent of cultures.

Plate 4

Pollen pistil interaction after *in vivo* stigmatic pollination

4a. 40 X magnification

4b. 40 X magnification

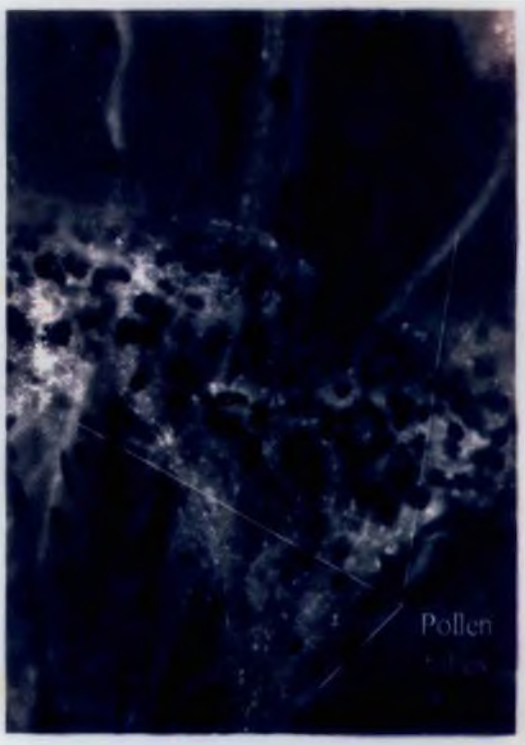


Table 34. Influence of illumination in the ovule development of kacholam after *in vitro* pollination

Sl. No.	Treatments	Ovule swelling	Percentage of cultures showing ovule development *
1.	Kept in diffused light after pollination	+++	83.33
2.	Kept in dark after pollination	++	80.00
3.	Kept in dark upto 10 DAP and then transferred to light	+++	80.00

* Average of 18 observations
Basal medium: ½ MS + BA 0.5 mg l^{-1} + NAA 3.0 mg l^{-1} + 3% sucrose
In vitro placental pollination
Scoring: +++: good, ++: moderate, +: low

4.5.10 Standardization of *in vitro* pollination technique

Different *in vitro* pollination techniques including stigmatic, stylar, intra-ovarian, placental, modified placental and ovular pollination as described by Bhojwani and Razdan (1983) were carried out. Among these, ovules developed in intra-ovarian, placental and modified placental pollination techniques (Table 35). In the intra-ovarian pollination technique, ovary as well as ovules developed. Ovary development was noticed in 72.22 per cent of cultures while ovules developed in lesser number of cultures (55.55 %). Placental pollination was the most successful technique with good ovule swelling and the highest percentage of ovule development (83.33 %) followed by modified placental pollination with 77.77 per cent cultures showing ovule development.

4.6 Pollen pistil interaction

Gynoeciums after *in vivo* stigmatic pollination and after *in vitro* placental pollination were observed for pollen germination and pollen tube growth under fluorescence microscope. In case of *in vivo* stigmatic pollination, pollen germination occurred on the spiny stigma but the extent to which the pollen tube might be growing through the style was not clear (Plate 4a, 4b). In case of *in vitro* placental pollination, the pollen tube growth was sufficient to cover the ovule length and to effect fertilization.

Table 35. Standardization of *in vitro* pollination technique in kacholam

Sl. No.	Method of pollination	Ovary or ovule swelling	Percentage of cultures with ovary development*	Percentage of cultures with ovule development*
1.	Stigmatic pollination	-	0.00	0.00
2.	Stylar pollination	-	0.00	0.00
3.	Intra-ovarian pollination	++	72.22	55.55
4.	Placental pollination	+++	NA	83.33
5.	Modified placental pollination	++	NA	77.77
6.	Ovular and test tube fertilization	-	0.00	0.00

* Average of 18 observations 20 DAP
Basal medium: ½ MS + BA 0.5 mg l^{-1} + NAA 3.0 mg l^{-1} + 3% sucrose
In vitro placental pollination
Scoring: +++: good, ++: moderate, +: low

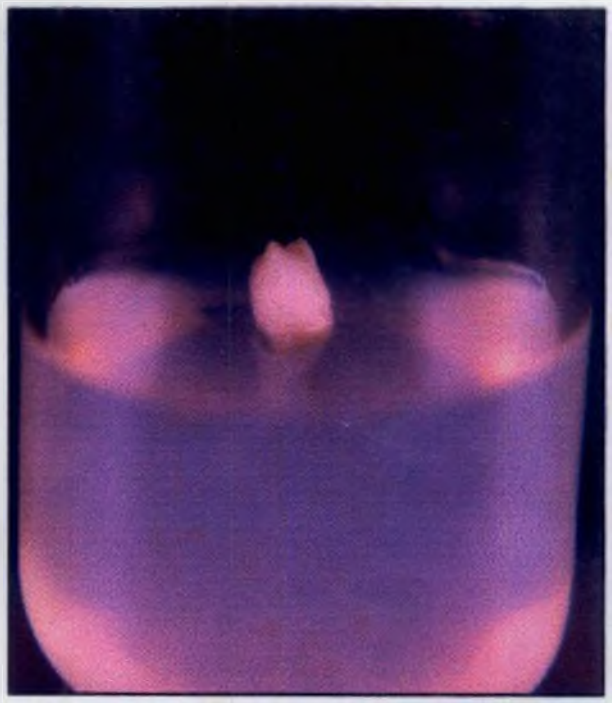
4.7 Post pollination changes

The ovary developed into fruit after intra-ovarian pollination (Plate 5a) and attained a maximum size (length 5.5 mm, breadth 3.5 mm in the centre) 20 DAP (Table 36). Fruits were creamy white in colour. Ovule/seed development was noted in the cultures after intra-ovarian, placental and modified placental pollination techniques. The ovules were white in colour at the time of pollination and turned creamy white within three DAP, remained so until 10 DAP after which, they turned brown, and became darker until 20 DAP (Plate 5b). The ovule size increased continuously until 15 DAP with faster growth in the initial 10 days.

Microscopic measurements of the seeds were done with an ocular micrometer to quantify the development of ovules into seeds (Table 36). Three different media were compared for the seed size attained therein at 20 days after *in vitro* placental pollination. (Table 36, Figure 3). The ovules developed to a largest mean size in the medium of ½ MS + 3 % sucrose + 2,4-D 0.2 mg l^{-1} and recorded a mean length of 1.82 mm and a mean breadth of 0.78 mm. In the medium of ½ MS + 3 % sucrose + BA 0.5 + NAA 3.0 mg l^{-1}, the ovules recorded a mean length of 1.58 mm and a mean breadth of 0.72 mm at 20 DAP. In the medium of ½ MS + 3 % sucrose + BA 1.0 + kinetin 3.0 + 2,4-D 0.2 mg l^{-1}, the ovules recorded a mean length of 1.44 mm and a mean breadth of 0.62 mm at 20 DAP.

Plate 5

5a. Developing ovary 20 days after *in vitro* intraovarian pollination



5b. Developing ovules 20 days after *in vitro* placental pollination

Table 36. Post pollination changes in ovules and ovaries of kacholam

Sl. No.	Treatments*	Ovule size				Ovary size			
		Before pollination		After pollination		Before pollination		After pollination	
		Mean length (μm)	Mean breadth (μm)	Mean length (mm)**	Mean breadth (mm)**	Mean length (mm)	Mean breadth (mm)	Mean length (mm)**	Mean breadth (mm)**
1.	2,4-D 0.2	96.33	61.16	1.82	0.78	4.11	2.68	-	-
2.	BA 0.5 + NAA 3.0			1.58	0.72			5.5	3.5
3.	BA 1.0 + Kinetin 3.0 + 2,4-D 0.2			1.44	0.62			-	-
		Mean		1.61	0.71				

* Hormone concentrations in mg l^{-1}

** Average of 18 observations 20 DAP

Basal medium: ½ MS + 3% sucrose; *In vitro* placental pollination

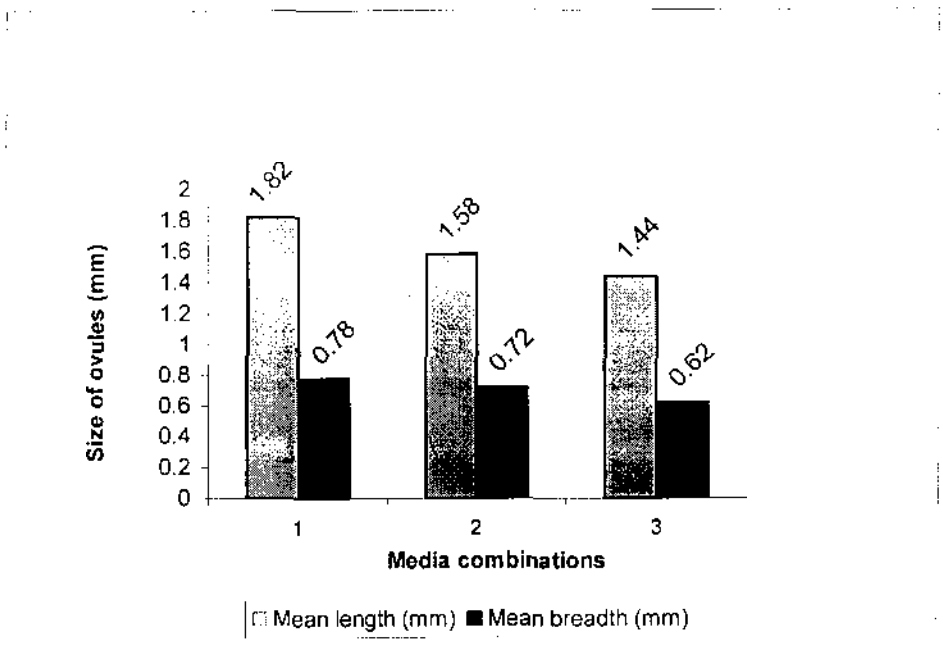


Fig.3. Size of developing ovules of kacholam 20 days after *in vitro* placental pollination in various media

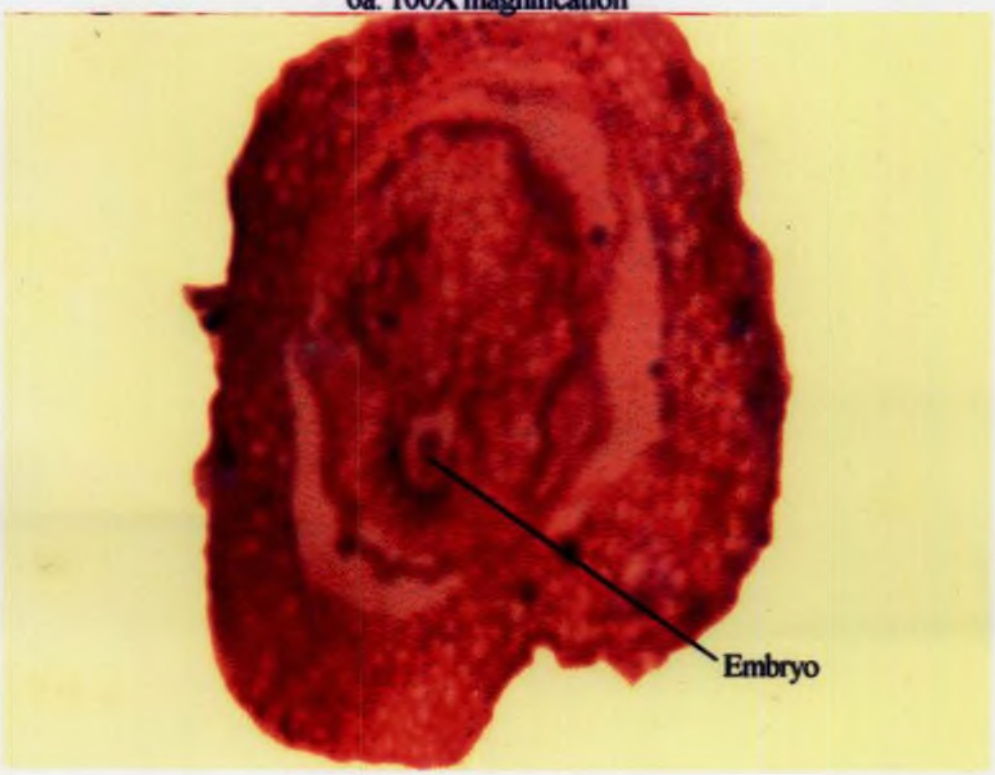
Media combinations:

1. ½ MS + 3 % sucrose + 2,4-D 0.2 mg l^{-1}
2. ½ MS + 3 % sucrose + BA 0.5 + NAA 3.0 mg l^{-1}
3. ½ MS + 3 % sucrose + BA 1.0 + Kinetin 3.0 + 2,4-D 0.2 mg l^{-1}

Plate 6

Longitudinal section of fertilized ovule 2 days after *in vitro* placental pollination

6a. 100X magnification



6b. 400X magnification

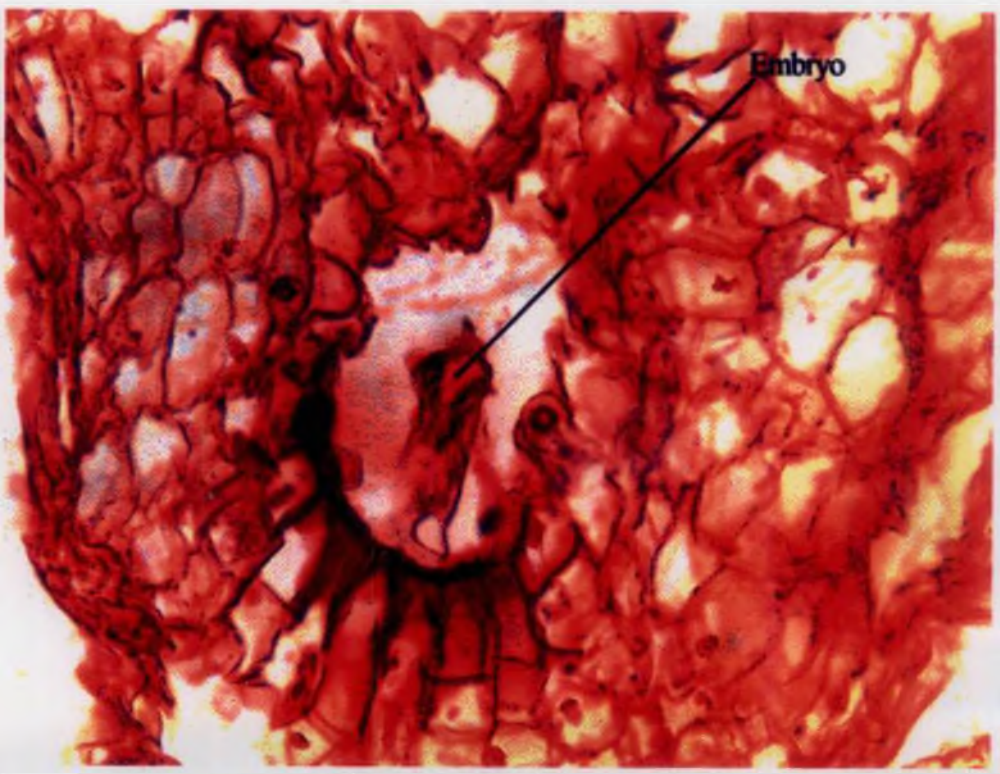
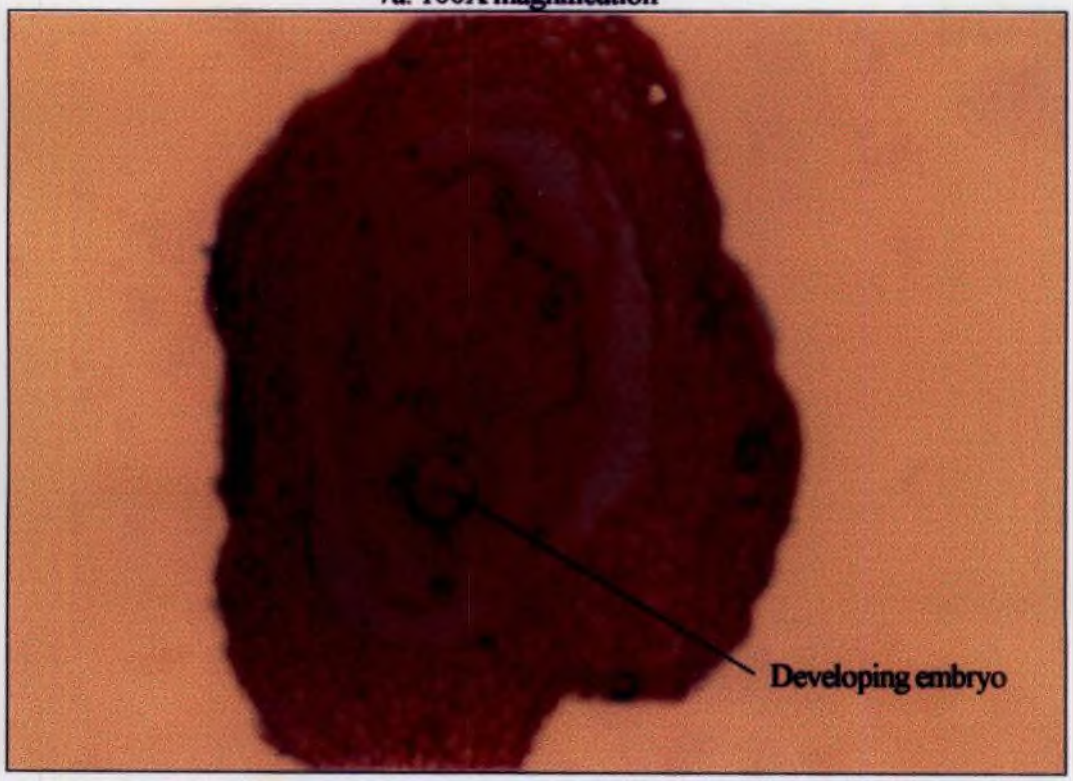


Plate 7

Longitudinal section of fertilized ovule 5 days after *in vitro* placental pollination

7a. 100X magnification



7b. 400X magnification

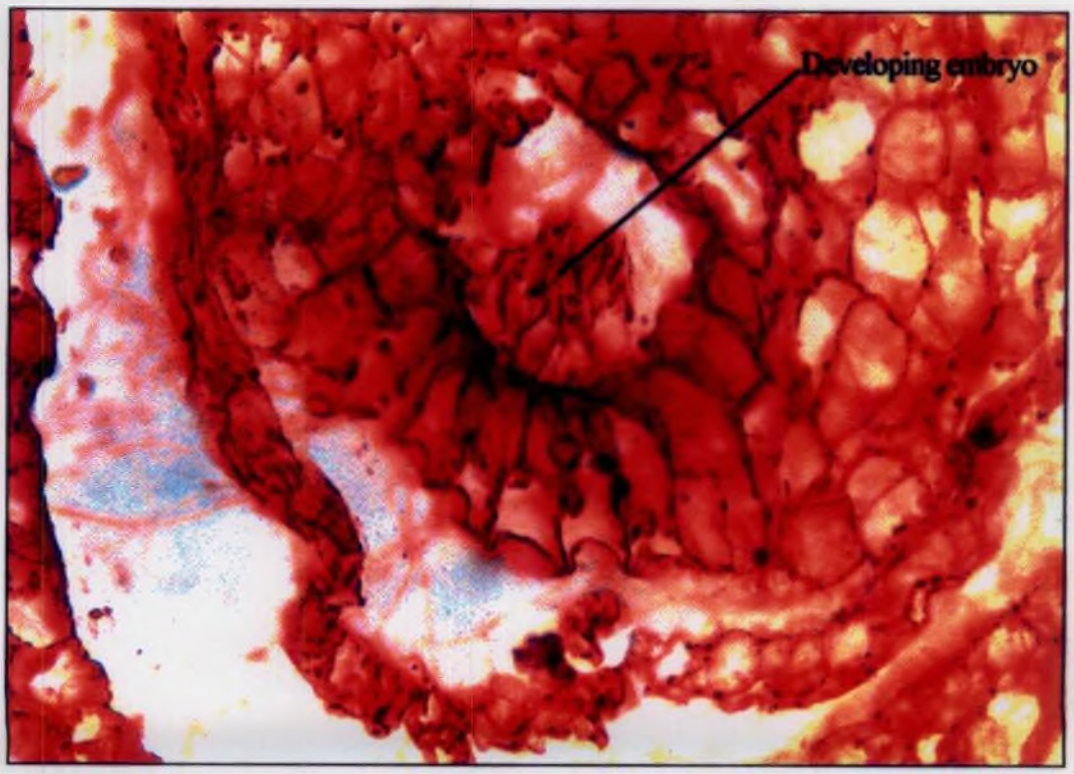
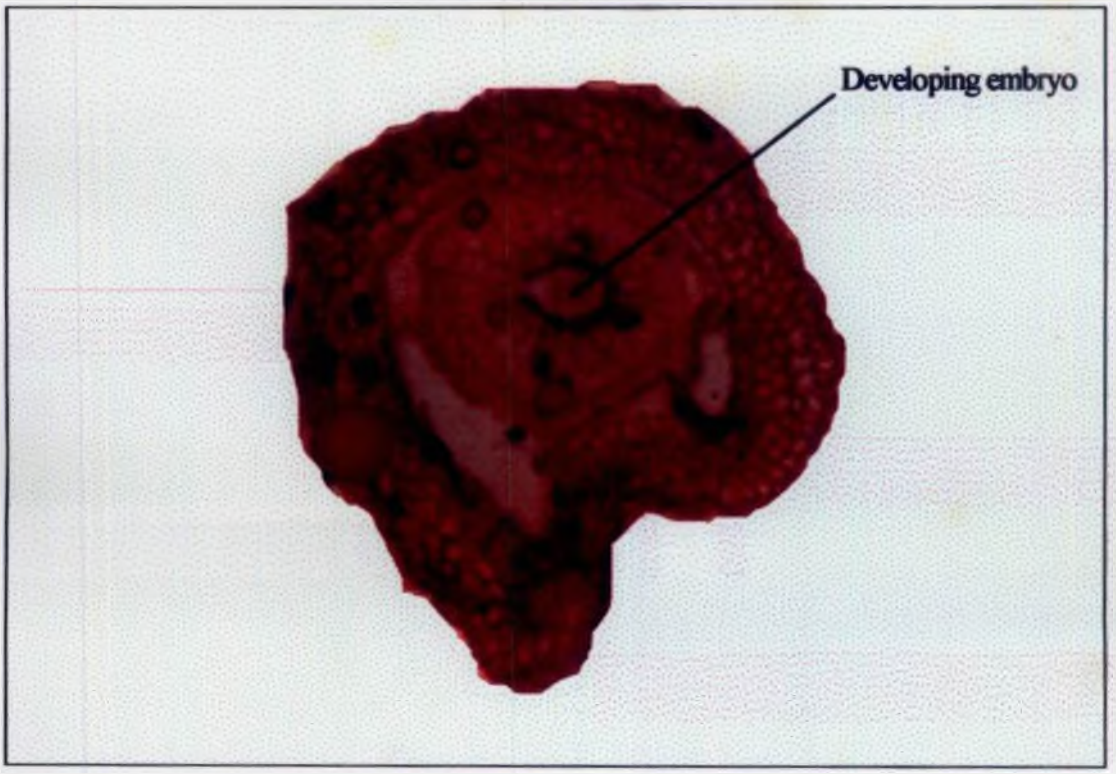


Plate 8

Longitudinal section of fertilized ovule 10 days after *in vitro* placental pollination

8a. 100X magnification



8b. 400X magnification

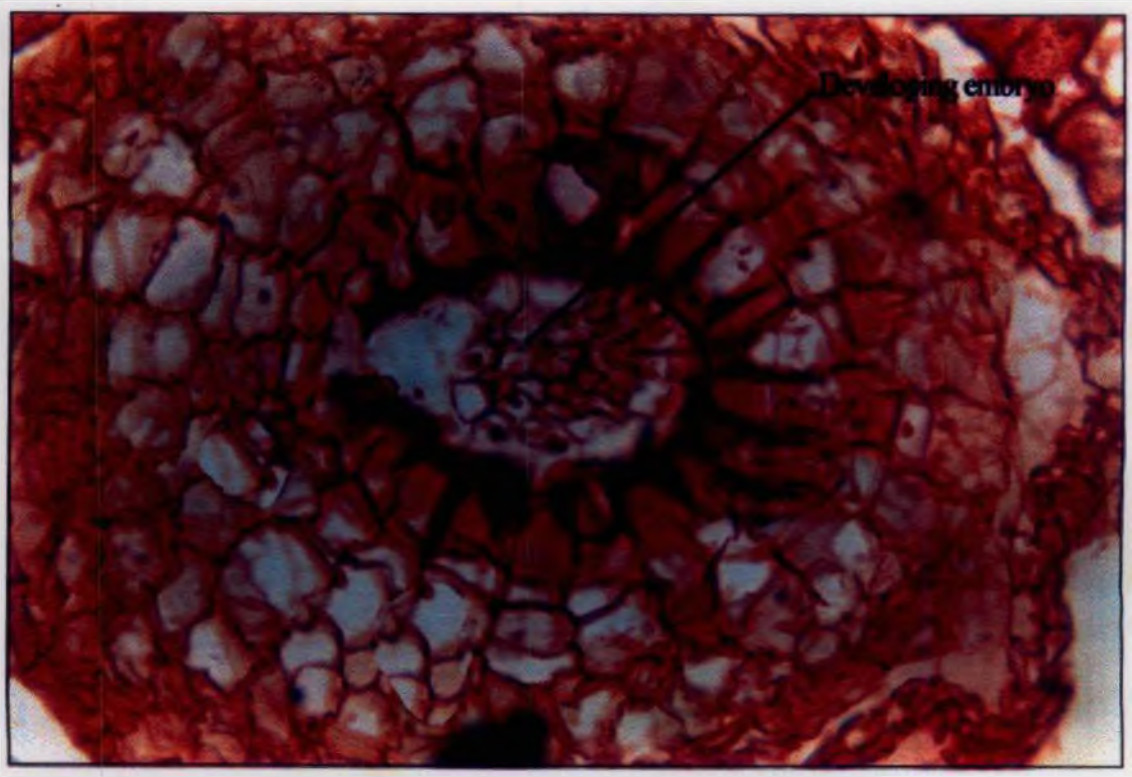
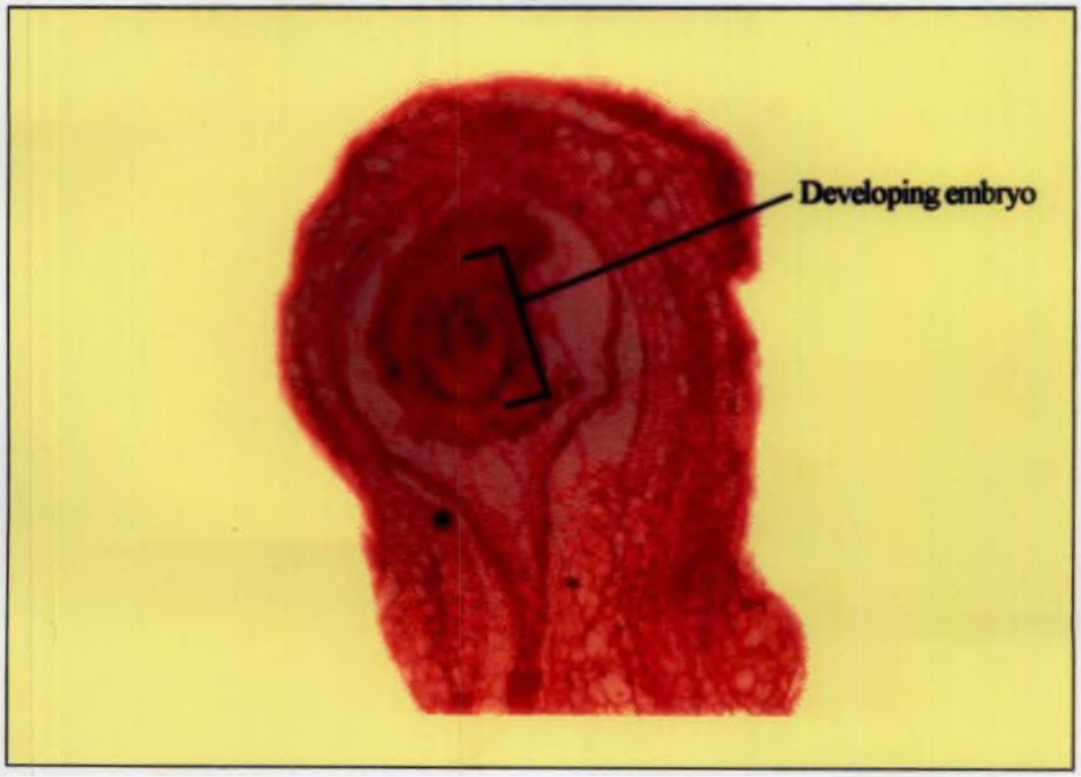


Plate 9

Longitudinal section of fertilized ovule 25 days after *in vitro* placental pollination

9a. 100X magnification



9b. 400X magnification

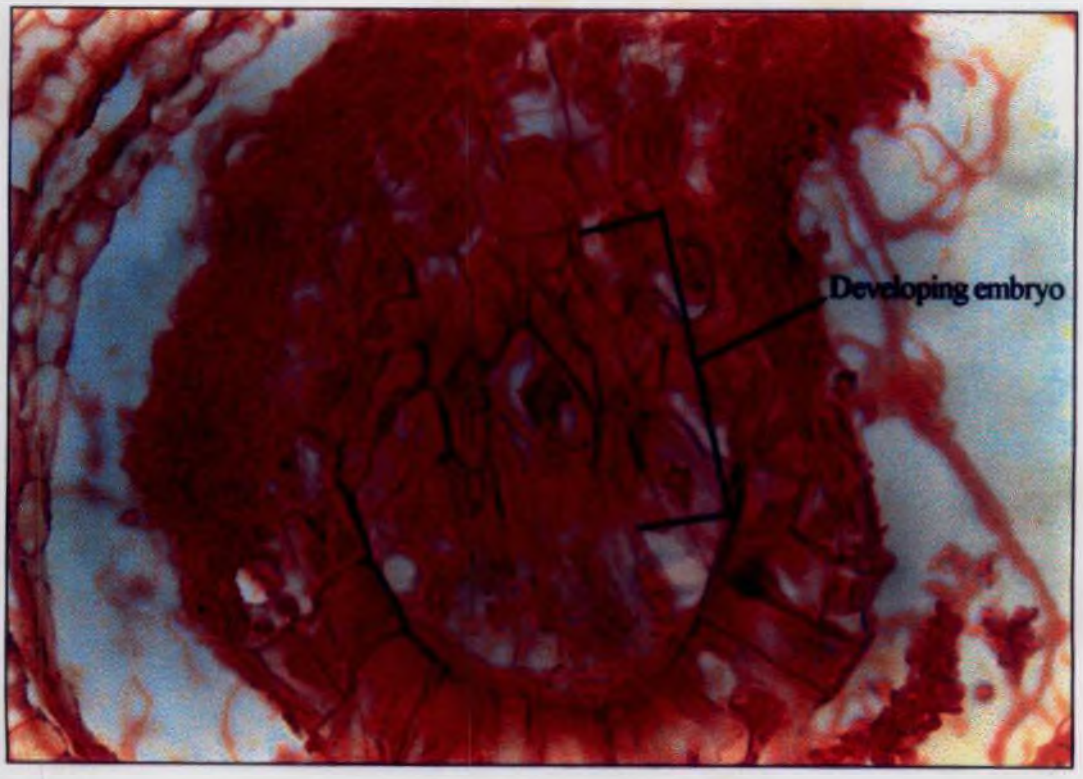
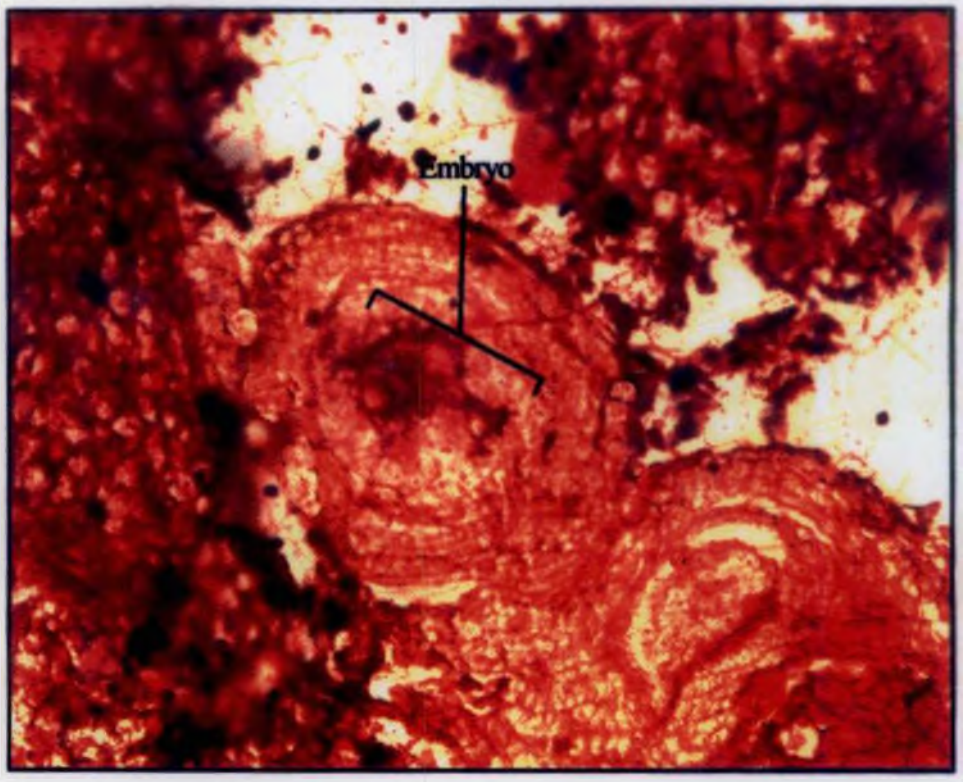


Plate 10

Longitudinal section of fertilized ovules 25 days after *in vitro* intraovarian pollination

10a. 100X magnification



10b. 400X magnification

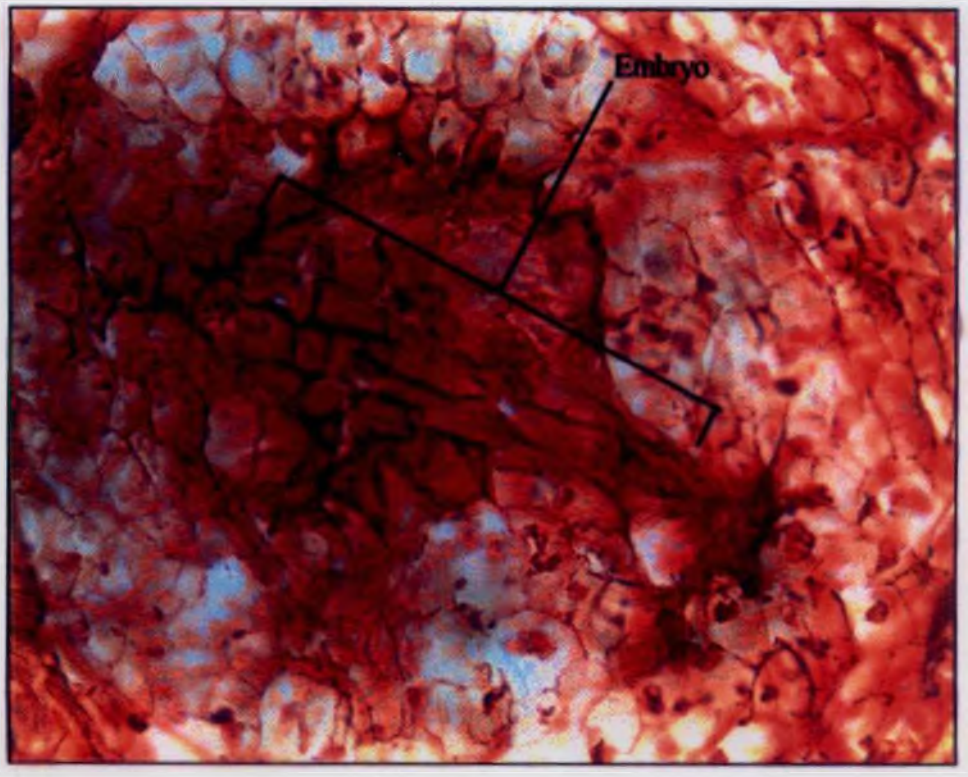
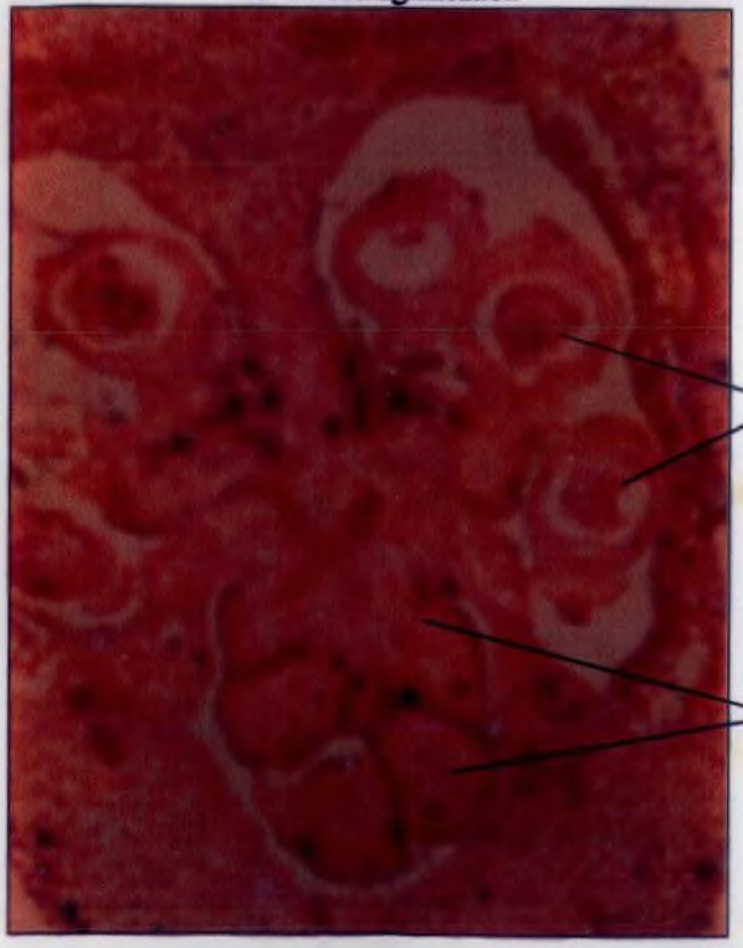


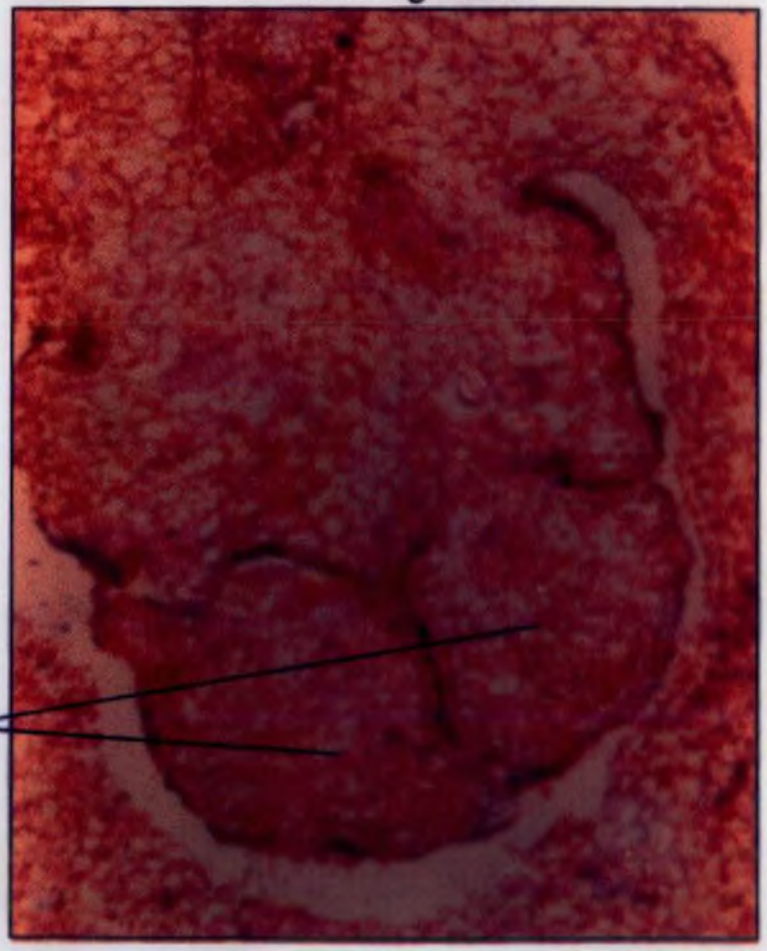
Plate 11

Longitudinal section of ovary 25 days after *in vitro* intraovarian pollination showing fertilized and unfertilized ovules

11a. 100X magnification



11b. 400X magnification



4.8 Histological examination of ovules

The longitudinal microtome sections of the ovules at 2, 5, 10 and 25 DAP were observed under a microscope at 40, 100 and 400 X magnifications. The sections showed deeply stained thick outer integument, thin inner integument and lightly stained nucellar tissue. The dividing embryo was distinguished by deep staining. The embryo covered a small area in the ovule at 2 DAP (Plate 6a, 6b) and subsequently enlarged at 5 DAP (Plate 7a, 7b) and 10 DAP (Plate 8a, 8b) and covered a substantial area of the ovules at 25 DAP (Plate 9a, 9b).

The sections of ovary 25 days after *in vitro* intra ovarian pollination showed embryo covering a substantial area of the ovule (Plate 10a, 10b). At 25 DAP, with intra ovarian pollination, some ovules in a locule of the ovary showed distinct darkly stained embryonic tissue along with endosperm, while in another locule of the same ovary, the ovules were devoid of it (Plate 11a, 11b).

4.9 Seed germination studies

4.9.1 *In vivo* treatments

Various pre-sowing treatments like soaking seeds in water for 24 h, keeping on moist filter paper, acid scarification and soaking in GA_3 solution (100 mg l^{-1}) for 12 h did not result in germination (Table 37, Plate 12a).

4.9.2 *In vitro* germination studies

Half strength MS medium with and without hormones did not result in seed germination (Table 38). A medium of ½ MS + BA 0.5 mg l^{-1} + NAA 3.0 mg l^{-1} + GA_3 1.0 mg l^{-1} + 3per cent sucrose did not result in germination. Knudson C medium did not produce germination. Taira and Larters' modified Norstog medium and Tomato embryo culture medium did not produce germination (Plate 12b).

Table 37. Influence of *in vivo* treatments on germination of kacholam seeds

Sl. No.	Treatments	Germination (%)
1.	Soaking seeds in water for 24 h	Nil
2.	Keeping on moist filter paper	Nil
3.	Acid scarification i) HCl (50%) for 5 min ii) HNO_3 (25%) for 5 min	Nil
4.	Soaking in GA_3 solution (100 mg l^{-1}) for 12 h	Nil

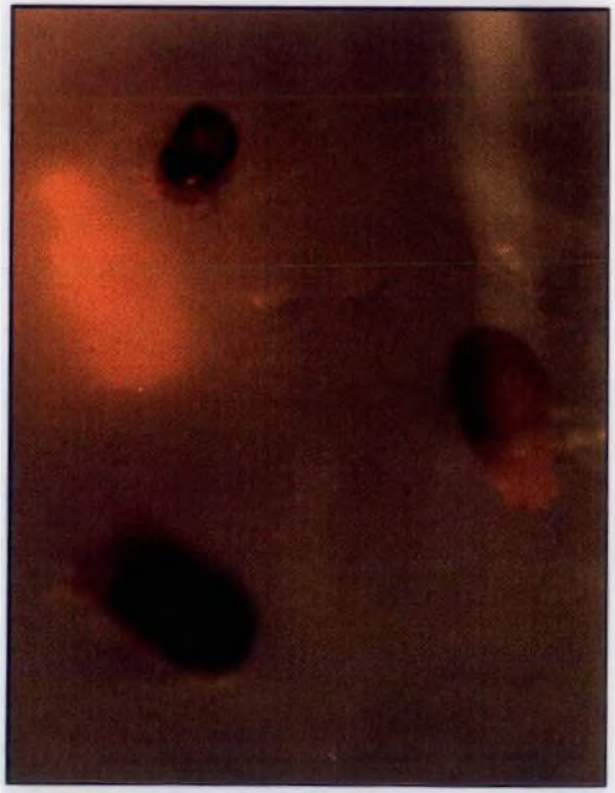
Table 38. Influence of *in vitro* treatments on germination of kacholam seeds

Sl. No.	Treatments	Germination (%)
1.	½ MS (solid) + 3% sucrose	Nil
2.	½ MS (liquid) + 3% sucrose	Nil
3.	½ MS + BA 0.5 mg l^{-1} + NAA 3.0 mg l^{-1} + GA_3 1.0 mg l^{-1} + 3% sucrose	Nil
4.	Knudson C medium	Nil
5.	Taira and Larters' modified Norstog medium	Nil
6.	Tomato embryo culture medium	Nil

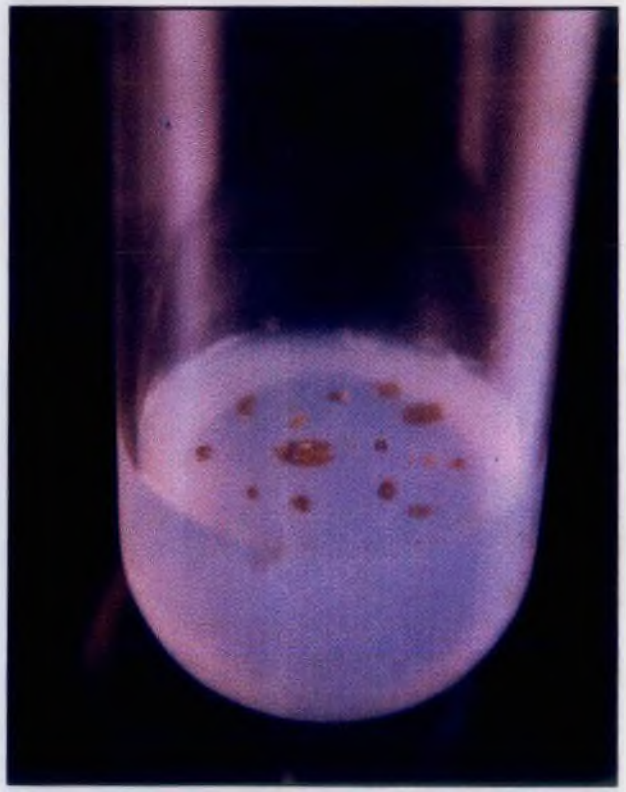
Plate 12

In vitro developed seeds of *Kaempferia galanga* kept for germination

12a. Seeds kept for *in vivo* germination



12b. Seeds kept for *in vitro* germination



DISCUSSION

Discussion

The investigations on "*In vitro* pollination in kacholam (*Kaempferia galanga* L.) for seed set" were carried out at the Department of Plantation Crops & Spices and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 1999-2001.

Kacholam is an important medicinal and aromatic plant of Zingiberaceae. Recently, the exploitation of rhizomes in the medicinal and perfumery industry has shot up and the demand for the same has increased. Limited natural genetic diversity and absence of seed set are the major constraints for crop improvement in kacholam. In this context, an attempt for seed set through *in vitro* pollination for induction of variability was carried out. *In vitro* pollination and fertilization is an effective tool in the situations of incompatibility and where pre-fertilization barriers prevent seed set.

Valsala (1994) successfully obtained seed set in ginger (*Zingiber officinale* Rosc.) of the family Zingiberaceae through *in vitro* pollination and fertilization. Similar attempt in turmeric (*Curcuma domestica* Val.) of Zingiberaceae resulted in seed set (Renjith, 1999).

The present investigations were aimed at standardizing a technique for seed set through *in vitro* pollination in kacholam. Success in seed set will open up new possibilities of crop improvement in kacholam wherein the conventional crop improvement strategies have not been hitherto successful due to lack of seed set. The limited variability available in the crop has further reduced the possibility of getting high yielding types through selection.

Hence, *in vitro* pollination could be utilized to create variability and combine the desirable characters to create hybrids, once the protocol is standardized.

The major aspects of investigations were

1. Variability studies in the selected ecotypes
2. Investigations for improvement of flowering
3. Floral biology and morphology of the crop
4. Pollen fertility and viability studies
5. Standardization of media for culture establishment
6. Response to *in vitro* pollination
7. Histological studies of ovules after *in vitro* pollination
8. Seed germination studies

The results obtained from the studies are discussed in this chapter.

5.1 Variability in the ecotypes

The variability in the selected ecotypes was examined for rhizome yield, dry recovery percentage, oil and oleoresin content and quality of oleoresin.

In the selected ecotypes, fresh rhizome yield ranged from 46.87 to 29.03 g/plant. The dry recovery percentage ranged from 24.99 to 29.15. The dry rhizome yield ranged from 29.03 to 13.33 g/plant. The oil content ranged from 1.40 to 2.40 per cent. The oleoresin percentage ranged from 2.66 to 3.71 (Table 14).

The results showed that the ecotypes differed in yield and quality characters and the economic characters; dry recovery percentage, oil, and oleoresin content remained scattered among the selected ecotypes. The fresh rhizome yield, dry rhizome yield, oil content and oleoresin quality were the highest in Kuruppanthara while dry recovery percentage was highest in Palakkad. Similarly, the oil content was the highest in Muttungal while the oleoresin content was the highest in Kothamangalam. The genetic materials identified for high dry rhizome yield were Kuruppanthara and Palakkad. The genotype identified for high dry recovery percentage was Palakkad. Muttungal was identified as genetic material having high oil content. Kothamangalam and Palakkad were identified as materials

having high oleoresin content. Even though the crop is maintained in cultivation through vegetative propagation, it shows variability. Therefore, there is scope for further improvement of selected types through hybridization and exploitation of hybrid vigour in kacholam.

5.2 Investigations for improvement of flowering

5.2.1 Influence of date of planting on flowering

Investigations on improvement of flowering showed that planting dates at monthly interval from 15th May to 15th July influenced the percentage of flowering and the flowering season of the ecotypes.

Planting on 15th May recorded the highest mean percentage flowering i.e. 61.11 and it got reduced to 23.61 when planted on 15th July (Table 15). In 15th May planting, the flowering season was from 5th July to 16th August and it got extended up to first July to 5th October by adopting the 15th and 15th July planting (Table 16). This showed that in subsequent plantings of one month interval, there was a corresponding shift in the flowering season.

With staggered planting using 5-10 g seed bits, the normal flowering season of kacholam i.e. June to August (Table 16) could be extended up to October. Planting after 15th July may not be advisable because in July planting three ecotypes did not flower while the other three showed up to 50 per cent flowering only.

5.2.2 Influence of seed bit size and biennial habit on flowering

The biennials (T_3) from normal see bit size (5-10 g) showed higher percentage of flowering, higher number of inflorescences per plant, longer duration of flowering and early flowering as compared to the annuals T_1 and T_2 (Tables 17, 18 and 19). The percentage of flowering was the highest in T_3 (87.49) followed by T_2 (20-25 g) with a mean of 66.55 per cent and T_1 (5-10 g) with 62.49 per cent.

The number of inflorescences per plant in T_3 was not statistically significant over T_1 and T_2. The plants from T_3 showed the longest duration of flowering with a mean of 17.85 days followed by T_1 (5-10 g) with a mean of 12.74 days and T_2 (20-25 g) with a mean of 10.75 days. The biennials started flowering early in the second year, by the first week of June while the annuals started flowering in the fourth week of June (Table 20). This investigation revealed that the biennial habit influences the percentage of flowering, duration of flowering and the flowering season.

There was no marked difference in T_1 and T_2 in flowering behaviour. So using seed bits larger than 25 g may be tried for enhancing flowering in annual crop. Biennials from the normal size seed bits are advisable for extending the flowering season as well as for getting higher number of flowers.

These experiments showed that the flowering season of kacholam could be extended by resorting to staggered planting and biennial crop. By staggered planting up to mid July, the flowering could be extended up to first week of October, while the biennial crop started flowering in the first week of June. Therefore, the flowering season of kacholam could be extended from first week of June to first week of October as against fourth week of June to first week of August in normal planting. This finding is of interest and relevance for the *in vitro* pollination work to get a continuous supply of flowers for a longer period.

Okwuowulu (1988) observed that large setts confer an advantage for flowering and produced significantly higher number of inflorescences than smaller setts in ginger. This may be so because with large setts the demand for the photosynthetic products during the early crop growth is low and hence the crop can afford a higher number of inflorescence bearing shoots. The results of the present study in kacholam show a similar trend.

Valsala (1994) observed that the size of the seed bit used for planting ginger and biennial habit positively influenced the flowering intensity.

5.3 Study of floral biology and morphology

Kacholam started flowering within a period of 48-68.5 days after planting. All the six ecotypes planted for the study flowered. The ecotypes Kuruppanthara, Kuruppallure and Palakkad were early to flower while Chimmoni and Kothamangalam were late in flowering. Since all the ecotypes flowered, lack of flowering will not be a problem in the hybridization work.

The inflorescence of kacholam is a scape covered by a leathery sheath and bears flowers in bracts, which open in succession. The inflorescence arises directly from the rhizome. The flower has only one fertile stamen.

The pistil of the flower is characterized by a long style (Plate 2b) of mean length 4.52 cm, which passes through the groove present between the two anther lobes and ends in a spiny stigma reaching just above the anther lobes. Ovary recorded a mean length of 4.11 mm and diameter of 2.68 mm in the middle. The mean number of ovules in the ovary was 20. The ovule measured a length of 96.33 µm and 61.16 µm in the middle (Table 21). Since the ovary contains 20 ovules, in successful seed set and development, 20 seeds per ovary can be expected. The observations on the ovary and ovule size will be helpful for monitoring their growth *in vitro*.

The anthesis occurred between 4 am and 5 am and the anther dehiscence occurred between 4.30 am and 5.15 am. Unlike in other crops, here the flower buds are not visible on the previous day of anthesis. So only opened flowers can be used for *in vitro* pollination work. This will lead to washing of some pollen grains during surface sterilization. Collection of flowers during the early hours of the day is also important considering the caducous nature of the flower.

5.4 Pollen studies

5.4.1 Pollen fertility, viability and pollen tube growth in kacholam ecotypes

The mean pollen fertility was high (76.33 %) as per the acetocarmine stain test (Table 22) and varied among the ecotypes from 64.96 to 86.33 per cent.

The mean pollen viability was the highest in ME_3 medium (78.79 %) followed by Brewbaker and Kwack's medium (67.57 %) and Rekha's medium (67.31 %) (Table 23, Figure 1). There was no pollen germination in distilled water.

The mean pollen tube growth was also the highest in ME_3 medium (809.17 µm) followed by that in Brewbaker and Kwack's medium (758.33 µm) and Rekha's medium (765.67 µm) (Table 24, Figure 2).

High pollen fertility (72.8 %) and viability (68.9 %) were observed in kacholam by Rekha (1993) also. The mean pollen tube length observed in the corresponding medium in the present study (765.67 µm) is in tune with her observations. The lack of germination in distilled water may be due to lack of osmoticum and essential micronutrients (Brewbaker and Kwack, 1963).

In ME_3 medium, the pollen germination percentage and mean pollen tube length were found to be higher than that in the other two media. Hence, ME_3 can be considered as the best medium among the media tried. The other two media can also be used for *in vitro* pollination since there is not much reduction in the germination and pollen tube growth. Moreover, these two media are relatively easier to prepare due to their simple compositions.

5.5 *In vitro* pollination

5.5.1 Basal medium for culture establishment

Both full strength MS and half strength MS with plant growth regulators supported the ovule development after *in vitro* placental pollination. The percentage of cultures showing ovule development was more in half MS. On visual assessment of the cultures, half MS was superior to that of full MS for increasing the size of the ovules (Table 25).

The suitability of MS medium with plant growth substances for *in vitro* culture of *Kaempferia* has been reported earlier (Joseph, 1997; Anand *et al.*, 1997; Vincent *et al.*, 1992a; Babu *et al.*, 1997).

5.5.2 Control of bacterial interference in the culture establishment

Culture establishment was difficult due to bacterial contamination. Four types of bacteria were isolated from the cultures, the white gram negative bacteria being the most damaging one. *In vitro* studies showed that copper oxychloride (2500 mg l^{-1}) could suppress all the three bacteria tested. It also showed that the yellow and the pink bacteria could be completely suppressed by various antibiotic treatments and mercuric chloride (1000 mg l^{-1}). However, the white bacterium could be controlled only by copper oxychloride (2500 mg l^{-1}).

This study reveals that copper oxychloride can be included in the surface sterilization procedure of the flowers but the pollen viability has to be warranted. Its efficacy to suppress the bacteria may be examined at lower concentrations also. Field spraying or drenching of plants with copper oxychloride can be planned to reduce the bacterial load on the plants.

In the present study, antibiotics were examined singly for their effect on bacteria. Combinations of antibiotics and combinations of antibiotics and fungicides may be tried as indicated by Thurston *et al.* (1979).

Since the kacholam plant is lying flat on the ground, avoiding the practices of topdressing with organic manures and earthing up will reduce the microbial load. Maintaining the plants under a protective structure and mulching with black polythene sheet can also be done for reducing the bacterial interference in the cultures.

5.5.3 Influence of auxins and cytokinins in ovule development

Auxins alone, cytokinins alone as well as the combinations of auxins and cytokinins induced ovule development. Out of the various hormone combinations tested, the following four media combinations proved to be the best for maximum ovule swelling (Plate 3a, 3b, 3c and 3d).

i) ½ MS + 3 % sucrose + 2,4-D 0.2 mg l^{-1}
ii) ½ MS + 3 % sucrose + BA 1.0 + kinetin 1.0 mg l^{-1}
iii) ½ MS + 3 % sucrose + BA 0.5 + NAA 3.0 mg l^{-1}
iv) ½ MS + 3 % sucrose + BA 1.0 + kinetin 3.0 + 2,4-D 0.2 mg l^{-1}

The medium of half MS + 2,4-D 0.2 mg l^{-1} was exceptionally good for ovule development.

The above results show that the cultures vary in their requirements of hormones for development.

Joseph (1997) found that the half MS medium supplemented with BA 0.5 mg l^{-1} and NAA 3.0 mg l^{-1} gave rise to somatic embryos from calli of kacholam.

The role of auxins in supporting ovary development into fruit is well documented. The developing seeds are a rich source of auxin that is utilized for normal fruit growth, as in apple, pear and strawberry (Nitsch, 1952). Synthetic auxins such as NOA and 2,4-D can replace the stimulus provided by pollination.

Raghavan and Torrey (1963) demonstrated that low concentrations of IAA and kinetin aided globular *Capsella* embryos. The cytokinin activity at the early stage of embryogenesis is responsible for enhanced seed size by increasing cell number (Michael and Seiler-Kelbitsch, 1972). Studies with isogenic mutants of barley, which vary in grain weight, demonstrated that large grain lines contain higher amounts of cytokinin at the very early stage of seed development than small grain lines (Seiler-Kelbitsch *et al.*, 1975). Eeuwens and Schwale (1975) have

reported that in general high auxins (IAA) and GA levels have been associated with active seed growth and fruit growth by cell expansion. In a number of plants, both GA and auxins are highest during early to mid embryo development, at a stage when cytokinins are decreasing rapidly.

According to Tollennar (1977), cytokinins are found in relatively high concentration in the liquid endosperm stage of early seed growth and their presence coincides with the higher rate of mitosis. Philips (1982) found that moderate levels of auxin with low levels of cytokinin aided the growth and survival of heart shaped interspecific hybrid embryos in *Trifolium*. Neal and Topoleski (1985) found combinations of kinetin and GA_3 or kinetin and IAA to be most beneficial for the growth of tomato embryos excised 12 days after pollination. Quartrano (1987) reported that hormones play an important role in the stages of seed development and germination. Brar *et al.* (1991) reported about the requirement of IAA and BAP for in ovulo embryo culture in seedless grapes.

Valsala (1994) reported that auxins as well as cytokinins are essential for the development of *in vitro* pollinated ginger ovules. Renjith (1999) also reported that the auxins and cytokinins are essential for the development of *in vitro* pollinated ovules of turmeric.

These reports explain the requirement of auxins and cytokinins for proper development of ovules.

The seeds formed in the four aforesaid media were examined for seed filling / endosperm development and were found to be completely filled. Since there was no germination, these four media combinations may be used to produce more number of seeds for testing various seed germination treatments. The best medium among these four media can be further ascertained from the results of the seed germination studies.

5.5.4 Influence of sucrose concentration on ovule development

The ovules developed at all levels of sucrose tried (3.0,6.0,9.0 and 12.0 %) along with hormones but the maximum development was observed at 3 per cent level and there was a gradual reduction in the ovule development up to 12 per cent level (Table 31).

The sucrose concentration has been reported to influence ovary development, ovule development and germination of seeds following *in vitro* pollination. In most of the studies, sucrose has been used at a concentration of 4.0-5.0 per cent (Kanta and Maheshwary, 1963; Usha, 1965; Rangaswamy and Shivanna, 1967 and Dhaliwal and King, 1978).

In Lilium, swelling of the capsule was highest at sucrose concentration of 10.00 per cent but embryos were mostly found in ovaries cultured at 6.0-8.0 per cent sucrose (Van Tuyl *et al.*, 1991).

Since endosperm filling and embryo development was obtained at 3.0 per cent sucrose level, along with hormones, 3 per cent sucrose can be considered as optimum for *in vitro* pollination of kacholam.

5.5.5 Influence of vitamin supplementation and organic supplements in ovule development

The addition of double the quantity of the vitamin stock of MS medium in the medium of ½ MS + 3 % sucrose + BA 0.5 + NAA 3.0 mg l^{-1} enhanced ovule development (Table 33). This was not seen in case of the other two favourable media combinations tried.

Other supplements tried i.e. casein hydrolysate, coconut water, yeast extract and L-glutamine did not favour ovule development (Table 32).

Vitamins such as B_1 and B_6 or even a mixture of vitamins (Maheshwari and Lal, 1961) stimulated ovary growth of *Iberis amara*. Vitamin E (tocopherol acetate) increased seed fertility in *Dendrobium nobile* (Ito, 1966). Castano and DeProft (2000) reported that vitamin stock of MS medium supported the ovule development in chicory (*Cichorium intybus* L.). Vijayasree (2001) reported that addition of double the quantity of the vitamin stock of MS medium enhanced ovule development in turmeric after *in vitro* pollination.

Literature shows that casein hydrolysate (Zeibur *et al.*, 1950; Bajaj, 1964), malt extract (Blakslee and Satina, 1944), coconut water (Shantz and Steward, 1952; Letham, 1954; Kapoor, 1959 and Valsala, 1994) and L-glutamine (Emershad and Ramming, 1984; Collins and Grosser, 1984) favour embryo and ovule development under *in vitro* condition. In the present study, this favourable effect was not manifested.

The present study reveals that double the quantity of vitamin stock of MS medium can be added to the medium of ½ MS + 3 % sucrose + BA 0.5 + NAA 3.0 mg l^{-1}. It could not be added to the other two favourable media combinations i.e. 2,4-D 0.2 mg^{-1} and BA 1.0 + kinetin 3.0 + 2,4-D 0.2 mg l^{-1} in half MS medium with 3 % sucrose.

5.5.6 Influence of illumination in ovule development

The ovaries of kacholam were subjected to three treatments in relation to illumination after *in vitro* placental pollination. The ovule development was satisfactory in diffused light. Keeping in dark up to 10 DAP and then transferring to light was equally good. The ovule development was not optimum in dark.

The literature on the effect of illumination on ovule development is scanty. Cultures were usually stored in darkness or near darkness (Rangaswamy and Shivanna, 1967; Balatkova *et al.*, 1977 and Dhaliwal and King, 1978). The results of *in vitro* pollination were the same whether the cultures were incubated in light or dark (Zenkteler, 1969). Lech *et al.* (1994) reported that embryo development in

plum after *in vitro* pollination was not influenced by light condition Valsala (1994) observed that *in vitro* pollinated ovules of ginger developed in dark, light and light intensities of 500 to 1000 lux.

The present study indicates that the kacholam cultures after *in vitro* pollination can be kept in diffused light or can be kept in dark up to 10 DAP and then be transferred to light for development.

5.5.7 Standardization of *in vitro* pollination method

Ovules developed in intra-ovarian, placental and modified placental pollination techniques (Table 35). In all the three methods, pollen grains along with pollen germination medium were applied over the ovules or on the exposed part of the ovary, as the case may be. The ovule development is a result of fertilization and suitable culture medium. Histological examination of placental pollinated ovules at various time intervals from 2 DAP to 25 DAP showed actively dividing embryo of increasing size. This supports fertilization consequent to *in vitro* placental pollination. In the sections of intra ovarian pollination at 25 DAP, fertilized and unfertilized ovules were observed. In the fertilized ones, actively dividing embryo was observed in the embryo sac, while the unfertilized ovules were devoid of it. This also suggests that in the intra ovarian pollination, fertilization occurs in some ovules but some ovules may remain unfertilized. In the modified placental pollination also, ovule development occurs after fertilization since pollen grains are applied on the exposed ovules alongwith the pollen germination medium. Among the three methods, placental pollination is the best since chances of getting fertilized seeds are the maximum.

Valsala (1994) reported seed set and seed development through placental, modified placental and test tube fertilization technique in ginger. Renjith (1999) reported successful seed set in turmeric through intra ovarian, placental and modified placental technique.

5.6 Pollen pistil interaction

Pollen pistil interaction studies after *in vivo* stigmatic pollination showed pollen germination on the spiny stigma but the extent of pollen tube growth through the style was not clear (Plate 4a, 4b). Under *in vivo* condition, lengthy style and caducous nature of the flower may be the contributing factors for lack of seed set. Pollen pistil interaction studies after *in vitro* placental pollination revealed that pollen tube growth is sufficient to cover the ovule length and to effect fertilization. Hence, there is chance of fertilization in case of the *in vitro* placental pollination.

5.7 Post pollination changes

5.7.1 Fruit development after *in vitro* pollination

The ovary on the day of anthesis measured a mean length of 4.11 mm and a mean diameter of 2.68 mm. The ovary developed into fruit after *in vitro* intra-ovarian pollination and attained a maximum size (length 5.5 mm, breadth 3.5 mm) at 20 DAP in the medium of ½ MS + 3 % sucrose + BA 0.5 + NAA 3.0 mg l^{-1}.

The size increase in the ovary development into fruit was not substantial within 20 DAP and colour changes indicating maturation and ripening were also not visible under culture condition. Since natural seed set and fruit development are not available, conclusions regarding the normal size and maturity of the fruit could not be drawn.

5.7.2 Seed development after *in vitro* pollination

The ovules on the day of anthesis measured a length of 96.33 µm and breadth of 61.16 µm. The growth of ovules was rapid in the initial 10 days and slowed subsequently. The ovules had a mean length of 1.61 mm and a mean breadth of 0.71 mm at 20 DAP in the three selected media. The mean size increase of the ovules 20 DAP was more than 10 times the original size. The colour of the

ovules was white on the day of anthesis and turned creamy white within three DAP and remained so until 10 DAP after which it turned brown and became darker until 20 DAP. The maximum size increase was observed in the medium of ½ MS + 3 % sucrose + 2,4-D 0.2 mg l^{-1} (Figure 3).

In the seed development, the size increase within 20 DAP was substantial. This may be due to the influence of the method of pollination i.e. placental pollination and the culture medium. In turmeric, in the natural seed set, the maturation period of the seed is 28-30 days. Kacholam under *in vitro* condition may also require more days or media manipulations for the maturation of the embryo. Since in kacholam, there is no natural seed set, the embryo development cannot be compared with that of the *in vitro* development.

5.7.3 Description of kacholam fruit and seed

The kacholam fruit developed after *in vitro* intra-ovarian pollination was a thick walled capsule with small light brown coloured seeds. At 20 DAP; the fruits recorded a mean length of 5.5 mm and breadth of 3.5 mm.

The small arillate seeds had two seed coats. The outer one being thick and the inner being thin. The seed coat encloses a cavity, which is typical of monocots. In the cavity, endosperm with embedded embryo was seen.

5.8 Histological examination of ovules

The longitudinal microtome sections of the ovules at 2, 5, 10 and 25 DAP showed dividing embryo in the embryo sac surrounded by nucellar / endospermous tissue, inner integument and outer integument. The actively dividing embryo was smallest in sections of 2 DAP (Plate 6a, 6b) and showed maximum size in sections of 25 DAP (Plate 9a, 9b). This supports fertilization consequent to *in vitro* placental pollination.

Even though the sections showed various stages of embryo development, well-developed embryo with distinctly differentiated parts was not clearly visible. Kacholam under *in vitro* condition may require more days or media manipulations for the maturation of the embryo. Since there is no natural seed set, embryo development cannot be compared with that of the *in vitro* development. Germination of kacholam seeds would have given conclusive results.

5.9 Seed germination studies

In vitro developed seeds were subjected to various *in vivo* treatments for germination. Soaking of seeds in water for 24 h and keeping on moist filter paper did not favour germination. Acid scarification with HCl 50 per cent and HNO_3 25 percent also did not favour germination. Seeds soaked in GA_3 solution (100 mg l^{-1}) for 12 h did not germinate.

In vitro treatments also failed to bring about germination. Half MS medium with three per cent sucrose tried for seed germination in solid and liquid states did not favour germination. Seeds did not germinate in the same medium supplemented with BA 0.5 + NAA 3.0 + GA_3 1.0 mg l^{-1}. Seeds did not germinate in Knudson C medium, which is known for orchid seed germination. Taira and Larters' modified Norstog medium (1978) reported for supporting the intergeneric hybrid embryos of wheat and rye (Raina, 1984) did not support germination. Seeds failed to germinate in tomato embryo culture medium (Neal and Topolcski, 1983).

Valsala (1994) reported *in vitro* germination of ginger seeds on half MS medium supplemented with three per cent sucrose, 2,4-D 8.0 + BAP 9.0 and then subcultured on half MS supplemented with 2,4-D 0.1 mg l^{-1} but the germination percentage was five per cent only.

Detailed study on media and hormone manipulations for seed germination could not be conducted owing to limited number of seeds available. *In vitro* developed seeds of ginger germinated *in vitro* with hormones. It implies that media and hormonal manipulations may be necessary for seed germination, which can be

explored further. Eventhough there was no seed germination in the present study, the chances of success in seed germination are high in kacholam. This is supported by high pollen fertility and viability as well as the active embryo development as ascertained by the histological studies of the seeds.

Conclusions

The six ecotypes of kacholam selected for the study exhibited variability in yield and quality characters and these characters remained scattered among the ecotypes. The practices of staggered planting and keeping the crop as biennial, together could extend the flowering season up to June to October as against June to August in the normal planting. High mean pollen fertility (76.33 %) and pollen viability (78.80 %) were observed in the ecotypes. ME_3 medium of pH 6 was the best medium for pollen germination and tube growth with a mean pollen tube length of 809.17 µm. Seed set and development was observed through *in vitro* pollination. Placental pollination was found best for seed development. *In vitro* fertilization was confirmed by histological examination of ovules. Four media combinations were identified for ovule development. Among them ½ MS + 3 % sucrose + 2,4-D 0.2 mg l^{-1} was the best. The seeds developed after *in vitro* pollination did not germinate by various *in vivo* and *in vitro* germination treatments.

Future lines of work

i) Mass production of seeds using the four most favourable media combinations and testing various seed germination treatments. Embryo rescue can also be tried.

ii) After obtaining seed germination, planned hybridization programme based on variability studies could be undertaken.

SUMMARY

Summary

Investigations on "*In vitro* pollination in kacholam (*Kaempferia galanga* L.) for seed set" were carried out at the Department of Plantation Crops and Spices and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 1999 to 2001. The salient findings of the study are summarized as follows:

1. The kacholam ecotypes under study exhibited variability in yield and quality characters. The fresh rhizome yield ranged from 46.87 g/plant in Chimmoni to 103.00 g/plant in Kuruppanthara with a mean of 86.40 g/plant. The dry recovery percentage ranged from 24.99 in Kothamangalam to 29.15 in Palakkad with a mean of 26.99. The dry rhizome yield ranged from 13.33 g/plant in Chimmoni to 29.03 g/plant in Kuruppanthara with a mean of 23.22 g/plant. The oil content ranged from 1.40 per cent in Kothamangalam to 2.40 per cent in Muttungal with a mean of 1.90 per cent. Oleoresin content ranged from 2.66 per cent in Muttungal to 3.71 per cent in Kothamangalam with a mean of 3.21 per cent. The oleoresin of Kuruppanthara had the most pleasing aroma.

2. In kacholam, the flowering season ranged from June to August when planting was done on 5th May. The ecotypes flowered within a period of 48.0 to 68.5 days after planting. Kuruppanthara was the earliest to flower while Chimmoni and Kothamangalam were late in flowering. Kacholam inflorescence took 13.58 days from the visual initiation to flower opening and the blooming completed within 11.75 days.

3. The flowers are characterized by long style of mean length 4.52 cm ending in a spiny stigma. The ovary measured a mean length of 4.11 mm and diameter of 2.68 mm and recorded a mean ovule number of 20.0. The ovules measured a mean length of 96.33 µm and breadth of 61.16 µm at the middle. The anthesis in different kacholam ecotypes started by 4 am and continued up to 5 am. Anther dehiscence took place between 4.30 am and 5.15 am.

4. The influence of date of planting on flowering was examined by planting at monthly intervals from 15th May to 15th July. All the six ecotypes flowered when planted on 15th May with a mean percentage of flowering 61.11. The mean percentage of flowering reduced in the subsequent plantings. With the aforesaid staggered planting from 15th May to 15th July, the flowering season of kacholam could be extended up to first week of October as against the normal flowering season from June to August. The study on the influence of seed bit size on flowering showed that the large seed bit size (20-25 g) did not confer a marked advantage for flowering over the normal size of 5-10 g. It was also observed that maintaining the plants from normal seed bit size as biennials was favourable for extending the flowering season by early flowering (first week of June) in the second year.

5. The pollen grains of kacholam were sticky, white and spherical in shape. The mean pollen fertility in kacholam ecotypes as per acetocarmine stain test was 76.33 per cent.

6. Modified ME_3 medium was the best for *in vitro* pollen germination and pollen tube growth and recorded a mean pollen germination of 78.80 per cent and mean pollen tube length of 809.17 µm. The other two media, Brewbaker and Kwack's medium and the medium standardised by Rekha (1993) were also found favourable.

7. Surface sterilization procedure of flowers included cleaning of the flowers in tap water and dipping in streptocycline solution (500 mg l^{-1}) for 1 h followed by wiping with 70 per cent alcohol and finally treating with mercuric chloride (0.1 %) for three minutes inside the laminar flow. The sterilants were completely removed by three washings in sterile distilled water.

8. Occurrence of various endophytic bacteria caused difficulty in culture establishment. Four most commonly occurring bacteria were isolated and subjected to gram stain test. An *in vitro* sensitivity test revealed that the three

types of bacteria under study varied in their response to the antibiotics and chemicals tried. Fytolan (copper oxychloride) at 2500 mg l^{-1} suppressed all the three types of bacteria and the yellow coloured bacteria were suppressed in all the treatments. Fytolan (copper oxychloride) can be included in the surface sterilization procedure of the flowers and for spraying of plants in the field to reduce the microbial load.

9. Murashige and Skoog (1962) basal medium at half and full strengths along with hormones was suitable for the culture establishment of kacholam ovary but half strength MS was superior to full strength MS medium. In the basal medium of half MS with 3 per cent sucrose, cytokinins BA 1.0 to 4.0 mg l^{-1} and kinetin 1.0 to 4.0 mg l^{-1} induced ovule development singly and in combinations after *in vitro* placental pollination. Similarly, auxins NAA 0.5 to 1.0 mg l^{-1}, IAA 0.2 mg l^{-1}, IBA 0.2 mg l^{-1} and 2,4-D 0.2 to 1.0 mg l^{-1} also induced ovule development. Maximum ovule swelling was observed in cultures with hormone combinations of

   i) ½ MS + 2,4-D 0.2 mg l^{-1}

   ii) ½ MS + BA 1.0 + kinetin 1.0 mg l^{-1}

   iii) ½ MS + BA 0.5 + NAA 3.0 mg l^{-1}

   iv) ½ MS + BA 1.0 + kinetin 3.0 + 2,4-D 0.2 mg l^{-1}. Hence, these media can be taken as a template for the refinement of media requirements and for mass production of seeds to test various seed germination treatments.

10. The ovules developed at three, six, nine and 12 per cent sucrose levels after *in vitro* placental pollination in the medium of ½ MS + BA 0.5 + NAA 3.0 mg l^{-1}. Three per cent sucrose level was found superior to other levels on visual assessment of cultures.

11. Vitamin supplementation by adding the vitamin stock of MS medium in double the normal quantity was beneficial for ovule development in the medium of ½ MS + 3 % sucrose + BA 0.5 + NAA 3.0 mg l^{-1}. The organic supplements casein hydrolysate 250 mg l^{-1}, coconut water 15 per cent v/v,

yeast extract 250 mg l^{-1} and L-glutamine 100 mg l^{-1} were not favourable for the ovule development.

12. Different *in vitro* pollination techniques, stigmatic, stylar, intra- ovarian, placental, modified placental and test-tube fertilization were attempted. Pollination was done with pollen grains suspended in ME_3 medium. Ovules developed in intra ovarian, placental and modified placental pollination techniques. Among these techniques, placental pollination was found to be the best with maximum ovule swelling and highest percentage of cultures with ovule development. Ovules / seeds developed after *in vitro* pollination were creamy white during the initial stages of development and changed to brown and then darkened up to 20 DAP.

13. Pollen pistil interaction studies after *in vivo* stigmatic pollination showed pollen germination in spiny stigma but the extent of pollen tube growth in the entire style was not confirmed. The lengthy style and caducous nature of the flower may be preventing seed set *in vivo*. Pollen pistil interaction studies after *in vitro* placental pollination showed that the pollen tube growth covers the entire length of the ovules and there is chance of fertilization.

14. The small arillate seeds had two seed coats. The seed coats enclosed endosperm with embedded embryo. In the intra ovarian pollination, the ovary developed into fruit and attained a maximum size (length 5.5 mm, breadth 3.5 mm in the middle) at 20 DAP.

15. Histological studies of the ovules at 2, 5, 10 and 25 DAP revealed the developing embryo of increasing size in the embryo sac surrounded by nucellar tissue, inner integument and outer integument. This confirmed fertilization after *in vitro* placental pollination. Sections of intra ovarian pollinated ovules showed fertilized and unfertilized ovules.

16. The seeds formed after *in vitro* pollination were subjected to various *in vivo* and *in vitro* treatments. Under *in vivo* condition, various treatments like

soaking the seeds in water for 24 h, keeping on moist filter paper, acid scarification with HCl (50 %) and HNO_3 (25 %) and soaking the seeds in GA_3 100 mg l^{-1} did not bring about seed germination. Under *in vitro* condition, Knudson C medium, ½ MS liquid, ½ MS solid, Taira and Larters' modified Norstog medium, tomato embryo culture medium and the medium of ½ MS + BA 0.5 + NAA 3.0 + GA_3 1.0 mg l^{-1} with 3 per cent sucrose did not induce germination.

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IN VITRO POLLINATION IN KACHOLAM (*KAEMPFERIA GALANGA* L.) FOR SEED SET

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ABSTRACT OF THE THESIS

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Abstract

Investigations on "*In vitro* pollination in kacholam (*Kaempferia galanga* L.) for seed set" were carried out at the Department of Plantation Crops and Spices and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 1999 to 2001.

The six kacholam ecotypes under study exhibited variability in yield and quality characters and these characters remained scattered among the ecotypes.

The flowering season of kacholam ranged from June to August when planted on 5th May. The practices of staggered planting at monthly intervals from 15th May to 15th July and maintaining the crop as biennial, together could extend the flowering season up to June to October as against June to August in the normal planting. Increasing the rhizome bit size from 5-10 g to 20-25 g did not confer any marked advantage for flowering.

The study of floral biology and morphology of kacholam showed that the ecotypes took 48.0 to 68.5 days for flowering from planting. The inflorescences produced 4.0 to 11.0 flowers and the blooming period ranged from 9.3 to 14.9 days. The anthesis started by 4.00 am and continued up to 5.00 am. Anther dehiscence occurred shortly after the anthesis and took place between 4.30 am to 5.15 am. The flowers are characterized by a spiny stigma and a long style of mean length of 4.52 cm, which passes through the groove present in the only fertile stamen. The ovary measured a mean length of 4.11 mm and a diameter of 2.68 mm and recorded a mean ovule number of 20.0. The ovules recorded a mean length of 96.33 µm and breadth of 61.16 µm at the middle.

The mean pollen fertility with acetocarmine stain in the ecotypes was 76.33 per cent. The study for selection of a medium to support pollen germination and

tube growth resulted in the identification of ME_3 medium of pH 6 as the best medium while Brewbaker and Kwack's medium and the medium standardized by Rekha (1993) were also found favourable. The mean pollen viability in the ME_3 medium was 78.80 per cent.

Seed set and development was obtained in kacholam through *in vitro* pollination. The flowers were collected in the early hours of the day (6.30 to 7.30 am) on the day of anthesis. They were surface sterilized by dipping in streptocycline solution (500 mgl^{-1}) and wiping with 70 per cent alcohol followed by rinsing in mercuric chloride (0.1 %) for 3 minutes in the laminar flow. The sterilants were completely removed by three washings in sterile distilled water. Bacterial contamination interfered culture establishment. *In vitro* sensitivity studies revealed that copper oxychloride 2500 mg l^{-1} could effectively suppress gram negative white bacteria and gram positive pink and yellow bacteria occurring in the cultures. Among the various methods of pollination tried, ovary/ovules developed in intra ovarian pollination, placental pollination and modified placental pollination. Pollination was done with pollen grains suspended in ME_3 medium. The placental pollination method was found best for ovule development.

The experiments on culture establishment showed that MS medium at half and full strength supplemented with hormones can support ovary / ovule development but half MS medium was superior. The cultures varied in their hormone requirements and four media combinations were identified for ovule development.

i) ½ MS + 3 % sucrose + 2,4-D 0.2 mg l^{-1}

ii) ½ MS + 3 % sucrose + BA 1.0 + kinetin 1.0 mg l^{-1}

iii) ½ MS + 3 % sucrose + BA 0.5 + NAA 3.0 mg l^{-1}

iv) ½ MS + 3 % sucrose + BA 1.0 + kinetin 3.0 + 2,4-D 0.2 mg l^{-1}

The maximum ovule development was observed in the medium of ½ MS + 3 % sucrose + 2,4-D 0.2 mg l^{-1}. Addition of double the quantity of the vitamin

stock of the MS medium to the medium of ½ MS + 3 % sucrose + BA 0.5 + NAA 3.0 mg l^{-1} was good for ovule development. The supplements casein hydrolysate, coconut water, yeast extract and L-Glutamine did not favour ovule development.

The pollen pistil interaction studies after *in vitro* placental pollination showed that pollen tube growth is sufficient to cover the entire length of the ovule. Histological examination of placental pollinated ovules at various stages from 2 DAP to 25 DAP showed embryo of increasing size. This confirmed fertilization in placental pollination. The pollinated ovules developed into dark brown arillate seeds at 20 DAP. The size of the seeds was more than 10 times the size of the ovules. The kacholam ovary after intra-ovarian pollination developed into a thick walled capsule with light brown seeds but the size increase was not substantial.

The small arillate seeds had an outer thick seed coat and an inner thin seed coat. The seed coat enclosed a cavity, which is typical of monocots. In the cavity, endosperm with embedded embryo was seen.

The seeds developed after *in vitro* pollination did not germinate even after subjecting to various *in vivo* and *in vitro* germination treatments.