CYTOGENETIC ANALYSIS IN KACHOLAM, *Kaempferia galanga* L.

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "**Cytogenetic analysis in Kacholam, Kaempferia galanga L.**" is a bonafide record of work done by me during the course of research work and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Certified that this thesis is a record of research work done independently by **Miss.Rekha, K.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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CERTIFICATE

We the undersigned, members of the Advisory Committee of **Miss.Rekha, K.,** a candidate for the degree of Master of Science in Agriculture with major in Plant Breeding and Genetics, agree that the thesis entitled **"Cytogenetic analysis in Kacholam, Kaempferia galanga L."** may be submitted by Miss.Rekha,K., in partial fulfilment of the requirements for the degree.

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INTRODUCTION

Kaempferia galanga L. popularly known as kacholam is an important medicinal plant belonging to the genus Kaempferia of the family Zingiberaceae. The genus is widely distributed in the tropics and subtropics of Asia and Africa and in Philippine Islands. Of the 55 species reported in the genus only 10 are known in India among which, K. galanga L. and K. rotunda L. are the economically important ones.

Rhizomes of Kaempferia galanga L. are used in bulk quantities in ayurvedic medicines and agarbathi industry. It finds an important place in indigenous medicine as a stimulant, expectorant, diuretic and carminative. Powdered rhizome mixed with honey is administered against coughs and, decoction of the rhizome is prescribed for dyspepsia headache, rheumatism and malaria. It is used in hairwashes because of its antidandruff property. The rhizome is also used in the curing of inflammatory wounds. The rhizome yields 2.40-3.84 per cent volatile oil the active ingredients of which are n-pentadecane and ethyl-p-methoxy-cinnamate. The oil is utilised in the manufacture of perfumes and curry flavourings. Antifungal and larvicidal properties are also reported for this plant. Recently some anticancerous principles have also been identified in Kaempferia galanga L. from Japan.

Because of these multivarious desirable attributes the export value of this crop increased rapidly in the recent years. One quintal of dried rhizomes fetch a market price of about 20,000-30,000 rupees. The simple cultivation practices and the suitability for intercropping in coconut gardens have attracted a good number of farmers in Kerala for undertaking the cultivation of kacholam. Acute scarcity is hence being felt for quality planting material.

Systematic breeding programmes have not been undertaken in this crop mainly because it has gained importance only in recent years. No promising varieties have been reported in kacholam and the types available with farmers are only local selections. Improvement of this crop by conventional methods of breeding is also not possible for want of seed production. Seedlessness also contributes to the limited variability available with the crop.

In any crop cytogenetical studies serve as an essential prelude to scientific crop improvement. Such studies have been successful in many crop plants to understand the phylogeny, evolution and domestication of various species. Improvement of Kaempferia galanga L. requires thorough elucidation of cytogenetical details and our present knowledge on this aspect is not complete. Various authors have reported diverse chromosome numbers for this species. The reports available on basic chromosome number as well as level of ploidy are also conflicting. Eventhough seedlessness is the main hurdle for breeding of this crop, no serious attempts

have been made to elucidate the various factors contributing to the lack of seed production.

Being a crop with tremendous potential for the present, cytogenetic analysis in kacholam (Kaempferia galanga L.) was undertaken with the following objectives:

To confirm the chromosome number by mitotic and meiotic studies and to work out the karyomorphology of Kaempferia galanga L.

To find out the reasons for seedlessness in the crop by detailed studies on cytology, floral biology, pollen morphology and pollination mechanisms leading to fertilization.

Review of Literature

REVIEW OF LITERATURE

2.1 Origin, distribution and taxonomy

The genus Kaempferia is supposed to have been originated in South East Asia, probably in Burma, where it is considered to have been grown as an evergreen in shady forest conditions and from there, it appears to have migrated across most of the tropical Asia, and right across 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.

Kaempferia galanga L. is a monocotyledonous plant, belonging to the family Zingiberaceae of the order Zingiberales. It comes under the series Epigynae (Bentham and Hooker, 1894). According to Willis (1960) the family Zingiberaceae, contains 45 genera and about 800 species. According to Hutchinson (1934), there are four tribes in the family, viz., Costea, Hedychia, Globeae and Zingiberea. Panchaksharappa (1966) opined that among the families of Scitaminales, Zingiberaceae consists about 47 genera and 1400 species.

Schumann (1904) divided the family Zingiberaceae into two subfamilies, viz., Zingiberoideae and Costoideae. The Zingiberoideae

is further divided into three tribes viz., Globeae, Hedychiae and Zingibereae. The genus Kaempferia comes under the tribe Hedychieae. Hooker (1892) recognised four sub-genera under the genus Kaempferia namely Sincorus, Protanthium, Monolophus and Stachyanthesis. Among this, K. galanga L. comes under the subgenera Sincorus. The subgenera Sincorus includes 11 species other than K. galanga L. They are Kaempferia marginata, K. angustifolia, K. ovalifolia, K. speciosa, K. pandurata, K. prainiana, K. roscoeana, K. parviflora, K. involucrata, K. andersoni and K. cocinna. The only variety reported in K. galanga L. is K. latifolia.

2.2 Morphology, propagation and cultivation aspects

According to Hooker (1892) Kaempferia is a plant with tuberous root stock. Leaves are three to six inches long, spreading flat on the ground, deep green in colour with deltoid tip. Leaf margin is not thickened or coloured. Petioles are short and channelled. Kirthikar and Basu (1935) described the morphology of K. galanga L. as follows. The plant is a stemless herb, with tuberous, aromatic root stock which possess fleshy cylindric nonaromatic root fibres. Leaves are horizontally spreading and lying flat on the surface of the ground and having a length of approximately 6.3 - 12.5 cm and a breadth of 4.5 - 9.0 cm. They are deep green in colour, rotund, ovate, deltoid and acuminate. Leaf margins are neither thickened, nor coloured. Flowers arise

from the centre of the plant between the leaves. Drury (1978) described K. galanga L. as a plant with biennial tuberous rhizome, stemless stalked leaves, spreading flat on the surface of the earth, which are either ovate, rotund or cordate in shape. Leaf margins are membraneous and wavy. Biswas and Chopra (1982) described K. rotunda L. as a perennial stemless herb, about one foot high. Root stock is tuberous with very thick root fibers. Leaves are oblong and erect, with short channelled petiole.

Gopalaswamiengar (1951) reported that propagation of Kaempferia is by the division of the rhizome and it can be potted in light soil and that liquid manure promotes its growth. The Aromatic and Medicinal Plants Research Station, Odakkali has undertaken a study to find out the response of K. galanga L. to different spacing and levels of FYM. Preliminary trials have shown that a spacing of 20 x 15 cm and application of FYM at the rate of 30 tonnes per hectare gave maximum rhizome yield (Annual Report, 1982). Rajagopalan (1983) carried out an investigation to standardise the propagation method, planting time and harvesting time of K. galanga L. and reported that mother rhizomes planted during the third week of May and harvested after six months, were significantly superior.

Phytochemical analysis and nutrient uptake studies on K. galanga L. conducted by Rajagopalan et al. (1989) revealed that mother rhizome planted during the 3rd week of May and harvested

at six months maturity recorded the maximum essential oil and oleoresin yield. The mean nutrient uptake by the crop was 22.8 kg N, 28 kg P_2O_5 and 36.9 kg K_2O per ha.

A micropropagation trial on Kaempferia galanga L. was conducted by Vincent et al. (1992) and they reported that the axillary bud explant of K. galanga L. had the potential to induce multiple shoots as well as roots in the medium containing BA alone or BA + Kinetin. The mortality rate of tissue culture derived plants was low and about 90 per cent plants survived on transfer to soil. These results demonstrated that, K. galanga L. can be micropropagated easily.

An experiment was conducted to study the effect of gamma irradiation on Kaempferia galanga L. by Viswanathan et al. (1992) and it has been found that irradiation treatments at lower dosages viz., 0.5, 0.75 and 1.0 K rad produce stimulatory effect on the germination of Kaempferia rhizome. Inhibitory effect on germination was observed at higher doses. LD 50 was found to be 2.5 K rad and no germination was observed at 10.0 K rad and above. Bushy type mutants were noticed in 7.5 K rad and they reported that it can be used as an ideal raw material for crop improvement programmes of Kaempferia.

2.3 Economic importance of the crop

Medicinal properties of Kaempferia galanga L. have been

described by many workers. The herb is used as a flavouring for rice. Rhizomes and leaves are employed as a perfume in hair washes, powders and other cosmetics. They are used by women for fragrance and also used for protecting cloths against insects. They are also eaten along with betel as a masticatory (Burkill, 1935 and Quisumbing, 1951).

The rhizome is used as a stimulant, expectorant, carminative and diuretic. They are used in the preparations of gargle. They are administered with honey in coughs and pectoral affections. In Philippines, a decoction of rhizome is used for dyspepsia, headache and malaria. The rhizomes, boiled in oil, are applied externally, to remove nasal obstruction. Roasted rhizomes are applied hot for rheumatism and for hastening the ripening of inflamatory tumours. They are also used as a wash in dandruff and for relieving irritation produced by stinging caterpillars. Mixed with oil, rhizomes are used as a cicatricant. In Malaya, they are used for chills in elephants. The juice of the plant is an ingredient of some tonic preparations. The leaves are used in lotions and poultrices for sore eye, sore throat, swelling, rheumatism and fevers (Kirthikar and Basu, 1935; Burkill, 1935; Brown, 1941 and Quisumbing, 1951). Recently larvicidal and anti-cancerous principles have been obtained from the rhizome extract of K. galanga L. (Kiuchi et al., 1988; Kosuga et al., 1985). The hot water extract of Kaempferia rhizome showed strong larvicidal

activity against the larvae of dog round worm Toxocara canis (Kiuchi et al., 1985).

2.4 Chemical composition of rhizome

Panicker et al. (1926) analysed the dried and powdered rhizome and found that it contains 2.4 - 3.88 per cent of a volatile oil. The tuberous rhizome possesses a camphoracious odour with somewhat bitter aromatic taste resembling that of Hedychium spicatum. The compounds such as n-pentadecane, ethyl-P-methoxy cinnamate, ethyl cinnamate, carene, camphene, borneol and p-methoxy styrene are reported to be present in the volatile oil (Guenther, 1975; Panicker et al., 1926). Pillai and Warrier (1962) conducted investigations on the aromatic resources of Kerala and reported that petroleum ether extract of the tubers of K. galanga L. contained 2.05 per cent of ethyl-p-methoxy cinnamate and 2.87 per cent of a residual essential oil.

Seven species of Kaempferia such as K. galanga L., K. parviflora, K. angulstifolia and K. rotunda L. and three other unnamed Kaempferia species have been studied by Tuntiwachwuttikul (1991) and reported 4 major classes of chemicals viz., cinnamate esters, flavanoids, diterpenoids and cyclohexane oxide derivatives. Tuntiwachwuttikul et al. (1992) again studied 4 species of Kaempferia such as K. parviflora, K. angustifolia and 2 unnamed

Kaempferia species and reported that the rhizomes of K. parviflora yielded sixteen flavanoids. The major constituent, 5,7 - dimethoxy flavone was found to be anti-inflamatory and the activity was comparable to aspirin. Work on K. anquistifolia and on unnamed Kaempferia species lead to the isolation of the two cyclohexane diepoxide analogues.

An attempt has been made to compare some of the gene products in 2 monotypes of K. galanga L. viz., prohom and jangang using isozyme eletrophoresis by Suvachittanom (1991) and he found out that prohom showed three superoxide dismutase bands, whereas jangang showed only two. With esterase, prohom showed 2 bands while jangang showed only one. Based on these findings, he suggested that the 2 types should be regarded as different species since they exhibit different gene products.

2.5 Cytogenetics

The chromosome number of Kaempferia has been studied only by a few workers and the reports available are conflicting. Since the literature available on this crop is scanty, the reports available on related crops also are included in this section.

Raghavan and Venkatasubhan (1943) studied the cytology of 4 species of Kaempferia viz., K. gibsonii, K. gilbertii, K. rotunda L. and K. galanga L. and reported that they showed a regular polyploid series having chromosome number 24, 36, 54

and 54 respectively. Since the chromosome numbers are all multiples of 6, he ascertained '6' as the basic chromosome number for the genus. Eventhough there were difference in chromosome numbers among the 4 species, the chromosomes in all the 4 were fairly alike and the size of the chromosomes were more or less the same. Hence they reported that the increase in chromosome number in this genus is not by the diminution of the size of the chromosome. In addition to this, Raghavan and Venkatasubhan (1943) could find 2 chromosomes with subterminal constriction in K. gibsonii and a pair of SAT chromosomes in K. rotunda L. Based on these findings they concluded that, the speciation in this family is due to polyploidy, aneuploidy and structural changes. Raghavan and Arora (1958) assigned a chromosome number of 2n = 54 for K. galanga L. Chakravorthi (1948) studied the cytology of 5 species of Kaempferia such as K. gibsonii, K. gilbertii, K. rotunda L., K. cienkowskya and K. atrovirens and opined that there is a clear evidence of polyploidy. He describes K. atrovirens as a diploid species showing normal pairing and 11 bivalents. For K. rotunda L. and K. gilbertii he assigned a chromosome number of 2n = 33, and considered them as triploids. He again found chromosome number of 2n = 28 for K. cienkowskya and concluded that the genus Kaempferia had all the probabilities to have two distinct polyploid series, based on x = 11 and x = 14. Eventhough Sharma and Bhattacharya (1959) reported a chromosome number of 22 for K. galanga L, Ramachandran (1969) had

the same opinion as Raghavan and Venkatasubhan (1943). Ramachandran studied the chromosome numbers of 27 species belonging to 11 genera of the family Zingiberaceae and reported a chromosome number of 2n = 44 for K. rotunda L. and 2n = 54 of K. galanga L. According to him K. galanga L. is presumably and aneuploid pentaploid. Chromosome numbers of 56 species including 14 species of Kaempferia were studied by Mahanthy (1971) and he described the chromosomes in detail. He found that the 2n number of K. anguistifolia is 22 and there were 3 pairs of long chromosomes and 5 pairs of medium length chromosomes and three pairs of short chromosomes. All the long chromosomes have their primary constriction in a sub-median position. Among the median chromosomes, one pair had subterminal constriction, 2 pairs showed a median constriction and other two had a submedian constriction. All the short chromosomes showed submedian primary constriction. In Kaempferia brachystemon the somatic number was found to be 2n = 26. A similar species K. ethelae having a chromosome number of 2n = 26 is characterised by the abscence of a pair of SAT chromosomes which was very prominent in African Kaempferias. It is also noticeable that these 26 chromosomes consist of 2 sets of 13 each which is also intermediate between Asiatic and African Kaempferias. A chromosome number of 2n = 22 had been assigned for the species K. elegans and K. rosea and also for an unnamed species. For another species of Kaempferia, a chromosome number of 2n = 42 was also reported. For K. gilbertii and K. rotunda.L.

he reported a chromosome number of 2n = 33. Based on the studies of floral morphology, geographical distribution and cytology the author suggested that the African representatives of Kaempferia should be assigned the status of a separate genus, Cienkowskya, and also the basic chromosome number x = 11 can be considered as the original one for zingiberales as a whole.

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

Many cytogeniticists confirmed the chromosome number of Zingiber officinale as 2n = 22. Eventhough Takahashi (1931) reported a chromosome number of 2n = 24 for the species, Raghavan and Venkatasubhan (1943) worked on the cytology of Zingiber officinale Rosc., Z. cassumunar Rose. and Z. zerumbet and reported that all of them had a somatic chromosome number of 2n = 22. They also found difference in the chromosome morphology in these species and concluded that, the chromosomes of Z. officinale were different from that of the other 2 species in morphology. Darlington and Janakiammal (1945) observed 2 B chromosomes in certain types of Z. officinale, in addition to the normal complement of 2n = 22. Chakravorti (1948) concluded that, in view of the bivalent association in diploid species Z. cassumunar and Z. zerumebt, Z. mioga Rose. with 2n = 55

chromosomes has to be considered as a pentaploid. Sato (1960) also emphasized that, Z. mioga with 2n = 55 is to be considered as a pentaploid with basic number x = 11. Sharma and Bhattacharya (1959) reported widespread occurrence of inconsistency in chromosome number, in several species of Zingiberaceae, including Z. officinale. Ramachandran (1969) reported the cytology of five species of Zingiber, including Z. officinale, Z. macrostachyum, Z. roseum, Z. whitianum and Z. zerumbet and found out that, meiosis was normal in the last three, where as, bridges and laggards were observed in Z. officinale and Z. macrostachyum. He found evidence for structural hybridity involving interchanges and inversions in Z. officinale. Mahanthy (1971) in an extensive cytological investigation in Zingiberaceae reported chromosome number of 2n = 22 for Z. spectable Griff and Z. cylindricum. Suzuka and Mitsuoka (1978) conducted cytological studies in Zingiber mioga and reported it as an autopentaploid with 2n = 55. Pillai et al. (1978) observed meiosis as highly irregular in ginger, with only 46.6 per cent of the PMCs showing bivalents and the rest showing univalents, trivalents and quadrivalents.

Karyomorphology of 32 cultivars of Z. officinale and three species of Zingiber viz., Z. zerumbet, Z. macrostachyum and Z. cassumunar were investigated by Ratnambal (1979). She found considerable difference in gross morphology of chromosomes, based on the studies of karyotype of 13 genera, including 24 species.

A classification of karyotype, according to degree of asymmetry in Zingiberaceae showed that, karyotype '1b' was represented in most of the cultivars of Z. officinale while '1a' was found in Z. zerumbet, Z. macrostachyum and Z. casumunar. Ratnambal et al. (1983) observed the formation of quadrivalent in most of the cultivars of Z. officinale. Ramachandran (1983) found that tetraploids 2n = 44 could be produced in ginger, by colchicine treatment. Beltran and Kam (1984) reported the somatic chromosome number of 33 species including nine ginger species and noticed aneuploidy (2n = 24), polyploidy (2n = 26) and B chromosomes (22 + 2B) in Z. officinale. They also remarked that Indian and Malayan Zingiber species were diploids while the Japanese species Z. mioga was a pentaploid with 2n = 55. Dutta and Biswas (1985) observed cytological irregularities such as fragments and ring formations in Z. officinale. Omanakumari and Mathew (1985) presented a detailed karyotypic data on Z. officinale, Z. zerumbet, Z. macrostachyum all having 2n = 22 chromosomes. Cytology of nine ginger varieties was studied by Sathiabhama (1988) and it was observed that all the nine varieties have a somatic chromosome number of 2n = 22. She also reported that the chromosomes showed difference in morphological features among the cultivars. Bridges and laggards during anaphase of meiosis was also reported.

Sugiura (1936) reported, a somatic chromosome number of 2n = 64 for Curcuma longa. Chromosome numbers of 24 species

in the family Zingiberaceae, including that of C. aromatica, C. amada and C. longa were studied by Raghavan and Venkatasubhan (1943) and reported that, C. aromatica and C. amada had 2n = 42 whereas C. longa and C. zedoaria had 63 somatic chromosomes. They also reported that, C. longa and C. zedoaria were triploids and the chromosome numbers such as 62, 64 etc. are found occassionally, due to somatic aberrations. Cytology of six species of curcuma and seven cultivars of C. longa was reported by Ramachandran (1961). A chromosome number of 2n = 86 for C. aromatica was also reported by him and he concluded that the species is a tetraploid. He also studied in detail, the meiosis of two species C. decipiens (2n = 42) and C. longa (2n = 63) and concluded that, the sterility in C. longa was probably due to its autotriploid nature. C. decipiens showed normal bivalents at metaphase 1, whereas in C. longa a higher percentage of trivalent association was found, indicating the presence of autotriploidy. A further report on the chromosome numbers in Zingiberaceae and genus Curcuma was published by Ramachandran (1969), wherein he reported the chromosome number for C. amada (2n = 20, 42), C. decipiens (n = 21; 2n = 42), C. neilgherrensis (2n = 42), C. aromatica (2n = 63, 86), C. longa (2n = 63) and C. zedoaria (2n = 63). A high basic number of n = 21 was assumed for Hitchenia and Curcuma. Ramachandran (1969) concluded that, the genus Curcuma might have been derived either by dibasic

amphidiploidy [x = (9+12)] or by secondary polyploidy. Microsporogenesis and megasporogenesis in C. aurantiaea and C. lorgengii were reported by Sastrapradja and Aminah (1970). Fruit set was observed only in C. aurantiaea by these workers and they concluded that, the abscence of fruit set in C. lorgengii was deu to pollen abortion. Detailed cytological investigation by Nambiar (1979) revealed that, all cultivars of C. aromatica had somatic chromosome numbers of 2n = 84 and C. longa had a chromosome number of 2n = 63. This along with 2n = 42 for C. amada, indicated a polyploid series, with multiples of n = 21. Nambiar (1979) presumed that the earlier reports of chromosome numbers viz., 2n = 32, 62 and 64 for C. longa and 42, 63 and 86 for C. aromatica were probably exceptional cases and the correct chromosome numbers for these species are 2n = 63 and 2n = 84 respectively.

2.8 Flowering

In Kaempferia the inflorescence is reported to be a short scape (Gamble, 1926). The floral morphology has been described by a few workers (Hooker, 1892; Kirthikar and Basu, 1935 and Drury, 1978). According to them, six to 12 flowers are produced from an inflorescence which is situated within the sheath of the leaves. Flowers open successively and are fugacious and fragrant, with three lanceolate bracts. They are white in colour with a purple spot in the centre of each of the division of the inner

series. Calyx is having the same length as that of the bracts. Corolla tube is very long with filiform stigma. Both the essential whorls are trimerous. There are 2 lateral staminodes which are cuneate, obovate and are situated at the base of the ovary. Floral characters of K. rotunda L. also have been described by many workers (Hooker, 1892; Gamble, 1926; Kirthikar and Basu, 1935). According to them in this, flowers are born on radical scapes one cm long with spreading linear petals nearly as long as the tube. Staminodes are oblong, acute, white in colour and having a length of about 3.8 - 5.0 cm, lip is lilac or reddish in colour, and is bifid. Anther crest is deeply bifid and anther lobes are lanceolate.

Rajagopalan (1983) described the flowering behaviour in K. galanga L. He observed that flowering started in June and ended in September and the peak occurred during July-August. It was also reported that flowers are produced directly from the rhizome and they open in succession.

In a related crop ginger, very rare flowering was reported by Hooker (1892). Holtum (1950) stated that flowering in ginger occur under certain conditions only. Pillai et al. (1978) reported that, of the 35 germplasm collections maintained at CPCRI, all but 6 flowered and that flowering started in October and lasted till early December, the peak being in November.

Jayachandran et al. (1979) reported that it took 20-25 days from the bud initiation to full bloom and that a period of 23-28 days was required for the completion of blooming in an inflorescence in ginger. However, Usha (1983) estimated that it took 29 days from the bud initiation to full bloom and about nine to 18 days for the completion of blooming in an inflorescence in ginger. She also reported that flower opening is in an acropetal succession and that the flowers fall off the next day of anthesis. Pillai et al. (1978) observed that anther dehiscence take place simultaneously with the flower opening.

Patnaik et al. (1960) studied the floral biology of turmeric and reported that the first flush of flowering in a spike is generally completed within five to 12 days. Opening of flowers takes place in the morning between 6.00 - 6.30 a.m. at a temperature of about 24°C, during which period the humidity of the atmosphere remains above 70 per cent in the locality. Dehiscence of anthers takes place just at the time of flower opening and pollination is observed to be brought about by insects.

2.7 Pollen studies

No previous reports on pollen characterisation is available in Kaempferia. However similar studies in ginger are presented below.

Pillai et al. (1978) reported that pollen grains in ginger are heteromorphic, round and with a diameter ranging from 77 to 104 µm, the average being 91 µm. A striking feature observed by the workers was the very thick exine of pollen grain. Jayachandran et al. (1979) stated that pollen grains are spherical with a size ranging from 90 µm - 100 µm, the mean being 95.5 µm. Jayachandran et al. (1979) also mentioned that anther lobes in ginger are filled with plenty of pollen grains. However, Usha (1983) estimated pollen production per flower in ginger as 1,87,500 in variety Rio-de-Janeiro and 1,91,500 in the variety Maran. According to her, the inadequacy of pollen grains was not a limiting factor in ginger breeding. Sathiabhama (1988) also reported that ginger flower produced sufficient pollen, but a high percentage of pollen sterility has been observed. Pillai et al. (1978) reported 35 per cent fertility while Usha (1983) reported only 12.48 per cent in Rio-de-Janeiro and 16.42 per cent in Maran.

Germination of the pollen grains in ginger has been found to be very low by different investigators. Nair et al. (1975) reported that pollen grains of varieties like Rio-de-Janeiro, Wynad local, Maran, Burdwan and Assam germinated in a media containing 15 per cent sucrose, 300 ppm calcium nitrate, 100 ppm boric acid, 200 ppm potassium nitrate, 100 ppm magnesium nitrate and 1 per cent agar. However, germination was less than 1.6 per cent. Pillai et al. (1978) found that addition of boric acid was helpful in

breaking the exine and thereby achieving pollen germination. Of the different media tried the one containing 8 per cent sucrose, 3 per cent gelatin, 60 ppm boric acid and kept in a moist chamber at 26°C gave maximum germination of 14.5 per cent. Jayachandran et al. (1979) observed pollen sterility as high as 76 per cent. They obtained only 2.5 per cent germination in dextrose agar medium. Among the various media tried 8 per cent sucrose with 3 per cent gelatin and 60 ppm boric acid gave maximum pollen germination of 6.2 per cent. Sathiabhama (1988) reported 60-80 per cent pollen sterility in ginger. Pollen germination was also poor and was only 11.8 per cent in a medium containing 8 per cent sucrose, 60 ppm boric acid and 1 per cent gelatin.

No reports on seed set are available so far both in ginger and Kaempferia galanga L. However in Kaempferia rotunda L. fruiting in each rains in Himalayas was reported by Biswas and Chopra (1982). Pillai et al. (1978) reported that flower structure of ginger manifests an adaptation suitable for entomophily. Hand pollination using large quantities of pollen grains in variety Rio-de-Janeiro by Jayachandran et al. (1979) and Usha (1983) could not achieve seed set. According to Hooker (1892) ginger is a species never setting seed. East (1940) and Fryxell (1957) suspected that the lack of seed set may be due to self incompatibility. Ramachandran (1961) and Ratnambal (1979) remarked that chromosomal aberrations

were responsible for lack of seed set in ginger. Pillai et al. (1978) suspected three possible reasons for the abscence of seed set viz., defects in micro and megasporogenesis, lack of suitable pollinating agents and failure of pollen germination on stigma or due to incompatibility. Jayachandran and Vijayagopal (1979) reported that in the event of incompatibility the inhibitory action may not be located on the stigma, on the other hand, Usha (1983) was of the opinion that incompatability reaction may not be the factor causing failure of seed set in ginger, as she failed to get seed by bud pollination or with stigma and style removal. Sathiabhama (1988) conducted an experiment using fluorescent microscopy in order to study the pollen tube growth through the style. But no growth of pollen tube was observed in the style.

Materials and Methods

MATERIALS AND METHODS

The present study on cytogenetics of Kaempferia galanga L. in relation to seed set was undertaken at the Department of Agricultural Botany, College of Horticulture, Vellanikkara during the period 1991-93. A local selection 'Vellanikkara' grown in the garden of AICRP on Medicinal and Aromatic Plants, was used for the study (Plate 1 and 2).

3.1 Mitotic studies

In order to find out the chromosome number of Kaempferia, mitotic studies were carried out using root tip squash method.

3.1.1 Standardisation of the procedure for mitotic studies

3.1.1.1 Time of collection of roots for mitotic studies

Since the number of dividing cells was found to be varying, the time of collection of roots giving the highest mitotic index was found out. For this, roots were collected in the active growth stage during the months of May and June, at two hours interval throughout the full day cycle of 24 hours and fixed in Carnoy's fluid. The fixed roots were washed thoroughly and stained with Feulgen stain for 45 minutes after hydrolysis. The number of dividing cells in 25 fields were counted and the mitotic index was calculated by the formula.

Plate 1. A flowering plant of Kaempferia galanga L.

Plate 2. A view of the kacholam field

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

3.1.1.2 Pre-treatmens, fixatives and stains

Different pre-treatment agents, fixatives and stains were tried so as to find out the best suited among them for obtaining good cytological preparations. The four pre-treatment agents tried were paradichlorobenzene, 8-hydroxy quinoline, colchicine and α-bromonaphthalene. Duration of pre-treatment varied from 1 to 4 hours. Different fixatives viz., acetic-alcohol (1:3), Carnoy's fluid and Ostergren and Heneens fixative were tried for the present study.

The three different stains tried were acetocarmine, Feulgen and Snow's carmine. The above mentioned pre-treatments, fixatives and stains were used in different combinations, the details are presented in the Tables 1, 2 and 3. The duration of different treatments also varied. The best combination was used for further studies. The procedure adopted is discribed as follows.

3.1.2 Karyotype analysis

For karyotype analysis squash preparations were made as follows. Young and actively growing roots were excised after 2 weeks of planting. They were washed to remove the adhering soil particles and pre-treated in saturated aqueous solution of α-bromonaphthalene for a period of four hours at 4°C. The pre-

Table 1. The pre-treatments, fixatives and stains used for mitotic studies in kacholam (Kaempferia galanga L.)

*Pre-treatment	Fixative	Stains
1. Para-dichlorobenzene (saturated aqueous solution)	Acetic alcohol (3:1)	Acetocarmine Feulgen
	Carnoy's fluid II	Acetocarmine Feulgen
2. 8-hydroxyquinoline (saturated aqueous solution)	Acetic alcohol (3:1)	Acetocarmine Feulgen
	Carnoy's fluid II	Acetocarmine Feulgen
3. Colchicine (1 per cent)	Acetic alcohol (3:1)	Acetocarmine Feulgen
	Carnoy's fluid II	Feulgen Acetocarmine
	Ostergren and Heneen's fixative	Acetocarmine Feulgen
4. α-bromonaphthalene (saturated aqueons solution)	Acetic alcohol (3:1)	Acetocarmine Feulgen Snows carmine
	Carnoy's fluid II	Acetocarmine Feulgen Snows carmine
	Ostergren and Heneen's fixative	Acetocarmine Feulgen Snows carmine

* Each pre-treatment was tried for different durations ranging from 1-4 hours

Table 2. The composition of different fixatives used for mitotic studies in kacholam (Kaempferia galanga L.)

Fixatives	Composition
1. Acetic alcohol 1:3	1 part acetic acid + 3 parts ethyl alcohol
2. Carnoy's fluid	1 part acetic acid + 3 parts chloroform + 6 parts ethyl alcohol
3. Ostergren and Heneen's Fixative (1963)	Methanol - 60 ml Chloroform - 30 ml Distilled water - 20 ml Picric acid - 1 g Mercuric chloride - 1 g

Table 3. Preparation of the stains used for mitotic studies in kacholam (Kaempferia galanga L.)

Stain	Components		Preparation
1. Acetocarmine 1%	Carmine powder 45% acetic acid	1 gm 100 ml	Hundred ml of 45 per cent acetic acid was heated in a conical flask until it boiled. Then 1 gm of carmine powder was added to it with constant stirring. Boiling was continued for 2-3 minutes until the dye got dissolved and the colour changed to grape red. The stain was cooled to room temperature, filtered and stored in a glass stoppered bottle (Sharma and Sharma, 1980).
2. Feulgen reagent	Basic fuchsin 1N HCl Potassium meta-bisulphate Activated charcoal Distilled water	1 gm 30 ml 3 gm 2 gm 100 ml	Dissolved 1 gm of basic fuchsin gradually in 100 ml boiling distilled water. Cooled to 58°C filtered and again cooled upto 26°C. Thirty ml 1N HCl and 3 gm potassium metabisulphite were added followed by vigorous shaking for three minutes, the bottle was sealed well, wrapped with black paper and kept overnight in a cool dark chamber. Next day the stain was taken and the colour was observed. If straw yellow coloured, it was used as such and if otherwise, 2 gm charcoal was added, shaked vigorously and filtered. The stain was kept in dark coloured bottle in a refrigerator (Sharma and Sharma, 1980).
3 Snow's carmine	Carmine 85% alcohol Concentrated HCl Distilled water	4 gm 95 ml 1 ml 15 ml	Four gm of carmine powder was dissolved in 15 ml boiling distilled water to which 1 ml of concentrated HCl had been added. Then 95 ml of 85 per cent ethyl alcohol was added to it. The stain was then cooled and stored in tightly stoppered bottles in order to prevent the evaporation of the alcohol (Snow, R., 1963)

treated roots were washed thoroughly in four to five changes of distilled water and fixed in Carnoy's fluid (6:3:1) to which a little ferric acetate had been added. After 24 hours the roots were washed thoroughly and drained on a filter paper and stained in Snow's Carmine for 24 hours. A liberal quantity of stain, i.e., about two to three times as much as needed to cover the material was used for getting good penetration. The excess stain was poured back into the stock bottle. The stained roots were squashed in 45 per cent acetic acid. After keeping the coverglass, the slide was warmed slightly and pressed under the folds of a filter paper. The slides were sealed with nail polish and screened for mitotic chromosomes. Camera lucida drawings of the good plates were made and photomicrographs were taken using an orthoplan automatic camera unit.

Observations

Number of chromosomes from 25 well spread cells at metaphase stage were counted and the somatic chromosome number in this crop was confirmed. Camera lucida drawings and photomicrographs were also utilised for further confirmation of the number.

Karyomorphological measurements were recorded from photographs of 5 well spread cells, at metaphase stage. Care was taken to measure the chromosome from cells at identical stage. Measurements of length of long arm (l), short arm (s) and satellite of individual chromosomes were tabulated and identical chromosomes

were identified. All the measurements were converted to microns and arm ratio i.e., long arm/short arm (l/s) for each chromosome was calculated. The chromosomes were classified into the following groups (Giorgi and Bozzini, 1969) based on the position of centromere and presence or absence of satellite.

SAT - Satellite chromosome - the arm ratio of which is calculated by leaving out the satellite length

M - Median chromosome - the arm ratio of which is between 1.00 - 1.25

SM - Submedian chromosome - the arm ratio of which is between 1.26 - 1.75

ST - Subterminal chromosome - the arm ratio of which is 1.76 and above

Total chromatin length (TCL) of the basic complement and also the relative chromosome length which is expressed as the percentage of individual chromosome length over the total length of basic complement were estimated. Average chromosome length (ACL) was also calculated.

Categorisation of karyotype asymmetry of somatic complement was made according to the method of Stebbins (1958). Stebbins classified the karyotype under 12 classes, taking into account, both the position of the centromere in the chromosome and the

degree of difference between the largest and smallest chromosomes of a complement.

Table 4. Stebbins classification (1958)

Largest chromosome length / Smallest chromosome length	Proportion of chromosomes with arm ratio more than 2:1			
	0.00	0.01-0.50	0.50-0.99	1.00
2:1	1a	2a	3a	4a
2:1 - 4:1	1b	2b	3b	4b
4:1	1c	2c	3c	4c

Total form per cent (TF %) was calculated, using the formula given by Huziwara (1962).

$$TF\ \% = \frac{\text{Total sum of short arm length}}{\text{Total chromosome length}}$$

3.1.3 Karyotype and Idiogram

Photographs of well spread cells were used for the preparation of karyotype. The chromosome figures were cut and arranged in an order according to their lengths. Chromosomes having equal length and arm ratio were pasted together.

Idiogram was also constructed for this crop. Chromosomes were designated as 1 to 11 in the decreasing order of size and increasing order of asymmetry. Idiograms based on actual length

of the chromosome and also based on the relative length of chromosomes were drawn.

Plants were raised in the field during May 1993 to August 1993 adopting routine cultural operations. Profuse flowering was observed in July - August. During this period meiotic studies were carried out and detailed studies on floral morphology were also undertaken, so as to investigate the reasons for seedlessness in this crop.

3.2 Meiotic studies

3.2.1 Standardisation of time of fixation of flower buds

From the preliminary observations it was found that microsporogenesis is highly dependant on temperature. In order to standardise the time of fixation of buds they were collected at two hour intervals during the day time between six a.m. and six p.m. After 24 hours of fixation smear preparation were made and ideal time for fixation was standardised based on division of the pollen mother cells (PMCs).

3.2.2 Standardisation of maturity and size of buds

Inflorescence in Kaempferia is a radical scape, young flower buds being concealed in the leaf sheath and remaining underground until anthesis. Hence it is not possible to pluck single buds. So the whole inflorescence was scooped out and

fixed. Inflorescences at different stages of maturity from different plants were fixed and examined. The emergence of the first flower bud above the soil level indicated initiation of flowering in this crop.

3.2.3 Studies on meiotic chromosomes

Meiotic studies were carried out following routine fixation and staining technique as described below.

Flower buds were collected between 11 a.m. and 12 noon and fixed in acetic alcohol (1:3) for 24 hours.

The single anther was dissected out from the bud, kept on a clean slide and smeared well in a drop of 1 per cent aceto-carmine to which a little ferric acetate had been added. Coverglass was placed and the slide was gently warmed and pressed under the folds of the filter paper. The slides were examined under the microscope and those with dividing cells were sealed well using nail polish and examined for the meiotic chromosomes. Photo-micrographs of different stages of meiosis were taken using an orthoplan automatic camera unit.

The excess buds fixed were stored in 70 per cent ethyl alcohol for future use.

Observations

Camera lucida drawings were made from 25 well spread PMCs and observations were recorded.

1) Chromosome associations like univalents, bivalents and multivalents per pollen mother cells (PMCs) at diakinesis and metaphase I stage
2) Mean chiasmata per PMC and mean chiasmata per chromosome
3) Percentage of cells with bridges and laggards if any at anaphase stage
4) Percentage of cells with micronuclei if any at telophase stage

3.3 Pollen studies

3.3.1 Anthesis and anther dehiscence

With the objective of understanding the exact time of flower opening, 10 inflorescences were observed at 15 minutes interval from 12.30 a.m. onwards until the cessation of flower opening. The inflorescences tagged for determining the time of anthesis were also utilized for ascertaining the time of anther dehiscence.

3.3.2 Pollen production per flower

This was estimated adopting haemocytometer method. Haemocytometer employed in this study was Neubeuer type, having improved double ruling. Flowers possessing mature anthers, about to dehisce, were collected at their peak anthesis time (3 a.m.).

Five anthers were separated with a dissection needle and were gently crushed and suspended in 2.5 ml of sucrose solution taken in a test tube. Contents of the test tube were stirred well to get an even suspension of the pollen grains. A drop of the suspension was drawn in a fine pipette and transferred to each of the two counting chambers. The pollen grain in each of the four corner squares of the counting chamber was counted using a low power objective of the microscope. The number of pollen grains in a flower was calculated as per the following expression

$$N = \frac{\alpha \times v \times 10^4}{n}$$

where,

N = Number of pollen grains per flower

α = Mean number of pollen grains counted per corner square

v = Volume of suspension made with anther in ml (in the present investigation it is 2.5 ml)

n = Number of anthers with which the suspension was made up, it being equal to five in the present experiment

The experiment was repeated and the mean number of pollen grains per flower was worked out.

3.3.3 Pollen fertility, size and morphology

Fertility of pollen was assessed on the basis of stainability of pollen grains in acetocarmine-glycerine mixture. The inflorescence was bagged on the previous day of opening of the flowers. Pollen

grains were collected from the newly opened flowers at 6 a.m. and stained in a drop of acetocarmine-glycerine mixture on a clean slide and kept aside for one hour. All the pollen grains that were well filled and stained were counted as fertile and others as sterile. Pollen grains were counted from 10 different samples, scoring 10 fields from each. The values were expressed in percentage.

Pollen diameter was measured using an ocular micrometer, after calibration. Mean as well as range of size were calculated. Photomicrogrphs of pollen grains were taken showing fertile and sterile grains as well as different size ranges. Morphology of the pollen grains were also studied.

3.3.4 Pollen viability and pollen tube length

To find out the ideal medium for germination of pollen grains different combinations were tried as listed below. These included different levels of sucrose along with 60 ppm boric acid and 1 per cent gelatin. Distilled water was used as control. The treatments were fixed based on previous report in ginger (Sathiabhama, 1988). The percentage of germination of pollen grains was recorded in the seven media.

1. Distilled water
2. 8 per cent sucrose + 60 ppm boric acid
3. 8 per cent sucrose + 60 ppm boric acid + 1 per cent gelatin

4. 15 per cent sucrose + 60 ppm boric acid
5. 15 per cent sucrose + 60 ppm boric acid + 1 per cent gelatin
6. 30 per cent sucrose + 60 ppm boric acid
7. 30 per cent sucrose + 60 ppm boric acid + 1 per cent gelatin

Pollen grains isolated from freshly opened flowers were kept in a drop of the medium contained in a cavity slide. The slide was then incubated in a moist-chamber for 24 hours. Observations on germination were recorded in 20 fields in each media after 24 hours. The most suitable media for pollen germination was then worked out.

The slides used to study pollen germination were also utilized to study the length of the pollen tube, after 24 hours of incubation. The length of the pollen tubes were measured from the well germinated pollen grains using an ocular micrometer, in ten randomly selected fields of the seven treatments. The mean values were expressed in μm. Photomicrographs of the germinated pollen grains were also taken.

3.4 Stigma receptivity, style length and number of ovules

For finding out the stigma receptivity flowers were hand pollinated at two hour intervals after opening. Fifty flowers were covered with polythene bags on the previous day of opening. Freshly opened flowers with sufficient pollen grains were collected in the early morning and reserved in a petriplate lined with moist filter paper as the source of pollen. Five freshly opened flowers each were pollinated at an interval of two hours starting from 6 a.m. One hour after pollination the flower was plucked and stigma along with a small portion of the style was separated and kept in a clean slide. A cover glass was placed above it, the gynoecium was gently pressed and observed under the microscope for the germination of pollen grains on the stigma.

A few quantitative measurements such as style length, ovary length and number of ovules were recorded using a finely divided scale and observing through a dissection microscope. The size of the ovule was also measured using an ocular micrometer. Photographs showing the length of the style was taken.

3.5 Hand pollination

While analysing the different aspects contributing to seedlessness within the crop artificial pollination was also tried to overcome the barriers if any.

3.5.1 Artificial pollination to induce seed set

Fifty randomly selected flowers protected prior to anthesis were pollinated using freshly collected pollen in the morning. These flowers were labelled properly and observed for one week for the development of ovary and ovule.

3.5.2 Stub pollination

Since the style was too long compared to the pollen tube, stub pollination was attempted in order to produce seeds in Kaempferia. In fifty randomly selected flowers the style was cut back to half the length and pollen grain were applied on the cut surface. The same was repeated after removing 3/4th of the style.

3.6 Pollen-pistil interaction

Since all the above methods failed to get any positive result on seed set, the following experiment was conducted to study pollen tube penetration and growth through the style. The fluorescence technique given by Kho and Baer (1968) was adopted for the study. Inflorescences were covered with polythene bags on the previous day of experiment. The next-day flowers were pollinated at 6 a.m. The pollinated flowers were fixed in FAA (Formalin 5 ml, acetic acid 5 ml and ethyl alcohol 90 ml) at 3, 6, 9, 12 and 24 hours after pollination. After 24 hours the

fixed buds were transferred to IN NaOH for eight hours at room temperature. They were washed thoroughly with distilled water and stained in 0.1 per cent aniline blue in 0.1 N K_2HPO_4 for a period of 18 hours. The pistil was later macerated in 90 per cent glycerol. In vivo pollen tube growth was then examined under the fluorescent lamp of an orthoplan microscope.

The above experiment was also repeated by pollinating the flowers after wetting the stigma with a drop of the pollen germination medium.

Results

RESULTS

Investigations on cytogenetics, floral biology and pollen studies in Kaempferia galanga L. were undertaken at the College of Horticulture, Vellanikkara during 1992-93 and the important results are presented below.

4.1 Mitotic studies

In order to find out the chromosome number of the crop, root tip squash preparations were made and examined. The results are presented below.

4.1.1 Standardisation of the procedure for mitotic studies

4.1.1.1 Time of collection of roots

Roots were fixed at different time intervals and they were examined for the presence of dividing cells. Maximum cells at metaphase I were observed in the early morning as well as evening hours. Mitotic index was also worked out for the roots fixed at different time intervals. It was found that maximum mitotic index was at 6 p.m. (24 per cent) followed 6 a.m. (19 per cent) (Fig. 1). Hence the roots for mitotic studies were fixed at 6 p.m. (Table 5).

4.1.1.2 Pre-treatments, fixatives and stains

Para-dichlorobenzene, α-bromonaphthalene, colchicine and

Table 5. Mitotic index of Kaempferia galanga at different time intervals of the day

Time	Number of dividing cells	Number of undividing cells	MI (per cent)
12	60	642	8.5
2 a.m.	34	816	4.1
4 a.m.	54	611	8.8
6 a.m.	95	385	19.8
8 a.m.	70	655	9.6
10 a.m.	51	640	7.3
12 noon	24	435	5.2
2 p.m.	57	740	7.1
4 p.m.	54	611	8.1
6 p.m.	118	363	24.0
8 p.m.	46	956	4.7
10 p.m.	28	916	2.9

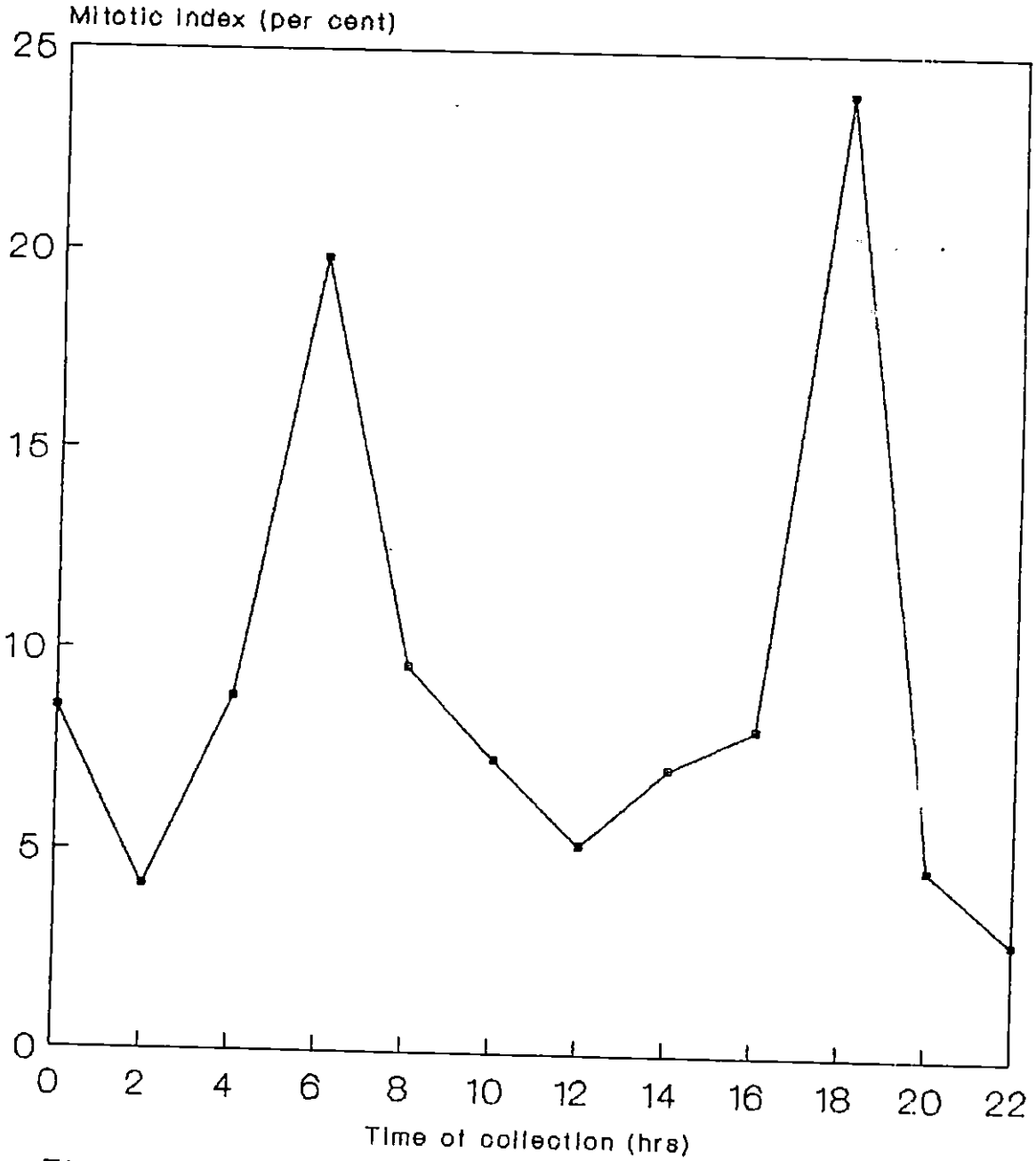


Fig.1 Mitotic Index of ***Kaemferia galanga*** L. at different time intervals of the day

8-hydroxy quinoline were the four pre-treatment agents attempted. When para-dichlorobenzene was used, most of the cells were at anaphase stage. Clumping of the chromosome was also noticed. 8-hydroxy quinoline was not found to be effective in giving good separation of the chromosome. Staining was also not satisfactory with this reagent. Eventhough colchicine gave good separation of the chromosome, proper staining was not achieved. Using α-bromonaphthalene, proper condensation and separation of the chromosomes were obtained. Number of dividing cells was also maximum with this reagent which averted most of the cells at metaphase stage. Among the different durations of pre-treatment a low temperature treatment (4°C) for four hours was found to be the most effective.

Three fixatives such as Carnoy's fluid, acetic alcohol and Ostergren and Heneens fixative were utilized for the study. Among them Carnoy's fluid was found to be the most satisfactory.

Among the three stains used Snows carmine as well as Feulgen stain was equally effective in giving good cytological preparations. Acetocarmine was defective because of the staining of the cytoplasm.

For Feulgen staining the hydrolysis of the roots in IN HCl for five minutes was found to be the best. Adequate staining was achieved within a duration of 45 minutes. The squash

preparations of the roots were made in one per cent acetocarmine with a little ferric acetate being added to it. Staining as well as chromosome spread was satisfactory with this. However, some difficulties were met with during the preparation of permanent slides using this stain. The colour of chromosomes faded gradually. Moreover, the preparation as well as storage of the stain was difficult.

Staining in Snows carmine for 24 hours and squashing the roots in 45 per cent acetic acid was found to give very good cytological preparation. In this case the decrease in the intensity of colour in permanent mounts was not noticed as happened in the case of Feulgen stain. In addition to the most satisfactory results obtained, 'Snows carmine' has got several advantages over others as listed below.

1. Preparation and storage of the stain are easy
2. The procedure for staining is simple as it does not involve steps like hydrolysis
3. The stain can be reused
4. Stained roots can be stored for two to three days
5. Staining of cytoplasm was not observed

Pre-treatment in α-bromonaphthalene for four hours at 4°C followed by fixation in Carnoy's fluid for 24 hours and over-night staining in Snows carmine followed by squashing in 45 per cent acetic acid was found to be the best method for the study

of mitotic chromosomes in Kaempferia. Addition of a little ferric acetate to the fixative also improved the stainability of chromosomes.

4.1.2 Karyotype analysis

Root tip squash studies in Kaempferia galanga L. using Snows carmine stain revealed that somatic chromosome number in this crop is 55. These 55 chromosomes could be classified into eleven sets of five structurally similar chromosomes (Plate 3a & b). Measurements of total chromosome length and arm ratio also showed that chromosomes can be grouped into five sets of 11 each. The mean values of long arm length, short arm length and arm ratio are presented in Table 6. The basic chromosome complement is characterised by the presence of two SAT chromosomes, six median chromosomes and three submedian chromosomes. The length of short arm ranged from 0.39 to 0.57 μm and the average short arm length was 0.48 μm. The long arm length varied from 0.49 to 0.76 μm and the average was 0.57 μm. The length of chromosome ranged from 0.88 μm to 1.33 μm and average chromosome length was 1.05 μm. The average arm ratio was 1:1.7. Total length of the chromosome complement was 11.53 μm. The average length of satellites was 0.14 μm. The relative chromosome length varied from 6.77 to 11.53 per cent (Table 7).

Table 6. Karyotype analysis and chromosome classification in Kaempferia galanga L.

Chromosome No.	Long arm (L) length (um)	Short arm (S) length (um)	Total length (um)	Arm ratio	Chromosome type
1	0.76 ± 0.04	0.57 ± 0.07	1.33 ± 0.08	1.35	SM
2	0.65 ± 0.04	0.57 ± 0.05	1.22 ± 0.07	1.15	M
3	0.61 ± 0.05	0.55 ± 0.06	1.16 ± 0.11	1.10	M
4	0.64 ± 0.08	0.46 ± 0.04	1.10 ± 0.10	1.40	SM
5	0.59 ± 0.05	0.49 ± 0.03	1.08 ± 0.07	1.10	M
6	0.56 ± 0.05	0.50 ± 0.04	1.06 ± 0.07	1.10	M
7	0.51 ± 0.05	0.49 ± 0.04	1.0 ± 0.07	1.00	M
8	0.56 ± 0.03	0.43 ± 0.04	0.99 ± 0.06	1.30	SM
9	0.51 ± 0.01	0.43 ± 0.06	0.94 ± 0.07	1.19	SAT
10	0.47 ± 0.05	0.41 ± 0.03	0.88 ± 0.05	1.10	M
11	0.49 ± 0.08	0.39 ± 0.04	0.88 ± 0.06	1.05	SAT

SM - Sub median

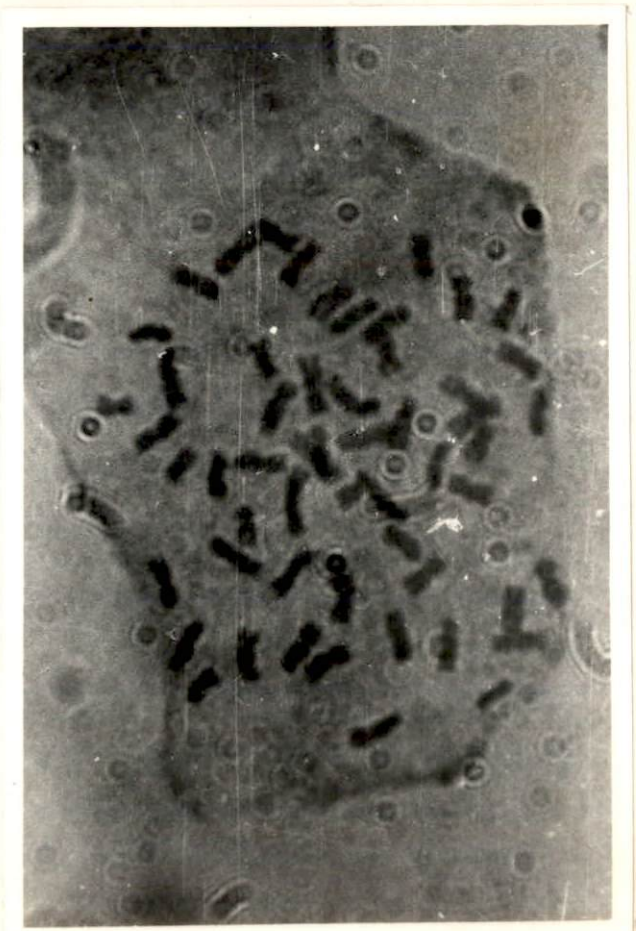
M - Median

SAT - Satellite

Table 7. Total chromosome length and relative chromosome length in Kaempferia galanga L.

Chromosome number	Total length of chromosome μm	Relative chromosome length μm
1	1.33 ± 0.08	11.53 ± 0.08
2	1.22 ± 0.10	10.58 ± 0.10
3	1.16 ± 0.10	10.01 ± 0.10
4	1.10 ± 0.10	9.54 ± 0.10
5	1.07 ± 0.07	9.28 ± 0.07
6	1.06 ± 0.07	9.19 ± 0.07
7	1.01 ± 0.07	8.73 ± 0.07
8	0.99 ± 0.06	8.58 ± 0.06
9	0.94 ± 0.07	8.18 ± 0.07
10	0.88 ± 0.05	7.60 ± 0.05
11	0.88 ± 0.06	7.60 ± 0.06

Plate 3a & b. Somatic chromosomes of Kaempferia galanga L.
(a x 3500, b x 6300)



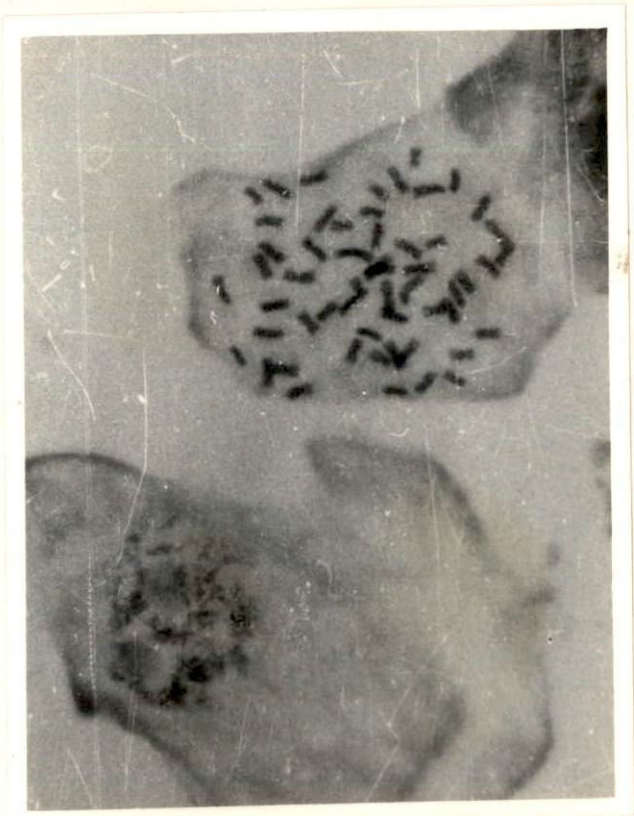


Fig. 2. Idiogram of Kaempferia galanga L. based on actual chromosome length

Fig. 3. Idiogram of Kaempferia galanga L. based on relative chromosome length

The karyotype classification according to the degree of asymmetry, (Stebbins, 1958) was worked out for this crop and the data are presented in Table 8. Kaempferia galanga L. belonged to the symmetrical group '1a' because none of the chromosomes had arm ratio more than 2:1 and the ratio between largest/smallest chromosome was only 1.7:1. The total form percentage was 45.86.

4.1.3 Karyotype and Idiogram

Photographs of well spread cells at Metaphase I were taken, enlarged and the chromosome figures were cut. They were arranged in an order according to their lengths. The structurally similar chromosomes were pasted together. The chromosomes could then be arranged in 11 groups as shown in Plate 4.

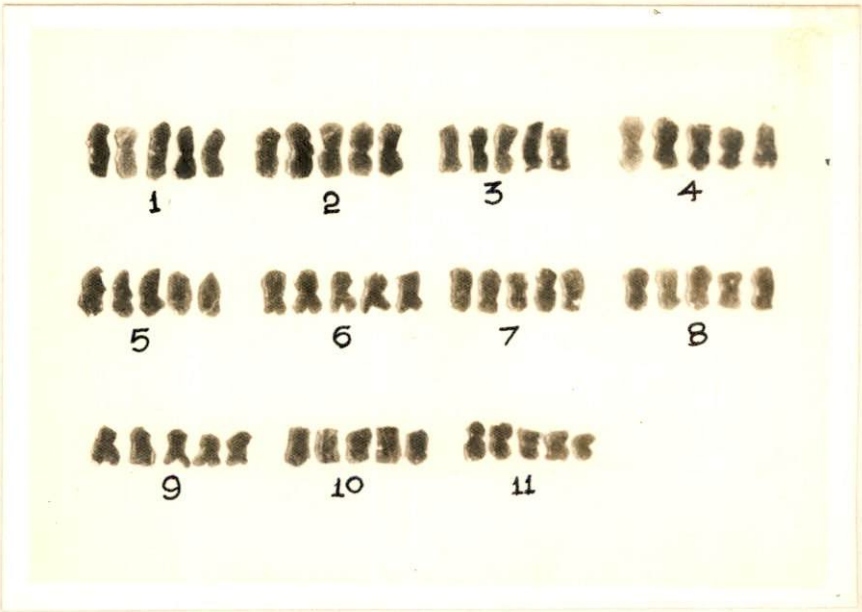
Idiogram of K. galanga L. is presented in Fig. 2. Chromosomes were designated as one to eleven in the decreasing order of size and increasing order of asymmetry. Idiogram based on relative length of chromosome is also presented in Fig. 3.

4.2 Meiotic studies

4.2.1 Flowering behaviour

In Kaempferia galanga L. flowering occurs only once in a year. Flowering started in June and completed by September. The peak flowering was noticed during July and August months. Flowers were found to be produced in central clusters directly

Plate 4. Karyotype of Kaempferia galanga L.



KARYOTYPE OF *Kaempferia galanga* L.

$2n = 5x = 55$

Table 8. Karyotype classification according to Stebbins in kacholam (*Kaempferia galanga* L.)

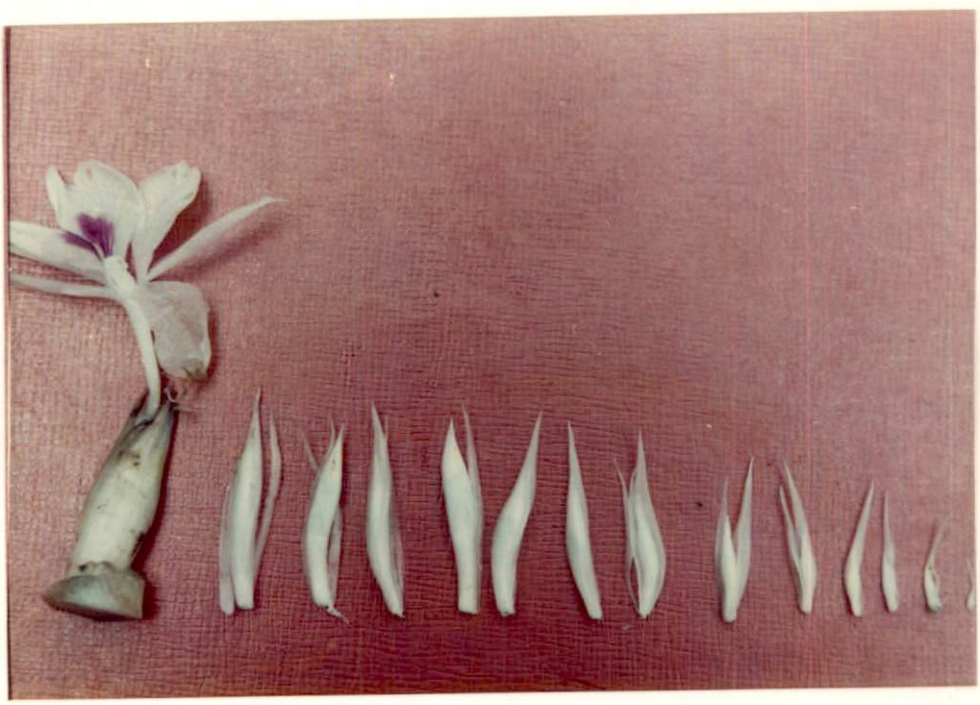
No.	Proportion of chromosome with arm ratio more than 2:1	Largest/smallest chromosome ratio	Stebbins classification	Total Form (%)
1	0.00	1.70	1a	45.86

from the rhizome and are enclosed by the sheathy leaf petiole. Flower opening was in succession and the outermost one opened first. In Kaempferia galanga L. flowering started 45-55 days after planting. First flower opened 15-20 days after initiation. A period of eight to ten days was required for the completion of blooming in a cluster.

4.2.2 Floral morphology

Detailed floral morphology of the crop is depicted in Fig.4. Inflorescence is a scape covered by a leathery sheath, directly arising from the rhizome. An inflorescence contains 2-13 buds of which the outer one opened first (Plate 5). The number of flower buds per inflorescence varied with season the maximum being in the peak flowering period and minimum during the early and late periods. Each flower is subtented by a bract and one or two bracteoles. Flowers are bisexual, complete, and zygomorphic. The floral parts are arranged on the trimerous ground plan of the monocotylendous. The five whorls of floral parts alternate with one another. Perianth is having six segments which are in two whorls and can be distinguished into calyx and corolla. The outer whorl of the perianth is connate at the base and free at the apex. The inner whorl is tubular at the base forming a long tube and free at the apex. Of the inner whorl the posterior lobe is absent. The outer whorl of stamens is represented by two staminodes which are situated at the base of the tubular

Plate 5. A complete inflorescence in Kaempferia galanga L. and flower buds of different stages of maturity



perianth. The posterior stamen of the inner whorl is the only fertile stamen. The other two stamens are united and form a large, biolobed showy labellum, which is the most conspicuous part of the flower. The fertile stamen is bilobed and the connective forms a hood above the stamen. The anther lobes form a groove through which the style passes so that the stigma comes very close to the anthers. Gyneociem consists of tricarpellary syncarpous, inferior ovary with 16 to 19 ovules arranged in axile placentation. Style is very long, about 4 cm in length and ends in a spiny stigma (Plate 12).

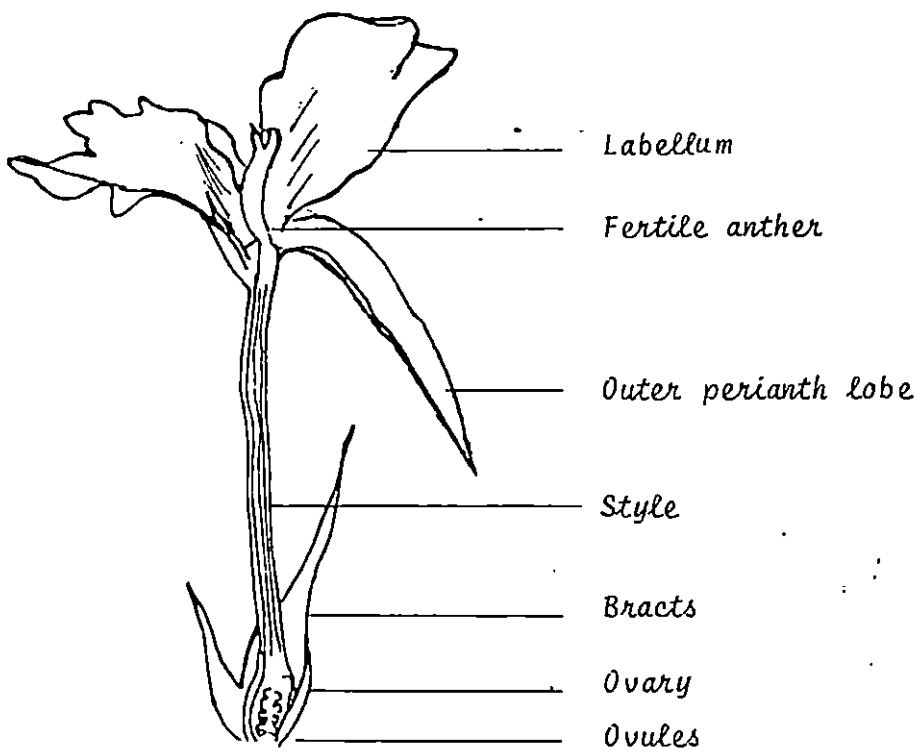
4.2.3 Time of fixation of flower buds

From the Table 9 it can be seen that, the time of fixation of flower buds varied with the weather condition. In May the best time for fixing flower buds was between 9 and 10 a.m. In June and July the time of fixation was at 2 p.m. on a rainy day and between 11-12 a.m. on a sunny day. In August the ideal time of fixing the buds was between 10-11 a.m.

4.2.4 Size and maturity of the flower bud

The inflorescence is produced directly from the rhizome and remains enclosed by the leaf sheath. The distinction of floral and vegetative buds could be made only by experience. While the vegetative buds emerged singly at a time the floral bud initiation was demarkated by the simultaneous appearance of two

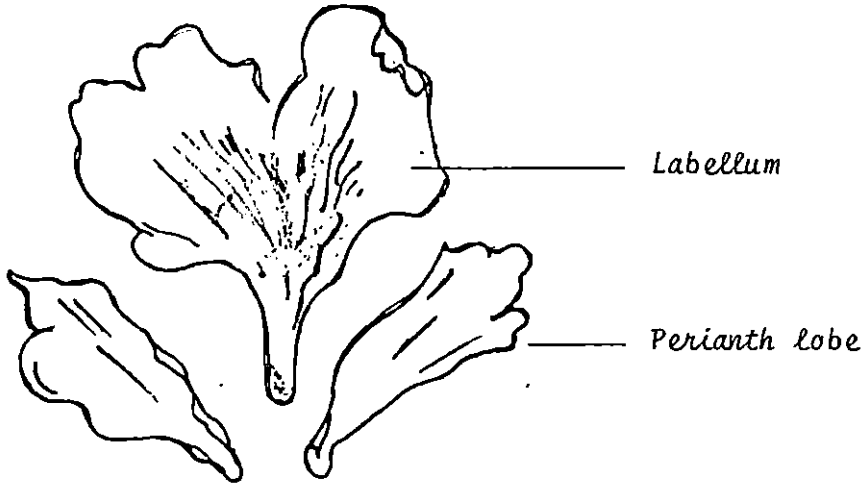
Inflorescence with single flower

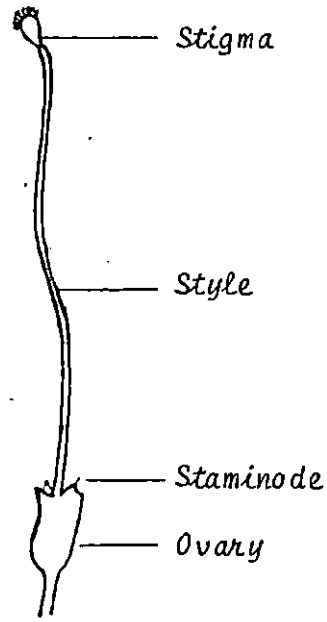


L.S. of a single flower

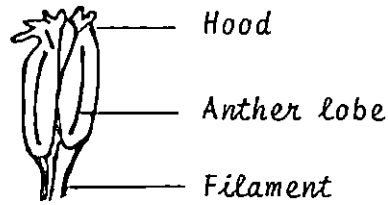
Fig. 4. Floral morphology of *Kaempferia galanga* L.

Fig. 4. Continued

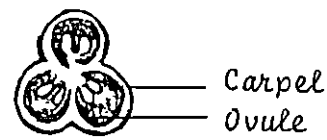




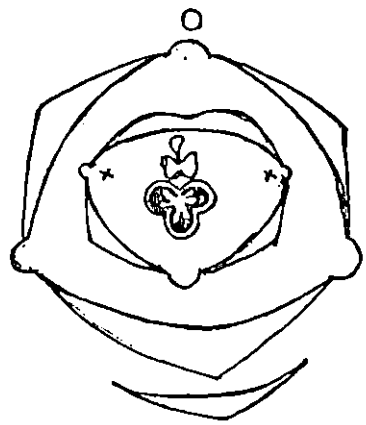
Pistil



Fertile stamen



C.S. of ovary



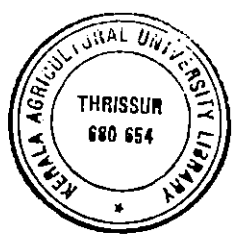
Floral diagram

$+ \; \underset{\varphi}{\underset{\updownarrow}{0}} \; K_{(3)} \; \overset{\frown}{C_{(3)}} \; A_1 \; \underline{G}_{(3)}$

Floral formula

Table 9. Ideal time of fixation of flower buds in Kaempferia galanga L. during different weather conditions

Month	Weather condition	Time of fixation
May	Hot day	9 - 10 a.m.
June	Rainy day	2 p.m.
	Clear day	11 - 12 noon
July	Rainy day	2 p.m.
	Sunny day	11 - 12 noon
August	Clear day	10 - 11 a.m.



bract tips above the soil level. The vegetative buds were normally pale green in colour while the flower sheaths were reddish tinted. The best stage of fixation was before the opening of the first flower, when 1/4th of the inflorescence tip came above the soil level. Dividing pollen mother cells could be obtained from 3rd or 4th bud from the base. The different stages of maturity of the flower bud as well as a complete inflorescence is presented in the Plate 5.

4.2.5 Studies on meiotic chromosomes

For meiotic studies flower buds were fixed in acetic alcohol (1:3). Duration of fixation was standardised as 24 hours. Acetocarmine (1 per cent) blended with a little ferric acetate was the ideal stain for meiotic studies. Fixed flower buds could be stored in 70 per cent ethyl alcohol for one or two weeks.

Dividing pollen mother cells were examined for different meiotic stages. Abnormalities wherever observed were recorded. Twentyfive pollen mother cells at diakinesis/metaphase I stage were thoroughly examined to study their chromosome associations. Camera lucida drawings were made. Photomicrographs were taken (Plate 6). Mean number of univalents, bivalents and multivalents per PMC, mean number of rod and ring bivalents and mean chiasmata/chromosome were worked out from these cells. These values are presented in Tables 10 and 11.

Table 10. Chromosome configurations at diakinesis in Kaempferia galanga L.

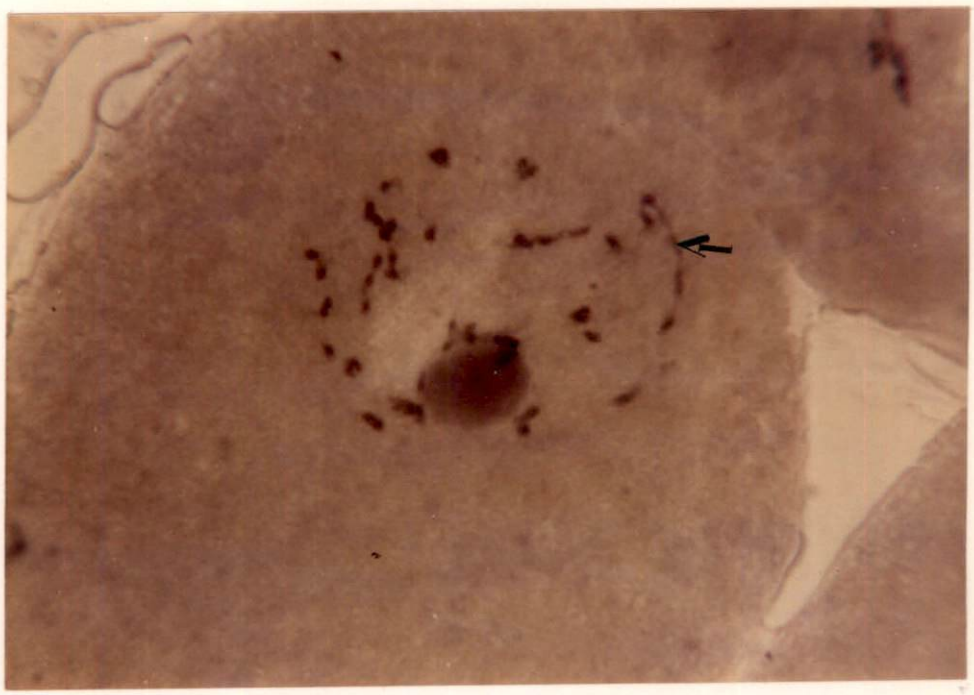
Cell No.	Multivalents				Bivalents	Univalents
	Hexa	Penta	Tetra	Tri		
1	0	0	3	5	11	6
2	0	0	2	1	17	10
3	0	0	0	4	15	13
4	0	1	3	4	8	10
5	0	1	0	1	18	11
6	0	0	0	5	17	6
7	0	1	3	5	8	7
8	0	1	2	3	13	7
9	0	1	2	4	9	12
10	0	0	1	2	14	17
11	0	1	3	3	11	7
12	0	0	0	5	12	16
13	0	2	2	4	10	5
14	0	0	1	5	14	8
15	0	0	1	5	15	6
16	0	2	1	5	10	6
17	0	0	1	4	16	7
18	1	2	3	1	11	2
19	0	0	2	5	12	8
20	0	0	1	6	12	9
21	0	0	2	7	9	8
22	0	2	1	4	13	3
23	0	1	2	4	13	4
24	1	0	2	3	15	2
25	0	2	2	1	16	2
Mean	0.08	0.5	1.64	3.84	13.16	7.8
Range	0-1	0-2	0-3	1-7	8-18	2-17

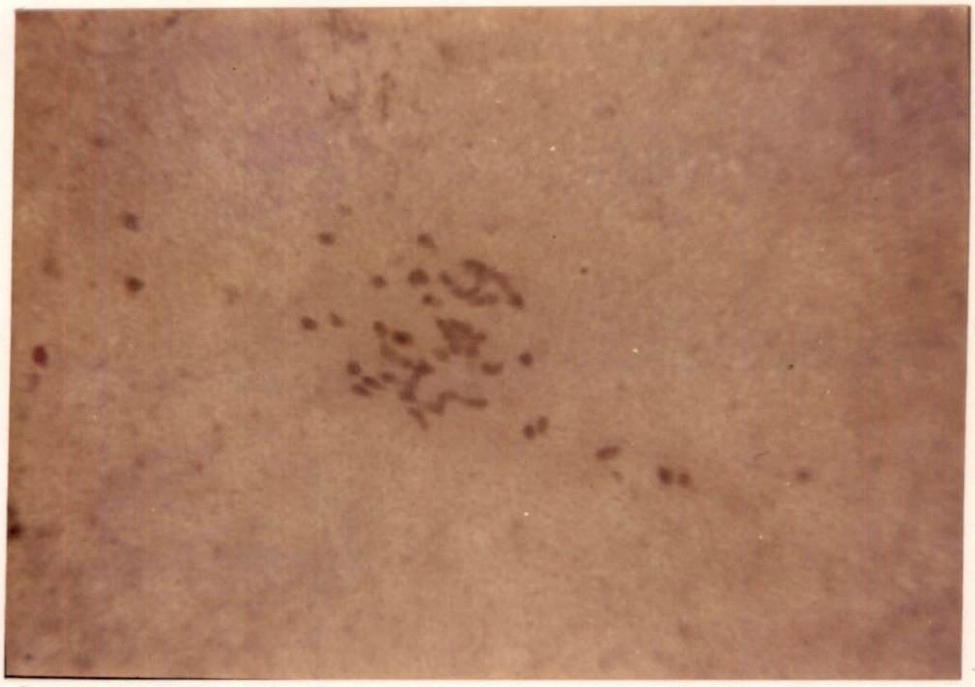
Table 11. Chromosome configurations at diakinesis and the chiasma frequency in Kaempferia galanga L.

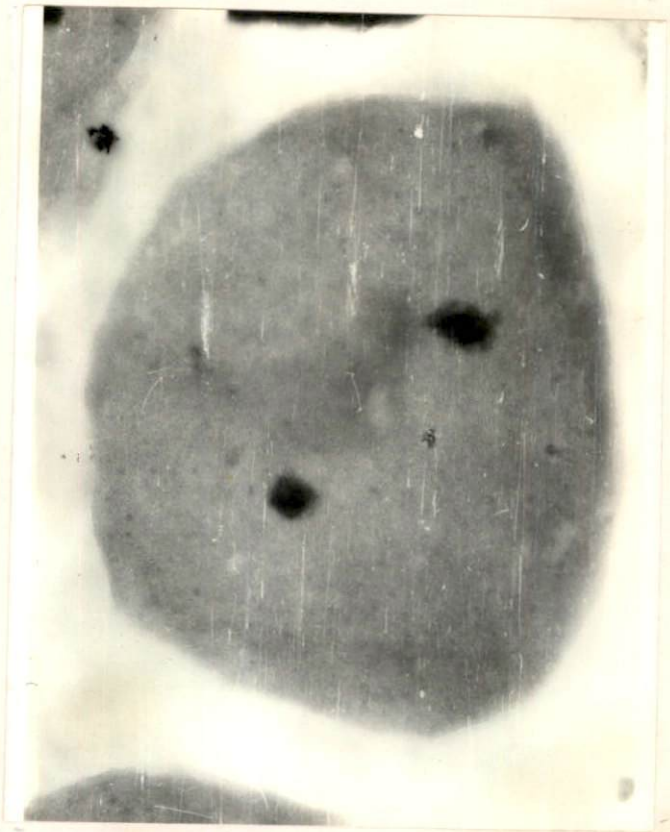
Cell No.	Bivalent		Trivalent		Quadrivalent		Pentavelent		Xta/ PMC	Xta/ chromo-some
	Ring	Rod	2 xta*	3 xta	3 xta	4 xta	4 xta	5 xta		
1	0	8	3	1	3	0	1	0	30	0.54
2	10	5	0	1	0	0	1	0	32	0.58
3	6	11	4	1	0	0	0	0	35	0.63
4	2	6	5	0	1	2	1	0	35	0.63
5	6	7	3	0	2	0	1	0	35	0.63
6	2	7	3	1	1	1	1	0	31	0.56
7	3	11	2	0	1	0	0	0	24	0.43
8	2	9	2	1	1	1	1	0	31	0.56
9	4	7	4	0	0	0	0	0	26	0.47
10	3	7	3	1	2	0	1	0	32	0.58
11	4	10	5	0	0	1	0	0	32	0.58
12	5	10	4	1	1	0	0	0	34	0.61
13	4	6	5	0	1	0	2	0	35	0.63
14	3	8	0	1	2	1	0	0	34	0.61
15	3	9	3	2	1	1	0	0	34	0.61
16	1	11	2	4	1	0	0	0	32	0.58
17	0	9	5	2	2	0	0	0	31	0.56
18	5	8	3	1	1	0	2	0	33	0.60
19	1	12	4	0	1	1	1	0	33	0.60
20	3	9	2	1	2	1	0	0	32	0.58
21	0	16	1	0	1	1	2	0	34	0.61
22	1	15	3	0	0	0	0	0	23	0.41
23	2	13	2	0	0	1	2	0	33	0.60
24	0	13	5	0	2	0	0	0	29	0.52
25	1	9	4	0	0	0	1	0	23	0.41
Mean	2.84	9.44	3.08	0.72	1.04	0.44	0.68	0	31.32	0.57
Range	0-10	5-16	0-5	0-4	0-3	0-2	0-2	-	23-35	

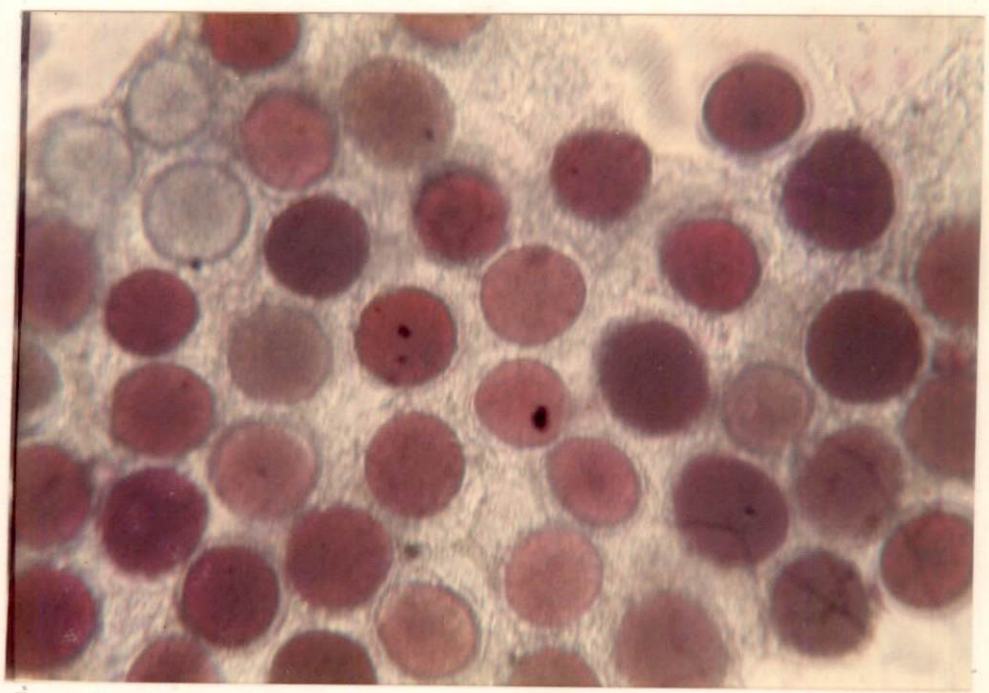
Plate 6. Pollen mother cell showing multivalents involving 3-6 chromosomes at diakinesis (x 1800)

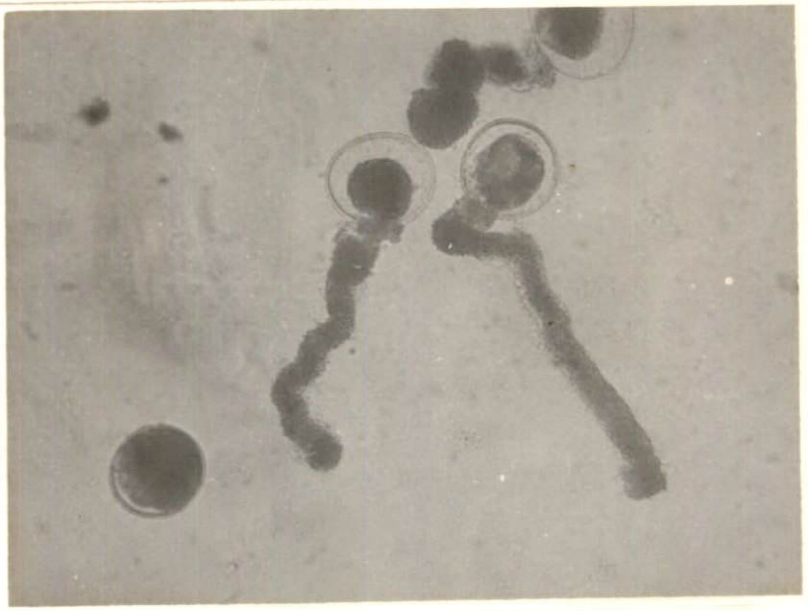
Plate 7. Pollen mother cell showing multivalents involving 3-6 chromosomes at metaphase I (x 1800)

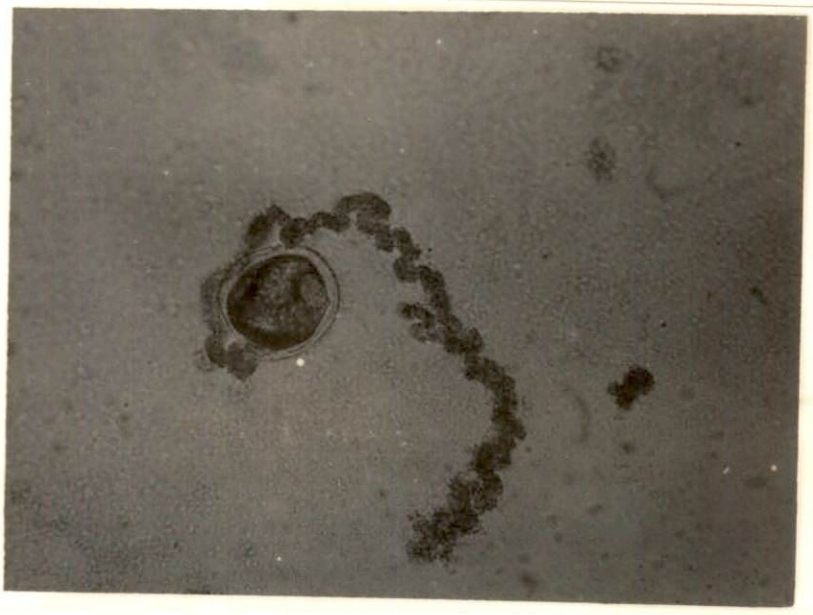












Cytogenetical analysis revealed the presence of associations involving three to six chromosomes at metaphase I in every PMC. Bivalents and univalents were also frequent (Plate 7).

A moderate frequency of bivalents was observed in all the pollen mother cells. Their mean value was 13.16 and the number varied from eight to 17 per PMC. Trivalents were of more frequent occurrence as compared to quadrivalents, pentavalents and hexavalents. They were present in all the PMCs observed and the mean number per cell was 3.84 and the value ranged from one to five. Associations involving four chromosomes were also observed in most of the cells but were only in lower frequencies than trivalents. The mean value of quadrivalents per PMC was only 1.64 and the range was 0 to three. Pentavalents were present in more than 40 per cent cells observed and range of occurrence varied from 0 to two per cell. Hexavalents were only of rare occurrence and their mean value was only 0.08. However, frequency of univalents was very high in most of the cells observed. The mean number of these unpaired chromosomes per PMC was 7.80 and the value ranged from two to 17.

Among the bivalents both ring bivalents and rods bivalents were present. Rods with a frequency of nine to twelve per PMC were more frequent than rings. Mean number of ring bivalents was only 2.84 per cell.

The mean number of trivalents showing two chiasmata was 3.03 per PMC, whereas only 0.72 trivalents per PMC showed three chiasmata, 1.04 quadrivalent per PMC showed three chiasmata and 0.44 per PMC showed four chiasmata. All the pentavalents showed four chiasmata. The mean chiasmata recorded per PMC was 31.32 and it ranged from 23-35. The mean chiasmata per chromosome was 0.57.

A large number of cells were also screened in order to verify the presence of meiotic abnormalities at later stages like anaphase I and telophase I. Bridges, fragments or laggards could never be observed in any of the cells. Anaphase separation was perfectly normal without any lagging chromosomes (Plate 8). During telophase I also no micronuclei could be seen in any of the daughter cells. Stages of meiosis II also could not be observed in the large number of cells screened.

4.3 Pollen studies

4.3.1 Anthesis and anther dehiscence

The flower opening in Kaempferia galanga L. was observed between three and four a.m. The mature flower buds were closely observed from 12.30 a.m. onwards at a regular interval of 15 minutes and the peak opening of flowers was in the early morning hours between three and four a.m. Anther dehiscence also coincided

Plate 8. Normal separation of chromosomes at late anaphase I without any lagging chromosomes (x 1800)

Plate 9. Pollen grains of Kaempferia galanga L. stained with acetocarmine glycerine mixture (x 400)

with anthesis and the pollen grains were released by longitudinal dehiscence.

4.3.2 Pollen production

Using haemocytometer the pollen production in Kaempferia was estimated as 54000 pollen grain per flower. The value ranged from 50000-60000.

4.3.3 Pollen fertility, size and morphology

Pollen fertility among different samples as assessed by stainability in acetocarmine ranged from 46.9-91.9 per cent. Mean fertility of pollen grains was 72.8 per cent. Slight variation in the pollen size also was noticed. It ranged from 106-128 μm. The mean being 114.8 μm (Table 12). In Kaempferia pollen grains were exactly spherical in shape (Plate 9). The exine was smooth, nonspiny and having two germpores (Plate 14).

4.3.4 Pollen viability

Pollen viability was estimated using seven different media. From the Table 13, it is evident that the maximum viability of pollen grains (68.9) was noticed in the medium containing 8 per cent sucrose, 60 ppm boric acid and 1 per cent gelatin. The maximum length of the pollen tube (732 μm) was also obtained in the same medium (Plate 10). The percentage of germination varied from 14.0 to 68.9 per cent. Minimum germination was

Plate 10. Germination of pollen grains of Kaempferia galanga L. (x 400)

Plate 11. Coiling of pollen tube often observed in the germination media (x 400)

Table 12. Fertility and size of pollen grains in Kaempferia galanga L.

Sample No.	No. of pollen grains observed	Fertility (percentage)	Mean pollen size (µm)
1	148	72.9	112.0
2	143	70.6	112.0
3	132	59.8	119.0
4	130	46.9	106.0
5	247	91.9	128.0
6	180	78.3	114.0
7	172	87.8	122.0
8	225	74.0	107.0
9	154	57.0	112.0
10	123	70.6	116.0
Mean		72.8	114.8
Range		46.9-91.9	106-128

Table 13. Pollen germination of Kaempferia galanga L. in different media

Media	Total no. of pollen grains counted	Mean germination (percentage)	Mean pollen tube length (μm)
1. Distilled water	120	14.8	352
2. 8% sucrose + 60 ppm boric acid	140	43.0	694
3. 8% sucrose + 60 ppm boric acid + 1% gelatin	182	68.9	732
4. 15% sucrose + 60 ppm boric acid	160	44.4	656
5. 15% sucrose + 60 ppm boric acid + 1% gelatin	134	54.0	452
6. 30% sucrose + 60 ppm boric acid	223	50.6	452
7. 30% sucrose + 60 ppm boric acid + 1% gelatin	187	14.0	236

observed in distilled water (14.8) as well as in media with 30 per cent sucrose, 60 ppm boric acid and 1 per cent gelatin. Coiling of the pollen tube was often observed in the germination media (Plate 11).

4.4 Stigma recepitivity, style length and number of ovules

Flowers withered off within 12 hours of opening. The stigma remained receptive for nine hours after anthesis, as indicated by germination of pollen grains on the stigmatic surface.

The style length, ovary length and the number of ovules varied with flower. The mean style length was 4.37 cm and it ranged from 3.9 to 4.7 cm (Table 14). Ovary length varied from 0.30 to 0.40 cm, the mean being 0.34. Sixteen to 19 ovules were present in a flower, arranged in three locules on axile placentation.

4.5 Hand pollination

4.5.1 Artificial pollination

All the hand pollinated flowers withered after 12 hours of flower opening. Rotting of the ovary was noticed after two to three days and no instance of seed set could be observed.

4.5.2 Stub pollination

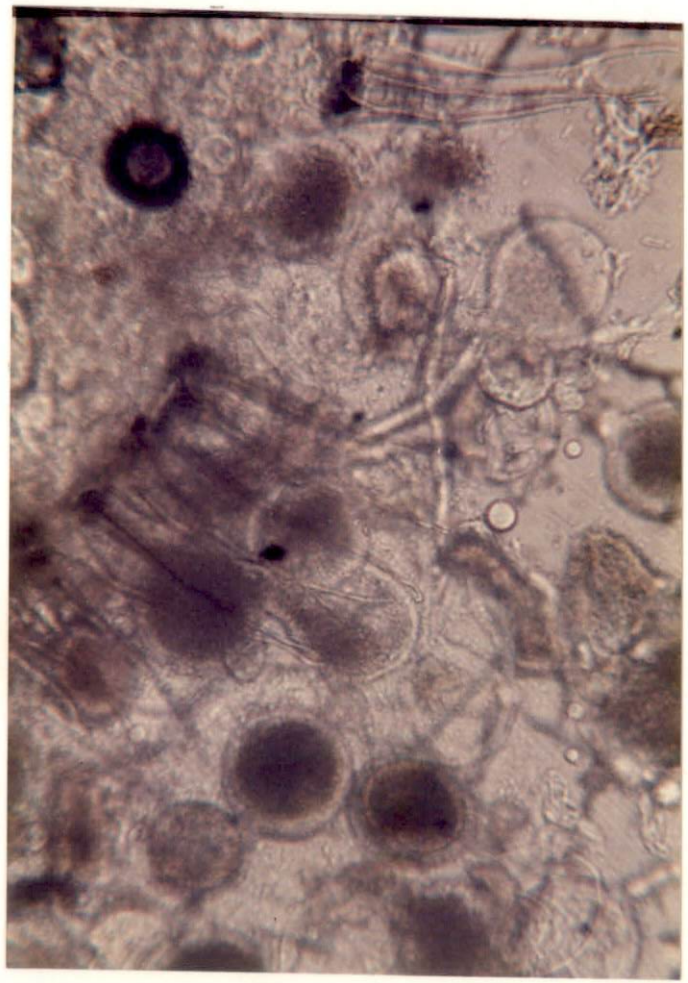
By stub pollination also no positive result on seed set was obtained since the highly sensitive flowers could not withstand the mechanical injury.

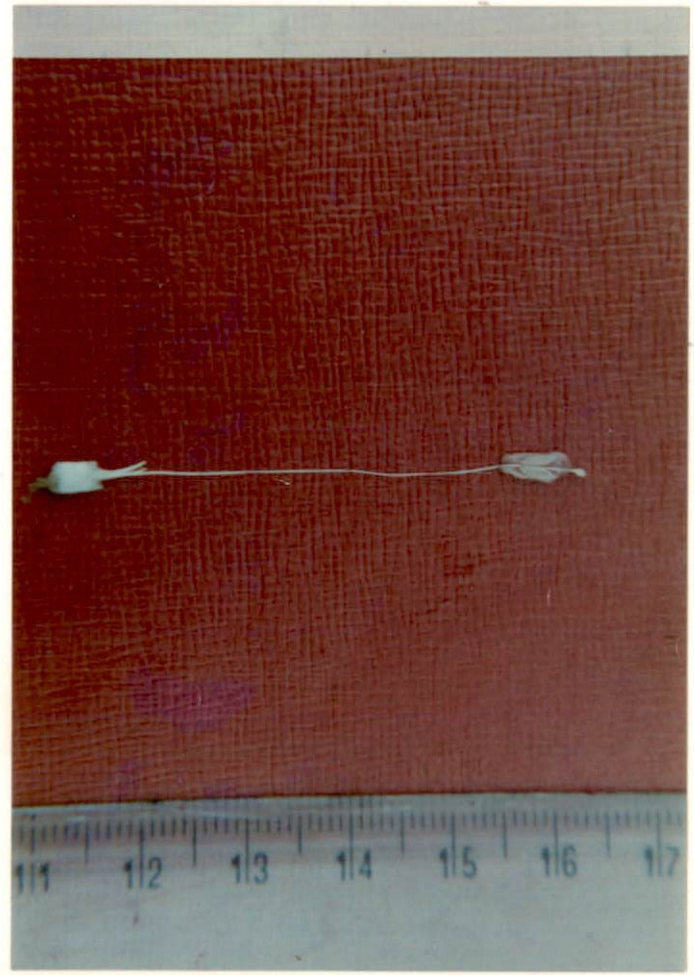
Table 14. Style length, ovary length and number of ovules in Kaempferia galanga L.

Flower No.	Style length (cm)	Ovary length (cm)	Number of ovules
1	4.2	0.31	19
2	3.9	0.30	18
3	4.7	0.40	16
4	4.8	0.30	16
5	4.5	0.40	17
6	4.3	0.32	18
7	4.5	0.40	19
8	4.2	0.34	18
9	3.9	0.30	16
10	4.7	0.40	18
Mean	4.37	0.34	17.5
Range	3.9-4.7	0.3-0.4	16-19

Plate 12. The lengthy style in the flower of Kaempferia galanga L. (x 400)

Plate 13. A few pollen grains germinating on the spinous stigma of Kaempferia galanga L. (x 400)




1|1
1|2
1|3
1|4
1|5
1|6
1|7

4.6 Pollen pistil interaction

Since the pollination methods failed in producing seed set, pollen germination on the stigmatic surface was carefully studied. It was observed that pollen grains germinate on the stigmatic surface but the number of germinating grains was found to be very low (Plate 13). The spines on the stigmatic surface may be causing some hindrance for pollen germination. Pollen tube penetration and growth through the stigmatic and stylar tissues were studied using fluorescent microscopy. The results showed that under natural conditions pollen tube penetration through the style did not occur. It was also found that when chemically aided pollination was done using the pollen germination medium pollen tube grows through the style (Plate 15). The maximum length of the pollen tube measured was 350 μm after 24 hours of pollination.

Plate 14. Germination of viable pollen grains *in vitro* as observed under fluorescent microscopy (x 400)

Plate 15. Pollen tube growth through the style under chemically aided pollination as observed under fluorescent microscopy (x 400)

Discussion

DISCUSSION

Kaempferia galanga L. is an important medicinal plant belonging to the family Zingiberaceae. The plant is gaining importance now a days because of its multivarious desirable attributes and increasing export demand for the dried rhizomes. At present production of this crop is not able to cop up with the demand. Improvement of the crop by conventional methods of breeding is not possible due to seedless nature of the plant. Existing variability is also much limited for want of seed production. The reasons for seedlessness in this crop are yet to be unraveled. This may be due to several reasons such as cytological, genetic, physiological or mechanical. Systematic improvement in any crop is possible only after elucidation of the different aspects of its seedlessness. Regarding chromosome numbers also conflicting reports are available. At this context the present study was undertaken in order to confirm the chromosome number of the species and to find out the various reasons for seedlessness.

Studies on mitotic index revealed that the maximum cell division occurs in the evening hours. In most other crops highest mitotic index is observed in the morning hours. In a related plant Zingiber officinale also maximum mitotic index was reported in the morning (Sathiabhama, 1988). Our results do not agree with the above finding. This difference in the timing of cell division

in the present study may be due to the following facts. Plantlets for the study were raised indoor in trays filled with sand devoid of any additional nutrient source. The diffuse light and relatively low temperature under indoor conditions would also have weakened these plantlets. The relatively low mitotic index observed may also have resulted from the limited availability of nutrients and light under indoor conditions restricting the active growth of the plant.

Pre-treatments, fixatives and stains

For mitotic studies the best suited pre-treating agent, fixatives and stains were standardised. Out of the four pre-treating agents tried, α-bromonaphthalene was found to be the best. In most of the slides prepared using paradichlorobenzene as the pre-treating agent, majority of the cells were in the anaphase stage. The presence of anaphase stage indicated the probable inefficiency of the pre-treating agent for arresting the cells at metaphase Using 8-hydroxy-quinoline also similar results were obtained. The marked defect of this agent was the decreased intensity of staining. When colchicine was used for pre-treatment chromosome separation was better but the staining was not satisfactory. Thus while lack of proper separation and adequate staining were the major draw backs with above mentioned three pre-treatment agents these defects were minimum when α-bromonaphthalene was used. The present study thus indicates that the best pre-treating chemical

suited for the mitotic studies of Kaempferia is ∝-bromonaphthalene. Mahanty (1971) also recommended ∝-bromonaphthalene as the pre-treatment agent for members of Zingiberaceae. However, the duration of treatment recommended by him is only 2 hours against 4 hours found ideal in the present study.

Among the fixatives Carnoy's II fluid was found to be the most suitable. It makes the cytoplasm more clearer than acetic alcohol and Ostergrens - Heneen's fixative. Among the different stains used, acetocarmine was not acceptable because of excessive staining of the cytoplasm. Feulgen stain and Snow's carmine were equally effective in getting good cytological preparation. Snow's carmine was found to have some added advantage over Feulgen staining. These are the easyness in preparation and storage of the stain, simplicity with the staining procedure, reusability and the ability for prolonged storage of stained roots. The gradual decrease in the intensity of colour of chromosomes in the permanent slides with Feulgen staining also was not observed with Snow's carmine. Because of all these advantages Snow's carmine was identified as the best stain suited for the study of mitotic chromosomes in Kaempferia galanga L. For the best cytological preparations a low temperature pre-treatment in ∝-bromonaphthalene for four hours, fixation in Carnoy's II fluid for 24 hours and staining overnight in Snow's carmine were found to be the best. Incorporation of a little ferric acetate in the fixative was found to improve staining.

This procedure is different from that used by earlier workers. Raghavan and Venkatasubhan (1943) used Navashins fluid as fixative and Newtons iodine - Gentian - violet as stain for the study of mitosis in the family Zingiberaceae. This is a very old method suited for the study of chromosomes by sectioning of tissues and not suited for modern squash techniques. Ramachandran (1969) used chilling of the plant material as pre-treatment, Carnoy's fluid as fixative and acetocarmine as stain for the chromosome studies in Zingiberaceae. The acetocarmine as mentioned earlier had the defect of excessive staining of the cytoplasm. Improvement in staining as well as spread of chromosomes could be achieved by the present method developed by us.

Karyotype analysis

Karyotype analysis in Kaempferia galanga L. showed that the somatic cells contained 55 chromosomes. Detailed studies on the morphology of individual chromosomes has shown that these 55 chromosomes could be classified into 11 sets of five each (Plate 4). The occurrence of five sets of structurally similar chromosomes indicates that K. galanga L. is a pentaploid with basic number 11. Mahanthy (1971) reported a basic chromosome number x = 11 in the family Zingiberacea. Beltran and Kam (1984) also reported the basic number as 11 in Asiatic Kaempferia. Our studies also fully support these findings on the basic number.

The length of chromosome measured showed that there is not much variation in the length of the chromosomes. The ratio between smallest and largest chromosome was only 1:1.7. The classification of karyotype according to the length of the arms and position of centromere showed that there are six sets of chromosomes having long and short arm ratio below 1.25 and they are classified as median. Two sets of chromosome were having satellites and were named as SAT chromosomes. Another two sets were having the primary constriction in a submedian position and arm ratio between 1.25 to 1.75 and were classified as submedian chromosomes. Karyotype classification according to Stebbins (1958) revealed that none of the chromosome is having long arm: short arm ratio more than 2:1 and the karyotype comes under '1a' group which is the most primitive one. The karyotype can be called as a symmetric one.

Present studies also revealed the presence of ten SAT chromosomes in the karyotype of Kaempferia. These 10 SAT chromosomes represent the chromosome numbers nine and eleven of the idiogram. In Alpinia allughias and A. calcarata the attachment of four prochromosomes to a single large nucleolus and the organization of four nucleoli at somatic telophase has been interpreted as the possible evidence for this species being reported as tetraploid (Raghavan and Venkatasubhan, 1943). In our studies also eight to ten prochromosomes were detected during diakinesis. The presence of ten SAT chromosomes was also indicated by the

presence of numerous nucleoli at somatic telophase. This is yet another evidence to the fact that the plant is a polyploid especially a pentaploid. Sharma and Bhattacharya (1959) reported that K. galanga L. is a diploid with 2n = 22. Our results are in total disagreement with this report since all the 55 chromosomes could be clearly counted in good preparations (Plate 3). The chances of occurrence of diploids with 2n = 22 in nature, however, cannot be ruled out. Therefore it is assumed that such plants would have contributed to the pentaploid genome in their natural crosses with unreduced gametes of tetraploids. However, no systematic work has been undertaken so far to elucidate the phylogeny of this species.

Meiotic studies

Meiotic studies in polyploids may help to determine the nature of ploidy as well as the level of ploidy (Schaeffer, 1980). Pairing of chromosomes in a polyploid is indicative of chromosome relationship. Meiotic studies in Kaempferia galanga L. led to the conclusion that the material used in our studies is a polyploid and with all probability a pentaploid. The evidences for this conclusion are the following.

During meiosis multivalent formation was prevalent in almost all the PMCs at metaphase I eventhough the number of chromosomes involved in these associations varied. Trivalents, quadrivalents and pentavalents were prevelant in most of the cells in addition

to the bivalents and univalents (Plate 6). Hexavalents also were rarely observed. The occurence of multivalents is the most reliable evidence for the fact that this species is a polyploid. Lack of structural abnormalities as indicated by the absence of bridges, laggards and micronuclei during anaphase I also leads to the conclusion that multivalent formation was due to polyploidy alone. Polyploidy in the genus Kaempferia has been reported by many earlier workers (Raghavan and Venkatasubhan, 1943; Chakravarthi, 1948; Ramachandran, 1969 and Mahanthy, 1971). Chakravorti (1948) reported the formation of 11 bivalents during meiosis in a diploid Kaempferia species K. atrovirens. He also reported that K. rotunda L. and K. gilbertii were triploids with a chromosome number 2n = 33. All these reports support our finding that the basic chromosome number for Kaempferia is x = 11 and K. galanga L. is a pentaploid.

Both meiotic and mitotic studies undertaken in the present investigations lead to the conclusion that K. galanga L. has 55 chromosomes in the somatic cells. Raghavan and Venkatasubhan (1943), Raghavan and Arora (1958) and Ramachandran (1969) reported that chromosome number in this plant is 2n = 54. All the 55 chromosomes could be clearly recognised in the well spread cells of both mitotic and meiotic metaphase stage in our studies. Hence we do not agree to the above finding especially with the material used in the present study. The report of 2n = 54 may be due to aneuploidy and the plant might be a monosomic (2n-1).

Many of the naturally occuring polyploids such as wheat, datura etc. are having surviving aneuploid plants. Kaemperfia galanga.L. being a polyploid have all the probability to have aneuploid plants having chromosome numbers ranging from 50 to 54. Since Kaempferia is vegetatively propagated, the presence of aneuploidy and the absence of seed set usually do not pose any problems. Ramachandran (1969) also does not rule out the chance for the occurrence of pentaploidy in this crop as he has already suspected aneuploid pentaploidy in his report of 54 chromosomes.

Stebbins (1950) suggested that all the naturally occuring polyploids are of allopolyploid or segmental allopolyploid nature. Segmental allopolyploids are characterised by homoelogy or partial homology (Stebbins, 1947). This type of homology may indicate that only some of the members of the chromosome set are homologous with those of the other sets while the others are nonhomologous or partially homologous. The occurrence of multivalents involving varied number of chromosomes in the PMCs at metaphase I showed that the degree of affinity varied from cell to cell leading to preferential pairing of the chromosome. If all the five chromosomes were homologous they would definitely have formed pentavalents in most of the cells. Eventhough pentavalents were formed in many cells their frequency did not indicate complete homology in five each of eleven basic chromosomes. The affinity of chromosomes as expressed by multivalents indicated partial homology or homoelogy rather than complete homology

between them. Hence the possibility that this crop may be a segmental allopolyploid also cannot be ruled out. This assumption is based on the statement by Stebbins (1950) that 'most naturally occuring polyploids are either allopolyploids or segmental allopolyploids'. The genomic constitution of this species can thus tentatively be indicated as $A_1A_2A_2A_2A_2$. This might have originated by natural hybridization between a diploid race (A_1A_1) and an unreduced gamete of a natural autotetraploid ($A_2A_2A_2A_2$) race. Natural crossing would also have taken place between plants of other ploidy levels. Since the races involved in the original hybridization have generally grown in separate environments they very likely have undergone different cytological and genetic changes. However, no definite conclusions can be arrived at since Riley and Law (1965) suggested that 'pairing of chromosomes is not entirely a measure of chromosome relationship since this process is independently under a rigid genetic control'.

The prevalence of multivalents in the meiotic cell division leads to unequal separation during anaphase I resulting in gametes with unequal number of chromosomes. According to Jackson et al. (1979) odd number of genomes may result in several types of orientations at metaphase I with one or more genomes completely unpaired, unpaired chromosomes with some special disjunctional mechanism or a mixture of multivalents, bivalents and univalents. Indicating the classical example in Rosa canina (2n = 5x = 35), Jackson et al. (1979) suggested that an odd number of genomes

may not appreciably decrease fertility in cases with preferential disjunction of univalents. Gillie and Randolph (1912) noted a satisfactory significant reduction in quadrivalent frequency in tetraploid maize after ten generations of sexual reproduction. They also suggested that increased fertility is correlated with an increase in percentage of cytologically balanced gametes produced. In populations with the highest percentage of seed set the tetravalents fall apart at late diakinesis and the chromosomes reach the metaphase plate as bivalents. Later meiotic movements were regular and cytologically balanced gametes were produced. Thus natural polyploids behaved cytologically as diploids. Present studies also did not indicate any abnormalities during anaphase I or later stages of meiosis and the percentage fertility of pollen grains was also appreciable. The pentaploid genome in Kaempferia galanga L. appear to be comparatively balanced producing viable gametes especially due to the preferential disjunction of chromosomes.

From all the above observations we report for the first time that Kaempferia galanga L. is a pentaploid with $2n = 5x = 55$. The plant can be considered as a segmental allopolyploid as indicated by the preferential pairing of chromosomes.

Problems associated with seed set

When the absence of seed set and associated problems are considered Kaempferia deserves special mention. As a general rule

in almost every crop the seedlessness is found to be associated with sterility occuring due to poor pollen fertility resulting from meiotic abnormalities. In Zingiber a related genus of Kaempferia one of the main reasons for seedlessness is reduced pollen fertility associated with irregular meiosis (Ratnambal, 1979). In the present study eventhough multivalents were prevelant during diakinesis, bridges, laggards and micronuclei were not detected in the anaphase indicating the absence of structural aberrations. The present studies also revealed a mean pollen fertility of 73 per cent in Kaempferia. A fertility level of 43 to 91 per cent cannot be considered as a low value. However, all the stained pollen grains may not be viable. As Vazhintsanya (1960) pointed out most stains are not sufficiently accurate when compared to germination tests to give other than crude estimates of pollen viability. Kihara (1919) also reported that nonviable pollen grains of hybrid wheat stained as normal ones. Some variation was also observed in size of the pollen grains. The range in size was 106 to 128 µm. The variation in size as well as difference in fertility of different samples of pollen can be substantiated on the basis of meiotic behaviour in this crop. The prevalence of univalents during the meiotic cell division leads to random distribution of chromosomes to poles (Schaeffer, 1980). This can result in gametes having unequal number of genomes. However, an odd number of genomes may not appreciably decrease fertility in cases with preferential disjunction of univalents as reported in Rosa canina (Jackson et al., 1979). The relation between pollen size variation

and chromosome number is a well established fact. As early as 1919 Renner reported that pollen grain size is related to chromosome number. In polyploids the pollen grains can survive the deficiency of one or more chromosomes because of the presence of more than two homologous sets. The extra-genomes function as genetic buffers in such a case (Schaeffer, 1980). This factor might have contributed to the variation in fertility as well as size in the different samples of pollen in Kaempferia.

The contribution of cytological abnormalities and pollen sterility towards seedlessness being very meagre, the investigations were extended to identify other possible reasons for seedlessness. Usually such factors involve inadequacy of pollen, lack of pollination, lack of pollen germination, inadequacy of tube length or other incompatibility factors.

The estimates of pollen production Kaempferia flower showed that a single fertile stamen produces sufficient quantity of pollen grains, the number varying from 50,000 to 60,000. This number does not indicate the inadequacy of pollen production in this crop especially with respect to the number of ovules present. Studies on floral morphology also revealed that the floral characters are well adapted for entomophily. White showy tepals with purple coloured labellum, mild fragrance of the flower, presence of nectar and nocturnal anthesis are all adaptations for insect pollination. Adaptations favouring self pollination are also

prevelant in the flower viz., proximity of anther and stigma as well as homogamy i.e., simultaneous maturity of anther and stigma. Eventhough the flowers are well adapted for pollination by any means no seed set has so far been reported in Kaempferia galanga L. In the present study hand pollinations using pollen grains from the same flower or flower of a different plant belonging to various morphotypes were also done so as to confirm whether lack of pollination was the factor responsible for seedlessness. This attempt was also unsuccessful as no seed set could be obtained from artificial pollination. All these factors show the possibility for some floral adaptations to prevent seed set in Kaempferia.

Germination of the pollen grains was studied both under in vitro and in vivo conditions. Of the different media tried for pollen germination the one containing eight per cent sucrose, 60 ppm boric acid and one per cent gelatin was the most ideal for promoting satisfactory growth of the pollen tube in 69 per cent of the grains. Even in this medium the maximum pollen tube growth attained was only 732 μm. Bursting of the pollen as well as coiling of the pollen tube were also observed in some pollen grains in the germination media. Pollen germination studies in ginger revealed only 14 per cent germination under in vitro conditions (Sathiabhama, 1988). Compared to ginger the germination was satisfactory in K. galanga L. but even the maximum tube length attained was insufficient to reach the ovary surpassing the lengthy style.

Detailed studies on the morphology of stigma showed that the stigmatic surface was spiny. In vivo growth of the pollen tube was also examined using fluorescent microscopy. Under natural pollination the percentage of germination was found to be low and the penetration of the pollen tube through the style also was lacking (Plate 13). Many of the pollen grains seemed suspended on the spines without getting proper adherence to the stigmatic surface for germination. Presence of spines hindering pollen germination was also observed in ginger (Sathiabhama, 1988). In Kaempferia the length of style comes to about 4 cm (Plate 12). Assisted pollination studies revealed that even in presence of media promoting germination applied to the stigma, the pollen tube could reach only upto 1/5th of the length of the style. Fluorescent microscopic studies revealed that penetration of the pollen tube beyond the stigma occurs only under chemically aided pollination. Even after 24 hours the tube length attained was only 350 µm. Hence the chance of the pollen tube reaching the ovary before withering of the flower is quite remote. The retention of the flower in this crop was only for 12 to 16 hours. Hence the style length may be the major hindrance for fertilization in this crop. Similar results are also available in the related crop ginger in which the excessive length of the style was the major hindrance to achieve fertilization. Cutting off a part of the style and then applying pollen on the cut surface was also tried to induce seed set in this crop. However, the attempt failed as this flimsy

flower being unable to withstand the mechanical injury caused by stub pollination, withered without any seed set. Failure of stub pollination has also been reported in ginger by Usha (1983).

Moderately high germination of pollen grains was obtained under in vitro conditions. But even after 24 hours the tube length was not sufficient so as to surpass the lengthy style and to reach the ovary below. Another deformity noticed in different media was the coiling of the pollen tubes (Plate 11) as reported in plants having incompatibility reactions. Kho and Baer (1970) observed the pollen tube have thickened tips or coil in the vicinity of the embryosac in incompatible crosses. Different degrees of coiling could be observed under in vitro conditions coupled with bursting of the pollen grains. Even though the coiling of the pollen tube could not be traced in the style by fluorescent studies, similar deformities may be preventing the pollen tube from reaching the ovule. Hence the factors relating to seedlessness in this crop may be mainly due to the incompatibility reactions prevelant in the style and stigma. Since the crop is vegetatively propagated the chances for prevalence of incompatibility is also high, due to the accumulation of like alleles in different cultivars.

Based on the present studies the reasons for seedlessness in this crop appear to be one or more of the following.

1. Presence of spinous stigma which prevent adherence of pollen to the stigmatic surface
2. Poor germination of the pollen grains under in vivo conditions
3. Inability of the pollen tubes to penetrate the stigmatic surface
4. Slow growth of the pollen tube through the style so that it does not reach the ovary before withering of the flower
5. Coiling of the pollen tube
6. Bursting of the contents of the pollen tube before reaching the ovule
7. Caducous nature of the flower i.e., withering in a short span

The presence of incompatibility reactions contributing to many of the above factors are being reported for the first time in this crop. No serious studies on these aspects were undertaken so far. However, further studies are needed to investigate into the details of these and the present results are based only on preliminary observations.

From all the observations mentioned above the following conclusions can be arrived at.

Kaempferia galanga L. is a natural polyploid having a chromosome number of 2n = 5x = 55. The karyotype belongs to '1a' group of Stebbins classification. The prevalance of multivalents with varying numbers instead of pentavalents may be due to homoelogous nature of the chromosomes. This indicates that the plant may be a segmental allopolyploid, derived from partially

related genomes by natural hybridization. The abscence of meiotic abnormalities like laggards and resulting micronuclei may be because of the preferential disjunction of the chromosomes during anaphase I. A moderately high fertility of pollen grains also supports this view. The structural abnormalities like inversions and translocations were absent in this crop as indicated by the abscence of bridges during anaphase I. Thus the chance of contribution of cytological abnormalities and resultant pollen sterility towards seedlessness is meagre.

The seedlessness in this crop can mainly be attributed to the presence of incompatibility factors in the style. The sterility of the pollen grains and poor pollen germination on the stigmatic surface might also be contributing towards seedlessness.

Future lines of work suggested are

Further studies should be conducted in this crop in order to confirm the nature of ploidy as well as to unravel the phylogeny of this species. Investigations to confirm the nature of incompatibility reactions should also be taken up. The experiment using chemically aided pollination should be strengthened for studying the possibility for further growth of the pollen tube through the style and possible fertilization. In vitro pollination, can also be attempted in this crop in order to induce seed set by techniques like intra-ovarian, placental or ovular pollination.

Summary

SUMMARY

Investigations on cytogenetic aspects and floral biology of Kaempferia galanga L. were carried out in the Department of Agricultural Botany, College of Horticulture, Vellanikkara during the year 1992-93. The major objectives were to standardise the procedure for mitotic and meiotic studies and to verify the existing reports on chromosome numbers and also to find out the reasons responsible for seedlessness with respect to chromosome pairing as well as floral biology. The salient findings of the study are summarised below.

1. For mitotic studies roots were collected from plants grown under indoor conditions. Maximum number of dividing cells were observed in roots collected during the evening hours.

2. Among the four pre-treatment agents tried viz., Paradichlorobenzene, α-bromonaphthalene, colchicine and 8-hydroxyquilonine, the one which averted most of the cells at metaphase stage was α-bromonaphthalene. The duration of pre-treatment was four hours at 4°C.

3. Carnoy's fluid (II) was identified as the most satisfactory fixative compared to acetic alcohol (1:3) and Ostergren-Heneen's fixative.

4. The best stain suited for karyotype studies in Kaempferia galanga was standardised as 'Snows carmine'. This stain has several

advantages like easiness in preparation and storage, simplicity in staining, reusability of the stain and ability for storage of stained roots for longer periods. Stainability with this was better than acetocarmine and Feulgen stain.

5. Root tip squash studies revealed that the number of chromosomes in somatic cells of Kaempferia galanga L. was 55. This number is being reported for the first time in this species.

6. The 55 chromosomes could be classified into five sets of eleven chromosomes based on chromosome morphology. Hence it was concluded that the basic chromosome number (x) is 11 and that the species is a pentaploid.

7. The genome in Kaempferia galanga L. is characterised by 2 SAT chromosomes, 6 median chromosomes and 3 submedian chromosomes.

8. According to Stebbins (1958) classification this karyotype was identified as a symmetrical one belonging to '1a' group.

9. Photomicrographs were taken and karyotype was constructed for the first time for this species. Idiograms based on actual length of chromosome as well as relative lengths of chromosomes are also presented.

10. Flowering in Kacholam occurred during July-August during which period the flower buds were fixed for meiotic studies. The time of fixation varied with weather conditions, best

fixative was identified as acetic alcohol and good staining was achieved with 1 per cent acetocarmine blended with a few drops of ferric acetate.

11. Meiotic studies revealed the presence of associations involving 2 or more chromosomes in addition to the univalents during diakinesis/metaphase I. Pentavalents, quadrivalents and trivalents were observed in most of the PMCs and their frequencies varied from cell to cell.

12. Irregularities during later meiotic stages were absent including bridges, fragments, laggards or micronuclei. This indicated the abscence of structural abnormalities including inversions and translocations in the chromosome complement.

13. The frequency of pentavalents was much less than expected and the chance for autopentaploidy in this crop was less suspected.

14. Mitotic and meiotic studies in K. galanga L. indicated the possibility for segmental allopolyploidy in the species.

15. Pollen fertility or viability was not much affected by the meiotic abnormalities. This might be due to the preferential pairing of chromosomes and their random separation during the formation of gametes.

16. The cytological factors contributing to sterility were found to be meagre and hence the morphological features responsible for seedlessness were examined.

17. The floral characters showed adaptations for autogamy by the proximity of anther and stigma as well as by homogamy.

18. The species also exhibited adaptations for entomophily by the presence of scented and showy flowers opening at night.

19. The single fertile stamen produced sufficient quantity of pollen grains (about 54,000/flower) to pollinate the limited number of ovules present in the flower.

20. Pollen studies revealed that the percentage fertility varied from 46 to 91 with a mean value of 73.

21. The ideal medium for pollen germination contained 8 per cent sucrose, 60 ppm boric acid and 1 per cent gelatin.

22. Maximum germination of pollen grains achieved under in vitro conditions was 68 per cent and the length of the pollen tube attained was 732 μm.

23. The presence of spines on the stigma prevented proper adherence of pollen to the stigmatic surface and only sparse germination was observed under in vivo conditions.

24. The average length of the style was about 40000 um while the maximum length of the pollen tube achieved even under chemically aided pollination was only 350 μm. Fluorescent microscopy studies revealed the abscence of pollen tube growth through the style except under chemically aided pollination.

25. Neither artificial pollination nor stub pollination induced seed set in Kaempferia galanga L.

26. Growth of the pollen tube was arrested in the style and often coiling of the pollen tubes or bursting of the pollen were also observed. The flower withered within 12 hours of opening much before the pollen tube reached the ovary surpassing the lengthy style.

27. The possible reasons for seedlessness identified were spiny stigma, poor pollen germination, slow growth of the pollen tube, excessive length of the style and presence of incompatibility factors.

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* Originals not seen

Appendix

APPENDIX-I

Meteorological parameters of the experimental site at the College of Horticulture, Vellanikkara for the period from May to August 1993

Weak No.	Month and date	Temperature		Relative humidity		Rainfall (mm)	Sunshine hours
		Maximum	Minimum	Forenoon	Afternoon		
19	May 7-13	35.4	25.9	82	56	1.4	8.6
20	14-20	34.1	23.8	86	65	0	6.0
21	21-27	34.5	24.4	88	62	0	6.6
22	28-3	32.8	24.0	90	69	0	4.3
23	June 4-10	29.6	23.3	95	80	236	1.8
24	11-17	29.2	23.8	95	81	237.9	1.8
25	18-24	30.4	24.5	94	73	85.5	4.4
26	25-11	29.2	23.6	94	82	186.4	2.9
27	July 2-8	28.6	22.7	95	78	188.9	2.0
28	9-15	28.7	22.6	92	83	167.8	1.8
29	16-22	28.9	22.9	94	76	128.1	2.8
30	23-29	28.0	23.1	94	80	101.0	2.9
31	30-5	29.1	23.7	95	76	96.4	3.6
32	August 6-12	29.9	23.5	95	75	54.9	4.6
33	13-19	29.2	25.1	93	78	66.3	3.3
34	20-26	29.8	23.2	96	74	61.9	5.6
35	27-2/9	29.8	23.5	95	73	33.6	7.2

CYTOGENETIC ANALYSIS IN KACHOLAM, *Kaempferia galanga* L.

By

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ABSTRACT OF A THESIS

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ABSTRACT

The present study on cytogenetics of Kaempferia galanga L. in relation to seed set was undertaken at the Department of Agricultural Botany, College of Horticulture, Vellanikkara during the period 1991-93. A local selection 'Vellanikkara' grown under garden of AICRP on Medicinal and Aromatic Plants was used for the study. The major objectives were to confirm the existing reports on chromosome numbers, to determine the nature of ploidy based on meiotic studies and to find out reasons for seedlessness in the crop in relation to cytology and floral biology.

The procedure for karyotype studies in Kaempferia was standardised. Pre-treatment of the roots in -bromonaphthalene for four hours at 4°C followed by fixation in Carnoy's fluid for 24 hours and staining overnight in Snow's carmine were effective in getting best cytological preparations. Mitotic studies revealed that this species is a polyploid and with all probability a pentaploid with 2n = 5x = 55. This somatic chromosome number is being reported for the first time in Kaempferia galanga L. The karyotype was found to be a symmetric one and belonged to '1a' group of Stebbins (1958) classification.

Meiotic studies revealed the presence of associations involving three, four, five and six chromosomes in addition to the bivalents and univalents. However, the number of multivalents were much less than expected and later meiotic abnormalities were

rather almost absent. Pollen grains also exhibited reasonable fertility and viability. Based on both mitotic and meiotic studies it was indicated that Kaempferia galanga L. is a segmental allopolyploid with five sets of genomes designated as $A_1A_2A_2A_2A_2$.

Studies on floral morphology and artificial pollinations to induce seed set led to the conclusion that seedlessness in the crop is mainly due to the incompatibility factors in the style and stigma. The spiny stigma does not permit the proper adherence and germination of the pollen grains and the pollen tube growth attained was not sufficient to surpass the lengthy style and to reach the ovary. Attempts to induce seed set by hand pollination and stub pollination also failed.