REFINEMENT OF *IN VIVO* AND *IN VITRO* POLLINATION TECHNIQUES IN TURMERIC (*Curcuma domestica* Val.)

By

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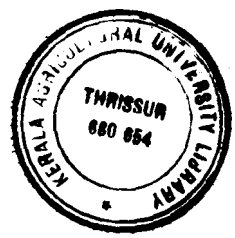
THESIS

Submitted in partial fulfilment of the requirement for the degree of



Master of Science in Horticulture

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DECLARATION

I hereby declare that this thesis entitled **"Refinement of *in vivo* and *in vitro* pollination techniques in turmeric (*Curcuma domestica* Val.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara

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CERTIFICATE

Certified that this thesis, entitled **"Refinement of *in vivo* and *in vitro* pollination techniques in turmeric (*Curcuma domestica* Val.)"** is a record of research work done independently by **Miss.Vijayasree, P.S.,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Vijayasree, P.S.

Affectionately dedicated to
my loving parents and grand mother

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF ABBREVIATIONS

BAP	- Benzylaminopurine
CH	- Casein hydrolysate
CW	- Coconut water
DAP	- Days after pollination
2,4-D	- 2,4-Dichloro Phenoxy Acetic Acid
FAA	- Formalin acetic acid mixture
GA	- Gibberellic acid
g	- gram
h	- hour
IAA	- Indole 3-Acetic Acid
IBA	- Indole Butyric Acid
Kg	- kilogram
Kin	- Kinetin, N^6-furfuryl acetone
mg l^{-1}	- milligram per litre
mm	- millimeter
MS	- Murashige and Skoog's (1962) medium
NAA	- α-Naphthalene Acetic Acid
PEG	- Poly ethylene glycol – 4000
ppm	- parts per million
t ha^{-1}	- tonnes per hectare
v/v	- volume in volume
µm	- micrometer

INTRODUCTION

INTRODUCTION

Turmeric (*Curcuma domestica* Val.) a native of Indo Malayan region, characterized by bright orange yellow rhizomes is very much important to mankind as a spice, food colourant, drug and cosmetic.

Turmeric is the third important spice crop of India next to black pepper and chilli. It is being cultivated in an area of 1.69 lakh ha with an annual production of 6.98 lakh t. India is by far the largest producer and exporter (35,560 t) of turmeric in the world and earns a foreign exchange to the tune of Rs.121.69 crores annually (Sasikumar and Sarma, 2001).

Production of turmeric in Kerala is limited to the tune of 9100 t of dry turmeric from an area of 3800 ha. However, the Alleppey Finger Turmeric (AFT) from Kerala is famous in the international market on account of its high curcumin, the yellow colouring pigment (6 to 7%).

Turmeric is very much identified with human civilization, religion, customs and it finds use both in developed and under developed countries. The curcumin is gaining importance in food industry, pharmaceuticals, preservatives and in medicine as antiseptic and anticancerous. With the ban of artificial colours in food industry, the use of curcumin has become wide spread. These facts point to the future prospects of turmeric cultivation in the Country. To take up cultivation in a competitive manner, high yielding varieties with high quality and disease resistance is a must.

So far, crop improvement in turmeric is limited to clonal selection and mutation breeding only. The existing population of turmeric shows tremendous variability for dry yield, curing percentage, curcumin content and disease resistance. The available genepool is not so far fully exploited for crop improvement as hybridization technique is not yet perfected for this crop.

Cultivated turmeric consists of short, medium and long duration types. Short duration types are generally fertile tetraploids (2n=84) while medium and long duration types are sterile triploids (2n=63). Seed set in controlled crosses involving short duration types has been reported by Nazeem and Menon (1994) and Renjith (1999) but posed difficulty with medium and long duration types as one of the parents is triploid.

According to Renjith (1999), medium duration types are not sterile. Pollen germination and tube growth occur in medium duration types in a limited scale, but not to the extent of short duration types. So there is possibility of getting seed set in medium and long duration types when short duration types are used as male parents or when pollination is done following cut style method or just above the ovary. He has also reported seed development in turmeric through *in vitro* pollination.

Present study "Refinement of *in vivo* and *in vitro* pollination techniques in turmeric (*Curcuma domestica* Val.)" aims at refining the hybrization technique in turmeric either through *in vivo* or *in vitro* pollination. Success in this line will

open up new vistas of crop improvement in long duration types which occupy 93 percentage of cultivated area of turmeric. Alleppey turmeric, which is rich in curcumin content can be further improved for curing percentage. Establishment and maintenance of hybrid population will be easier as micropropagation is standardized in this crop.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Turmeric (*Curcuma domestica* Val.) shows wide variability for duration, yield components quality,components like curcumin, curing percentage and reaction to major pests and diseases. Hybridization is a viable tool for assembling desirable economic characters in a genotype for evolving superior varieties. But the crop improvement in turmeric through hybridization is not realized yet due to various reasons. The present study aims at perfecting a hybridization technique in turmeric either through *in vivo* or *in vitro* pollination. The accumulated literature on various aspects relevant to the subject matter of the present investigation is reviewed hereunder:

2.1 Cultivars

In India a number of cultivars are distinguished by the names of the localities in which they are grown (Purseglove *et al.,* 1981). Menon (1975) found that the turmeric produced in different localities vary in quality, Alleppey, Rajpuri, Guntur and Madras being popular trade names of Indian turmeric.

Turmeric types can be grouped into three, based on maturity period as short, medium and long duration types. Short duration types are known as Kasturi. They mature in seven months, rhizomes possess pleasant aroma, good yielders of dried turmeric and rich in volatile oil content but low in curcumin and used in culinary preparation. Flowering is common in these types and seeds can produce gametic seedlings. Medium duration Kesari types (Bontha) mature in eight months

referred as intermediary types and are high yielders of fresh rhizomes than Kasturi types and rich in curcumin and volatile oil. Long duration types mature in nine months and are moderately good both for rhizome yield and other quality constituents. Flowering and seed set are rare in medium and long duration types (Rao *et al.,* 1975 and Rao and Rao, 1988).

All short duration types are not in confirmity with the above statements. Exceptions occur. Released short duration varieties, Suguna, Sudharsana are low in curing percentage (12 to 14) but some what high in curcumin content (>5%) (Edison *et al.,* 1991).

Long duration types are economically more important accounting for 96.4 per cent of total area under turmeric cultivation and the remaining 3.6 being under short duration types (Pruthy, 1976).

Some of the popular Kasturi types of Andhra Pradesh are Kasturi Kothapeta, Kasturi Tanuka, Kasturi Amalapuram, Chayapasupu, Gummalakshipuram etc. They have an yield potential of 10 to 15 t of raw turmeric per ha. The curing percentage of Kasturi Kothapeta, Kasturi Tanuka and Kasturi Amalapuram is about 24 to 25 per cent while in Chayapasupu it is about 19 per cent and in Gummalakshmipuram 14 per cent. Gummalakshmipuram has a high curcumin content of 5 per cent (Rao and Rao, 1994).

Popular Kesari types of Andhra Pradesh are Kesari Deevur and Amrutapani Kothapeta (Rao and Rao, 1994). Popular long duration types are

Duggirala, Tekurpet, Mydukur, Armoor, Sugandham, Vontimitta, Nandyal, Avanigadda. They have an yield potential ranging from 15 to 30 t ha^{-1} and curing percentage 15 to 22.4 (Rao and Rao, 1994).

Erode and Salem are popular cultivars of Tamil Nadu with curcumin 3.9 and 4.5 per cent respectively (Muthuswamy and Ahmed Shah, 1982).

Shillong and Tall Karbi are popular cultivars of Assam (Rathiah, 1986). Shillong is high yielder of fresh rhizomes (40 t ha^{-1}) and low in curing percentage (14) but good in curcumin (4.1%). Tall Karbi is also a good yielder (30 to 40 t ha^{-1}) but low in curing percentage (17.6) and curcumin content (3.06%).

Rajpuri and Evaigon are the popular cultivars in Maharashtra and Gujarat. Rajpuri is a moderate yielder (20 t ha^{-1}) with 3.4 per cent curcumin content, while Evaigon is high green yielder of (45 t ha^{-1}) with low curing percentage (17) (Pujari *et al.,* 1987).

The popular cultivars in Orissa are Duhgi, Jabedi and Katingia. Duhgi is a low yielder (10 t ha^{-1}) but has good curing percentage (26%) and poor in curcumin content (1.2%). Jabedi is low in curcumin content (1.2%), while Katingia has high curing percentage (36) but poor in yield (8 t ha^{-1}) and curcumin content (1%) (Rao and Rao, 1994).

Gorakhpur, the popular cultivar of Uttar Pradesh has a green yield of 15 t ha^{-1} and moderate curing out turn (18%) and curcumin content (3.5%) (Rao and Rao, 1994).

Alleppey turmeric has an yield potential of 25 t ha^{-1} and high curcumin content of 6.5 per cent but with slightly low curing percentage of 19 (Lewis, 1973 and Purseglove *et al.,* 1981).

Mannuthy Local from Central Kerala is a good yielder of rhizome with 24 t ha^{-1} and rich in curcumin content of 7.58 per cent (Philip, 1978).

The superiority of Mannuthy Local cultivar was also reported by Kurian and Valsala (1995). They found that the local cultivars from Kuruppampadi (Ernakulam District) and Mupliyam (Thrissur District) are rich in curcumin content with 7.82 and 7.69 per cent respectively.

Turmeric produced in different localities vary in quality and price. Alleppey, Salem, Erode, Rajpuri and Nizamabad fingers are popular trade names of Indian turmeric (Spices Market, 1999). Velayudhan *et al.* (1994) classified 568 collections of *Cucurma longa* into 21 morphotypes based on the aboveground and underground characters.

2.2 Genetic variability

Evaluation of 19 turmeric types collected from different parts of the Country revealed the presence of significant variation among types with regard to morphological characters, yield of fresh turmeric, curing percentage, oleoresin, curcumin content, leaf blotch, leaf spot and shoot borer infection. The yield of cured produce varied between 8558.4 kg ha^{-1} (VK 5 - Mannuthy Local) and 1504.1 kg ha^{-1} (Thekurpet). The curing percentage ranged from 14.06 (Nandyal) to

28.17 (Dindrigam Ca 69). The oleoresin content varied from 12.1 per cent (Kasturi Tanuka) to 21.1 per cent (Duggirala Cll-325) and the variation in curcumin content was from 2.33 per cent (Duggirala) to 6.55 per cent (VK 5 - Mannuthy Local). The incidence of leaf blotch disease ranged from 1.16 per cent (G.L – Puram 11) to 5.27 per cent (Armoor CII-325), leaf spot from 1.54 per cent (VK 5 - Mannuthy Local) to 4.07 per cent (Rajpuri) and shoot borer infection from 18.1 per cent (VK 5) to 84.5 per cent (G.L. Puram). Heritability estimates were higher for curing percentage, curcumin and oleoresin content (Philip, 1978; Philip *et al.,* 1980; Philip and Nair, 1983 and Philip and Nair, 1986). The investigations concluded that significant variation among the turmeric types grown under the same cultural and agroclimatic conditions can be attributed to the genetic factors.

Occurrence of genetic variability in turmeric for morphological characters, yield, curing percentage, curcumin content and disease resistance were also reported by a number of workers (Philip, 1978; Mohanty, 1979; Mukhopadhyay *et al.*, 1986; Pujari *et al.*, 1987; Jalgaonkar *et al.*, 1988; Reddy and Rao, 1988; Jalgaonkar *et al.*, 1997; Yadav *et al.*, 1996; Kurien and Valsala, 1995 and Kurien and Nair, 1996).

The open pollinated seedlings of turmeric *C. aromatica* viz. Amalapuram and Dindrigam showed variability with respect to fresh yield, curing percentage, curcumin and oleoresin content (George, 1981).

Menon *et al.* (1992) evaluated 39 open pollinated progenies of turmeric variety, Nandyal and found significant differences among the progenies with

respect to all plant characters except the number of tillers/plant, rhizome characters, yield, curing percentage and curcumin content.

2.3 Crop improvement in turmeric

So far, the crop improvement in turmeric is limited to clonal selection and mutation breeding. The genepool of cultivated turmeric consists of high yielding types like Dindrigam and G.L. Puram with high curing percentage of 35 and high curcumin types like Sobha and Mannuthy Local (> 7 per cent). The available wide variability for yield and quality characters are not so far exploited for crop improvement, as hybridization technique is not perfected.

2.3.1 Clonal selection

Varietal improvement through clonal selection has resulted in the release of a few high yielding cultivars with good quality.

Suguna, Suvarna and Sudharsana are high yielding varieties released for Kerala (Rao and Rao, 1994 and Mohanakumaran and Rao, 1996).

Suvarna has a green yield potential of 43.5 t ha^{-1} curing percentage 20 and curcumin content 4 per cent and oleoresin 12.91 per cent. Suguna has a raw yield potential of 60.3 t ha^{-1}, curcumin of 4.9 per cent curing percentage 24.6 and oleoresin 13.85 per cent. Sudharsana has a high yield potential of 54.8 t ha^{-1} with curcumin content of 7.9, curing percentage of 25.3 and oleoresin 13.63 per cent (Edison *et al.*, 1991). According to Renjith (1999) the curing percentage of Suguna is 12, while that of Sudarsana 11.5.

Kanthi is a clonal selection from Mydukur cultivar of Andhra Pradesh and has curing percentage 20.15, Curcumin content 7.18 per cent and oleoresin 12.13 per cent. Sobha is a clonal selection form the local cultivar of Methala region of South Kerala. It has a curing percentage of 19.28, curcumin content 7.39 percentage and oleoresin 15.95 per cent (Mohanakumaran and Rao, 1996).

According to Renjith (1999) curing percentage of Kanthi is 15 and curcumin content is 7.02 per cent. The variety Sobha has a curing percentage of 14 and curcumin 7.43 per cent.

Prabha is an open pollinated progeny selection from Kerala. It has a green yield potential of 37.47 t ha^{-1} with curcumin content of 6.52 per cent, curing percentage 15.0 and oleoresin content 15 per cent. Pratibha is also an open pollinated progeny selection from Kerala. It has a green yield potential of 39.12 t ha^{-1}, curing, curcumin and oleoresin percentage are 18.5, 6.21 and 16.20 respectively.

The other released varieties for the various states of the Country are Krishna, Sugandham, BSR-1, Suroma, Rajendra Sonia, Ranga, Rasmi, Mega Turmeric and RCT-1 (Edison *et al.*, 1991 and Peter, 1997).

2.3.2 Hybridisation

Nazeem and Menon (1994) have reported seed set for the first time in controlled crosses of *C. aromatica* types (short duration) and *C. domestica* (long duration) types. Seed set was noticed in eight of the eleven crosses tried. The

hybrids are of the crosses Amalapuram x Nandyal, Amalapuram x Amrithapani Kothapetta and its reciprocal, Kodur and Amalapuram.

2.3.3 Mutation breeding

Co-1 and BSR-1 are the two varieties recommended for cultivation in Tamil Nadu, which were developed from Erode local by X-ray irradiation (Khader and Vedamuthu, 1989).

2.4 Cytology

Sugiura (1936) was the first to report the chromosome number in *C. longa* as 2n=64.

Raghavan and Venkatasubhan (1943) reported chromosome number for *C. longa* as 2n = 62,and for *C.aromatica* as 2n=42.

A chromosome number of 2n = 32 for *C. longa* was reported by Sato (1960) and based on karyomorphology he concluded that the species seems to be allotetraploid with a basic number of x=8. According to Nair *et al.* (1980) this can be a miscount as later works suggest a chromosome number of 63.

Cytology of six species of *Curcuma* and seven cultivars of *C. longa* was reported by Ramachandran (1969). A chromosome number of 2n = 86 for *C. aromatica* (short duration types) was 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 auto triploid nature.

A further report on the chromosome number in Zingeberaceae and genus *Curcuma* was published by Ramachandran (1969) wherein he reported the chromosome number for *C. aromatica* (2n = 63,86) and *C. longa* (2n = 63). A high basic number of n = 21 was assumed for *Curcuma* and Ramachandran (1969) concluded that the genus *Curcuma* might have been derived either by dibasic amphidiploidy (x = 9 + 12) or by secondary polyploidy.

Detailed cytological investigations by Nambiar (1979) revealed that all the cultivars of *C. aromatica* had chromosome number of 2n = 84 and *C. longa*, 2n = 63. This along with 2n = 42 for *C. amada* indicated a polyploid series with multiples of n = 21.

Renjith (1999) reported that the somatic chromosome number of short duration cultivars VK 70 and Suvarna was found to be 2n = 84 and that of medium duration cultivar Kanthi as 2n = 63.

2.5 Flowering

2.5.1 Flowering behaviour

Valeton (1918) reported that all the cultivars in Java came to flowering but the fruit set was observed only in two. Aiyer (1954) mentioned that flowering was scarce in turmeric.

Patnaik *et al.* (1960) and Pai (1961) described the flowering behaviour of *C. longa*. The flowering period ranged from June to October.

Aiyadurai (1966) mentioned that the climatic conditions influenced flowering to a great extent. He also stated that in Andhra Pradesh cultivars of the *longa* species flowered very rarely and viable seeds could be collected from the flowering types.

Pillai and Nambiar (1975) noticed flowering and fruit set in 9 *longa* types and 8 *aromatica* types. Philip (1978) observed that out of the 19 types studied, 15 had flowered under Vellanikkara conditions. The percentage of flowering ranged from 1.2 to 95.28 per cent with the maximum in VK-14 (Dindrigam Ca-69) and the minimum in VK-16 (Rajpuri). Flowering was high in Amalapuram (92.7%) also.

Under Kasaragod conditions, the flowering period was July to September in *C. aromatica* and September to December in *C. longa* (Nambiar *et al.*, 1982).

Renjith (1999) reported flowering in turmeric from July to October. The plants flowered within a period of 105 to 155 days after planting.

2.5.2 Inflorescence

The inflorescence of turmeric is a compound spike born terminally on the leafy shoot and partially enclosed by leaf sheath. The spike bears bracts with

spiral arrangement and dark green at the base which are fertile i.e., carry flowers while the bracts on the top are pale green and sterile i.e., do not carry flowers. Each lower fertile bracts carry three flowers which open in succession. The mean number of flowers per inflorescence ranged between 26 and 35.2 (Philip, 1978; Lad, 1993; Nazeem and Menon, 1994; Renjith, 1999).

2.5.3 Floral morphology

The flowers of turmeric are yellowish white in colour, zygomorphic, bisexual, bracteolate and epigynous. The perianth is in two whorls, consisting of six segments, making a gamosepalous calyx and a gamopetalous corolla. Calyx is tubular and short dividing above into three short teeth. Corolla tube is funnel shaped having three ovate/oblong lobes.

Androecium consists of six stamens which are epipetalous arising from the throat of the corolla tube in two whorls. The outer whorl of three stamens is represented by petalloid, trilobed labellum, the most conspicuous part the flower. In the inner whorl, the posterior stamen was fertile and had a prominent two lobed anther with a sickle shaped spur, having broad connectives projected beyond the anther. The other two are reduced and seen at the base of the flower. Style is elongated and slender lying in the channel. The stigma is seen projected above the anther and is bifid. Ovary is inferior, sincarpous, trilocular and with axile placentation. Ovary measured a mean length of 0.26 cm and diameter of 0.24 cm at the middle. The average number of ovules per ovary was 29.31. The ovules showed a mean length of 611.44 µm and breadth of 436.65 µm at the middle. Fruit

is a capsule with numerous arillate seeds (Philip, 1978; Purseglove *et al.*, 1981; Lad, 1993 and Renjith, 1999).

2.5.4 Floral biology

Patnaik *et al.* (1960); Pai (1961) and Nazeem and Menon (1994) have described the floral biology of *C. longa.*

According to Renjith (1999), the inflorescence primodium of 4 to 5 cm length developed into a blooming inflorescence within a period of 9.6 days and that anthesis in different turmeric cultivars started by 5 am and continued upto 6 am. The anther dehiscence took place between 7.15 am and 7.45 am.

2.6 Pollen studies

2.6.1 Pollen morphology

The pollen grains are ovoid to spherical in shape, highly heterogeneous in size, light yellow in colour and sticky (Nazeem and Menon, 1994). They are highly heterogeneous in size among cultivars with length ranging from 59.48 µm in Kanthi to 82.61 µm in VK 70 and breadth ranging from 54.29 µm in Sobha to 63.87 µm in Suguna (Renjith, 1999).

2.6.2 Pollen fertility and germination

According to Nambiar *et al.* (1982) the pollen stainability in acetocarmine varied between 45 to 48 per cent in *C. longa* and 68 to 75 per cent in *C. aromatica.*

Nazeem and Menon (1994) have reported pollen fertility ranging from 71.1 per cent (Kodur) to 84.46 per cent (Kuchipudi) based on stain test.

According to Renjith (1999) the mean pollen fertility of turmeric cultivar as per acetocarmine stain test was 78.51 per cent. Pollen fertility was high in the flowers at the lower portion (90.32%) and low at the middle (82.38%) and upper portions (61.13%). He also stated that the cultivars differed significantly with respect to pollen germination percentage. Germination was influenced by the position of flowers in the inflorescence and the germination was high in the flowers at the lower portion (32.55%) and low in the upper portion (22.93%).

2.6.3 Pollen storage

Pollen storage becomes necessary for intercrossing genotypes of asynchronous flowering or growing in different regions. Viable stored pollen will be available whenever the female parent flowers. In turmeric flowering of short, medium and long duration types are scattered. So the use of stored pollen can be thought of for conducting crosses between specific short, medium and long duration types.

Storage of pollen in organic solvents avoids the problem of maintaining relative humidity and may be useful technique for transporting pollen without drying or in refrigeration (Iwanami, 1971). He reported pollen storage of *Lilium* sp. in various organic solvents such as acetone, benzene, petroleum benzene, benzyl alcohol, methanol, isopropanol etc.

Kobayashi *et al.* (1978) tried nine organic solvents for the storage of citrus pollen. The pollen viability could be retained for four months at –20°C in most of the organic solvents. He also reported that alcohols have generally been found unfavourable for pollen storage.

In chrysanthemum, Ikeda *et al.* (1998) reported pollen storage in organic solvent benzene at 1°C prolonged pollen germination ability maintaining 70 per cent viability after 35 days.

2.6.4 Pollination and seed set

Patnaik *et al.* (1960) reported that in turmeric pollination is brought about by insects.

Seed setting in turmeric has been reported by Aiyadurai (1966) and Pillai and Nambiar (1975).

Seed set was noticed in *C. aromatica* types while in the triploid species i.e., *C. longa* types, fruit set as well as seed set was not observed (Nambiar *et al.*, 1982).

Nazeem and Menon (1994) observed seed set in crosses involving *Curcuma domestica* and *C. aromatica* types. In crosses involving the aromatica types, seed set was high while none of the varieties produced seeds on selfing.

Renjith (1999) reported seed set in crosses involving short duration cultivars (*C. domestica*). But failed to obtain success in crosses involving short and

medium duration cultivars. The fruits matured within a period of 20 to 22 days. The number of seeds per fruit ranged from 4 to 6.

Two distinct type of seeds, dark heavy and light brown were extracted from mature capsules of *C. aromatica*. The seeds had a white aril, smooth surface and an apical micropylar ring with a wavy line (Nambiar *et al.*, 1982).

Lad (1993) observed seed setting in turmeric cultivars Ca-92/1 and Ca-17/1. Ca-92/1 produced 27 seeds/plant whereas Ca-17/1 only 4 to 5 seeds per plant. According to him turmeric seeds are triangular and of bright chocolate, lustrous colour with white placenta persisting at the base.

2.6.5 Seed germination studies

Nambiar *et al.* (1982) reported that percentage of germination varied from cultivar to cultivar and even plant to plant. Within 8 to 20 days after sowing 70 to 90 per cent germination was recorded. There was practically no germination, after the 20th day. Seedlings raised from seeds of individual plants had remarkable morphological similarities to the parental clones.

Nazeem *et al.* (1993) observed the seed germination in 17 to 26 days after sowing and duration of germination ranged from 10 to 44 days in various crosses.

Seed germination ranged from 17.22 to 100 per cent and seedlings produced only root tubers during the first year of growth and the weight ranged between 14.18 and 49.4 g (Nazeem and Menon, 1994).

Renjith (1999) reported that seeds produced *in vivo* germinated under *in vitro* condition on moist filter paper.

2.7 *In vitro* pollination and fertilization

The technique of *in vitro* pollination and fertilization is an effective tool for getting seed set in species where prefertilization barriers block seed set. The most important application of the technique is the production of rare hybrids (Bhojwani and Razdan, 1983).

2.7.1 *In vitro* pollination and fertilization technique

The pre-requisites and procedures to be adopted for the successful *in vitro* pollination had been described by Kanta and Maheswari (1963).

The technique consists of (1) A detailed study of floral biology of the crop i.e., time of anthesis, anther dehiscence, mode of pollination etc. (2) Pollen germination and pollen tube growth has to be determined (3) Standardisation of a surface sterilization technique for flowers and flower buds so that the viability of pollen grains and receptivity of gynoecium is not affected (4) Identification of a suitable medium which support the development of the ovule or ovary into mature seed or fruit (5) Standardization of appropriate pollination technique so that fertilization and growth of the ovule takes place (6) Identification of suitable culture conditions which will promote the growth of the ovule to viable seeds. (7) Histological examinations of ovule or ovary at various stages to know the

fertilization and development of embryo and endosperm. (8) Standardization of culture conditions for germination of seeds.

2.7.2 Methods of *in vitro* pollination

The different methods of *in vitro* pollination as per Bhojwani and Razdan (1983) are *in vitro* stigmatic pollination, intraovarian pollination, *in vitro* placental pollination and *in vitro* ovular pollination. *In vitro* stylar pollination was reported by Niimi (1976), Tuyl *et al.* (1991) have reported *in vitro* grafted style pollination.

2.7.2.1 *In vitro* stigmatic pollination

The pollen grains are applied on the stigma. Usha (1965) obtained seeds in *Antirrhinum majus* by *in vitro* stigmatic pollination. In Lilium interspecific hybrids were successfully obtained through *in vitro* stigmatic pollination (Tuyl *et al.*, 1991).

2.7.2.2 *In vitro* stylar pollination

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

2.7.2.3 Grafted style pollination

Here pollen is applied in a compatible stigma while the style with the germinated pollen is attached to the ovary of another plant which is incompatible.

In Lilium crosses style and stigma were joined using a piece of drinking straw filled with stigmatic exudates or were stuck together only with the exudates (Tuyl *et al.*, 1991).

2.7.2.4 Intraovarian pollination

Pollen grains are directly applied into the ovary in intra ovarian pollination.

In *Papaver rhoeas,* Kanta (1960) developed seeds through modified intraovarian pollination. In this technique the pollen suspended in distilled water having boric acid (100 ppm) was injected into the ovary through a hole using a hypodermic syringe. This technique was also successfully adopted in *Papaver somniferum* and *Eschscholzia californica.*

The pollen grain can also be introduced into the ovary through a slit made on the ovary wall. This technique has given positive result both in *Argemone mexicana* and in *Argemone ochroleuca.* By adopting this technique inter specific hybrids between these two species could be produced (Kanta and Maheswari, 1963).

2.7.2.5 *In vitro* placental pollination

In, *in vitro* placental pollination the whole placenta bearing ovules attached to short pedicel is generally used (Rangaswamy and Shivanna, 1967 and 1971a). The placenta may be cut into two or more pieces each carrying a certain number of ovules.

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

Castano (2000) reported *in vitro* pollination of isolated ovules of *Cinchorium intybus* L. In this ovules were developed and he could obtain seed set and seedlings at a low percentage (0.76%).

Fernando *et al.* (1998) described the success with in *in vitro* fertilization of the conifer *Pseudotsuga menziessii.*

2.7.2.6 *In vitro* ovular pollination/test tube fertilization

Pollen is directly applied to the excised ovules in *in vitro* ovular pollination.

This technique has successfully adopted in *Papaver somniferum, Argemone mexicana, Eschscholzia california, Nicotiana rustica* and *Nicotiana tabacum* (Kanta and Maheswari, 1963).

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

By this technique embryo were developed in plums (Lech *et al.*, 1994).

Slusarkiewicz (1984) also reported the test tube fertilization of ovules in some species of Solanaceae.

2.7.3 Factors affecting seed set

2.7.3.1 Age of flower buds

Generally the flower buds on the day of anthesis or one or two days after anthesis respond to *in vitro* pollination. The occurrence of seed set was reported to be higher when the ovules were excised 1 or 2 days after anthesis than on the day of anthesis (Kanta *et al.,* 1962, Kanta and Maheswari, 1963, Rangaswamy and Shivanna, 1967 and Balatkova *et al.,* 1977a).

In maize the spike, 3 to 4 days after silking is found suitable for *in vitro* pollination (Gengenbach, 1977). The flower buds one day prior to anthesis were ideal for *in vitro* pollination in Gossypium (Refaat *et al.,* 1984).

In Lilium, for the production of interspecific hybrids, the seed set was observed when the flowers pollinated three days prior to anthesis. Success rate was often reduced when pollination was done two days prior to anthesis.

In Chichory seed set was obtained after *in vitro* pollination when the flower buds at developmental stage before anthesis were pollinated (Castano, 2000).

2.7.3.2 Surface sterilization

A suitable surface sterilization technique, harmless to pollen viability and receptivity of the ovules, is necessary for the success of *in vitro* pollination.

Surface sterilization of the ovaries of *Papaver somniferum* which is relatively hard is done by dipping in rectified spirit and flaming. The ovaries of *Argemone mexicana, Eschcholzia californica, Nicotiana rustica* and *Nicotiana tabacum* which are more delicate were surface sterilized with chlorine water (Kanta and Maheswari, 1963).

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

In Vaccinium with 1 per cent sodium hypochlorite solution for 15 minutes, satisfactory surface sterilization was achieved (Munoz and Lyrene, 1985).

In Lilium, flower buds in ethyl alcohol (70%) for one minute followed by a commercial bleach (2% Cl) treatment for 15 minutes give proper surface sterilization (Tuyl *et al.*, 1991).

In maize surface sterilization was found unnecessary since the ovaries are protected by several layers of husks. The removal of inner husks with sterile forceps in the laminar flow chamber is sufficient (Gengenbach, 1977).

In chichory, flower buds were disinfected by an immersion in 1 per cent NaOCl for five minutes and then rinsed with sterile distilled water (Castano, 2000) gave proper surface sterilization.

2.7.3.3 Nature of explant

In the intraovarian pollination of *Papaver rhoeas* L. the retention of petals and sepals were found to accelerate the development of the ovary (Kanta, 1960).

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

In *Petunia axillaris in vitro* pollination on excised ovules or a group of ovules attached to a piece of placenta did not result in seed set. The pollen grains germinated normally, but it failed to enter the ovules. Normal seed development resulted when pollination was done on intact placenta with undisturbed ovules (Rangaswamy and Shivanna, 1971a).

In *Petunia hybrida* complete removal of the style had a deleterious effect on seed set following placental pollination. Consequently the entire pistil with exposed ovules by peeling the ovary wall alone was used for *in vitro* pollination (Wagner and Hess, 1973).

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

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

2.7.3.4 The culture medium

An appropriate culture media combination is necessary for the successful development of *in vitro* fertilized ovule into mature seed.

In the initial years of *in vitro* pollination and fertilization technique, the Nitsch medium (1951) identified for ovary development was used. (Kanta and Maheswari, 1963, Usha 1965, Rangaswamy and Shivanna, 1967 and Jarzina and Zenkteler, 1983).

In maize Gengenbach and Green (1975) reported modified MS medium to support *in vitro* seed development. Later it was found that MS medium is also suitable for the development of maize karyopsis (Dhaliwal and King, 1978, Bajaj, 1979 and Havel and Novak, 1981). MS medium was also suitable for the production of interspecific hybrids in *Gossypium*, *Nicotiana* and *Lilium* following the *in vitro* pollination (Refaat *et al.,* 1984; Slusarkiewicz, 1984; Zhou *et al.,* 1991 and Tuyl *et al.,* 1991). It was suitable for the crops plum and chichory also (Lech *et al.,* 1994 and Castano, 2000).

2.7.3.5 Sucrose concentrations

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

Generally sucrose has been used at a concentration of 4 to 5 per cent (Kanta and Mahewsari 1963; Usha, 1965; Rangaswamy and Shivanna, 1967 and Castano, 2000).

In maize a high concentration of 15 to 17 per cent sucrose has given successful results (Sladky and Havel, 1976 and Gengenbach, 1977a). But Dhaliwal and King (1978) could obtain viable seeds even with 5 per cent sucrose. Bajaj (1979) found that 7 per cent sucrose is optimum for maize.

In the production of interspecific hybrids of *Lilium* by *in vitro* pollination it was found that the sucrose concentration affect the capsule development. The swelling of the capsule was highest at a sucrose concentration of 10 per cent but embryos were mostly found in ovaries cultured at 6 to 8 per cent sucrose. Best results were obtained with MS medium supplemented with 7 per cent sucrose (Tuyl *et al.*, 1991).

Lech *et al.* (1994) observed that in plum, 2 per cent sucrose along with other supplements can support embryo development consequent to *in vitro* fertilization.

2.7.3.6 Effect of growth regulators and supplements

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

In plum, GA_3 0.5 ppm promoted the development of zygote after *in vitro* pollination (Lech *et al.*, 1994).

Casein hydrolysate at 500 ppm was found to enhance ovule development (Kanta and Maheswari, 1963, Wagner and Hess, 1973 and Zubkova and Sladky, 1975 and Castano, 2000).

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

Balatkova *et al.* (1977a) observed the influence of growth regulators like IAA, kinetin, tomato juice, coconut milk and yeast extract on seed development in tobacco after placental pollination. Coconut milk, tomato juice and yeast extract inhibited the seed development while the growth regulators like IAA 10 ppm or kinetin 0.1 ppm improved the number of seeds per ovary. Kinetin at a higher concentration (1 ppm) had an inhibitory effect.

2.7.3.7 Culture conditions

Cultures were usually stored in darkness or near darkness (Rangaswamy and Shivanna, 1967; Balatkova *et al.*, 1977a and Dhaliwal and King, 1978).

In *Petunia diffusa* daylight (10-12 ft candle at 25 ± 2°C) was found optimum for *in vitro* seed development (Rangawsamy and Shivanna, 1967). The result of *in vitro* pollination was same whether the cultures were incubated in light or dark (Zenkteler, 1969).

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

Castano (2000) reported ovule development in chichory when pollinated isolated ovules were placed in culture room under 16 h day length at 22° ± 2 C, 60 ± 5 per cent relative humidity and 40 μ mol $m^{-2}s^{-1}$ irradiance. The light was provided by fluorescent lamps.

2.7.4 Germination of *in vitro* produced seeds

The *in vitro* raised seeds may often require special pre-treatments, media and culture conditions for its germination.

In vitro seeds of *Papaver somniferum* did not germinate when they were attached to the placenta. If separated from the placenta, they germinated within 15 days after planting in new medium. Even though the endosperm development was not normal, *Argemone mexicana* germinated within three months after starting the culture. The seeds of *Nicotiana rustica* and *N. tabacum* germinated *in situ* as well as in basal medium (Kanta *et al.*, 1962).

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

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

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

The karyopsis of maize produced under *in vitro* condition were dried at room temperature for several days and then made to germinate on moist filter

paper at 28°C. They germinated within seven days after incubation (Gengenbach, 1977).

2.7.5 *In vitro* pollination in ginger

Seed set and seed germination in ginger through *in vitro* pollination and fertilization has been reported for the first time by Valsala (1994), Valsala and Nair (1996) and Valsala *et al.* (1996). The protocol standardized could be summarized as given below.

Flower buds on the day of anthesis and one day after anthesis were suitable for *in vitro* pollination.

Flower buds had to be surface sterilized before anthesis i.e., 2.30 pm. Surface sterilization was done by dipping the flower buds in streptocyclin 500 mg l^{-1} for one hour followed by wiping with 70 per cent alcohol and rinsing with mercuric chloride 0.1 per cent for 3 minutes.

In vitro pollination could be done by 5.30 pm as by this time pollen grains from anthers could be easily scooped out for pollination. Placental, modified placental and ovular pollination along with pollen germination medium (modified ME_3 medium) brought out fertilization. Placental pollination was the best as it registered maximum number of seeds per culture.

Seed set and seed development could be obtained in the medium ½ MS + 6 per cent sucrose + NAA 0.5 mg l^{-1} + BAP 2.5 mg l^{-1} + 15 per cent v/v coconut water. The effect of coconut water could be replaced by casein hydrolysate.

The *in vitro* produced seeds of ginger germinated, when 80 days old seeds were incubated initially in the medium of half MS with 2,4-D 8 mg l^{-1} for two months and then in hormone combination of BAP 9 mg l^{-1} and 2,4-D 0.2 mg l^{-1}.

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

2.7.6 *In vitro* pollination in turmeric

Seed set was obtained in turmeric (*C. longa* L.) through *in vitro* pollination (Renjith, 1999).

Flowers on the day of anthesis and one day prior to anthesis were suitable for *in vitro* pollination.

The flower buds and the flowers after removing the unwanted floral parts were surface sterilized by soaking in streptocyclin 350 mg l^{-1} for one hour followed by rinsing with 0.1 per cent $HgCl_2$ for 3 minutes.

Placental, modified placental and intraovarian pollination along with pollen germination medium (modified ME_3 medium) brought out ovule/seed development. Among them placental pollination was found to be the best. The culture medium ½ MS + BAP 1 mg l^{-1} + kinetin 1 mg l^{-1} + NAA 0.5 mg l^{-1} + 3 per cent sucrose along with organic supplements CW (15% v/v) and CH (200 mg l^{-1}) supported ovule development after *in vitro* pollination.

2.8 Micropropagation

Micropropagation of *Curcuma spp.* was first reported by Nadgauda *et al.* (1978). They got success with young sprouting buds in Murashige and Skoog medium supplemented with coconut milk 10.0 per cent, kinetin 0.1 mg l^{-1}, and BAP 0.2 mg l^{-1}. Smith's medium with above supplements was also suitable. Rooting was obtained in White's liquid medium supplemented with 2 per cent sucrose.

Kuruvinashetty *et al.* (1982) reported successful micropropagation in modified MS medium containing sucrose 4 per cent and kinetin 0.2 to 0.5 mg l^{-1}. Kuruvinashetty and Iyer (1982) was able to get a multiplication rate of eight plantlets from every bud cultured for two months which works out to over two lakh plantlets per bud per year. The medium used was MS supplemented with 0.2 mg l^{-1} kinetin and 0.4 mg l^{-1} BAP.

Winnaar and Winnaar (1989) cultured emerging buds of 5 to 10 mm long trimmed to contain the meristem with a few leaf primordia in a nutrient medium containing BAP mg l^{-1} and sucrose 2 per cent for one month. Later they were subcultured to fresh medium of the same composition. Shoot growth as well as multiple shoots and root formation occurred after 3 to 4 weeks.

Young sprouting buds of turmeric cultivars Co-1 and BSR-1 were cultured on MS medium supplemented with kinetin 1 mg l^{-1}, BAP 1 mg l^{-1} and 4 per cent sucrose. Multiple shoots were obtained on an average 2.11 and 2.50 respectively in BSR-1 and Co-1 (Kesavachandran and Khader, 1989).

In vitro clonal multiplication of turmeric was reported by Balachandran *et al.* (1990). Rhizome buds excised from *C. domestica, C. aeruginosa* and *C. caesia* were inoculated on MS medium with different combinations of benzyladinine and kinetin. For shoot multiplication 3 ppm BAP was found to be optimum for all the species.

Vidya *et al.* (1989) conducted micropropagation studies in turmeric and enhanced release of axillary buds were recorded, when inoculated on a medium containing cytokinins. A multiplication ratio of 1:2240 was obtained from single rhizome in a year as against 5 to 6 plants under field conditions.

Rajan (1997) reported micropropagation of turmeric by *in vitro* microrhizomes. MS medium supplemented with 0.3 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, 0.5 mg l^{-1} ancymidol and 10 per cent sucrose was best for induction of microrhizomes.

Sit and Tiwari (1996) reported that best sprouting in turmeric obtained through axillary bud culture in half MS media supplemented with BAP and Kinetin (both at 1 mg l^{-1}).

Babu *et al.* (1997) reported that multiple shoots could be induced from vegetative buds as well as rhizome explants in turmeric on MS medium with 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA.

2.8.1 Callus mediated organogenesis

Kuruvinashetty and Iyer (1982) reported initiation of callus cultures, when buds were grown on MS medium supplemented with 2 mg l^{-1} IAA or 0.5 mg l^{-1} 2,4-D in dark. These calli were soft and friable. When exposed to light, IAA induced calli underwent differentiation to produce several plantlets.

Rejuvenation of plantlets from the calli separated from the base of the shoot bud explant, were obtained when the separated calli inoculated on MS medium supplemented with kinetin 0.2 mg l^{-1}, 0.4 mg l^{-1} BAP, 0.01 mg l^{-1} GA_3 and sucrose 4 per cent (Kuruvinashetty *et al.*, 1982).

Nadgauda *et al.* (1980) reported that, on repeated subculture of shoots, a swelling was observed at the base of the leaves. These swelling when carefully removed and cultured on MS medium supplemented with 0.1 mg l^{-1} kinetin, 0.2 mg l^{-1} BAP and 10 per cent coconut water, formed a callus under dark incubation. This callus readily differentiated into shoots, when incubated in light and could be maintained indefinitely by monthly sub culture.

Yasuda *et al.* (1988) reported that callus was successfully induced from rhizome tissues of *C. zeodoria, C. domestica* and *C. aromatica* on MS medium supplemented with 1 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin.

MATERIALS AND METHODS

MATERIALS AND METHODS

The investigations on "Refinement of *in vivo* and *in vitro* pollination techniques in turmeric (*Curcuma domestica* Val.)" were carried out at the Department of Plantation Crops and Spices and the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 1998 to 2000. Details regarding the experimental materials and methodology adopted for conducting various aspects of the study are presented in this chapter.

3.1 Plant materials

Ten cultivars of turmeric viz. VK 70, VK 55, VK 76, Sudharsana, Suvarna, Suguna, Kanthi, Sobha, Prabha and Pratibha which are expected to show wide variation in crop duration, curcumin content and curing percentage were selected for conducting the studies. The cultivars represent different pedigree and geographic region (Table 1). Among the selected cultivars, VK 70, VK 55, VK 76, Sudharsana, Suvarna and Suguna are short duration types and Kanthi, Sobha, Prabha and Pratibha are medium duration cultivars.

3.2 Variability in plant materials

The selected cultivars were raised in the field under open condition during the cropping season of April, 1999 to January, 2000. Planting was done on raised beds of size 2 x 1 m taken at a spacing of 50 cm. Rhizome bits of 15-20 g were used for planting. The cultural, manurial and other plant protection operations were carried out as per package of practices recommendations of KAU (1996). The

cultivars were examined for variation in salient morphological characters, yield and quality parameters i.e., curing percentage, curcumin and oleoresin content. Observations were recorded in twelve plants of each cultivar at 6 months after planting and were analysed statistically in CRD design.

Morphological characters recorded include height, number of leaves, number of tillers per plant, length, breadth and area of leaves. Height of the plant was measured from the ground level to the top of longest leaf. The number of leaves and number of tillers per plant were also measured. The length of leaf was measured from the bottom of leaf lamina to the tip and breadth was measured at the middle portion of the leaf. The individual leaf area was estimated by multiplying the product of maximum length and maximum breadth of leaf with the factor 0.7 (Randhawa *et al.,* 1982).

The mother rhizome characters recorded include number of mother rhizomes, length, circumference, number of nodes, internodal length at the middle of the rhizomes. These observations were also recorded for the primary and secondary fingers. Mother finger ratio of each variety was also recorded.

Raw yield and dry yield of selected cultivars were recorded as kg/2 m^2. Curcumin content was quantitatively extracted by refluxing the material in methanol and was estimated spectrometrically at 425 nm (Sadasivam and Manikam, 1991). Curing percentage was estimated by boiling and drying one kg of

raw turmeric. Oleoresin content in each cultivar was estimated by solvent extraction method using acetone as solvent.

Table 1. Source and pedigree of different cultivars of turmeric

Sl. No.	Cultivars	Pedigree	Source
1	VK 70	Dindigram D. 182	Andhra Pradesh
2	VK 76	Dindigram CLNo.625	Andhra Pradesh
3	VK 55	Amalapuram CI 320	Andhra Pradesh
4	Suguna	PCT – 13	Andhra Pradesh
5	Suvarna*	PCT – 8	Assam
6	Sudharsana	PCT – 14	Manipur
7	Kanthi	Single plant selection from Mydukur	Andhra Pradesh
8	Sobha	Single plant selection from Methala local	Kerala
9	Prabha	Open pollinated progeny selection	Kerala
10	Pratibha	Open pollinated progeny selection	Kerala

* Suvarna was not included for pollination studies since it did not flower during the course of investigation.

3.3 Investigations for the improvement of flowering

3.3.1 Influence of size of seed material for staggered flowering

Rhizome bits of three different sizes by weight were used for the study

1) Rhizome bits of 15-20 g

2) Rhizome bits of 40-50 g

3) Whole clump of : 200 g

In each group vigour of the plants in terms of height, number of leaves, number of tillers, length, breadth and area of leaf per plant were recorded. Duration and intensity of flowering was also noted. The intensity of flowering was recorded as percentage of plants flowered.

3.3.2 Influence of light and shade on flowering

The selected turmeric cultivars were planted in open and shade of coconut garden of age, 23 years after planting. In each cultivar, observations on duration and percentage of flowering were noted.

3.4 Pollen studies

3.4.1 Pollen fertility and viability studies

Studies were carried out to determine pollen fertility and viability of selected cultivars. Flowers from the lower 1/3rd portion of the inflorescence were taken for fertility and viability studies. The pollen viability was observed in the beginning , middle and end of the blooming season of each variety. The observations recorded were statistically analysed for interpretation.

3.4.2 Estimation of pollen fertility

The fertility of pollen grains was estimated by staining with acetocarmine stain (Zirkle, 1937). The pollen grains were collected from freshly dehisced anthers on the day of anthesis and were stained and viewed at 100X magnification. All the stained, well filled pollen grains were considered as fertile and others as sterile. The fertility percentage was calculated using the formula.

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

The mean of a sample from 10 microscopic fields served as a replication.

3.4.3 Estimation of pollen viability

The *in vitro* pollen germination and tube growth of various turmeric cultivars viz. VK 70 VK 76, VK 55, Suguna, Sudharsana, Kanthi, Sobha, Prabha and Pratibha were studied in the modified ME_3 medium at pH 6 (Leduc *et al.*, 1990). The modified ME_3 medium at pH 6 was selected for the study based on the investigations done by Renjith (1999).

The composition of ME_3 medium is given in Table 2. The ME_3 medium was prepared by dissolving the required quantity of chemicals in distilled water and sterilizing before use. The pH was adjusted prior to sterilization.

Pollen grains were collected from the freshly dehisced anthers on the day of anthesis and were incubated in a humid chamber along with a drop of modified ME_3 medium taken on a glass slide. Observations were recorded 24 h after incubation. Total number of pollen grains and also the number of germinated pollen grains were counted on 10 microscopic fields and the mean germination percentage was calculated. Mean and maximum pollen tube growth attained were also measured using a calibrated ocular micrometer and was expressed in µm.

3.5 Pollen storage

Non polar organic solvents hexane, cyclohexane,isopropanol, methanol and acetone were used for pollen storage studies. The fresh pollen grains collected on the day of anthesis from the selected cultivars especially from the lower $1/3^{rd}$ portion of inflorescence were used for this purpose. Before storage, viability of the

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

Constituents	Concentration ($mg\ l^{-1}$)
Macronutrients	
$MgSo_4.\ 7H_2O$	370.00
KNO_3	950.00
H_2PO_4K	85.00
$CaCl_2.\ 2H_2O$	880.00
NH_4NO_3	412.00
KCl	175.00
Na_2EDTA	7.45
$FeSO_4.7H_2O$	5.55
Micronutrients	
H_3BO_3	50.00
$MnSO_4.H_2O$	16.80
$ZnSO_4.7H_2O$	10.50
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Thiamine	1.00
Pyridoxine	1.00
Others	
PEG – 4000	120,000

pollen grains in the modified ME_3 medium was estimated. The collected pollen grains were air dried for five minutes in a laminar air flow cabinet. They were then transferred to precooled solvents taken in small airtight screw cap bottles and kept in cold temperature at –15°C provided in the freezer of a refrigerator for one month. Viability of the stored pollen in ME_3 medium was monitored. For this, samples were drawn after one month and solvents were driven off by evaporation. The pollen grains thus obtained were rehydrated and incubated in ME_3 medium for 24 h for germination. The pollen viability was examined by viewing the pollen grains under the microscope. The efficacy of the different organic solvents for pollen storage at –15°C were compared.

3.6 Natural seed set

Natural seed set in the selected ten cultivars were recorded. In each cultivar 54 plants were observed. The mode of pollination was also examined. The time taken for fruit set was ascertained by observing pollinated flowers for ovary swelling daily for five days. The maturity of fruit was decided by examining the colour change of fruit and seeds. On maturity the ovary wall become dry and seeds turn dark brown.

3.7 Controlled *in vivo* pollinaiton

Controlled *in vivo* stigmatic, stylar and intraovarian pollination were carried out in different cross combinations among the selected turmeric cultivars. The stigmatic pollination was done as per the method described by Nazeem and Menon (1994). Emasculation was done 15 h prior to anthesis, in the previous

evening, from 4 to 6 pm using a needle and forceps. The rest of the floral buds including the developing ones present in the same bract were pulled out and removed. After emasculation, the inflorescence was bagged with butter paper cover. The next day morning from 6 to 8 am fresh pollen from the opened flowers of the desired male parent was collected using a needle and applied to the receptive stigma of the emasculated female parent. The pollinated flowers were labelled, tagged and whole inflorescence was bagged after pollination.

In vivo stylar pollination was done in minimum 20 plants of each cultivar by cutting styles at different lengths and by applying pollen grains along with pollen germinating medium at the cut end. *In vivo* produced turmeric fruit and seeds were described.

3.7.1 Pollen pistil interaction after *in vivo* and *in vitro* pollination

Pollen germination and tube growth after *in vivo* pollination and *in vitro* placental pollination in selected cross combinations (Table 3) were examined using fluorescence microscopic technique proposed by Kho and Baer (1968) and Kho *et al.* (1980). The pollinated flowers after *in vivo* pollination and pollinated ovules with placenta after *in vitro* pollination were fixed in FAA mixture (formalin 10 ml, acetic acid 10 ml and ethyl alcohol 80 ml) at 24 h after pollination. After 24 h of fixation, the material was transferred into glass vials containing 1N NaOH for 8 h at room temperature in order to soften the tissues. The softened material was washed thoroughly with distilled water and then transferred to 0.1 per cent aniline blue in 0.1 N K_2HPO_4. It was retained in aniline blue solution for 18 h for staining.

The prepared specimen was then mounted on a microscopic slide with a drop of glycerine and viewed through fluorescence microscope and documented by photomicrography.

Table 3. Cross combinations tried to assess pollen pistil interaction

Sl. No.	*In vivo* & *in vitro* cross combinations
1	VK 70 x VK 76
2	VK 70 x VK 55
3	VK 70 x Suguna
4	Kanthi x VK 70

3.8 Refinement of *in vitro* pollination

The investigations were done as a follow up of work done by Renjith (1999).

3.8.1 Preparation of explant

The flower buds 15 h prior to anthesis and opened flowers on the day of anthesis were used for *in vitro* pollination studies. Such flowers and flower buds were scooped out with the help of forceps and needle with out injuring the ovary. The calyx surrounding the base of the corolla tube was removed and were quickly transferred to a conical flask containing sterilized water.

3.8.2 Surface sterilization

The flower buds and opened flowers collected in the sterile water were initially washed in teepol solution and then drained. From the opened flowers the unwanted floral parts such as petals and part of labellum were dissected out with a scalpel. The washed explants were treated with streptocyclin 350 mg l^{-1} for opened

flowers and 450 mg l^{-1} for buds for one hour. During this period they were continuously agitated manually. After one hour, they were taken out, air dried and wiped with 70 per cent alcohol and surface sterilized at the laminar flow air cabinet with 0.1 per cent $HgCl_2$ for three minutes. The sterilants were removed by washing thrice in water.

3.8.3 Preparation of media

The chemicals used for preparing various media were of analytical grades obtained from British Drug House (BDH), Sisco Research Laboratories (SRL), Merck or Sigma. Standard procedures were adopted (Gamborg and Shyluk, 1981) for the preparation of media. The pH of the medium was adjusted to 5.8. Semisolid media were prepared by adding good quality agar (0.75%). Sterilization of medium was done by subjecting the media to a temperature of 121°C at a pressure of 1.06 kg cm^2 for 20 minutes (Dodds and Roberts, 1982). After sterilization, the media were stored in air conditioned culture room.

3.8.4 Basal medium for culture establishment

The basal media, ½ MS (Murashige and Skoog, 1962) and modified ½ MS (Monnier, 1976) along with BAP 1 mg l^{-1}, Kin 1 mg l^{-1}, NAA 0.5 mg l^{-1} and 3 per cent sucrose were used for this experiment. The composition of media are given in Table 4. The pollination technique followed was placental pollination. The observations for swelling of ovary/ovules were recorded 20 DAP.

Table 4. Composition of various culture media used for culture establishment

Constituents	Quantity ($mg\ l^{-1}$)	
	MS	Modified MS
Macronutrients		
KNO_3	1900.0	1900.0
NH_4NO_3	1650.0	825.0
KH_2PO_4	170.0	170.0
$MgSO_4.7H_2O$	370.0	370.0
$Ca(NO_3)_2.4H_2O$	440.0	-
$CaCl_2.2H_2O$	440.0	880.0
Micronutrients		
H_3BO_3	6.200	12.4
$MnSO_4.4H_2O$	22.300	33.6
$ZnSO_4.7H_2O$	8.600	8.6
$Na_2MoO_4.2H_2O$	0.250	0.5
$CuSO_4.5H_2O$	0.025	0.05
$CoCl_2.6H_2O$	0.025	0.025
KI	0.830	1.66
Na_2EDTA	33.600	14.9
$FeSO_4.7H_2O$	27.8	11.1
Vitamins		
Thiamine, HCl	0.10	0.10
Pyridoxine, HCl	0.50	0.50
Nicotinic acid	0.50	0.50
Others		
Glycine	2.00	2.00
Myo-inositol	100.00	100.00
Sucrose	30000.00	30000.00
pH	5.80	5.80

3.8.5 Refinement of culture medium for ovule development

The basal media ½ MS and modified ½ MS with 3 per cent sucrose were used for this experiment. The basal media were supplemented with different levels of auxins and cytokinins as shown in Table 5. The influence of double the quantity of vitamin stock of MS medium, CH (200 mg l^{-1}), coconut water (15% v/v) and different levels of coconut milk extract (0.5, 1.0, 2,5, 10.0, 15.0, 20.0 per cent) were also studied.

Table 5. Cytokinin and auxin combinations tested for ovule development

Basal media	Cytokinin mg l^{-1}		Auxin mg l^{-1}
	BAP	Kinetin	NAA
½ MS	1.0	1.0	0.5
,,	0.5	0.5	2.0
,,	1.0	0.5	0.5
,,	1.0	0.5	1.0
,,	1.0	1.0	1.0
Modified ½ MS	0.5	0.5	1.0

3.8.6 Refinement of *in vitro* pollination techniques

The flower buds 15 h prior to anthesis and on the day of anthesis were used for pollination studies. The pollen grains collected from the unopened flower buds along with modified ME_3 medium at pH 6 were used for pollination. The gynoecium for pollination was taken from the opened flowers dehisced on the plant. The following techniques of *in vitro* pollination were tried.

3.8.6.1 Stigmatic pollination

The pollination was effected by applying the fresh pollen grains on the stigma along with pollen germination medium after partial removal of floral appendages like corolla and anther lobes.

3.8.6.2 Stylar pollination

Style was cut at different lengths (¾, ½, ¼) and pollen grains were deposited on the stylar surface along with pollen germination medium.

3.8.6.3 Intraovarian pollination

Ovary was cut just below the junction of the style and ovary and pollination was done on the opened surface of the ovary.

3.8.6.4 Placental pollination

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

3.8.6.5 Modified placental pollination

Pollination was done on the exposed ovules after the partial removal of the ovary wall.

3.8.6.6 Test tube/ovular fertilization

The ovules were separated and pollen grains were applied on the individual ovules.

3.8.7 Post pollination changes

Observations were made for ovary/ovule changes at 10 days interval and seed development was also examined in the selected media combinations. The ovule/seed develoment was assessed by scoring as low (+), medium (++), good (+++) and maximum (++++).

3.9 Seed germination studies

3.9.1 *In vivo* studies

Seeds collected through *in vivo* pollination were kept in moist sterile sand for germination.

3.9.2 *In vitro* studies

Seeds obtained from *in vivo* crosses were graded on the basis of maturity (light brown and dark brown) and weight of the seeds (floats, sinks). The sorted seeds were surface sterilized with 0.1 per cent $HgCl_2$ for eight minutes and then kept for germination in dark on moist filter paper bridge over distilled water in test tubes.

3.9.3 *In vitro* seed germination

The *in vitro* seeds produced were incubated in dark on moist filter paper and cultured in semisolid basal medium for germination.

3.10 *In vitro* multiplication of *in vivo* produced hybrid seedling

The *in vitro* germinated seedling was allowed to grow in the same germinating medium for 20 days. Later, they were subcultured to a medium of ½ MS + BAP 2.5 mg l^{-1} + NAA 0.5 mg l^{-1} + 3% sucrose. Forty days later seedlings were topped at a height of 2 cm and were kept for multiple shoot production in the same medium. Then multiple shoots were subcultured to two media combinations as given below and growth of plantlets were compared.

T_1 – ½ MS + BAP 2.5 mg l^{-1} + NAA 0.5 mg l^{-1} + 3% sucrose

T_2 – ½ MS + BAP 1 mg l^{-1} + NAA 0.5 mg l^{-1} + 3% sucrose

Ten months after germination, six plantlets were planted out in small pots in sterilized sand, after washing with Indofil M-45 0.1 per cent for five minutes. Later they were repotted to the medium of sand, soil and FYM at 1:1:1 proportion. The growth of seedlings 20 days after repotting was further recorded for height, number of leaves and tillers.

RESULTS

RESULTS

The results of the experiment on "Refinement of *in vivo* and *in vitro* pollination techniques in turmeric (*Curcuma domestica* Val.)" conducted at the Department of Plantation Crops and Spices and the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 1998 to 2000 are presented in this chapter.

4.1 Variability in plant materials

The plant materials selected for the study were six short duration cultivars i.e., VK 70, VK 76, VK 55, Sudharsana, Suvarna, Suguna and four medium duration cultivars ie, Kanthi, Sobha, Prabha and Pratibha.

4.1.1 Pseudostem characters

The selected cultivars differed significantly with respect to height, number of leaves per plant, length, breadth and area of leaf (Table 6). Cultivar Suvarna has recorded maximum height of 93.90 cm and Sobha the minimum (57.50 cm). The difference observed with respect to number of tillers per plant was not significant. Maximum number of leaves per plant was noticed in Prabha (9.20) and the minimum in Pratibha (9.00). The cultivar Suvarna has recorded maximum length, breadth and leaf area of 60.50 cm, 15.63 cm and 667.01 cm^2 respectively, while VK 55 has recorded minimum length of leaves (40.38 cm). The leaf breadth and area was minimum in cultivar Sudharsana i.e. 12.00 cm and 342.04 cm^2 respectively.

Table 6. Variability in pseudostem characters of turmeric cultivars at six months after planting

Sl. No.	Cultivars	Height of the plant (cm)	No. of leaves/ plant	No. of tillers/ plant	Leaf length (cm)	Leaf breadth (cm)	Leaf area (cm^2)
1	VK 70	73.40	13.40	1.80 (1.45)	49.75	14.63	508.38
2	VK 55	84.40	13.80	1.70 (1.42)	40.38	12.63	361.55
3	VK 76	72.20	9.90	1.50 (1.28)	49.00	14.63	501.38
4	Sudharsana	69.60	11.90	1.10 (1.20)	40.50	12.00	342.04
5	Suvarna	93.90	13.30	4.05 (1.49)	60.50	15.63	667.01
6	Suguna	75.20	9.20	2.60 (1.13)	42.25	12.63	377.39
7	Sobha	57.50	12.30	1.40 (1.31)	45.25	13.50	431.81
8	Kanthi	75.80	13.80	1.40 (1.31)	43.63	13.00	397.25
9	Prabha	64.60	15.20	2.60 (1.75)	41.63	13.00	382.46
10	Pratibha	73.10	9.00	1.40 (1.22)	43.25	13.00	396.73
Mean		73.97	12.18	1.82 (1.36)	45.61	13.46	436.60
CD at 5%		10.31	3.74	NS	4.05	1.60	80.99

NS – Not Significant

4.1.2 Rhizome characters

4.1.2.1 Mother rhizome characters

There was significant difference among the cultivars regarding the number of mother rhizomes, length, circumference and internodal length of mother rhizomes (Plates 1a, b, c, d and e). Prabha has recorded maximum number of mother rhizomes (2.80) per clump and Sudharsana minimum (1.00). The cultivar Sudharsana has showed maximum length of mother rhizome (7.90 cm) while VK 76 the minimum (5.10 cm). Maximum internodal length was noticed in Suvarna (7.20 cm) while minimum in VK 55 (4.60 cm). Significant difference existed with regard to number of nodes per mother rhizome. Maximum number of nodes was observed in Pratibha (12.60) whereas minimum in VK 76 (8.60). Kanthi recorded maximum circumference of mother rhizome (12.70 cm) while minimum was in Pratibha (8.60 cm) (Table 7).

4.1.2.2 Finger characters

There were significant differences among cultivars with respect to all finger characteristics i.e., number of primary fingers, length, circumference and internodal length of primary fingers. Maximum number of primary fingers was noticed in cultivar Suvarna (6.20) and minimum in VK 76 (2.20). The cultivar VK 76 (9.60 cm) recorded the maximum length of primary fingers and minimum in Prabha (4.10 cm). The cultivar Sudharsana has showed maximum circumference (8.00 cm) while minimum in VK 76 (5.30 cm). The internodal length was maximum in Sudharsana (9.27 cm) whereas minimum was recorded in Prabha

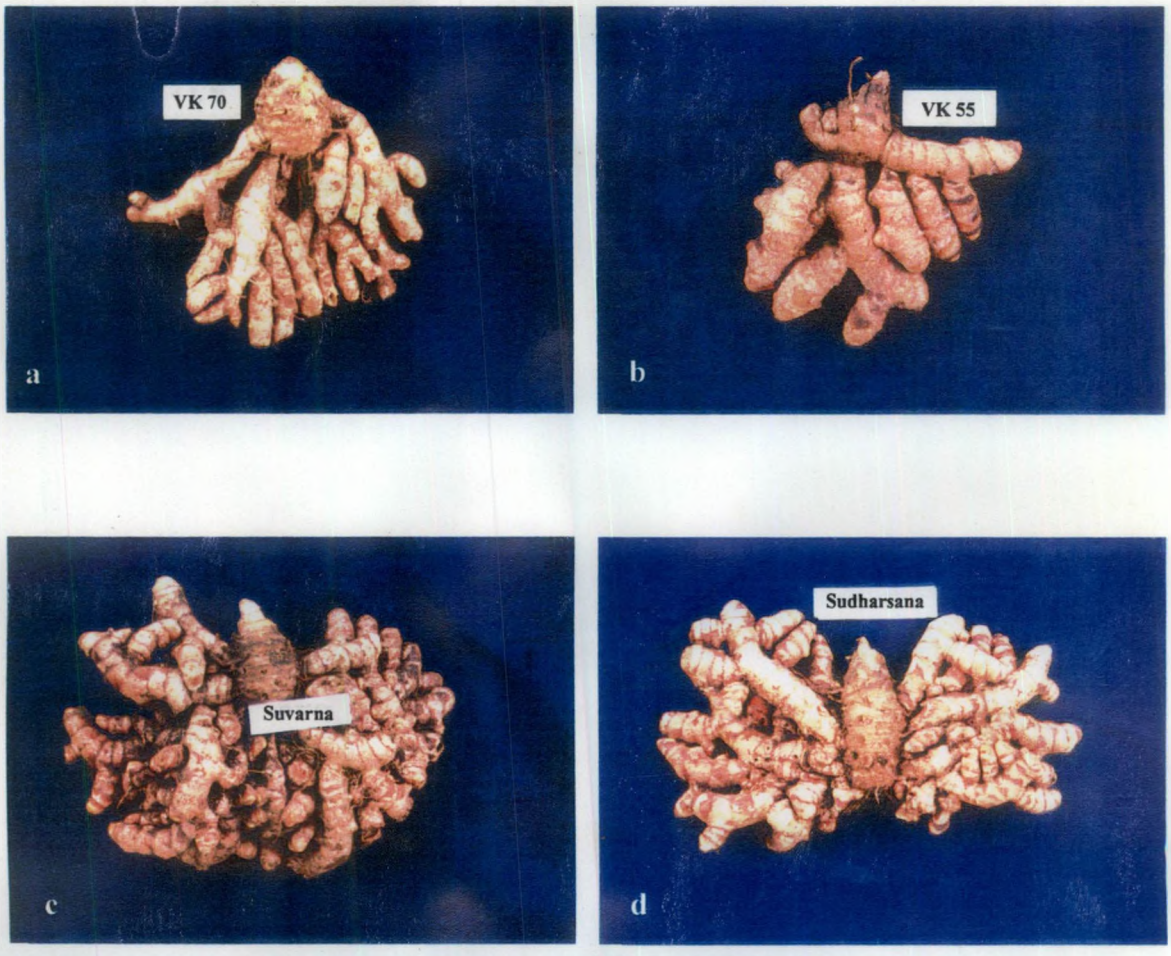

Plate 1a,b,c and d. Short duration cultivars – VK 70, VK 55, Suvarna and Sudharsana

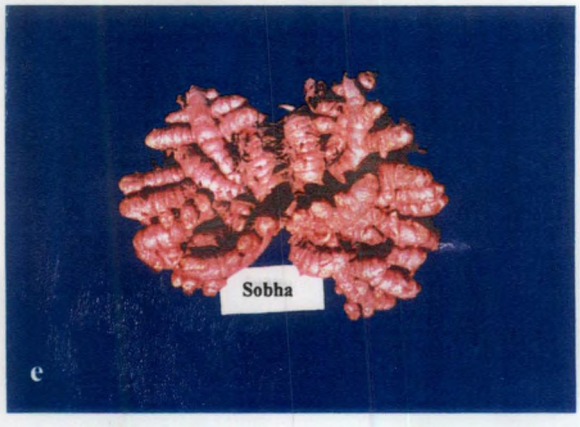

Plate 1e. Medium duration cultivar – Sobha

(4.60 cm). Significant difference was also observed with respect to number, length, circumference and internodal length of longest secondary finger. Maximum number of secondary fingers was noticed in cultivar Suvarna (21.20 cm) while minimum in Prabha (3.00 cm). Sudharsana had longest secondary fingers (5.90 cm) whereas Sobha had the shortest (1.60 cm). Maximum circumference of secondary finger was noticed in Suguna (7.30 cm) while Sobha recorded minimum (2.70 cm). Internodal length of secondary finger was maximum in Sudharsana (9.80 cm) while minimum in varieties Sobha and Prabha (2.40 cm). There was no significant difference among cultivars with respect to mother finger ratio.

4.1.3 Variability in quality characters

In the ten cultivars studied, raw yield/2 m^2 ranged from 9.20 kg in Suvarna to 3.60 kg in Sobha (Table 8).

Curing percentage ranged from 8.60 in VK 55 to 22.00 in VK 70 (Plate 2). The cultivar Suvarna and VK 76 also recorded high curing percentage of 19.17 and 18.02 per cent respectively. In medium duration cultivars Pratibha, Prabha, Sobha and Kanthi it ranged from 13.91 to 17.52 per cent. The cultivar Suvarna recorded maximum dry yield of 1.70 kg/2 m^2 and minimum in VK 55 (0.43 kg/2 m^2). Curcumin content ranged from 2.61 per cent in VK 70 to 7.24 per cent in Sobha. Curcumin content in Kanthi, Prabha, VK 76 and Pratibha were 6.98, 6.31, 6.37 and 5.91 per cent respectively. All others recorded below 5.5 per cent. Oleoresin percentage varied from 11.80 in VK 55 to 21.60 in VK 70. The cultivars Suguna, Suvarna and Sudharsana showed high oleoresin percentage of

Table 8. Variability in yield and quality parameters of turmeric cultivars

Sl. No.	Cultivars	Raw* yield kg/2 m²	Curing (%)	Dry yield* kg/2 m²	Curcumin (%)	Oleoresin (%)
1	VK 70	5.00	22.00	1.10	2.61	21.6
2	VK 55	5.00	8.60	0.43	5.00	11.8
3	VK 76	5.75	18.02	1.05	6.31	16.6
4	Sudharsana	6.10	12.11	0.70	5.01	18.0
5	Suvarna	9.20	19.17	1.70	3.97	18.6
6	Suguna	7.60	12.30	0.93	5.40	21.2
7	Sobha	3.60	16.17	0.58	7.24	14.4
8	Kanthi	5.20	17.52	0.90	6.98	14.4
9	Prabha	5.60	15.41	0.86	6.37	13.0
10	Pratibha	4.00	13.91	0.55	5.91	17.4
Mean		5.71	15.52	0.80	5.48	16.7

*Mean of 18 plants

21.20, 18.60 and 18.00 per cent respectively. In medium duration cultivars it ranged from 13.00 to 17.40 per cent.

4.2 Investigations for improvement of flowering

4.2.1 Influence of seed size on biometric characters of turmeric cultivars

Biometric observations like number of leaves, height, number of tillers, length, breadth and leaf area of plants recorded in various cultivars from three groups of plants T_1 (15-20 g), T_2 (40-50 g) and T_3 (200 g) were analysed statistically and are presented in (Tables 9 and 10).

The results showed that the growth rate of plants in the groups T_1, T_2 and T_3 differed significantly with respect to height in cm, number of leaves, length, breadth and leaf area per plant. But not significant with respect to number of tillers per plant. At 180 days after planting, the plants from T_3 group registered a plant height of 92.11 cm while it was only 73.97 cm in T_1 and 76.19 cm in T_2. The difference among them were significant. Similarly number of leaf production was also more in T_3 group of plants (16.96), while it was only 12.18 in T_1 and 14.2 in T_2. The number of tillers per plant at six months after planting ranged from 1.32 to 1.64. But the data pertaining to this character were not significant. The size of leaves in terms of length was larger in T_1 and T_2 groups of plants i.e., 45.61 cm and 45.68 cm respectively while it was lower in T_3 group of plants (38.99 cm). Breadth of leaf was also more in T_1 and T_2 group of plants (13.46 cm, 14.37 cm) than T_3 group of plants (13.03 cm). Leaf area in cm^2 was also more in T_1 (436.60) and T_2 (478.64) and minimum in T_3 group of plants (363.60).

Plate 2. Cured turmeric of VK 70

Table 9. Influence of seed size on pseudostem characters of turmeric cultivars at six months after planting

Sl. No.		Height in cm				No. of leaves per plant				No. of tillers per plant			
		Seed size				Seed size				Seed size			
		T_1 15-20 g	T_2 40-50 g	T_3 200 g	Mean	T_1 15-20 g	T_2 40-50 g	T_3 200 g	Mean	T_1 15-20g	T_2 40-50 g	T_3 200 g	Mean
1	VK 70	73.40	74.60	92.80	80.27	13.40	12.50	14.70	13.53	1.80(1.45)	1.20(1.22)	1.30(1.25)	1.43(1.31)
2	VK 55	84.40	71.10	81.10	78.87	13.80	15.90	13.80	14.50	1.70(1.42)	1.50(1.36)	1.60(1.36)	1.60(1.38)
3	VK 76	72.20	80.80	93.10	82.03	9.90	13.50	17.50	13.63	1.50(1.28)	1.10(1.60)	1.90(1.51)	1.50(1.32)
4	Sudharsana	69.60	83.00	102.50	85.03	11.90	13.90	19.40	15.07	1.10(1.20)	1.80(1.47)	1.70(1.42)	1.53(1.36)
5	Suvarna	93.90	82.80	104.20	93.63	13.30	13.00	17.50	14.60	1.90(1.49)	1.20(1.20)	1.50(1.32)	1.53(1.34)
6	Suguna	75.20	76.90	92.50	81.53	9.20	12.00	17.50	12.90	1.20(1.13)	1.00(1.19)	1.60(1.39)	1.27(1.24)
7	Sobha	57.50	69.80	92.40	73.23	12.30	15.60	17.30	15.07	1.40(1.31)	1.60(1.39)	1.40(1.31)	1.47(1.33)
8	Kanthi	75.80	88.20	92.40	85.47	13.80	17.40	16.80	16.00	1.40(1.31)	1.90(1.51)	2.00(1.55)	1.77(1.45)
9	Prabha	64.60	72.00	88.30	74.97	15.20	12.20	14.80	14.08	2.60(1.75)	1.10(1.21)	1.40(1.33)	1.70(1.43)
10	Pratibha	73.10	62.70	81.80	72.53	9.00	16.00	20.30	15.10	1.40(1.22)	1.90(1.47)	2.00(1.50)	1.77(1.40)
Mean		73.97	76.19	92.11	80.76	12.18	14.20	16.96	14.45	1.60(1.36)	1.43(1.32)	1.64(1.39)	1.56(1.36)

CD (5%) for comparing varieties	- 6.1440	CD (5%) for comparing varieties	- NS	CD (5%) for comparing varieties	- NS
CD (5%) for comparing seed wt.	- 3.1616	CD (5%) for comparing seed wt.	- 0.966	CD (5%) for comparing seed wt.	- NS
CD (5%) for comparing interaction	- 10.642	CD (5%) for comparing interaction	- 3.539	CD (5%) for comparing interaction	- NS

NS – Not Significant

Table 10. Influence of seed size on leaf characters of turmeric cultivars at six months after planting

Sl. No.	Cultivars	Leaf length (cm)				Leaf breadth (cm)				Leaf area (cm^2)			
		Seed size				Seed size				Seed size			
		T_1 15-20 g	T_2 40-50 g	T_3 200 g	Mean	T_1 15-20 g	T_2 40-50 g	T_3 200 g	Mean	T_1 15-20g	T_2 40-50 g	T_3 200 g	Mean
1	VK 70	49.75	36.25	32.25	39.42	14.63	12.19	10.88	12.56	508.38	313.51	245.79	355.89
2	VK 55	40.38	49.13	41.25	43.58	12.63	16.25	12.38	13.75	361.55	562.01	355.78	426.45
3	VK 76	49.00	38.06	41.88	42.98	14.63	13.31	14.63	14.19	501.38	355.45	435.66	430.83
4	Sudharsana	40.50	61.94	32.25	44.90	12.00	18.94	11.88	14.27	342.04	818.30	272.48	477.60
5	Suvarna	60.50	47.00	55.63	54.38	15.63	15.25	16.13	15.67	667.01	506.01	629.65	600.89
6	Suguna	42.25	37.50	36.13	38.63	12.63	11.10	12.00	11.91	377.39	294.05	304.24	325.23
7	Sobha	45.25	41.81	40.50	42.52	13.50	12.81	12.25	12.85	431.81	375.03	345.89	384.24
8	Kanthi	43.63	67.25	39.13	50.00	13.00	17.50	15.38	15.29	506.01	833.44	422.36	551.02
9	Prabha	41.63	36.00	39.38	39.00	13.00	12.63	13.50	13.04	382.46	325.94	375.11	361.17
10	Pratibha	43.25	41.81	31.50	38.85	13.00	13.69	11.25	12.65	396.73	402.65	249.03	349.47
Mean		45.61	45.68	38.99	43.43	13.46	14.37	13.03	13.62	436.60	478.64	363.60	426.28

CD (5%) for comparing varieties - 2.6845

CD (5%) for comparing seed wt. - 1.6018

CD (5%) for comparing interaction - 4.6497

CD (5%) for comparing varieties - 0.950

CD (5%) for comparing seed wt. - 0.418

CD (5%) for comparing interaction - 1.637

CD (5%) for comparing varieties - 49.5017

CD (5%) for comparing seed wt. - 22.8440

CD (5%) for comparing interaction - 85.7394

4.2.2 Influence of seed size on duration of flowering in turmeric cultivars

The size of seed material used for planting influenced the days for first flowering and thereby the flowering season of turmeric cultivars (Table 11). The plants of short duration cultivars from T_3 group (200 g) was the earliest to flower followed by the same in T_2 (45-50 g) and T_1 (15-20 g). The same pattern was observed for the medium duration variety Sobha also. But in the case of medium duration cultivars Kanthi and Prabha, plants from T_2 group flowered first followed by T_1 and then only from T_3 group. In the case of Pratibha plants from T_1 group flowered first followed by T_2 and T_3. The flowering season in T_3 was from 15-8-99 to 8-11-99 i.e., 85 days, while in T_2 it was from 4-9-99 to 30-10-99 i.e., 57 days. In T_1 group it was from 15-9-99 to 1-11-99 i.e., 48 days. The plants from extra big seed size (T_3) flowered one month earlier to normal plants (T_1). The duration of flowering in individual cultivars in three sets of planting ranged from 17.2 days in T_1 to 20.11 days in T_3.

In all the three sets of planting short duration cultivars were early to flower and the cultivars flowered during the period from 15-8-99 to 27-9-99. All the medium duration cultivars except Pratibha were late to flower. In T_1 group (normal seed size) Pratibha flowered by 25-9-99. All other medium duration types (Sobha, Kanthi and Prabha) flowered between the period 8-10-99 and 21-10-99. In medium duration cultivars, first flowering was delayed by 55 days compared to short duration types.

Table 11. Influence of seed size on duration of flowering in turmeric cultivars

Sl. No.	Cultivars	Seed size 15-20 g (T_1)				Seed size 40-50 g (T_2)				Seed size 200 g (T_3)			
		Date of				Date of				Days of			
		First flowering	End of flowering	Flowering duration (days)	No. of days for first flowering	First flowering	End of flowering	Flowering duration (days)	No. of days for first flowering	First flowering	End of flowering	Flowering duration (days)	No. of days for first flowering
1	VK 70	15-9-99	2-10-99	18	146	4-9-99	21-9-99	18	135	15-8-99	1-9-99	16	115
2	VK 55	27-9-99	18-10-99	22	158	25-9-99	15-10-99	21	156	9-9-99	28-9-99	20	140
3	VK 76	27-9-99	12-10-99	16	158	24-9-99	13-10-99	20	155	22-9-99	15-10-99	24	153
4	Sudharsana	27-9-99	14-10-99	18	158	27-9-99	19-10-99	23	158	5-9-99	26-9-99	22	136
5	Suguna	27-9-99	12-10-99	16	158	20-9-99	10-10-99	21	151	10-9-99	30-9-99	21	141
6	Sobha	18-10-99	1-11-99	15	178	16-10-99	30-10-99	15	175	10-10-99	4-11-99	26	171
7	Kanthi	15-10-99	29-10-99	15	176	10-9-99	22-9-99	13	141	17-10-99	4-11-99	19	178
8	Prabha	10-10-99	28-10-99	19	171	8-10-99	23-10-99	16	169	21-10-99	8-11-99	19	182
9	Pratibha	25-9-99	10-10-99	16	156	12-10-99	27-10-99	16	173	19-10-99	1-11-99	14	181
Flowering duration		15-9-99	1-11-99	17.22	162.11	4-9-99	30-10-99	18.11	157	15-8-99	8-11-99	20.11	155.22

Flowering duration in T_1 : 15-9-99 to 1-11-99 – 48 days
„ in T_2 : 4-9-99 to30-10-99 – 57 days
„ in T_3 : 15-8-99 to 8-11-99 – 85 days

The mean number of days for first flowering of cultivars in T_3 group of plants was 155.22 days while it was 157 days in T_2 and 162.11 in T_1. Generally short duration cultivars took less number of days for flowering and it ranged from 115 days (VK 70) to 158 days (VK 55, VK 76, Sudharsana and Suguna). In medium duration cultivars, the number of days for flowering ranged from 141 (Kanthi) to 182 days (Prabha).

4.2.3 Influence of seed size on intensity of flowering in turmeric cultivars under open/shaded conditions

Influence of seed size on intensity of flowering in turmeric cultivars under open and shade are presented in Table 12. The use of seed material of different size did not significantly influence the intensity of flowering of turmeric cultivars both under open and shade. The trend was same for short and medium duration cultivars. Under open condition the mean flowering in T_1, T_2 and T_3 group of plants was 76.41, 72.65 and 83.65 respectively. In shade it was only 25.31, 25.31 and 27.78 per cent respectively. The intensity of flowering under open in T_1, T_2 and T_3 group of short duration cultivars was 89.76, 85.22 and 96.58 per cent respectively. It was only 59.72, 56.95 and 54.00 per cent in medium duration cultivars. Under shade in short duration cultivars it was only 32.22, 33.33 and 56.66 per cent respectively while it was 16.67, 15.28 and 20.83 percentage in medium duration cultivars.

The mean flowering in turmeric cultivars was significantly high in open condition (77.60%) compared to shade (26.13%). In all nine tested cultivars this

Table 12. Influence of seed size on intensity of flowering in turmeric cultivars under open and shaded condition

Sl. No.	Cultivars	Percentage of flowering in open condition				Percentage of flowering in shade condition				Percentage of flowering open/ shade Mean
		Seed size				Seed size				
		T_1 15-20 g	T_2 40-50 g	T_3 200 g	Mean	T_1 15-20 g	T_2 40-50 g	T_3 200 g	Mean	
1	VK 70	94.00	94.00	100.00	96.00	50.00	33.33	61.11	48.15	72.07
2	VK 55	94.00	88.88	100.00	94.29	16.67	22.22	22.22	20.37	57.33
3	VK 76	100.00	94.00	100.00	98.00	55.56	55.56	33.33	48.15	73.08
4	Sudharsana	83.00	77.00	94.00	84.67	16.67	27.78	27.78	24.08	54.37
5	Suguna	77.78	72.22	88.88	79.63	22.22	27.78	22.22	24.07	51.85
Mean of short duration cultivars		89.76	85.22	96.58	90.52	32.22	33.33	56.66	32.96	61.74
6	Sobha	61.11	55.56	86.67	68.11	16.67	16.67	22.22	18.52	43.32
7	Kanthi	61.11	55.56	61.11	59.26	16.67	11.11	22.22	16.67	37.96
8	Prabha	55.56	55.56	66.67	59.26	22.22	16.67	22.22	20.37	39.82
9	Pratibha	61.11	61.11	55.56	59.26	11.11	16.67	16.67	14.82	37.04
Mean of medium duration cultivars		59.72	56.95	54.00	61.47	16.67	15.28	20.83	17.60	39.54
Mean		76.41	72.65	83.65	77.61	25.31	25.31	27.78	26.13	51.87

CD at 5% for comparing seed weight - NS
CD at 5% for comparing varieties - 8.0239
CD at 5% for comparing open/shade - 11.3475
NS – Not Significant

was true. Both under shade and open condition, flowering intensity was more in short duration types compared to medium duration types. The intensity of flowering under open condition in short duration types was 90.52 per cent while it was only 61.47 in medium duration types. Under shade, flowering intensity in short duration types was only 32.96 per cent while it was only 17.60 per cent in medium duration cultivars.

4.3 Pollen studies

4.3.1 Influence of season on pollen viability of turmeric cultivars

The data regarding the influence of season on germination of pollen grains in turmeric cultivars in modified ME_3 medium at pH 6 are presented in Table 13 and Plate 3. The results showed that the pollen viability was low during early flowering season (18.88%) compared to mid (25.05%) and late (25.84%). In the early season, the pollen viability ranged from 11.05 per cent in Pratibha to 35.40 per cent in Suguna. The mean pollen viability in short duration cultivars was 24.82 per cent while it was only 11.44 per cent in medium duration cultivars. During the middle of the season viability percentage ranged from 12.81 per cent in Kanthi to 48.54 per cent in VK 70. The mean pollen viability in short duration cultivars was 33.51 per cent and it was only 14.48 per cent in medium duration cultivars. At the end of the season it ranged from 13.51 per cent in Kanthi to 56.47 in Suguna. The mean pollen viability in short duration cultivars was 34.48 per cent while it was only 15.03 per cent in medium duration cultivars. The mean pollen viability in turmeric cultivars irrespective of seasonal variation was 23.25 per cent

Table 13. Seasonal variation of pollen viability in turmeric cultivars

Sl. No.	Cultivars	Viability in percentage			
		Beginning of the season	Middle of the season	End of the season	Mean
1	VK 70	27.53	42.44	37.26	35.74
2	VK 55	18.08	23.10	19.84	23.34
3	VK 76	24.66	30.88	31.38	28.97
4	Sudharsana	18.45	22.58	27.45	22.83
5	Suguna	35.40	48.54	56.47	46.80
Mean of short duration cultivars		24.82	33.51	34.48	31.54
6	Sobha	11.43	13.49	14.79	13.24
7	Kanthi	11.90	12.81	13.51	12.74
8	Prabha	11.38	16.88	14.43	14.23
9	Pratibha	11.05	14.74	17.40	14.40
Mean of medium duration cultivars		11.44	14.48	15.03	13.65
Mean		18.88	25.05	25.84	23.25

CD (5%) for comparing varieties - 4.633
CD (5%) for comparing season - 2.734
CD (5%) for comparing interaction - Not significant

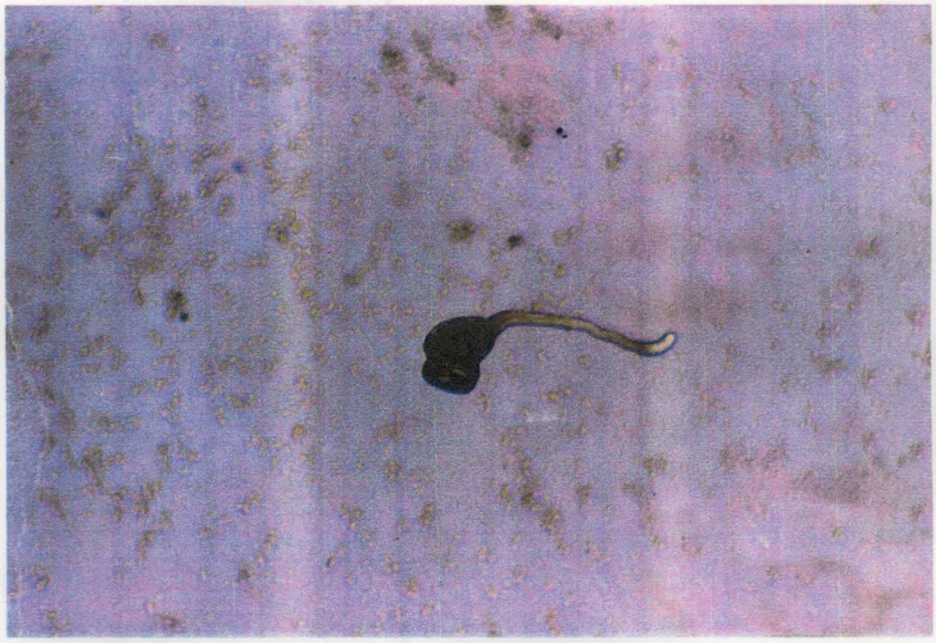

Plate 3. Pollen germination and tube growth in modified ME_3 medium (100X)

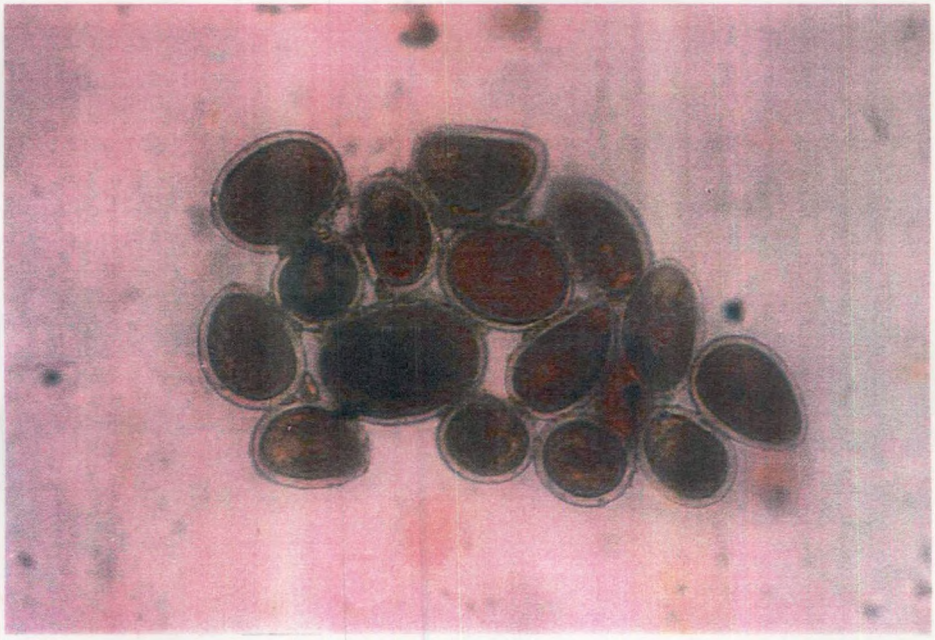

Plate 4. Pollen grains stained with acetocarmine (100X)

ranging from 12.74 in Kanthi to 46.80 in Suguna. In short duration cultivars it was 31.54 per cent while it was only 13.65 per cent in medium duration cultivars.

During the whole flowering season the pollen viability was significantly higher in short duration cultivars compared to all medium duration cultivars except for Pratibha during the end of the season.

4.3.2 Pollen fertility, viability and pollen tube growth in turmeric cultivars

The mean pollen fertility, viability and pollen tube growth in turmeric cultivars recorded during the middle of the flowering season are presented in Table 14. The pollen fertility assessed by the acetocarmine stain had showed significant difference among certain turmeric cultivars (Plate 4). The mean pollen fertility observed was 76.93 per cent. Pollen fertility in the cultivars studied ranged from 53.79 per cent in VK 70 to 89.96 per cent in VK 55. The fertility percentage was comparatively high in cultivars like VK 55, Sudharsana and Suguna. The mean pollen fertility in short duration cultivars was 77.44 per cent while it was slightly lower in medium duration cultivars (75.79%).

The mean pollen viability in the cultivars in modified ME_3 medium was only 25.01 per cent. In cultivars it ranged from 12.81 per cent in Kanthi to 48.54 per cent in Suguna. The mean pollen viability in short duration cultivars was 33.5 per cent while it was only 14.30 per cent in medium duration cultivars.

The mean pollen tube growth in cultivars ranged from 56.33 µm in Sobha to 230.45 µm in VK 70. The mean pollen tube growth in short duration

Table 14. Pollen fertility, viability and pollen tube growth of turmeric cultivars

Sl. No.	Cultivars	Pollen fertility (%)	Pollen viability (%)	Longest pollen tube length (µm)	Mean pollen tube length (µm)
1	VK 70	74.43	42.44	452.61	230.45
2	VK 55	89.96	23.10	214.35	152.31
3	VK 76	53.79	30.88	431.72	218.76
4	Sudharsana	85.46	22.58	253.33	128.55
5	Suguna	83.56	48.54	420.14	210.18
Mean of short duration cultivars		77.44	33.51	354.43	188.05
6	Sobha	77.04	13.49	196.45	56.33
7	Kanthi	77.24	12.81	218.96	81.22
8	Prabha	74.99	16.88	112.21	65.16
9	Pratibha	73.92	14.74	169.03	68.40
Mean of medium duration cultivars		75.79	14.30	174.16	67.78
Mean		76.93	25.05	274.34	134.60
CD (5%) for comparison of varieties		- 10.87	6.22		

cultivars was more (188.05 µm) compared to medium duration cultivars (67.78 µm).

The mean of longest pollen tube length in the nine cultivars was 274.34 µm and it ranged from 112.21 µm in Prabha to 452.61 µm in VK 70. The mean of longest pollen tube length in short duration cultivars was 354.43 µm while it was 174.16 µm in medium duration cultivars. The medium duration cultivar Kanthi has produced longer pollen tubes (218.96 µm) than short duration cultivar VK 55 (214.35 µm).

The cultivar VK 70 noted for profuse seed set recorded highest values for mean pollen tube length (230.45 µm) and longest pollen tube length (452.61 µm).

4.3.3 Pollen storage

4.3.3.1 Effect of storage on pollen viability of different turmeric cultivars

The influence of storage on pollen viability of turmeric cultivars in various organic solvents at –15°C for a period of one month is given in Table 15. The mean pollen viability before storage was 25.96 per cent. The pollen grains of all tested cultivars recorded varying percentages of pollen viability one month after storage. The mean pollen viability in various organic solvents one month after storage was 17.73 per cent and registered a viability loss to the extent of 31.70 per cent. When individually considered, the organic solvent cyclohexane registered maximum pollen viability of 20.18 per cent and viability loss was 22.26 per cent. It was followed by methanol with a pollen viability percentage of 20.09 and viability

Table 15. Effect of storage on pollen viability of different turmeric cultivars

Cultivars	Viability before storage (%)	Viability after storage (%)					
		Hexane	Acetone	Cyclohexane	Isopropanol	Methanol	Mean
VK 70	37.26	30.60	23.38	24.20	15.20	34.00	25.48
VK 55	19.84	22.40	25.76	16.60	14.60	17.98	19.47
VK 76	31.88	15.60	12.25	30.86	11.43	18.04	17.63
Sudharsana	27.08	20.80	22.20	14.00	13.60	27.80	19.68
Suguna	56.72	17.00	28.80	35.00	14.80	23.20	23.76
Sobha	14.79	13.20	17.20	14.40	8.00	15.40	13.64
Kanthi	13.51	15.40	17.20	14.60	8.20	15.80	14.24
Prabha	18.15	12.20	11.00	16.20	8.60	16.80	12.96
Prathiba	14.43	11.20	13.40	15.80	11.40	11.80	12.72
Mean (%)	25.96	17.60	19.02	20.18	11.76	20.09	17.73
Percentage loss of viability		32.20	26.73	22.26	54.70	22.61	31.70

CD (5%) for the comparison of solvents - 3.58
CD (5%) for the comparison of varieties within solvents - 4.39

loss was 22.61 per cent. The solvents acetone and hexane were also good for pollen storage as they have registered a pollen viability percentage of 19.02 and 17.60 respectively. The organic solvent isopropanol recorded the lowest pollen viability of 11.76 per cent and the corresponding viability loss was 54.70 per cent.

4.4 Natural seed set in turmeric cultivars

Among the selected turmeric cultivars, natural fruit set and seed set were observed in all short duration cultivars viz. VK 70, VK 55, VK 76 and Suguna, except Sudharsana. The insects act as natural pollinating agent. No seed set was noted in medium duration cultivars. In VK 76 and VK 55 fruits matured in 21 days while it was only 20 days in VK 70 and Suguna. The cultivars VK 70 produced 10 seeds per fruit while it was nine in VK 76, VK 55 and Suguna. Seed set was high in VK 76 while in the other cultivars, only limited seed set was observed (Table 16).

4.5 Controlled *in vivo* pollinatiton

Different pollination methods i.e., stigmatic, stylar and intraovarian pollination were done. Among stigmatic and stylar pollination, seed set was obtained only in stigmatic pollination. Observations related to this aspect are presented in Table 17. Seed set was observed in three of 15 crosses tried viz., VK 70 x VK 76, VK 70 x VK 55 and VK 70 x Suguna. But failed to get seeds in combination with medium duration cultivars viz., Kanthi, Sobha, Prabha and Pratibha. The crosses involving VK 70 as the female parent only yielded seed set.

Table 16. Details of natural seedset in turmeric cultivars

Sl. No.	Characters	Cultivars								
		VK 70	VK 55	VK 76	Sudharsana	Suguna	Kanthi	Sobha	Prabha	Pratibha
1	Natural seedset	Yes	Yes	Yes	Nil	Yes	Nil	Nil	Nil	Nil
2	Mode of natural pollination	IP	IP	IP	-	IP	-	-	-	-
3	Days to fruit set	4	4	5	-	4	NA	NA	NA	NA
4	Days to fruit maturity	20	21	21	-	20	NA	NA	NA	NA
5	No. of seeds/fruit	10	9	9	-	9	NA	NA	NA	NA
6	Percentage of germination	Nil	Nil	Nil	-	Nil	NA	NA	NA	NA

IP - Insect pollination
NA - Not applicable

Table 17. Effect of controlled *in vivo* crosses in turmeric cultivars

Crosses	Success/failure
VK 70 x VK 76	Success
VK 70 x VK 55	,,
VK 70 x Suguna	,,
VK 70 x Sudharsana	Failure
VK 76 x VK 70	,,
VK 55 x VK 70	,,
Suguna x VK 70	,,
VK 70 x Sobha	,,
VK 70 x Kanthi	,,
VK 70 x Prabha	,,
VK 70 x Pratibha	,,
Kanthi x VK 70	,,
Sobha x VK 70	,,
Sobha x Kanthi	,,
Kanthi x Sobha	,,

No seed set was observed when cultivar other than VK 70 was used as female parent.

In vivo stylar and intraovarian pollination with modified ME_3 medium did not produce any seed set.

4.5.1 Pollen pistil interaction studies after *in vivo* and *in vitro* cross pollination

The pollen pistil interaction studies after *in vivo* cross pollination involving short duration cultivars (VK 70 x VK 76), (VK 70 x VK 55) and (VK 70 x Suguna) showed that the pollen grains of VK 76, VK 55 and Suguna germinate on stigma of VK 70 and pollen tube passes through the style and reaches the ovule (Plates 5a, b, c and d). The pollen pistil interaction studies involving medium and short duration cultivars (Kanthi x VK 70) showed that the pollen grains of VK 70 germinate on the stigma of Kanthi and pollen tube passes through the style and reaches the ovules.

The pollen pistil interaction studies in the aforesaid crosses after *in vitro* placental pollination showed that pollen grains germinate on the gynoecium under *in vitro* condition. In crosses involving short duration cultivars, VK 70 x VK 76, VK 70 x VK 55, VK 70 x Suguna, the pollen tube length was sufficient to cover the entire length of ovule. In crosses involving medium and short duration cultivars also (Kanthi x VK 70) the pollen tube length was sufficient to cover the entire length of ovule (Plate 5e).

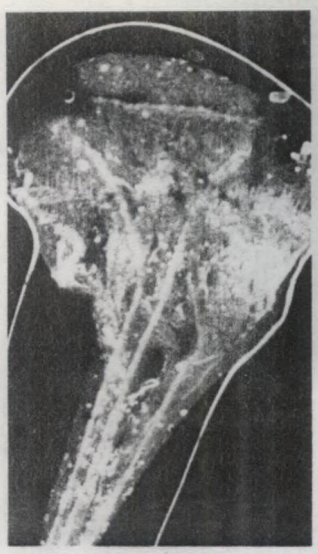

Plate 5a. Pollen germination on stigma and tube growth through the style in the *in vivo* cross of Kanthi × VK 70 (50X)

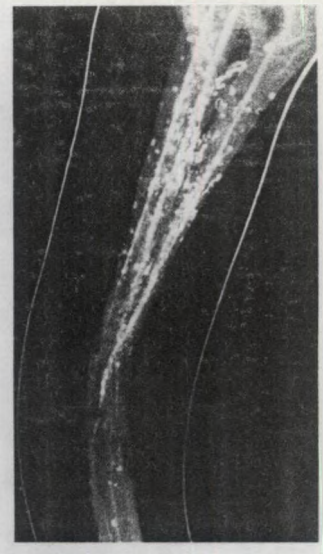

Plate 5b. Pollen tube growth through the style in the *in vivo* cross of Kanthi × VK 70 (100X)

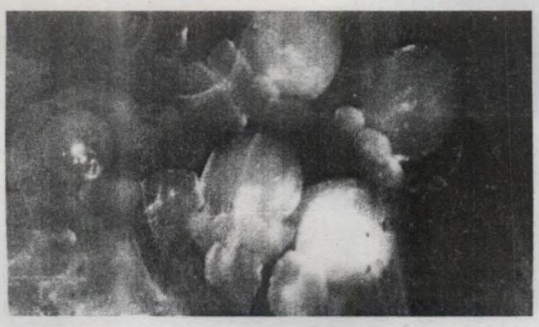

Plate 5c. Pollen tube covering the ovule length in the *in vivo* cross of Kanthi × VK 70(40X)

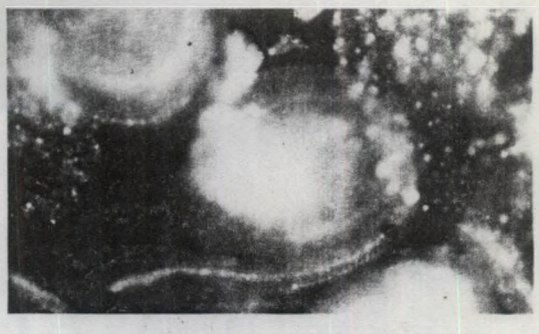

Plate 5d. Pollen tube covering the ovule length in the *in vivo* cross of VK 70 ×VK 76 (100X)

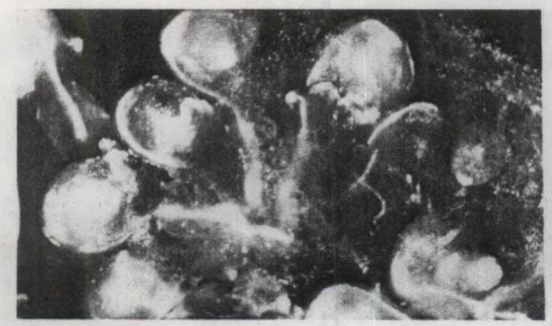

Plate 5e. Pollen tube covering the ovule length in the *in vitro* cross of Kanthi ×VK 70 (50X)

4.5.2 Description of turmeric fruit and seed produced after *in vivo* cross pollination

The turmeric hybrid fruit developed after *in vivo* cross pollination (VK 70 x VK 76) was a thin walled trilocular capsule with arillate seeds (Plates 6a, b and c). The seeds were creamy white at immature stage and turned black on maturity i.e., 21 DAP. The number of seeds per fruit was nine. The arillate seeds are triangular with two seed coats. The outer layer being highly lignified and thickened. Seeds are filled with massive endosperm and the embryo is seen oriented towards the upper side of the ovule.

4.6 Refinement of *in vitro* pollination techniques

4.6.1 Identification of basal medium for culture establishment

In order to get initial *in vitro* establishment, *in vitro* placental pollinated ovules on the day of anthesis were incubated on basal media ½ MS and modified ½ Ms with growth regulators. The results are presented in Table 18. The ovules developed in both the media when supplemented with BAP, NAA and Kinetin. The percentage of cultures showing ovule development was more in modified ½ MS (80.33%) when supplemented with BAP 0.5 mgl^{-1}, Kinetin 0.5 mg l^{-1} and NAA 1 mg l^{-1} than ½ MS (75.54%). As per visual observation, ½ strength MS was superior to modified ½ MS for ovule development in terms of size.

4.6.1.2 Influence of auxins/cytokinins in the ovule development

In the ½ MS medium with BAP and kinetin each at 1 mg l^{-1} along with NAA (0.5 mg l^{-1}) favoured maximum ovule swelling and the response was

Table 18. Influence of ½ MS and modified ½ MS in the ovule development

Sl. No.	Basal medium	Ovule swelling	Percentage of cultures * showing ovule development (%)
1	Modified ½ MS	++	80.33
2	½ MS	+++	75.54

* Average of 12 observations
Supplements - BAP 1 mg l^{-1} + Kin 1 mg l^{-1} + NAA 0.5 mg l^{-1} + 3% sucrose
Explant - Ovule after *in vitro* placental pollination

Plate 6a. Turmeric fruit developed after *in vivo* cross pollination (VK70×VK76)

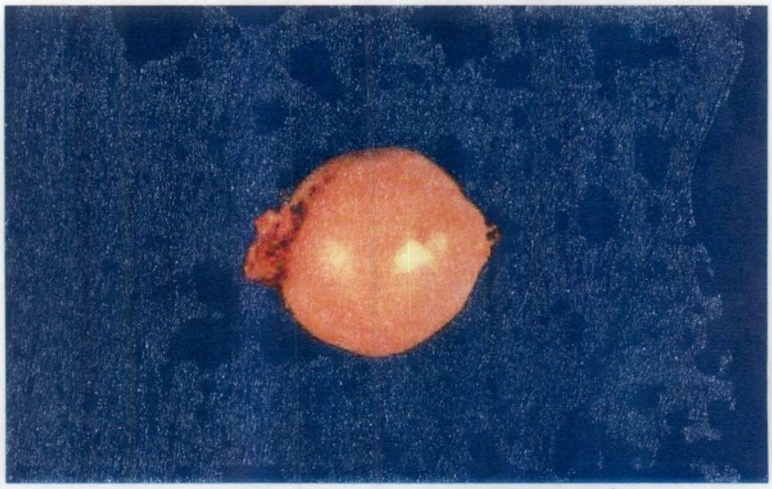

Plate 6b. Turmeric fruit

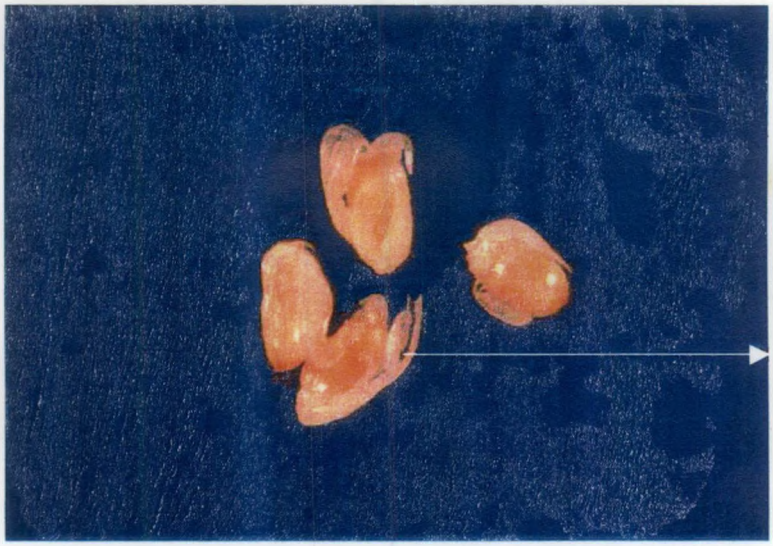

Plate 6c. Arillate seeds of turmeric

observed in 80.16 per cent of cultures. The ovule swelling got reduced when NAA was enhanced from 0.5 mg l^{-1} to 1 mg l^{-1} and percentage of cultures showing response also got reduced to 52.76 per cent. Similarly reduction in Kinetin from 1 mg l^{-1} to 0.5 mg l^{-1} also reduced ovule swelling and percentage of cultures showing response was 62.15 per cent. Reducing the kinetin to 0.5 mg l^{-1} and enhancing NAA from 0.5 to 1 mg l^{-1} also reduced ovule swelling and percentage of cultures showing response was 58.00 per cent.

In the modified ½ MS medium BAP, kinetin (each at 0.5 mg l^{-1}) and NAA 1 mg l^{-1} favoured ovule swelling and the response was observed in 83.01 per cent of cultures (Table 19).

The culture of ovary after intraovarian pollination in the medium of ½ MS + 3 per cent sucrose + BAP 0.5 mg l^{-1} + Kin 0.5 mg l^{-1} + NAA 2.0 mg l^{-1} caused swelling of ovary but no ovule development was observed (Plate 7a).

4.6.1.3 Influence of vitamins, coconut water and CH in the ovule development

The data related to this aspect are presented in Table 20. Addition of double the quantity of vitamin stock of MS medium along with hormones enhanced ovule development (++++) and the response was observed in 80.16 per cent of cultures. The organic supplement CW 15 per cent v/v did not favour much ovule development. But the cultures showed some ovule development due to the presence of hormones and the response was observed in 30.16 per cent of cultures. The organic supplement CH 200 mg l^{-1} also did not promote ovule development

Table 19. Influence of cytokinins and auxins in the ovule development of turmeric after *in vitro* pollination

Pollination technique	Media composition		Response		
	Basal medium	Growth regulators	Ovule swelling	Ovary swelling	Percentage* of cultures showing ovule develop-ment (%)
Placental pollination	½ MS	BAP 1 mg l^{-1} + Kin 1 mg l^{-1} + NAA 0.5 mg l^{-1}	+++	NA	80.16
,,	,,	BAP 1 mg l^{-1} + Kin 1 mg l^{-1} + NAA 1 mg l^{-1}	+	NA	52.76
,,	,,	BAP 1 mg l^{-1} + Kin 0.5 mg l^{-1} + NAA 0.5 mg l^{-1}	+	NA	62.15
,,	,,	BAP 1 mg l^{-1} + Kin 0.5 mg l^{-1} + NAA 1 mg l^{-1}	+	NA	58.00
,,	Modified ½ MS	BAP 0.5 mg l^{-1} + Kin 0.5 mg l^{-1} + NAA 1 mg l^{-1}	++	NA	83.01
Intra ovarian pollination	½ MS	BAP 0.5 mg l^{-1} + Kin 0.5 mg l^{-1} + NAA 2 mg l^{-1}	+	Ovary swelling	00.00

* Average of 12 observations
Explant – Pollinated gynoecium or part of it
Sucrose – 3 per cent

Table 20. Influence of vitamin stock, CW and CH in the ovule development

Treatments	Media additives			Response	
	Vitamin stock	CW (%)	CH mg l^{-1}	Ovule swelling	Percentage * of cultures showing ovule development
Control	0.5x	-	-	+++	45.60
1	1x	-	-	++++	80.16
2	-	15	-	+++	30.16
3	-	-	200	+++	12.43

*Average of 12 observations
Basal medium - ½ MS + 3% sucrose
,, - BAP 1 mg l^{-1} + Kin 1 mg l^{-1} + NAA 0.5 mg l^{-1}
Explant – Ovules after placental pollination
0.5x - ½ MS
1x - Full MS

substantially. But as in the former case the cultures showed some ovule development and it was observed in 12.43 per cent of cultures.

4.6.1.4 Influence of coconut milk extract in the ovule development

The influence of coconut milk extract in ovary/ovule development was studied by culturing ovary after intraovarian pollination in media combinations containing coconut milk extract at concentrations ranging from 0.5 to 20 per cent (Table 21). The addition of coconut milk made the medium much oily. Coconut milk extract promoted visually 25 per cent ovary development at concentrations ranging from 0.50 to 20.00 per cent and the response was observed in 12.91 to 48.12 per cent of cultures. The concentration of 5.00 per cent coconut milk extract seems to be the best as the percentage of cultures showing response was maximum (48.12%) in this treatment and the size of ovary was also more (++).

4.7 Refinement of *in vitro* pollination techniques in turmeric

Different *in vitro* pollination techniques stigmatic, stylar, intraovarian, placental, modified placental and ovular pollination as described by Bhojwani and Razdan (1983) were tried. Among these, ovules were developed in intraovarian, placental and modified placental pollination techniques (Table 22). In intraovarian pollination technique, ovary as well as ovule development was noticed in 70 per cent of cultures. Ovule development alone was noticed in 30 per cent of cultures. Percentage of cultures showing maximum ovule development (80.49%) was in placental pollination (Plate 7b) followed by modified placental pollination (77.98%). The number of ovules developed per culture in intraovarian, placental

Plate 7a. Developing ovary 15 days after intraovarian pollination

Plate 7b. Developing ovules eight days after *in vitro* placental pollination (50 X)

Table 21. Influence of coconut milk extract in the ovary/ovule development

Sl. No.	Treatments	Ovary swelling	Ovule swelling	Percentage * of cultures showing ovary development
1	0.5% coconut milk extract	+	-	12.91
2	1% „	+	-	16.18
3	2% „	+	-	35.33
4	5% „	++	-	48.12
5	10% „	+	-	21.56
6	15% „	+	-	12.10
7	20% „	+	-	10.15

* Average of 12 observations
Basal medium - ½ MS + BAP 1 mg l^{-1} + Kin 1 mg l^{-1} + NAA 0.5 mg l^{-1} + 3% sucrose
Explant - Ovary after intraovarian pollination

Table 22. Response to different *in vitro* pollination techniques in turmeric for crossing

Sl. No.	Mode of pollination	Percentage * of cultures with ovary development	Percentage* of cultures with ovule development	No. of ovules developed/ culture
1	Stigmatic	0.00	0.00	-
2	Stylar	0.00	0.00	-
3	Intraovarian	70.00	30.00	4.00
4	Placental	NA	80.49	12.10
5	Modified placental	NA	77.89	8.00
6	Ovular or test tube fertilization	0.00	0.00	-

* Average of 12 observations
Culture medium - ½ MS + BAP 1 mg l^{-1} + Kin 1 mg l^{-1} + NAA 0.5 mg l^{-1} + 3% sucrose
Explant - Pollinated gynoecium or part of it

and modified placental pollination was 4.0, 12.10 and 8.00 respectively. Placental pollination is the best as rate of seed production was more in this method.

4.7.1 Post pollination changes

Ovules/seeds developed in the intraovarian placental and modified placental pollination techniques were creamy white during the initial stage of development and changed to dark brown colour with in a period of 20 to 30 DAP.

4.8 Seed germination studies in turmeric

4.8.1 *In vivo* studies

The seeds kept in moist sterile sand did not germinate.

4.8.2 *In vitro* studies

The seeds kept in moist filter paper only germinated while those kept in moist sand, mixture of moist sand and vermiculite in 1:1 proportion and basal medium did not germinate.

4.9 *In vitro* germination of turmeric seed produced under *in vivo* cross pollination

Immature light brown coloured, heavy as well as light seeds germinated on moist filter paper in test tubes under dark condition (Plate 8a). The germination percentage was 25. Dark brown heavy seeds failed to germinate. The number of days required for germination ranged from 15.00 to 16.00 days (Table 23).

4.9.1 *In vitro* multiplication of *in vivo* produced seedling

The *in vitro* germinated seedlings 20 days after germination were subcultured to the medium of ½ MS + 3 per cent sucrose + BAP 2.5 mg l^{-1} + NAA 0.5 mg l^{-1} in distilled H_2O. It got established in the above medium and produced two leaves within a period of 20 days. The seedlings were made free from bacterial infestation by washing with streptomycin (250 mg l^{-1}). Multiple shoots were produced by detopping the seedlings at 40 days after germination and subculturing in the same medium (Plate 8b).

The establishment and growth of multiple shoots in the two media combinations were studied and presented in Table 24. The results showed that average number of multiple shoots, number of leaves, mean height (cm), and average number of roots are more in the medium of T_2 (½ MS + 3% sucrose + BAP 1 mg l^{-1} + NAA 0.5 mg l^{-1}) than T_1 (½ MS + 3% sucrose + BAP 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}). In T_2 medium the subcultured individual multiple shoots produced an average of 3.2 multiple shoots, roots (4.2), number of leaves (3.2) and attained a height of (3.6 cm) within a period of 30 days. In T_2 medium root growth was significantly more (4.2) compared to T_1 (2.6). The multiplication rate recorded was 3.4 multiple shoots/subculture of 20 days interval. The cultures were maintained by serial subculturing.

4.9.1.1 Hardening and planting out

After 10 months culture period, six seedlings of average height of 4 cm, tillers (2.0), leaves (4.4) and roots (4.0) were successfully planted out in small pots

Table 23. *In vitro* germination of turmeric seeds produced under *in vivo* condition

Sl. No.	Medium	Seed character	Germination (%)	Days taken for germination
1	Distilled H_2O	Immature (light brown		
		a) Heavy	25	15
		b) Light	25	16
		Mature (dark brown)		
2	,,	Heavy	Nil	Nil

Substratum - Moist filter paper
Condition - Dark

Table 24. *In vitro* multiplication of *in vivo* produced turmeric hybrid seedlings

Basal media	Growth regulator mg l^{-1}	*Average no. of multiple shoots	*Average no. of roots	*Average no. of leaves	Mean height (cm)
½ MS + 3% sucrose	BAP (2.5 + NAA 0.5)	3.0	2.6	2.5	3.13
,,	BAP (1) + NAA (0.5)	3.2	4.2	3.2	3.60

* Observations recorded in the 3rd subculture, 60 days after germination

containing sterilized sand (Plate 8c). After 15 days they were transferred to small pots containing sand, soil and FYM in 1:1:1 proportion. Twenty five days after repotting, the seedlings recorded an average height of 11 cm, 2 tillers and 8 leaves.

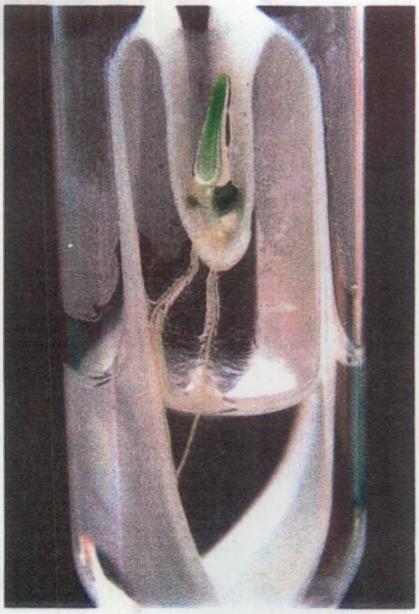

Plate 8a. *In vitro* germination of turmeric seed on moist filter paper

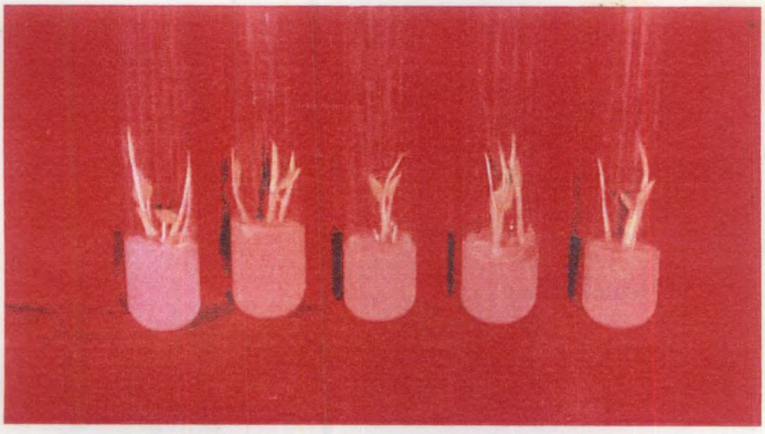

Plate 8b. *In vitro* multiplication of *in vivo* produced turmeric hybrid seedlings

Plate 8c. *In vitro* produced turmeric seedling planted out

DISCUSSION

DISCUSSION

The investigations on "Refinement of *in vivo* and *in vitro* pollination techniques in turmeric (*Curcuma domestica* Val.)" were carried out at the Department of Plantation Crops and Spices and the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 1998 to 2000.

Turmeric is a monocot and belongs to the family Zingiberaceae. At present the rhizome of the same is valued as spice, natural food colourant, medicine, cosmetic and dye in addition to its use in religious ceremonies.

Turmeric is exclusively propagated by vegetative means. Even then tremendous variability is available in this crop and the population shows variation for yield from 2 t to 8 t, curing percentage from 14 to 31 and curcumin content from 3.0 to 7.5 per cent (Philip, 1978). This available variability is not fully exploited for crop improvement as hybridization programme is not perfected in this crop. Cultivated turmeric consists of fertile tetraploids (2n=84) and sterile triploids (2n=63). Generally tetraploids are high yielders of cured turmeric and low in curcumin content but exceptions are also available. Long duration types are high yielders but moderate in curing percentage and curcumin content. Medium duration types are rich in curcumin content but low in curing percentage. Theoretically seed set could be obtained in crosses involving short duration types

as they are tetraploids. There can be difficulty in getting seed set in crosses involving short and medium duration cultivars.

Nazeem and Menon (1993) have reported successful *in vivo* pollination in turmeric. Seed set could be obtained in different parental combinations of short duration types. But in crosses involving short and long duration types, only one particular combination could give seed set.

Renjith (1999) also recorded seed set through *in vivo* pollination in crosses involving short duration cultivars. But failed in crosses involving short and medium duration cultivars. So it can be assumed that certain incompatability mechanism may be hindering the seed set. The mechanism of incompatability has to be examined.

In vitro pollination and fertilization is an effective tool in situations where incompatability reactions prevent seed set. In ginger, an important spice crop of Zingiberaceous family, natural seed set is not occurring because of the incompatability operating in stigma and style. Valsala (1994) was able to get seed set in this crop through *in vitro* pollination and fertilization. Renjith (1999) reported ovule/seed development in turmeric through *in vitro* pollination.

The present investigations were aimed at refining a technique for successful seed set through *in vivo and in vitro* pollination among turmeric cultivars. The research was carried out as the follow up of investigation done by Renjith (1999). Success in this line will open up new vistas of crop improvement

in turmeric especially for the improvement of medium and long duration types, which occupies 93 percentage of cultivated area of turmeric. Alleppey turmeric which is rich in curcumin content can be further improved for curing percentage. Similarly, short duration types with high cured yield and curcumin content suitable for specific climatic conditions can also be developed.

The major aspects of the investigation were

1. Variability studies in the selected plant materials
2. Pollen fertility and viability studies
3. Pollen storage
4. Response of selected cultivars to *in vivo* pollination
5. Refinement of medium for ovule/seed development
6. Response of selected cultivars to *in vitro* pollination
7. Seed germination and multiplication studies

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

5.1 Variability in plant materials

The variability in selected cultivars was examined for salient morphological characters and quality characters.

The results showed that the cultivars differed significantly with respect to various pseudostem characters (Table 6), mother rhizome characters and finger rhizome characters (Table 7).

In the selected ten cultivars, raw yield in kg/2 m^2 ranged from 3.6 in Sobha to 9.2 in Suvarna (Table 8). Curing percentage ranged from 8.6 in VK 55 to

22 in VK 70. The cultivars Suvarna and VK 76 recorded high curing percentage of 19.17 and 18.02 respectively. The cultivar Suvarna recorded maximum dry yield of 1.7 kg/2 m^2 and minimum in VK 55 (0.43 kg/2 m^2). Curcumin content ranged from 2.61 per cent in VK 70 to 7.24 per cent in Sobha and it was high in medium duration cultivars i.e., Kanthi (6.98%) and Prabha (6.37%) and short duration cultivar VK 76 (6.31%) while all others recorded below 5.50 per cent. Oleoresin percentage varied from 11.80 in VK 55 to 21.60 in VK 70. The short duration cultivars Suguna, Suvarna and Sudharsana recorded high oleoresin percentage of 21.20, 18.60 and 18.00 per cent respectively (Table 8).

Significant variation among turmeric cultivars with regard to morphological and quality characters were reported by Philip (1978), Geetha and Prabhakaran (1987), Jalgaonkar *et al.* (1986), Mukhopadhyay *et al.* (1987), Reddy and Rao (1988), Menon *et al.* (1992), Yadav *et al.* (1996), Kurian and Nair (1996) and Renjith (1999).

As per Rao *et al.* (1975) short duration types are low in curcumin content but VK 76, Sudharsana and VK 55 in the present study are exceptions to this. They recorded > 5.1 per cent curcumin content.

The cultivar Sobha take its pedigree to the Alleppey turmeric and hence high curcumin content (7.24%) can be expected.

Above study revealed that plant materials selected for the study are having adequate variability for the exploitation of hybrid vigour.

The results also indicate that the cultivars VK 76, Kanthi, Sobha and Prabha can be identified as the source of genetic material for high curcumin content. The cultivars Suvarna, VK 70 and VK 76 can be selected for their high curing percentage and VK 70 and Suguna for high oleoresin content. It was also observed that the released medium duration varieties Kanthi and Sobha can be improved for their curing percentage by conducting suitable crosses with VK 70 and VK 76. The elite type VK 76 possesses good curing percentage, curcumin content and it can be further improved for yield by crossing with other high yielding cultivars. In the present study, Suvarna was the most high yielding cultivar with high curing percentage (19.17%) and its low curcumin content (3.97%) has to be improved for better exploitation. However, the possibility of using Suvarna in crop improvement programme is blocked by its non flowering nature. Efforts can be made to induce flowering in this cultivar.

5.2 Investigations for improvement of flowering

5.2.1 The influence of seed size on duration of flowering in turmeric cultivars

The size of seed material used for planting influenced the days for first flowering and thereby the flowering season of turmeric cultivars. The plants from T_3 (200 g) group was the earliest to flower followed by T_2 (45-50 g) and T_1 (15-20 g). The flowering season of T_3 was from 15-8-99 to 8-11-99 i.e., 85 days while in T_2, it was from 4-9-99 to 30-10-99 i.e., 57 days. In T_1 group it was from 15-9-99 to 1-11-99 i.e., 48 days.

This experiment enabled to extend the flowering season of turmeric to about 85 days in place of 48 days in normal plants. The distributed flowering of cultivars in three sets of planting helped to conduct crossing in specific combinations of cultivars.

5.2.2 Influence of seed size on intensity of flowering in turmeric cultivars under open and shaded condition

The use of seed material of different size viz., T_1 (15-20 g), T_2 (40-50 g) and T_3 (200 g) did not significantly influence the flowering intensity of turmeric cultivars both under open and shade. The mean flowering of turmeric cultivars were significantly high in open condition (77.61%) compared to shade (26.13%). Flowering intensity was more in short duration types compared to medium duration types. It is advisable to raise the plants in open if the breeder wants to get more flowers for breeding work.

5.3 Pollen studies

5.3.1 Influence of season on pollen viability of turmeric cultivars

The results showed that the pollen viability was influenced by season and the cultivar from which it was collected. The viability was significantly high during the mid (25.05%), and late (25.84%) flowering season compared to early (18.88%) flowering season (Table 13).

From the aforesaid observations it can be suggested that pollination during mid and late seasons may result in better seed set in turmeric.

Bindu (1997) reported that in ginger, the pollen viability was influenced by season and it was high in early compared to mid and late flowering seasons.

5.3.2 Pollen fertility, viability and pollen tube growth in turmeric cultivars

The mean pollen fertility was high (76.93%) as per the acetocarmine stain test (Table 14). But viability in the modified ME_3 medium was only 25.05 per cent.

The mean pollen viability was high in short duration cultivars (33.51%) while it was low in medium duration cultivars (14.30%) (Table 14). The mean pollen tube length for the various cultivars was 134.60 µm. It was comparatively longer in short duration types (188.05 µm) and was short for medium duration types (67.78 µm). The observations on maximum pollen tube length also followed the same trend. But the cultivar Kanthi has produced longer pollen tubes (218.96 µm) than seed setting short duration cultivar VK 55 (214.35 µm). Renjith (1999) also reported similar results in turmeric.

Even if pollen fertility was fairly high in turmeric cultivars, the viability in modified ME_3 medium was not correspondingly high. This may be due to the fact that acetocarmine stain test may not be fully efficient in revealing the exact fertility of pollen grains.

The better pollen germination and tube growth justifies seed set in short duration types. The study also brings out the fact that seed set will be more in

crosses involving Suguna, VK 70 and VK 76 as male parents and these cultivars could be effectively utilized for future breeding programmes.

Since the cultivar Kanthi has produced longer pollen tubes than seed setting short duration cultivar VK 55, chances of natural seed set exist in some medium duration cultivars. The reason for lack of seed set may be explored by pollen pistil interaction study and using better medium for pollen germination.

5.3.3 Pollen storage

5.3.3.1 Effect of storage on pollen viability of different turmeric cultivars

The organic solvents used for pollen storage were hexane, acetone, cyclohexane, isopropanol and methanol. The mean pollen viability in nine turmeric cultivars before storage was 25.96 per cent. Mean pollen viability one month after storage in the selected organic solvents at -15°C was 17.73 per cent and the corresponding viability loss was 31.70 per cent. Among the solvents tested, cyclohexane registered the highest mean pollen viability of 20.18 per cent followed by methanol (20.09%) (Table 15).

The study revealed that dry pollen grains can be successfully stored in organic solvents at -15°C with partial loss of pollen viability. Among the five organic solvents tested, cyclohexane was best for pollen storage followed by methanol and acetone. This information is valuable for hybridizing short duration cultivars with medium and long duration cultivars. In simultaneous planting, the short duration cultivars flower earlier to medium and long duration types. So there

is difficulty for conducting crosses between specified cultivars. This could be overcome by pollen storage.

5.4 Natural seed set in turmeric cultivars

Among the selected cultivars, natural fruit set and seed set were observed in all the short duration cultivars viz. VK 70, VK 55, VK 76 and Suguna except Sudharsana and the natural pollinating agent was insect (Table 16). Seed set was high in VK 70. Since they are tetraploids with high pollen viability, natural seed set can occur. Fruit set and seed set were not noticed in any of the medium duration cultivars viz., Kanthi, Sobha, Prabha and Pratibha which are said to be triploid cultivars.

The aforesaid results are in agreement with the reports of Nambiar *et al.* (1982). They observed seed set in *C. aromatica* types and no seed set in *C. longa* types.

Renjith (1999) observed similar results in turmeric, and he observed seed set in short duration cultivars including Sudharsana.

The fruit of turmeric matured in 20 to 21 days. There were 9 to 10 seeds in the fruit. Seed set was high in VK 70 but in other cultivars the seed set was limited. This variety recorded maximum mean pollen tube length of 230.45 µm.

5.5 Controlled *in vivo* pollination

Even though stylar, stigmatic and intraovarian pollination were done, seed set was obtained only in stigmatic pollination. Seed set was observed in three

of fifteen crosses tried viz. VK 70 x VK 55, VK 70 x VK 76 and VK 70 x Suguna (Table 17).

Seed set was not observed in crosses involving other short duration cultivars, even though they are tetraploids. The crosses involving medium duration cultivars i.e., Kanthi x Sobha, Sobha x Kanthi, VK 70 x Sobha, VK 70 x Kanthi, VK 70 x Prabha, VK 70 x Pratibha failed to set seeds.

Similar reports were reported by Renjith (1999) in *Curcuma domestica*. He could obtain seed set in crosses involving short duration cultivars.

Nazeem and Menon (1994) have reported high seed set in crosses involving *C. aromatica* cultivars. But in the present investigation, only some crosses were successful. Since they are tetraploids, seed set could be obtained. The failure may be due to the decay of the developing fruits due to bacterial infestation, as the inflorescence structure i.e., presence of open bracts promote harbouring of bacterial inoculum along with rain water. Hence the crosses have to be repeated for conclusive results. *In vitro* pollination and fertilization may be tried in the absence of seed set under *in vivo* condition.

The hybrids of the combination, VK 70 x VK 76 could be promising as they can combine high curing percentage of VK 70 (22.00) and high curcumin content of VK 76 (6.31%). Crosses involving Kanthi and Sobha and vice versa can also be promising as it may combine high yield potential of Kanthi with high curcumin content of Sobha. The crosses involving VK 70 and Prabha and vice

versa may also be promising as it can combine high curing percentage of VK 70 with high curcumin content of Prabha. The cross involving Suvarna with Sobha is good as it can combine high yield, curing percentage and oleoresin content of Suvarna with high curcumin content of Sobha. The improvement of released varieties Suguna, Sudharsana and high yielding accession VK 55 pose difficulty as they register curcumin content below 5.5 per cent and curing below 13.0 per cent.

The combinations involving VK 70 as the female parent only yielded seed set. These results indicate that VK 70 could be successfully employed as a female parent in future hybridization programme, since the cultivar easily set seeds and possesses good curing percentage. Hence an effective hybridization programme could be chalked out with this cultivar with that of Sobha and VK 76, which are rich in curcumin content.

5.6 Pollen pistil interaction studies

The pollen pistil interaction studies after *in vivo* cross pollination involving short duration cultivars (VK 70 x VK 76), (VK 70 x VK 55) and (VK 70 x Suguna) showed that the pollen grains of VK 76, VK 55 and Suguna germinate on the stigma of VK 70 and pollen tube passes through the style and reaches the ovule.

The pollen pistil interaction studies involving medium and short duration cultivars (Kanthi x VK 70) showed that the pollen grains of VK 70 germinate on the stigma of Kanthi and pollen tube passes through the style and

reaches the ovule. So it is possible to conduct crosses between short and medium duration types provided the short duration types are taken as male parent.

In the present investigation similar crosses i.e., medium duration types as female and short duration types as male failed to set seeds. This aspect needs further research.

5.6.1 Description of turmeric fruit and seed

The turmeric fruit developed after *in vivo* cross pollination, VK 70 x VK 76 was a thin walled trilocular capsule with small black arillate seeds. These observations are similar to the reports made by Nambiar *et al.* (1982), Lad (1993) and Renjith (1999).

5.7 Refinement of *in vitro* pollination

5.7.1 Identification of basal medium for culture establishment

MS and Modified MS at half strength with plant growth regulators supported the development of ovules after *in vitro* placental pollination. The percentage of cultures showing ovule development was more in modified ½ MS. But to visual assessment, half strength MS was best for increasing the size of the ovules.

The suitability of MS medium with plant growth substances for *in vitro* culture of turmeric has been reported earlier (Nadgauda, 1978; Kuruvina Shetty *et al.*, 1982; Kuruvina Shetty and Iyer, 1982; Winnaar and Winnaar, 1989;

Kesavachandran and Abdulkhader, 1989; Balachandran *et al.*, 1990; Vidya *et al.*, 1989 and Rajan, 1995).

Renjith (1999) has also reported that basal medium ½ MS along with BAP, kinetin and NAA can support ovule development after *in vitro* placental pollination. According to him ½ MS medium was more favourable for ovule development than full strength.

5.7.1.1 Influence of auxins / cytokinins in the ovule development

In the half strength MS with BAP and kinetin (each at 1 mg l^{-1}) and NAA 0.5 mg l^{-1} favoured maximum ovule development and the response was evident in 80.16 per cent of cultures. Modified ½ MS with BAP and kinetin, each at 0.5 mg l^{-1} and NAA 1 mg l^{-i} also supported the ovule development in 83.01 per cent of cultures. But to visual assessment, first media combination was best for increasing the size of the ovules.

Since the flowering season was over, the influence of modified ½ MS along with BAP and kinetin, each at 1 mg l^{-1} and NAA 0.5 mg l^{-1} was not studied.

Renjith (1999) has also observed that in the ½ MS with hormone combination of BAP and kinetin, each atl mg l^{-1} along with NAA 0.5 mg l^{-1} is optimum for ovule development in turmeric after *in vitro* pollination.

Valsala (1994) also reported favourable effect of BAP ranging from 2.0 to 10 mg l^{-1} along with NAA 0.5 to 1 mg l^{-1} for ovule development after *in vitro* pollination in ginger, another rhizomatous crop of Zingiberaceous family.

Quartrano (1987) reported that hormones play an important role in the stage of seed development and germination. The cytokinin, auxin, gibberelin (GA) and abscisic acid (ABA) were found relatively high in extracts from seeds of different development stages.

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

Eeuwens and Schwale (1975) have reported that, in general high auxins (IAA) and GA levels have been associated with active seed growth and fruit growth by cell expansion. In a number of plants, both GA and auxins are highest during early to mid embryo development, at a stage when cytokinins are decreasing rapidly.

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

Castano (2000) reported that auxin (IAA), cytokinin (Kinetin) and GA_3 play an important role in the ovule as well as seed development in chicory.

5.7.1.2 Influence of vitamins, coconut water and CH in the ovule development

The addition of double the quantity of vitamin stock of MS medium along with the hormones enhanced ovule development (Table 21).

Castano (2000) reported that vitamin stock of MS medium supported the ovule development in chicory (*Cinchorium intibus* L.).

The coconut water at 15% v/v did not favour ovule development. Generally coconut water supports ovule development after fertilization under *in vitro* condition (Shantz and Steward, 1952; Letham, 1974 and Valsala, 1994).

In the present study CH at 200 mg l^{-1} only was examined. CH at 200 mg l^{-1} did not promote ovule development. Hence lower and higher levels may also be tested.

The coconut milk extract at 5 per cent level favoured some ovary development in the intraovarian pollination. But the presence of coconut milk made the medium much oily and it did not promote much ovule development. The effect of lower doses can be examined for ovule and endosperm development.

5.7.2 Refinement of *in vitro* pollination techniques in turmeric

Ovules have developed in intraovarian, placental and modified placental pollination (Table 22). Pollen grains along with modified ME_3 medium were applied over the ovules or ovary as the case may be.

Valsala (1994) had also reported seed set and seed development through placental and modified placental pollination techniques in ginger.

Renjith (1999) reported seed set through placental and modified placental pollination in turmeric.

Ovule development can be the effect of suitable medium and fertilization. Pollination along with modified ME_3 medium enhances the chances of seed set. For confirming the fertilization and embryo development, pollen pistil interaction studies after pollination and microtome sectioning are needed. In the present investigation, pollen pistil interaction was studied. It showed that in crosses of medium and short duration cultivars Kanthi x VK 70, pollen tube growth is sufficient to effect fertilization. But pollen pistil interaction was not studied in short x medium and medium x medium duration cultivars. These aspects need further investigation. For confirming fertilization, microtome sectioning of pollinated ovules can be done.

5.7.3 Post pollination changes

In the intraovarian pollination technique, size of the ovary was increased and were creamy white in diffused light. Ovules developed in the intraovarian,

placental and modified placental pollination techniques. They were creamy white during the initial stage of development and changed to dark brown colour with in a period of 20 to 30 DAP.

Under *in vivo* condition, maturity period for seed development was 20 to 21 days and under *in vitro* condition it was 20 to 30 days.

5.8 Seed germination studies in turmeric

Seeds kept in moist sterile sand did not germinate under *in vivo*.

Under *in vitro* condition only immature light brown coloured, heavy as well as light seeds germinated on moist filter paper kept in test tubes under dark condition. Only 25 per cent seed germination was recorded (Table 23).

Renjith (1999) has reported seed germination on moist filter paper under *in vitro*. This shows that the seeds produced under *in vivo* can be germinated under *in vitro* condition successfully with adequate moisture and air. There was no necessity of any complex media requirements and growth regulators. The reasons for low germination percentage has to be examined i.e., embryo and endosperm development. The seed viability test can also be undertaken.

The reasons for lack of seed germination under *in vivo* condition may be due to the absence of satisfactory conditions required for germination or high percentage of defective seeds.

5.9 *In vitro* multiplication of hybrid seedling

In vitro germinated seedling from *in vivo* produced seed got established in the medium of ½ MS + 3 per cent sucrose BAP 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}. Multiple shoots were produced by detopping the seedling at 40 days after germination in the same medium itself. For further elongation and multiple shoot production, reducing the BAP from 2.5 mg l^{-1} to 1.0 mg l^{-1} was found good. A multiplication rate of 3.4 shoots per subculture of 20 days interval was obtained. This can be further increased by refinement of micropropagation technique.

Multiplication of hybrid plants through micropropagation enables to produce the minimum required population size of 12 plants for the preliminary evaluation trial in the first year itself. In the *in vivo* system of multiplication, tuberisation in the seedling occurs only during the second year. The hybrid plants established under *in vitro* can be easily maintained by vegetative propagation through rhizomes.

Future lines of work suggested

Improvement of short duration cultivars of turmeric is possible through *in vivo* crossing. So large scale breeding could be undertaken for producing desirable ideotypes. Crosses involving medium and short duration cultivars may be repeated for getting seed set. The pollen pistil interaction studies may be done after crossing short duration cultivars as female and medium duration cultivars as male to know the chances of seed set. The possibility of using Suvarna in crop improvement programme is blocked by its non flowering nature. Efforts can be made to induce flowering in this cultivar. Germination studies for the *in vitro* produced seeds of turmeric should be undertaken.

SUMMARY

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Investigations were carried out at the Department of Plantation Crops and Spices and the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 1998 to 2000 on "Refinement of *in vivo* and *in vitro* pollination techniques in turmeric (*Curcuma domestica* Val.)". The work undertaken and the results obtained could be summarized as follows.

1. The plant materials used for the study consisted of six short duration cultivars i.e., VK 70, VK 55, VK 76, Suguna, Sudharsana, Suvarna and four medium duration cultivars i.e., Kanthi, Sobha, Prabha and Pratibha. Studies conducted among them showed significant variability with respect to pseudostem, rhizome and quality characters. The raw yield in the cultivars selected ranged from 3.6 kg/2 m^2 in Sobha to 9.2 kg/2 m^2 in Suvarna. Curing percentage ranged from 8.6 in VK 55 to 22 in VK 70. The cultivars Suvarna and VK 76 recorded high curing percentage of 19.17 and 18.02 respectively. The cultivar Suvarna recorded maximum dry yield (1.7 kg/2 m^2) and minimum in VK 55 (0.43 kg/2 m^2). Curcumin content ranged from 2.61 per cent in VK 70 to 7.24 per cent in Sobha and it was high in other medium duration cultivars i.e., Kanthi (6.98%), Prabha (6.37%) and short duration cultivar VK 76 (6.31%) while all others recorded below 5.50 per cent. Oleoresin percentage varied from 11.80 in VK 55 to 21.60 in VK 70. The short duration cultivars Suguna,

Suvarna and Sudharsana recorded high oleoresin percentage of 21.20, 18.60 and 18.0 per cent respectively.

2. Investigations for the improvement of flowering showed that the size of seed material used influenced the days for first flowering. The plants of short duration cultivars from T_3 group (200 g) was the earliest to flower followed by T_2 (45-50 g) and T_1 (15-20 g). The flowering season in T_3 group was from 15-8-99 to 8-11-99, i.e., 85 days, while in T_2 it was from 4-9-99 to 30-10-99, i.e., 57 days. In T_1 group it was from 15-9-99 to 1-11-99, i.e., 48 days. Flowering was significantly high in open condition (77.61%) compared to shade (26.13%). Flowering intensity was more in short duration types (61.74%) compared to medium duration types (39.54%).
3. Pollen viability was found influenced by season and it was low during early flowering season (18.88%) compared to mid (25.05%) and late (25.84%).
4. Mean pollen fertility in turmeric cultivars as per acetocarmine stain test was 76.93 per cent and the corresponding mean viability in the modified ME_3 medium at pH 6 was only 25.05 per cent. The mean pollen tube length was 134.60 µm and the mean of longest pollen tube length was 274.34 µm. The pollen viability and tube growth were high in short duration cultivars compared to medium duration cultivars.
5. Viability of pollen grains one month after storage in organic solvents i.e., hexane, acetone, cyclohexane, isopropanol and methanol at −15°C was examined. The mean pollen viability in different cultivars before storage was 25.96 per cent and it was reduced to 17.73 per cent after storage. The

reduction in viability over a period of one month was 31.70 per cent. Among the different solvents studied, cyclohexane was best for pollen storage followed by methanol.

6. In the turmeric cultivars studied, natural fruit set and seed set were observed in short duration cultivars viz. VK 70, VK 55, VK 76 and in Suguna. No natural seed set was observed in medium duration cultivars and short duration cultivar Sudharsana. The fruits matured with in a period of 20 to 21 days. The number of seeds per fruit ranged from 9 to 10.

7. In controlled *in vivo* cross pollination, seed set was obtained only through stigmatic pollination. Seed set was obtained in three of fifteen crosses tried viz., VK 70 x VK 76, VK 70 x VK 55 and VK 70 x Suguna. Seed set could not be achieved in crosses involving short and medium duration cultivars and medium and medium duration cultivars. *In vivo* stylar and intraovarian pollination did not produce any seed set.

8. The turmeric fruit developed after *in vivo* cross pollination (VK 70 x VK 76) was a thin walled trilocular capsule with nine black arillate seeds.

9. The pollen pistil interaction studies after *in vivo* cross pollination of short duration cultivars (VK 70 x VK 76), (VK 70 x Suguna) showed pollen germination on stigma and pollen tube passing through the style and reaching the ovules. Similarly crosses involving medium and short duration cultivars (Kanthi x VK 70) showed pollen germination on stigma and pollen tube passing through the style and reaching the ovule.

10. The medium identified for the development of *in vitro* pollinated ovules are (1) ½ strength MS + 3% sucrose + BAP 1 mg l^{-1} + kinetin 1 mg l^{-1} + NAA 0.5 mg l^{-1}. (2) Modified ½ strength MS + 3% sucrose + BAP 0.5 mg l^{-1} + kinetin 0.5 mg l^{-1} + NAA 1 mg l^{-1}. The first medium was the best for increasing the size of the ovule. The organic supplement, coconut milk extract at five per cent concentration and addition of double the quantity of vitamin stock of MS medium favoured ovule development. But coconut water at 15 per cent v/v and CH 200 mg l^{-1} did not favour ovule development.
11. Different *in vitro* pollination techniques, stigmatic stylar, intraovarian, placental, modified placental and test tube fertilization with pollen grains suspended in modified ME_3 medium of pH 6 were attempted. Ovules/seeds developed in intraovarian, placental and modified placental pollination techniques. Among them placental pollination is the best. These techniques can be used for conducting crosses involving short duration cultivars and short and medium duration cultivars provided medium duration cultivars are used as female parents.
12. Ovules/seeds developed after *in vitro* pollination were creamy white during the initial stage of development and changed to dark brown colour with in a period of 20 to 30 DAP. However the seeds failed to germinate under *in vitro*.
13. The seeds produced under *in vivo* germinated *in vitro* in dark on moist filter paper in test tubes and recorded germination percentage of 25. The seeds failed to germinate in the medium of moist sand, mixture of moist sand and vermiculite at 1:1 proportion and in basal medium.

14. *In vitro* germinated seedlings got established and produced multiple shoots in the medium, ½ MS + 3 per cent sucrose + BAP 2.5 mg l^{-1} + NAA 0.5 mg l^{-1} under light intensity of 1000 lux. For further elongation and multiplication, reducing the BAP from 2.5 mg l^{-1} to 1 mg l^{-1} was found good. A multiplication rate of 3.4 shoots per subculture within a period of 20 days was obtained. Ten months after germination, six plantlets have been successfully planted out and their performance was found satisfactory.

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*Originals not seen

APPENDIX

APPENDIX

Abstract of analysis of variance for the effect of different treatments

Sl.No.	Table details and characters	Treatment MSS	Error MSS
I	Table 6. Variability in pseudostem characters of turmeric cultivars		
	1. Height in cm for plants of different cultivars	992.47**	138.10
	2. Number of leaves per plant	46.00**	18.30
	3. Number of tillers per plant	7.93	4.64
	4. Leaf length (cm)	302.82**	17.02
	5. Leaf breadth (cm)	10.28**	2.74
	6. Leaf area (cm^2)	76986.92**	6831.28
II	Table 7. Variability in rhizome characters of different turmeric cultivars		
	1. Number of mother rhizome	1.95**	0.72
	2. Length of mother rhizomes (cm)	3.47*	1.41
	3. Circumference of mother rhizomes (cm)	8.29*	3.06
	4. Internodal length of mother rhizomes (cm)	4.00*	1.55
	5. Number of nodes	10.80**	3.34
	6. Number of primary fingers	6.08**	1.73
	7. Length of primary fingers (cm)	18.20**	2.06
	8. Circumference of primary fingers (cm)	3.98**	1.35
	9. Internodal length of primary fingers (cm)	17.28**	3.25
	10. Number of secondary finger	13.01	51.21
	11. Length of secondary fingers (cm)	11.27**	1.98
	12. Circumference of secondary fingers (cm)	10.18**	2.95
	13. Internodal length of secondary fingers (cm)	36.78**	4.58

Contd.....

Appendix continued

Sl. No.	Table details and characters	Mean squares		
		Seed size	Varieties	Seed size x varieties
III	Table 9. Influence of seed size on pseudostem characters of turmeric cultivars			
	1. Height of plant (cm)			
	2. Number of leaves/plant	9790.57**	1233.59**	447.66**
	3. Number of tillers/plant	575.77**	25.56	49.09**
	4. Leaf length (cm)	0.14	0.12	0.24
	5. Leaf breadth (cm)	1181.56**	655.78**	502.82**
	6. Leaf area (cm^2)	37.43**	36.44**	25.40**
		271081.49**	202070.31**	152598.64**
		Seed size	Open/shade	Varieties
IV	Table 12. Influence of seed size on intensity of flowering in turmeric cultivars			
	Percentage of flowering	2.62	423.07**	24.90**
		Seed size	Varieties	Seed size x varieties
V	Table 13. Seasonal variation of pollen viability in turmeric cultivars			
	Pollen viability	14.94**	50.23**	1.52
		Solvents	Varieties	Varieties x solvents
VI	Table 15. Effect of storage on pollen viability of different turmeric cultivars	8.96**	7.32**	1.41

Level of significance ** $P < 0.01$
* $P < 0.05$
NS $P > 0.05$

REFINEMENT OF *IN VIVO* AND *IN VITRO* POLLINATION TECHNIQUES IN TURMERIC (*Curcuma domestica* Val.)

By

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ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

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ABSTRACT

Investigations on "Refinement of *in vivo* and *in vitro* pollination techniques in turmeric (*Curcuma domestica* Val.)" were carried out at the Department of Plantation Crops and Spices and the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period from 1998 to 2000.

The plant materials used for the study consisted of six short duration cultivars viz., VK 70, VK 55, VK 76, Suguna, Sudharsana and Suvarna and four medium duration cultivars viz., Kanthi, Sobha, Prabha and Pratibha. Studies conducted among them showed significant variability with respect to pseudostem, rhizome and quality characters. The genotypes identified for high curcumin are Sobha (7.24%), Kanthi (6.98%), VK 76 (6.31%) and Prabha (6.37%) and those identified for high curing percentage are VK 70 (22.00%), Suvarna (19.17%) and VK 76 (18.02%).

The investigations to improve flowering showed that the size of the seed material used influenced flowering behaviour of turmeric cultivars. The plants of short duration cultivars from T_3 group (200 g) was the earliest to flower followed by T_2 (45-50 g) and T_1 (15-20 g). The flowering season in T_3 group was extended for 85 days while it was only 48 days in T_1 group and 57 days in T_2 group. The flowering intensity in turmeric cultivars were significantly high in open condition (77.61%) compared to shade (26.13%).

Pollen viability studies were conducted in modified ME_3 medium at pH 6. Viability was low during early flowering season (18.88%) compared to mid (25.05%) and late (25.84%). The mean pollen viability in short duration cultivars was 33.51 per cent while it was low in medium duration cultivars (14.30%). The mean pollen tube growth also followed the same trend.

Dry pollen grains can be stored in cyclohexane and methanol at –15°C for one month with mean pollen viability percentage of 20.18 and 20.09 respectively.

Natural fruit set and seed set were observed in short duration cultivars and not noticed in medium duration cultivars.

Through controlled *in vivo* pollination, seed set was obtained in crosses involving short duration cultivars. But failed in crosses involving short and medium duration cultivars. Seed set was not obtained through *in vivo* stylar and intraovarian pollination.

Pollen pistil interaction studies after *in vivo* cross pollination of short and medium duration cultivars showed pollen germination on stigma and pollen tube passing through style and reaching the ovules. So lack of seed set can be due to some other factors.

In vitro pollination was done by pollen grains suspended in the modified ME_3 medium. Among the various methods of pollination tried, ovule/seed

development was observed in intraovarian, placental and modified placental pollination techniques. Among them, placental pollination is the best as number of ovules developed per culture was maximum in this. These techniques can be used for conducting crosses involving short and medium duration cultivars provided medium duration cultivars as female parents.

The medium identified for the development of *in vitro* pollinated ovules are (1) ½ strength MS + 3% sucrose + BAP 1 mg l^{-1} + kinetin 1 mg l^{-1} + NAA 0.5 mg l^{-1}. (2) Modified ½ strength MS + 3% sucrose + BAP 0.5 mg l^{-1} + kinetin 0.5 mg l^{-1} + NAA 1 mg l^{-1}. The first medium was the best for increasing the size of the ovules. The organic supplement coconut milk extract at five per cent concentration and addition of double the quantity of vitamin stock of MS medium favoured ovule development.

Two hybrid seeds from the *in vivo* cross (VK 70 x VK 76) germinated under *in vitro* on moist filter paper in test tubes. They were multiplied under *in vitro* and six plantlets were successfully planted out in the field and the rest were multiplied further under *in vitro*.