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STANDARDIZATION OF *IN VITRO* POLLINATION AND FERTILIZATION TECHNIQUE FOR HELICONIA

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DECLARATION

I hereby declare that this thesis entitled "**Standardization of *in vitro* pollination and fertilization technique for heliconia**" 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 of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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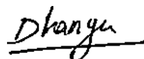

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Certified that this thesis entitled "**Standardization of *in vitro* pollination and fertilization technique for heliconia**" is a record of research work done independently by **Ms. Dhanya, A.** (2004-12-09) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Dhanya

Dhanya, A.

Dedicated to
Lord Krishna &
My Family

CONTENTS

	Page No.
1. INTRODUCTION	1 - 2
2. REVIEW OF LITERATURE	3 - 28
3. MATERIALS AND METHODS	29 - 41
4. RESULTS	42 - 66
5. DISCUSSION	67 - 76
6. SUMMARY	77 - 81
7. REFERENCES	82 - 94
APPENDICES	
ABSTRACT	

LIST OF TABLES

Table No.	Title	Page No.
1	Treatments to assess the pollen germination in Brewbaker and Kwack medium	31
2	Treatment to assess the pollen germination in ME_3 medium	31
3	Surface sterilization treatments to assess the culture establishment	33
4	Treatment for the management of bacterial interference in cultures	33
5	Treatments to assess the age of flower buds for *in vitro* pollination	36
6	Treatments to asses the influence of auxin concentrations on ovule development	37
7	Treatments to assess the combination of cytokinin and auxin concentrations on ovule development	38
8	Treatments to asses the effect of organic supplements on ovule development	39
9	Treatments for the germination of heliconia seed	40
10	Pollen fertility of different heliconia cultivars	43
11	Effect of PEG in Brewbaker and Kwack medium on *in vitro* pollen germination percentage of heliconia cultivars	43
12	Effect of PEG in ME_3 medium on *in vitro* pollen germination in heliconia cultivars	45
13	Effect of incubation period on *in vitro* pollen germination in heliconia cultivars	45
14	Effect of p^H in ME3 medium on pollen germination in heliconia cultivars	46
15	Pollen tube length of different heliconia cultivars	46
16	Morphological characters of gynoecium in different heliconia cultivar	49

LIST OF TABLES CONTINUED

Table No.	Title	Page No.
17	Effect of various surface sterilants on culture establishment of heliconia cultivars	49
18	Effect of management of bacterial interference on culture establishment of heliconia cultivars	51
19	Effect of addition of $CuSO_4$ on culture medium for controlling the bacterial interference in culture establishment of heliconia cultivars	51
20	Effect of basal media on ovule development of heliconia after *in vitro* pollination	52
21a.	Effect of different *in vitro* pollination techniques in heliconia	52
21b.	Effect of different *in vitro* pollination techniques in heliconia continued	53
22	Effect of sucrose on ovule development of heliconia after *in vitro* pollination	53
23	Effect of auxins on ovule development of heliconia after *in vitro* pollination	57
24	Effect of cytokinin on ovule development of heliconia after *in vitro* pollination	57
25	Effect of auxin and cytokinin on ovule development of heliconia after *in vitro* pollination	58
26	Effect of gibberllic acid on ovule development of heliconia after *in vitro* pollination	60
27	Effect of organic supplements on ovule development of heliconia after *in vitro* pollination	60
28	Effect of illumination on ovule development of heliconia after *in vitro* pollination	62
29	Effect of physical form of the media on ovule development in heliconia after *in vitro* pollination	62
30	Effect of *in vitro* treatments on germination of heliconia seeds	62

LIST OF FIGURES

Sl. No.	Title	Between pages
1	Comparison of effect of PEG in Brewbaker and Kwack medium and ME_3 medium on in vitro pollen germination	68 & 69
2	Effect of incubation period on percentage of pollen germination in heliconia cultivars	68 & 69
3	Comparison of pollen fertility in acetocarmine stain test vs pollen viability in ME_3 medium	68 & 69
4	Comparison of pollen tube length of three selected heliconia cultivars in Brewbaker and Kwack medium and ME_3 medium	68 & 69
5	Effect of auxins on ovule development of heliconia after *in vitro* pollination	73 & 74
6	Effect of cytokinin on ovule development of heliconia after *in vitro* pollination	73 & 74
7	Combination effect of auxin and cytokinin on ovule development of heliconia after *in vitro* pollination	73 & 74
8	Effect of casein hydrolysate and yeast extract on ovule development of heliconia after *in vitro* pollination	73 & 74

LIST OF PLATES

Sl. No.	Title	Between pages
1	*Heliconia psittacorum* selected for experiment	29 & 30
2	Floral morphology of heliconia cultivars	32 & 33
3	Pollen fertility of heliconia cultivars	43 & 44
4	*In vitro* pollen germination	43 & 44
5	Ovary development in MS medium	55 & 56
6	*In vitro* modified placental pollination	55 & 56
7	Ovule development 4 days after modified placental pollination	55 & 56
8	*In vitro* seed formation after modified placental pollination	55 & 56
9.	*In vitro* formed seeds after *in vitro* pollination 5x	63 & 64
10.	Ovule development after *in vitro* pollination 5x	63 & 64
11.	Longitudinal sections of *in vitro* formed seeds 5x	63 & 64
12.	Dieing of embryo 90 days after pollination 5x	64 & 65
13.	Completely stained seeds after saffranin staining 5x	64 & 65
14.	Embryos formed after *in vitro* pollination	65 & 66
15.	*In vitro* embryo germination	65 & 66

LIST OF APPENDICES

Sl. No.	Title	Appendix No.
1	Composition of Brewbaker and Kwack medium	I
2	Composition of ME_3 medium (Leduc *et al.*, 1991)	II
3	Composition of MS, SH and Nitsch basal media	III

LIST OF ABBREVIATIONS

%	–	Per cent
°C	–	Degree Celsius
μm	–	micrometer
½ MS	–	Half strength Murashige and Skoog
2,4-D	–	2,4-dichloro phenoxy acetic acid
BA	–	Benzyl adenine
BAP	–	Benzyl amino purine
CD	–	Critical difference
CH	–	Casein Hydrolysate
cm	–	Centimeter
cv	–	Cultivar
CW	–	Coconut water
DAP	–	Days after pollination
g	–	Gram
GA_3	–	Gibberellic Acid
h	–	Hour
i.e.	–	That is
IAA	–	Indole 3- Acetic Acid
IBA	–	Indole 3- Butyric Acid
kg	–	Kilogram
KIN	–	Kinetin
l	–	Litre
mg	–	Milligram
min	–	Minute
MS	–	Murashige and Skoog (1962)
NAA	–	1-Napthyl Acetic Acid
PEG	–	Polyethylene glycol
pH	–	Per hydrogen
s	–	Second
SH	–	Schenk and Hildebradt (1972)
viz.,	–	Namely
YE	–	Yeast Extract

Introduction

1. INTRODUCTION

Heliconias belonging to the family Heliconiaceae, are among the most provocative of all exotic tropical flowering plants. *Heliconia* is the sole genus of family Heliconiaceae, but formerly included in the family Musaceae. The family comprises of 250-300 species distributed mainly in Neotropical areas from the North of Mexico to the South of Brazil (Kress, 1990). They are native to Central and South America, the Caribbean Islands and some of the islands of the South Pacific.

Heliconia is now gaining popularity as one of the most important commercial cut flower crops of the modern world. They are medium to large erect herbs often with extensive rhizomatous growth with large, colourful, bracteate inflorescence. Species of *Heliconia* are utilized both as landscaping plants and as cut flowers. The number of species commercially used has increased, mainly as cut flowers, because of the colour and the considerable durability of their floral bracts.

In India heliconia is gaining importance as annual production of about one lakh stems which accounts for less than one per cent of the total flowers production of the country (Sheela, 2006). Fifty per cent of the production of heliconia comes from West Godavari district of Andhra Pradesh and gaining importance as a commercial flower crop in states like Karnataka and Kerala in recent times with great potential for export.

As most of the cultivated *Heliconia* species set few fruits or are sterile and the germination of the seed is low, the vegetative propagation by division of rhizomes is preferred in its commercial production (Criley, 1988; Lee *et al.*, 1994; Simao and Scatena, 2003). It has been found that cross-fertilization between *Heliconia* species are generally unsuccessful due to problems of incompatibility. There is no evidence that any hybrids of heliconia have been artificially made (Berry and Kress, 1991). Existence of

pre-fertilization crossability barrier *i.e.*, stigmatic, stylar or ovarian, act as the ultimate mechanism preventing hybridization (Kress, 1983a). Sanjeev (2005) also reported that cross incompatibility existed in heliconia leading to problems in seed set.

For evolving novel varieties suitable for domestic and international market hybridization is essential. The technique of *in vitro* pollination and fertilization is very promising for overcoming pre-fertilization barriers due to compatibility and for raising new genotypes through seed propagation (Bhojwani and Razdan, 1983). The purpose of *in vitro* pollination and fertilization is to bring pollination and embryogenesis under artificial control so as to produce hybrid embryos among plants that cannot be crossed by conventional method of plant breeding.

Hence the present study was undertaken with the objective of standardize the various steps in *in vitro* pollination and fertilization technique in heliconia.

Review of Literature

2. REVIEW OF LITERATURE

Heliconia is an emerging tropical cut flower crop. They are medium to large erect herb with extensive rhizomatous growth. The most distinguishing characteristic of this group is the large, colourful, bracteate inflorescence. The inflorescence of heliconia is nearly always terminal and may last from several days to several months. Heliconias have several common names including lobster's claws, parrot flowers, False-bird of paradise, parrot plantain and false plantain. There are two main types of heliconia *viz.*, erect heliconia and pendent heliconia. Erect heliconias stand straight with bract pointing up and pendent heliconias hang with bracts pointing down.

Heliconia are propagated by rhizomes as well as suckers and rarely from seed. Most species of *Heliconia* have been self compatible on the other hand, it has been found that cross fertilization between species is generally unsuccessful due to pre-fertilization barrier (Kress, 1983a; Berry and Kress, 1991; Sanjeev, 2005). So far there is no solid evidence for any artificial *Heliconia* hybrid.

Conventional method of artificial hybridization is not possible in heliconia because of several pre-zygotic barriers. But in ornamental breeding hybridization is an important tool to create new cultivars. Pollination and fertilization under *in vitro* condition offer an opportunity for producing hybrid embryos among plants that cannot crossed by conventional methods of plant breeding. Through *in vitro* pollination and fertilization intergeneric and interspecific hybrids can be produced, which are unknown in nature due to pre-fertilization barriers. According to Shivanna and Moharam (2005) in flowering plant *in vitro* fertilization would provide an effective means of overcoming cross ability barriers imposed by the stigma and style.

A review of research work done is heliconias as well as attempts made on *in vitro* pollination and fertilization in different crops are attempted here.

2.1 SPECIES AND CULTIVARS

There are about 250-300 species of *Heliconia* distributed primarily in Neotropical areas from the North Mexico to the South of Brazil (Dahlgren *et al.*, 1985; Kress, 1990). They are banana like plants with rhizomes or underground stems, propagated by bits of rhizomes as well as suckers. There are too types of heliconia erect and pendent with respect to the orientation of inflorescence.

Some of the important erect species of *Heliconia* are *Heliconia psittacorum, H. stricta, H. latispatha, H. bihai, H. distance, H. angustifolia, H. angusta, H. bourgeana, H. subulata, H. velloziana, H. hirsuta, H. rivularis* and *H. spathocircinata*. Pendent species of *Heliconia* include *Heliconia rostrata, H. collinsiana, H. chartacae, H. nutans* and *H. combinata*.

Other species of *Heliconia* include *H. solomenensis, H. orthotricha, H. magnifica, H. xanthovillosa, H. metallica, H. moriae, H. caribaea, H. secunda, H. clonophila, H. marginata, H. schiedeana, H. spissa, H. bourgeana, H. touruosa, H. irrasa, H. thomasiana* and *H. imbricata.*

In *Heliconia* there are more than 500 cultivars (Berry and Kress, 1991). Among these *H. psittacorum* was considered the most useful for cut flower production became of its upright stem, moderately vigorous growth habit, long flowering season and long lasting flowers. Important cultivars of *H. psittacorum* are Andromeda, Lady Di, Sassy, Parakeet, St. Vincent Red, Pascal, Choconiana, Nikeriensis, Rosi and Blush.

2.2 FLORAL BIOLOGY

The varied and fantastic forms and rich colour of the different species of *Heliconia* make them an important florist item. Once a plant is mature, each shoot produced from rhizome has the potential to generate a single inflorescence; each inflorescence may last from several days to several

months (Berry and Kress, 1991). The inflorescence of *Heliconia* is terminal and composed of several cincinni, each cincinnus has floral buds and fruits are different stages of development and partially or totally subtended by a several colourful bract (Simao *et al.*, 2006).

Watson and Dallwitz (1991) had given the following description for the floral biology of heliconia. Flower are bisexual each flower open only for single day. Floral nectories present at the base of the style. The inflorescence is cymose and terminal in habit. Flower bracteate, very regular and zygomorphic perianth of six tepals, two whorled which are fused to form a five lobed, boat shaped upper lip. When the flower open, a single sepals become free from outer perianth part and allows pollinators to enter the flower. The colour of the perianth is species specific. Androecium six in number with five stamens and one staminode, are free and basifixed. Pollen grains are three celled. Gynoecium inferior, syncarpous and 3-carpelled ovary, basal placentation with one ovule per locule, which is anatropous. One style with one or three wet type stigma. The mature fruit of heliconia is a drupe with three seeded. The outer layer of fruit is fleshy and at maturity the surface layer becomes bluish colour. The colourful fruits are very attractive to the birds and mammals that disperse the seeds. Seeds are triangular, endospermic with straight embryo.

2.3 ANTHESIS IN HELICONIA

The process related to anthesis varied with species and environment. Mc Dade (1983) reported that in *Heliconia irrasa* anthesis occurs at or shortly after dawn and flower last for a day. Kress (1985) reported that the green and white odorless flower of *Heliconia solomonensis* open in the evening.

According to Pederson (1999) the anthesis time for *Heliconia laufao* and *Heliconia paka* just before dawn.

Criley *et al.* (1999) reported that *Heliconia wagneriana* and *H. stricta* 'Dwarf Jamaica' are short days species, reached anthesis 15 to 19 weeks after the start of short day. They also reported that *Heliconia angusta* a long day species, required day length excelled 13.3 hours and reached anthesis after 15 to 17 weeks of long days.

According to Sanjeev (2005) the peak anthesis time estimated to be between 3.00 am and 6.00 am. He also reported that variety Collinsiana, anthesis was found distributed from 8.00 pm to 7.00 am, there was no flower opening for Petra Orange, Petro Ortiz, Guyana, Golden Torch and Alan Carle.

2.4 POLLINATION BIOLOGY

Humming birds are the primary pollinators of *Heliconia* (Linhart, 1973; Stiles, 1975; Mc Dade, 1983; Dobkin, 1984; Gill, 1987; Berry and Kress, 1991; Mandell, 1999 and Yoshioka, 2003). The tubular flowers tend to have a 'lock and key' fit with the beak of the humming birds that pollinate most of the heliconia.

Rose and Barthlott (1995) described the thread like structures connecting the pollen grains of heliconia. These threads ensure that pollen becomes embedded in the feathers or attached to the smooth, unsculptured beak of pollinating humming birds.

Pederson (1999) reported that the primary pollinator of Polynesian heliconias, *Heliconia laufao* and *H. paka* are the wattled honey eater (*Foulehaio carunculata*). Altshuller (2003) reported that pollination in heliconia is mainly Ornithophillous. Kress (1985) reported that in the Salmon Islands *Heliconia solomonensis* pendent, green in conspicuous inflorescence is pollinated by the bat (*Melonyeteris woodfordi*).

Sanjeev (2005) also reported that other than humming bird, Lotus Sun bird (*Netarina lofenia*), stingless bees (*Melipona iridipennis*) and ants are possible pollinators of heliconia.

2.5 ARTIFICIAL POLLINATION AND HYBRIDIZATION

Hybridization in *Heliconia* species is probably uncommon in nature. Most species of heliconia are self compatible but cross fertilization is unsuccessful due to biochemical incompatibility (Berry and Kress, 1991).

Kress (1983a) studied artificial hybridization among species of neotropical, heliconia. He reported that cross ability barriers between the majority of species are strong and foreign pollen tubes are inhibited at the stigmatic surface, within the style or within the ovary.

Kress (1983b) also studied self incompatibility in 19 species of Central American heliconia reported that response ranged from total self rejection in one species to full self-incompatibility in the majority of taxa studied.

Lee *et al.* (1994) selected six cultivars of *Heliconia psittacorum* for studies on that natural fruit hearing ability, pollen formation and pollination under the tropical climatic conditions of Singapore. Three of them, namely Tay, Andromeda and Lady Di were partially fertile with a very low rate of fruit set, ranging from 2.8 to 4.7 per cent. The poor fruit set of these three cultivars was attributed to poor pollen germination on stigma rather than poor pollen formation. The other three cultivars namely Petra, Sassy and Iris were completely sterile.

Berry and Kress (1991) reported some of the natural hybrids of heliconia. E.g., *Heliconia* cv. Golden Torch is the natural hybrid between *H. psittacorum* and *H. spathocircinata* other hybrids between species include *H. caribaea* x *H. bihai*, *H. secunda* x *H. clinophila*, *H. psittacorum* x H. *marginata*.

Yoshioko (2003) also reported that heliconia hybrids are common in Puerto Rico due to several reasons such as environmental factors, flowering seasonality and difference in pollinators.

2.6 SEED DEVELOPMENT IN HELICONIA

With regards to Heliconiaceae very few reports about seed development studies Humphrey (1896) described part of the ovule and seed development in 16 species of Zingibrales including *Heliconia psittacorum* and *Heliconia bihai*.

Mauritzon (1936) also described aspects of seed development in 17 species for Zingibrales including *Heliconia aurantiacea*.

Simao *et al.* (2006) studied the developmental anatomy and morphology of the seed in several species of *Heliconia*. They reported that the zygote was round after syngamy and enveloped by a slightly thickened wall, with a prominent nucleus and nucleolus. The first transversal division resulted in the formation of the proembryo, with apical and basal cells, both of which had prominent nucleoli. The proembryo underwent several divisions and became globular and later reniform. In ripe and almost ripe fruit, the embryo was longitudinally elongated slightly curved on the apex. Most of the embryo formed by the cotyledon, especially the haustorial part at the apical and the endosperm started to develop in the zygote stage with successive nuclear division. In ripe seeds, the endosperm occupied a large part of the seeds and contained a substantial quantity of starch and numerous spherical bodies, probably lipid deposits. The drupaceous fruits usually had three seeds, with each one totally filling the ovule.

2.7 HELICONIA SEED GERMINATION

In most of the *Heliconia* species set few fruits or are sterile and the germination of the seed is slow, the vegetative propagation by division of rhizome, is preferred in its commercial production (Montgomery, 1986; Criley, 1988; Lee *et al.*, 1994; Simao and Scatena, 2003). In species of *Heliconia* there have been few detailed studies about seed and seedling morphology (Humphrey, 1896; Tillich, 1995; Simao and Scatena, 2003).

Germination of heliconia seed is erratic in nature. Seeds of the same species under close to identical conditions present different germination intervals (Simao and scatena, 2004; Tejedor, 2003). Different species have different germination intervals which could vary from weeks to many months (Tejedor, 2005).

Some species of *Heliconia* took three years to germinate (Montgomery, 1986; Criley, 1988). Montgomery (1986) reported that the range in the germination period of Heliconia (from three months to three years), depended on the degree of the embryo development at the time of the fruit maturity.

Specified technique has been reported for inducing growth in seeds of heliconia. The fleshy part of the fruit removed and the seeds were sterilized in 10 per cent sodium hypochlorite for five minutes, then washed in distilled water. The seeds were stored in plastic bags with moist vermiculite in a greenhouse at 25-30°C, until germination was observed in some seeds (Criley, 1988; 1995; Simao and Scatena, 2003).

2.8 POLLEN STUDIES

2.8.1 Pollen Morphology

The pollen grains of heliconia and many of its relatives in the Zingibrales are virtually devoid of a conspicuous, protective exine (Kress *et al.*, 1978). Watson and Dallwitz (1991) described that pollen grains of *Heliconia* are non-aperturate and 3-celled. In heliconia process of pollen formation is normal and pollen grains are uniform in size and appeared normal (Lee *et al.*, 1994). Babu (2005) reported that pollen size of different *Heliconia* species varied from 360 μ (Wagneriana Yellow) to 238 μ (*H. latispatha*).

2.8.2 Pollen Fertility

Zirkle (1937) described a method for assessing pollen fertility by using acetocarmine stain, the pollen grains which stained well and looked plump and normal were considered as viable and unstained shrivelled one as non-viable. A high percentage of pollen fertility has been reported in heliconia. Babu (2005) reported that pollen fertility of *Heliconia* species varied from 90.81 per cent to 16.25 per cent. She recorded higher fertility percent in *H. latispatha* (90.8 %) and in Lady Di (82.7 %). Sanjeev (2005) also reported that pollen fertility of *Heliconia* species varied from 23.23 per cent (Guyana) to 89.39 per cent (Lady Di).

2.8.3 Pollen Germination

Germination is the first critical morphogenetic event in the pollen towards fulfilling its ultimate function of discharge of male gametes in the embryosac. It is possible to germinate pollen grains of a number of taxa *in vitro*, using a simple nutrient medium and to achieve a reasonable length of tube growth.

2.8.3.1 Pollen Germination Media

Brewbaker and Kwack (1963) proposed a medium suitable for pollen germination and tube growth of 86 species. But the optimal concentration of sucrose is variable between these species.

According to Shivanna and Johri (1985) two-celled pollen grains are more amenable to germination than three-celled pollen, hydration phase is critical for subsequent process of pollen germination and tube growth. According to them controlled hydration seems to be more suitable or even essential for successful germination and tube growth particularly to three-celled and stored pollen. They also reported that germination requirements of pollen vary from species to species, apart from hydration, they generally require a carbohydrate source, boron and calcium for satisfactory germination and tube growth.

Leduc *et al.* (1990) developed a new medium for the germination of tri-nucleate pollen *i.e.*, Monnier's medium. According to them Brewbaker and Kwack medium only 16 per cent of the pollen grains germinated and produce pollen tubes having a maximum length of 25 µm, but in Monnier's medium 47 per cent germination and 160 µm long pollen tubes were produced.

Germination medium containing polyethylene glycol was most effective for inducing pollen germination by acting as osmoticum in *Brassica* (Ferrari and Wallace, 1975) and in oil palm pollen (Tandon *et al.*, 1999). Leduc *et al.* (1990) also reported that PEG was superior to sucrose as an osmoticum in germination and tube growth of pollen.

Valsala (1994) reported the ME_3 medium support significantly better pollen germination and pollen tube length in ginger (*Zingiber officinale* Rosc.) with 12 per cent PEG as osmoticum. She recorded maximum pollen tube length of 1042.17 µm. Bindu (1997) also reported germination of ginger pollen grain in ME_3 medium varied from 2.9 per cent to 7.4 per cent among cultivars. She recorded the maximum pollen germination of 7.4 per cent in Rio-de-Janeiro.

Renjith (1999) reported that modified ME_3 medium with pH 6 was best artificial media for pollen germination in turmeric (*Curcuma domestica*).

Bhurke (2002) reported that in kacholam (*Kaempferia galanga*) also modified ME_3 medium was the best for *in vitro* pollen germination and pollen tube growth. He recorded 78 to 80 per cent pollen germination and 809.17 µm pollen tube length in this medium. He also reported that Brewbaker and Kwack medium was favourable for pollen germination in kacholam.

2.9 *IN VITRO POLLINATION AND FERTILIZATION*

Pioneer attempts in *in vitro* pollination and fertilization was made by embryologist, led by Maheshwari in Delhi in early 1960's. First successful test tube fertilization was reported in *Papaver somniferum* (Kanta *et al.*, 1962).

The most important application of *in vitro* pollination and fertilization is in raising hybrids which are unknown because of the pre-fertilization barriers (Bhojwani and Razdan, 1983). This can be employed where fertilization barriers like, inability of pollen to germinate on foreign stigma, failure of pollen tube to reach the ovule due to excessive length of style or slow growth of the pollen tube, so that ovary abscises before the pollen tube reaches the base of the style or bursting of pollen tube in the style.

In the research of sexual plant reproduction and in the improvement of higher plants *in vitro* fertilization play an important role (Yilan, 2003).

2.9.1 *In vitro* Pollination and Fertilization Technique

The pre-requisites and procedures to be adopted for the successful *in vitro* pollination and fertilization was described by Kanta and Maheshwari (1963a). According to them technique essentially consists of the following:

1. A thorough understanding of floral biology of crop *i.e.*, time of anthesis, anther dehiscence, pollination etc.
2. Pollen germination and tube growth has to be ascertained.
3. Standardization of surface sterilization technique for flower/flower buds. So that the viability of pollen grains or receptivity of gynoceium is not affected.
4. Identification of appropriate nutrient medium for ovule/ovary development into seed.
5. Development of appropriate pollination technique
6. Identification of suitable culture conditions for the viable seed development
7. Histological examination of ovule/ovary at various stages to know the fertilization and development of embryo and endosperm.
8. Standardization of cultural condition for germination of seeds.

Kumar and Kumar (1996) also described some of the conditions for achieving successful *in vitro* fertilization *viz.*, identification of correct physiological and morphological state of pollen grains and ovules, identification of appropriate cultural conditions and *in vitro* pollination technique and choice of nutrient medium at different process *i.e.*, germination of pollen grains, fertilization and growth of embryo in the seed.

2.9.2 Different Kinds of *In vitro* Pollination

Bhojwani and Razdan (1983) described the following kinds of *in vitro* pollination.

2.9.2.1 In vitro Stigmatic Pollination

In this, pollination done on cultured whole pistil. The pollen grains are aseptically deposited on the receptive stigma of cultured pistil. Stigma should not be in contact with sterilizing agent otherwise exudates on the stigma may be dissolved. Also the wetting of stigma lead to poor pollen germination (Balatkova *et al.*, 1977b).

Usha (1965) reported the successful *in vitro* stigmatic pollination in *Antirrhinum majus* a member a member of scrophulariceae.

Successful *in vitro* stigmatic pollination in *Nicotiana rustica* reported by Rao (1965) and Rao and Rangaswamy (1972).

Shivanna (1965) reported successful *in vitro* stigmatic pollination and seed set in *Petunia violaceae.* According to Rangaswamy and Shivanna (1967), self incompatibility of *Petunia* species could be eliminated by *in vitro* stigmatic pollination.

2.9.2.2 Intra-ovarian Pollination

In the intra-ovarian pollination pollen grains are directly introduced into ovary. Here pollen grains either placed on the cut surface of the ovary or transferred through a hole in the wall to the ovary.

Kanta (1960) reported a successful intra–ovarian pollination in *Papaver rhoeas* L. This technique is also successful in *Eschscholzia californica* Chem., *Argemona mexicana* L., *A. ochroleuca* and *Papaver somniforum* (Maheshwari and Kanta, 1961; Kanta and Maheshwari, 1963b).

Using this technique, Kanta and Maheshwari (1963b) developed a hybrid between *Argemone mexicana* and *A. ochroleuca.* Pollen were collected from dehiscing anthers and suspended in sterile double distilled water containing 100 mg l^{-1} boric acid, injected into the ovary through a hole using a hypodermic syringe.

In *Trifolium repens* intra-ovarian pollination resulted in fertilization and the formation of torpedo embryo (Leduc *et al.*, 1992).

2.9.2.3 In vitro Placental Pollination

In this, pollination is done on excised ovules together with its placenta. Alternatively the placenta may be cut into two or more pieces each carrying a certain number of ovules. Rangaswamy and Shivanna (1971a) further modified the technique of placental pollination. They cultured the entire pistils after exposing the placenta bearing ovules by removing the ovary wall and pollination was done on the exposed ovules.

Balatkova *et al.* (1977a) got viable seeds of *Narcissus pseudonarcissus* through *in vitro* placental pollination. Balatkova *et al.* (1977b) reported that in *Nicotiana tabacum* the fertilizing capacity of the pollen culture was found to increase by placental pollination during the first hour of cultivation.

Olson and Cass (1981) reported successful *in vitro* placental pollination in *Papaver nudicule.* In this fertilized ovules normally developed into germinable seeds.

Janson (1993) studied the interaction between pollen tube and placenta with ovule in *Lilium longiflorum.* He reported that the pollen tube growth between the ovules seems directed and the pollen tubes do find the

inner integument, a reaction to the micropyle is observed but ovule penetration was low.

Zenkteler (2000) reported hybridization through *in vitro* placental pollination in *Nicotiana tabacum, Melandrinum album* and *Allium moly* with *Pinus Wallichiena.*

Vervaeke *et al* (2002) reported that placental grafted style pollination resulted in higher fertilization per cent in *Aechmea fasciata.* Placentae of *Melandrinum album* excised from ovaries of unopened flower were pollinated. Successfully *in vitro* with pollen grains of *Lychnis coronaria* (Zenkteler *et al.*, 2005).

2.9.2.4 In vitro Ovular Pollination / Test-Tube Fertilization

In this pollination, application of pollen to the excised ovules is done. This technique was successful is *Papaver somniferum, Argemone mexicana, Eschscholzia californica, Nicotiana rustica* and *Nicotiana tabacum* (Kanta and Maheshwari, 1963a).

Successful test tube fertilization in *Dicranostigma franchetianum* was reported by Rangaswamy and Shivanna (1969). Zenkteler (1970) reported that by growing pollen grains directly around the excised ovules in axenic cultures, fertilization was successfully achieved among unrelated taxa such as members of Solanaceae (*Datura stramonicum*) and caryophyllaceae (*Melandrinum album*).

Faurea *et al.* (1994) reported a successful *in vitro* pollination and fertilization in maize. Castano and Deproft (2000) succeeded in overcoming self-incompatibility in *Cichorium intybus* L. through *in vitro* pollination of isolated ovules.

Chi (2000) reported successful interspecific crosses in *Lilium* species through *in vitro* ovular pollination. Successful *in vitro* self-pollination of isolated ovules of sunflower (*Helianthus annuus* L.) was reported by Popielarska (2005).

2.9.2.5 In vitro Stylar Pollination

The style is cut at various heights and pollination done on the cut surface. This was successful employed for *in vitro* seed production in *Petunia hybrids* (Nimi, 1976).

Successful interspecific crosses through *in vitro* cut-style pollination were developed in *Lilium* species (Van Tuyl *et al.*, 1991). Veraeke *et al.* (2002) also studied. The effect of cut style pollination in *Aechmea fasciata*. They reported that after cut style pollination the per cent of ovule penetration of pollen tubes increased by guidance of pollen tube growth through the style.

2.9.3 *In vitro* Pollination and Fertilization in Zingiberales

Attempts have not been reported regarding *in vitro* pollination and fertilization in heliconia. But the work reported in this line in some of the Zingiberales are reviewed below.

In ginger (*Zingiber officinale* Rosc.) formation of viable seeds through *in vitro* pollination and fertilization was first reported by Valsala (1994). The various methods of *in vitro* pollination tried in ginger, seed set was obtained in placental pollination, modified placental pollination and ovules / test - tube fertilization. Among these successful methods *in vitro* placental pollination is the best with maximum number of seed set (Valsala, 1994; Valsala *et al.*, 1996).

Seed set through *in vitro* placental pollination in both selfing and crossing of ginger cvs. were also reported by Bindu (1997) Devi (2005) also assessed seed set and seed development in various crosses in ginger involving auto tetraploid and diploids through *in vitro* placental pollination. The embryo culture studies conducted with these seeds did not give progressive results.

In turmeric (*Curcuma domestica*) also, different *in vitro* pollination techniques have been tried. Seed development was observed in the intra-

ovarian, placental and modified placental pollination technique (Renjith, 1999; Vijayasree, 2001). Among these *in vitro* pollination technique, placental pollination is found to be more suitable (Vijayasree, 2001).

In kacholam (*Kaempferia galanga*) as a result of different *in vitro* pollination techniques, seed development occurred in intra-ovarian, placental and modified placental pollination (Bhurke, 2002). He also reported that among these placental pollination technique is found to be best.

2.9.4 Factors Affecting Seed Set in *In vitro* Pollination

2.9.4.1 Collection of Explant

The physiological state of the pistil at the time of excising or pistil may also influence seed set *in vitro* pollination.

The incidence of seed set is higher when the ovules are excised 1-2 days after anthesis than on the day of anthesis (Kanta *et al.*, 1962; Kanta and Maheshwari, 1963b). Successful *in vitro* pollination was reported in *Antirrhinum majus* L. when pollination was done on the day of anthesis (Usha, 1965). In *Narcissus psuedonarcissus* a significantly higher number of seed per ovary was obtained on placentae excised from flower collected two days after anthesis as compared with those taken on the day of anthesis (Balatkova *et al.*, 1977a).

In the case of maize (*Zea mays*) optimal stage for *in vitro* pollination was 3-4 days after the silking (Gengenbach, 1977). Olson and Cass (1981) reported that in *Papaver nudicule* placentae with attached ovules were dissected out of unpollinated gynoecia 1-4 days after anthesis, dusted with pollen and cultured *in vitro* resulted in germinable seeds. Rafaat *et al.* (1984) reported that in *Gossypium* flower buds taken one day prior to anthesis were found suitable for *in vitro* pollination.

Van Tuyl *et al.* (1991) observed that in interspecific hybrids of *Lilium* most embryos were formed when flowers were pollinated three days prior to anthesis. In the case of ginger (*Zingiber officinale* Rosc.) flower buds on the

day of anthesis were found suitable for *in vitro* pollination (Valsala, 1994; Valsala *et al.*, 1996; Bindu, 1997; Devi, 2005). Seed set was observed in turmeric (Renjith, 1999; Vijayasree, 2001) and kacholam (Bhurke, 2002) through *in vitro* pollination on the day of anthesis.

Castano and Deproft (2000) reported that in the case of *Cichorium intybus* ovules were isolated from flower buds before anthesis for *in vitro* pollination. In Bromeliaceae interspecific and intergeneric pollination were performed well when pollen and stigma were at anthesis stage (Partone *et al.*, 2001). Zenkteler and Rozak (2003) reported that flower buds collected shortly before anthesis were suitable for *in vitro* pollination. For successful for *in vitro* pollination in *Melandrium album* pistils were isolated from flower buds 24 or 48 h before opening (Zenkteler *et al.*, 2005).

2.9.4.2 Nature of Explant

The most critical step in *in vitro* pollination technique is the development of viable seeds from ovules. For this the selection of suitable explant is highly important.

When stigmas is used as explant wetting the surface of the ovule and stigma (in stigmatic pollination) should be avoided because it lead to poor pollen germination and tube growth resulting poor seed set (Balatkova and Tupy, 1968; Zenkteler, 1980).

In *Petunia axillaris in vitro* pollinated excised ovules or a group of ovules attached to a piece of placenta did not form viable seed because the pollen tubes failed to enter the ovules. However when intact placenta with undisturbed ovules were pollinated, normal pollen germination leading to development of viable seeds occurred (Rangaswamy and Shivanna, 1971b).

Wagner and Hess (1973) reported that in *Petunia hybrida* complete removal of the style had a deleterious effect on seed set following placental pollination. They cultured the entire pistil and exposed the ovules by simply peeling the ovary wall.

In maize the ovaries attached to cob tissue give better result than single ovaries (Sladky and Havel, 1976; Gengenbach, 1977; Dhaliwal and King, 1978). In *Brassica* species removal of entire ovary during *in vitro* pollination was highly detrimental to seed set whereas removal of only a part of the ovary gave the best results (Zenkteler *et al.*, 1987). Leduc *et al.* (1992) reported that in *Trifolium repens* besides the excision of calyx and corolla tubes, excessive injury to the ovary wall was detrimental to *in vitro* development of seed set.

Vervaeke *et al* (2003) reported that in Bromeliaceae very low fertilization occurred in placental pollination technique because pollen tubes are not guided to the ovules. When style was present the pollen tube growth was guided through this and fertilization per cent increased.

In the case of *Melandrium album* pollinated placenta along with a part of calyx cultured resulted in *in vitro* seed development (Zenkteler *et al.*, 2005).

2.9.4.3 Surface Sterilization

A principle requirement for *in vitro* pollination is the maintenance of reasonable sterility during pollen and ovule collection. Many scientists have tried various surface sterilization techniques without affecting pollen viability and receptivity of the ovules.

The ovaries of *Argemone mexicana, Eschcholzia californica, Nicotiana rustica* and *Nicotiana tabacum* surface were sterilized in fresh chlorine water and washed in sterile water (Kanta and Maheshwari, 1963b). Rangswamy and Shivanna (1969) also reported successful test tube fertilization in *Discranostigma fragchetianum* after surface sterilization using chlorine water.

For *in vitro* pollination in *Antirrhinum majus* pistils with the calyx intact were surface sterilized by dipping them in 70 per cent alcohol and later in strong chlorine water (Usha, 1965). In *Lilium* this was achieved by

treating the flower buds in ethanol 70 per cent for 1 min followed by a commercial bleach (2 per cent) treatment for 15 min (Van Tuyl *et al.*, 1991).

In ginger unopened flower buds were soaked in streptocyclin 500 mg l^{-1} for 1 h followed by wiping with 70 per cent alcohol and rinsing with mercuric chloride 0.1 per cent for 3 min. The procedure gave satisfactory microbial sterilization of flower buds for *in vitro* pollination (Valsala, 1994; Valsala *et al.*, 1996).

Zenkteler and Rozak (2003) used flower buds of angiosperm shortly before anthesis surface sterilized with chlorine water and sterile water.

In *Melandrium album* the stigma were cut off and the ovaries along with part of the calyx and part of pedicel were disinfected in 70 per cent ethanol for 45 sec and in one per cent chlorine water for 8-10 min and rinsed three times in sterile water (Zenkteler *et al.*, 2005).

In the case of *Helianthus annuus* before surface sterilization, the sepals and the corolla tube with style and sterilized in diluted commercial bleach solution for 10 min and washed three times with sterile water (Popielarska, 2005).

2.9.4.4 Culture Medium

The efficiency of *in vitro* pollination technique depends on the composition of the medium which can support two process *viz.*, germination of pollen grains and pollen tube growth leading to fertilization and development of the fertilized ovules into mature seeds with viable embryo.

During *in vitro* pollination pollen grains are sprinkled on the ovules where they germinate and pollen tubes enter into the ovules. Whereas pollen grains fail to germinate on the surface of the ovules they may be separately grown on a suitable medium and applied to the ovules (Balaktova and Tupy, 1968). Application of pollen grains along with suitable medium also reported in ginger (Valsala, 1994; Valsala *et al.*, 1996; Bindu, 1997; Devi, 2005), turmeric (Renjith, 1999; Vijayasree, 2001) and kacholam, (Bhurke, 2002).

The Nitsch medium identified for ovary development was most widely used medium during the initial years of *in vitro* pollination (Kanta and Maheshwari, 1963a; Usha, 1965; Rangaswamy and Shivanna, 1967; Olsen and Cass, 1981; Jarzina and Zenkteler, 1983).

Rangaswamy and Shivanna (1969) reported that agar nutrient medium containing Knop's mineral salts, vitamins, glycine and sucrose supported successful test-tube fertilization in *Dicranostigma fangchetianum.*

Sladky and Havel (1976), who tested different basal media *i.e.*, White (1943), Murashige and Skoog (1962) and Nitsch (1951), did not find a significant difference in the response of the *in vitro* pollinated ovaries.

MS medium was ideal for the *in vitro* pollination of *Gossypium* (Refaat *et al.*, 1984), *Nicotiana* (Zhou *et al.*, 1991), Lilium (Van Tuyl *et al.*, 1991; Chi, 2000) and *Helianthus annuus* (Popielarska, 2005). Zenkteler *et al.* (2005) also reported that MS medium was ideal for the culture of *in vitro* pollinated ovules of *Melandium album* x *Lychnis coronaria.*

Half strength MS medium was most suitable for *in vitro* pollination and fertilization in ginger (Valsala, 1994; Valsala *et al.*, 1996; Bindu, 1997; Devi, 2005), turmeric (Renjith, 1999; Vijayasree, 2001) and kacholam (Bhurke, 2002).

2.9.4.5 Sucrose Concentration

The sucrose concentration influences ovary development, ovule development and germination of seeds following *in vitro* pollination. Various concentration of sucrose has been tried by many workers.

In most of the cases sucrose has been used at a concentration of 4-5 per cent (Kanta and Maheshwari, 1963a; Usha, 1965; Rangaswamy and Shivanna, 1967 and 1969).

In maize some workers used 15-17 per cent sucrose for *in vitro* pollination (Sladky and Havel, 1976; Gengenbach, 1977). Dhaliwal and King (1978) obtained viable seeds following intra and interspecific

pollination with five per cent sucrose. Bajaj (1979) reported seven per cent sucrose to be optimum for maize.

Van Tuyl *et al.* (1991) reported that for interspecific hybridization of *Lilium* through *in vitro* pollination seven per cent sucrose was found to be optimum.

Fernandez *et al.* (1998) and Chi (2000) successfully cultured *in vitro* pollinated interspecific crosses of *Lilium* ovules in six per cent sucrose medium.

Valsala (1994) reported that 6-8 per cent sucrose was optimum for *in vitro* pollination and fertilization in ginger. According to Devi (2005) three per cent sucrose was ideal for *in vitro* ovule development in ginger.

Three per cent sucrose concentration was also reported to be ideal for *in vitro* ovule development in turmeric (Renjith, 1999; Vijayasree, 2001), kacholam (Bhurke, 2002), sunflower (Popielarska, 2005) and *Melandrium album* (Zenkteler *et al.*, 2005).

2.9.4.6 Effect of Growth Regulators

The information on the effect of various growth regulators added to the basal medium on seed development from cultured ovules is very meager.

Balatkova *et al.* (1977c) studied the effect of IAA and kinetin on seed development following placental pollination in tobacco. They reported that the presence of 10 $\mu g\ l^{-1}$ IAA or 0.1 $\mu g\ l^{-1}$ kinetin significantly improved the number of seeds per ovary and higher level of kinetin (1 $\mu g\ l^{-1}$) were inhibitory. Van Tuyl *et al.* (1991) reported that auxin appears to be essential at the initial stages of ovule development following *in vitro* pollination of *Lilium*.

The use of growth regulators including auxin (IAA, NAA, IBA and 2,4-D), cytokinin (Kinetin, BA and BAP) and GA_3 have been reported by several scientists in ovule development *in vitro* pollination and fertilization

(Waldt, 1976; Valsala, 1994; Valsala *et al.*, 1996; Bindu, 1997; Renjith, 1999; Chi, 2000; Vijayasree, 2001; Bhurke, 2002; Popielarska, 2005; Zenkteler *et al.*, 2005; Devi, 2005).

2.9.4.7 Other Media Supplements

Casein hydrolysate 500 mg l^{-1} was reported to promote ovule development (Kanta and Maheshwari, 1963b; Zubkova and Sladky, 1975). Rangaswamy and Shivanna (1971b) reported that in *Petunia axillaris* did not find any beneficial effect of casein hydrolysate for seed development after placental pollination.

Balatkova *et al.* (1977c) reported that coconut milk, tomato juice and yeast extract were inhibitory to seed development after placental pollination in tobacco. Usha (1965) used 25 per cent coconut milk for the *in vitro* pollination of *Antirrhinum majus.*

Valsala (1994) reported that CW (10 to 12 per cent) and CH (200 to 500 mg l^{-1}) enhanced ovule development in ginger along with cytokinin and auxin.

In turmeric CW 15 per cent and CH 200 mg l^{-1} enhanced ovule development (Renjith, 1999), whereas Vijayasree (2001) reported that CW and CH did not favour ovule development in turmeric. Bhurke (2002) reported that CH, CW, YE and L-glutamine did not find any beneficial effect for ovule development in kacholam.

2.9.4.8 Culture Conditions

There are hardly any data on the precise effect of light on the response of *in vitro* pollinated ovules cultures are usually stored in dark or near darkness.

Rangaswamy and Shivanna (1967) reported the use of diffused day light (10 – 12 for candle light at 25 ± 2°C) for *in vitro* seed development in *Petunia.*

Zenkteler (1969) could not find any difference in the results of *in vitro* pollination whether the cultures were incubated in light or dark.

Rangaswamy and Shivanna (1969) incubated *in vitro* pollinated cultures of *Dicranostigma franchetianum* under 200 lux diffused day light at 22 ± 2°C. Waldt (1976) incubated the ovule at 26°C and eight hours photo period for *in vitro* pollination of barely with rye and bread wheat.

Balaktova *et al.* (1977a) observed that in *Narcissus* incubation at 15°C instead of the usual 25°C resulted in increase in the number of seed developed in each ovary.

Van Tuyl *et al.* (1991) incubated cultures of *Lilium* in light intensity 12 Wm^{-2}, 16 h photo period at 24°C for the production of interspecific hybrids. Zhou *et al.* (1991) kept the cultures of interspecific hybrids of *Nicotiana* in continuous illumination (3000 lux) at 28°C for ovary development and ovule development.

Valsala (1994) and Valsala *et al.* (1996) reported that ovule development was promoted after *in vitro* pollination cultures of ginger were incubated at dark or differed light of 500 and 1000 lux at 26 ± 2°C.

Popielarska (2005) placed incubated *in vitro* pollinated ovules of sunflower in a culture room under a 16 h photoperiod (cool white fluorescent tube 700 – 100 μ mol m^{-2} s^{-1} at 25 ± 3°C and 60 per cent RH.

Zenkteler *et al.* (2005) incubated *in vitro* pollinated placentae of *Melandrium album* at 23 ± 2°C with continuous cool white fluorescent light (55 α mol $m^{-2}s^{-1}$).

2.9.4.9 Genotype

There is some evidence of genotypic variation in the response of *in vitro* pollinated ovaries of maize (Gengenbach, 1977; Bajaj, 1979).

2.9.5 *In vitro* Post Pollination Changes

Kanta *et al.* (1962) first reported test-tube fertilization in *Papaver somniferum*. The pollen grains lodged on the ovules germinated within 15 min and fertilization occurred after 1-2 DAP. Within five days the fertilized ovules enlarged, become turgid and opaque, contained a four celled pro-embryo and free nuclear endosperm. Fully developed dicotyledonous embryo developed 22 DAP. Kanta and Maheshwari (1963a) reported similar success with some other taxa belonging to Papaveraceae (*Argemone mexicana* and *Eschscholfzia california*) and solanaceae (*Nicotiana rusticana* and *Nicotiana tabaccum*).

In *Antirrhinum majus in vitro* pollinated pistils started enlarging in 4-5 DAP. The ovules from the developing ovaries were examined periodically *viz.*, 8, 12, 18 and 22 DAP showed globular, heart shaped, cotyledony and fully formed embryo respectively (Usha, 1965).

Rangaswamy and Shivanna (1969) described that in *Dicranostigma franchetianum* the pollen of *in vitro* pollinated cultures readily germinated and the pollen grew amidst the ovules were seen. Four DAP endosperm nuclei and a zygote *i.e.*, double fertilization *in vitro*. In 15 DAP, mature seeds containing dicotyledonous embryo formed.

Valsala (1994) reported that the *in vitro* produced seeds and fruits of ginger grew rapidly at the initial stage of 20 DAP and latter growth was slow. Ginger produced black arillate seed with endosperm and embryo 80 DAP. According to Devi (2005) *in vitro* pollinated ginger seeds endosperm was soft and jelly like in 10 DAP and it became firm 20 DAP and hardness of endosperm progressed up to 50 DAP. She also reported that embryo was seated at chalazal end and endosperm constituted the major portion of seed.

Zenkteler *et al.* (2005) reported that pollen grains germinated and pollen tubes were found entering micropyle 18 h after pollination in *Melandrium album* x *Lychnis coronaria*. They also observed double

fertilization *in vitro* and mature hybrid embryo at different stages of development up to third week of culture.

In *Helianthus annuus* L. ovules 10 days after *in vitro* pollination developed into a globular embryo and cellular endosperm were observed. Six weeks after inoculating embryos at different stages of development (globular, heart and torpedo shaped) were dissolved and it was observed that globular embryos were most frequent (Popielarska, 2005).

2.9.6 Germination of *In vitro* Produced Seeds

In vitro raised seeds of *Papaver rhoeus* soaked in tap water for 24h germinated in modified white medium (Kanta, 1960). Seeds of *Papaver somniferum* did not germinate when they are attached to placenta. They germinated within 15 days after planting in new medium when separated from placenta.

In the case of *Argemone mexicana* seeds 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 and Maheshwari, 1963).

The seeds of *in vitro* pollinated *Antirrhinum majus* germinated in Nitsch medium with four per cent sucrose (Usha, 1965). *In vitro* produced *Petunia* seeds germinated in water soaked filter paper and in the same nutrient agar medium on which it was developed (Rangaswamy and Shivanna, 1967).

In vitro formed seeds of *Dicranostigma franchetianum* germinated *in situ* as well as in fresh nutrient medium (Rangaswamy and Shivanna, 1969). Maize caryopsis produced *in vitro*, germinated on moist filter paper at 28°C within seven days after incubation.

The *in vitro* produced seed of ginger germinated when 80 days old seeds were incubated initially in the medium of ½ 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 0.1 mg l^{-1} (Valsala, 1994; Valsala *et al.*, 1996).

In *Helianthus annuus in vitro* formed seeds produced seedlings 2-4 weeks after culture. Fifty per cent of seedlings germinated 12-21 days after inoculation (Popielaska, 2005).

2.9.7 Embryo Culture

The first systematic attempt to grow the embryo of angiosperms *in vitro*, under aseptic conditions was by Hanning (1904), who cultured mature embryos of two crucifers, *Corchleria* and *Raphanus*.

A suitable source of carbon energy is generally required for the cultivation of excised mature and immature embryos, sucrose is by far the best form of carbohydrate used for embryo culture (Burghardtova and Tupy, 1980). Sucrose is added to the embryo culture media not only as a source of energy but also to maintain a suitable osmolarity which is extremely important for immature embryos (Liu *et al.*, 1993). Monnier (1978) suggested that hormones should not be added to the embryo culture media because they bring about structural abnormalities.

Though *in vitro* pollination technique would help to induce seed set in interspecific and intergeneric crosses, further development of the seed may be limited due to post-zygotic factors. According to Van Tuyl (1997) an integrated approach of *in vitro* pollination followed by embryo rescue has been applied successfully in many crosses.

Successfully developed interspecific and intervarietal hybrids of *Lilium* sp. through cut-style pollination and culturing resulted embryo *in vitro* (Kim *et al.*, 1991; Okazaki *et al.*, 1992). Okazaki *et al.* (1994) studied the effect of sugars, phytohormones and amino acids in the growth of excised embryos from compatible and incompatible crosses of *Lilium* sp. According to them embryos from crosses were able to grow on culture medium containing 3-6 per cent sucrose. They also reported that cytokinin and high

concentration of auxins in dried abnormal growth of embryo and amino acid did not effect embryo growth.

Kim and Sung (1990) reported that fertilization in *Lilium* sp. was improved by cut style pollination followed by embryo rescue six weeks after pollination. They also reported that MS medium was suitable when supplemented with three sucrose, 0.7 per cent agar and 0.01 mg l^{-1} NAA under 16 h photoperiod.

Cotyledony embryo of *in vitro* pollinated *Melandrinum album* x *Lychnis coronaria* were excised from the ovules 25 DAP and incubated to MS medium with 1 mg l^{-1} IAA thereby hybrid plants grown normally (Zenkteler *et al.*, 2005).

Embryo Rescue in Heliconia

Carlos *et al.* (2005) excised embryo from mature fruits of *Heliconia rostrata* and cultured them in MS medium. They reported that sucrose was essential for embryo development, three per cent of sucrose were beneficial for embryo growth. They also reported that addition of kinetin, isopentenyl adenine and zeatin did not improve embryo growth and development. They suggested that the use of the basal medium with three per cent sucrose was effective for culture of embryos of *Heliconia caribae*, *H. episcopalism*, *H. psittacorum*, *H. chartaceae* and *H. bihai*.

Materials and Methods

3. MATERIALS AND METHODS

The present investigations on the "Standardization of *in vitro* pollination and fertilization technique for heliconia" were carried out in the Department of Pomology and Floriculture, utilizing the facilities available at Plant Tissue Culture Laboratory, Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2005-2006. The details regarding the materials and methods adopted for conducting various aspects of the study are presented here.

The experiment consisted of the following major studies.

1. Pollen studies
2. Morphological studies of gynoecium
3. *In vitro* pollination and fertilization
4. Embryo culture

3.1 PLANT MATERIALS

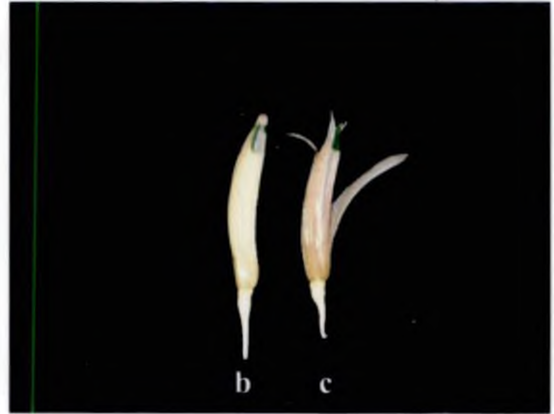
Three cultivars of *Heliconia psittacorum viz.*, Lady Di, Andromeda and Parakeet were selected for the study and planted in the field for sufficient supply of flowers.

3.2 POLLEN STUDIES

3.2.1 Pollen Fertility

The pollen fertility of selected three cultivars were estimated using acetocarmine stain. For the estimation of pollen fertility flower buds were collected at the time of anthesis and pollen grains were stained with 1:1 glycerin – acetocarmine (2 per cent) and viewed under 10 x magnification. Five slides were prepared for each variety and from each slide, ten microscopic fields were scored and the data recorded. All the pollen grains

cv. Lady Di



cv. Andromeda

cv. Parakeet

a - Inflorescence
b - Flower bud
c - Flower

Plate 1. ***Heliconia psittacorum*** **selected for experiment**

that were well filled and stained were counted as fertile and others as sterile. The pollen fertility was calculated as,

$$\text{Pollen fertility} = \frac{\text{Number of well filled and uniformly stained pollen grains}}{\text{Total number of pollen grains}}$$

3.2.2 *In vitro* Pollen Germination

Standardization of medium for pollen germination was attempted. *In vitro* pollen germination studies give a more dependable estimate of pollen fertility. For this fresh pollen grains collected from the flower at the time of anthesis. Fresh pollen grains were incubated in a moisture chamber along with a drop of medium tested for germination. After 24 hours of incubation germination counts were made under low power (10x) of microscope. The observations were made in ten different microscopic fields and the mean percentage of germination was worked out. Mean of pollen tube growth attained was also measured using a calibrated ocular micrometer.

3.2.3 Standardization of Media for Pollen Germination

Influence of two different media *i.e.*, Brewbaker and Kwack medium (1963) and ME_3 medium (Leduc *et al.*, 1990) were used for the standardization. The required quantity of chemicals on distilled water and were sterilized before use. The different treatments tried for these media were as follows.

3.2.3.1 Influence of Brewbaker and Kwack Medium

The medium along with different concentration of sucrose and polyethylene glycol (PEG) were tried (Table 1).

Table 1. Treatments to assess the pollen germination in Brewbaker and Kwack medium

Osmoticum	Concentration (%)
Sucrose	0
	5
	10
	20
	30
	40
PEG	5
	10
	12
	15

3.2.3.2 *Influence of ME_3 Medium (Leduc et al., 1990)*

The medium along with different concentration of PEG were tried (Table 2).

Table 2. Treatments to assess the pollen germination in ME_3 medium

Osmoticum	Concentration (%)
PEG	0
	5
	10
	12
	15

3.2.3.3 *Influence of pH on ME3 Medium*

Effect of different pH level (pH 4, 5, 6, 7 and 8) were also studied. For this ME_3 medium with 12 per cent PEG was used.

3.2.3.4 *Influence of Incubation Period on Pollen Germination*

Pollen grains from the selected cultivars were collected at the time of anthesis. ME3 medium with 12 per cent PEG used for the experiment. The pollen grains dusted on the above medium were incubated in a moist chamber

and observations made at periodic intervals (½, 1, 2, 3, 5 and 24 hours after incubation).

3.3 MORPHOLOGICAL STUDIES OF GYNOECIUM

The following characters of gynoecium of selected three varieties of *Heliconia psittacorum* were studied.

- The length of the style in centimeter
- The length of the ovary in millimeter
- The diameter of the ovary in millimeter
- Number of ovules per ovary
- The length of the ovule in micrometer
- The breadth of the ovule in micrometer

3.4 *IN VITRO* POLLINATION AND FERTILIZATION

3.4.1 Explant

Pollinated ovules/ovaries were used as explants.

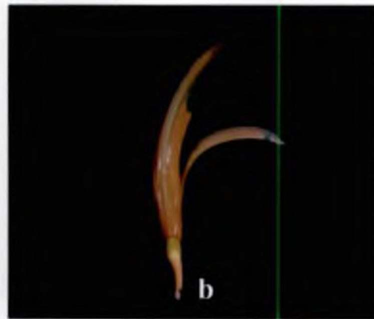
3.4.2 Collection and Preparation of Flower Buds for *In vitro* Pollination

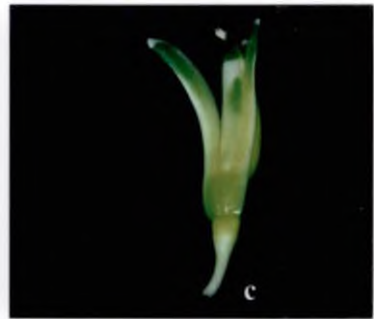
Flower buds were collected prior to anthesis. The flower buds ready to dehisce on next day, were collected with the help of a knife, along with its pedicel in the evening itself. These flower buds were quickly transferred into a beaker containing distilled water. Collected flower buds were immersed in 'Labolene' (1000 times diluted) solution for one minute, washed thoroughly using distilled water.

3.4.3 Standardization of Surface Sterilization Procedure

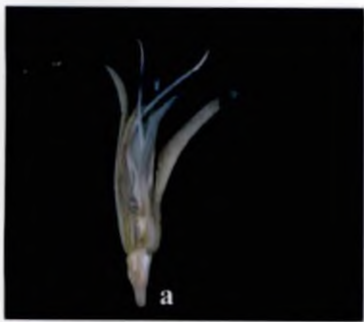
The flower buds collected and prepared as above are subjected to different surface sterilization treatments (Table 3). The surface sterilization treatments were carried out under aseptic conditions in a laminar air flow cabinet.

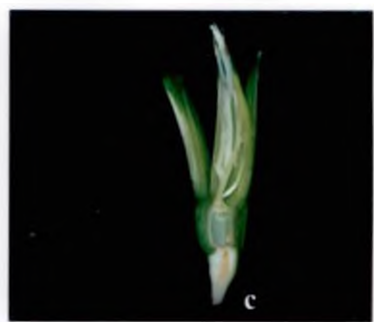
Flowers



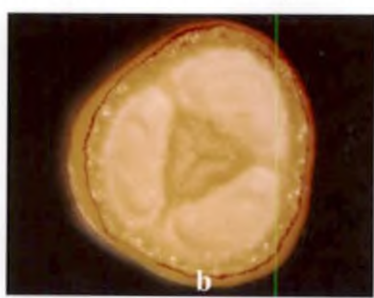


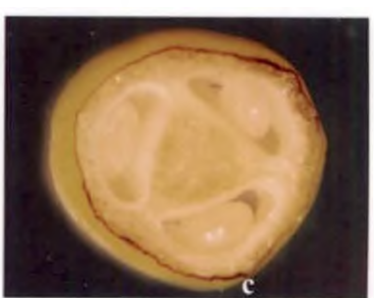
Longitudinal sections of flower



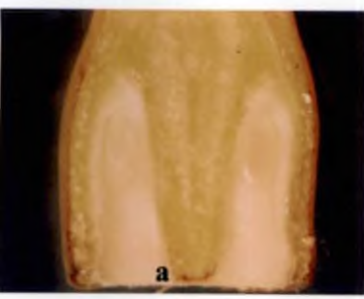


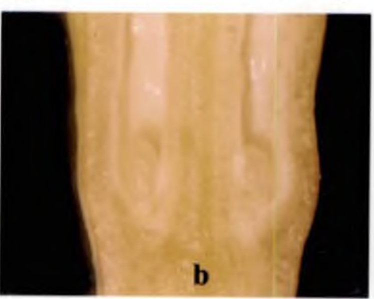
Cross sections of ovary 5x





Longitudinal sections of ovary 5x





a - Lady Di
b - Andromeda
c - Parakeet

Plate 2. Floral morphology of heliconia cultivars

Table 3. Surface sterilization treatments to assess the culture establishment

Basal medium : MS

Sl. No	Sterilant	Concentration	Duration
1	Ethanol	95 %	5 s & 10 s
2	Ethanol	70 %	25 s & 30 s
3	Mercuric chloride	0.1 %	3, 5 & 7 min
4	Mercuric chloride	0.08 %	3, 5 & 7 min
5	Mercuric chloride	0.06 %	3, 5 & 7 min
6	Ethanol + Mercuric chloride	75 % + 0.1 %	25 s + 3 min

3.4.4 Management of Bacterial Interference in Cultures

For controlling the bacterial interference *in vitro* culture, two experiments were tried.

3.4.4.1 Using Antibiotic

Treating the explants using antibiotic for controlling the bacterial interference. The treatment details are given in the Table 4.

Table 4. Treatment for the management of bacterial interference in cultures

Basal Medium : MS

Sterilant	Concentration	Duration
Mercuric chloride	0.1 %	7 min
		8 min
		10 min
		12 min
Streptomycin + mercuric chloride	250 mg l^{-1} + 0.1 %	1 hr + 3 min
	500 mg l^{-1} + 0.1%	1 hr + 3 min
	750 mg l^{-1} + 0.1%	1 hr + 3 min
	1000 mg l^{-1} + 0.1%	1 hr + 3 min

3.4.4.2 Addition of Chemical in the Culture Media

Addition of different strength of copper sulphate (10, 15 and 25 mg l^{-1}) into the medium was tried for controlling bacterial interference in the culture. Copper sulphate added along with other chemical during media preparation. Sterilized ovaries (Mercuric chloride 0.1 per cent for 3 min) were inoculated into these culture media.

3.4.5 Basal Media for Culture Establishment

The basal media used for the study were MS (full & half) (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972) and Nitsch (Nitsch, 1951). The basal media alone and supplemented with hormones (BA 2.5 mg l^{-1} and NAA 0.5 mg l^{-1}) served as treatment. The whole ovaries were cultured, survival and swelling of the ovary was scored 20 days after pollination.

3.4.6 Media Preparation

The chemicals used for the preparation of the culture media were of analytical grade obtained from British Drug House (Bombay), Sisco Research Laboratory (Bombay) and Merck (Bombay). Standard procedures were followed for the preparation of media (Thorpe, 1980). Stock solution for major and minor nutrients and plant growth substances were prepared in double glass distilled water, were stored under refrigerator (+4°C). Sucrose and myo-inositol were added fresh and dissolved. Coconut water when used was collected from freshly harvested tender coconut (eight month old). The pH of the medium was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl with the aid of an electronic pH meter (Global Electronic model DPH 500). Semi solid media were prepared by adding good quality agar (0.6 %). In case of liquid media, strips of No. 1 Whatsman filter paper were used to support the explant. Sterilization of the media was done by autoclaved at 121°C temperature and 1.06 kg cm^{-2} preserve for 20 minutes. After sterilization, the culture vessels were transferred to the culture room.

3.4.7 Culture Concentrations

Cultures were established at light or darkness at 26 ± 2°C.

3.4.8 Standardization of *In vitro* Pollination Methods

In vitro pollination techniques compiled by Bhojwani and Razdan (1983) were adopted. Flower buds were collected one day before anthesis, surface sterilized and allowed to dehisce naturally. After anthesis the gynoecium was prepared depending upon the pollination technique followed. The pollen grains were scoped out with a needle and deposited on the specific part of the gynoecium along with a drop of pollen germination medium. The pollinated ovary/ovule were cultured in the medium (MS + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}) and observed swelling of ovary/ovule 20 days after pollination. Self as well as cross pollination of selected three varieties were tried. Self pollination was done in Lady Di only. In cross pollination Lady Di was used as female parent and Andromeda and Parakeet as male parent. In the case of cross pollination flower buds were emasculated before anthesis. The following pollination techniques were tried.

3.4.8.1 Stigmatic Pollination

The surface sterilized flower buds were allowed to dehisce naturally and the floral appendages like tepals and anthers were then removed. On the stigma of these flowers, pollen grains were deposited and implanted in the culture medium.

3.4.8.2 Stylar Pollination

The flowers were prepared as stigmatic pollination. The style of these prepared flowers were cut at different levels and pollen grains applied on the style along with the pollen germination medium.

3.4.8.3 Ovarian Pollination

In this pollination technique, pollen grains along with pollen germination medium, deposited over the excised ovary.

3.4.8.4 Intra-Ovarian Pollination

In this, the style and corolla tube were removed just above the ovary. Pollen grain along with pollen germination medium either placed on the cut surface of the ovary or transferred into the ovary through a hole in ovary wall.

3.4.8.5 Placental Pollination

In placental pollination, direct pollination of excised ovules together with placenta was done.

3.4.8.6 Modified Placental Pollination

In modified placental pollination, along with ovule bearing placenta, a portion of ovary wall was also dissected out.

3.4.8.7 Ovular Pollination / Test Tube Fertilization

In this pollination technique, pollen grains are applied on excised ovules. The separated ovules were put in a cavity slide containing pollen suspension medium. After 24 hours this was transferred to cultured medium.

3.4.9 Standardization of Age of Flower Buds for *In vitro* Pollination

Flower buds of different age (Table 5) were inoculated in the MS medium supplemented with BA 2.5 mg l^{-1} and NAA 0.5 mg l^{-1}. The modified placental pollination technique was followed. The cultures were observed for swelling of ovules after 20 DAP.

Table 5. Treatments to assess the age of flower buds for *in vitro* pollination

Treatment	
T_1	1 day prior to anthesis
T_2	2 days prior to anthesis
T_3	On the day of anthesis
T_4	1 day after anthesis
T_5	2 days after anthesis

3.4.10 Standardization of Media Supplements for Enhancing Ovule Development

For the standardization of media supplements, modified placental pollination was followed. Various supplements tried as follows.

3.4.10.1 Concentration of Carbon Source

The effect of varying strength of sucrose *i.e.*, 3, 6, and 9 per cent studied. The medium of MS + BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1} was used for supplementing various concentration of sucrose.

3.4.10.2 Plant Growth Substances

The effect of plant growth substances like cytokinin (BA) and auxin (IAA and NAA), alone as well as combinations were tried. Influence of GA were also studied. The treatment details are given below.

3.4.10.2.1 Influence of Auxin

The treatment details are given in Table 6.

Table 6. Treatments to asses the influence of auxin concentrations on ovule development

Basal medium	Treatment	
	Auxin	Concentration (mg l^{-1})
MS	IAA	0.1, 1.0, 2 .0 and 5.0
MS	NAA	0.1, 1.0, 2.0 and 5.0

3.4.10.2.2 Influence of Cytokinin

Basal media MS supplemented with BA 0.1, 1.0, 2.0, 2.5 and 5.0 mgl^{-1} were tried.

3.4.10.1.3 Influence of Cytokinin and Auxin Combinations

Treatment combinations are given in the Table 7.

Table 7. Treatments to assess the combination of cytokinin and auxin concentrations on ovule development

Basal medium : MS

Treatment (mg l^{-1})	
T_1	BA 0.1 + NAA 0.1
T_2	BA 0.1 + NAA 1.0
T_3	BA 0.1 + NAA 2.0
T_4	BA 1.0 + NAA 0.1
T_5	BA 1.0 + NAA 1.0
T_6	BA 1.0 + NAA 2.0
T_7	BA 2.0 + NAA 0.1
T_8	BA 2.0 + NAA 1.0
T_9	BA 2.0 + NAA 2.0
T_{10}	BA 2.5 + NAA 0.1
T_{11}	BA 2.5 + NAA 1.0
T_{12}	BA 2.5 + NAA 2.0
T_{13}	BA 5.0 + NAA 0.1
T_{14}	BA 5.0 + NAA 1.0
T_{15}	BA 5.0 + NAA 2.0
T_{16}	BA 1.0 + IAA 0.1
T_{17}	BA 1.0 + IAA 1.0
T_{18}	BA 1.0 + IAA 2.0
T_{19}	BA 2.5 + IAA 0.1
T_{20}	BA 2.5 + IAA 1.0
T_{21}	BA 2.5 + IAA 2.0
T_{22}	BA 5.0 + IAA 0.1
T_{23}	BA 5.0 + IAA 1.0
T_{24}	BA 5.0 + IAA 2.0

3.4.10.2.4 Influence of GA

The effect of GA at 0.1, 1.0, 2.0 and 10.0 mg l^{-1} was studied in basal MS medium.

3.4.10.3 Influence of Organic Supplements

The influence of organic supplements like CW, CH and YE were studied. The treatments are given in Table 8.

Table 8. Treatments to asses the effect of organic supplements on ovule development

Basal medium : MS + BA 2.5 mg l^{-1} + NAA 2.0 mg l^{-1}

Supplement	Concentration
CW	5, 10, 15 and 20% v/v
CH	250, 500 and 1000 mg l^{-1}
YE	250, 500 and 1000 mg l^{-1}

3.4.11 Standardization of Physical Conditions for Ovule Development

The cultures were kept in light as well as in dark in order to assess the effect of light. Light (3000 lux, 16 hours photoperiod) was provided using cool white fluorescent tubes. Darkness was provided by keeping the cultures in controlled dark room. Different temperature ranges (26 ± 2°C, 30 ± 2°C) were tested. Physical forms of the media *i.e.*, liquid and solid (0.6 per cent agar) were also studied.

3.4.12 Post Pollination Changes

For assessing the post pollination changes, detailed observations were made for the ovule development. The size of the ovule was measured using ocular micrometer. Seed set was also examined.

3.4.13 Histological Examination of Pollinated Ovules

The longitudinal sections of pollinated ovules were observed under stereomicroscope. For this study, the pollinated ovules were dissected at various intervals (20, 40, 80 and 90 DAP) to understand the embryo and endosperm development of the fertilized ovules.

3.4.14 Germination of Seeds

The seeds of 60 days after pollination were incubated in culture media. The treatment details are given in Table 9.

Table 9. Treatments for the germination of heliconia seed

Treatment	Concentration of growth regulator (mg l^{-1})
Kept in moist filter paper	-
Sand + ½ MS spray	-
MS	-
MS + GA	0.1
	1.0
	2.0
	10.
MS + BA	1.0
	2.0

3.4.15 Embryo culture

3.4.15.1 Excision of Embryo

Under aseptic condition, *in vitro* formed seeds of heliconia had been removed from placenta. Pericarp was removed and the embryo had been excised by dissecting the seed, with the aid of pointed needle and knife. The excised embryo immediately inoculated in the culture media.

3.4.15.2 Age of Embryo

Seeds of different age 20, 40, 60, and 80 DAP were dissected out and implanted in the culture medium.

3.4.15.3 Concentration Sucrose in the Media

The effect of three levels of sucrose were tried *i.e.*, 3, 6 and 9 per cent.

3.4.15.4 Cultural Condition

The cultures were incubated at 26 ± 2°C under photoperiod (3000 lux) for 12 hours and also in darkness.

3.4.16 Statistical Analysis

The data generated from various experiments were subjected to statistical analysis in completely randomized design wherever possible as prescribed by Panse and Sukhatme (1985).

Results

4. RESULTS

The results of the investigation carried out for standardization of *in vitro* pollination and fertilization technique for heliconia are presented in this chapter as follows:

- Pollen studies
- Morphological studies of gynoecium
- *In vitro* pollination and fertilization
- Embryo culture

4.1 POLLEN STUDIES

4.1.1 Pollen Fertility of Heliconia Cultivars

Pollen fertility of selected three *Heliconia psittacorrum* cvs were determined using acetocarmine stain (Plate 3). The results obtained are presented in Table 10.

Among the cultivars evaluated, the highest pollen fertility was observed in cultivar Lady Di (93.04 %). This was followed by Andromeda (83.61 %) which as statistically on par with Parakeet (83.92 %).

4.1.2 Standardization of Media for Pollen Germination

4.1.2.1 Influence of Brewbaker and Kwack medium

The pollen grains were incubated in Brewbaker and Kwack medium along with different concentration of sucrose and PEG as osmoticum. Pollen germination percentage was observed after 24h of incubation. No pollen germination was recorded in the medium without osmoticum and when sucrose was the osmoticum. Pollen grains germinated in Brewbaker and Kwack medium with PEG as osmoticum (Plate 4a).

Table 10. Pollen fertility of different heliconia cultivars

Sl. No	Cultivar	Fertility (%)
1	Lady Di	93.04 (9.65)
2	Andromeda	83.61 (9.16)
3	Parakeet	83.92 (9.14)
	CD	0.211
	F	17.36**

Value in parenthesis $\sqrt{x}$ transformed

**Significant at 1 per cent level

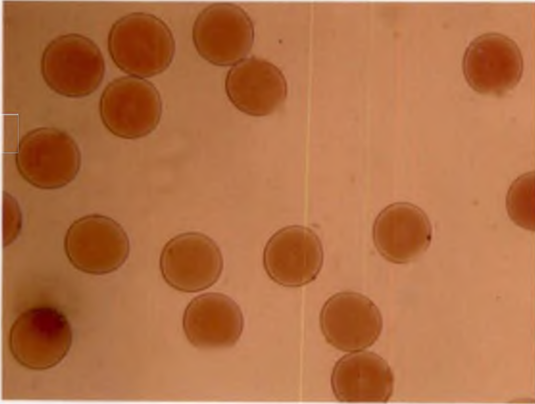
Table 11. Effect of PEG in Brewbaker and Kwack medium on *in vitro* pollen germination percentage of heliconia cultivars

Concentration of PEG (%)	Pollen germination (%)			Mean value of treatment
	Lady Di	Andromeda	Parakeet	
5	8.10 (2.85)	7.99 (2.83)	7.70 (2.77)	7.95 (2.82)
10	14.33 (3.79)	15.44 (3.93)	14.74 (3.84)	14.82 (3.85)
12	23.87 (4.89)	21.67 (4.66)	21.63 (4.65)	22.37 (4.73)
15	12.78 (3.57)	12.19 (3.49)	9.78 (3.13)	11.56 (3.40)
Mean value of cultivar	14.21 (3.77)	13.91 (3.73)	12.96 (3.60)	

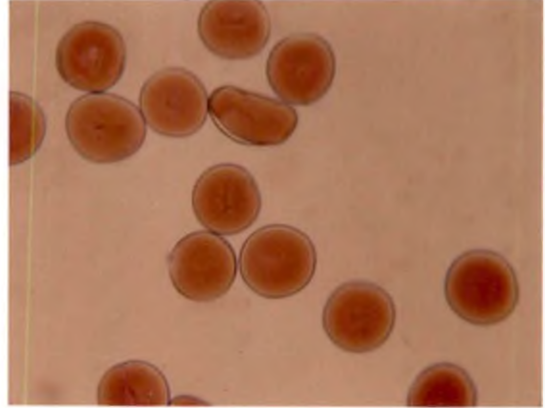
CD for concentration of PEG 0.155 F 220.38**
CD for cultivars 0.134 F 3.67**
CD for interaction 0.268
Value in parenthesis $\sqrt{x}$ transformed

**Significant at 1 per cent level

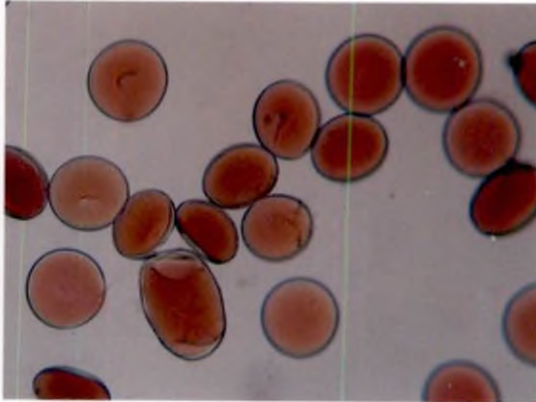
Lady Di 10x



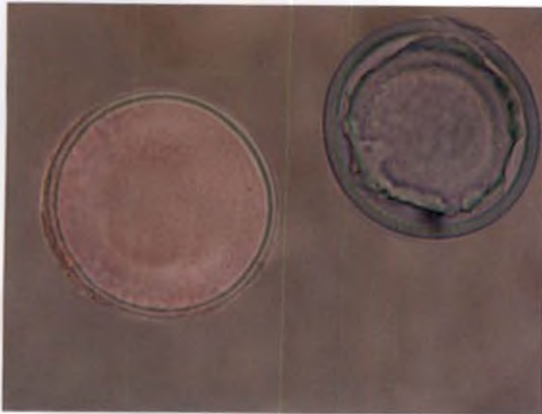
Andromeda 10x



Parakeet 10x



Unfertile pollens 40x



Fertile pollen 40x

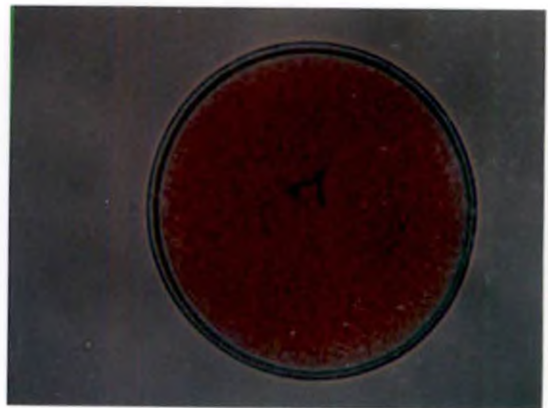
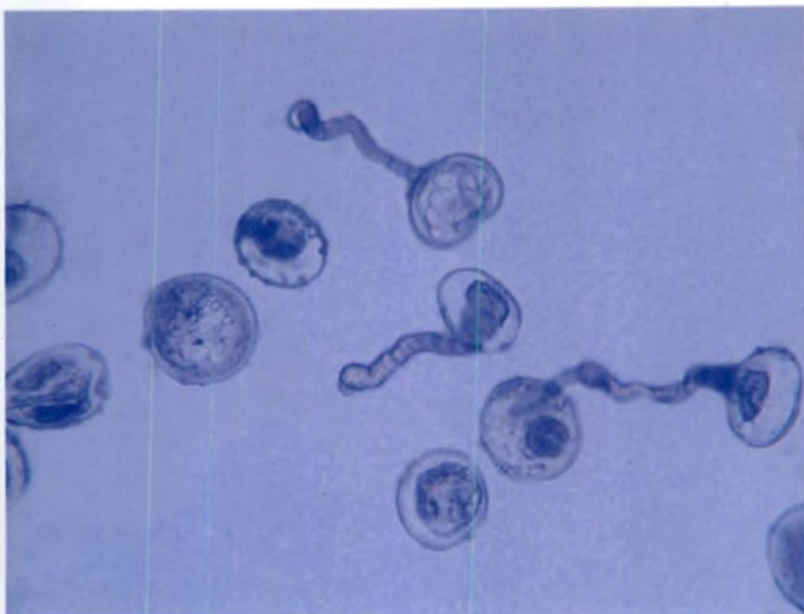
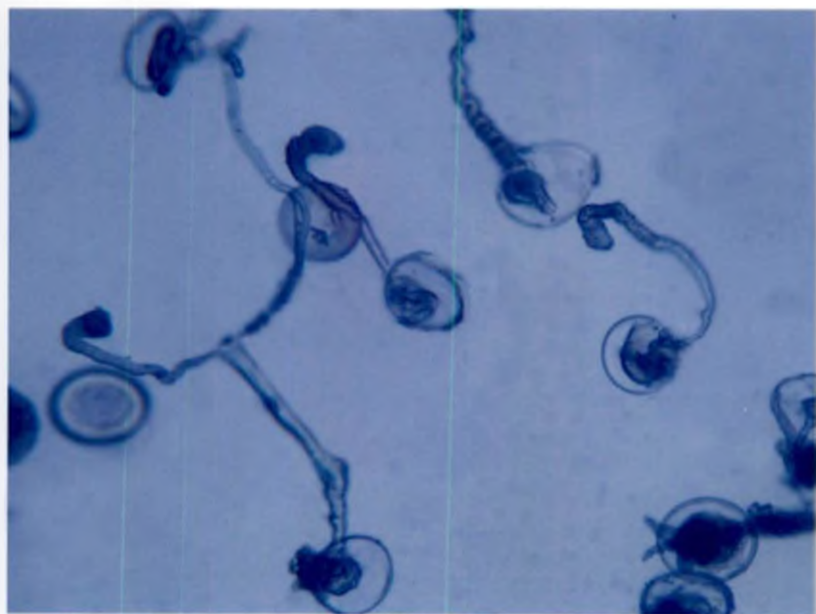


Plate 3. Pollen fertility of heliconia cultivars



a. Brewbaker and Kwack medium 10x



b. ME_3 medium 10x

Plate 4. ***In vitro*** **pollen germination**

Four treatments were tried to assess the effect of PEG in Brewbaker and Kwack medium on *in vitro* pollen germination per cent in heliconia cultivars (Table 11).

With respect to the concentration of PEG significant difference was noticed among the treatments. The higher percentage of pollen germination was observed in medium with 12 per cent PEG (22.37%). This was followed by treatments 10 per cent PEG and 15 per cent PEG. Lower pollen germination was recorded in 5 per cent PEG with mean value of 7.95 per cent. The cultivar Lady Di recorded 14.21 per cent mean germination per cent, Andromeda 13.91 per cent and in Parakeet 12.96 per cent. Interaction between concentration of PEG and cultivars was not significant with reference to pollen germination per cent.

The results of the investigation showed that an increase in concentration of PEG to 12 per cent resulted in increase in percentage of pollen germination. A further increase in PEG level to 15 per cent was not favourable for pollen germination.

4.1.2.2 Influence of ME_3 Medium on Pollen Germination in Heliconia Cultivars

The pollen grains were incubated in ME_3 medium along with four different concentration of PEG (Table 12).

The results of the study showed that an increase in concentration of PEG to 12 per cent resulted in increase in percentage of pollen germination (Plate 4b). There was no further increase in percentage of pollen germination with the enhancement of PEG level up to 15 per cent.

Significant difference was noticed among the different concentration of PEG with respect to pollen germination in ME_3 medium. ME_3 medium with 12 per cent PEG was superior to other treatments with mean germination of 61.31 per cent. Lowest germination (20.43%) recorded in medium with 5 per cent PEG. Significant difference was noticed between the

Table 12. Effect of PEG in ME_3 medium on *in vitro* pollen germination in heliconia cultivars

Conc. Of PEG (%)	Pollen germination (%)			Mean value of treatment
	Lady Di	Andromeda	Parakeet	
5	20.62 (4.54)	20.78 (4.56)	19.86 (4.46)	20.43 (4.52)
10	37.85 (6.11)	32.77 (5.72)	34.65 (5.89)	34.93 (5.91)
12	65.51 (8.09)	59.07 (7.69)	59.49 (7.71)	61.31 (7.83)
15	27.31 (5.23)	24.35 (4.94)	21.79 (4.67)	24.40 (4.94)
Mean value of cultivar	35.88 (5.99)	32.83 (5.73)	32.26 (5.68)	

CD for treatments 0.134 F 983.76**
CD for cultivars 0.116 F 17.23**
CD for interaction 0.232 F 2.85**
Value in parenthesis $\sqrt{x}$ transformed

**Significant at 1 per cent level

Table 13. Effect of incubation period on *in vitro* pollen germination in heliconia cultivars

Time (h)	Pollen germination (%)			Mean value of treatment
	Lady Di	Andromeda	Parakeet	
0.5	30.40 (33.44)	30.47 (33.49)	30.89 (33.75)	30.6 (33.56)
1	48.56 (44.16)	45.80 (42.58)	48.44 (44.09)	47.67 (43.61)
2	56.05 (48.46)	53.51 (46.99)	55.17 (47.95)	54.87 (47.80)
3	63.22 (52.64)	58.43 (49.83)	56.53 (48.73)	59.36 (50.40)
5	63.72 (52.94)	58.55 (49.91)	56.71 (48.84)	59.64 (50.56)
24	63.75 (52.96)	58.57 (49.91)	56.83 (48.91)	59.69 (50.59)
Mean value of cultivar	54.23 (47.43)	50.78 (45.45)	50.66 (45.38)	

CD for time 1.351
CD for cultivar 0.955
CD for interaction 2.34
Value in parenthesis $Sin^{-1}\sqrt{x}$ transformed.

Table 14. Effect of p^H in ME3 medium on pollen germination in heliconia cultivars

pH	Germination (%)			Mean value of treatment
	Lady Di	Andromeda	Parakeet	
4	62.17	57.37	53.30	57.62
5	62.15	56.94	54.84	57.97
6	62.11	56.28	55.03	57.81
7	62.28	56.40	55.00	57.89
8	61.69	56.63	53.65	57.32
Mean value of cultivar	62.08	56.72	54.37	

CD for treatment (pH) 1.205
CD for cultivars 0.933 F 145.66**
CD for interaction 0.732

**Significant at 1 per cent level

Table 15. Pollen tube length of different heliconia cultivars

Media	Pollen tube length (μm)			Mean value of treatment
	Lady Di	Andromeda	Parakeet	
Brewbaker and Kwack's medium with 12% PEG	201	217.5	210.83	209.78
ME_3 medium with 12% PEG	454.5	450	457.5	454.00
Mean value of cultivars	327.75	333.75	334.167	

CD for treatment 36.371
CD for cultivars 44.545

CD for interaction 21.815

cultivars with respect to pollen germination per cent in different media tried. Maximum mean germination was recorded in Lady Di (35.88%) followed by Andromeda (32.83%) and Parakeet (32.26%).

ME_3 medium with 12 and 15 per cent showed significant difference in interaction between concentration of PEG and cultivars with respect to percentage of pollen germination while five per cent PEG was on par with 10 per cent PEG. On comparing the 12 per cent PEG among different varieties. Lady Di showed significant difference while the Andromeda and Parakeet were on par with each other. However in 15 per cent PEG, three varieties showed significant difference with respect to pollen germination.

4.1.2.3 Influence of pH in ME_3 Medium on in vitro Pollen Germination

The pollen grains were incubated in ME_3 medium with 12 per cent PEG at five different levels of p^H. Pollen germination per cent were recorded after 24h of incubation (Table 14).

Among the different pH level tried in ME_3 medium, significant difference was not noticed with respect to percentage of pollen germination. However significant difference was noticed among the cultivars. In cultivars Lady Di recorded highest germination percentage (62.08 %) followed by Andromeda 56.72 per cent and in Parakeet 54.37 per cent. The influence of P^H level on cultivars, significant difference was observed with regard to the pollen germination.

In all treatment Lady Di showed higher percentage of germination followed by Andromeda and Parakeet. The results of the study showed that the change in P^H of ME_3 medium from 4 to 8 did not interference the pollen germination per cent.

4.1.2.4 Influence of Incubation Period on in vitro Pollen Germination

Pollen grains of selected cultivars were incubated in ME_3 medium with 12 per cent PEG at pH 6. Observations were made at periodic intervals (Table 13).

Pollen germination started within $^{1}/_{2}$ h after incubation with a mean value of 30.6 per cent. There was significant increase in the pollen germination percentage with respect to incubation period till 3 h. After 3 h there was no significant difference in pollen germination per cent (Fig.2). The cultivar Lady Di recorded significantly higher pollen germination percentage compared to Andromeda and Parakeet. On comparing incubation time with different cultivars there was no significant difference upto 2 h of incubation in pollen germination percentage. However after 2 h of incubation significant difference was shown by the cultivars with regard to pollen germination.

4.1.2.5 Pollen Tube Length of Heliconia Cultivars

The pollen grains were incubated in two different pollen germination media, after 24 h of incubation the pollen tube length of the different cultivars were recorded and presented in Table 15. With respect to the pollen tube length significant difference was noticed between the two media tried (Fig.4). ME_3 medium recorded mean pollen tube length of 454 μm while Brewbaker and Kwack medium recorded 209.78μm. There was no significant difference among the cultivars with respect to the pollen tube length. The results of the study showed ME_3 medium with 12 per cent PEG to be suitable for pollen germination and pollen tube growth in heliconia cultivars.

4.2 MORPHOLOGICAL CHARACTER OF GYNOECIUM

4.2.1 Style Length

Among the three selected cultivars Parakeet recorded maximum style length of 4.82cm, which was on par with Andromeda (4.7cm). Lady Di recorded lowest mean style length of 4.25cm (Table 16).

4.2.2 Ovary Length

Significant difference was not noticed among the cultivars with respect to ovary length (Table 16). Parakeet recorded maximum mean ovary length of 4.58mm while Lady Di the lowest (4.25mm).

Table 16. Morphological characters of gynoecium in different heliconia cultivar

Particulars	Lady Di	Andromeda	Parakeet	CD
Style length (cm)	4.23	4.7	4.82	1.169
Ovary length (mm)	4.25	4.5	4.58	0.459
Ovary diameter (mm)	3.08	3.58	3.5	0.440
Number of ovules / ovary	3	3	3	-
Length of ovule (µm)	1251	1287	1282.5	159.221
Breadth of ovule (µm)	877.5	931.5	1108.5	152.252

Table 17. Effect of various surface sterilants on culture establishment of heliconia cultivars

Treatment	Duration	Survival 20 DAP (%)*
Ethanol 70%	25 s	-
	30 s	-
Ethanol 95%	5 s	-
	10 s	-
$HgCl_2$ 0.06%	3 min	-
	5 min	-
	7 min	-
$HgCl_2$ 0.08%	3 min	-
	5 min	-
	7 min	-
$HgCl_2$ 0.1%	3 min	28.72
	5 min	28.58
	7 min	28.79
Ethanol 70% + $HgCl_2$0. 1%	25 s + 3 min	28.03
Ethanol 95% + $HgCl_2$ 0.1%	10 s + 3 min	28.04

*Mean of 15 observations

4.2.3 Ovary Diameter

The cultivars showed no significant difference in ovary diameter (Table 16). The cultivar Andromeda recorded highest ovary diameter with mean value of 3.58 mm while Lady Di the lowest (3.08 mm).

4.2.4 Number of Ovules/Ovary

There was no difference in number of ovules/ovary in the selected cultivars. All cultivars recorded same value *i.e.*, 3 ovules/ovary (Table 16).

4.2.5 Length of Ovules (μm)

There was no significant difference in length of ovule. The cultivar Parakeet recorded highest length of ovule with mean value of 1282.5 μm while Lady Di the lowest 1251μm (Table 16).

4.2.6 Breadth of Ovule (μm)

There was no significant difference between the cultivars Lady Di and Andromeda whereas cultivar Parakeet showed significant difference from other two varieties (Table 16). The cultivar Parakeet recorded highest mean value for breadth of ovule (1108.5 μm).

4.3 *IN VITRO* POLLINATION AND FERTILIZATION

4.3.1 Standardization of Surface Sterilization Techniques

Flower buds collected one day before anthesis were subjected to various surface sterilization treatments. Among the various surface sterilants tried culture establishment was obtained in mercuric chloride 0.1 per cent and combination treatments with ethanol (70 & 95%) and mercuric chloride 0.1 per cent (Table 17). Treating the flower buds either in mercuric chloride 0.1 per cent or combination treatment with ethanol was ineffective in controlling the bacterial contamination. Hence bacteria contaminated most of the cultures.

172631

Table 18. Effect of management of bacterial interference on culture establishment of heliconia cultivars

Treatments	Duration	Survival 20 DAP (%)
$HgCl_2$ 0.1%	8 min	26.13 (5.11)
	10 min	26.31 (5.13)
	12 min	26.99 (5.20)
Streptomycin 250 mgl^{-1} + $HgCl_2$ 0.1%	1h + 3 min	27.01 (5.20)
Streptomycin 500 mgl^{-1} + $HgCl_2$ 0.1%	1h + 3 min	32.54 (5.70)
Streptomycin 750 mgl^{-1} + $HgCl_2$ 0.1%	1h + 3 min	37.48 (6.12)
Streptomycin 1000 mgl^{-1} + $HgCl_2$ 0.1%	1h + 3 min	41.71 (6.46)

CD Value 0.164

Value in parenthesis $\sqrt{x}$ transformed

Table 19. Effect of addition of $CuSO_4$ on culture medium for controlling the bacterial interference in culture establishment of heliconia cultivars

Concentration of $CuSO_4$ (mgl^{-1})	Survival 20 DAP (%)*
10	50.00
15	66.51
25	100

*Mean of 15 observations

Table 20. Effect of basal media on ovule development of heliconia after *in vitro* pollination

Treatment	Ovary swelling	Culture showing ovary development 20 DAP (%)
MS + BA 2.5 mgl^{-1} + NAA 0.5 mgl^{-1}	+++	85.40 (67.51)
½ MS + BA 2.5 mgl^{-1} + NAA 0.5 mgl^{-1}	++	53.12 (46.77)
SH + BA 2.5 mgl^{-1} + NAA 0.5 mgl^{-1}	++	19.53 (26.21)
Nitsch + BA 2.5 mgl^{-1}+ NAA 0.5 mgl^{-1}	++	32.90 (34.99)

CD value 3.629

Scoring;+++ Good ++ Moderate

Value in parenthesis $Sin^{-1}\sqrt{x}$ transformed

Table 21a. Effect of different *in vitro* pollination techniques in heliconia

Method of *in vitro* pollination	Cultures showing ovary development 20 DAP			Cultures showing ovule development 20 DAP			Mean value of treatment
	L x L	L x A	L x P	L x L	L x A	L x P	
Stigmatic pollination	52.86 (7.27)	49.42 (7.03)	50.53 (7.11)	-	-	-	50.98 (7.14)
Stylar pollination	63.69 (8.29)	70.85 (8.42)	70.67 (8.41)	-	-	-	70.06 (8.37)
Ovarian pollination	82.18 (9.07)	81.83 (9.05)	82.24 (9.07)	-	-	-	82.08 (9.06)
Intra-ovarian pollination	80.64 (8.98)	82.07 (9.10)	82.33 (9.07)	-	-	-	81.90 (9.05)
Mean value of cross	70.56 (8.40)	70.56 (8.40)	70.73 (8.41)				

CD 5% comparison of crosses 0.176

CD 5% for comparison of treatments 0.204

CD 5% for comparing crosses x treatments 0.353

Value in parenthesis are $\sqrt{x}$ transformed

Table 21b. Effect of different *in vitro* pollination techniques in heliconia continued

Method of *in vitro* pollination	Cultures showing ovary development 20 DAP			Cultures showing ovule development 20 DAP			Mean value of treatment
	L x L	L x A	L x P	L x L	L x A	L x P	
Placental pollination	NA	NA	NA	55.06 (7.42)	55.16 (7.43)	56.42 (7.51	55.50 (7.45)
Modified placental pollination	NA	NA	NA	78.26 (8.85)	79.04 (8.89)	77.85 (8.82)	78.32 (8.85)
Ovular/Test tube fertilization	NA	NA	NA	36.17 (6.01)	30.62 (5.53)	33.38 (5.78)	33.41 (5.78)
Mean value of cross				55.20 (7.43)	53.00 (7.28)	54.32 (7.37)	

CD 5% for comparison of crosses 0.345

CD 5% for comparison of treatment 0.345

CD 5% for comparing crosses x treatment 0.597

Value in parenthesis are $\sqrt{x}$ transformed.

Table 22. Effect of sucrose on ovule development of heliconia after *in vitro* pollination

Sl. No.	Sucrose (%)	Ovule swelling	Cultures showing ovule development 20 DAP * (%)
1.	3	+++	81.82
2.	6	++	66.66
3.	9	++	55.56

Scoring +++ Good ++ Moderate

*Mean of 15 observations

Increasing the duration of mercuric chloride (0.1%) from 3 to 7 min did not increase the survival percentage. The treatment of mercuric chloride (0.1 %) for 3 min registered 28.72 per cent survival and for 7 min registered 28.79 per cent survival (Table 17). The combination treatment of ethanol (95% and 75%) with mercuric chloride (0.1 %) also registered equal survival per cent (Table17). The treatment of ethanol 70 per cent and mercuric chloride 0.1 per cent registered 28.03 per cent survival and ethanol 95 per cent and mercuric chloride 0.1 per cent registered 28.04 per cent. Hence among the different treatment tried mercuric chloride 0.1 per cent for 3 minute was the best.

4.3.2 Management of Bacterial Interference in Cultures

The treatments tried for management of bacterial interference in cultures and the results are presented in Tables 18 and 19.

Increasing the duration of mercuric chloride (0.1%) treatments from 8 minutes to 12 minutes did not register any control against bacterial infection (Table 18). The combination treatment of streptomycin for 1 h followed by mercuric chloride 0.1 per cent for 3 minutes registered more survival percentage (Table 18). Higher survival percentage (41.71%) was recorded in streptomycin at 1000 mgl^{-1}. However addition of copper sulphate (2.5 mg l^{-1}) in the culture medium during media preparation could control the bacterial interferences at 100 per cent survival rate (Table 19). The results showed that flower buds surface sterilized with 0.1 per cent mercuric chloride for 3 minutes, followed by inoculation in the media containing 25 mg l^{-1} copper sulphate registered 100 per cent survival of the cultures.

4.3.3 Basal Media for Culture Establishment of Heliconia

In order to get the initial *in vitro* establishment surface sterilized ovaries on the day of anthesis were inoculated in four different media *i.e.*, MS, ½ MS, Nitsch and SH with or without supplements. There was no ovary development in the simple basal medium. However, ovaries developed in all

four media when they were supplemented with BA 2.5 mgl^{-1} + NAA 0.5 mg l^{-1} (Table 20). Different media tried for ovule development showed significant difference. On comparing the different media MS medium was superior to other three media with high percentage of culture establishment and good ovary development (Plate 5).

4.3.4 Standardization of *In vitro* Pollination Technique for Heliconia

For the standardization of *in vitro* pollination and fertilization technique for heliconia different *in vitro* pollination technique were tried (Tables 21a and b). Both selfing and crossing were tried. Selfing was done only in Lady Di whereas crossing was done with Lady Di as the female parent and Andromeda and Parakeet as the male parents.

There was no significant difference noticed between the three crosses. But significant difference was noticed between the different *in vitro* pollination techniques tried.

In the self and cross pollination ovary development was observed in stigmatic, stylar, ovarian and intra-ovarian pollination (Table 21a). In these techniques 52.86 to 82.18 per cent of cultures in self-pollination and 49.42 to 82.24 per cent of cultures in cross-pollination showed ovary development. Highest per cent of ovary development was registered by ovarian pollination (82.08%) and was on par with intra-ovarian pollination (81.90%). Lowest per cent of ovary development was observed in stigmatic pollination (50.98%). But none of these pollination techniques showed ovule development in self and cross pollination.

Among the various *in vitro* pollination method tried, ovules were found to develop in placental pollination, modified placental pollination and test-tube fertilization (Table 21b). Ovule development was varied from 36.17 to 78.23 per cent in the case of self pollination and 30.62 to 79.04 per cent in cross pollination. In these highest per cent of ovule development was registered by modified placental pollination (78.32%) where as lowest by test

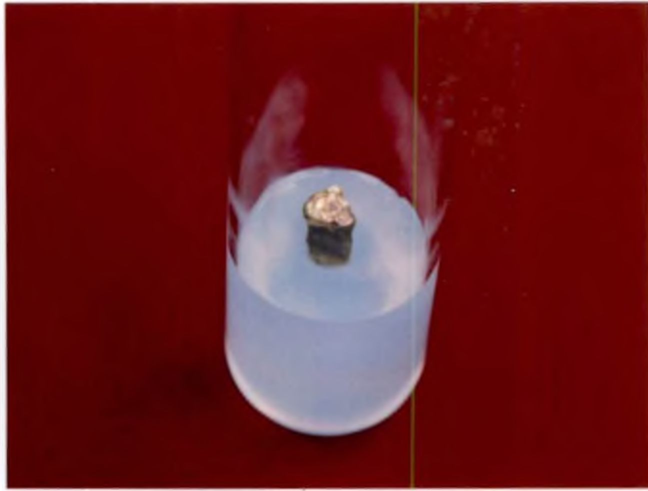


Plate 5. Ovary development in MS medium

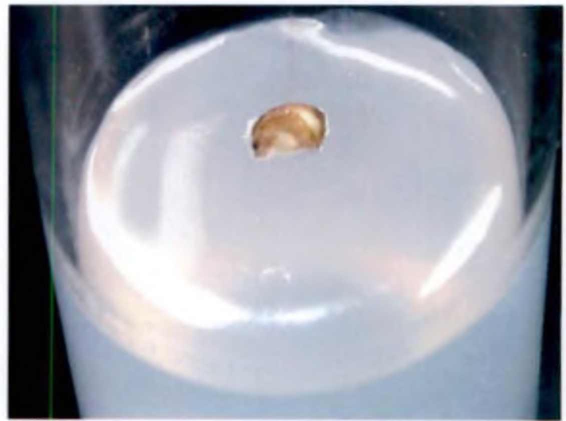


Plate 6. *In vitro* modified placental pollination

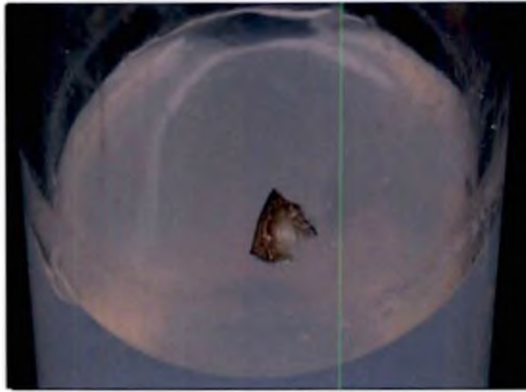
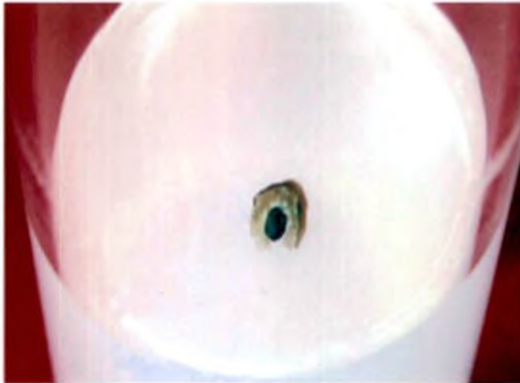


Plate 7. Ovule development 4 days after modified placental pollination



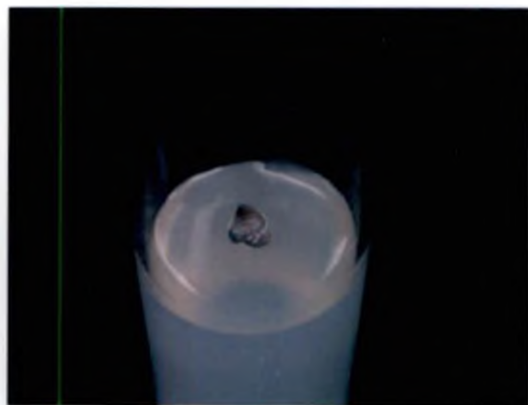


Plate 8. *In vitro* seed formation after modified placental pollination

tube fertilization (33.4%). Placental pollination registered 55.5 per cent ovule development.

The results showed that in the case of heliconia modified placental pollination was found to be easiest and best for *in vitro* pollination and fertilization with high per cent of ovule development (Plate 6a and b).

4.3.5 Standardization of Age of Flower Buds for *in vitro* Pollination

After standardization *in vitro* pollination and fertilization technique attempts were made to optimize the age of flower buds for *in vitro* pollination. For this flower buds were collected at five different time intervals *i.e.*, one day prior to anthesis, two day prior to anthesis, on the day of anthesis, one day after anthesis and two day after anthesis. Among these ovule development occurred only in the flower buds collected one day prior to anthesis and *in vitro* pollination done on the day of anthesis. The result showed that for the successful *in vitro* pollination in heliconia, flower buds collected one day before anthesis kept for the natural dehiscence after surface sterilization and pollination done at the time of anthesis was best.

4.3.6 Standardization of Media Supplement for Enhancing Ovule Development

4.3.6.1 Effect of Sucrose Concentration on Ovule Development

Three different levels of sucrose were tried for studying the effect of sucrose on ovule development of heliconia after *in vitro* pollination. Maximum ovule development (81.82%) was observed at three per cent sucrose level with good ovule swelling (Table 22). An increase in the sucrose level to 6 per cent resulted in decrease in ovule development (66.66%) and extend of ovule swelling. A further increase in sucrose level to 9 per cent also resulted in the decrease in ovule development (55.56%) and extend of ovule swelling. The results showed that three per cent sucrose was ideal for the ovule development in heliconia after *in vitro* pollination.

Table 23 Effect of auxins on ovule development of heliconia after *in vitro* pollination

Treatment (mg l^{-1})	Ovule swelling	Culture showing ovule development 20 DAP (%)
IAA 0.1	+	19.28
IAA 1.0	+	21.97
IAA 2.0	+	26.94
IAA 5.0	+	33.66
NAA0.1	+	44.87
NAA1.0	++	58.52
NAA 2.0	++	56.76
NAA 5.0	++	56.79

CD at 5% 2.833

Scoring + low, ++ moderate

Table 24. Effect of cytokinin on ovule development of heliconia after *in vitro* pollination

Treatment (mg l^{-1})	Ovule swelling	Cultures showing ovule development 20 DAP (%)
BA 0.1	+	26.2
BA 1.0	++	44.68
BA 2.0	++	52.61
BA 2.5	++	58.66
BA 5.0	++	58.48

CD at 5% level 2.871

Scoring + Low, ++ Moderate

Table 25 Effect of auxin and cytokinin on ovule development of heliconia after *in vitro* pollination

Treatment (mg l^{-1})		Ovule swelling	Culture showing ovule development 20 DAP (%)
T_1	BA 0.1 + NAA 0.1	+	54.05 (7.35)
T_2	BA 0.1 + NAA 1.0	+	63.45 (7.95)
T_3	BA 0.1 + NAA 2.0	+	64.87 (8.05)
T_4	BA 1.0 + NAA 0.1	+	62.53 (7.91)
T_5	BA 1.0 + NAA 1.0	+++	68.12 (8.25)
T_6	BA 1.0 + NAA 2.0	+++	68.40 (8.27)
T_7	BA 2.0 + NAA 0.1	+	64.73 (8.05)
T_8	BA 2.0 + NAA 1.0	+++	78.96 (8.89)
T_9	BA 2.0 + NAA 2.0	+++	78.63 (8.87)
T_{10}	BA 2.5 + NAA 0.1	+	62.95 (7.93)
T_{11}	BA 2.5 + NAA 1.0	+++	82.78 (9.10)
T_{12}	BA 2.5 + NAA 2.0	+++	83.05 (9.11)
T_{13}	BA 5.0 + NAA 0.1	+	61.92 (7.87)
T_{14}	BA 5.0 + NAA 1.0	+	71.98 (8.48)
T_{15}	BA 5.0 + NAA 2.0	++	71.93 (8.48)
T_{16}	BA 1.0 + IAA 0.1	-	42.59 (6.53)
T_{17}	BA 1.0 + IAA 1.0	+	53.11 (7.29)
T_{18}	BA 1.0 + IAA 2.0	+	53.51 (7.31)
T_{19}	BA 2.5 + IAA 0.1	-	42.57 (6.52)
T_{20}	BA 2.5 + IAA 1.0	+	53.00 (7.28)
T_{21}	BA 2.5 + IAA 2.0	++	53.60 (7.32)
T_{22}	BA 5.0 + IAA 0.1	-	45.74 (6.76)
T_{23}	BA 5.0 + IAA 1.0	+	54.25 (7.37)
T_{24}	BA 5.0 + IAA 2.0	+	54.62 (7.39)

CD at 5% level 31 0.254

Scoring - Poor; + Low; ++ Moderate; +++ Good

Value in parenthesis are $\sqrt{x}$ transformed

4.3.6.2 Plant Growth Substances

4.3.6.2.1 Influence of Auxins on Ovule Development of Heliconia

Significant difference was noticed among the treatment with respect to the concentration of auxins (Fig.5). On comparing the effect of auxins NAA was found to be superior to IAA in ovule development of heliconia. IAA 0.1 to 5 mgl^{-1} induced low ovule swelling in 19.28 to 33. 66 per cent of cultures whereas NAA 0.1 to 5 mgl^{-1} recorded moderate ovule swelling in 44.87 to 56.79 per cent of cultures. IAA recorded maximum of 33.66% ovule development at $5mgl^{-1}$. In the case of NAA, $5mgl^{-1}$ recorded higher per cent of ovule development (56.79%). It was on par with NAA 2 mgl^{-1} (56.76%) (Table 23). The results showed that auxins induced ovule development in heliconia.

4.3.6.2.2 Influence of Cytokinin on Ovule Development of Heliconia

Significant difference was noticed among the treatments with respect to the concentration of BA (Fig.6). An increase in the concentration of BA resulted in increase in the ovule development also. BA 0.1 mg l^{-1} to $5mgl^{-1}$ induced low (+) to moderate (++) ovule development in 26.2 to 58.66 per cent of the cultures. Highest per cent of ovule development (Table 24) was observed in BA 2.5 mgl^{-1} (58.66%). It was on par with BA 5 mgl^{-1} (58.48%). The results of the investigation showed that BA favoured ovule development in heliconia after *in vitro* pollination.

4.3.6.2.3 Influence of Auxin and Cytokinin on Ovule Development of Heliconia

Significant difference was observed among the treatments (Table 25) with respect to concentration of cytokinin (BA) and auxin (NAA and IAA). The combinations of BA with NAA and IAA favoured ovule development in 42.57 to 83.05 per cent cultures (Fig.7). The treatment T_{12} (BA 2.5 mgl^{-1} + NAA2 mgl^{-1}) favoured maximum ovule development in 83.05 per cent of the cultures. It was on par with treatments T_{11} (BA 2.5 mgl^{-1} + NAA 1 mgl^{-1}),

Table 26 Effect of gibberllic acid on ovule development of heliconia after *in vitro* pollination

Treatment (mg l^{-1})	Ovule swelling	Culture showing ovule development 20 DAP (%)*
GA 0.1	-	-
GA 1.0	-	19.2
GA 2.0	-	19.3
GA 10.0	-	20.9

Scoring – Poor

*Mean of 15 observations

Table 27 Effect of organic supplements on ovule development of heliconia after *in vitro* pollination

Treatments	Ovule swelling	Culture showing ovule development 20 DAP (%)
CW 5% v/v	+	76.77 (8.76)
CW 10% v/v	+	76.42 (8.74)
CW 15% v/v	+	76.31 (8.74)
CW 20% v/v	+	76.12 (8.73)
CH 250 mgl^{-1}	++	81.05 (9.00)
CH 500 mgl^{-1}	+++	85.96 (9.27)
CH 1000 mgl^{-1}	++	77.25 (8.79)
YE 250 mgl^{-1}	+++	88.23 (9.39)
YE 500 mgl^{-1}	+++	87.08 (9.83)
YE 1000 mgl^{-1}	++	80.05 (8.95)

CD value at 5% 0.202

Scoring + Low; ++ Moderate; +++ Good

Value in parenthesis are $\sqrt{x}$ transformed

T_8 (BA 2 mgl^{-1} + NAA 1mgl^{-1}) and T_9 (BA 2 mgl^{-1} + NAA 2 mgl^{-1}). Treatments with reduced level of NAA and BA resulted low ovule swelling (T_1, T_2, T_3, T_4, T_7, T_{10}, T_{13}, and T_{14}). BA and NAA in equal concentration (T_5, T_9 and T_{12}) and 1:2 ratio and *vice versa*. (T_6, T_8, T_{11} and T_{15}) resulted good ovule swelling.

Higher concentration of BA and very low concentration of NAA resulted in low ovule development (T_4, T_7, T_{10}, T_{13} and T_{14}) and *vice versa* (T_2 and T_3). Replacement of NAA with IAA produced ovule development in 42.57 to 53.62 per cent but the ovule swelling was not up to the level as that in the optimum combination of BA and NAA. Among these, the combination of BA 2.5 mgl^{-1} + NAA 2 mgl^{-1} was found to be the best and produced optimum ovule swelling in 88.05 per cent of the cultures.

4.3.6.2.4 Influence of GA on ovule development of heliconia

Four treatments were tried to assess the effect of GA on ovule development in heliconia (Table 26). An increase in concentration of GA (1 to 10 mg l^{-1}) did not induce ovule development in heliconia. No ovule development was observed in 0.1 mg l^{-1} GA. The results showed that GA had no effect on ovule development it produced very poor ovule swelling with maximum of 20.9 per cent (GA 10 mg l^{-1}) cultures.

4.3.6.3 Influence of Organic Supplements on Ovule Development of Heliconia

For studying the effect of organic media supplements three different organic supplements were used viz. coconut water (CW), casein hydrolysate (CH) and yeast extract (YE).

Four treatments were tried to assess the effect of CW on ovule development of heliconia after *in vitro* pollination (Table 27). Significant difference was not noticed among these treatments. The results indicated that CW was not favourable for ovule development as the ovule swelling was low.

Table 28. Effect of illumination on ovule development of heliconia after *in vitro* pollination

Treatment	Ovule swelling	Culture showing ovule development 20 DAP (%)
Kept in diffused light	+++	83.36 (9.13)
Kept in dark	-	14.68 (3.83)

CD value at 5% 0.369
Scoring : - Poor ; +++ Good
Value in parenthesis are $\sqrt{x}$ transformed

Table 29 Effect of physical form of the media on ovule development in heliconia after *in vitro* pollination

Treatment	Ovule swelling	Culture showing ovule development 20 DAP (%)
Liquid	-	14.80 (3.85)
Semi solid	+++	85.29 (9.23)

CD Value at 5% 0.283

Scoring: - Poor ; +++ Good
Value in parenthesis are $\sqrt{x}$ transformed

Table 30. Effect of *in vitro* treatments on germination of heliconia seeds

Sl. No.	Treatments	Germination (%)
1	MS	Nil
2	MS + GA 0.1 mg l^{-1}	Nil
3	MS + GA 1.0 mg l^{-1}	Nil
4	MS + GA 2.0 mg l^{-1}	Nil
5	MS + GA 5.0 mg l^{-1}	Nil
6	MS + GA 10.0 mg l^{-1}	Nil
7	MS + BA 1.0 mg l^{-1}	Nil
8	MS + BA 2.0 mg l^{-1}	Nil

In the case of CH three different treatments were tried and significant difference was noticed among these treatments (Table 27). The medium containing CH (250 mg l^{-1}) induced moderate ovule development in 81.05 per cent of cultures. The CH supplementation (500 mg l^{-1}) was found to enhance ovule development by achieving the maximum percentage of development (85.96%) with good ovule swelling. A further increase in CH level to 1000 mgl^{-1} was not favourable for ovule swelling.

When YE as organic supplements (Table 27) recorded maximum of 88.23 per cent ovule swelling in 250 mg l^{-1}, it was on par with YE 500 mg l^{-1} (87.08%). Increasing the concentration of YE (1000 mg l^{-1}) resulted in decreased percentage of ovule swelling (80.05%).

The results showed that among these, YE 250 mg l^{-1} was found to be the best and produced optimum ovule swelling in 88.23 per cent of the cultures (Fig.8).

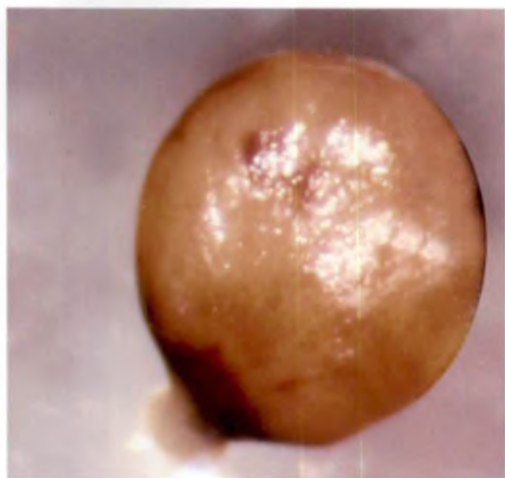
4.3.7 Influence of Illumination on Ovule Development

To study the effect of illumination on ovule development, the *in vitro* pollinated ovules were subjected to two different conditions (Table 28). The results showed that continuous diffused light (12h) was the best treatment in 88.36 per cent with good ovule development. The cultures kept in dark favoured ovule development in 14.66 per cent cultures with poor ovule swelling.

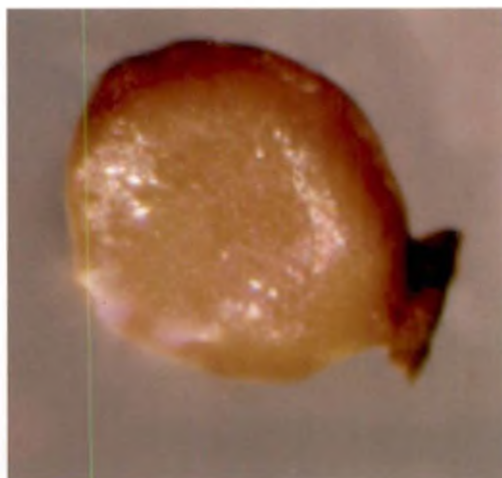
4.3.8 Influence of Physical Condition of Media on Ovule Development

The effect of solid and liquid media on ovule development in heliconia after *in vitro* pollination differed significantly (Table 29). The results showed that solid medium was the best treatment resulting in 85.29 per cent of cultures with good ovule swelling. In the case of liquid medium ovule development was induced only in 14.80 per cent of cultures with poor ovule swelling.

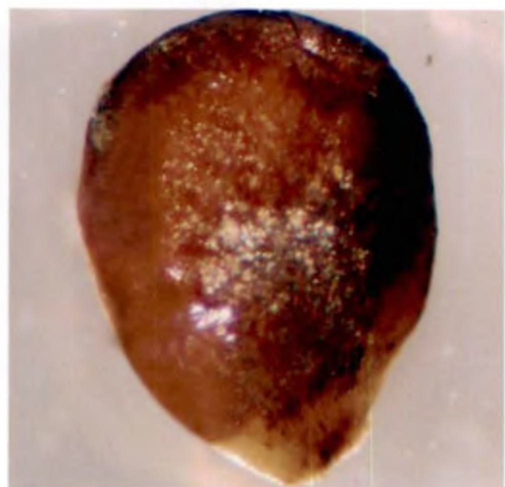
a. 20 DAP



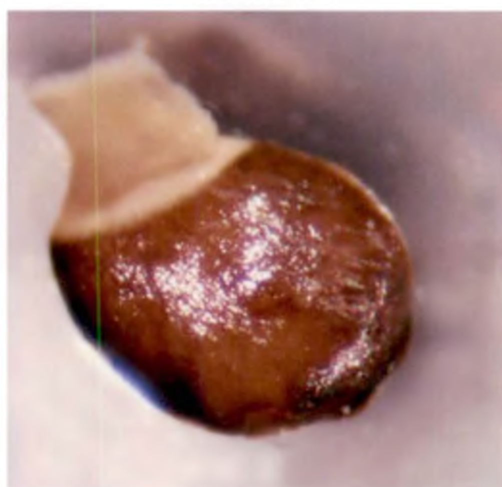
b. 40 DAP



c. 60 DAP



d. 90 DAP



e. 100 DAP

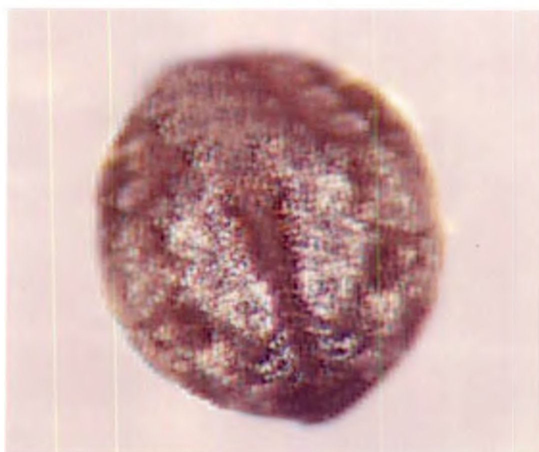
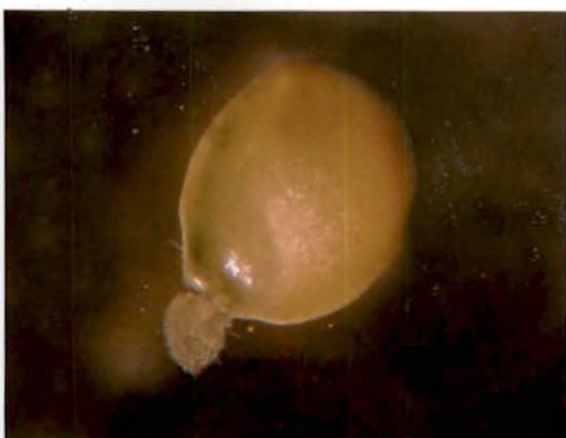


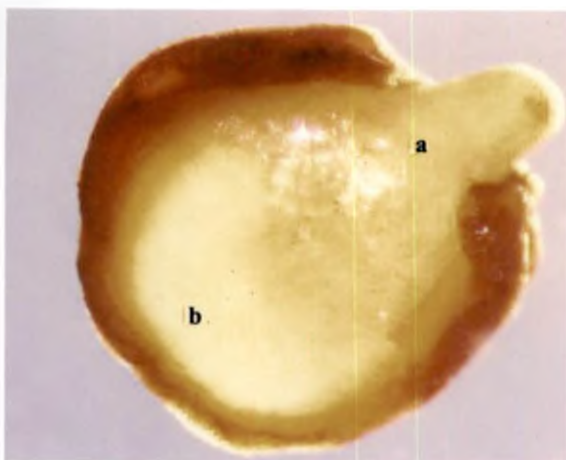
Plate 9. *In vitro* formed seeds after *in vitro* pollination 5x

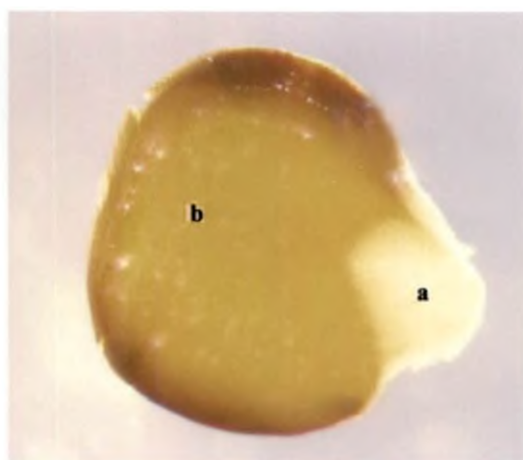
4 DAP

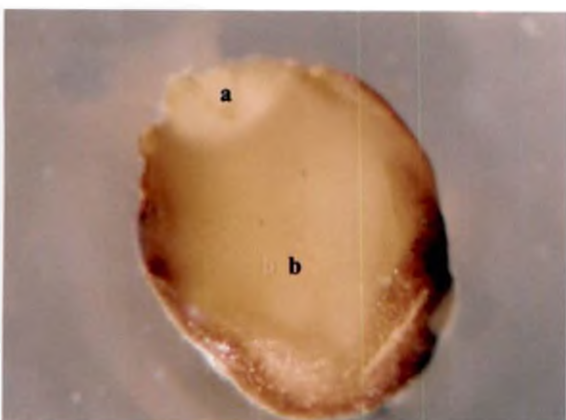


8 DAP

Plate 10. Ovule development after *in vitro* pollination 5 x







a. Embryo
b. Endosperm

Plate 11. Longitudinal sections of *in vitro* formed seeds 5 x

4.3.9 Influence of Temperature on Ovule Development of Heliconia after *in vitro* Pollination

For studying the effect of temperature two treatments were tried *i.e.*, cultures were kept at 26 ± 2°C and 30 ± 2°C. Among these ovule development was observed only in cultures were kept at 26±2°C (84.13%) and was no ovule development at 30±2°C.

4.3.10 Post Pollination Changes

Among the different pollination technique tried ovule/seed development was noted in the cultures after placental pollination, modified placental pollination and test tube fertilization. After *in vitro* pollination the fertilized ovules enlarged and developed into seeds. For visual observation, swelling of ovules and change in colour of ovules interpreted as fertilization reaction. Pollinated ovules started enlarging in four to five days after culturing (Plate 7 and 10). Unpollinated ovules showed no sign of ovule swelling it became persisted as such and on aging it become shriveled and dead.

The seed development showed that the growth was very rapid during the initial days. Within 20 days it became maximum size of the seed (Plate 8). The colour of the ovule during the initial stage was creamy white turned to light brown within 20 days after pollination (Plate 9a). Latter it turned to brown 40 to 60 days after pollination (Plate 9b and c) and then dark brown to the period of 60 to90 days after pollination (Plate 9d). 90 DAP the seeds turned to completely black (Plate 9e).

Microscopic measurements of the seeds were done with an ocular micrometer to quantify the development of ovules into seeds. The ovules on the day of anthesis mean length of 1251 μm and breadth of 877.5 μm. Twenty days after pollination the mean length of the ovule was 1800 μm and mean breadth of 1575 μm. This results showed that an increase in breadth and length of the ovules after *in vitro* pollination.

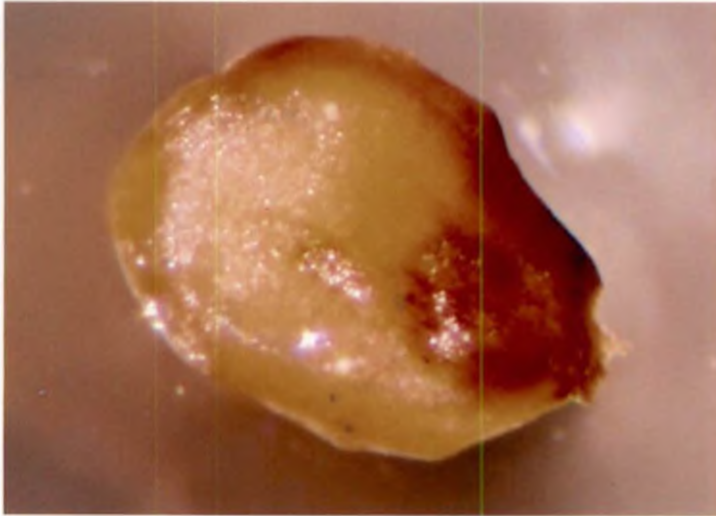


Plate 12. Dieing of embryo 90 days after pollination 5x

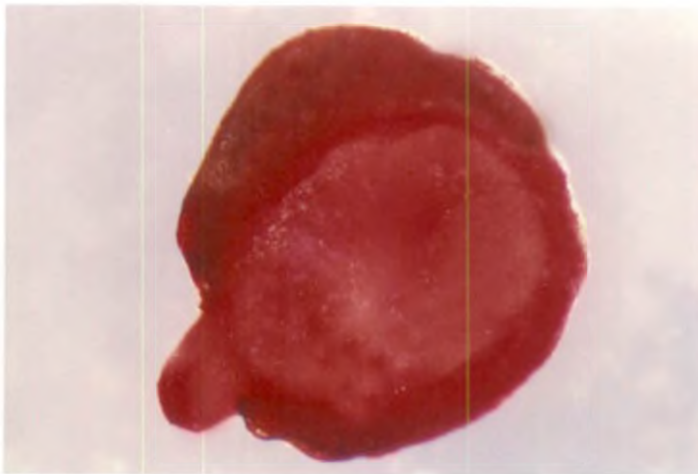


Plate 13. Completely stained seed after saffranin staining 5x

4.3.11 Histological Examination of Ovules

The longitudinal sections of the ovules were observed under stereomicroscope at 5x magnitude. Actively growing embryos were seen in the pollinated ovules. The embryos obtained from the dissected ovules at various intervals showed globular, reniform and elongated embryos. Embryos are seated at the chalazal end (Plate 11). Ovules dissected from 20 DAP showed globular embryo (Plate 14a), 40 DAP had reniform shaped (Plate 14b) and 80 DAP possessed elongated embryo (Plate 14c) ovules dissected after 90 DAP showed embryo turned brown in the darkened seeds (Plate 12).

Endosperm filling was also monitored. Well developed endosperm also seen in the pollinated ovules. The endosperm was soft and jelly like in the early days and it became cellular and developed well by 20 DAP. Endosperm constitute major portion of the seed with very good filling inside the seed (Plate 11). The seeds were fully filled with endosperm and embryo and were readily stained by saffranin indicating their viability (Plate 13).

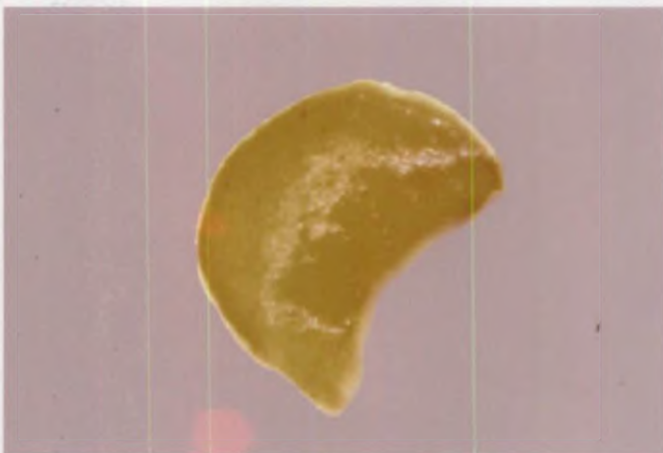
Embryological studies of the ovules revealed that presence of well developed embryo and endosperm in the *in vitro* pollinated ovules. *In vitro* culture did not stimulate egg cell induction and autonomous endosperm development in unpollinated ovules cultured on the same media.

4.3.12 Seed Germination Studies

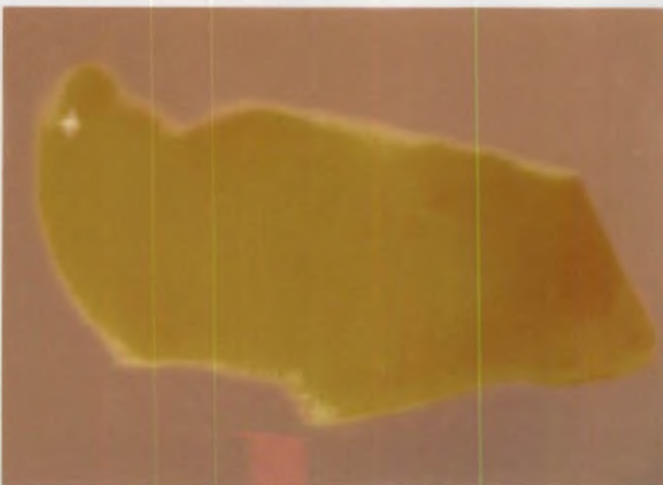
4.3.12.1. In vivo Treatments

In vitro formed seeds of heliconia were kept on moist filter paper for germination. And some seeds were sown in sterile sand and spray ½ MS one-week intervals. But these treatments did not give positive result.

a. Globular embryo

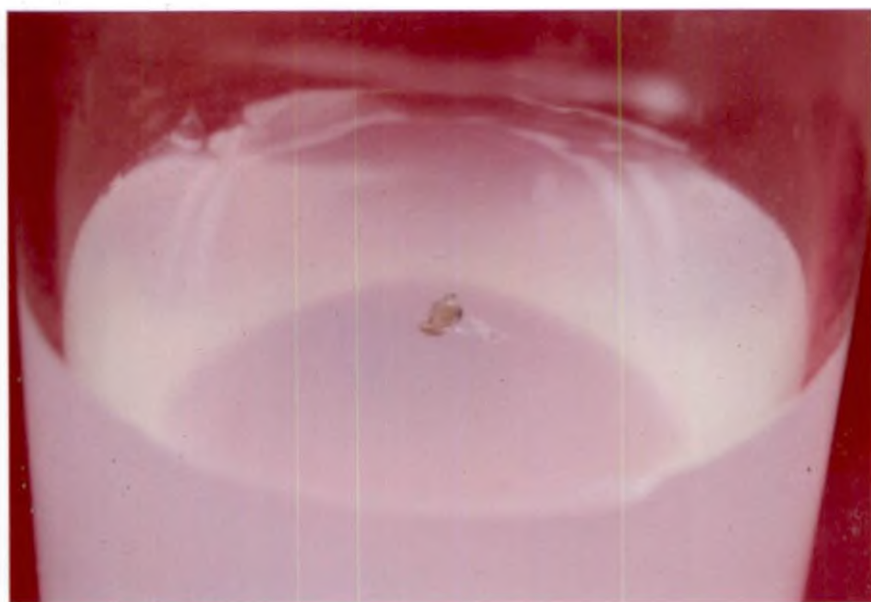


b. Reniform embryo

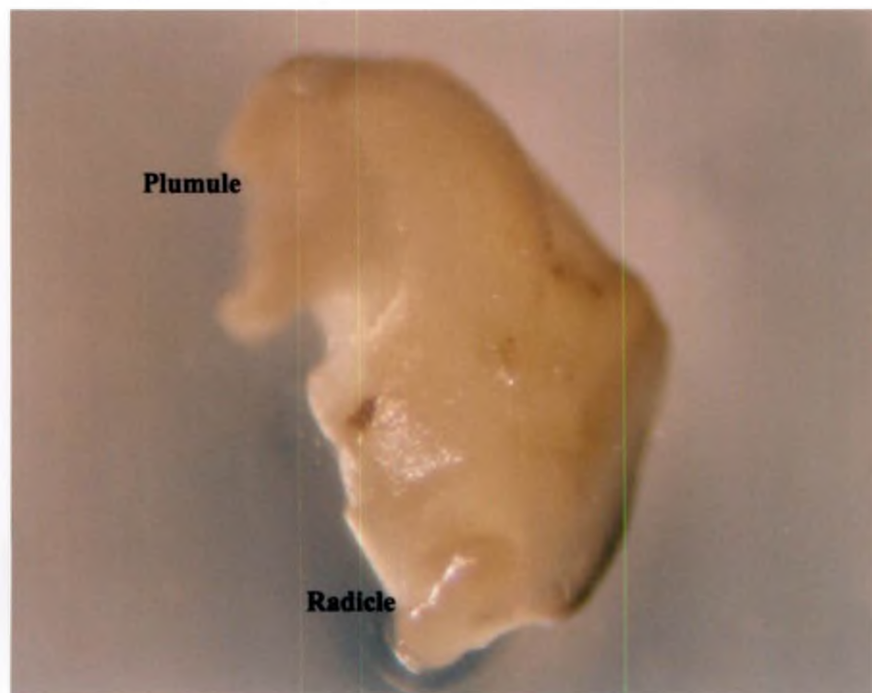


c. Elongated embryo

Plate 14. Embryos formed after *in vitro* pollination



a. 60 days after embryo culture



b. 90 days after embryo culture 5x

Plate 15. ***In vitro*** **embryo germination**

4.3.12.2 In vitro Seed Germination Studies

MS medium alone or with hormones (GA and BA) did not result in seed germination (Table 30). The results showed that GA and BA not favoured the germination of *in vitro* produced seeds of heliconia.

4.4 EMBRYOCULTURE

In the present study, attempts were to integrate the *in vitro* pollination and fertilization with subsequent embryo rescue. For this embryos of different ages (20, 40, 60 and 80 DAP) were dissected out and cultured. Among this embryos dissected 40 DAP showed germination indices (Plate 15). None of the embryos dissected on 20 DAP, 60 DAP and 80 DAP showed germination indices.

For the optimization of media MS medium with three different levels of sucrose (3, 6 and 9 per cent) were tried. Among these embryos survived only in three per cent sucrose concentration. An increase in sucrose concentration to 6 to 9 per cent resulted embryo dieing. Studies showed that MS medium was suitable when supplemented with three per cent sucrose.

For standardizing the incubated condition for germination, the cultures were kept at two different conditions (dark and diffused light) at 26 ± 2°C. Among these embryo germination indices started cultures kept at dark (26 ± 2°C) after 60 days after embryo culture. Ninety days after embryo culture, embryos showed initiation of radicle and plumule (Plate 15b). This embryo was transferred under diffused light and afterward subcultured into MS medium supplemented with three per cent sucrose and BA 0.1 mg l^{-1} and IBA 0.1 mg l^{-1}. Thereafter size of the embryo increased and greenish tinge was observed. The length of the radicle and plumule increased the embryo growth was slow.

Discussion

5. DISCUSSION

Heliconia is an important exotic tropical cut flower crop. This is recently introduced cut flower crop in Kerala. Their brilliant colour, exotic form, long straight peduncle and excellent post harvest characteristics make them an important flower for florist trade. Though many species and cultivars are grown, consumers are interested in newer and different types of flower. Hence hybridization is essential in heliconia. Till now there was no reports on artificial hybridization in heliconia. Hybridization in heliconia is unsuccessful due to the pre-fertilization barriers *viz.*, stigmatic, stylar or ovarian (Kress, 1983).

The technique of *in vitro* pollination and fertilization has been proved promising for overcoming pre-fertilization barriers to compatibility and for raising new genotypes. This pollination technique could bring fertilization under controlled condition and development of hybrid embryo even in wide crosses. This hybrid embryo developed into normal hybrid plants when grown in a suitable medium.

The present investigation was aimed at standardizing an appropriate *in vitro* pollination techniques thereby achieve successful seed set in heliconia. Success in this line will open up new vistas of crop improvement in heliconia. Three cultivars of *Heliconia psittacorum viz.*, Lady Di, Andromeda and Parakeet were selected for the study. The results obtained from the studies are discussed in this chapter.

5.1 POLLEN STUDIES

A reliable method for testing pollen viability is essential for the study of pollination biology. In most of the studies on staining techniques, the effectiveness of staining methods was evaluated based on this correlation with pollen germination *in vitro* (Khatun and Flowers, 1995; Riano and Dayni, 2000). *In vitro* pollen germination is believed to provide the best

estimate of pollen viability. In order to standardize a suitable medium for pollen germination, studies were carried out using two different media *viz.*, Brewbaker and Kwack medium and ME3 medium. Among these media tried, ME3 medium with 12 per cent PEG was reported , high pollen germination in heliconia cultivars (Fig.1). ME3 medium with 12 per cent PEG was superior with mean germination per cent of 61.31. Significant difference was noticed between the cultivars with respect to percentage of pollen germination. Maximum mean germination was in Lady Di followed by Andromeda and Parakeet. ME3 medium was also identified as the best artificial medium for the *in vitro* pollen germination in ginger (Valsala, 1994; Bindu, 1997; Devi, 2005), turmeric (Renjith, 1999; Vijayasree, 2001) and kacholam (Bhurke, 2002).

The change in pH of ME3 medium from 4 to 8 did not influence the percentage of pollen germination (Table 14). This was also supported by Valsala (1994) in ginger. With regard to the incubation time of pollen in the ME3 medium, pollen germination started within ½ h after incubation and continued upto 3 h. After 3 h of incubation there was no significant difference in percentage of pollen germination (Fig.2). ME3 medium recorded significantly more pollen tube length. The ME3 medium recorded a mean pollen tube length of 454 μm (Fig.4).

In the case of Brewbaker and Kwack medium when sucrose was used as osmoticum there was no pollen germination, while pollen grains germinated when PEG used as osmoticum. Leduc *et al.* (1991) emphasize that in germination medium PEG was superior to sucrose as an osmoticum in germination and tube growth of pollen. PEG has been well recognized as a non-penetrating osmotic agent and it decreases the water potential of the culture medium. PEG has also been suggested to regulate the permeability of the plasma membrane of the growing tip (Read *et al.*, 1993). Sucrose on the other hand, enters the cell and increases the osmotic potential of the cell which may not be conducive for tube growth. In Brewbaker and Kwack

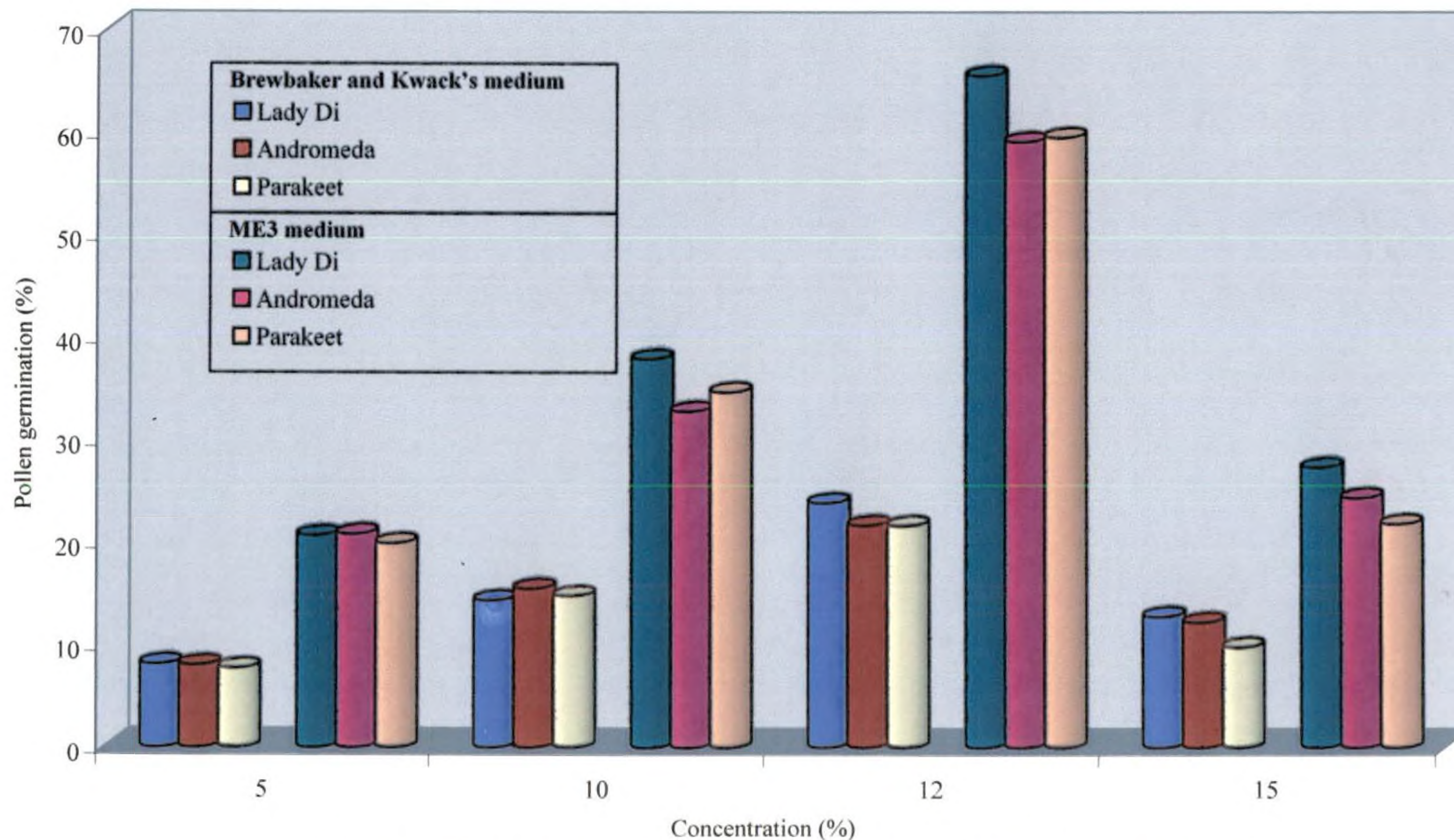


Fig. 1. Comparison on effect of PEG in Brewbaker and Kwack medium and ME_3 medium on *in vitro* pollen germination

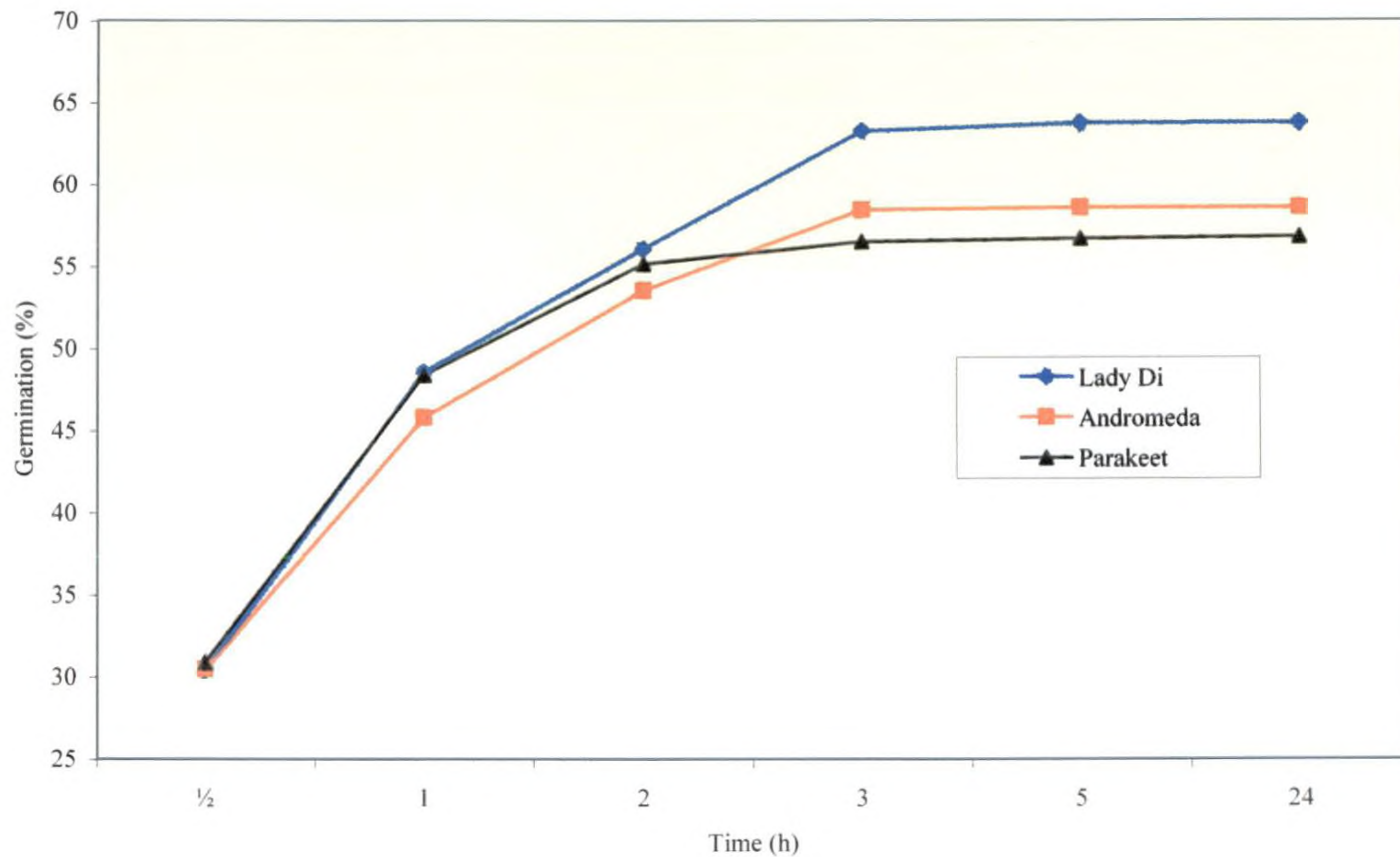


Fig. 2. Effect of incubation period on percentage of pollen germination in heliconia cultivars

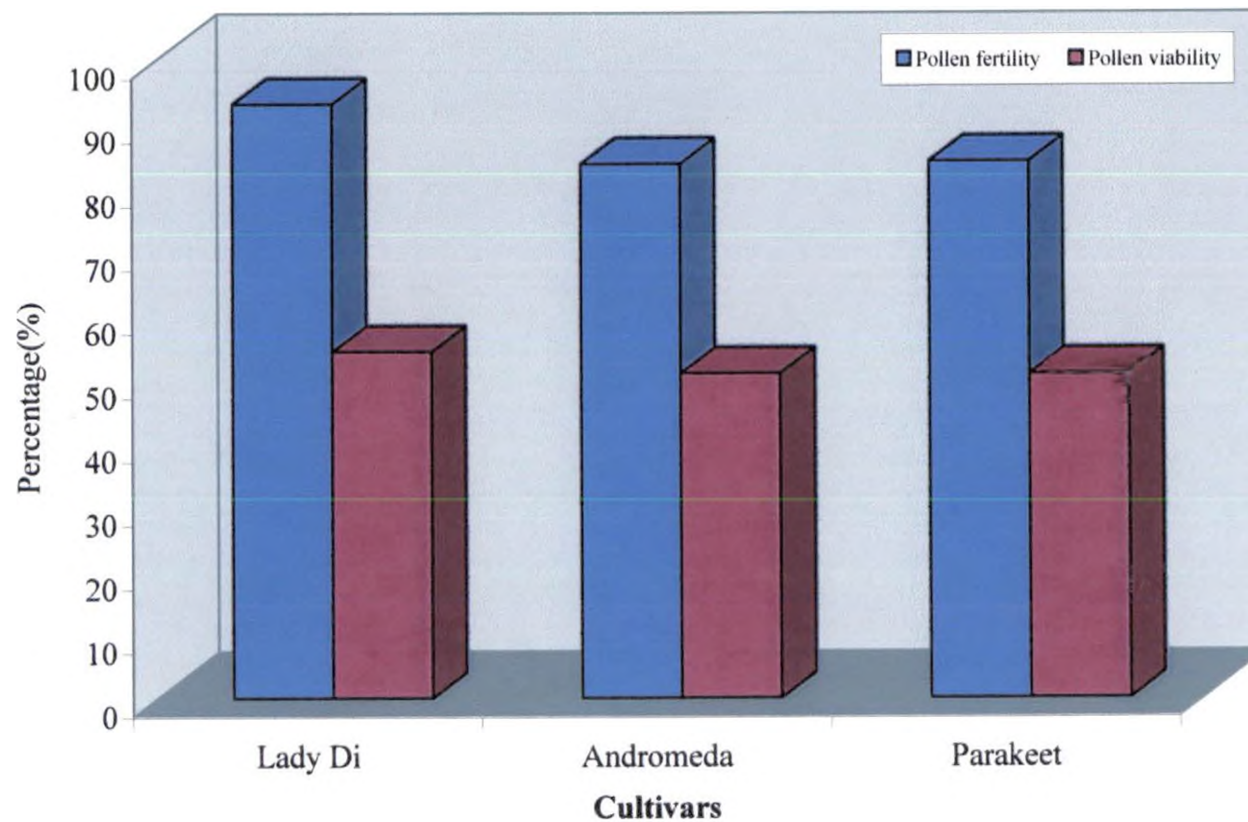


Fig. 3. Comparison of pollen fertility in acetocarmine stain test vs pollen viability in ME_3 medium

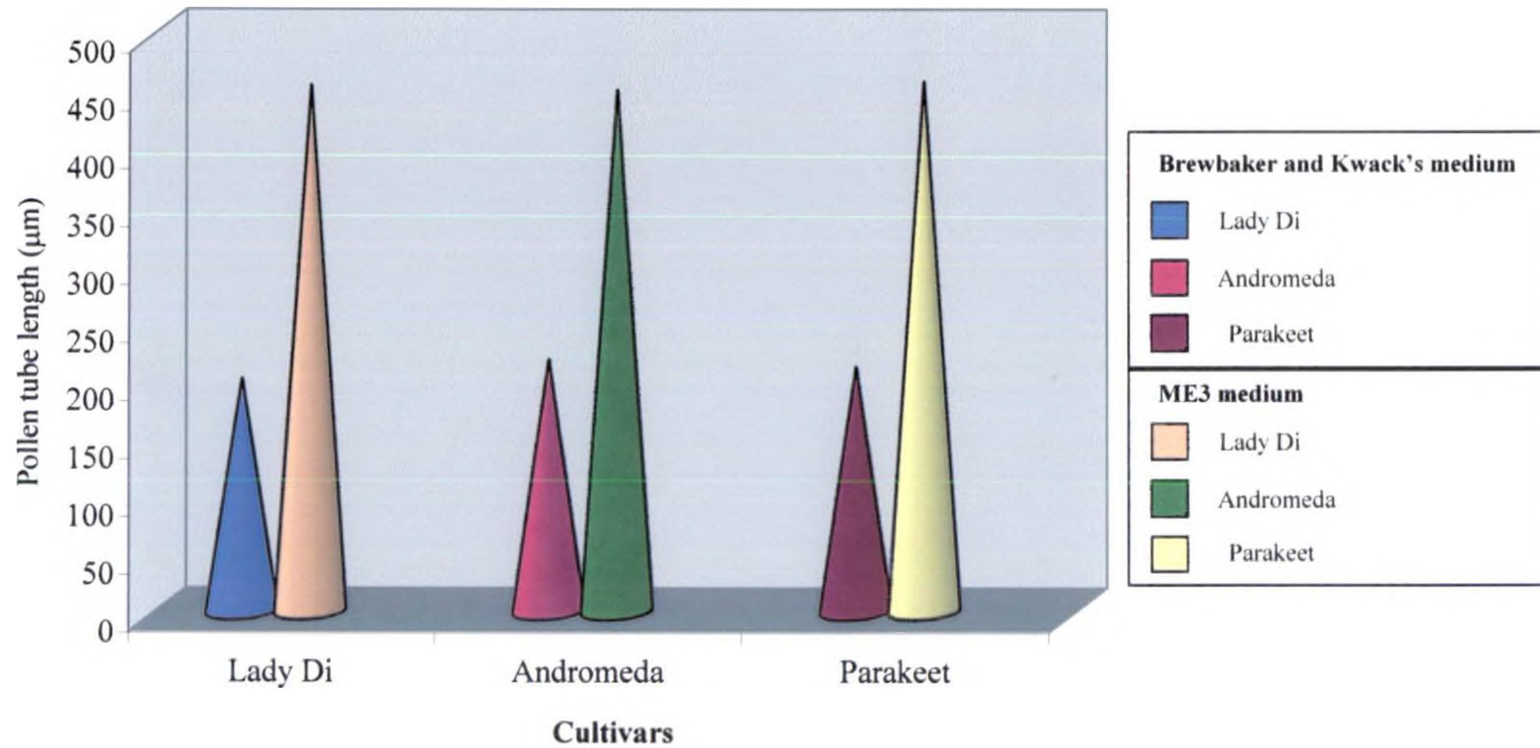


Fig. 4. Comparison of pollen tube length of three selected heliconia cultivars in Brewbaker and Kwack medium and ME_3 medium

medium recorded higher percentage of pollen germination 22.37 per cent (Table 11) and mean pollen tube length of 209.73 μm (Table 15). In Brewbaker and Kawak medium only four nutrients are involved (Boron, Calcium, Magnesium and Potassium), whereas in ME_3 medium other than these four nutrients, more number of macro and micro nutrients are present. ME3 medium, also contain two vitamins (Thiamine and Pyrodoxine). Because of these superiority in composition of nutrients may be the increase in percentage pollen germination and tube length in ME_3 medium.

There was no pollen germination in both medium without osmoticum. The requirement of hydrationphase in subsequent processes of pollen germination and tube growth was also supported by Shivanna and Johri (1985). Pollen grains are desiccated units at the time of shedding and generally show higher osmotic potential compared to other cells. After pollen germination, the resultant pollen tubes are fully hydrated and their osmotic potential is expected to be lower than that of pollen grain. Presence of osmotic agents in the medium maintains the required osmotic potential of the medium (Shivanna, 2003). The present studies indicated that ME_3 medium with 12 per cent PEG was ideal for *in vitro* germination of heliconia pollen germination.

The pollen fertility of selected three cultivars were estimated using acetocarmine stain. The mean pollen fertility varied among the cultivars from 83.61 to 93.04 per cent. A high percentage of pollen fertility has been reported in heliconia as per acetocarmine test (Babu, 2005; Sanjeev, 2005). But when the pollen viability was estimated using ME_3 medium, the mean germination per cent recorded from 54.37 to 62.08. This showed that pollen fertility was high in heliconia cultivars but the viability (pollen germination) in ME_3 medium was not correspondingly high (Fig.3). This studies indicate that acetocarmine stain test may not be fully efficient in revealing pollen fertility. It may only give an indication of the fertility of pollen. So the correct assessment of pollen viability is estimated through *in vitro* germination studies using suitable media like ME3 medium.

5.2 MORPHOLOGICAL STUDIES OF GYNOECIUM

The difference in morphological characters of gynoecium of selected cultivars were examined. The results showed that there was no significant difference between the selected cultivars with respect to various characters of gynoecium *viz.*, style length, ovary length, ovary diameter, number of ovules/ovary, length of ovule and breadth of ovule.

In the present morphological studies of gynoecium showed that heliconia has single long tubular style often somewhat curved with single stigma. This was also reported earlier by Watson and Dallwitz (1992).

The ovary was inferior and epicarp adhered tightly to the ovary. The outer colour of pericarp is species specific *i.e.*, Lady Di it was creamy yellow, Andromeda orange and greenish in Parakeet. Ovary trilocular has basal placentation with one ovule per locule. Mature ovules of heliconia were anatropous and creamy white colour.

5.3 *IN VITRO* POLLINATION AND FERTILIZATION

For an effective hybridization programme, attempts were made for standardizing an *in vitro* pollination and fertilization technique for heliconia. The outcomes of the investigations are discussed below.

Attempts for successful *in vitro* pollination were made after standardizing surface sterilization procedure for flower buds. For the *in vitro* pollination flower buds were surface sterilized before flower opening. This is advisable as contact of surface sterilant may reduce the viability of pollen. In order to standardize a suitable steriliant for culture establishment, studies were carried out using various surface sterilants (Table 17) and also the management of bacterial interference in the culture (Tables 18 and 19). Among these flower buds surface sterilized with 0.1 per cent mercuric chloride for 3 min, followed by inoculation in the media contained 25 mg l^{-1} copper sulphate registered 100 per cent survival of the cultivars.

The basal media requirement is highly essential for culture establishment and subsequent growth of *in vitro* pollinated ovary/ovule. In the present investigation, MS medium was superior to ½ MS, SH and Nitsch (Table 20) with respect to the ovary development (Plate 5). However, the above media devoid of plant growth regulators did not support ovule development. The suitability of MS medium with plant growth regulators for *in vitro* culture of heliconia has been reported earlier (Nathan *et al.*, 1993; Goh *et al.*, 1995; Yihjuh *et al.*, 1999; Taludkdar *et al.*, 2002; Babu, 2005). MS medium was also reported as ideal for the *in vitro* pollination of many other crops (Rejaat *et al.*, 1984; Zhou *et al.*, 1991; Van Tuyl *et al.*, 1991; Chi, 2000; Popielarska, 2005; Zenkteler *et al.*, 2005).

Among the various methods of pollination ovule development and seed set was observed in placental pollination, modified placental pollination and test tube fertilization (Tables 21a and b). In all these cases pollen grains along with pollen germination media were applied over the ovules. In the case of stigmatic pollination, stylar pollination, ovarian pollination and intra-ovarian pollination there was ovary development but none of them showed ovule development. In these cases *in vitro* pollinated ovaries turned dark and shrivelled within 30 DAP. This may be due to the inability of the pollen tubes to reach ovule due to the excessive length of style or some of the biochemical factors that prevent the fertilization. This was also supported by Kress (1983) and Berry and Kress (1991). But in the case of placental pollination, modified placental pollination and test tube fertilization, stigmatic, stylar and ovary walls were almost completely removed from the path of the pollen tube. Hence pollen grains come in direct contact with the ovules and germinated pollen grains fertilized the ovules developed into seeds. Placental pollination and test tube fertilization/ovular pollination were induced in the seed set in heliconia but compared to modified placental pollination its efficacy was less with regards to ovule development. In heliconia, three ovules per ovary with one ovule per locule are in basal placentation. Hence, modified placental pollination was found to be the easiest and best for producing *in vitro* seed set

in heliconia (Plate 7 and 8). In the present study, modified placental pollination registered the maximum *in vitro* seed set in heliconia. Seed set through *in vitro* modified placental pollination was also reported in Zingiberales (Valsala, 1994; Renjith, 1999; Bhurke, 2002).

The successful *in vitro* pollination was reported when the flower buds were collected one day before anthesis and pollination done at the time of anthesis. After standardizing *in vitro* pollination technique, attempts were made for standardizing the media supplements for refining the protocol.

The sucrose concentration influences ovary development, ovule development and germination of seeds following *in vitro* pollination. In the present study, a comparison of three different levels of sucrose showed that three per cent sucrose was superior for ovule development. An increasing concentration of sucrose, 6 to 9 per cent reduced ovule development (Table 22). Three per cent sucrose concentration was also reported to be ideal for the ovule development after *in vitro* pollination in turmeric (Renjith, 1999; Vijayasree, 2001), kacholam (Bhurke, 2002), sunflower (Popielarska, 2005), *Melandrinum album* (Zenkteler *et al.*, 2005) and ginger (Devi, 2005).

The plant growth substances are required for ovary and ovule development. Quartrano (1987) reported that hormones play an important role in the stages of seed development and germination. The cytokinin activity at the early stage of embryogenesis is responsible for enhanced seed size of increasing cell number (Michael and Kelbitsch, 1972). Eeuwens and Schwala (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. However in the present study, GA was no effect on the ovule development. Requirement of auxin and cytokinin for the development of *in vitro* pollinated ovules were reported earlier (Valsala, 1994; Bindhu, 1997; Renjith, 1999; Vijayasree, 2001; Bhurke, 2002; Devi, 2005).

In order to standardize a suitable hormone combination for better ovule development after *in vitro* pollination in heliconia, studies were carried

out using BA, NAA and IBA. The individual effect of cytokinin, auxins and combination of both were also studied. Individual effect of auxin and cytokinin caused low to moderate ovule development (Tables 23 and 24). Similar effect was also reported by Renjith in turmeric (1999). Among the auxins tried, NAA was found to be superior to IAA with respect to the ovule development (Fig.5). The combination treatment of cytokinin and auxin was found to be superior to their individual effects. The combination of BA (0.1 to 5 mg l^{-1}) with NAA (0.1 to 2 mg l^{-1}) or IAA (0.1 to 2 mg l^{-1}) were effective for ovule development (Table 25). But the combination of BA (0.1 to 5 mg l^{-1}) with NAA (0.1 to 2 mg l^{-1}) was seen to be better in promoting ovule development (Fig.7). Among these BA 2.5 mg l^{-1} along with NAA 2 mg l^{-1} was best for the good ovule development in heliconia. Similar effects was also obtained in the treatments BA 2.5 mg l^{-1} + NAA 1 mg l^{-1}, BA 2.0 mg l^{-1} + NAA 1.0 mg l^{-1} or BA 2.0 mg l^{-1} + NAA 2.0 mg l^{-1}.

Coconut water contains number of cell division factors and large number of amino acids (Shantz and Steward, 1952; Letham, 1974; Van-Standen and Drewes, 1975). In the present study, coconut water in the medium (5, 10, 15 and20 % v/v) did not support the ovule development (Table 27). But favourable effects of coconut water in ovule development have been reported earlier (Valsala, 1994; Renjith, 1999).

Casein hydrolysate (500 mg l^{-1}) promoted ovule development in heliconia. Casein hydrolysate contains more than 20 amino acid which might have favoured the ovule development in the present study. The favourable effect of casein hydrolysate in ovule development has been reported earlier (Kanta and Mahashwari, 1963; Wagner and Hess, 1973; Zubkova and Sladky, 1975; Valsala, 1994; Renjith, 1999).

In the present investigation, yeast extract in the medium supported ovule development at concentration 250 and 500 mg l^{-1}. The favourable effect of yeast extract in ovule development has been reported earlier by Maheshwari and Lal (1961). Among the organic supplements tried, ovule

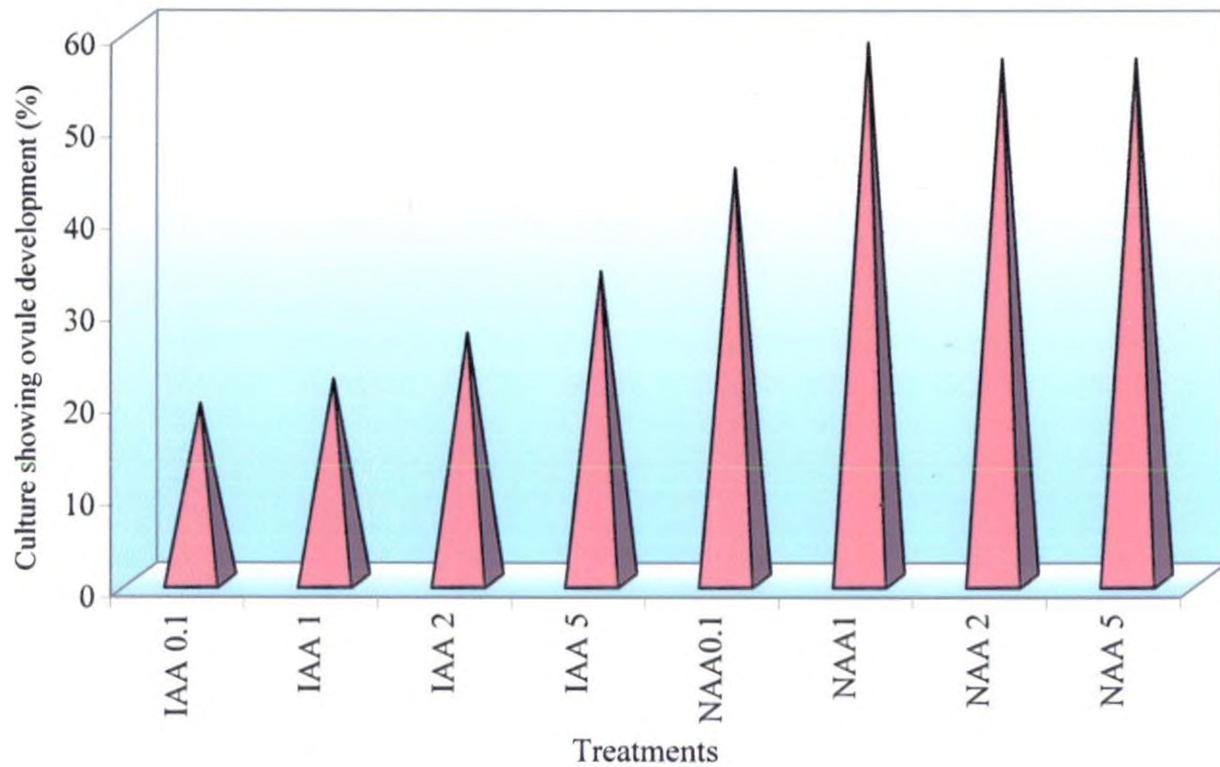


Fig. 5. Effect of auxins on ovule development of heliconia after *in vitro* pollination

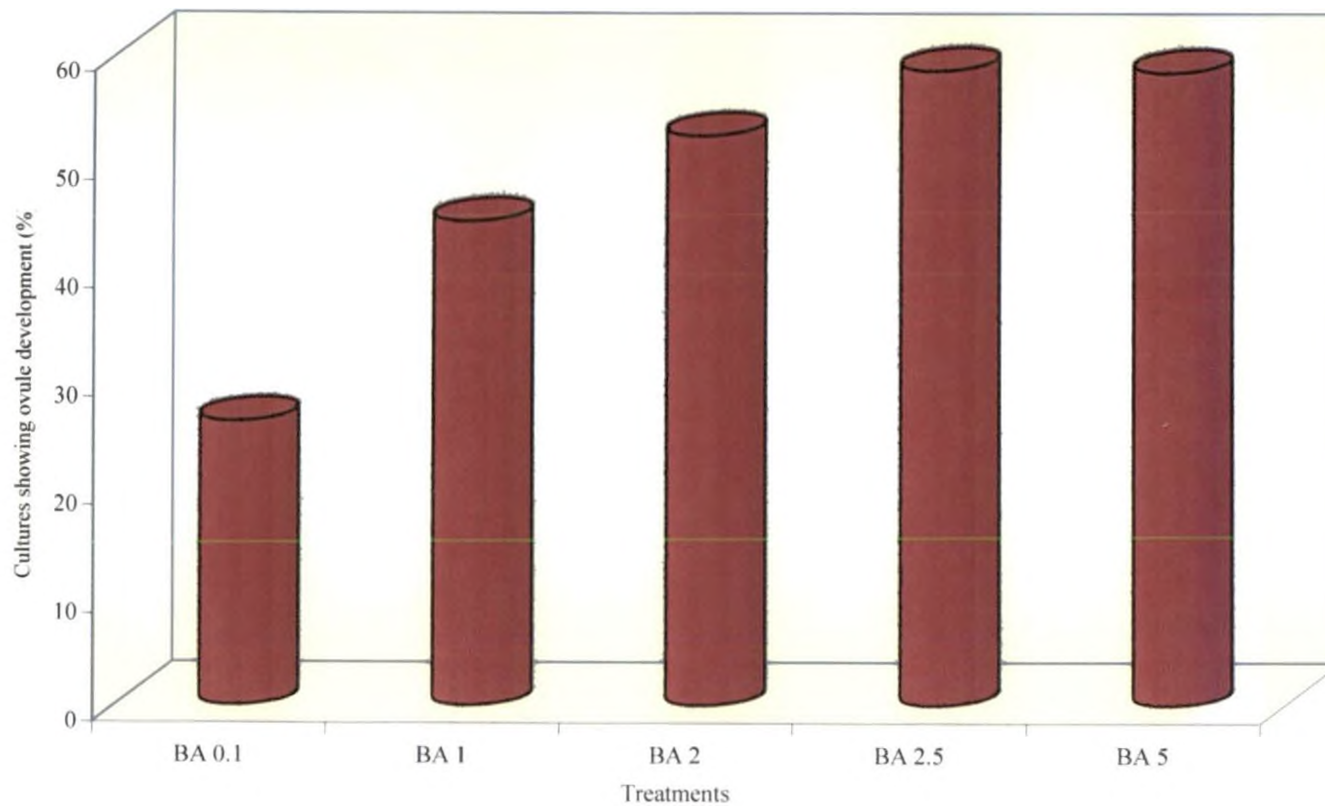


Fig. 6. Effect of cytokinin on ovule development of heliconia after *in vitro* pollination

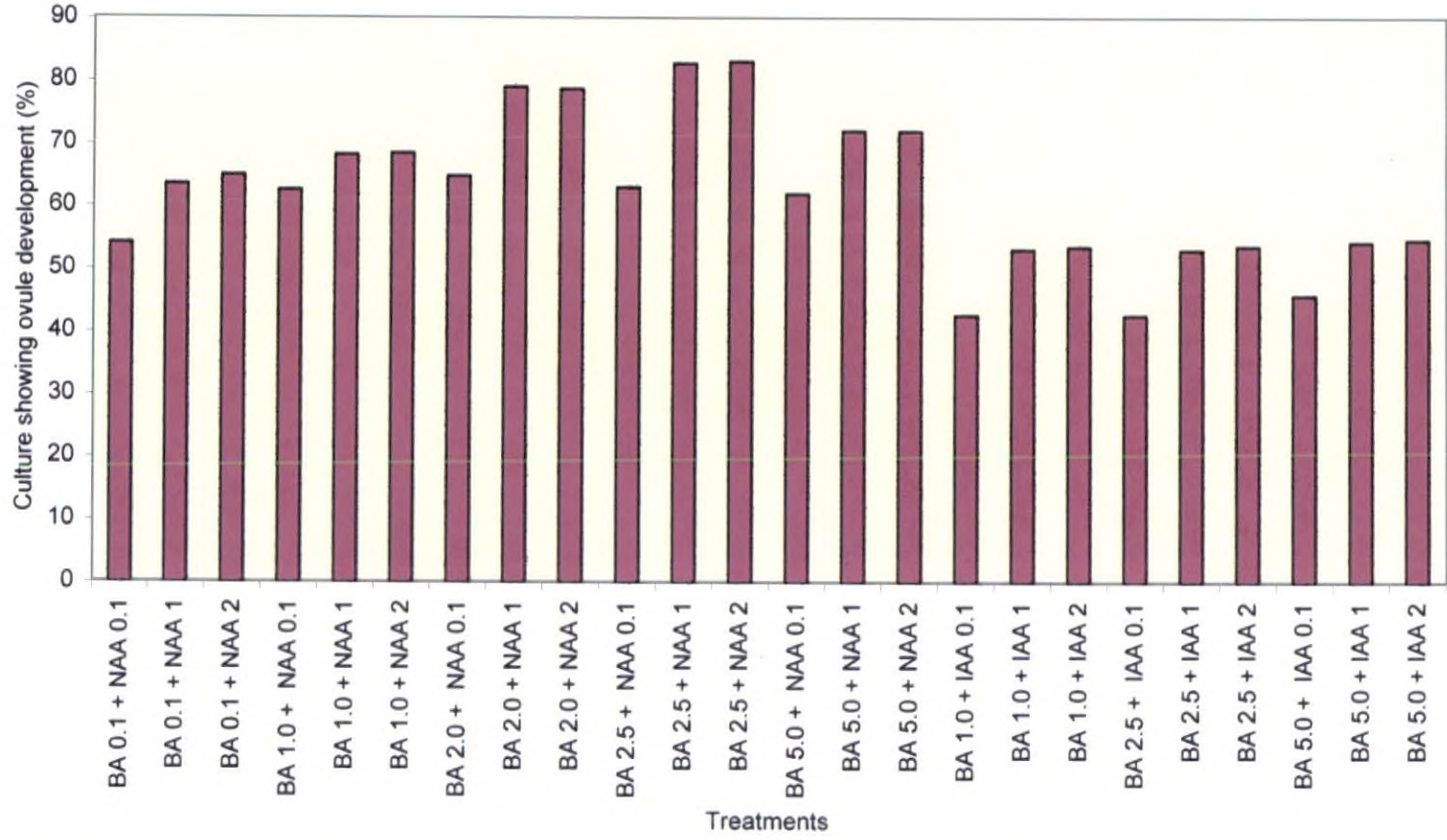


Fig. 7. Combination effect of auxin and cytokinin on ovule development of heliconia after *in vitro* pollination

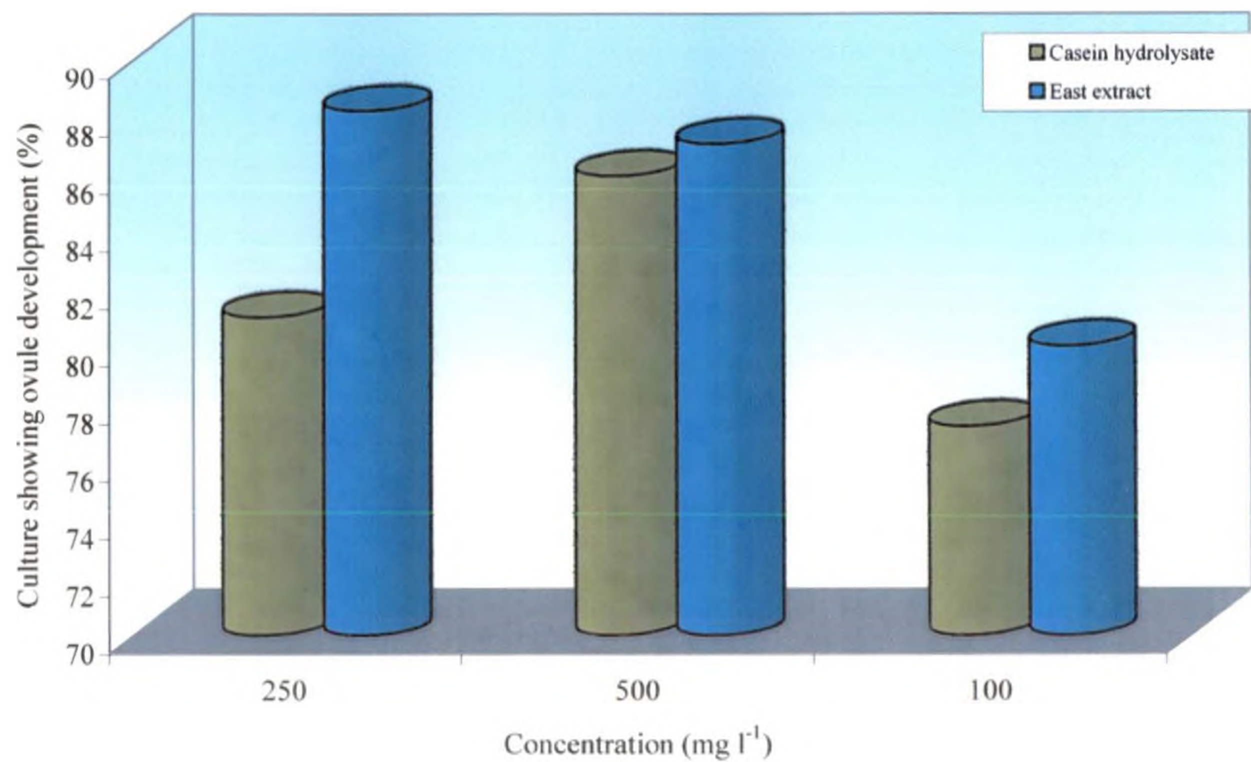


Fig. 8. Effect of casein hydrolysate and yeast extract on ovule development of heliconia after *in vitro* pollination

development was promoted by CH and YE when supplemented along with growth regulators. However, very good result was obtained when media was supplemented with yeast extract (Fig. 8).

The solid medium supported very good ovule development in heliconia. Light had significant influence on ovule development. Under diffused light condition very good ovule development was obtained. Development of *in vitro* pollinated ovules under diffused light was also reported earlier (Rangaswamy and Shivanna, 1967; Rangaswamy and Shivanna, 1969; Van Tuyl *et al.*, 1991; Popielarska, 2005). The pollinated ovules kept at 26 ± 2°C were developed into seeds. This was also supported by Waldt (1976), Balaktova (1977c), Van Tuyl *et al.* (1991), Valsala (1994) and Papielarska (2005). These studies emphasized that controlled conditions are needed for the seed development after *in vitro* pollination in heliconia.

5.3.1 Post Pollination Changes

After *in vitro* pollination, fertilized ovules developed into seeds. The *in vitro* formed seeds of heliconia were elongated oval shape, covered with well developed seed coat. However the size of the *in vitro* seed was less than the *in vivo* formed seeds. Likewise the seed coat was not so hardy in *in vitro* formed seeds, but it is very hardy in *in vivo* seeds.

The colour of the seed was light brown in the initial 20 DAP (Plate 9a). The colour changed to brown within 40-60 DAP (Plate 9b and c). And colour changed to dark brown within 90 DAP (Plate 9d). The seeds turned completely black after 90 DAP (Plate 9e). Both sides of the seed were convex, sometime ventral side become depressed. The size of the seed measured 1800 μm in length and 1575 μm in breadth.

The histological examination of ovules showed well developed endosperm and embryo inside the seed. The embryos formed in vitro are seated at the chalazal end (Plate 11). Three progressive stages of embryos were identified globular, reniform and elongated shape. The size of the seed

increases only upto 20th day. Thereafter maturation of the embryo and endosperm occurs. In the case of embryo the shape of the embryo varied during maturation. In the initial stage it was globular (20th days) (Plate 14a) which changed to reniform on the 40th day (Plate 14b). Later it become elongated shape on 80th DAP (Plate 14c). There were no abnormalities noted in the embryo development after *in vitro* pollination. But the embryo after 90 DAP turned brown (Plate 12) and finally dark *i.e.*, embryo became dead 90 DAP if they were retained in the seed. So there may be none of the seeds germinated in the *in vivo* / *in vitro* seed germination studies. The seeds kept for germination retain as such for some days thereafter it turned black.

In the present work, attempts have been made to integrate *in vitro* pollination and fertilization and embryo rescue. In all crosses where viable seeds are not formed, to avoid embryo abortion with the purpose of producing viable plant, it may be appropriate to excise this embryo and culture in a suitable nutrient medium. Successfully developed interspecific and intervarietal hybrids of *Lilium* sp. through embryo culture by Kim *et al.* (1991) and Okazaki *et al.* (1992) have been reported.

In embryo culture, embryos of three different age (20, 40, 60 and 80 DAP) were dissected out and cultured in a basal MS medium with three different levels of sucrose (3, 6 and 9 %). Among these, embryos of 40 days old in three per cent sucrose medium showed indication of germination, 60 days after embryo culture (Plate 15a). The suitability of MS medium with three per cent sucrose for embryo culture in heliconia was reported earlier by Carlos *et al.* (2005). Embryo germination indices started in 60 days after culturing in the cultures kept at 26 ± 2°C in dark. Ninety days after embryo culture, embryos showed initiation of radicle and plumule (Plate 15b).

After observing germination indices, the embryos were transferred under diffused light conditions. The embryo was subcultured into MS medium supplemented with three per cent sucrose and BA 0.1 mg l^{-1} and IBA 0.1 mg l^{-1}. The hormones may be able to induce further growth of the embryo

into viable plants. The size of the embryos increased very slowly, and greenish tinge was seen on the embryos. There was also increase in length of the radicle and plumule. This can be considered as a positive indication for the progress in this direction. So for the crop improvement programme of heliconia, *in vitro* pollination and fertilization followed by embryo rescue is ideal. Success in this line depends on the refining of the media composition for the embryo rescue, using various media supplements also tried the effect of various basal media, for the growth enhancement of embryo. Since the growth of embryo is at a very slow pace, application of growth enhancing hormones combined with other media supplements will hopefully accelerate the pace of development of the embryo. Further experiments are to be continued in this direction.

To conclude, the results of the present study, among the different *in vitro* pollination techniques, modified placental pollination technique is ideal for *in vitro* pollination and fertilization in heliconia when suspended pollen grains in ME_3 medium used for pollination. The cultural conditions for the development of fertilized ovules to mature seeds have been standardized. Histological examination of *in vitro* pollinated ovules showed well developed embryo and endosperm. However, none of the seeds germinated under *in vivo*/ *in vitro* treatments because embryo became dead 90 DAP, if retained in the seed. Thus in the present study, *in vitro* pollination and fertilization was integrated with embryo culture. The embryo rescued, 40 DAP in MS medium with three per cent sucrose showed germination indices after 60 days of embryo culture. Ninety days after embryo culture showed initiation of plumule and radicle in the embryo. This can be considered as a turning point in the crop improvement programme of heliconia. Embryos can be successfully developed into the viable plants by refining the media requirement for embryo rescue. *In vitro* pollination and fertilization followed by embryo rescue, opens up new vistas for the hybridization programme in heliconia.

Summary

6. SUMMARY

Attempts were carried out to "Standardize *in vitro* pollination and fertilization technique for heliconia" at the Department of Pomology and Floriculture utilizing the facilities available at Plant Tissue Culture Laboratory Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2005-2006.

The salient findings of the above studies are summarized below:

1. The pollen fertility of selected three cultivars, Lady Di, Andromeda and Parakeet were 93.04 per cent, 83.61 per cent and 83.92 per cent, respectively.
2. Pollen grains germinated in Brewbaker and Kwack medium when PEG was used as osmoticum. But there was no pollen germination in the medium without osmoticum and when sucrose was the osmoticum.
3. In Brewbaker and Kwack medium, pollen germination was significantly more in media containing 12 per cent PEG. Maximum pollen germination recorded was 22.37 per cent.
4. ME_3 medium with 12 per cent PEG recorded maximum pollen germination of 61.31 per cent. Significant difference was observed between the cultivars with respect to pollen germination. Among the cultivars Lady Di recorded the highest pollen germination followed by Andromeda and Parakeet.
5. An increase in concentration of PEG upto 12 per cent in medium resulted increase in percentage of pollen germination. However, further increase in PEG level to 15 per cent was not favourable for pollen germination.

6. Among the different pH level (pH 4 to 8) tried in ME3 medium, significant difference was not observed with respect to percentage of pollen germination, However significant difference was observed among the cultivars. Among the cultivars Lady Di recorded highest germination (62.08 %) followed by Andromeda (56.72 %) and Parakeet (54.37 %).
7. Monitoring of pollen germination at various time intervals showed that pollen germination started within ½ h of incubation and progressed upto 3 h of incubation. After 3 h, there was no significant difference in percentage of pollen germination.
8. ME3 medium recorded pollen length of 454 µm whereas Brewbaker and Kwack medium recorded mean pollen tube length of 209.78 µm.
9. Among the two media tried, ME3 medium with 12 per cent PEG was found to be suitable for pollen germination and pollen tube growth in the three heliconia cultivars studied.
10. Morphological studies of gynoecium showed that there was no significant difference between the selected cultivars with respect to various characters of gynoecium *viz.*, style length, ovary length, ovary diameter, number of ovules per ovary, length of ovule and breadth of ovule.
11. The flower buds surface sterilized with 0.1 per cent mercuric chloride for 3 min, followed by inoculation in the medium containing 25 mg l^{-1} copper sulphate, registered 100 per cent survival of the cultures.
12. Out of four basal media tried for ovary development in heliconia, MS medium was the best when supplemented with growth regulators.
13. Among various methods of pollination tried, ovule development was observed in placental pollination, modified placental pollination and test tube fertilization.

14. Even though ovary development after *in vitro* pollination occurred in stigmatic pollination, stylar pollination, ovarian pollination and intra-ovarian pollination, none of them showed ovule development.
15. Among the various methods of *in vitro* pollination tried, modified placental pollination was found to be convenient and best for *in vitro* pollination and fertilization with high percentage of ovule development (78.32 %).
16. The flower buds collected one day before anthesis were suitable for successful *in vitro* pollination in heliconia.
17. MS medium with three per cent sucrose was ideal for the ovule development in heliconia after *in vitro* pollination.
18. The ovule development was maximum after *in vitro* pollination when combination of auxins (NAA and IAA) and cytokinin (BA) in the culture medium supplemented with three per cent sucrose.
19. Replacement of NAA with IAA produced ovule development in 42.57 to 53.62 per cent but the ovule swelling was not upto the level as that in the optimum combination of BA and NAA.
20. Among the combination treatments of auxin and cytokinin, BA 2.5 mg l^{-1} + NAA 2 mg l^{-1} was found to be the best and it produced optimum ovule swelling in 88.05 per cent of the cultures.
21. The GA levels tried (0.1 to 10 mg l^{-1}) did not induce ovary or ovule development in heliconia after *in vitro* pollination.
22. The CW (5, 10, 15 and, 20 per cent v/v) did not favour the ovule development after *in vitro* pollination in heliconia.
23. The organic supplement CH (500 mg l^{-1}) enhanced ovule development in heliconia after *in vitro* pollination, when supplied with MS + BA 2.5 mg l^{-1} + NAA 2 mg l^{-1} with three per cent sucrose.

24. The YE (250 and 500 mg l^{-1}) recorded maximum ovule development in heliconia after *in vitro* pollination when supplied along with MS + BA 2.5 mg l^{-1} + NAA 2 mg l^{-1} with three per cent sucrose.

25. The cultures kept under diffused light showed very good ovule development.

26. Solid medium was ideal for the ovule development of heliconia after *in vitro* pollination.

27. The ovule developed when incubated at 26 ± 2°C after *in vitro* pollination

28. The seed development showed that the growth was very rapid during the initial days. Seeds became maximum size within 20 days. Twenty days after pollination the mean length of the ovule was 1800 μm and breadth of 1575 μm.

29. The colour of the ovule during initial stages was creamy white, which turned to light brown within 20 DAP changing to brown 45-60 DAP and then dark brown to 60-90 DAP. The seeds turned completely black 90 DAP.

30. Histological examination of *in vitro* pollinated ovules showed different stages of embryos *viz.*, globular, reniform and elongated shape.

31. Ovules dissected from *in vitro* pollinated seeds 20 DAP showed globular embryo, 40 DAP reniform shaped embryo and 60 DAP elongated embryos.

32. Endosperm constitute major portion of the seeds with very good filling inside the seed. The endosperm was soft and jelly like in the early days and it became cellular and well developed 20 DAP.

33. Embryological studies of the ovules revealed presence of well developed embryo and endosperm in the *in vitro* pollinated ovules. *In vitro* culture did not stimulate egg cell induction and autonomous endosperm development in unpollinated ovules cultured on the same media.

34. The seeds formed after *in vitro* pollination were subjected to various *in vivo* and *in vitro* treatments. But these treatments did not result in seed germination.

35. Embryo culture studies conducted with *in vitro* formed seeds, resulted in embryos of 40 days maturity in MS medium with three per cent sucrose, showed germination indices 60 days after embryo culture.

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*Original not seen

Appendices

APPENDIX – I

Composition of Brewbaker and Kwack medium

Nutrient	**Quantity mg l^{-1}**
Boric acid (H_3BO_3)	100
Calcium nitrate (CaNO3)	300
Magnesium sulphate (MgSO4)	200
Potassium nitrate (KNO_3)	100

APPENDIX – II

Composition of ME_3 medium (Leduc *et al.*, 1991)

Nutrient	Quantity ($mg\ l^{-1}$)
Macronutrients	
$MgSO_4 . 7H_2O$	370.00
KNO_3	950.00
$KH_2 PO_4$	85.00
$CaCl_2 . 2H_2O$	880.00
$NH_4 NO_3$	412.50
KCl	175.00
Na_2EDTA	7.45
$FeSO_4 . 7H_2O$	5.55
Micronutrients	
H_3BO_3	50.00
$MnSO_4 . H_2O$	16.80
$ZnSO_4 . 7H_2O$	10.50
KI	0.83
$Na_2MoO_4 . 2H_2O$	0.25
$CuSO_4 . 5H_2O$	0.25
$CoCl_2 . 6H_2O$	0.25
Vitamins	
Thiamine	1.00
Pyridoxine	1.00

APPENDIX – III

Composition of MS, SH and Nitsch basal media

Nutrients	Quantity (mg l^{-1})		
	MS	SH	Nitsch
Macronutrients			
NH_4NO_3	1650.00	-	-
$(NH_4)_2SO_4$	-	-	-
$NH_4H_2PO_4$	-	300.00	-
KNO_3	1900.00	2500.00	1250
KH_2PO_4	170.00	-	1250
$MgSO_4.7H_2O$	370.00	400.00	1250
$CaCl_2.2H_2O$	440	20.00	-
$CaNO_3.4H_2O$	-	-	500
Micronutrients			
H_3BO_3	6.20	5.00	5.0
$MnSO_4.4H_2O$	22.30	13.20	3.0
$ZnSO_4.7H_2O$	8.60	1.00	5.0
KI	0.83	1.00	-
$Na_2MoO_4.2H_2O$	0.25	0.10	0.25
$CuSO_4.5H_2O$	0.025	0.20	0.25
$CoCl_2.6H_2O$	0.025	0.10	-
$FeSO_4.7H_2O$	27.85	15.00	-
$Na_2EDTA.2H_2O$	37.25	20.00	-
$FeC_6O_5H_7.5H_2O$	-	-	10.00

APPENDIX – III Continued

Nutrients	Quantity (mg l^{-1})		
	MS	SH	Nitsch
Vitamins			
Thiamine, HCl	0.10	5.00	0.25
Pyridoxine, HCl	0.50	0.50	0.25
Nicotinic acid	0.50	5.00	1.25
Ca-Pantothenate	-	-	0.25
Amino acid			
Glycine	2.00	-	7.5
Others			
Inositol	100.00	1000.00	-
Sucrose*	30.00	30.00	30.00
Agar*	6.00	6.00	6.00

*gl^{-1}

STANDARDIZATION OF *IN VITRO* POLLINATION AND FERTILIZATION TECHNIQUE FOR HELICONIA

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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 "Standardization of *in vitro* pollination and fertilization technique for heliconia" were carried out at the Department of Pomology and Floriculture utilizing the facilities available at Plant Tissue Culture Laboratory Department of Plant Biotechnology, College of Agriculture, Vellayani during 2005-2006 with the objective to standardize various steps of *in vitro* pollination technique in heliconia.

Three cultivars of *Heliconia psittacorum viz.*, Lady Di, Andromeda and Parakeet were selected for the study.

The mean pollen fertility with acetocarmine stain in the selected cultivars was high ranged which from 83.61 to 93.04 per cent.

Attempts to develop a medium which will support pollen germination and tube growth in heliconia resulted in the identification of ME_3 medium with 12 per cent PEG. The pH reaction ranging from 4 to 8 did not influence the pollen germination. Pollen germination started within ½ h of incubation and continued upto 3 h. Regarding pollen germination, ME_3 medium with 12 per cent PEG, cultivar Lady Di recorded highest germination per cent (62.08 %) followed by Andromeda (56.72 %) and Parakeet (54.37 %).

Among the selected cultivars, there was no significant difference in morphological characters of gynoecium.

Successful *in vitro* pollination was obtained when the flower buds were collected one day before anthesis and pollination done at the time of anthesis. The flower buds surface sterilized with 0.1 per cent mercuric chloride for three minutes, followed by inoculation in the medium containing 25 mg l^{-1} Copper Sulphate registered 100 per cent survival of the cultures.

MS medium was superior to ½ MS, SH and Nitsch medium with respect to the ovule development when supplemented with growth regulators.

Among the various methods of pollination, ovule development and seed set was observed in placental pollination, modified placental pollination and test-tube fertilization. In all these cases pollen grains along with pollen germination media were applied over the ovule. In the case of stigmatic pollination, stylar pollination, ovarian pollination and intra-ovarian pollination, there was ovary development, but none of them showed ovule development. Among the successful *in vitro* pollination techniques, modified placental pollination was found to be convenient and recorded maximum ovule development in heliconia.

Studies of three different levels (3, 6 and 9 %) of sucrose showed that three per cent sucrose was superior for ovule development.

Among the auxins, NAA was found to be superior to IAA. The combination treatments of auxin and cytokinin were found to be superior to their individual effect. Among these, combination of BA with NAA was seen to be better in promoting ovule development. The treatment BA 2.5 mg l^{-1} + NAA 2 mg l^{-1} was recorded highest ovule development in heliconia after *in vitro* pollination.

Other supplements like CH (500 mg l^{-1}) and YE (250 and 500 mg l^{-1}) enhanced ovule development along with auxin and cytokinin. However in the present study, coconut water (5, 10, 15 and 20 % v/v) did not support the ovule development after *in vitro* pollination.

Solid medium supported the ovule development after *in vitro* pollination than liquid medium. Cultures kept at 26 ± 2°C developed seeds under diffused light condition.

In vitro formed seeds of heliconia were elongated oval shape. The colour of the seed was light brown in the initial 20 days, turned brown within 40-60 days and become completely black after 90 days. The seed measured 1800 µm in length and 1575 µm in breadth.

The histological examination of ovules showed well developed endosperm and embryo. The embryo seated at chalazal end and three progressive stages of embryos were identified *viz.*, globular, reniform and elongated shape. The embryos become dead 90 days after pollination when they were retained in the seeds.

Hence attempts have been made to integrate *in vitro* pollination and fertilization and embryo rescue. When 40 days old embryos were cultured in MS medium with three per cent sucrose, germination indices were observed 60 days after embryo culture, kept at 26 ± 2°C in dark.

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