VALIDATION OF TESTS FOR SEX DETERMINATION IN PAPAYA (*Carica papaya* L.)

by

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(2011-12-103)

THESIS

submitted in partial fulfilment of the

requirement for the degree of

MASTER OF SCIENCE IN HORTICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF POMOLOGY AND FLORICULTURE

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM- 695 522

KERALA, INDIA

2014

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I hereby declare that this thesis entitled **"Validation of tests for sex determination in papaya (*Carica papaya* L.)"** 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, fellowship or other similar title of any other University or Society.

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ACKNOWLEDGEMENT

This thesis is the end of my journey in obtaining my Master's degree. I have not travelled in a vacuum in this journey. This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people including my well-wishers and my friends. At the end of my thesis, it is a pleasant task to express my thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me.

I bow my head before ***God Almighty*** *for rendering His unseen hands that held me during difficult times in my life and lighted all roads to help me achieve my goal.*

At this moment of accomplishment, first of all I pay homage to my guide, ***Dr. C. S. Jayachandran Nair,*** *Professor and Head, Department of Pomology and Floriculture for his valuable and affectionate guidance, constructive criticism and constant encouragement. This work would not have been possible without his guidance, support and encouragement. Under his guidance I successfully overcame many difficulties and learned a lot.*

I wish to express my sincere gratitude to ***Dr. Sabina George****, Professor, Department of Pomology and Floriculture and member of Advisory Committee, for her valuable suggestions and critical evaluation during the course of this work.*

I sincerely extend my profound gratitude to ***Dr. V. L. Sheela****, Professor, Department of Pomology and Floriculture and member of Advisory Committee, for her guidance and suggestions rendered to me in formulating the thesis.*

I am particularly grateful to ***Dr. K. B. Soni,*** *Associate Professor, and Department of plant biotechnology and member of Advisory Committee, for her kind support in molecular work which has been of great value.*

I cordially offer my sincere and heartfelt gratitude to ***Dr. Thomas George,*** *Associate Professor, Department of Soil Science and Agricultural Chemistry for his helpful suggestions and guidance in conducting the chemical analysis.*

I am highly thankful to ***Dr. R.V. Manju****, Associate Professor, Department of Plant Physiology for her moral support and encouragement throughout my research work.*

I take this opportunity to express my obligations to farm officer Ajith sir, Grancis chechi and all other non teaching staff of Pomology and Floriculture Department for their sincere co-operation and assistance rendered during the course of investigation.

All the teaching and non-teaching staff of Plant Biotechnology department, Plant physiology department and Agronomy department who had helped me during the course of my study, I express my indebtedness and thanks.

I owe immense gratitude to Kerala Agricultural University for providing an opportunity to be a research scholar in my career.

Words fail to express my whole hearted thanks to ***Niya chechi*** *(PhD student, Plant Physiology) and* ***Asish*** *(PG student, Plant Biotechnology) for their timely help and support by scarifying their valuable time during biotechnology related work and for providing all the necessary facilities in Biotechnology and Physiology departments. Without their help this thesis would not have been realized.*

My loving and wholehearted thanks to my all time batch mates and dear friends Mekha, Asha, Abhi, Shruthy, Manju, Amla, Neethu and Cuckoo for being with me from beginning to end, lending me a helping hand whenever I needed it most. Their care and constant encouragement has gone a long way in helping me to overcome the problems I had to face during the course of my work.

The inspiration, moral support, encouragement and good-will bestowed on me by my friends Noelin Chinnu Thomas, Harikrishnan, Aswini, Divya, Angela,

Jyolsna, Nilesh, Dattathrey, Gajanan, Anand, Vineeth and Praveen are acknowledged with greatest gratitude. Special thanks to my UG mate Devendra Mahawar for his help in collecting literature.

I find special pleasure in expressing whole hearted thanks to my seniors Lekshmi chechi (PhD student, Plant Biotechnology), Shimi chechi (PhD student, Agronomy), Anis chechi (PhD student Entomology), Sheena chechi, Vikram chettan and Shajuma chechi for their help, valuable advice and guidance during course of my work.

I wish to express my gratefulness to juniors Sreejith, Aryamba, Jacob Shemon, Henry Nicholas, Hemanth and Jayalekshmi for their timely help and support.

No words are left to express my feelings for my beloved ***Achan, Amma and Kunjummay*** *for their infinite love, blessings, prayer and selfless sacrifices made for my better and bright future without which this venture would have remained a dream. Last but not the least; I express my never ending love and gratitude to my* ***brother Vishnu*** *who always contributes to the best part of me.*

Meera Mohan, N.

Dedicated to

My Family and Guide

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LIST OF ABBREVIATIONS

Abbreviation	Expansion
%	per cent
µg	micro gram
Cm	centimeter
et al.	And others
Fig.	Figure
G	gram
H	hour
Ha	hectare
I.U	International Unit
KAU	Kerala Agricultural University
Kg	kilogram
M	meter
Mg	milligram
Min	minutes
Ml	milliliter
Mm	millimeter
Nm	nanometer
^{o}C	degree celsius
RAPD	Random Amplified Polymorphic DNA
S	seconds
Var.	variety
V	volts

INTRODUCTION

1. INTRODUCTION

Papaya (*Carica papaya* L.) which belongs to the family Caricaceae is one of the major fruit crops cultivated in the tropics and subtropics. It has long been known as a 'wonder fruit of the tropics' and is grown primarily for its delicious fruits and for extraction of its digestive constituent papain. The tree bears fruit round the year and has the highest productivity next to banana. Ripe fruits of papaya serve as a cheap source of carbohydrates, minerals, vitamins, pectin and alkaloids in daily diet. The milky latex which is abundant in the immature fruit of papaya is rich in the proteolytic enzyme papain which has medicinal value and a variety of industrial uses.

India is one of the leading papaya producers in the world with an area of 1,06,000 hectares and an annual production of 41,96,000 MT (Anon., 2011). In India the major papaya growing states are Andhra Pradesh, Gujarat, West Bengal, Karnataka, Maharashtra and Tamilnadu. In Kerala, papaya is cultivated in an area of 16,156 hectares with an annual production of 1,01,000 MT (Anon., 2011).

In Kerala, papaya is commonly cultivated as a homestead crop. The major hurdle in commercial cultivation of papaya is its varied sex forms. Three basic sex types seen in papaya are male, female and hermaphrodite. There are no sex linked morphological characters identified so far and sex cannot be predicted till mid development stage when the plants start flowering. In order to overcome this problem, growers are forced to maintain three plants per pit till flowering so that at least one of them may be female. Rouging unwanted male plants from dioecious papaya orchards is a cumbersome procedure, usually followed in cultivation which results in wastage of inputs, time and labour cost. The identification of sex type prior to transplanting would help to solve this problem.

Colorimetric tests based on phenol content are reported to be helpful in identifying male and female seedlings at pre-flowering stage. However, the accuracy of these tests varies from one another and from one sex form to the

other. Comparison of these tests may help to arrive a method to attain a better level of accuracy in sex determination. The best test can be utilized for determining the sex form of papaya plant in the pre-flowering stage.

Sex determination in papaya based on morphological and agronomic traits, biochemical markers, isozyme analysis etc. are influenced by environmental conditions and stage of development of seedlings. Recently, DNA based molecular markers using PCR technology was introduced as a more reliable strategy, since it is not affected by environmental conditions and growth stage of the plant. Among these RAPD (Random Amplified Polymorphic DNA) marker technique is a quick, easy to assay and widely accepted one. It is used for sex determination, characterization of genetic variability, determination of somaclonal variants and hybrids as well as in taxonomic studies.

The present study, therefore, was initialised to compare the efficiency of the chemical tests developed for identification of sex forms in papaya in order to find a reliable test for sex of papaya plants in the pre flowering stage. It also aimed at identification of sex forms in papaya using RAPD markers.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 SEX EXPRESSION IN PAPAYA

Papaya is a polygamous plant and has three basic sex types - staminate, hermaphrodite and pistillate. Though considered dioecious in nature, papaya expresses many sex forms. In papaya, flowers fall into continuous series of forms with regard to morphology and sex expression. On one extreme are the unisexual staminate flowers with 10 stamens and a rudimentary pistil. On the other extreme are the unisexual pistillate flowers devoid of stamens, but with a large functional pistil. In between these are numerous types, mostly bisexual, but differing in the number of carpels and stamens. According to the classification of Storey (1958), papaya has 40 sex types which can be classified into 8 working groups - typical male, typical female and six intermediate (hermaphrodite) sex forms viz, teratological staminate, reduced elongata, elongata, carpelloid elongata, pentandria and carpelloid pentandria.

2.1.1 Genetics of sex expression in papaya

The inheritance of sex in papaya is complicated. Hofmeyr (1938) and Storey and Jones (1941) suggested that sex in papaya is controlled by a single gene with 3 alleles. Accordingly, male and hermaphrodite are heterozygous for sex and female is homozygous. Sex determination in papaya was elucidated by Hofmeyr (1939) forwarding the genic balance hypothesis. The sex in papaya was determined by not a single gene, but rather a complex of genes or a series of allelic genes which lie closely linked in differential segments occupying identical regions on sex chromosomes (Storey 1953., Horovitz 1954a). The sex determining segments behave in heredity as if they were unit factors. Out of the 40 types, 32 are heritable types. All combinations of the dominants are lethal to the zygote. Males are enforced heterozygotes. The genotype of the zygotes are therefore M_1m=Male, M_2m=Hermaphrodite and mm= female. Staminate forms

are sex reversing, while pistillate are more stable and hermaphrodite are most unstable forms.

Their crosses and progeny segregation were as follows:

mm x M_1M - 1 mm: 1 M_1m

mm x M_2m - 1mm:1M_2m

M_2mxM_2m - 1mm: 2M_2m: 1M_2M_2

M_2mxM_1m - 1mm: 1M_2m: 1M_1m: 1M_1M_2

2.1.2 Environmental factors related to sex expression

Storey (1958) opined that, sex expression was dependent on temperature and photoperiodism. There were two independent set of factors which modify sex expression in male and hermaphrodite under certain environmental conditions. One set was responsible for seasonal shift from female fertility to female sterility and *vice versa*. The other set caused stamens to become carpelloid usually with the fusion to the pistil. The set of factors either singly or in combination differentiate 15 sex forms in papaya. Trees carrying the factors produce mixtures of normal and teratological fruits and tree free from factors produce normal fruits. The studies conducted under Hawaii conditions showed that female sterility was enhanced by warm weather and long day conditions. Female fertility was promoted by cool weather and short days. Singh *et a*l. (1961 a) observed that in North Indian conditions female sterility was more in winter and female fertility was more in warm weather.

2.1.3 Physiological factors related to sex expression

Choudhary *et al.* (1957) found that the leaves of male plants were richer in total carbohydrate, phosphorus and chlorophyll a and chlorophyll b when compared with the female plants which were rich in nitrogen and potash. According to Kashinatan *et al.* (1965) female flowers were found to contain significantly more asparagin, arginine and histidine and less alanine and aspartic

acid than the male flowers. According to Jindal and Singh (1976 b), leaf petiole and bark of male plants are high in phenolic compounds. They also observed that application of TIBA enhanced femaleness.

2.2 SEX IDENTIFICATION IN PAPAYA

A sex linked character in seed or seedling which might be useful in early detection of sexes has not been discovered. The precocious separation of one pair of chromosome was found at anaphase -1 of meiosis in males and hermaphrodites (Kumar *et a*l., 1945). They did not observe such a separation in anaphase 1 of the female. The precocious separation of one pair of chromosome with complete 9:9 chromosomal separation was also found in males. Karyological analysis showed that there was a satellite chromosome in the male plant. This satellite chromosome determines male sex in papaya. The homologue chromosome was not satellite (Ram, 1982).

2.2.1 Morphological and biochemical markers for sex identification

Morphological characters

Bojappa and Singh (1974 a) made an attempt to identify sex of papaya at vegetative stage by root characters. Seedlings at 3-4 leaf stage were transplanted into 2 groups comprising those with a distinct tap root and normal secondary roots and those with no tap root but with laterals of almost equal thickness and length. They observed that plants with a branched root system flowered earlier and grew tall than those with a tap root. There were only slight differences in the percentage of pistillate, staminate and hermaphrodite plants in the two groups indicating that seedling root characteristics were not sex-linked.

Singh *et al.* (1977) conducted studies in papaya to correlate sex with seed characteristics and vegetative characteristics of plants. Seeds of papaya cultivar HoneyDew were divided into 27 grades depending up on their size, colour and density. Dark seed colour was associated with a high proportion of female and hermaphrodite plants. While taking two characteristics at a time, dark brown

seeds of medium size were found to be predominantly pistillate where as the same colour accompanied by large size was conducive to hermaphroditeness. They found that most seeds of the female group germinated in the first 20 days. No correlation was found to exist between hypocotyls/ epicotyl length of seedlings and their sex.

Rao *et al.* (1985) assessed leaf petiole characters in papaya to determine sex at seedling stage and found that these characters showed no relationship with sex.

Arango *et al.* (2008) evaluated seed characters of two papaya varieties namely the hybrid Tainung- 1 and Improved Sunrise Solo to find the possibility of sex identification at seedling stage either with isozyme pattern or physical seed characteristics. They evaluated the relationship of seed physical characters such as size and weight and the corresponding sex, relationship between seed vigour based on four seed emergence stages and patterns of three isozymes such as peroxidase (PER), esterase (EST), and leucine amino peptidase (leucyl amino peptidase - LAP) using leaf tissues of 4 to 5 leaf stage seedlings. There were no differences between seeds classified by size or weight and sex expression. In Tainung- 1 hybrid, seeds with higher initial vigour developed a higher proportion of hermaphrodite plants in contrast with a lower proportion of female plants from seed with less vigour. Seedlings with lower leaf number developed a higher number of hermaphrodite plants. PER, EST and LAP patterns did not help in the identification of sex of the two cultivars studied.

Biochemical characters

Choudhury *et al. (*1957) reported that male and female plants of papaya may be differentiated on the basis of leaf content of total carbohydrates, phosphorus, nitrogen, potash and chlorophyll a and b. Singh and Jindal (1972) observed a higher amount of free and bound phenolics in male plants compared to female ones.

In the opinion of Todokovo (1930) male plants indicated higher pH compared to the other two sex forms. Similar relations were established by Bojappa and Singh (1974 a) in different sex forms of papaya. They observed that in the case of male plants, 70 per cent of them showed the pH range of 5.5 – 5.8, while 85 per cent of females and 90 per cent of hermaphrodite plants showed a pH range of 4.0 – 5.4. On the contrary, Choudhury *et al.* (1957) reported higher pH in the leaves of female papaya plants compared to males. Such variations according to Bojappa and Singh (1974 a) may be due to the differing environmental conditions under which the plants are grown and also the parts tested and their age. The pH levels in the plant sap or their change with the stage of development may be explained by the metabolic changes in the quantities of buffering compounds.

Horovitz (1954 a) suggested that there are specific male and female florigenic substances produced by the dominant and recessive alleles and it may be possible that the genes governing maleness and femaleness may be producing some substances which react differently to different chemicals. From similar studies conducted in citrus, Halma and Haas (1929), Halma (1934), Marloth (1936), Jungwirth (1953), Benatena (1954) and Krishnamurthy *et al.* (1960) explained this phenomenon on the basis of glucosides containing phenol which react differently from each other with certain reagents.

In similar studies, it was observed that there is sharp increase in chlorogenic acid and p-hydroxy benzoic acid content in leaves associate with the shift from vegetative to flowering stage (Zucker *et al*., 1965 and Gasper *et al*., 1968) and these vegetative plants do not give such exact colour reactions as observed in the sexually differentiated plants. Jindal and Singh (1975) observed that in papaya the same kinds of phenolic could be isolated from male and female plants, although a marked difference was observed between different cultivars. The amount of free, acid-hydrolysable and alkali-hydrolysable phenolic compounds was considerably higher in male plants.

2.2.2 Colorimetric tests for sex identification

Singh *et al.* (1961 b) carried out colorimetric tests for sex identification of papaya in the nursery stage. Among these, tests using remodified semen reagent were very encouraging.

According to Singh *et al.* (1961 b), higher efficiency of forecast for femaleness was due to the absolute sex stability of female plants as against the unstable nature of male and hermaphrodite sex forms. None of the tests were hundred per cent correct in the forecast which needs further investigation to explain the basis for colour distinctions depicted by different sex forms. Similar observations were made by Storey (1953), Horovitz (1954 b) and Singh (1964) who opined that forecast of sex is more accurate in the case of female plants than of male plants. This may be due to greater stability of the female sex. It may also be due to the occurrence of 55 per cent female and only 45 per cent male plants in relatively small population.

According to Bojappa and Singh (1974 b), the same test sometimes showed varied colour reactions in the same sex form. Modified nitrous acid and mercuric nitrate tests have shown French blue, Cerulean blue or Capri blue in relation to expression of maleness where as French blue, Hyacinth blue or Gentian blue were seen in the case of femaleness. In hermaphrodites, Enamel blue or Porcelain blue colour was observed in different situations. Storey (1958) and Singh *et al*. (1963) also observed that the same test in some vegetative seedlings gave different shades of the same colour making exact prediction difficult.

In the opinion of Bojappa and Singh (1974 b), the validity of forecast differed depending upon the tests used. In general, forecast for male plants was less correct compared to the forecast for female and hermaphrodite plants. Tests with nitrous and ferrous compounds were more efficient, the latter being the most reliable one.

Singh *et al*. (1961 b) reported 67 per cent of efficiency in the forecast of male plants and 87 per cent in female seedlings with Modified Almen reagent test

in papaya variety Ranchi. In tests involving ferrous sulphate, the accuracy of prediction in the case of males was 48 per cent and in females 67 per cent. In Titanous chloride test, prediction of sex was 43 per cent accurate in the case of males, while 51per cent accuracy was observed in the case of females.

The levels of accuracy of prediction in the case of male and female sex forms were observed by different scientist. In the Almen reagent test, Bojappa and Singh (1974 b) observed that the accuracy of prediction for male plants was 63 per cent, while for females 77 per cent and for hermaphrodites 76 per cent. In the case of Modified Almen reagent test, the efficiency of forecast for male plants was 69 per cent, while it was 77 per cent for females and 74 per cent for hermaphrodites. Both the tests involved the use of mercury and nitric or nitrous acid. The efficiency of the forecast was higher for females compared to the males and accuracy of prediction for hermaphrodites was almost nearer to females. According to them, the accuracy of the forecasts ranged from 58-71% for male plants where as with female and hermaphrodite plants, the accuracy ranged from 77-90 per cent and 74-89 per cent respectively.

Jindal and Singh (1976 a) conducted colorimetric tests specific to phenolics in the leaves of both sexually differentiated and undifferentiated plants. The precision of sex forecasts with the "Prussian blue test" was 80% in female plants and 60% in male plants. They found that a colorimetric test for total phenolics could distinguish females (86%) from males (77%) but could not detect the hermaphrodites.

Rao *et al.* (1985) reported that modified semen reagent applied to the leaves induced a colour change that correctly identified 92.5% of female plants, 72.5% of hermaphrodites and 70.00% of males. The Prussian blue and ferrous sulphate tests were less efficient.

2.3. MOLECULAR CHARACTERIZATION

Molecular markers are genotypic markers (Bretting and Widrlechner, 1995) used for studying differences among strains at molecular level. Molecular markers are naturally occurring polymorphisms which includes proteins and nucleic acids that are detectably different (Thotapilly *et al.*, 2000). They have proven to be powerful tools in the assessment of genetic variation and in elucidation of genetic relationships within and among species (Chakravarthy and Naravaneni, 2006). Molecular markers include biochemical constituents like secondary metabolites in plants and macro molecules like proteins and DNA.

Biochemical markers that have been used since long for the characterization of variation in a plant are now considered to be inappropriate as universal markers (Cooke, 1984). DNA based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering etc. DNA based molecular markers provide the best estimate of genetic diversity that are not environmentally regulated and are detectable in all stages of plant growth. Because of its plasticity, ubiquity and stability, DNA is the ideal molecule for germplasm identification and characterization (Anolles *et al.*, 1991).

Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization based markers and Polymerase chain reaction (PCR) based markers (Joshi *et al*., 1999). In hybridization based DNA markers, DNA profiles are visualized by hybridizing the restriction enzyme digested DNA to a labelled probe, which is a DNA fragment of known origin or sequence (Kumar *et al*., 2009). RFLP which is one of the important hybridization based technique was used to detect variation at DNA level. However, over the last few years, Polymerase Chain Reaction (PCR) technology has had a significant impact in almost all areas of molecular biology (Saiki *et al*., 1988). PCR based DNA marker techniques are finger printing techniques that use an *invitro* enzymatic reaction to specifically amplify a

multiplicity of target sites in one or more nucleic acid molecules (Anolles and Trigiano, 1997). PCR technology has lead to the development of several novel genetic assays based on selective amplification of DNA (Bardakci, 2001).

Among the PCR based techniques, the important ones are Amplified Fragment Length Polymorphism (AFLP), Microsatellites, Sequence Characterized Amplified Region (SCAR) and Random Amplified Polymorphic DNA (RAPD).

2.3.1. RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

According to William *et al*. (1990), RAPD detects nucleotide sequence polymorphism in DNA by using a single primer of arbitrary nucleotide sequence, (mostly 10 bases long). RAPD was used for fingerprinting genomes (Welsh and Mc Clelland, 1990), for population biology studies (Astley, 1992) identification of genome specific markers and other uses (William *et al*., 1990 and Erlich *et al*., 1991). RAPD technique used in the estimation of genetic relatedness was found very efficient and reliable (Brown *et al*., 1994, Munthali *et al*., 1996).

2.3.1.1 RAPD in sex determination

RAPD was used for sex identification in papaya by Somsri (1998). A PCR based Seedling Sex Diagnostic Assay (SSDA) specially designed for early sexing of papaya seedlings was reported by Parasins *et al*. (2000). They have developed a male specific SCAR marker in papaya by cloning a male specific RAPD (831 base pair) fragment and designing longer primers.

Haoma *et al.* (2001) constructed a papaya BAC library with 13.7x genome equivalents to use for cloning and characterizing sex determination gene. A papaya linkage map was constructed with DNA markers. Five markers were tightly linked to sex determination gene, including 3 AFLP and 2 SCAR markers.

Urasaki *et al.* (2002) used the RAPD technique to determine the sex of *Carica papaya* with three sex forms - male, female and hermaphrodite. They found that a 450 bp marker fragment named Papaya Sex Determination Marker (PSDM) exists in all male and hermaphrodite plants but not in the female plants

so far analyzed. They developed a SCAR marker SCAR Ps from the PSDM which is a suitable marker for the precise and rapid diagnosis of sex in papaya.

Lemos *et al.* (2002) used RAPD technique to differentiate between the sexual forms of three commercial cultivars of *Carica papaya* belonging to the Solo group. Out of 152 primers selected for study, only one primer BC210 was able to detect hermaphrodite sex specific DNA amplification product of approximately 438 base pair length in all of the cultivars tested, while none of the female tested gave this product.

Deputy *et al.* (2002) developed molecular markers tightly linked to the gene that determines plant sex in papaya. Three RAPD products have been cloned and a portion of their DNA sequenced. Based on these sequences SCAR primers were synthesised. The sexing technique, using SCAR T12 and SCAR T1 as a positive control was used to correctly predict hermaphrodite papaya plants in a population of seedlings with an overall accuracy of 99.2%.

Neeta *et al.* (2005) used RAPD technique to identify the sex of papaya seedlings among two dioecious and three gynodioecious cultivars. Out of 280 random primers screened, only one primer OPE-6 elicited a DNA amplification product of 1000 base pair in males and hermaphrodites but not in females. They suggested that OPE-6 can be used to distinguish males in dioecious varieties and hermaphrodites in gynodioecious varieties at the seedling stage itself.

RAPD markers were used to determine the sex types of Colombian cultivars of papaya by Bedoya *et al.* (2007). They found an RAPD marker of 900 base pair in males but not in females or hermaphrodites. They also developed a SCAR marker from this RAPD which had the potential to amplify fragments from the genomes of male and hermaphrodite plants, but not females.

RAPD technique was used to identify male trait in papaya by Xingh *et al.* (2007). They found that a marker fragment Z18-1000 existed in all male plants

but not in the female plants so far analyzed. The fragment was cloned and sequenced and was successfully converted in to a SCAR marker designated as SD 1000.

Esfandiyari *et al*. (2010) used RAPD and SCAR marker to identify sex at juvenile stage in *Pistacia* species. Out of 20 primers screened, only one primer BC1200 amplified a specific bandof approximately 300 base pair length in all female plants but not in males.

Sujitha *et al.* (2012) used RAPD to distinguish sex types of five papaya cultivars in the seedling stage itself. Out of 20 RAPD primers screened using PCR, male and female were clearly distinguished by the profile generated by the marker OPL13. A marker fragment of approximately 2000 base pair exists in all females but not in males.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study, "Validation of tests for sex determination in papaya (*Carica papaya* L.)" was carried out in the Department of Pomology and Floriculture and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala from 2011 to 2013. Three varieties of papaya namely, Pusa Dwarf, Pusa Nanha and Washington were selected for the study.

3.1 RAISING SEEDLINGS

Breeder's seeds of the three varieties were sown separately in large pots and kept in glass house for germination. The two weeks old healthy seedlings were shifted to polythene bags filled with sand, soil and dried cow dung powder in the ratio 1:1:1. When they attained two months age, these seedlings were serially numbered and transplanted in the main field. Twenty seedlings of each variety were planted at a spacing of 2.0 x 2.0 m. The plants were raised as per the package of practice recommendations of Kerala Agricultural University (KAU, 2011).

3.2 CHEMICAL STUDIES

3.2.1 Procedure of leaf sample collection for chemical studies

Three newly emerged leaves were collected from one and a half months old nursery seedlings and dried at 60^0 C , powdered and the water extract (1 g leaf powder in 40 ml distilled water, shaken for 15 minutes and filtered as extract) of the sample was prepared.

The following colorimetric tests were done at pre-flowering stage.

3.2.2 Almen reagent test (Halma and Haas, 1929)

The reagent consists of 50g chemically pure mercury dissolved in 70 ml of red, fuming nitric acid and diluted with 200 ml of distilled water. 5 ml of the

leaf extract was taken and made alkaline adding 3 drops of 5% potassium hydroxide and to this, 10 drops of saturated copper sulphate was added. To this sample, ten drops of semen reagent was added and the solution was boiled for a few seconds. The colour obtained was recorded after an hour. Blue to purple colour and intermediate shades are indicative of male plants. Bluish green to green colour is indicative of female plants.

3.2.3 Ammonium molybdate test (Singh *et al*., 1961)

The ammonium molybdate reagent was prepared by dissolving 100 g of molybdic acid in a mixture of 144 ml of ammonium hydroxide and 271 ml of distilled water. This solution was then poured slowly in to a cool mixture of 489 ml of concentrated nitric acid and 1148 ml of distilled water. To 5 ml of the filtered leaf extract, 10 drops of the reagent was added. The solution was boiled for a few seconds and the colour was noted after an hour.

3.2.4 Ferric chloride test (Clarke and Nord, 1955)

Two drops of saturated aqueous 1% ferric chloride was added to the leaf extract and the colour change was noted**.**

3.2.5 Titanous chloride test (Clarke and Nord, 1955)

A readymade solution of 12.5% concentration was used as the reagent. Two drops of the reagent was added to 5 ml of the leaf extract and the resulting colour was then noted.

The colour developed by the tests was recorded with reference to Nickerson's colour fan distributed by the American Horticultural Council and published by Munsell Colour Co.

Based on the test results, the plants were labelled as male or female. After flowering, the same plants were observed to confirm the results already obtained. The accuracy of the tests was determined using observations

Plate 1. Over view of the experimental plot

3.3 MOLECULAR CHARACTERIZATION STUDIES

3.3.1 Isolation of Genomic DNA

DNA was extracted from young leaves using Cetyl trimethyl ammonium bromide (CTAB) method (Dellaporta *et al*., 1983). 0.5 gram of emerging leaves of papaya before fully unfurling was collected from plants in the morning. The leaves were first washed in running tap water. Leaves were then chopped coarsely and again washed three times in distilled water. Water was wiped off from the fresh leaves using tissue paper. The chopped leaves were then placed in a cool dry porcelain container and ground well to a fine mass in liquid nitrogen. One hundred milligrams of poly vinyl pyrollidone was added to this leaf powder and again ground well. Then this ground mass was transferred to 1 ml pre- heated extraction buffer and then placed in a water bath set at a temperature of 60º C for one hour with occasional shaking. The buffer contained 2 percent w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 0.2 percent β mercapto ethanol and 100 mM Tris HCl of pH 8.0. The homogenate was cooled to room temperature. To this 200 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 10,000 rpm for 10 minutes at 4 ^{0} C. The upper phase was collected and to this 200 µl of chloroform: isoamyl alcohol (24:1) was added. Again centrifugation was done at 10,000 rpm for 10 minutes at 4^0 C. The upper phase was collected. To this 200 µl of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 minutes at 4 ^{0} C. Clear upper phase was collected and to this 1/10 th volume of 3 M sodium acetate and 2 ml of cold absolute ethanol was added. On gentle mixing, DNA strings became visible. Again centrifugation was done at 10,000 rpm for 10 minutes at 4 ^{0} C and the supernatant decanted retaining the pellet. The DNA pellet was washed with 70 per cent ethanol then centrifuged at 10,000 rpm for five minutes at 4 ^{0} C and the supernatant was decanted retaining the pellet. The pellet was allowed to air dry and then dissolved in100 µl TE buffer (10 mM Tris HCl of pH 8.0 and 1 mM EDTA of pH 8.0) and stored at 4 ^{0} C.

All the glass wares and materials used in the preparation and storage of reagents and for the process of DNA isolation including reagent bottles, conical flasks, centrifuge tubes, glass rods, spatula, funnels and tips of micro pipettes were washed with labolin solution, rinsed with distilled water and autoclaved for 45 minutes before use. Phenol was saturated and equilibrated using Tris buffer and pH was adjusted to 8.0.

3.3.2 Quantification of DNA

DNA quantification was done with the help of UV- Vis Spectrophotometer (Spectronic Genesis 5). The absorbance was measured at 260 nm and 280 nm. The spectrophotometer was calibrated at 260 nm and 280 nm wave length using TE buffer in which DNA was dissolved. The optical density (OD) of the DNA sample dissolved in the buffer was recorded at 260 nm and 280 nm.

As optical density of 1.0 at 260 nm represents 50µg/ml of DNA, the quantity of DNA in the sample was estimated as follows:

Concentration of DNA (µg / ml) = A_{260} x50 x dilution factor (A_{260} = absorbance at 260 nm)

The quality of DNA was assessed from the ratio of the OD values recorded at 260 nm and 280 nm. A ratio between 1.8 and 2.0 indicates good quality of DNA.

3.3.3 Electrophoretic analysis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit supplied by the Bangalore Genei. Agarose concentration used was 0.8 per cent for visualising genomic DNA and 1.2 per cent for visualising PCR amplified products. Voltage level tried were 75V and buffer used was 1 x TAE buffer (0.04 M Tris acetate 0.01 M EDTA, pH 8.0).

The required quantity of agarose was weighed out (0.8 per cent for visualizing the genomic DNA and 1.2 per cent for visualizing the amplified

products) and \added to 1x TAE buffer. Agarose was dissolved by boiling. After cooling to 50 ^{0} C, ethidium bromide was added to a final concentration of 0.5µg $ml^{-1.}$ This mixture was immediately poured in to a template with appropriate comb. After solidification, the comb was removed and the gel was mounted in an electrophoresis tank filled with 1x TAE running buffer. The gel was completely covered on the surface by the buffer. The DNA sample was mixed with required volume of gel loading dye (6x loading dye viz., 40.00 per cent sucrose and 0.25 per cent bromo phenol blue). Each well was loaded with 20 µl of the sample. One of the wells was loaded with 1.50 µl of molecular weight marker, in required volume of gel loading dye. Electrophoresis was performed at 75 volts until the loading dye reached three fourth of length of the gel. The gel was visualised using an ultravisible (UV Vis) transilluminator (Appligene oncor, France).

3.3.4 RAPD Analysis

Random Amplified Polymorphic DNA analysis were performed using arbitrarily designed decamer primers supplied by Operon Inc, CA, USA

Genomic DNA (20 ng) isolated was amplified *in vitro* in a reaction mixture containing 2.5 µl 1.0 x buffer (10 mM Tris HCl of pH 9.0,(1.5mM $MgCl_2$ 50 mM KCl and0.01per cent gelatine), 5 pM Primer, 200µM each of deoxy nucleotides (dNTPs) and 0.6 units of Taq DNA polymerase (Banglore Genei, Pvt Ltd., Banglore). The PCR conditions vary with different set of primers. The amplification was done in a programmable Thermal controller (MJ Research Inc.) which was set as follows:

For operon primers: An initial denaturation at 95^{0} C for 3 minutes, followed by 45 cycles of denaturation at 95^{0} C for 1 minute, annealing at 36^{0} C for 1 minute 30 seconds and extension at 72^{0} C for 2 minutes was given. The synthesis step of final cycle was extended further by 6 minutes. Finally the products of amplification were cooled at 4^{0} C.

For SO primers: An initial denaturation at 94^0 C for 5 minutes, followed by 34 cycles of denaturation at 94^0 C for 1 minute, annealing at 39.7 ^{0} C for 1 minute and extension at 72^0 C for 2 minutes. The synthesis step of final cycle was extended further by 10 minutes. Finally the products of amplification were cooled at 4^0 C.

A negative control containing water instead of template DNA was also included in each reaction set.

Electrophoresis was carried out to separate the amplified products along with DNA molecular weight marker supplied by US biochemicals using 1.2 and 1.5 per cent agarose gel in 1x TAE buffer, which was stained with ethidium bromide (0.5 μg ml^{-1}). The DNA bands were visualised on a UV transilluminator (Appligene oncor, France) and the photographs taken using gel documentation system (Biorad, USA).

3.3.5 Number of Monomorphic and Polymorphic Bands

From the amplified products separated by electrophoresis, the number of monomorphic bands and number of polymorphic bands were recorded. The intensity of the fluorescence of the bands was also noted.

3.3.6 Selection of Primers

Those primers which produced maximum number of reproducible bands while amplification were selected.

3.3.7 Molecular characterization of papaya varieties and sex forms

Random amplified polymorphic DNA analysis was performed using the selected primers to amplify the DNA of all the three papaya varieties and their sex forms. Male and female plants of Pusa Nanha, Pusa Dwarf and Washington were subjected to Random amplified polymorphic DNA analysis using selected primers. Photographs of the amplification profile obtained were taken with the

help of gel documentation system. The RAPD bands were represented as “+” (for presence) and “-” (for absence) and recorded. PCR was repeated twice in order to check the reproducibility.

RESULTS

4. RESULTS

The present experiment on "Validation of tests for sex determination in papaya (*Carica papaya* L.)" was conducted at the Department of Pomology and Floriculture and Department of Plant Biotechnology, College of Agriculture, Vellayani during 2011-2013. The objective of the experiment was to compare the efficiency of the chemical tests developed for identification of sex forms in papaya in order to find out a reliable test for prediction of sex of papaya plants in the pre - flowering stage. The experiment also aimed at identification of sex forms in papaya using RAPD markers. The results of the investigation carried out are presented in this chapter.

4.1 CHEMICAL TESTS FOR SEX DETERMINATION IN PAPAYA

4.1.1 Almen reagent test

4.1.1.1 Results of Almen reagent test on sex identification in papaya variety Washington

The data presented in Table 1 shows the results of Almen reagent test on sex identification in papaya variety Washington. As notified in the methodology, blue to purple colour in Almen reagent test was expected to be male plants and bluish green to green to be female plants. Out of the 20 seedlings, nine were expected to be males based on the test. But observations after flowering showed that only six were males. The percentage of accuracy observed for male sex form was 66 per cent. Out of the remaining eleven plants which were expected to be females, only eight turned out to be females on flowering, giving 72 per cent accuracy.

Table 1. Results of Almen reagent test on sex identification in papaya variety Washington

Plant number	Colour obtained	Expected sex form	Sex form observed at flowering
W1	Blue	Male	Male
W2	Blue	Male	Male
W3	Blue	Male	Male
W4	Light green	Female *	Male
W5	Blue	Male	Male
W6	Light Green	Female	Female
W7	Light green	Female *	Male
W8	Dark green	Female *	Male
W9	Blue	Male *	Female
W10	Blue	Male	Female
W11	Blue	Male*	Female
W12	Blue	Male	Male
W13	Blue	Male	Male
W14	Bluish green	Female	Female
W15	Dark green	Female	Female
W16	Light green	Female	Female
W17	Bluish green	Female	Female
W18	Light Green	Female	Female
W19	Light Green	Female	Female
W20	Bluish green	Female	Female

* Difference noted between expected and actual sex form.

Inference of Table 1

Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Male	9	6	66.0
Female	11	8	72.0

Table 2. Results of Almen reagent test on sex identification in papaya variety Pusa Dwarf

Plant number	Colour obtained	Expected sex form	Sex form observed at flowering
PD1	Green	Female	Female
PD 2	Light blue	Male	Male
PD 3	Green	Female	Female
PD 4	Blue	Male	Male
PD 5	Blue	Male	Male
PD 6	Blue	Male *	Female
PD 7	Light blue	Male	Male
PD 8	Blue	Male *	Female
PD 9	Green	Female	Female
PD 10	Light Green	Female	Female
PD 11	Light green	Female *	Male
PD 12	Light blue	Male	Male
PD 13	Dark green	Female	Female
PD 14	Green	Female	Female
PD 15	Dark Green	Female *	Male
PD 16	Blue	Male	Male
PD 17	Light blue	Male	Male
PD 18	Light blue	Male	Male
PD 19	Blue *	Male	Male
PD 20	Light green	Female	Female

* Difference noted between expected and actual sex form.

Inference of Table 2

Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Male	11	8	72 .0
Female	9	7	77. 0

4.1.1.2 Results of Almen reagent test on sex identification in papaya variety Pusa Dwarf

The results of Almen reagent test in papaya variety Pusa Dwarf are presented in Table 2. Out of the 20 plants tested, eleven were expected to be males based on colour development. However, observation after flowering showed that only eight were males. Hence the accuracy obtained for male sex form was 72 per cent. Only seven plants were found to be females out of expected nine, based on colour reaction and therefore, the percentage of accuracy was 77 per cent.

4.1.1.3 Results of Almen reagent test on sex identification in papaya variety Pusa Nanha

Results of Almen reagent test in papaya variety Pusa Nanha are furnished in Table 3. Out of the eight predicted males based on the test, only five were found to be true on flowering. The percentage of accuracy of the test was found to be 64 per cent. Out of the rest twelve plants which were expected to be females, eight were found to be so on observation at flowering showing an accuracy level of 66 per cent.

Table 3. Results of Almen reagent test on sex identification in papaya variety Pusa Nanha

Plant number	Colour obtained	Expected sex form	Sex form observed at flowering
PN 1	Blue	Male	Male
PN 2	Light green	Female *	Male
PN 3	Light blue	Male	Male
PN 4	Green	Female*	Male
PN 5	Light blue	Male	Male
PN 6	Light green	Female	Female
PN 7	Light Green	Female	Female
PN 8	Light blue	Male *	Female
PN 9	Light green	Female*	Male
PN 10	Green	Female	Female
PN 11	Blue	Male	Male
PN 12	Blue	Male	Female
PN 13	Light blue	Male	Female
PN 14	Bluish green	Female	Female
PN 15	Blue	Male	Male
PN 16	Light green	Female	Female
PN 17	Bluish Green	Female	Female
PN 18	Dark green	Female *	Male
PN 19	Dark green	Female	Female
PN 20	Light Green	Female	Female

* Difference noted between expected and actual sex form.

Inference of Table 3

Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Male	8	5	62.0
Female	12	8	66.0

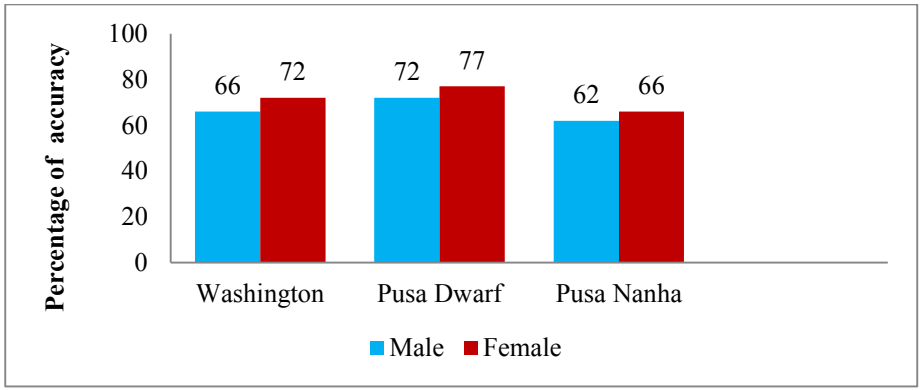


Fig.1. Comparison of Percentage of accuracy of Almen reagent test on sex identification in three papaya varieties namely Washington, Pusa Dwarf and Pusa Nanha

4.1.1.4 Overall assessment of accuracy of Almen reagent test on sex identification in all the three papaya varieties

Overall assessment of accuracy of Almen reagent test on sex identification in papaya varieties Washington, Pusa Dwarf and Pusa Nanha is furnished in Table 4. Out of the 60 seedlings tested, based on colour reaction, twenty eight were predicted to be males. But observations after flowering showed that only nineteen were males. Thus, Almen reagent test for maleness exhibited an accuracy of 67 per cent. Out of the remaining thirty two predicted female plants, only twenty three turned out to be females. Therefore, the percentage of accuracy obtained for female sex form was 71 per cent.

Table 4. Overall assessment of accuracy of Almen reagent test on sex identification in three papaya varieties Washington, Pusa Dwarf and Pusa Nanha

	Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Almen reagent test	Male	28	19	67.0
	Female	32	23	71.0

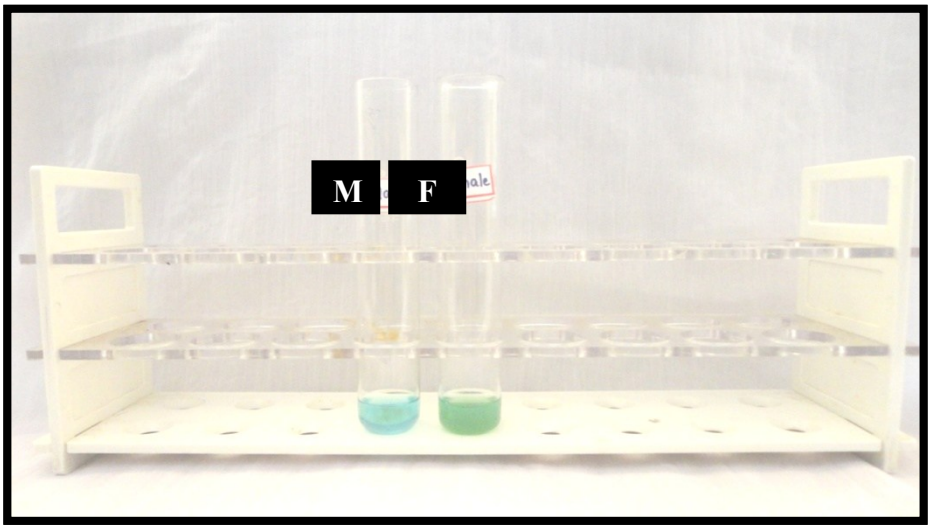


Plate 2. Almen reagent test showing the colour differentiation in male and female plants of papaya

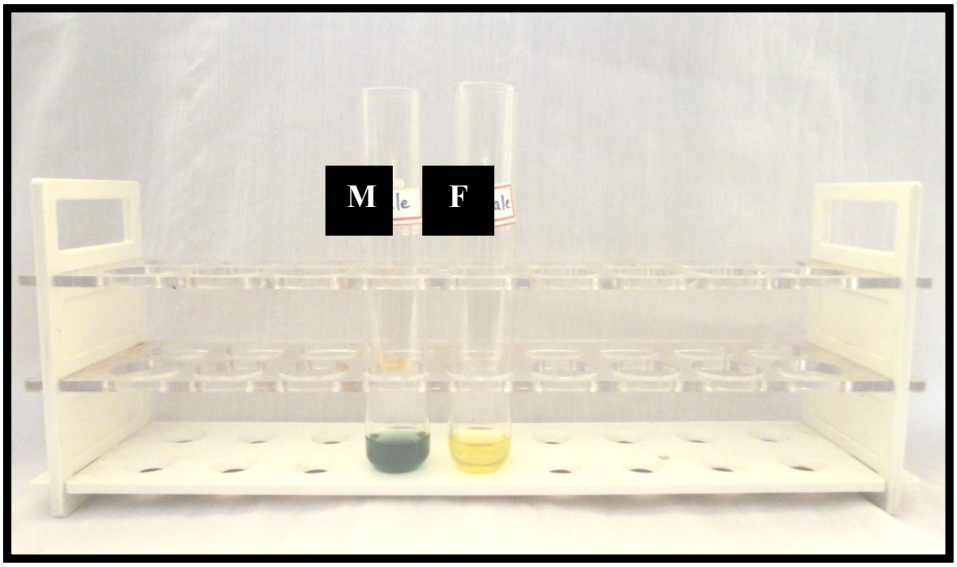


Plate 3. Ammonium molybdate test showing the colour differentiation in male and female plants of papaya

4.1.2 Ammonium molybdate test

4.1.2.1 Results of Ammonium molybdate test on sex identification in papaya variety Washington

On Ammonium molybdate test, males gave deep green colour and females gave yellow colour. The data furnished in Table 5 showed that out of 20 seedlings twelve were expected to be males based on the colour obtained in the test. But observations at flowering showed that only six were true. The accuracy of prediction was only 50 per cent. In the remaining eight plants, which were predicted to be females, only five were females, resulting in 62 per cent of accuracy.

4.1.2.2 Result of Ammonium molybdate test on sex identification in papaya variety Pusa Dwarf

Results of Ammonium molybdate test on sex identification in papaya variety Pusa Dwarf is presented in Table 6. Of the 20 seedlings tested, thirteen were expected to be males, but only nine were found to be males, on flowering. Out of the seven expected females, only four turned out to be females on flowering. Thus the percentage of accuracy obtained was 69 per cent for male sex form and 57 per cent for female sex form.

4.1.2.3 Results of Ammonium molybdate test on sex identification in papaya variety Pusa Nanha

The data presented in Table 7 shows the results of Ammonium molybdate test on sex identification in papaya variety Pusa Nanha. Out of the 20 seedlings analysed, based on the test results fifteen were predicted as males, but only seven expressed maleness at flowering. The percentage of accuracy obtained was thus 46 per cent for male sex form. The remaining five plants were expected to be females but only three were females among them. The percentage of accuracy obtained for females was 60 per cent

Table 5. Results of Ammonium molybdate test on sex identification in papaya variety Washington

Plant number	Colour obtained	Expected sex form	Sex form observed at flowering
W 1	Deep green	Male	Male
W2	Dark green	Male	Male
W3	Yellow	Female *	Male
W4	Deep green	Male	Male
W5	Deep green	Male	Male
W6	Deep green	Male *	Female
W7	Deep green	Male	Male
W8	Yellow	Female	Male
W9	Deep green	Male *	Female
W10	Yellow	Female	Female
W11	Yellow	Female	Female
W12	Yellow	Female *	Male
W13	Green	Male	Male
W14	Green	Male *	Female
W15	Yellow	Female	Female
W16	Green	Male *	Female
W17	Green	Male *	Female
W18	Green	Male *	Female
W19	Yellow	Female	Female
W20	Yellow	Female	Female

* Difference noted between expected and actual sex form

Inference of Table 5

Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Male	12	6	50.0
Female	8	5	62.0

Table 6. Results of Ammonium molybdate test on sex identification in papaya variety Pusa Dwarf

Plant number	Colour obtained	Expected sex form	Sex form observed at flowering
PD 1	Yellow	Female	Female
PD2	Deep green	Male	Male
PD3	Deep green	Male *	Female
PD4	Deep Green	Male	Male
PD5	Yellow	Female *	Male
PD6	Yellow	Female *	Female
PD7	Deep green	Male	Male
PD8	Deep green	Male *	Female
PD9	Deep green	Male*	Female
PD10	Light yellow	Female	Female
PD11	Deep Green	Male	Male
PD12	Deep Green	Male	Male
PD13	Yellow	Female	Female
PD14	Green	Male *	Female
PD15	Deep Green	Male	Male
PD16	Deep Green	Male	Male
PD17	Brownish green	Male	Male
PD18	Yellow	Female*	Male
PD19	Brownish green	Male	Male
PD20	Yellow	Female	Female

* Difference noted between expected and actual sex form.

Inference of Table 6

Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Male	13	9	69.0
Female	7	4	57.0

Table 7. Results of Ammonium molybdate test on sex identification in papaya variety Pusa Nanha

Plant number	Colour obtained	Expected sex form	Sex form observed at flowering
PN1	Deep green	Male	Male
PN2	Yellow	Female	Male
PN3	Yellow	Female *	Male
PN4	Deep green	Male	Male
PN5	Deep green	Male	Male
PN6	Deep green	Male *	Female
PN7	Deep green	Male*	Female
PN8	Deep green	Male*	Female
PN9	Deep green	Male	Male
PN10	Yellow	Female	Female
PN11	Deep green	Male	Male
PN12	Deep green	Male*	Female
PN13	Deep Green	Male*	Female
PN14	Yellow	Female	Female
PN15	Deep green	Male	Male
PN16	Deep green	Male*	Female
PN17	Deep green	Male*	Female
PN18	Deep green	Male	Male
PN19	Deep green	Male*	Female
PN20	Yellow	Female	Female

* Difference noted between expected and actual sex form.

Inference of Table 7

Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Male	15	7	46.0
Female	5	3	60.0

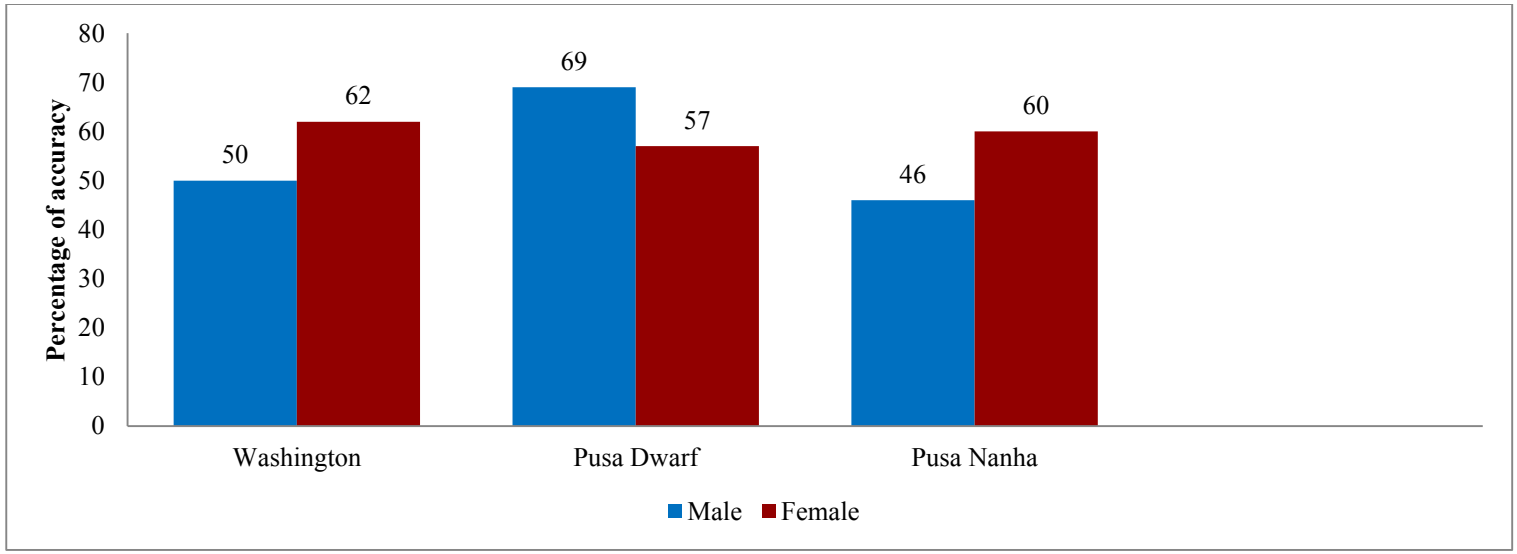


Fig.2. Comparison of Percentage of accuracy of Ammonium molybdate test on sex identification in three papaya varieties namely Washington, Pusa Dwarf and Pusa Nanha

4.1.2.4 Overall assessment of accuracy of Ammonium molybdate test on sex identification in all the three papaya varieties

Overall assessment of accuracy of Ammonium molybdate test on sex identification in papaya varieties Washington, Pusa Dwarf and Pusa Nanha are furnished in Table 8. Out of 60 seedlings belonging to three varietal groups, forty were predicted to be males, based on the test carried out at pre-flowering stage. But observations at flowering showed that only twenty two were males. Out of the remaining 20 plants, only twelve turned out to be females. Hence for Ammonium molybdate test, the accuracy level obtained for male sex form was 55 per cent and for females 60 per cent.

Table 8. Overall assessment of accuracy of Ammonium molybdate test on sex identification in three papaya varieties Washington, Pusa Dwarf and Pusa Nanha

	Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Ammonium molybdate test	Male	40	22	55.0
	Female	20	12	60.0

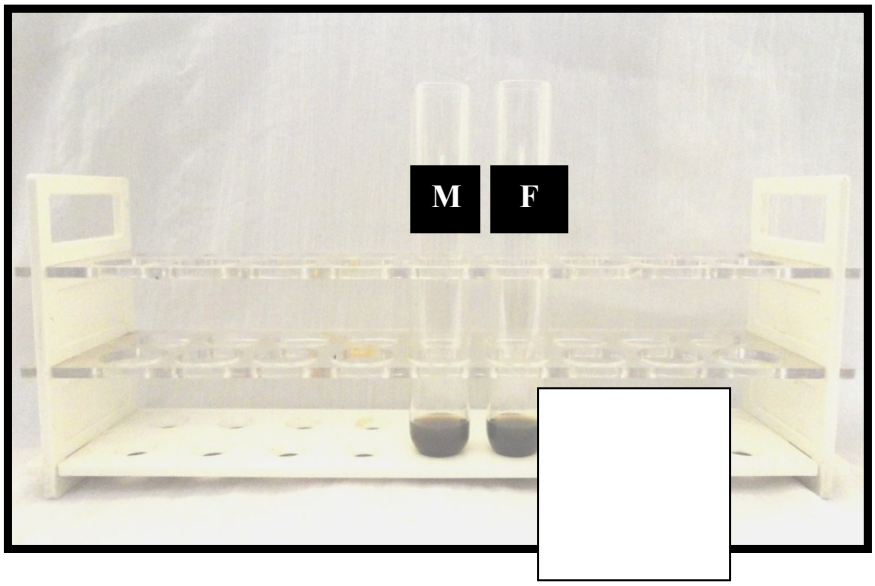


Plate 4. Ferric chloride test performed in male and female forms of papaya

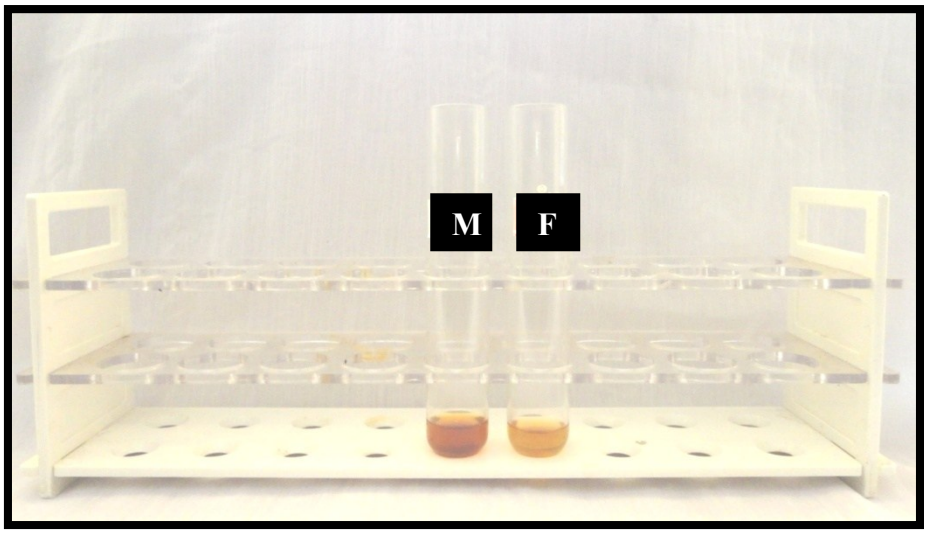


Plate 5. Titanous chloride test showing the colour differentiation in male and female plants of papaya

4.1.3 Ferric chloride test

Ferric chloride test failed to produce any characteristic colour difference in reaction and hence could not be effectively used to distinguish between male and female sex forms of papaya.

4.1.4 Titanous chloride test

4.1.4.1 Results of Titanous chloride test on sex identification in papaya variety Washington

On Titanous chloride test, males gave deep orange colour and females gave light brown colour. The results of Titanous chloride test on papaya variety Washington are presented in Table 9. Out of the 20 plants tested, seven were expected to be males based on colour development. However, observation after flowering showed that only three were males. Hence the accuracy of the test was 42 per cent for males. Only seven plants were found to be females out of expected thirteen, based on colour reaction and therefore, the accuracy was 53 per cent.

4.1.4.2 Results of Titanous chloride test on sex identification in papaya variety Pusa Dwarf

Results of Titanous chloride test on sex identification in papaya variety Pusa Dwarf is presented in Table 10. Of the 20 seedlings, eight were expected to be males, but on flowering, only four were found to be male. The percentage of accuracy obtained was 50 per cent. Out of the twelve expected females, only six turned out females on flowering giving 50 per cent accuracy.

4.1.4.3 Results of Titanous chloride test on sex identification in papaya variety Pusa Nanha

Results of Titanous chloride test in papaya variety Pusa Nanha are furnished in Table 11. Out of the 20 seedlings analysed, based on the test results eleven were predicted as males, but only five expressed maleness at flowering. The accuracy of the test was found to be 45 per cent. The remaining nine plants were expected to be females but only five were females among them. The percentage of accuracy obtained for females was 55 per cent.

Table 9. Results of Titanous chloride test on sex identification in papaya variety Washington

Plant number	Colour obtained	Expected sex form	Sex form observed at flowering
W1	Deep orange	Male	Male
W2	Deep orange	Male	Male
W3	Light brown	Female*	Male
W4	Light brown	Female*	Male
W5	Light brown	Female*	Male
W6	Light brown	Female	Female
W7	Light brown	Female*	Male
W8	Deep orange	Male	Male
W9	Light brown	Female	Female
W10	Deep orange	Male*	Female
W11	Light brown	Female	Female
W12	Light brown	Female*	Male
W13	Light brown	Female*	Male
W14	Deep orange	Male*	Female
W15	Light brown	Female	Female
W16	Deep orange	Male*	Female
W17	Light brown	Female	Female
W18	Deep orange	Male*	Female
W19	Light brown	Female	Female
W20	Light brown	Female	Female

* Difference noted between expected and actual sex form.

Inference of Table 9

Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of Accuracy
Male	7	3	42.0
Female	13	7	53.0

Table 10. Results of Titanous chloride test on sex identification in papaya variety Pusa Dwarf

Plant number	Colour obtained	Expected sex form	Sex form observed at flowering
PD1	Light brown	Female	Female
PD 2	Light brown	Female*	Male
PD 3	Light brown	Female	Female
PD 4	Deep orange	Male	Male
PD 5	Light brown	Female*	Male
PD 6	Light brown	Female	Female
PD 7	Deep orange	Male	Male
PD 8	Deep orange	male	Female
PD 9	Light brown	female	Female
PD 10	Deep orange	male	Female
PD 11	Light brown	Female *	Male
PD 12	Light brown	Female*	Male
PD 13	Deep orange	Male	Female
PD 14	Light brown	Female	Female
PD 15	Deep orange	Male	Male
PD 16	Light brown	Female*	Male
PD 17	Deep orange	Male	Male
PD 18	Light brown	Female*	Male
PD 19	Deep orange	Male	Male
PD 20	Light brown	Female	Female

* Difference noted between expected and actual sex form.

Inference of Table 10

Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Male	8	4	50.0%
Female	12	6	50.0%

Table 11. Results of Titanous chloride test on sex identification in papaya variety Pusa Nanha

Plant number	Colour obtained	Expected sex form	Sex form observed at flowering
PN 1	Light brown	Female*	Male
PN 2	Deep orange	Male	Male
PN 3	Light brown	Female*	Male
PN 4	Light brown	Female*	Male
PN 5	Deep orange	Male	Male
PN 6	Deep orange	Male*	Female
PN 7	Light brown	Female	Female
PN 8	Deep orange	Male *	Female
PN 9	Deep orange	Male	Male
PN 10	Light brown	Female	Female
PN 11	Deep orange	Male	Male
PN 12	Deep orange	Male*	Female
PN 13	Light brown	Female	Female
PN 14	Deep orange	Male*	Female
PN 15	Deep orange	Male	Male
PN 16	Deep orange	Male*	Female
PN 17	Light brown	Female	Female
PN 18	Light brown	Female *	Male
PN 19	Deep orange	Male*	Female
PN 20	Light brown	Female	Female

* Difference noted between expected and actual sex form.

Inference of Table 11

Sex form	Expected sex form (No. of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Male	11	5	45.0
Female	9	5	55.0

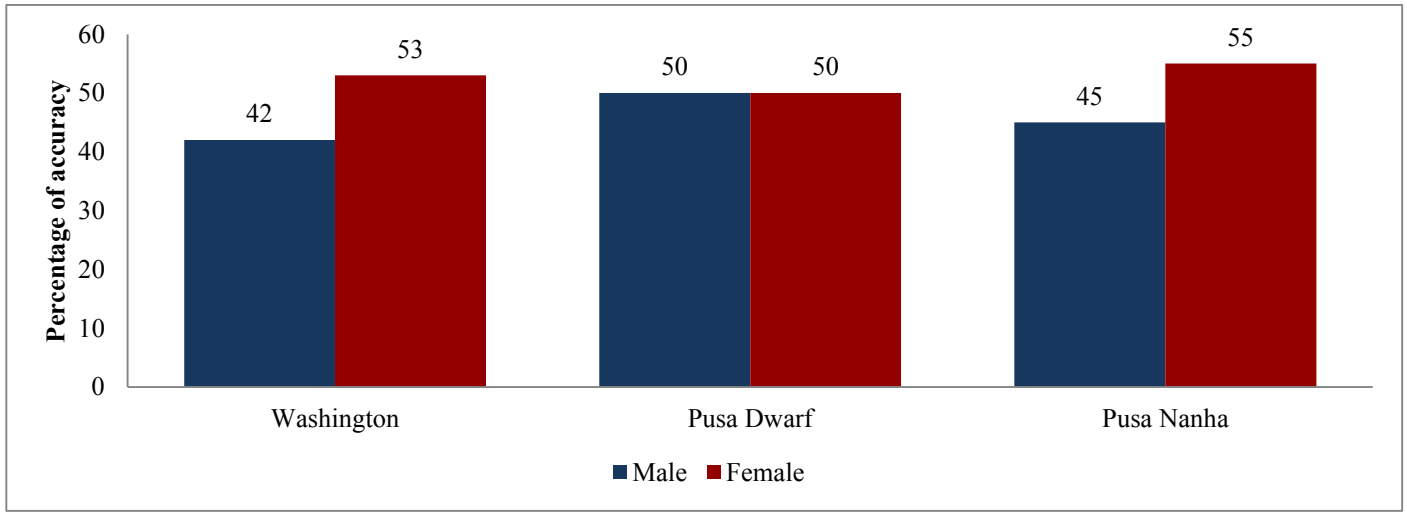


Fig.3. Comparison of Percentage of accuracy of Titanous chloride test on sex identification in three papaya varieties namely Washington, Pusa Dwarf and Pusa Nanha

4.1.3.4 Overall assessment of accuracy of Titanous chloride test on sex identification in all the three papaya varieties

Overall assessment of accuracy of Titanous chloride test on sex identification in all the three papaya varieties are furnished in Table 12. Out of the 60 seedlings belonging to three varietal groups, twenty six were predicted to be males based on the colour obtained in the test. But observations at flowering showed that only twelve were males. Out of the remaining thirty four plants predicted as females, only nineteen turned out to be females. Hence for Titanous chloride test, the percentage of accuracy obtained for male sex form was 46 per cent and 55 per cent for female sex form.

Table 12. Overall assessment of accuracy of Titanous chloride test on sex identification in three papaya varieties Washington, Pusa Dwarf and Pusa Nanha

	Sex form	Expected sex form (No.of plants)	Sex form observed at flowering (No. of plants)	Percentage of accuracy
Titanous chloride test	Male	26	12	46.0
	Female	34	19	55.0

Table 13. Comparison of efficiency of tests used for sex identification in papaya at seedling stage

Chemical tests	Varieties	Percentage of accuracy for male sex form	Percentage of accuracy for female sex form
Almen reagent test	Washington	66	72
	Pusa Dwarf	72	77
	Pusa Nanha	62	66
	Overall accuracy	**67**	**71**
Ammonium molybdate test	Washington	50	62
	Pusa Dwarf	69	57
	Pusa Nanha	46	60
	Overall accuracy	**55**	**60**
Titanous chloride test	Washington	42	53
	Pusa Dwarf	50	50
	Pusa Nanha	45	55
	Overall accuracy	**46**	**55**

Among the four chemical tests, Almen reagent test was found to be the best in determining the male and female sex forms in papaya varieties at seedling stage. With Almen reagent test, the percentage of accuracy obtained was 67 per cent for males and 71 per cent for females. On ammonium molybdate test, male sex form showed a percentage of accuracy of 55 per cent and 60 per cent for females. On the other hand, the percentage of accuracy of Titanous chloride test was only 46 per cent for males and 55 per cent for females.

4.2 MOLECULAR CHARACTERIZATION

4.2.1 Isolation and quantification of genomic DNA

DNA was isolated from the young emerging leaves of papaya using CTAB method (Dellaporta *et al*., 1983). Genomic DNA was analysed by electrophoresis using 0.8 per cent agarose gel. For most of the samples, DNA was observed as a crisp intact band. The yield of DNA from the different varieties and sex forms ranged from 3000 $\mu g\ ml^{-1}$ to 3840$\mu g\ ml^{-}$. The purity of DNA ranges from 1.6 to 2 (table 14).

4.2.2 RAPD analysis

A total of 10 random primers (OPA3, OPA13, OPB1, OPB4, OPB17, SO1, SO7, RY01, RPI01, and RPI02) were used for study. Out of these, six primers (OPA13, OPB1, OPB17, SO1, SO7 and RY01) yielded amplification products with the DNA from both male and female sex forms of all the three varieties viz, Pusa Nanha, Pusa Dwarf and Washington. Among these six primers, four primers (OPA13, OPB1, OPB17 and SO1) gave polymorphic banding pattern. The sequences of the primers are shown in the table 15.

The number of bands resolved per amplification was primer dependent and varied from a minimum of five to a maximum of nine bands. The nucleotide sequence of the four primers which gave polymorphic bands and number of informative RAPD markers given by each primer is shown in the table 16. The RAPD profiles were reproducible with all the primers.

Table 14. Quality and quantity of DNA isolated from different varieties and sex forms of papaya using CTAB method

Sl No	Variety	A260	A280	A260/280	DNA yield (µg/ml)
1	Washington Male	0.053	0.028	2.0	3180
2	Washington Female	0.056	0.034	1.6	3360
3	Pusa Dwarf Male	0.050	0.029	1.73	3000
4	Pusa Dwarf Female	0.064	0.036	1.77	3840
5	Pusa Nanha Male	0.057	0.031	1.83	3420
6	Pusa Nanha Female	0.060	0.037	1.71	3600

Table 15. Primer details that yielded polymorphic bands

Primer	Sequence	Number of informative RAPD markers
SO 1	5'CCACCACGAC 3'	9
OPB 1	5' GTTTCGCTCC 3'	7
OPA 13	5'CAGCACCCAC3'	7
OPB 17	5'AGGGAACGAC3'	5

Table 16. Primer associated banding patterns with the DNA of three varieties of papaya

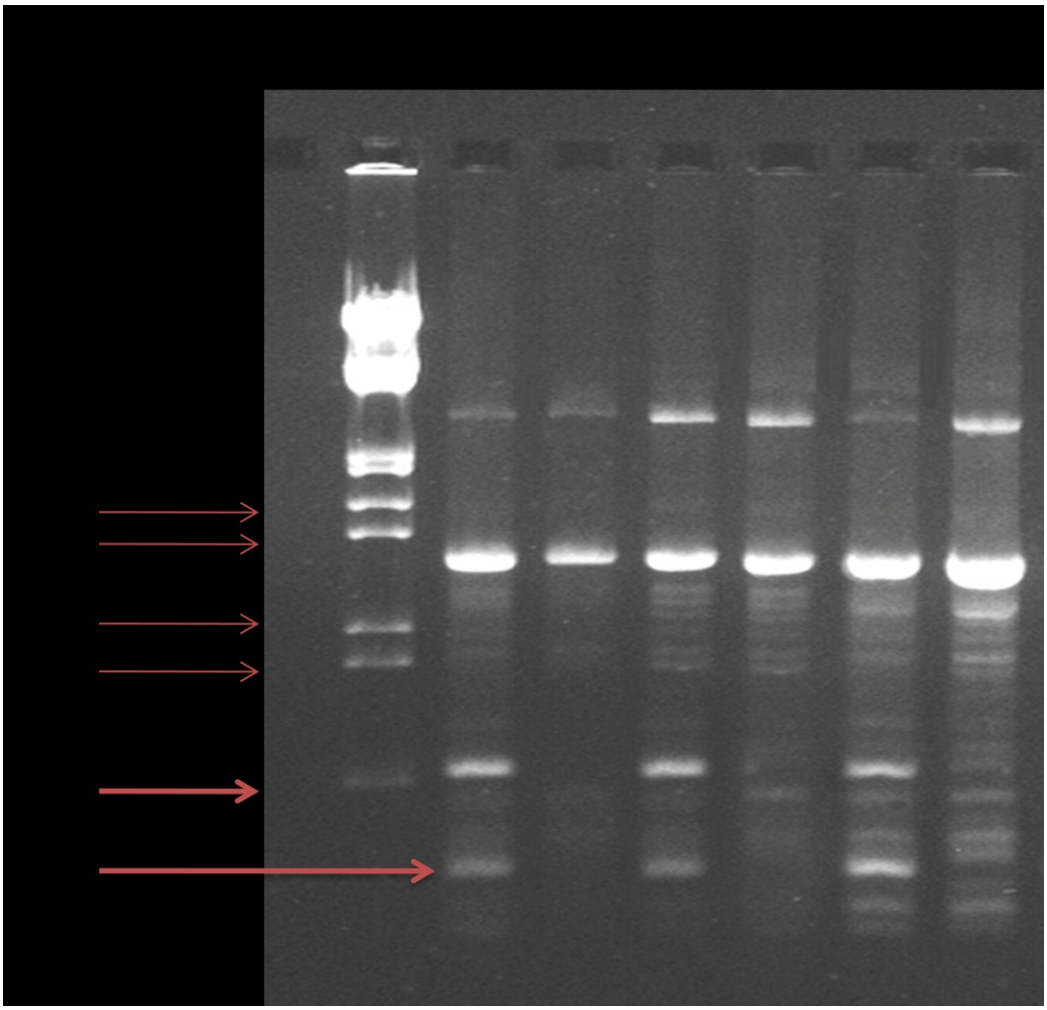
Sl no	Primers		Number of faint bands	Number of intense bands	Total number of bands
1	SO1	Pusa Nanha Female	1	3	4
		Pusa Nanha Male	1	1	2
		Pusa Dwarf Male	2	4	6
		Pusa Dwarf Female	4	2	6
		Washington Male	6	3	9
		Washington Female	5	2	7
2	OPB1	Pusa Nanha Male	4	3	7
		Pusa Nanha Female	2	4	6
		Pusa Dwarf Male	5	2	7
		Pusa Dwarf Female	4	2	6
		Washington Male	4	3	7
		Washington Female	5	2	7
3	OPA 13	Pusa Nanha Male	2	1	3
		Pusa Nanha Female	3	2	5
		Pusa Dwarf Male	5	2	7
		Pusa Dwarf Female	5	2	7
		Washington Male	5	2	7
		Washington Female	5	2	7
4	OPB 17	Pusa Nanha Male	2	2	4
		Pusa Nanha Female	2	2	4
		Pusa Dwarf Male	2	2	4
		Pusa Dwarf Female	3	1	4
		Washington Male	2	3	5
		Washington Female	3	2	5

With primer SO1, nine amplicons were produced out of which two bands were found to be monomorphic for both sex forms of three papaya varieties (Fig.4). Male plants of Pusa Nanha yielded a total of four bands of which three were intense and one was faint. Female Nanha gave only two bands (one intense and one faint). Male plants of all the three varieties yielded two intense bands, one with 500 bp and the other with a size approximately 400 bp. Male and Female Pusa Dwarf yielded six bands each out of which four were monomorphic. Two faint polymorphic bands were observed only in female plants of Pusa Dwarf (Plate 6).

When OPB1 was used for amplification, six bands yielded were monomorphic among male and female plants of the three varieties (Fig.5). Male Pusa Nanha produced a maximum of seven scorable bands while female Nanha gave six bands. An amplicon of size approximately 1250 bp was present in male forms of all the three varieties. But this band was observed in female plant of Washington. Male Pusa Dwarf gave seven bands while female gave six bands. Amplicons produced by OPB1 in male and female forms of Washington was all monomorphic (Plate 7).

When OPA13 was used for amplification, three bands were obtained for male Pusa Nanha while female plants yielded five bands. Two bands, one of size approximately 400 bp and the other with more than 1200 bp were found in female Nanha plants when compared to male plants (Plate 8). Male and female plants of Pusa Dwarf yielded a maximum of seven scorable bands of which five were faint and two were intense. None of the bands were polymorphic among male and female plants of Pusa Dwarf. Washington male and female plants also yielded seven bands. Among the male and female plants of Washington all the bands were monomorphic (Fig.6 andFig.7).

Plate 6. RAPD profiles of different sex forms of three varieties of papaya using primer SO1



1 – Pusa Nanha Male 3- Pusa Dwarf Male 5 –Washington Male

2 – Pusa Nanha Female 4- Pusa Dwarf Female 6- Washington Female

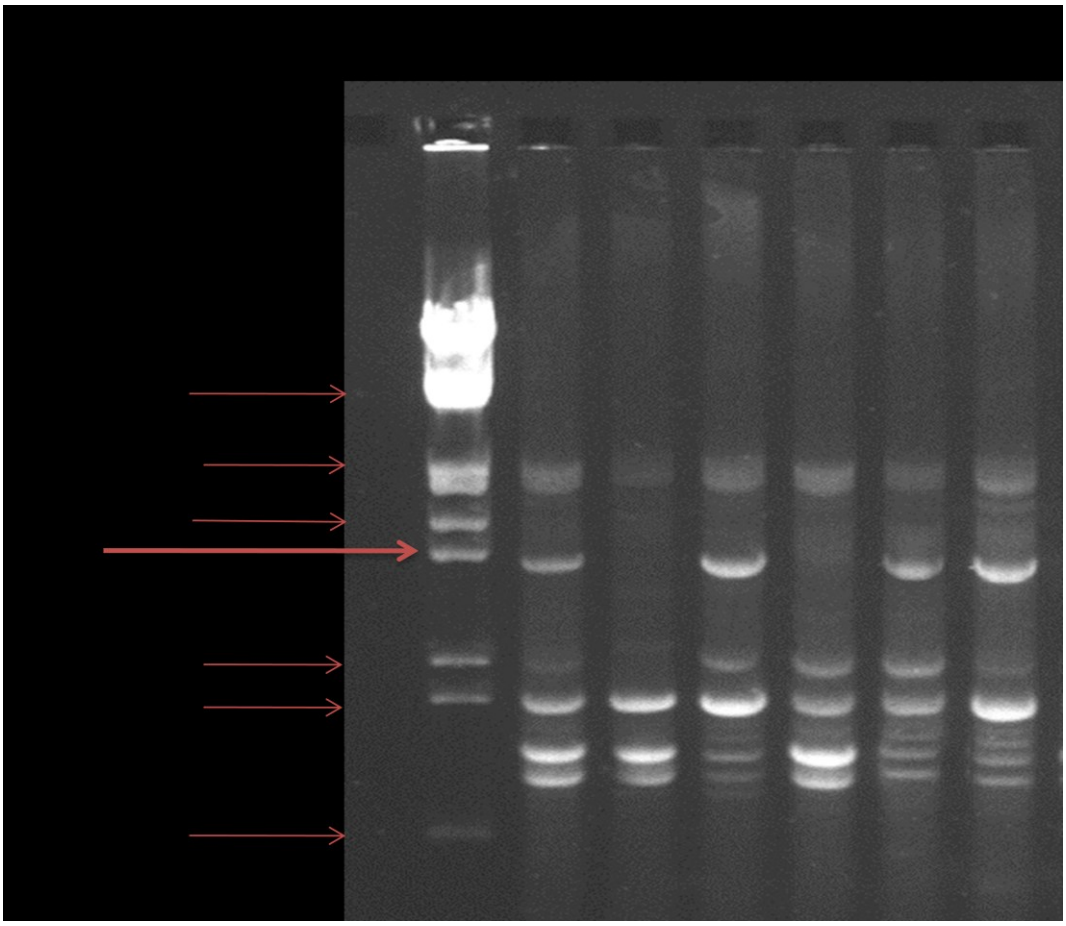
Fig. 4. Representation of RAPD profiles of different sex forms of three varieties using the primer SO1

	PN M	PN F	PD M	PD F	W M	W F
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	--	--	+	+	+	+
4	--	--	+	+	+	+
5	+	--	+	--	+	--
6	--	--	--	+	+	+
7	--	--	--	+	+	+
8	+	--	+	--	+	--
9	--	--	--	--	+	+

Fig.5. Representation of RAPD profiles of different sex forms of three varieties using the primer OPB1

	PN M	PN F	PD M	PD F	W M	W F
1	+	+	+	+	+	+
2	+	--	+	--	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+
7	+	+	+	+	+	+

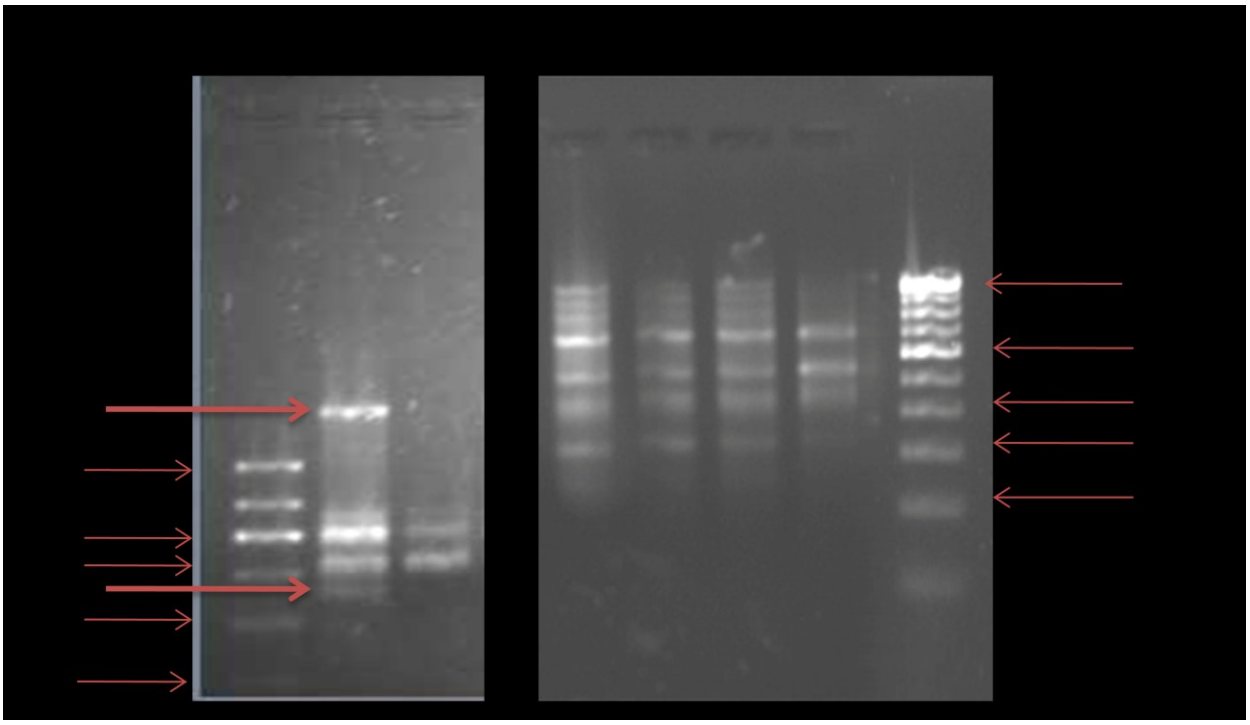
Plate 7. RAPD profiles of different sex forms of three varieties of papaya using primer OPB 1



1 – Pusa Nanha Male **3- Pusa Dwarf Male** **5 –Washington Male**

2 – Pusa Nanha Female **4- Pusa Dwarf Female** **6- Washington Female**

Plate 8. RAPD profiles of different sex forms of three varieties of papaya using primer OPA13



1 – Pusa Nanha Female **3- Pusa Dwarf Female** **5 –Washington Female**

2 – Pusa Nanha Male **4- Pusa Dwarf Male** **6- Washington Male**

Fig.6. Representation of RAPD profiles of different sex forms of papaya variety Pusa Nanha using the primer OPA13

	PN F	PN M
1	+	--
2	+	+
3	+	+
4	+	+
5	+	--

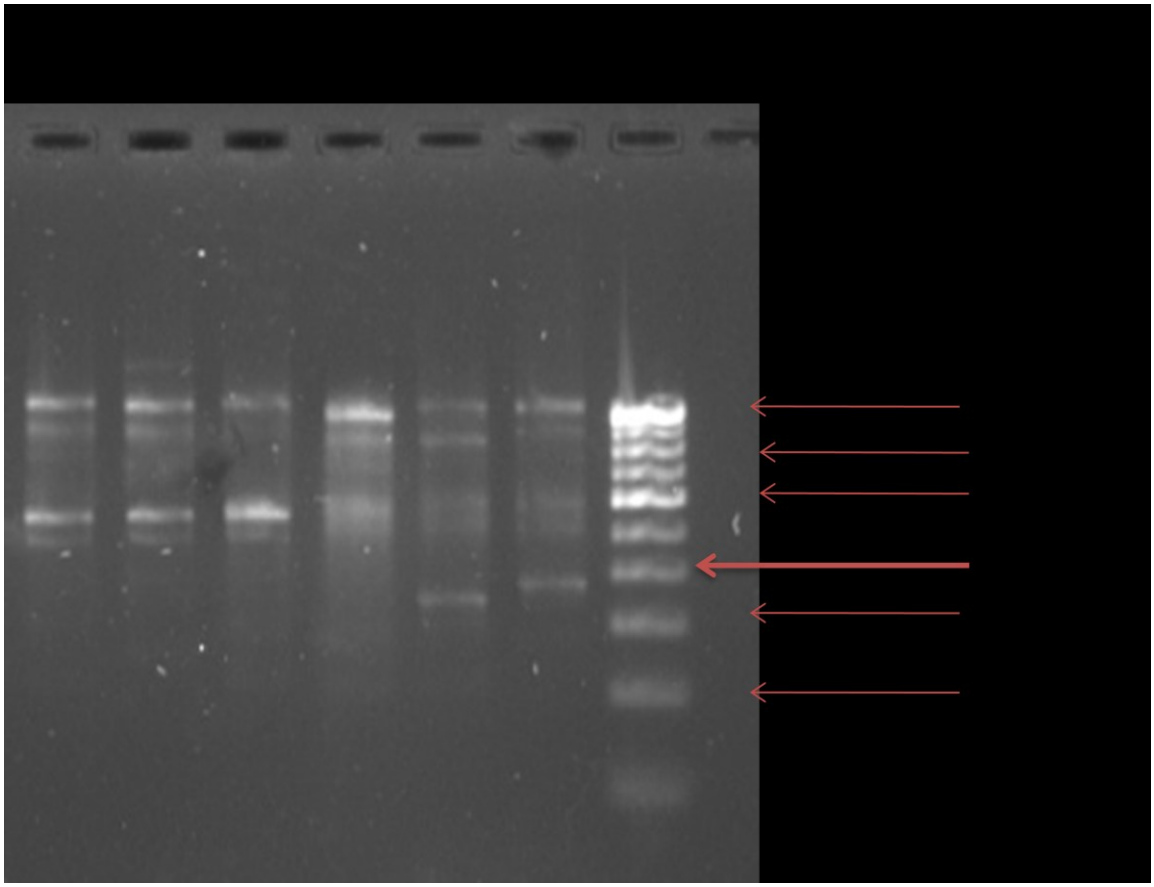
Fig.7. Representation of RAPD profiles of different sex forms of papaya varieties Pusa Dwarf and Washington using the primer OPA13

	PD F	PD M	W F	W M
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+

Fig.8. Representation of RAPD profiles of different sex forms of three varieties using the primer OPB17

	PN M	PN F	PD M	PD F	W M	W F
1	+	+	+	+	+	+
2	+	--	+	--	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	--	--	--	--	--	+
6	--	--	--	--	+	--

Plate 9. RAPD profiles of different sex forms of three varieties of papaya using primer OPB 17



1 – Pusa Nanha Male 3- Pusa Dwarf Male 5 –Washington Male

2 – Pusa Nanha Female 4- Pusa Dwarf Female 6- Washington Female

With OPB 17, a total of five scorable bands were obtained of which three were monomorphic for both sex forms of three papaya varieties (Fig.8). Male and female plants of Pusa Nanha yielded four bands each (Two faint bands and two intense bands). Female Pusa Dwarf produced four scorable bands of which three were faint and the other one was intense. Male Pusa Dwarf also yielded four scorable bands. In Pusa Nanha and Pusa Dwarf, all the bands were monomorphic for both male and female sex forms. Male and Female Washington gave five bands each. An amplicon of approximately 300 bp was found distinctly in the variety Washington (Plate 9).

The primer SO1 (5' CCACCACGAC 3') proved to be highly effective for the discrimination of male papaya plants of all the three varieties tested. RAPD profiles with SO1 primer showed two distinct bands, one with 500 bp and another with approximately 400 bp in male plants of all the three varieties. Primer OPB1 could differentiate sex forms of two varieties, viz. Pusa Dwarf and Pusa Nanha, while male and female plants of variety Washington could not be differentiated. With OPB1, one male specific band of approximately 1250 bp was present in Pusa Dwarf and Pusa Nanha while in Washington variety that band was present in both the sex forms. OPA13 helped to distinguish Pusa Nanha female plants with the presence of two bands at approximately 400 bp and more than 1.2 kb size. With OPB 17, an amplicon of approximately 300 bp was found distinctly in the variety Washington. But it was unsuccessful in differentiating sex forms in other two varieties.

DISCUSSION

5. DISCUSSION

The present study, "Validation of tests for sex determination in papaya (*Carica papaya* L.)" was carried out in the Department of Pomology and Floriculture and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala from 2011 to 2013. Three varieties of papaya namely, Pusa Dwarf, Pusa Nanha and Washington were selected for the study. Apart from colorimetric tests, molecular characterization was also attempted to differentiate male and female plants in the pre-flowering stage. The results obtained are discussed in this chapter.

5.1 CHEMICAL TESTS FOR SEX DETERMINATION IN PAPAYA

Several colorimetric tests to differentiate the male and female plants at pre-flowering stage were tried by research workers. From the experiments related to prediction of sex forms in papaya at pre-flowering stage, Horovitz (1954 a) suggested that there are specific male and female florigenic substances produced by the dominant and recessive alleles and it may be possible that the genes governing maleness and femaleness may be producing some substances which react differently to different chemicals. This view was supported by Jungwirth (1953), Benatena (1954) and Krishnamurthy *et al*. (1960) also. Choudhury *et al.* (1957) observed that male and female plants of papaya may be differentiated on the basis of leaf content of total carbohydrates, phosphorus, nitrogen, potash, chlorophyll a and b as well as certain florigenic substances. Singh and Jindal (1972) reported a higher amount of free and bound phenolics in male plants compared to females.

5.1.1 Almen reagent test for prediction of sex forms in papaya varieties Washington, Pusa Dwarf and PusaNanha

In the present studies, Almen reagent resulted in blue colour in the case of male plants and bluish green to green in the case of female plants.

The prediction of sex forms showed an accuracy level of 66 per cent in the case of male plants and 72 per cent in the case of female plants in papaya variety Washington at pre-flowering stage. In Pusa Dwaf the accuracy of prediction was 72 per cent in the case of males and 77 per cent in the case of females. Pusa Nanha showed 64 per cent accuracy of prediction in male sex form, while in females it was 66 per cent.

Overall assessment of accuracy of Almen reagent test on sex identification in papaya varieties Washington, Pusa Dwarf and Pusa Nanha was derived from the above results. Almen reagent test for maleness exhibited 67 percentage of accuracy and the percentage of accuracy obtained for female sex form was 71 per cent. In the tests using mercury and Nitrous / Nitric acid reagents, Bojappa and Singh (1974 b) observed that the accuracy of prediction for male plants was 63 per cent, while for females 77 per cent. In the case of Modified Almen reagent test, the efficiency of forecast for male plants was 69 per cent, while it was 77 per cent for females.

In the present studies, Almen reagent resulted in blue colour in the case of male plants and bluish green to green in the case of female plants and this was more or less same colour reaction obtained by earlier workers. According to Bojappa and Singh (1974 b), the same test showed varied colour reactions between sex the forms in papaya. They also noted that sometimes the shades of colour varied within the same sex form. Modified nitrous acid and mercuric nitrate tests have shown French blue, Cerulein blue or Capri blue in relation to expression of maleness. The same tests

showed French blue, Hyacinth blue or Gentian blue in the case of femaleness. In earlier studies, Storey (1958) and Sigh *et al*. (1963) also observed that the same test in some vegetative seedlings gave different shades of the same colour.

It was observed from the present studies that the efficiency of the forecast was higher for females compared to the males. This is supported by previous scientists also. According to Bojappa and Singh (1974 b), the accuracy of the forecasts ranged from 58-71% for male plants where as with female and hermaphrodite plants the accuracy ranged from 77-90 per cent and 74-89 per cent respectively. According to Singh *et al.* (1961 b), higher efficiency of forecast for femaleness was due to the absolute sex stability of female plants as against the unstable nature of male and hermaphrodite sex forms. Similar observations were made by Storey (1953), Horovitz (1954 b) and Singh (1964) who opined that forecast of sex is more accurate in the case of female plants than of male plants. This may be due to greater stability of the female sex. It may also be due to the occurrence of 55 per cent female and only 45 per cent male plants in relatively small population.

5.1.2 Ammonium molybdate test for prediction of sex forms in papaya varieties Washington, Pusa Dwarf and PusaNanha

In the present studies, Ammonium molybdate test showed deep green colour in males and yellow colour in females. Only 50 per cent accuracy of prediction was obtained in the case of male plants, while a higher level of 62 per cent was obtained in the case of female plants in papaya variety Washington. In the case of Pusa Dwarf, in male plants 69 percentage of accuracy of prediction was possible whereas in females, it was only 57 percent. Pusa Nanha showed 46 per cent accuracy with respect to male plants and 60 per cent with females.

Assessment of overall accuracy of Ammonium molybdate test in three varieties of papaya indicated that the accuracy level obtained for male sex form was 55 per cent and for females 60 per cent. No reviews of earlier works could be traced

in literature test, since most of them were currently not available, even though this test was reported to be useful by Singh *et al*., 1961. In fact, they found that this test was less effective compared to other tests like Almen reagent test. Bojappa and Singh (1974 b) also have expressed the same opinion. .

5.1.3 Ferric chloride test for prediction of sex forms in papaya varieties Washington, Pusa Dwarf and Pusa Nanha

In the present study, Ferric chloride test could not be effectively used to distinguish between male and female sex forms, since no characteristic colour difference in reaction was observed. In both the sex forms, only black colour was obtained when the test was performed.

According to Bojappa and Singh (1974 b), tests involving ferrous or ferric compounds did not give any characteristic color development in the case of different sex forms. With ferrous sulphate , they could observe only brownish yellow colour in all sex forms. Jindal and Singh (1976 a) could observe yellowish brown colour in the tests with a related compound ferrous sulphate in the case of male plants, but they also failed to get any distinct colour in the case of female plants. Only a turbid reaction mixture was obtained in the latter case. This may be due to the fact that the same test in some vegetative seedlings gives different shades of the same colour making exact prediction difficult. Such observations were made earlier by Storey (1958) and Sigh *et al*. (1963) also.

5.1.4 Titanous chloride test for prediction of sex forms in papaya varieties Washington, Pusa Dwarf and Pusa Nanha

In Titanous chloride test, males gave deep orange colour and females gave light brown colour in the present studies. In similar studies, Jindal and Singh (1976 a) observed creamy yellow colour in male plants, while female plants exhibited deep yellow colour. Thus, even though the shades differed in these studies, the basic

effect was almost the same. Such differences in shade of colour with the same test were reported by Bojappa and Singh (1974 b) also.

In papaya variety Washington, the accuracy of the test was 42 per cent with maleness and 53 per cent with females. At the same time, in Pusa Dwarf, the accuracy obtained in the present study was 50 per cent in the case of males and 50 percent in females. In Pusa Nanha, maleness showed only 45 per cent and femaleness 55 per cent accuracy.

Overall assessment of accuracy of Titanous chloride test for sex identification in papaya varieties Washington, Pusa Dwarf and Pusa Nanha showed that the accuracy level obtained for male sex form was 46 per cent and 55 per cent for females. These results agree with the findings of Jindal and Singh (1976 a) who also obtained accuracy levels of prediction close to the present studies.

Overall assessment of the results of the present investigations lead to the inference that even though the colour development differed from one sex form to other, the shades of colour developed can vary when tests are performed different situations. This observation is supported by earlier works in the same field by Storey (1958), Bojappa and Singh (1974 b) and Sigh *et al*. (1963) who observed that the same test in some vegetative seedlings gave different shades of the same colour.

None of the tests were hundred per cent accurate in prediction of sex form at pre-flowering stage in papaya as observed by previous workers in the same lines such as Singh *et al*. 1961(b), Bojappa and Singh (1974 b) as well as Jindal and Singh (1976 a).

In general, the present experiment showed that the accuracy of prediction was more in the case of female sex from compared to the male sex form. Same observations were made by Horovitz (1954), Storey (1953 and 1958),

Sigh *et al*. (1963), Singh (1964), Singh *et al*. 1961(b), Bojappa and Singh (1974 b) Jindal and Singh (1976 a) as well as Rao *et al.* (1985).

The assessment of efficiency of various tests performed indicated that Almen reagent showed higher levels of accuracy of prediction of both the sex forms at the pre-flowering stage. In this test, maleness could be predicted to an accuracy level of 67 per cent and femaleness to the level of 71 per cent. This is conformity with the findings of Singh *et al*. (1961 b), Bojappa and Singh (1974 b) as well as Jindal and Singh (1976 a) who could obtain high level of accuracy of prediction of sex form in papaya using reagents containing nitrous / nitric acid and mercuric nitrate.

5.2 MOLECULAR CHARACTERIZATION

One of the major problems encountered by papaya growers is its dioecious nature which leads to difficulty in identifying the sex of seedling at an early stage. Several studies have been reported which used molecular marker tools to identify sex form of papaya at early stages (Haoma *et al.,* 2001, Urasaki *et al.,* 2002, Lemos *et al.,* 2002, Deputy *et al*., 2002). The present study was undertaken to characterize the three varieties of papaya and their sex forms using RAPD markers. The results obtained are discussed in detail.

5. 2.1 Isolation and quantification of Genomic DNA

In the present study DNA isolation was done from young emerging leaves of papaya using Cetyl trimethyl ammonium bromide (CTAB) method (Dellaporta *et a*l., 1983). Mondal *et al.* (2000) opined that tender leaves contain actively dividing cells with lesser proportion of extra nuclear materials like proteins, carbohydrates and other metabolites that interfere with isolation of nucleic acids, in turn, improve the quality of DNA. Tender leaves also facilitate easy cell disruption for DNA extraction.

In the present study, the DNA yield of the different varieties and sex form ranged from 3000 µg ml^{-1} to 3840 µg ml^{-1}. Similar results has been shown by

Sereena (2004) with DNA yield of different varieties and sex forms of papaya that ranged from 600 µg ml^{-1} (male Pusa Dwarf) to 8035µg ml^{1} (hermaphrodite Solo).

5.2.2 RAPD analysis

Out of the ten primers tested, six primers yielded amplicons in both sex forms of all the three varieties. The total number of bands ranged from two to nine. No amplification was observed with the other four RAPD primers which indicated that there was no sequence complementarity to the sequence of these primers in the genome of three varieties of papaya.

In the present experiment out of the ten primers, four primers (SO1, OPB1, OPA13 and OPB17) gave polymorphic banding pattern. Two primers showed high level of polymorphism. The other primers SO7 and RY01 produced only monomorphic bands. The PCR reaction was repeated twice to check the reproducibility. The number of bands resolved per amplification was primer dependant and varied from a minimum of five to a maximum of nine bands.

The primer SO1 proved to be highly effective for the discrimination of male papaya plants of all the three varieties tested. RAPD profiles with SO1 showed two distinct bands, one with 500 bp and the other with approximately 400 bp in male plants of all the three varieties.

Primer OPB1 produced one male specific band of approximately 1250 bp in male plants of Pusa Dwarf and Pusa Nanha where as in Washington variety that band was present in both the sex forms.

RAPD primers have been used by Neeta *et a*l. (2005) to identify sex forms of papaya. They reported that out of 280 random primers screened only one primer OPE6 elicited a DNA amplification product of 1000 base pair in males and hermaphrodites but not in females of Tainung cultivars of papaya. Bedoya *et al*. (2007) used RAPD markers to determine the sex types of Columbian cultivars of

papaya and observed that primer OPY7 generated an amplicon of 900 base pair in all the male plants of the three cultivars tested but not in females or hermaphrodites. In another study by Xingh *et al*. (2007), it was found that primer Z18 produced an amplicon of 1000 bp in all male plants of papaya but not in the female plants so far analyzed.

In the present study, primer OPA13 helped to distinguish Pusa Nanha female plants with the presence of two bands, one with approximately 400 bp and the other with more than 1.2 kb size. The other two varieties Pusa Dwarf and Washington were found monomorphic between the two sex forms. With OPB 17, an amplicon of approximately 300 bp was found distinctly in the variety Washington. But it was unsuccessful in differentiating sex forms in other two varieties.

Esfandiyari *et al.* (2010) observed that in *Pistacia sp*, out of 20 RAPD primers screened, only one primer BC1200 amplified a specific band of approximately 300 base pair length in all female plants but not in males. In another study conducted by Sujitha *et al*. (2012) using five varieties of papaya, it was observed that the primer OPL13 yielded characteristic amplicons of approximately 2000 base pair in all female plants but not in males.

Of the four random primers tested, only SO1 yielded sex specific markers in the three varieties of papaya included in the study. The primer OPB1 could yield sex specific DNA profile only in two varieties. Other two primers could not produce any reproducible sex specific markers.

The present study suggests that RAPD profiling with the decamer primer SO1 can be used to identify the male plants of papaya varieties namely Pusa Dwarf, Pusa Nanha and Washington effectively. The male specific RAPD markers obtained in this study may be sequenced and SCAR (Sequence Characterized Amplified Region) primers could be developed in future for use as reproducible markers for sex identification in papaya varieties.

SUMMARY

6. SUMMARY

The present investigation entitled "Validation of tests for sex determination in papaya (*Carica papaya* L.)" was carried out to compare the efficiency of chemical tests developed for identification of sex forms in papaya in order to find out a reliable test for prediction of sex of papaya plants in the pre - flowering stage. Molecular characterization of the varieties and identification of sex forms in papaya using RAPD markers were also attempted. The experiment using three varieties of papaya namely Pusa Dwarf, Pusa Nanha and Washington was laid out at the Department of Pomology and Floriculture and Department of Plant Biotechnology, College of Agriculture, Vellayani during 2011-2013. Major findings of the study are summarized below.

6.1 CHEMICAL TESTS FOR SEX DETERMINATION IN PAPAYA

Two months old seedlings raised in polythene bags were serially numbered and transplanted into the main field. Two to three newly emerged leaves were collected from one and half months old nursery seedlings and the filtered aqueous extract was prepared. Chemical tests such as Almen reagent test, Ammonium molybdate test, Ferric chloride test and Titanous chloride test were carried out at pre-flowering stage and the results were compared with that from the field established plants after flowering.

Almen reagent test gave light blue to deep blue colour for males and light green to dark green colour for females. Out of the 60 seedlings tested, based on the colour reaction, twenty eight were predicted to be males. But observations after flowering showed that only nineteen were males. Out of the remaining thirty two plants predicted to be females, only twenty three turned out to be true on flowering. Thus Almen reagent test for maleness exhibited an accuracy of 67 per cent and 71 per cent for females.

On Ammonium molybdae test, males give deep green colour and females give yellow colour. Out of 60 seedlings belonging to three varietal groups, forty were predicted to be males, based on the test carried out at pre-flowering stage. But observations at flowering showed that only twenty two were males. Out of the remaining 20 plants, only twelve turned out to be females. Hence for Ammonium molybdate test, the percentage of accuracy obtained was 55 per cent for male sex form and 60 per cent for female sex form.

Ferric chloride test failed to produce any characteristic colour difference in reaction and hence could not be effectively used to distinguish between male and female sex forms of papaya.

On Titanous chloride test, males gave deep orange colour and females gave light brown colour. Out of 60 seedlings analysed, based on the test results, twenty six were predicted to be males. But observations at flowering showed that only twelve were males. Out of the remaining thirty four plants predicted as females, only ninteen turned out to be females. Thus the percentage of accuracy obtained was 46 per cent for males and 55 per cent for females.

Among the four chemical tests, Almen reagent test was found to be the best in determining the male and female sex forms in papaya varieties with accuracy of 67 per cent for males and 71 per cent for females.

6.2 MOLECULAR CHARACTERIZATION

DNA was isolated from the young emerging leaves of papaya using CTAB method with slight modification (Dellaporta *et al*., 1983). The DNA yield of the different varieties and sex forms ranged from 3000 µg ml^{-1} to 3840µg ml^{-}. A total of 10 random primers (OPA3, OPA13, OPB1, OPB4, OPB17, SO1, SO7, RY01, RPI01, and RPI02) were used for study. Out of these, six primers (OPA13, OPB1, OPB17, SO1, SO7 and RY01) yielded amplification products with the DNA from both male and female sex forms of all the three varieties. Among these six primers, four primers (OPA13, OPB1, OPB17 and SO1) gave polymorphic banding pattern.

The primer SO1 (5' CCACCACGAC 3') proved to be highly effective for the discrimination of male papaya plants of all the three varieties tested. RAPD profiles with SO1 primer showed two distinct bands, one with 500 bp and another with approximately 400 bp in male plants of all the three varieties. Primer OPB1 could differentiate sex forms of two varieties, viz. Pusa Dwarf and Pusa Nanha, while male and female plants of variety Washington could not be differentiated. With OPB1, one male specific band of approximately 1250 bp was present in Pusa Dwarf and Pusa Nanha while in Washington variety that band was present in both the sex forms. OPA13 helped to distinguish Pusa Nanha female plants with the presence of two bands at approximately 400 bp and more than 1.2 kb size. With OPB 17, an amplicon of approximately 300 bp was found distinctly in the variety Washington. But it was unsuccessful in differentiating sex forms in other two varieties.

The present investigation showed that the Almen reagent test and RAPD profiling with the decamer primer SO1 can be used for the determination of the sex forms of papaya in the seedling stage.

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VALIDATION OF TESTS FOR SEX DETERMINATION IN PAPAYA (*Carica papaya* L.)

by

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(2011-12-103)

ABSTRACT

submitted in partial fulfilment of the

requirement for the degree of

MASTER OF SCIENCE IN HORTICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF POMOLOGY AND FLORICULTURE

COLLEGE OF AGRICULTURE

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2013

ABSTRACT

Validation of tests for sex determination in papaya (*Carica papaya* L.)

An experiment on "Validation of tests for sex determination in papaya (*Carica papaya* L.)" was carried out at the Department of Pomology and Floriculture and Department of Plant Biotechnology, College of Agriculture, Vellayani during 2011-13 to compare the efficiency of chemical tests developed for identification of sex forms in papaya in order to find a reliable test for sex determination of papaya plants in the pre-flowering stage. Attempts were also made to identify the sex forms in papaya through molecular characterization using Random Amplified Polymorphic DNA (RAPD) technique. The colorimetric tests along with molecular analysis can be an efficient tool for more accurate prediction of sex forms in papaya.

Papaya varieties selected for the present study were Pusa Dwarf, Pusa Nanha and Washington. Two months old seedlings raised in polythene bags were serially numbered and transplanted into the main field. Two to three newly emerged leaves were collected from one and half months old nursery seedlings and the filtered aqueous extract was prepared. Chemical tests such as Almen reagent test, Ammonium molybdate test, Ferric chloride test and Titanous chloride test were carried out at pre-flowering stage and the results were compared with that from the field established plants after flowering. Almen reagent test was found to be the best in determining the male and female sex forms in papaya varieties. The accuracy of prediction of sex form was 67 per cent for males and 71 per cent for females.

For molecular analysis, genomic DNA was isolated from young leaves of all the varieties using Cetyl trimethyl ammonium bromide (CTAB) method. A total of 10 random primers (OPA3, OPA13, OPB1, OPB4, OPB17, SO1, SO7, RY01, RPI01, and RPI02) were used for study.Out of these, six primers (OPA13,

OPB1, OPB17, SO1, SO7 and RY01) yielded amplification products with the DNA from both male and female sex forms of all the three varieties. Among these six primers, four primers (OPA13, OPB1, OPB17 and SO1) gave polymorphic banding pattern.

The primer SO1 (5'CCACCACGAC 3') proved to be highly effective for the discrimination of male papaya plants of all the three varieties tested. RAPD profiles with SO1 primer showed two distinct bands, one with 500 bp and another with approximately 400 bp in male plants of all the three varieties. Primer OPB1 could differentiate sex forms of two varieties, viz. Pusa Dwarf and Pusa Nanha, while male and female plants of variety Washington could not be differentiated. With OPB1, one male specific band of approximately 1250 bp was present in Pusa Dwarf and Pusa Nanha while in Washington variety that band was present in both the sex forms. OPA13 helped to distinguish Pusa Nanha female plants with the presence of two bands at approximately 400 bp and more than 1.2 kb size. With OPB 17, an amplicon of approximately 300 bp was found distinctly in the variety Washington. But it was unsuccessful in differentiating sex forms in other two varieties.

The present investigation showed that the Almen reagent test and RAPD profiling with the decamer primer SO1 (5'CCACCACGAC 3') can be used for the determination of the sex forms of papaya in the pre-flowering stage.