# CHARACTERIZATION OF *COCCINIA GRANDIS* AS A MODEL SYSTEM TO STUDY SEX DETERMINATION IN PLANTS

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by

**Ghadge Amita Gautam**

**Reg No. 20081029**

Under the guidance of

**Dr. Anjan K Banerjee**

Assistant Professor, IISER, Pune

## TABLE OF CONTENTS

## CERTIFICATE

This is to certify that this dissertation entitled '**Characterization of *Coccinia grandis* as a model system to study sex determination in plants**' towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by **Ghadge Amita Gautam** at IISER Pune under the supervision of **Dr. Anjan K Banerjee**, Assistant Professor, Biology Division, IISER Pune during the academic year **2012-2013**.

**Dr. Anjan K Banerjee**

Assistant Professor

Biology Division,

IISER Pune

## DECLARATION

I hereby declare that the matter embodied in the thesis entitled '**Characterization of *Coccinia grandis* as a model system to study sex determination in plants**' are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of **Dr. Anjan K Banerjee**, Assistant Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

Student's Name: **Ghadge Amita Gautam**

BS-MS Dual Degree Student

IISER Pune

## ABSTRACT

*Coccinia grandis* (Ivy gourd), is a dioecious member of Cucurbitaceae family, bears male and female flowers on separate individuals. The male plants carry 22A + XY and female plants carry 22A + XX chromosomes. But rare hermaphrodites with chromosome constitution as 22A + XX, bearing perfect flowers are also observed in wild. Literature survey suggests that the molecular mechanism of sex determination varies across the plant kingdom and not well understood. Till date most of the efforts to understand the mechanisms of sex determination has led to the identification of “Sex Differentiation Genes” rather than “Sex determining Genes” i.e. effect but not the cause. In this study, we have attempted to characterize the morphological differences between male, female and hermaphrodite flowers at different stages of floral development. Our observation suggests that the development of male reproductive organs is arrested in female flowers. Further, we have taken a molecular approach to understand the phylogenetic relationship as well as the genetic diversities among sexual forms of *Coccinia grandis*. Using universal DNA barcodes, *rbcL* and *matK*, we could establish DNA-based identification of natural hermaphrodite. Through a RAPD analysis, we have determined the genetic markers to identify the sex of the plant. We have observed that the sex expression of *C. grandis* is not stable. Spraying of silver nitrate ($AgNO_3$) on female plant at an early stage of flower development produces bisexual flowers instead of female flowers. In order to understand the effect of silver nitrate on sex expression of *C. grandis*, we have undertaken a proteomic approach to study the male, female and $AgNO_3$ treated flowers. Our preliminary analysis, indicate that there is differential expression of proteins in all three sexual forms of *Coccinia grandis*. Future investigations in this regard would unravel the function of these differentially expressed proteins in sex determination.

## LIST OF FIGURES

**FIGURE** **PAGE**

## ACKNOWLEDGEMENT

I would like to express my gratitude towards my research mentor, Dr. Anjan K Banerjee for his guidance and mentorship. Without his consistent guidance and persistent help, this project would not have been possible. I would also like to thank Dr. Jayeeta Banerjee for her valuable contributions, guidance and for being such a great source of support throughout my research project.

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# 1. INTRODUCTION

Flowers are the reproductive organs of flowering plants. Thus, the reproductive fitness and fruit development is directly influenced by the growth and development of flowers. Most of the angiosperm flowers are hermaphrodites bearing both male (stamens) and female (carpels) reproductive organs (Colombo et al., 1995) whereas about 20-30% of angiosperms produce unisexual flowers (Yampolsky and Yampolsky, 1922; Richards, 1997). It is observed that the combination of unisexual flowers on a plant varies with the sexual forms (Kinney, 2008). One of the combinations is bearing both male and female unisexual flowers on the same plant (referred as monoecious) whereas in other sexual forms, both male and female unisexual flowers are produced by separate individual plants (referred as dioecious). In addition, a variety of sexual forms bearing a combinations of hermaphrodite and unisexual plants are also found, such as androdioecious (population bearing male and hermaphrodite plants), gynodioecious (population bearing female and hermaphrodite plants) and trioecious (population bearing male, female and hermaphrodite plants) (Ainsworth, 2000). The mechanism that determines the sex of the flower is still unknown in many of the above sexual forms. Through a literature review, we could find few reports about the mechanism of sex determination in some plant species (eg: *Silene latifolia* (Parker, 1990), *Rumex acetosa* (Ainsworth et al., 1995)).

In dioecious plant *Silene latifolia*, the sex of the plant is determined by the sex chromosomes (Parker, 1990). The male and female plants carry XY and XX sex chromosomes respectively (Westergaard, 1940). Y chromosome is reported to be 50% larger than the X chromosome (Lebel- Hardenack et al., 2011) and it consists of three sex determining regions such as a) Gynoecium- suppressing functional (GSF) region responsible for the suppression of female reproductive organ b) Stamen Promoting Function (SPF) region responsible for the development of male reproductive organs (stamens) c) Male Fertility Functional (MFF) region responsible for the maturation of pollen grains (Westergaard, 1958, Farbos et al. 1999, Lebel- Hardenback et al., 2002). Another well-studied plant in this context is *Rumex acetosa*. It is dioecious and has X to autosome ratio as a mechanism of sex determination, which is independent of the Y

chromosome (Ainsworth et al., 1995). The chromosome constitution of female is 12 + XX (X: A = 1) and of male is 12 + $XY_1Y_2$ (X: A = 0.5), where both $Y_1$ and $Y_2$ chromosome are required for the normal development of pollen mother cells (Stokes et al., 2003). Along with X to autosome ratio, the type of the autosome also influences the sex determination mechanism in *Rumex acetosa* (Ainsworth et al., 1995).

Other than the sex chromosomes-influenced sex determination mechanism, there are reports of environmental and hormone regulated sex determination in some plants as well. Though variation in sex expression according to the environment provides an advantage to the plant, only few plants undergoes this modification (Korpelainen, 1998). One of the examples of environmental regulation of sex is androdioecious *Mercurialis annua* (Euphorbiaceae), in which the population of the parental plant influences the sex of the progeny (Dorken and Pannell, 2008). It was observed that when there is low density of hermaphrodite plants, the number of male progeny in the subsequent generation is low (Adam et al., 2011). Day length and light intensity are also known to influence the sex ratios, example of which is monoecious *Atriplex halimus* (Chenopodiaceae), under short day length and less light intensity, plant produces more female flowers (Talamali et al., 2001, 2003). Besides variation in sex expression due to natural factors, there are some monoecious and dioecious plants that undergo sex modification on application of exogenous hormones or chemicals (Lal et al., 1988). As there is no common mechanism of sex determination across the sexual forms, there is no common hormone or chemical which causes the same sex modification in all the sex states (Law et al., 2002). Therefore, the application of growth regulators like cytokinins has a feminizing effect on mercury (*Mercuralis annua* L.) (Durand 1969; Durand and Durand, 1991) and spinach (*Spinacia oleracea* L.) (Chailakhyan, 1979) but has no such effect on cucumber (*Cucumis sativus* L.) and maize (Zea mays L.) (Chailakhyan, 1979; Law et al., 2002). Similarly, there are growth regulators that have masculinizing effect on plants. One of those growth regulators is gibberellins, which has masculinizing effect on spinach, cucumber and asparagus (*Asparagus officinalis* L.) (Lazarte and Garrison, 1980). In contrast to gibberellins, auxins have feminizing effect on cucumber and spinach (Heslop- Harrison, 1956;

Rudich. H. and Kedar, 1972). Certain chemicals such as silver nitrate ($AgNO_3$) are also used to influence the sex expression. In cucumber (*Cucumis sativus*), application of silver nitrate produces more male flowers than the female flowers (Beyer, 1976; Adams and Yang, 1979; Atsmon and Tabbak, 1979). It was later shown that silver nitrate is one of the chemicals that inhibit ethylene biosynthesis (Strader et al., 2009), where ethylene is known to promote development of female flowers (Wang et al., 2010). In some dioecious species like Mulberry (*Morus alba* L.), the application of silver nitrate leads to the development of hermaphrodite flowers rather than causing complete sex change from male to female flowers or vice versa (Thomas, 2004). Like Mulberry, sweet gourd (*Momordica cochinchinensis* Spreng.) produces hermaphrodite flowers on spraying of silver nitrate (Sanwal et al., 2011). Though it is being shown that application of silver nitrate causes sex modification in many sexual forms, the exact mechanism by which it affects the flower development is still not known. But it is evident that silver nitrate play a significant role in the basic mechanism of sex determination when sprayed on plants (V. Kumar et al., 2009).

From the above study, it is quite clear that even though there exist genetic mechanism of sex determination, the sex expression in plants may not be stable and can be influenced by a number of natural factors. Cucurbitaceae is one of the families in plant kingdom which consists of several members exhibiting various sexual forms (Kouonon et al., 2009). *Coccinia grandis* is one of the dioecious species in Cucurbitaceae family which has been shown to have chromosomal basis of sex determination (Vyskot et al., 2004). Similar to *Silene latifolia* (Caryophyllaceae), the sex in *C. grandis* is determined by the presence of Y chromosome (Kumar and Deodikar, 1940; Bhaduri and Bose, 1947 and Chakravarty, 1948). The chromosome constitution of male and female plants is 22A + XY and 22A + XX respectively, where Y chromosome is larger than the X chromosome (Sousa et al., 2012). The molecular mechanism by which sex determination takes place in *C. grandis* is still not known.

*Coccinia grandis* is dioecious in nature, bearing male and female unisexual flowers on separate plants. But rare natural hermaphrodites bearing perfect flowers, consisting of both male and female reproductive organs are also observed. The male

flower consists of three convoluted stamens and lack female reproductive organs; however, the female flower consists of three rudimentary stamens surrounding the three fused carpels with an inferior ovary. The reason of the retention of rudimentary stamens in female flowers is still not known. Thus, studying the morphological differences between the flowers of male, female and natural hermaphrodite might provide us with further understanding into the development of unisexual and hermaphrodite flowers in *C. grandis*. Additionally, analysis such as phylogenetic relationship, RAPD, metaphasic chromosomal cytology, between male, female and natural hermaphrodite flowers could potentially enhance our knowledge in understanding the molecular mechanism involved in sex determination of *C. grandis*. We have also seen that the sex expression in *C. grandis* is not stable similar to cucumber and sweet gourd as reported earlier. On application of silver nitrate on female plants of *C. grandis* causes sex modification in female unisexual flowers. The $AgNO_3$ treated flowers buds are hermaphrodites but morphologically different from the natural hermaphrodite. The mechanism by which this sex modification takes place in the female plant is not known. The question thus arises that how can a female plant, being genetically distinct from the male plant, produce male reproductive organs during sex change. In addition to our morphological analysis, we have opted for an approach as to compare the proteomes of male, female and $AgNO_3$ treated flowers buds, in order to understand the factors responsible for the sex modification.

## 2. OBJECTIVES

The main objective of this study is to understand the mechanism of sex determination in *Coccinia grandis*. In order to address this question, following approaches have been considered for the present study.

1) Morphological characterization of male, female and natural hermaphrodite plants.
    1.1) Analysis of morphological differences between male, female and natural hermaphrodite flowers.
    1.2) Understanding the development of unisexual and natural hermaphrodite flowers in *C. grandis.*
    1.3) Differences in the leaf epidermal morphology.

2) Molecular characterization of male, female and natural hermaphrodite plants.
    2.1) Phylogenetic relationship between the three sexes based on DNA barcodes.
    2.2) Development of genetic markers in order to identify the sex of the plant.
    2.3) Cytological differences between the three sexes.

3) Understanding sex modification in *C. grandis* on spraying of silver nitrate.
    3.1) Silver nitrate dose- dependent alterations in the sex expression of female flowers.
    3.2) Analysis of morphological differences between male, female, natural hermaphrodite and $AgNO_3$ treated flower buds.
    3.3) Comparative analysis of proteome profiles of male, female and $AgNO_3$ treated flower buds by 2-D gel electrophoresis.

# 3. MATERIALS AND METHODS

### Plant material

We have obtained the plant material from Agartala, Tripura and maintained throughout the year in the garden of National Chemical Laboratory (NCL), Pune. Flowers and leaves were collected from soil grown male, female and hermaphrodite *Coccinia grandis* plants and were stored at -80ºC for molecular analysis.

### Histology

A series of histology of flower buds at various developmental stages were performed according to Cai et al., 2006, with the following modifications. Flower buds of male and female, each of developmental stages from 1 to 10, were collected and fixed in 3:1 ethanol: acetic acid for overnight at 4ºC. The material was dehydrated with 75% ethanol for 40 mins, 95% ethanol for 40 mins and 100% ethanol with 3 changes, each with 45 mins intervals. The material was then treated with 50-50% ethanol-xylene for 45 mins, followed by clearing with 100% ethanol for 45 mins. The xylene was replaced with paraplast wax at 59ºC. Then the tissue was embedded in the paraplast blocks. The tissues were sectioned with 10 microns thickness and the paraplast sections were then mounted on the slides with water at 50ºC.

### Scanning Electron Microscopy (SEM)

The leaves from male, female and natural hermaphrodite plants were subjected to Scanning Electron Microscopy Quanta -200 3D (FEI) SEM (Environmental mode) at National Chemical Laboratory (NCL), Pune.

### Genomic DNA extraction

The leaf samples were crushed in liquid nitrogen into fine powder using mortar and pestle. Genomic DNA was extracted using DNeasy® Plant mini kit (Qiagen, Cat. No. 69104) and the DNA concentration was determined using Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, USA). The extracted genomic DNA was then stored at -20ºC.

**PCR for rbcl and matk loci**

A total volume of 30µl PCR reaction mixture containing 3µl of 10X Pfu buffer, 200µM of dNTPs , 1mM of $MgCl_2$, 0.16µM of primer, 100-150ng of genomic DNA, 0.16µl of 2.5U of Pfu DNA polymerase and the rest of the volume was adjusted with sterile Milli-Q water. The primer-pairs used for the PCR of *rbcL* loci are
*rbcL*F (5'-ATGTCACCACAAACAGAGACTAAAGC-3') and
*rbcL*R (5'-GAAACGGTCTCTCCAACGCAT-3'). The primer-pairs used for the PCR of *matK* loci are *matK*F (5'-ATCCATCTGGAAATCTTAGTTC-3') and
*matK*R (5'-CTTCCTCTGTAAAGAATTC-3'). PCR amplification was performed in thermocycler with the following PCR programme: initial denaturation at 95ºC for 1min; 35 cycles of denaturation at 95ºC for 30secs, annealing at TºC for 30secs, and extension at 68ºC for 1min; and final extension at 68ºC for 10min. The reaction mixtures were held at 4ºC. All the PCR conditions were same for all the primer-pairs except the annealing temperature (T). TºC for *rbcL*F and *rbcL*R primer pairs is 60ºC and for *matK*F and *matK*R primer pairs is 45ºC. The PCR amplified products were resolved on 1% agarose gel stained with ethidium bromide by electrophoresis.

**Phylogenetic analysis**

The sequences that were used from NCBI Nucleotide database for the construction of phylogenetic tree based on *rbcL* loci are as follows: *Coccinia microphylla* (HQ608505), *Coccinia rehmannii* (DQ535793), *Coccinia megarrhiza* (HQ608504), *Coccinia hirtella* (HQ608502), *Coccinia subsessiliflora* (HQ608510), *Coccinia barteri* (HQ608500), *Coccinia mildbraedii* (HQ608506), *Coccinia mackenii* (HQ608503), *Coccinia quinqueloba* (HQ608508), *Coccinia sessilifolia* (AY968520), *Coccinia adoensis* (HQ608509), *Coccinia ogadensis* (HQ608507) and *Coccinia grandiflora* (HQ608501). The sequences used for the development of phylogenetic tree based on *matK* loci were imported from NCBI Nucleotide database and are as follows: *Coccinia samburuensis* (HQ608264), *Coccinia rehmannii* (HQ608261), *Coccinia megarrhiza* (HQ608252), *Coccinia microphylla* (HQ608254), *Coccinia mildbraedii* (HQ608256), *Coccinia schliebenii* (HQ608265), *Coccinia grandiflora* (HQ608244), *Coccinia heterophylla* (HQ608246), *Coccinia keayana* (HQ608249), *Coccinia abyssinica* (HQ608224),

*Coccinia trilobata* (HQ608271), *Coccinia senensis* (HQ608267), *Coccinia aurantiaca* (HQ608236), *Coccinia ogadensis* (HQ608258), *Coccinia sessilifolia* (AY968446), *Coccinia adoensis* (HQ608225) and *Coccinia quinqueloba* (HQ608259). Finally, the *rbcL* and *matK* sequences were imported in MEGA5.1 software (Tamura, K. et al., 2011) and aligned using Multiple Sequence Alignment (ClustalW). Phylogeny was built using Neighbor-joining with bootstrap method and 500 replications.

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**Random Amplification of Polymorphic DNA (RAPD) analysis**

To identify the genetic marker in male, female and natural hermaphrodite, 150ng of genomic DNA from each sample were subjected to RAPD analysis. A total volume of 30µl PCR reaction mixture containing 3µl of 10X Taq buffer, 100µM of dNTPs , 1.5mM of $MgCl_2$, 0.4µM of primer, 0.6µl of 2.5U of Taq DNA polymerase and the rest of the volume was adjusted with sterile Milli-Q water. The RAPD primers used are as follows: OPC-10(5'-TGTCTGGGTG-3'), OPA-9(5'-GGGTAACGCC-3'), OPA-18(5'-AGCTGACCGT-3'), OPN-1(5'-CTCACGTTGG-3'), OPC-7(5'-GTCCCGACGA-3'), OPA-8(5'-GTGACGTAGG-3'), OPO-8(5'-CCTCCAGTGT-3'), OPC-14(5'-TGCGTGCTTG-3'), OPC-2(5'-GTGAGGCGTC-3'), OPC-5(5'-GATGACCGCC-3') and OPA-10(5'-GTGATCGCAG-3'). PCR amplification was performed in thermocycler with the following PCR programme: 1 cycle (94ºC for 4min; 35ºC for 1min; 72ºC for 2min) followed by 44 cycles (1 min at 94ºC, 1 min at 40ºC, 2 min at 72ºC) and final extension at 72ºC for 15 min. The reaction mixtures were then held at 4ºC. All the PCR conditions were same for all the RAPD primers. The PCR amplified products were then resolved on 3% agarose gel stained with ethidium bromide by electrophoresis.

**Standardization of silver nitrate doses**

We have carried out this experiment in collaboration with Prof. Sangram Sinha, Tripura University, Tripura. Two months old female *Coccinia grandis* plant grown in NCL, Pune ground was sprayed with different concentrations (4%, 5% and 6%) of silver nitrate (w/v) solution. The concentrations lower than 4% were also tested. Silver nitrate solution was sprayed on the basal leaves of female plant till the solution fall off the leaves. The observation was made 10 days after the foliar spray. Flower buds showing sex

conversion at the concentration of 5% of silver nitrate spray were collected, imaged and finally stored at -80ºC for protein isolation.

**Protein extraction**

For protein extraction, the flower buds from male, female and AgNO3 treated plants of developmental stage 5 to 10 were used. The total protein was extracted from the samples using the TCA method (Damerval et al, 1986) with minor modifications. The male, female and silver nitrate treated flower buds were crushed in liquid nitrogen into fine powder using mortar and pestle. Approximately 300 mg of the crushed sample was used further for the extraction. The crushed samples were properly resuspended in 1.5 ml of pre-chilled buffer containing 90% of acetone, 10% Trichloro-acetic acid and 0.07% β-mercaptoethanol. After resuspension, the above solution was centrifuged at 14000g for 30 minutes at room temperature. To the pellet another pre-chilled resuspension buffer containing 100% acetone, 0.07% β -mercaptoethanol and 2mM EDTA was added and centrifuged at 14000g for 30 mins at room temperature. The above step is repeated until the pellet becomes white. After centrifugation, the pellet was air-dried at room temperature and solubilized in rehydration buffer containing 8M urea, 2M thiourea, 4% CHAPS and 50mM DTT. The solubilized protein was then further precipitated using TCA precipitation protocol by Cold Spring Harbor, Link A. J. (2011). The protein yield was determined by Bradford assay according to the procedure of Bradford (Bradford, 1976) and estimated by plate reader (Varioskan flash, ThermoScientific). The protein aliquots were stored at -20ºC until further use.

**Two- dimensional gel electrophoresis**

The two- dimensional gel electrophoresis was performed as per manufacturer's protocol (Bio-Rad, USA). Isoelectric focusing was carried out using IPG strips (Biorad). Approximately, 350µg of the protein sample was loaded on the IPG strips (7cm, pH 3-10) and incubated at room temperature for 16hrs. After incubation, IPG strips were rehydrated for 9hrs in a stepwise manner: 250V for 20 min, 4000V for 2 hrs, 4000V until 10000 Volt-hrs are reached and finally hold at 1500V for 30 mins. After IEF run, the rehydrated strips were equilibrated in equilibrium buffer I containing 6M urea, 1.5M Tris-HCl (pH = 8.8), 2% SDS, 50% glycerol, 2% w/v DTT for 15 min and then in equilibrium

buffer II containing 6M urea,1.5M Tris-HCl (pH = 8.8), 2% SDS, 50% glycerol, 2.5% w/v iodoacetamide for 15 min with gentle shaking. After equilibration, the strips were applied to 12% w/v SDS-PAGE gels and sealed with low gelling agarose solution (0.5% w/v agarose in SDS buffer plus a few grains of Bromophenol Blue). Protein samples were separated with SDS gel electrophoresis in SDS buffer containing 250mM tris-base, 1.92M glycine and 1% SDS (pH = 8.3). The gel electrophoresis was carried out at 100V for 90 minutes. After electrophoresis, the gels were stained with CBB- R250 with gentle shaking for overnight and then transferred to destaining solution (60% distilled water, 30% methanol and 10% glacial acetic acid). The gels were destained for about 1 day till the gel background was clear. All the gels were pictured through LAS 4000 image documentation system. After imaging, the gels were stored in 5% Acetic acid solution until further use.

# 4. RESULTS

## 4.1) Characterization of morphological differences between male, female and natural hermaphrodite plants

### 4.1.1) Analysis of morphological differences in the flowers of male, female and natural hermaphrodite

Generally in wild, *C. grandis* is dioecious but rare hermaphrodites bearing perfect flowers are also observed. We have not observed any phenotypic differences between male, female and natural hermaphrodite plants other than flower morphology. The number of sepals and petals are same in male, female and natural hermaphrodite flowers. The *C. grandis* flowers consist of five petals with pointed tips and five small sepals (Figure 4.1.1.1). When the petals are removed from the flowers, the whorl 3 and whorl 4 can be then easily compared within male, female and natural hermaphrodite. In mature male flower (Figure 4.1.1.2 A), the three convoluted stamens are compactly packed and are connected to the single floral stalk at the base. There is no sign of rudimentary carpels in male flower. In mature female flower (Figure 4.1.1.2 B), the three fused carpels are connected to the single floral stalk leading to the single inferior ovary which is surrounded by three rudimentary stamens as found in our observations. In natural hermaphrodite (Figure 4.1.1.2 C), the morphology and structure of stamens is different from the male flower due to the presence of gynoecium at the centre of the flower. Because of the presence of carpels at whorl 4 we can observe the three distinct stamens.

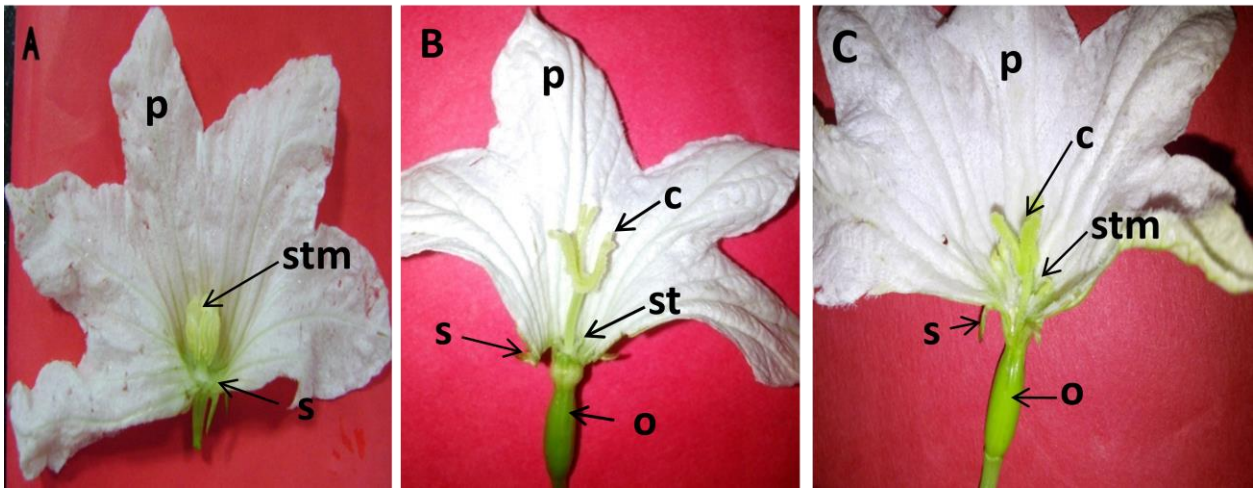


Figure 4.1.1.1: Flower morphology of *C. grandis*. (A) Mature male flower, (B) Mature female flower and (C) Mature natural hermaphrodite flower. s- sepals, p- petals, stm- stamens, st- rudimentary stamens, c- carpels, o- ovary.

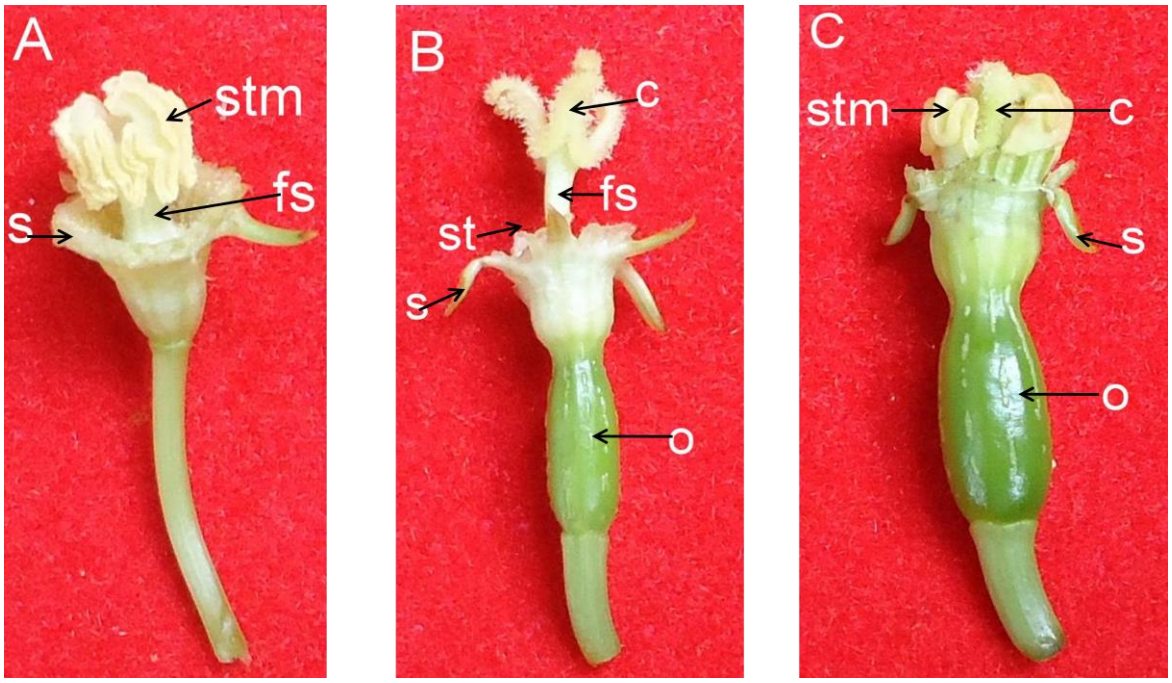


Figure 4.1.1.2: Lateral view of *C. grandis* flowers with petals removed. (A) Mature male flower (B) Mature female flower (C) Mature hermaphrodite flower. stm- stamens, st- rudimentary stamens, fs- floral stalk, c- carpels, o- ovary , s- sepals.

### 4.1.2) Development of unisexual and hermaphrodite flowers in *C. grandis*

In order to observe the morphology of different whorls at various developmental stages, we had arranged the flower buds of male, female and natural hermaphrodite according to their developmental stages from 1 to 10 (Figure 4.1.2.1). All these stages (1-10) of flowers were also subjected to histological analysis.

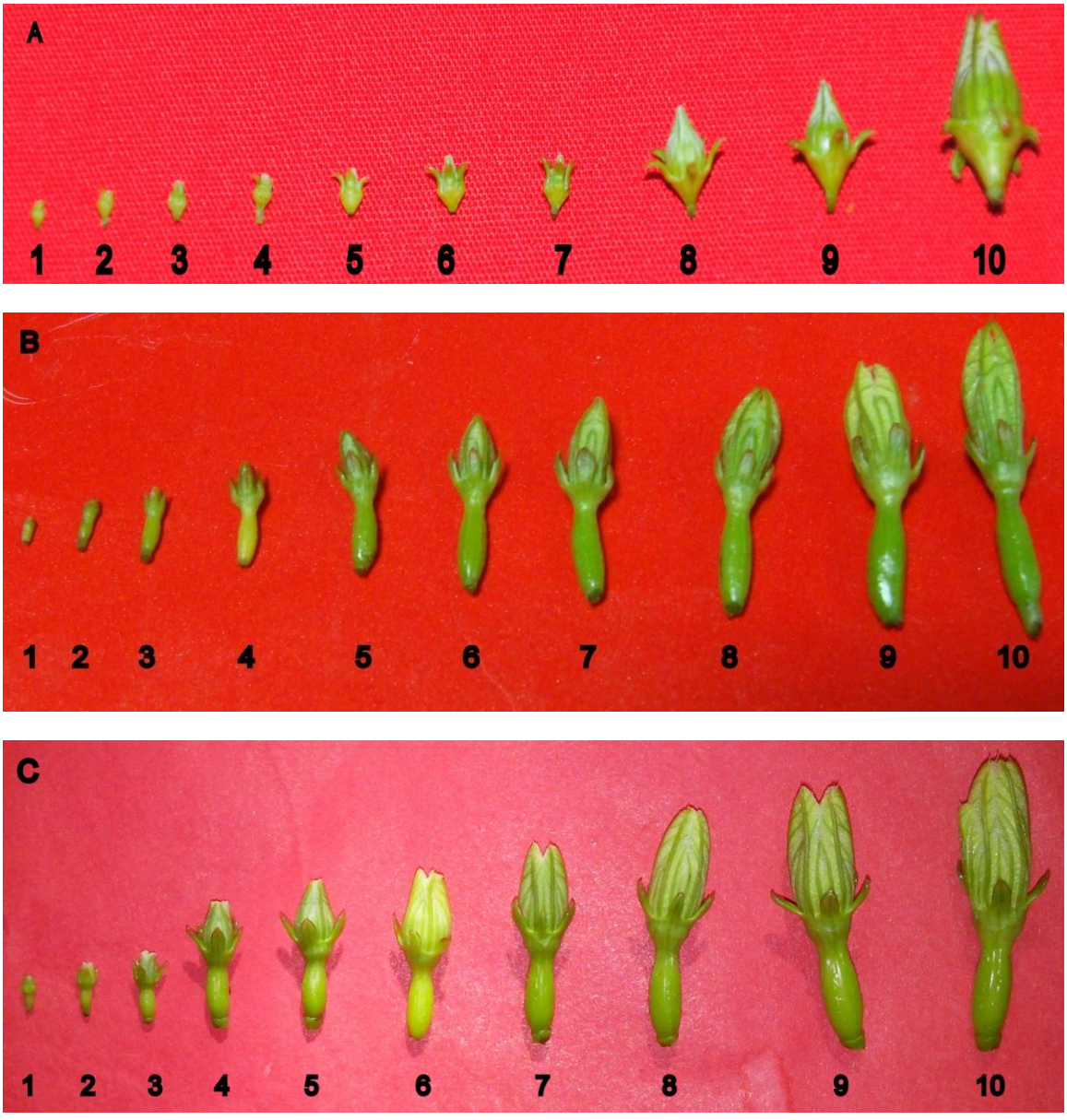


Figure 4.1.2.1: Different stages of floral development in *C. grandis*. (A) Male, (B) female and (C) natural hermaphrodite flower buds are arranged from stage 1 to 10.

Our morphological analysis suggests that there is no apparent phenotypic difference between female and natural hermaphrodite flowers. However, the morphology of male flower is phenotypically quite different from female and hermaphrodite flowers. To understand the growth patterns and also to find the mode of development of unisexual and hermaphrodite flowers in *C. grandis*, we analyze the developmental patterns of different whorls at various developmental stages through histology (Figure 4.1.2.2 A-B).

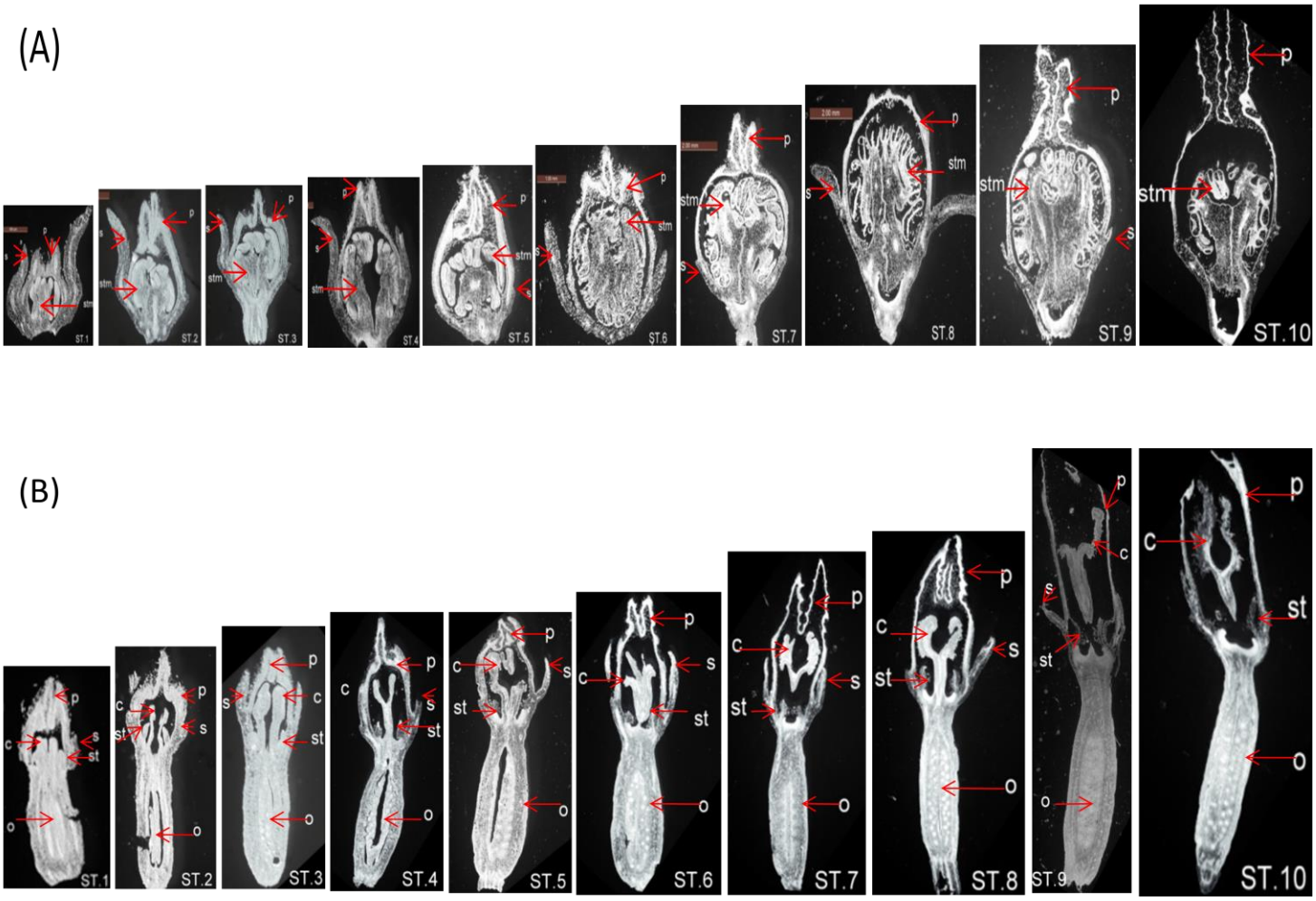


Figure 4.1.2.2: Longitudinal sections of *C. grandis* flower buds from stage 1 to 10. (A) Male, (B) Female and s- sepals, p- petals, stm- stamens, c- carpels, st- rudimentary stamens, o- ovary.

Our histology analysis showed that the morphology and development of sepals and petals is same in all the stages of male, female and natural hermaphrodite flower buds.

Stage1-3:

Male: There is initiation of stamen development at stage1 of male flower buds which is difficult to observe without the histological analysis. At this stage, there is development of stamens at the centre of the male flower bud, which in bisexual flower, consists of gynoecium. There are no signs of development of gynoecium in male Coccinia flower. At stage 2, we observed that the three convoluted stames are packed compactly. At this stage also there is no development of gynoecium in any of the whorls. At stage 3, there is development of floral stalk at the centre of the flower bud, supporting the three convoluted stamens.

Female: We have observed that the female flower bud of stage 1 shows initial development of carpels in whorl 4 and presence of an inferior ovary. The development of stamens is also seen in whorl 3 of female flower. At this stage, the female flower bud is hermaphroditic bearing both male (stamens) and female (carpels) reproductive organs. At stage 2 and stage 3, there is further development of carpels and inferior ovary in whorl 4 and stamens in whorl 3.

Stage 4-6:

Male: In stages 4-6, we could find that there is further development of floral stalk and stamens in the centre of the flower bud. At stage 4, we see that two stamens connected to a single floral stalk at the centre of the flower. There is clearly no carpel development in whorl 4 in contrast to the bisexual flower. The male flower buds are unisexual from stage 1 itself. At stage 6, the pollen development in the anthers is visible.

Female: At stage 4, there is development of floral stalk at the centre of the flower bud supporting the three fused carpels. The development of ovules can be seen in the ovary. Development of stamens in whorl 3 at stage 5 is similar to that of stage 4 and even in stage 6. It can be concluded that there is no further development of stamens in

female flowers after stage 4. Possibly there is an arrest of stamen development at stage 4.

Stage7-10:

Male: At these stages, we have observed that there is further maturation of stamens and elongation of the floral stalk. There is development of pollen grains in the anthers of the stamens and the floral stalk is distinctly seen.

Female: We have seen that from stages 7-10, there is further maturation of carpels and the elongation of floral stalk. Our observation shows that extended floral stalk supports the fused carpels. At this stage, the development of the ovules can be observed in the ovary.

The above observations indicate that the initial floral primordia of female flower are hermaphroditic but in later stages it becomes unisexual female flower. Therefore, we can conclude that the mode of development of female unisexual flower is by arresting the development of male reproductive organs. There is formation of stamens in whorl 3 of female flower but at later stages there is no further development of stamens. There is no abortion of stamens as well. Thus, *C. grandis* female flower does not follow the abortive mechanism of development of unisexual flower. The stamens in female flowers are sterile, therefore, we refer to them as staminodes (rudimentary stamens). The male flower of stage 1, is unisexual with no development of female reproductive organs. The initial floral primordia of male flower might also be hermaphroditic and the mode of development of male unisexual flower is by abortion of female reproductive organs. The other possible mechanism is that there is no development of female reproductive organs in male flowers right from the inception of formation of flower.

### 4.1.3) Differences in the leaf epidermal morphology of male, female and natural hermaphrodite plants.

In order to find the sex-related differences at the vegetative stage between male, female and natural hermaphrodite *Coccinia grandis*, the most easily available tissue that

can be used to study the vegetative difference is leaf. Like most of the other Cucurbitaceae species, in *C. grandis*, the stomata are present on both the abaxial and adaxial side of the leaf. Comparing the abaxial side of male, female and natural hermaphrodite leaves, we observed that the number of stomata is present in the increasing order of male, female and natural hermaphrodite plant. We noted differences in the morphology of epidermal cells of abaxial side between the sexes. The epidermal cells of abaxial side of male leaf looks like are less compact and bigger than that of female and natural hermaphrodite. The epidermal cells of abaxial side of female and natural hermaphrodite leaf are more compact and smaller than that of male leaf. Similar observations were seen when the adaxial side of the male, female and natural hermaphrodite leaves were compared.

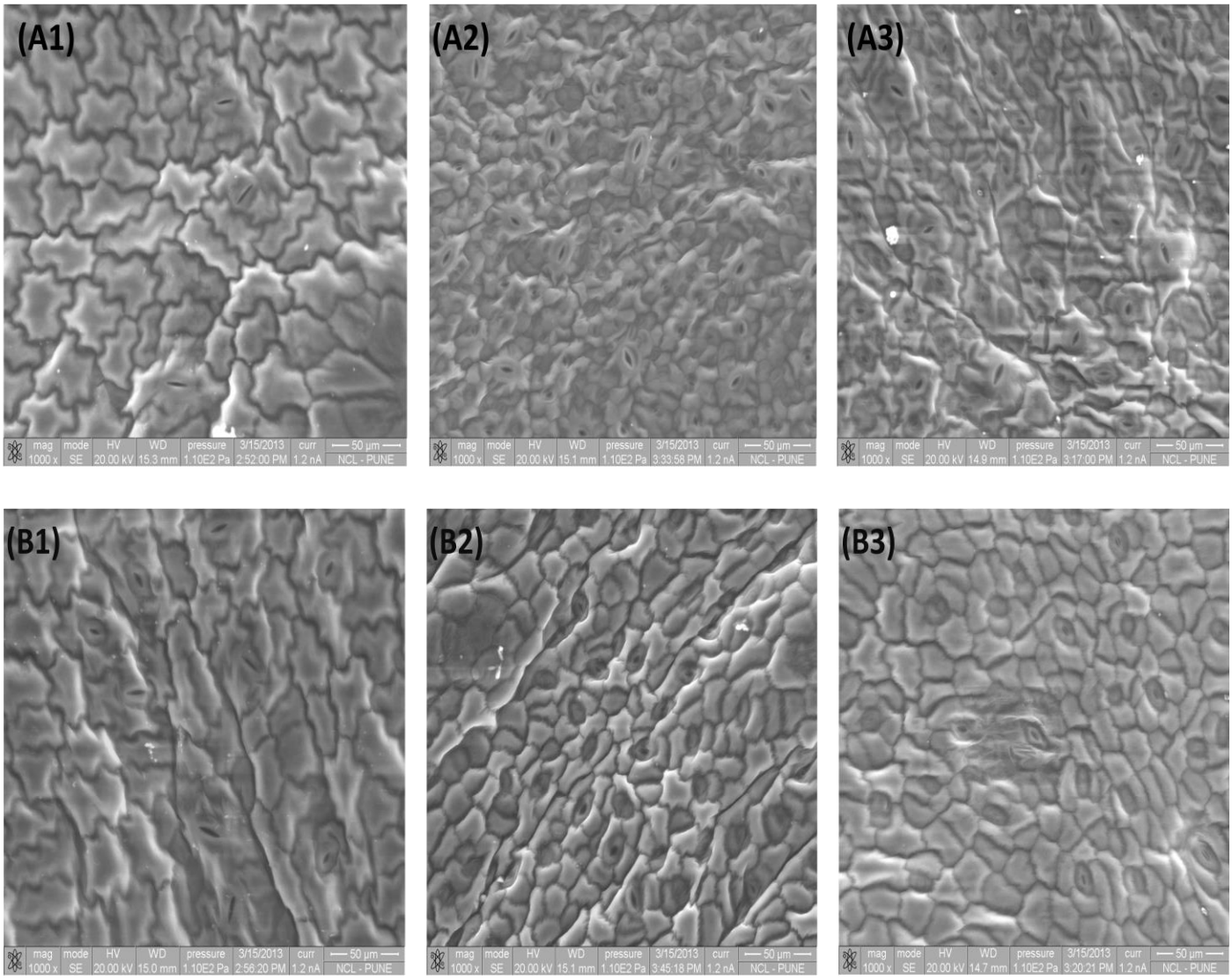


Figure 4.1.3.1: Leaf epidermal morphology in male, female and natural hermaphrodite of *C. grandis*. (A1-A3) is the abaxial and (B1-B3) is the adaxial side of the male, female and natural hermaphrodite leaf, respectively.

## 4.2) Molecular characterization of male, female and natural hermaphrodite *C. grandis*

### 4.2.1) DNA-based identification of natural hermaphrodite

Since morphology- based identification of species is not always reliable, so we used DNA sequences to establish the DNA-based identification of natural hermaphrodite. Plants in addition to nuclear genome, consists of chloroplast genome and mitochondrial genome. Most often it is the chloroplast genome, which is used for DNA barcoding because it is present in abundance in plants, easy to extract and

contains single copy genes (Burgess et al., 2011). The Consortium for the Barcode of Life (CBOL) plant working group recommended ribulose-1, 5- bisphosphate carboxylase oxygenase large subunit (*rbcL*) and maturase K (*matK*) as the standard plant barcodes (CBOL Plant Working Group, 2009). These two regions of chloroplast DNA were selected on the basis of: good recovery of sequences and better identification of species (Burgess et al., 2011). Therefore, in our study, we have used *rbcL* and *matK* sequences for identification of natural hermaphrodite by phylogenetic analysis.

Using universal primers, we were successful in amplifying ~650bp of *rbcL* and ~1Kb of *matK* loci from genomic DNA of male, female and natural hermaphrodite plants. Amplification results of *rbcL* and *matK* are shown in Figure 4.2.1.1.

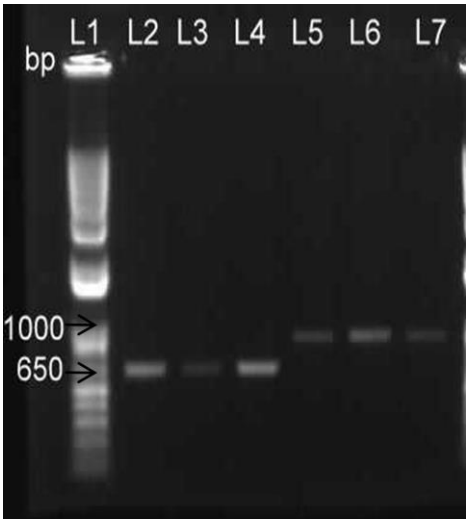


Figure 4.2.1.1: PCR amplification results of *rbcL* and *matK* loci in *C. grandis*. L1 is the 1Kb plus DNA ladder. L2- L4 are the PCR amplified products of *rbcL* using genomic DNA from male, female and natural hermaphrodite leaves. L5-L7 are the PCR amplified products of *matK* using genomic DNA from male, female and natural hermaphrodite leaves respectively.

### 4.2.1a) Phylogeny of *Coccinia* based on *rbcL* DNA sequences

The *rbcL* gene is a single copy gene in the chloroplast genome as reported earlier (Chase et al., 1993; Donoghue et al., 1993). The function of *rbcL* gene is to code for the large subunit of ribulose 1, 5- bisphosphate carboxylase/ oxygenase (RUBISCO).

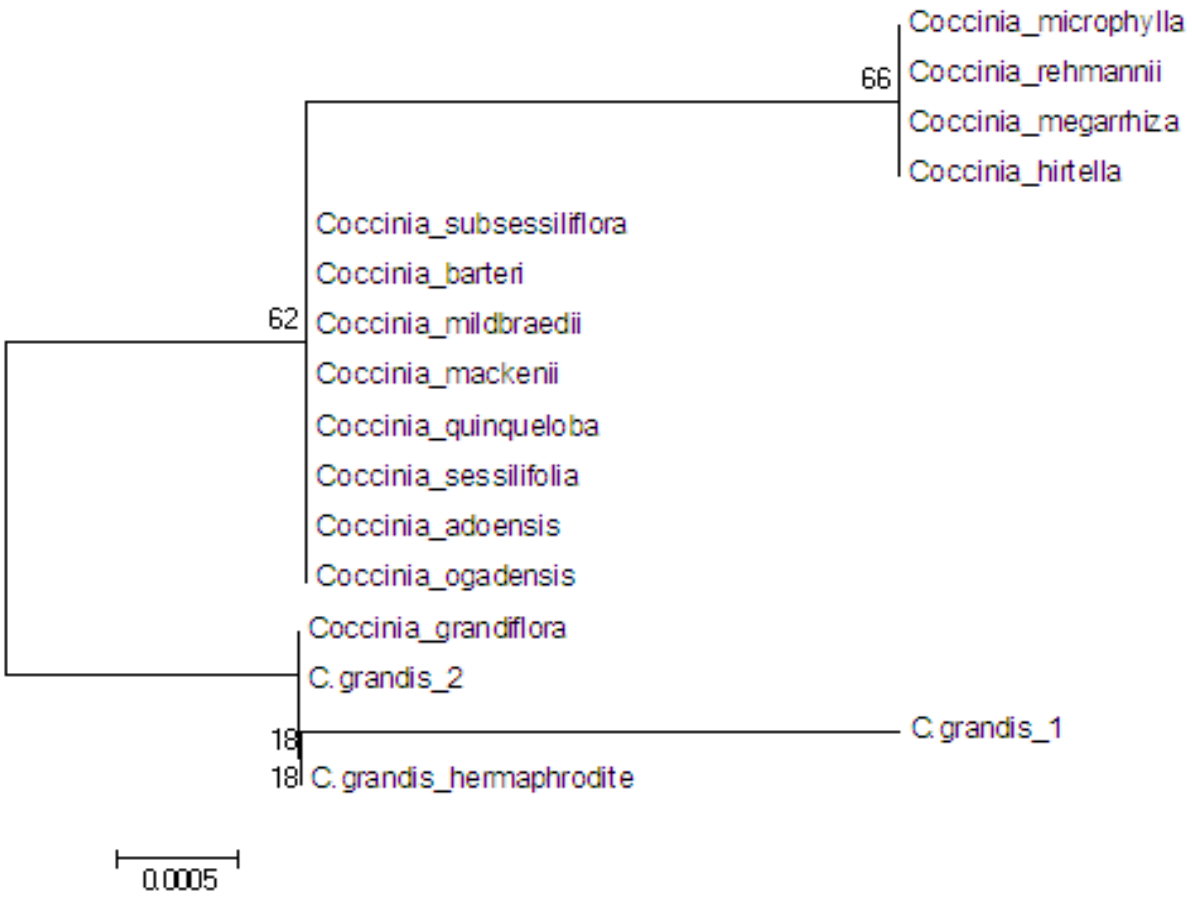


Figure 4.2.1.2: Neighbor-joining phylogeny of *Coccinia* based on *rbcL* DNA sequences.

From the phylogenetic tree (Fig 4.2.1.2) built on the DNA sequences of *rbcL* from various *Coccinia* species, we find that *rbcL* as a DNA barcode is not sufficient to resolve the phylogenetic relationship at the species level in this case. But still natural hermaphrodite forms a clade with the *C. grandis*.

### 4.2.1b) Phylogeny of *Coccinia* based on *matK* DNA sequences

To resolve the phylogenetic relationship, another candidate from the chloroplast genome - *matK* gene is used in the present study. The *matK* gene was first identified by Sugita et al. (1985) from tobacco (*Nicotiana tobacum*). It was hypothesized that the

putative function of maturase, *matK*, is to assist the splicing of group II introns other than the one in which it is normally encoded (Ems et al., 1995).

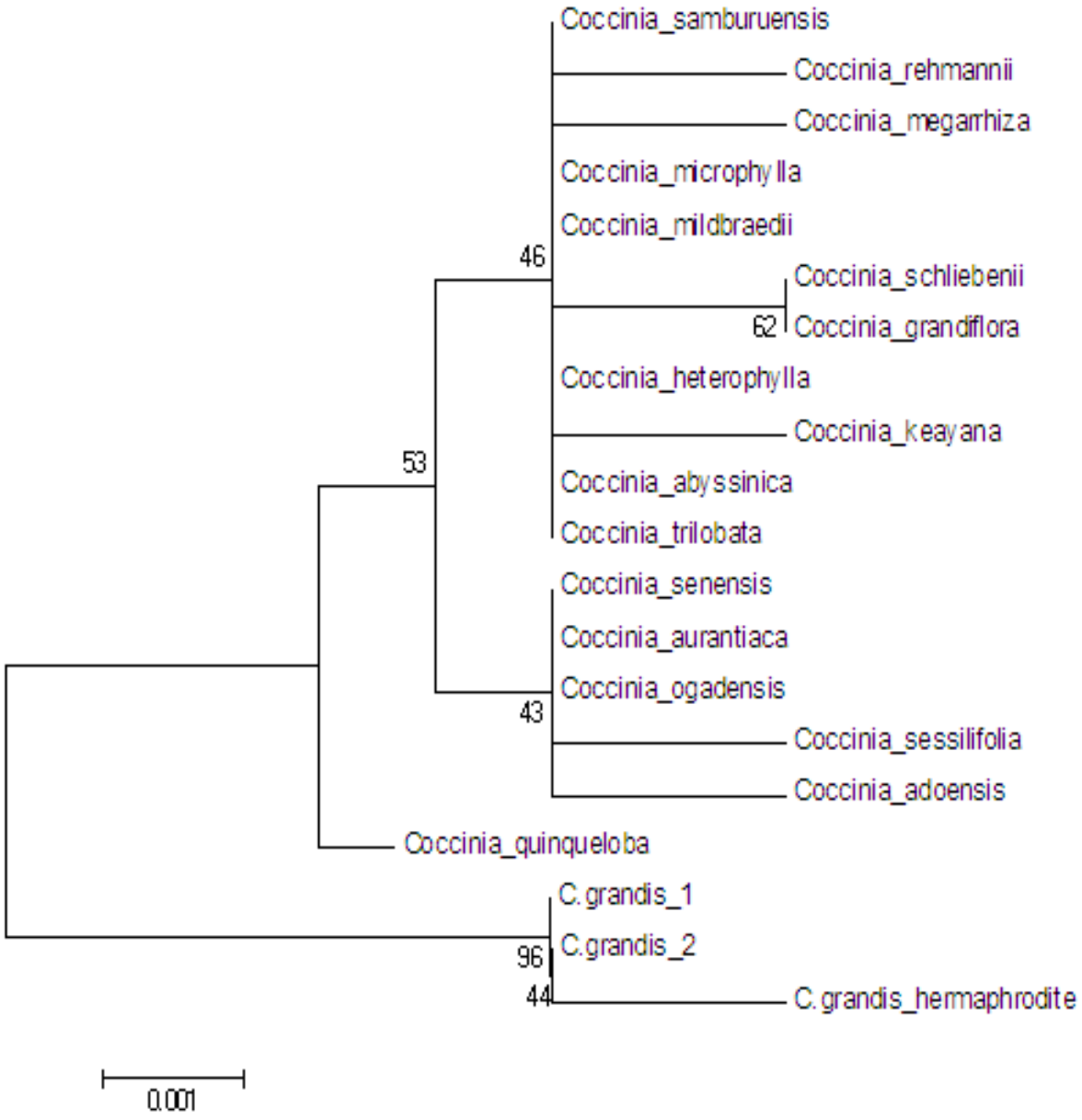


Figure 4.2.1.3: Neighbor joining phylogeny of *Coccinia* based on *matK* DNA sequences.

From the phylogenetic tree (Fig 4.2.1.3) built on the *matK* DNA sequences of various *Coccinia* species, we find that *matK* is a better DNA barcode than *rbcL* gene. When the overall results of phylogenetic relationships were compared, we were able to achieve DNA- based identification of natural hermaphrodite as another sexual form of *Coccinia grandis*.

### 4.2.2) Development of genetic markers through Random Amplification of Polymorphic DNA (RAPD)

Except the flowers, there exists no sex-related phenotypic difference between male, female and natural hermaphrodite. In order to identify the sex of the plant at the vegetative stage, we develop sex-related genetic markers using Random Amplification of Polymorphic DNA (RAPD). RAPD is widely used for detecting the genetic diversity among species. The following is the preliminary results of RAPD analysis. We have made eleven RAPD primers to amplify the genomic DNA extracted from the leaves of male, female and natural hermaphrodite *Coccinia* plants. Out of which, we identified three primers OPA-10, OPO-08 and OPN-01 which produced distinct DNA polymorphism and the probable sex-related bands.

Male –related marker: Figure 8B shows the amplification result of primer OPN-01 and the arrow pointed in the L2 is the male-related band of size ~ 530bp.

Female –related marker: Figure 8C shows the amplification result of primer OPA-10 and the two arrows pointed in the L2, one of size ~600bp and other of ~400bp are the probable female-specific bands.

Natural hermaphrodite –related marker: Amplification result of primer OPO-08 is shown in Fig. 8A. The arrow pointed in the L4 is the probable hermaphrodite-related band of size ~570bp.

The above experiment was repeated thrice to check the reproducibility of the result. In order to develop sex-related genetic markers, we need to screen more numbers of RAPD primers with a large number of identified male, female and natural hermaphrodite plant samples. Study in this regard is in progress.

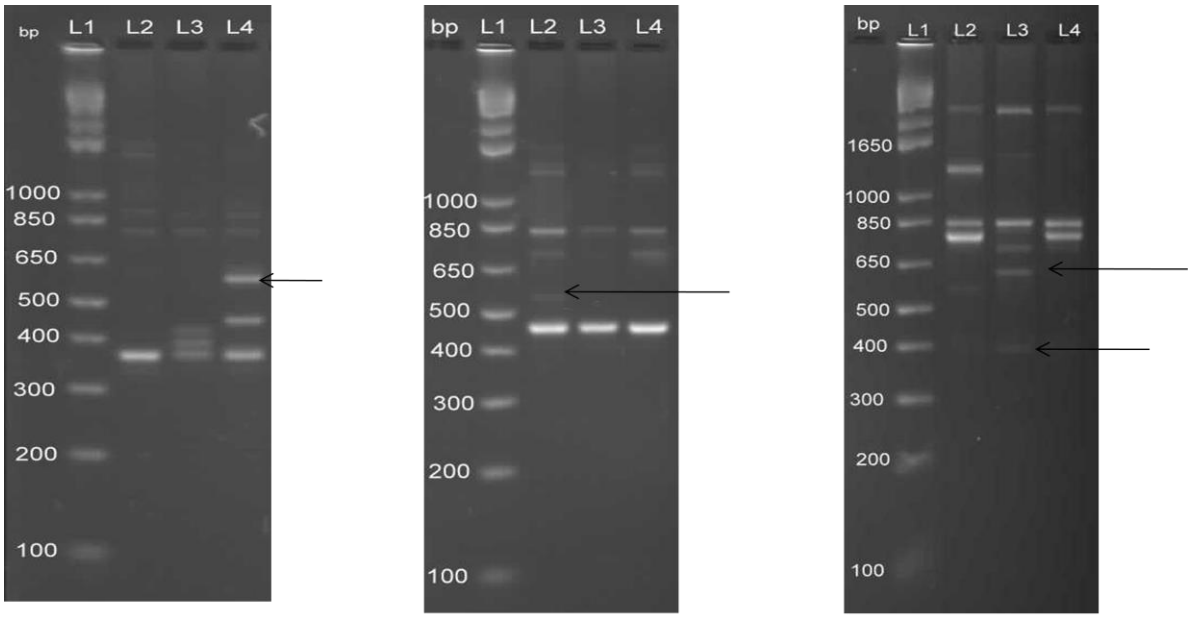


Figure 4.2.2.1: RAPD profiles of male, female and natural hermaphrodite *C. grandis*. L1 is the 1kb plus ladder, L2, L3 and L4 are the amplification results using genomic DNA of male, female and natural hermaphrodite respectively using RAPD primer (A) OPO-08, (B) OPN-01 and (C) OPA-10.

### 4.2.3) Cytological differences in male, female and natural hermaphrodite

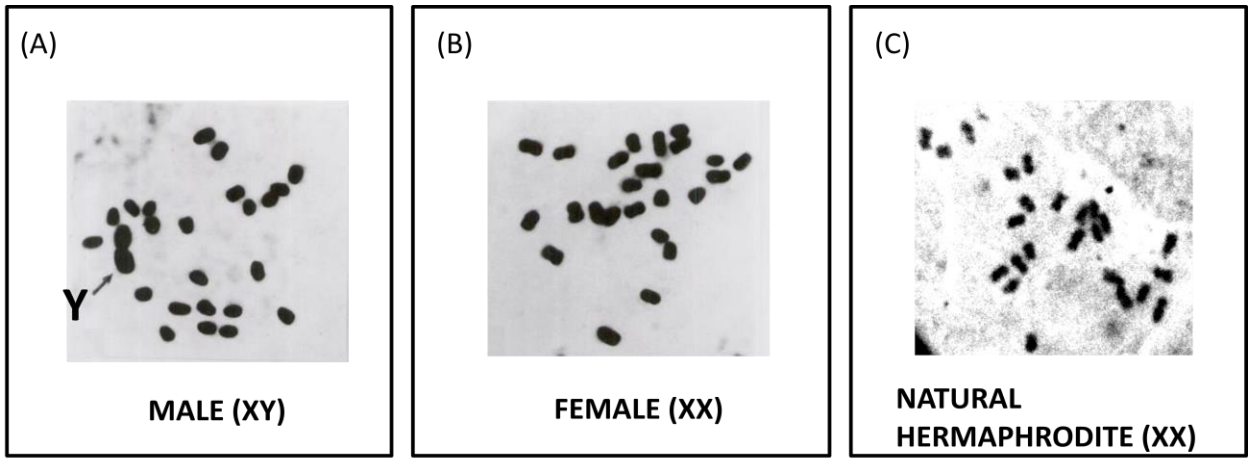


Figure 4.2.3.1: Metaphasic chromosome constitution of (A) male, (B) female and (C) natural hermaphrodite.

The above cytological analysis was carried out in collaboration with Prof. Sangram Sinha and Kanika Kamarkar in University of Tripura. From Figure 4.2.3.1 A,

we can see that the male plant is heterogametic with XY sex chromosomes and Y chromosome (arrow pointed) is much larger than the X chromosome, whereas the X chromosome is same as the rest of the autosomes. Our observations with male and female chromosome are in consistent with the previous report (Kumar L. S. S. and Viseveshwaraiah, S., 1952). Figure 4.2.3.1 (B) and (C) shows the chromosome constitution of female and natural hermaphrodite plant. Both consists of XX sex chromosomes and lack the Y chromosome. The plant of *C. grandis* that consists of Y sex chromosome is a male producing flower bearing male reproductive organs. But even though the natural hermaphrodite lacks Y chromosome, it is able to produce flowers bearing male reproductive organs (stamens). Therefore, it is quite possible that in addition to Y dependent mechanism that regulates sex determination in *C. grandis,* other factors could also play a major role in sex determination.

### 4.3) Understanding sex modification in *C. grandis* on spraying of silver nitrate

#### 4.3.1) Silver nitrate dose- dependent sex modification

Like other species of Cucurbitaceae family, there is alteration in the sex expression of *C. grandis* on application of silver nitrate. In our study, we have observed that the spraying of silver nitrate on the leaves of female plant caused the sex modification of developing flower buds. The female plant produces hermaphrodite flowers instead of female unisexual flowers for atleast 2 weeks post spraying of silver nitrate solution. We have observed that after a period of 2-3 weeks, there is a reversal of this sex modification and female plant produces female unisexual flowers again. This shows that the sex-modification in *C. grandis* is dependent on the amount and the frequency with which silver nitrate is sprayed. These $AgNO_3$ treated hermaphrodite flower buds are morphologically different than the natural hermaphrodite flowers.

The application of silver nitrate causes change in the sex expression of female plant but it is dependent on the amount of the doses and the frequency with which it is applied. We observed that there is change in the morphology of carpels in whorl 4 of female flower buds as shown in Figure 4. 3.1.1 A, after 10 days of an application of a 4% w/v AgNO3 solution. There is development of rudimentary stamens (arrowed) in

whorl 3 in the presence of carpels in whorl 4. When silver nitrate with the same concentration is sprayed on two alternate days, we observe that there is change in the morphology of carpels in whorl 4. There is alteration in the structure of one (arrowed) of the three fused carpels (fig 4.3.1.1 B). There was no flower bud which showed complete conversion from female flower to hermaphrodite flower with the above concentration tested. On spraying a higher concentration of $AgNO_3$ (5%), we observed sex conversion in the female flowers (Figure 4.3.1.1 C). However, our preliminary analysis with concentrations higher than 5 % was seen to be lethal for the plant which was evident from the necrosis of the leaves. The $AgNO_3$ treated flower buds consists of three convoluted stamens with an inferior ovary. As it contains both male (stamens) and female (ovary) reproductive organs, we refer it as hermaphrodite.

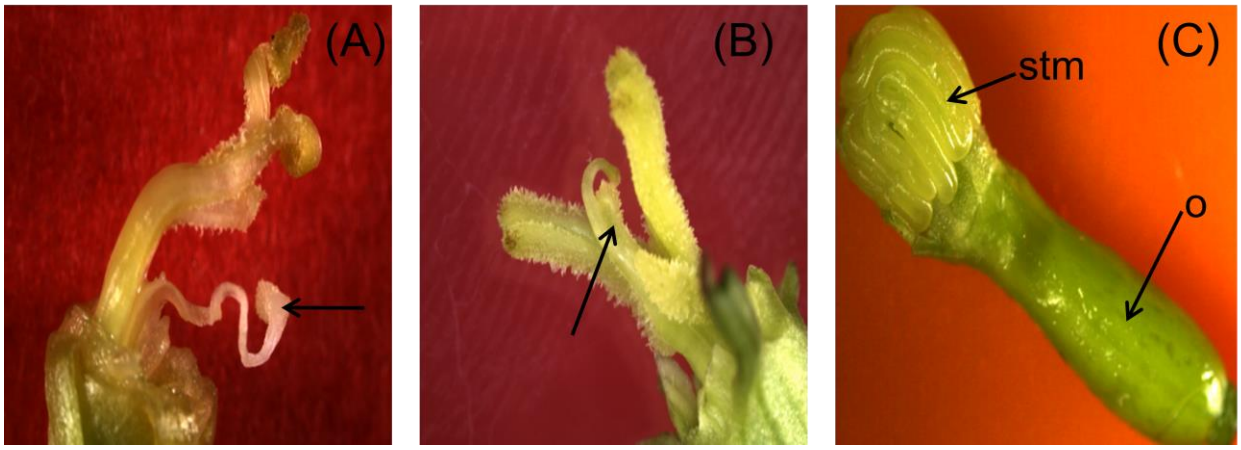


Figure 4.3.1.1: $AgNO_3$ dose dependent alteration in the sex expression of female *C. grandis* flowers. (A) 4% w/v, (B) 4% w/v sprayed on two alternate days and (C) 5% w/v.

### 4.3.2) Morphological differences between male, female and $AgNO_3$ treated hermaphrodite flower buds

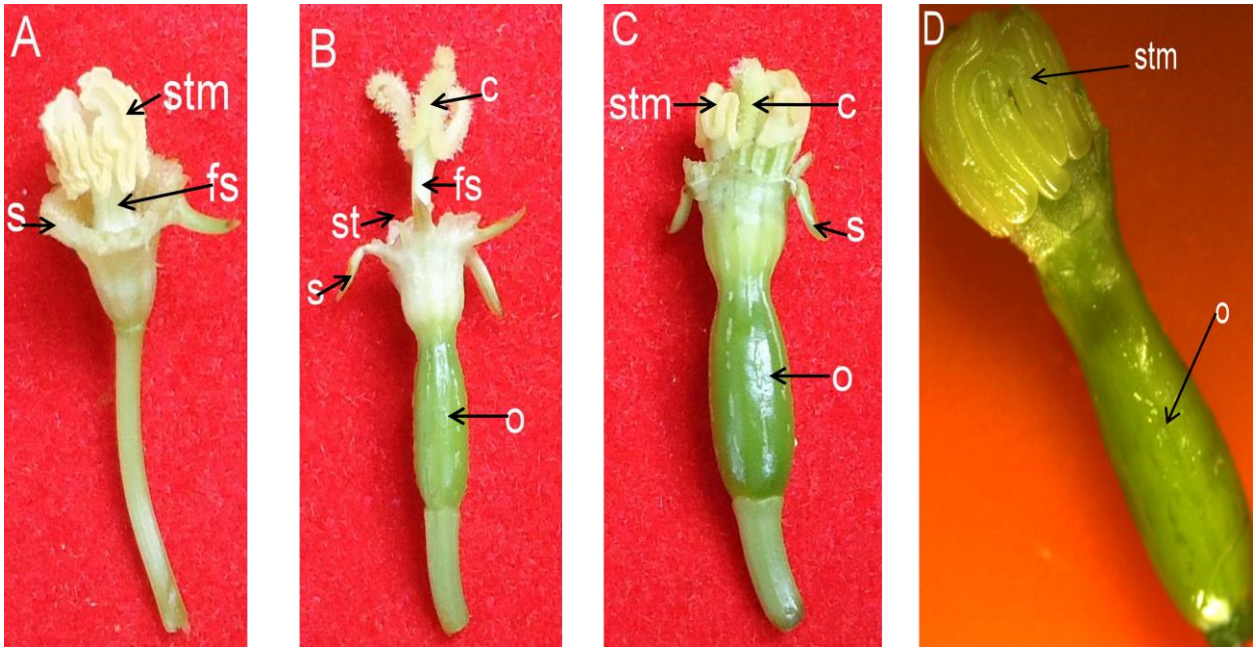


Figure 4.3.2.1: Morphological differences in male, female, natural hermaphrodite and $AgNO_3$ treated *C. grandis* flower buds. (A) Mature male flower, (B) Mature female flower, (C) Mature natural hermaphrodite flower and (D) Mature $AgNO_3$ treated flower. s- sepals, p- petals, stm- stamens, st- rudimentary stamens, fs- floral stalk, c- carpels and 0- ovary.

The number and morphology of sepals and petals is same in male, female and $AgNO_3$ treated hermaphrodite flowers. To investigate the changes in morphology of whorls, the petals were removed from the flowers of male, female and $AgNO_3$ treated hermaphrodite and our observation suggested that in mature $AgNO_3$ treated hermaphrodite, the three convoluted stamens looks similar to that of mature male flower. Unlike the natural hermaphrodite, the $AgNO_3$ treated hermaphrodite lack development of fused carpels in whorl 4 but it consisted of an inferior ovary.

### 4.3.3) Two-dimensional electrophoresis of male, female and $AgNO_3$ treated hermaphrodite flower buds:

In order to understand the effect of $AgNO_3$ on the flower development and also on the mechanism by which sex modification occurs in *C. grandis* female flowers, we compared the proteomes of male, female and $AgNO_3$ treated flower buds of stage 5 to 10. For this study, we have used flower buds of stage 5 to 10 with a hypothesis that the proteins responsible for the sex modification are possibly expressed at these stages. Even from the histological analysis, we observed that after stage 5, in both male and female flower buds, the identity of organs and whorls are well-established.

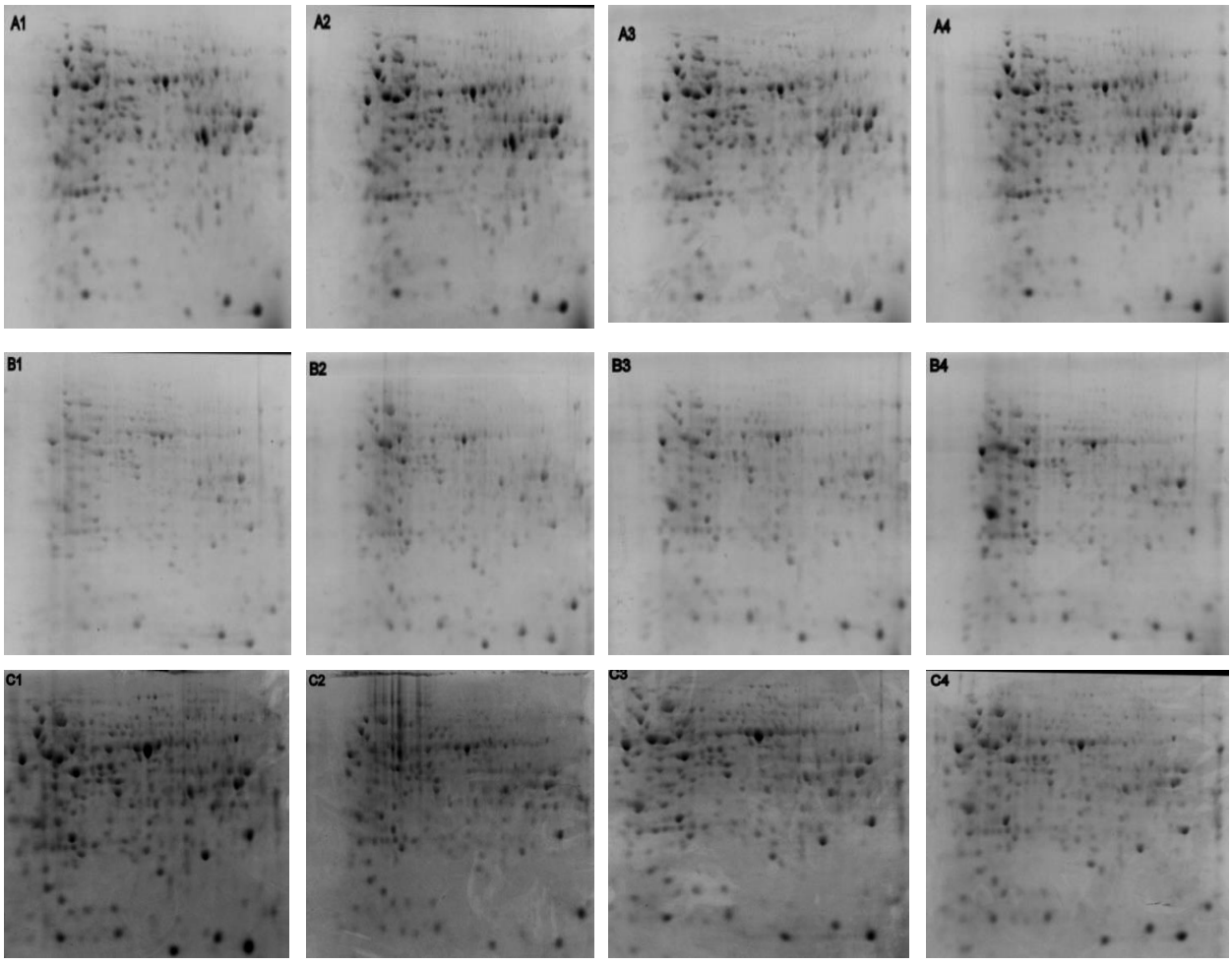


Figure 4.3.3.1: 2DE gel patterns of male, female and $AgNO_3$ treated hermaphrodite flower buds of stage 5 to 10. A1-A4 are the 2D replicates of male flower buds, B1-B4 are the 2D replicates of female flower buds and C1-C4 are the 2D replicates of $AgNO_3$ treated hermaphrodite flower buds.

## 5. DISCUSSION

Our study indicates that there exists an extent of flexibility in the sex expression of *C. grandis*. The sex expression in *C. grandis* is regulated by sex chromosomes but can be altered by silver nitrate ($AgNO_3$). Other than the male and female form of sex, there exists another sexual form – natural hermaphrodite being reported in the wild. The existence of natural hermaphrodite provides us an opportunity to study different characters in various sexual states of *C. grandis*. The morphological differences in the male, female and natural hermaphrodite flowers exists only in whorl 3 (stamens) and whorl 4 (carpels). The male flowers completely lack carpel development from stage 1. But female flower consists of rudimentary stamens in whorl 3. The rudimentary stamens are sterile in female flower, but the reason of its presence is still not known. So to find the stage at which the female flowers show arrest in development of stamens and to find whether male flowers also bear opposite sex organs (rudimentary carpels) during development, we analyzed various stages of flower development through histology. The histological analysis indicates that even though the initial floral primordia of female flower are hermaphroditic, at stage 4 it becomes unisexual female flower by arresting further development of stamens in whorl 3. There is no abortion of stamens, thus, *C. grandis* female flower do not follow the abortive mechanism for developing into unisexual flower. The male flower of stage 1 is unisexual with no development of female reproductive organs. The initial floral primordia of male flower might also be hermaphroditic and the mode of development of male unisexual flower can be by abortion of female reproductive organs. The other alternative is that there is no development of female reproductive organs in male flower from the inception of formation of flower. By analyzing the leaf epidermal morphology of male, female and natural hermaphrodite, we find the number of stomata is more in natural hermaphrodite than male and female. The increase in the stomata could be an adaptation for natural hermaphrodite plants.

To establish DNA-based identification of natural hermaphrodite, we used *rbcL* and *matK* as the DNA barcodes. By comparing the phylogenetic trees based on *rbcL* and *matK* DNA sequences, we find that *matK* leads to better resolution of phylogenetic

relationship of various *Coccinia* at the species level than *rbcL*. Also natural hermaphrodite belongs to the same clade as *C. grandis*, indicating that natural hermaphrodite is one of the sexual forms of *C. grandis*. Other than the morphological differences in male, female and natural hermaphrodite flowers, it is difficult to identify the sex of the plant. Here, we tried to develop genetic markers at the vegetative stage of the plant through RAPD. Analyzing the RAPD results, we observe that sex-related genomic differentiation exists among male, female and natural hermaphrodite.

From our cytological study, we observe that it is easy to identify the Y chromosome among the other male metaphasic chromosomes. But it is difficult to distinguish female from natural hermaphrodite on the basis of cytology because both consists of the same chromosome constitution (22A + XX). The natural hermaphrodite is called so, because it consists of both male (stamens) and female (carpels) reproductive organs. Earlier studies suggested that all the vital genes responsible for the maleness might be situated in the differential segment of the Y chromosome and also they cannot be transferred to the X chromosome since the male specific genes might be tightly linked (P. K. Agarwal and R. P. Roy, 1982). But the natural hermaphrodite plant lack Y chromosome, still it is able to produce male (stamens) reproductive organs. This indicates that the development of male reproductive organs in Y- independent. Probably, Y chromosome contains the genes that are responsible for the maturation of pollen grains. To prove this hypothesis, we need to analyze the gene constitution of the Y chromosome and also check the viability and fertility of pollen grains from the natural hermaphrodite flowers.

The sex expression in *Coccinia grandis* is not stable and undergoes sex modification on application of silver nitrate. Spraying of silver nitrate causes sex change in females and which has no effect on males. After spraying of silver nitrate, the female plant produces hermaphrodite flowers for 2-3 weeks. After that period there is sex reversal, the female plant again starts producing female unisexual flowers. By comparative analysis of morphology of male, female, natural hermaphrodite and $AgNO_3$ treated flowers, we observe that unlike natural hermaphrodite, the $AgNO_3$ treated flower lacks carpel development in whorl 4. Instead, there is development of three convoluted

stamens like the male flowers. But it consists of an inferior ovary. The $AgNO_3$ treated hermaphrodite consists of XX sex chromosome like the natural hermaphrodite and lacks Y chromosome. Here, the presence of male reproductive organs in $AgNO_3$ treated flower buds indicate that probably the genes responsible for the development of male reproductive organs is not situated on the Y chromosome. This also indicates that silver nitrate has a role to play in the basic mechanism of sex determination in *C. grandis*. Therefore, in order to understand the effect of silver nitrate on the flower development and also the mechanism of sex change, we compare the proteome of male, female and $AgNO_3$ treated hermaphrodite. With this objective, we need to perform further investigations to identify differentially expressed proteins in the above sex forms as a consequence of application of silver nitrate.

# 6. CONCLUSIONS

- From the morphological characterization of flowers, we could conclude that there is no phenotypic differences in whorl 1 (sepals) and whorl 2 (petals) of all three sexual forms. However, morphological differences were found in whorl 3 (stamens) and whorl 4 (carpels) in male, female and natural hermaphrodite flowers.

- Our observations suggest that female flowers develop by arresting stamen growth in initial hermaphroditic floral primordia. At stage 4 of flower development, it is seen that there is arrest of stamens in female flower. There is no abortion of the stamens at later stages of development and reason for retention of these stamens is also not known. The male flower is unisexual right from stage 1 with no development of female reproductive organs. One of the possible mechanisms of development of male unisexual flower is by abortion of opposite sex organs (carpels) in the initial hermaphroditic floral primordia. The other possible mechanism could be the initial floral primordia itself is unisexual with no development of opposite sex organs from the inception of flower formation.

- Leaf epidermal morphology analysis showed that the number of stomata are more in natural hermaphrodite than male and female plants. The increase in the number of stomata could be an adaptation for natural hermaphrodite plants.

- Phylogenetic analysis establishes that natural hermaphrodite belongs to the same clade as *C. grandis*, indicating that natural hermaphrodite is one of the sexual forms of *C. grandis*.

- We have developed a genetic markers which could be useful to identify sex at the vegetative stage of the plant through RAPD approach. Our results indicate that sex-related genomic differentiation exists among male, female and natural hermaphrodite.

- Y chromosome is seen to be morphologically distinct among the other male metaphasic chromosomes. But it is difficult to distinguish female from natural hermaphrodite on the basis of cytology because both consists of the same chromosome constitution (22A + XX).

- We have also observed that the sex expression in *C. grandis* can be altered by the application of silver nitrate ($AgNO_3$). Application of $AgNO_3$ on female plants leads to the formation of hermaphrodite flowers instead of female flowers.

- Sex modification in female flowers depends on the dosages as well as the frequency with which silver nitrate solution is applied.

- By morphological characterization of male, female, natural hermaphrodite and $AgNO_3$ treated flowers, we observed that unlike natural hermaphrodite, the flowers developed from plants treated with 5% $AgNO_3$, lacks carpel development in whorl 4. Instead, there is development of three convoluted stamens similar to male flowers. Higher dosage of AgNO3 found to be lethal for the plants.

- Comparative analysis of proteome profiles of male, female and $AgNO_3$ flower buds by two- dimensional gel electrophoresis suggests that there is differential expression of proteins in these three sexual states. Further investigations in this respect could unravel the effect of $AgNO_3$ on flower development in *C. grandis*.

# 7. REFERENCES


Adams, DO., Yang, SF., (1979). Ethylene biosynthesis: Identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc Natl Acad Sci USA 76: 170-174.

Adam, H., Collin, M., Richaud, F., Beuke, T., Cros, D., Omore, A., Nodichao, L., Nouy, B. and Tregear, J. (2011). Environmental regulation of sex determination in oil palm: current knowledge and insights from other species. Annals of Botany 108: 1529-1537

Ainsworth C. (2000). Boys and girls come out to play: the molecular biology of dioecious plant. Annals of Botany 86: 211-221.

Ainsworth, C., Crossley, S., Buchanan- Wollaston, V., Thangavelu, M. and Parker, J. (1995). Male and female flowers of the dioecious plant sorrel show different patterns of MADS box gene expression. The Plant Cell, 7: 1583-1598.

Ainsworth, CC., Lu J., Winfield, M., Parker, JS. (1999). Sex determination by X: autosome dosage: *Rumex acetosa* (sorrel). In: Ainsworth CC, ed. Sex determination in plants. Oxford: Bios Scientific Publishers, 121-136.

Atsmon, D. and Tabbak, C. (1979). Comparative effects of gibberellins, silver nitrate and aminoethoxyvinyl glycine on sexual tendency and ethylene evolution in the cucumber plant (*Cucumis sativus* L). Plant Cell Physiology, 20: 1547-1555.

Bais, H. P., George. J. and Ravishankar G. A. (1999). Influence of polyamines on growth of hairy root cultures of witl of chiocory (*Chichorium intybus* L cv Lucknow local) and formation of coumarins. Journal of Plant Growth Regulation, 18: 33-37

Bais, H. P., Sudha, G., Suresh, B. and Ravishankar, G. A. (2000a). $AgNO_3$ influences *in vitro* root formation in *Decalepis hamiltonii* Wight, Arn. Current Science, 79: 894-898.

Bais, H. P., Sudha G. S. and Ravishankar, G. A. (2000b). Putrescine and $AgNO_3$ influences shoot multiplication *In vitro* flowering and endogenous titres of polyamines in Chichorium intybus L cv Lucknow Local. Journal Plant Growth Regulation, 19: 238-248.

Bais, H. P., Sudha, G. S. and Ravishankar, G. A. (2001a). Influence of putrescine $AgNO_3$ and polyamine inhibitors on the morphogenetic response in untransformed tissues of *Chichorium intybus* and their regenerants. Plant Cell Reports, 20: 547-555.

Bais, H. P., Sudha, G. S. and Ravishankar, G. A. (2001b). Putrescine influences growth and production of coumarins in transformed and untransformed root cultures of witloof chicory (*Chichorium intybus* L cv Lucknow Local). Acta Physiologia Plantarum, 23: 319-327.

Bais, H. P., Venkatesh, R. T., Chandrasheka, A. and Ravishankar, G. A. (2001c). *Agrobacterium rhizogenes* mediated transformation of witl of chiocory *in vitro* shoot regeneration and induction of flowering. Current Science, 80: 83-87.

Bais, H. P. and Ravishankar, G. A. (2002). Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tissue and Organ Culture, 69:1-34

Beyer, E. M. (1976). A potent inhibitor of ethylene action in plants. Plant Physiology, 58: 268-271.

Bhaduri, P. N. and Bose, P. C. (1947). Cytogenetical investigations in some cucurbits with special reference to fragmentation of chromosomes as a physical basis of speciation. J. Genet. 48: 237-256.

Chakravarty, A. K. (1948). Cytology of *Coccinia indica* with reference to the behavior of its sex chromosomes. Proc. Ind. Acad. Sci. 'B' 27: 74-86.

Chailakhyan, M. K. H (1979). Genetic and hormonal regulation of growth, flowering and sex expression on plants. American Journal of Botany 66: 717-736.

Colombo, L., Franken, J., Koetje, E., Went, J., Hans, J. M., Angenent, G. and Tunen. A. (1995). The Petunia MADS box gene *FBP11* determines ovule identity. The Plant Cell, 7:1859-1868.

Diggle, P., Stilio, V., Gschwend, A., Golenberg, E., Moore, R., Russell, J. and Sinclair, J. (2011). Multiple developmental processes underlie sex differentiation in angiosperms. Trends in Genetics, 27: 368- 376.

Dorken, ME., Pannell, JR. (2008). Density-dependent regulation of the sex ratio in an annual plant. The American Naturalist 171: 824-830.

Durand, B. (1969). Selection de genotypes males de *Mercurialis annua* L. (2n = 16) en function de leur sensibilite aux cytokinines. Comptes Rendu de L' Academie des Science Paris, serie D 268: 2409-2051.

Durand, B. and Durand, R. (1991). Male sterility and restored fertility in annual mercuries, relations with sex differentiation. Plant Science 80: 107-118.

Farbos, I., Veuskens, J., Vyskot, B., Oliviere, M., Hinnisdaeles, S., Aghmir, A., Mouras, A. and Negrutiu, I. (1999). Sexual dimorphism in white campion: deletion on the Y chromosome results in a floral asexual phenotype. Genetics 151: 1187-1196.

Heslop-Harrison. J. (1956). Auxin and sexuality in *Cannabis sativa*. Physiological Planta 4: 588-597.

Kinney, M., Columbus, J. and Friar, E. (2008). Unisexual flower, spikelet, and inflorescence development in monoecious/ dioecious *Bouteloua Dimorpha* (Poaceae, Chloridoideae). American Journal of Botany 95(2): 123-132.

Korpelainen H. (1998). Labile sex expression in plants. Biological Reviews 73: 157-180

Kouonon, L., Jacquemart, A., Zoro Bi, A., Bertin, P., Baudoin, J. and Dje, Y. (2009). Reproductive biology of the andromonoecious *Cucumis melo* subsp. *agrestis* (Cucurbitaceae). Annals of Botany. 104(6): 1129-1139.

Kumar, L.S.S. and Deodikar, G. B. 1940. Sex chromosomes of Coccinia indica Wight et Arn. Curr. Sci. 9: 128-130.

Kumar, L. S. S. and Viseveshwaraiah, S. (1952). Sex mechanism in *Coccinia indica* Wight and Arn. Nature 170: 330- 331.

Kumar, V. Parvatam. G. and Gokare A. R. (2009). $AgNO_3$ - a potential regulator of ethylene activity and plant growth modulator. Electronic Journal of Biotechnology. Vol.12, No.2, ISSN: 0717-3458.

Lal, M. and Jaiswal, V. (1988). Modification of flower sex and acid phosphatase activity by phthalimides in female plants of *Morus nigra* L. Plant Growth Regulation 7: 29- 37.

Lardon, A., Georgiev, S., Aghmir, A., Le Merrer, G. and Negrutiu, I. (1999). Sexual dimorphism in white campion: complex control of carpel number is revealed by Y chromosome deletions. Genetics 151: 1173- 1185.

Law, T., Lebel- Hardenack, S. and Grant, S. (2002). Silver enhances stamen development in female white campion (*Silene latifolia* [Caryophyllaceae]). American Journal of Botany 89(6): 1014-1020.

Lazarte, J. E. and Garrison, A. (1980). Sex modification in *Asparagus officinalis* L. Journal of the American Society for Horticultural Science 105: 691-694.

Lebel- Hardenack, S., Hauser, E., Law, T., Schmid, J. and Grant, S. (2011). Mapping of sex determination loci on the White Campion (*Silene latifolia*) Y chromosome using Amplified Fragment Length Polymorphism. Genetics 160: 717-725.

Lebel- Hardenack, S., Hauser, E., Law, T. F., Schmid, J. and Grant, S. R. (2002). Mapping of sex determination loci on the white campion (*Silene latifolia*) Y chromosome using amplified fragment length polymorphism. Genetics 160: 717-725.

Link, A. and LaBaer, J. (2011). Trichloroacetic acid (TCA) precipitation of proteins. Cold Spring Harb Protoc; doi: 10.1101/ pdb. Prot5651.

Little, H., Ekaterina, P., Hammar, A., Grumet, R. (2007). The influence of ethylene perception on sex expression in melon (*Cucumis melo* L.) as assessed by expression of the mutant ethylene receptor, *At-etr1-1*, under the control of constitute and floral targeted promoters. Sexual Plant Reproduction (2007) 20: 123-136.

Parker, J. S. (1990). Sex chromosomes and sexual differentiation in flowering plants. Chrom. Today, 10: 187-198.

Pfent, C., Pobursky, K. J., Sather, D. N. and Golenberg, E. M. (2005). Characterization of SpAPETALA3 and SpPISTILLATA, B class floral identity genes in Spinacia oleracea, and their relationship to sexual dimorphism. Dev Genes Evol 215(3): 132-42.

Richards, A. J. (1997). Plant breeding systems, 2nd ed. Chapman and Hall, London, UK.

Roy, R. P. and Roy, P. M. (1971). Mechanism of sex determination in Coccinia indica. Jour. Ind. Bot. Soc. 50 A: 391-400.

Rudich, J.; Halevy, A. H. and Kedar, N. (1972). Ethylene evolution from cucumber plants as related to sex expression. Plant Physiology, 49: 998-999.

Sabine Hardenack, Ye, D., Saedler, H. and Grant, S. (1994). Comparison of MADS box gene expression in developing male and female flowers of the dioecious plant white campion. The Plant Cell, 6: 1775-1787.

Sanwal, S., Kozak, M., Kumar, S., Singh, B. and Deka, B. (2011). Yield improvement through female homosexual hybrids and sex genetics of sweet gourd (*Momordica cochinchinensis* Spreng.). Aceta Physiol Plant 33: 1991- 1996.

Sousa, A., Fuchs, J. and Renner, S. S. (2012). Molecular cytogenetics (FISH, GISH) of Coccinia grandis: A ca. 3 myr- old species of Cucurbitaceae with the largest Y/autosome divergence in flowering plants. Cytogenetics and Genome Research. DOI: 10.1159/000345370

Stokes, T., Croker, S. and Hanke, D. (2003). Developing inflorescences of male and female *Rumex acetosa* L. show differences in gibberellins content. Journal of Plant Growth Regulation. 22: 228-239.

Strader, L., Beisner, E. and Bartel, B. (2009). Silver ions increase auxin efflux independently of effects on ethylene responses. The Plant Cell, 21: 3585-3590.

Talamali A, Bajji M, Le Thomas A, Kinet JM, Dutuit P. (2003). Flower architecture and sex determination: how does Atriplex halimus play with floral morphogenesis and sex genes? New Phytologist 157: 105-113.

Talamali A, Dutuit P, Le Thomas A, Gorenflot R. (2001). Polygamy in *Atriplex halimus* L. (Chenopodiaceae). Comptes rendus de l'Acade´miedes Sciences 324: 107–113.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731- 2739.

Thomas, T. D. (2004). *In vitro* modification of sex expression in Mulberry (*Morus alba*) by ethrel and silver nitrate. Plant Cell Tissue Organ Culture, 77: 277-281.

Vyskot, B. and Hobza, R. (2004). Gender in plants: sex chromosomes are emerging from the fog. Trends in Genetics, 20: 432- 438.

Wang, D., Li, F., Duan, Q., Han, T., Xu, Z. and Bai, S.(2010). Ethylene perception is involved in female cucumber flower development. The Plant Journal 61, 862-872.

Westergaard, M. (1940). Studies on cytology and sex determination in polyploidy forms of *Melandrium album*. Dansk Bot. Arkiv, 5, 1- 131

Westergaard, M. (1958). The mechanism of sex determination in dioecious flowering plants. Adv. Genet. 9, 217-281.

Yampolsky, C., Yampolsky, H. (1922). Distribution of the sex forms in the phanerogamic flora. Bibliography of Genetics 3: 1 – 62.