# Understanding dioecy in *Cycas nayagarhensis* and *Putranjiva roxburghii* Wall.: A Morphological, Biochemical and Omics approach

Thesis submitted to the University of Hyderabad for the award of

**Doctor of Philosophy**

By

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# DECLARATION

I, **Rutuparna** hereby declare that this thesis entitled **"Understanding dioecy in *Cycas nayagarhensis* and *Putranjiva roxburghii* Wall.: A Morphological, Biochemical and Omics approach"** submitted by me, was carried out in the Department of Plant Science, School of Life Sciences, University of Hyderabad, under the guidance of **Dr. Irfan Ahmad Ghazi** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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This is certified that this thesis entitled **"Understanding dioecy in *Cycas nayagarhensis* and *Putranjiva roxburghii* Wall.: A Morphological, Biochemical and Omics approach"** is a record of bonafide work done by **Ms. Rutuparna Jena**, a research scholar for the Ph.D. program in Plant Science (15LPPH09), Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. This thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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*Dedicated to my beloved mother (Bou)*

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# ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ANOVA | Analysis of variance |
| APX | Ascorbate |
| µL | Microlitre |
| µg | Microgram |
| µM | Micromolar |
| ºC | Degree centigrade |
| CAT | Catalase |
| µg | Microgram |
| Bp | Base pair |
| BSA | Bovine serum albumin |
| CTAB | Cetyltrimethylammonium bromide, hexadecyltrimethylammonium bromide |
| DEPC | Diethylpyrocarbonate |
| DNA | Deoxy ribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTPs | Deoxy nucleotide triphosphates |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetra acetic acid |
| G | Gram |
| H | Hours(s) |
| H2O2 | Hydrogen peroxide |
| Kb | Kilobase pair |
| kDa | Kilodalton |
| L | Litre |
| LB | Luria-bertani |
| M | Molar |
| MS/MS | Tandem mass spectrometry |
| MALDI-TOF | Matrix-assisted laser desorption ionization - time of flight |
| Mg | Milligram |
| Min | Minute |
| Ml | Millilitre |
| mM | Millimolar |

| | |
|---|---|
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| PMSF | Phenylmethylsulfonylfluoride |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| Rpm | Revolutions per minute |
| SDS | Sodium dodecyl sulphate |
| Sec | Seconds |
| Tris | tris-(Hydroxymethyl) aminomethane |

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# Chapter 1: Introduction

# INTRODUCTION

## 1.1 Dioecy

"*Dioecious plants, however fertilised, have a great advantage over other plants in their cross-fertilisation being assured. But this advantage is gained ... with some risk ... of their fertilisation occasionally failing. Moreover, dioecious plants cannot spread so easily as emonoecious and hermaphrodite species, for a single individual, which happened to reach some new site, could not propagate its kind ... Monoecious plants also can hardly fail to be to a large extent dioecious in function, owing to the lightness of their pollen and to the wind blowing laterally, with the great additional advantage of occasionally or often producing some self-fertilised seeds. When they are also dichogamous, they are necessarily dioecious in function. Lastly, hermaphrodite plants can generally produce at least some self-fertilised seeds ... When their structure absolutely prevents self-fertilisation, they are in the same relative position to one another as monoecious and dioecious plants [except] that every flower is capable of yielding seeds*." - By Darwin (1876, p. 414)

Dioecy refers to having female and male flowers on separate unisexual plants (Devani et al., 2019). This sexual dimorphism provides an excellent opportunity to study and investigate sexual reproduction in dioecy plants. Dioecious plants are taxonomically significant for their diverse characteristics. Dioecy is identified less frequent (i.e., around 6%) among land plants (Renner et al., 1995). Due to the spatial segregation of sexes, there is a restricted distribution of each sex to different habitats. Hence, these plants are categorized as vulnerable (Timerman and Barett, 2019). Adverse conditions like fragmentation and habitat loss are two significant factors that mostly affect the plant survival rate. Diecious plants, because of their sexual dimorphic behavior, are more prone to this habitat loss compared to non-dioecious plants

(LeRoy et al., 2020). The difference in sex-specific characteristics favors the dimorphism in dioecious plants. Across the environmental gradients, reproductive allocation is plastically adjusted between males and females due to different reproductive costs (Tonnabel et al., 2017). Skewed sex ratios are noticed in several dioecious plant species because of their differences in several growth conditions like growth rate, reproductive age and mortality etc., (Stelkens and Wedekind, 2010).

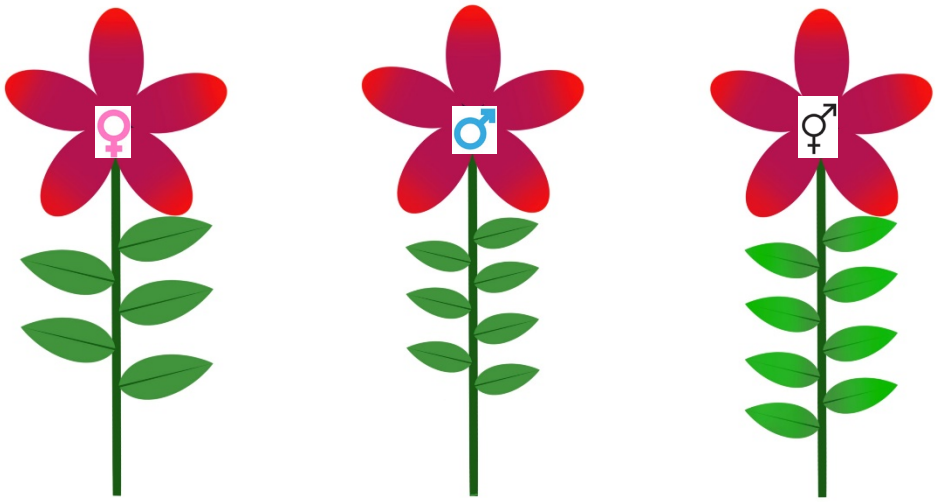


**Figure 1.** The representation of three different modes of plant reproduction.
A. Female plant (Dioecious) B. Male plant (Dioecious) C. Hermaphrodite Plant

It has also been reported that there is a difference in the number of male and female-specific plants in a single species in various habitats. Male plants have been exceeded than female plants in long-lived species, while the number of female plants is more in the clonal propagation system than the male plants (Barrett, 2015). Despite the vegetative difference, a varied sexual dimorphism has been seen among the dioecious plant species. It also varies among the population of a particular species. The allocation pattern of sexual reproduction in dioecious plants is controlled by the relative contribution of genetic and environmental factors (Wooten,

1971). The flowering sex ratio of female and male plants has been found to be significantly different. It has also been described that the male-biased flowering sex ratio is twice as compared to the female-biased sex ratio (Field et al., 2013).

## 1.2. Origin and evolution in Dioecy

Between 480 and 360 million years ago, in the mid-Paleozoic era, land plants originated, during which the terrestrial organisms gradually evolved. During their cause of evolution, plants evolved different sex determination systems (Fig. 2) (**L**eite Montalvão et al., 2021). Plants with unisexual character have evolved from hermaphroditic ancestors (Barrett et al., 2002). Dioecy has evolutionary significance and understanding this sexual specialization could provide more insights into the evolutionary link between monoecy and dioecy. It is useful to distinguish between developmental sex determination and genetic sex determination in plant reproduction. So, the evolution of genetic polymorphism for sex determination is required to transition from monoecy to dioecy, resulting in unisexual plants having either female or male flowers (Charlesworth and Charlesworth, 1978). It has also been found that plant sex determination could involve a single locus to switch the gene from a complex interacting gene network that influences each other's expression (Boualem et al., 2009). Hence, understanding the sex determination mechanism at the molecular level has long been an exciting area of research. Understanding whether or not the same genes are involved and regulated during reproduction could form a basis to further explore the reproduction system in dioecy species.

Secondary sexual dimorphism has been determined by morphological and physiological differences such as leaf size, water use, photosynthesis, hormonal contents etc, (Delph, 1999). Among all the species, functional loss of sexual organs occur with the same frequency at all of the four stages: Stage 1 - before initiation of early primordia; Stage 2 - early development of stamen or carpel; Stage 3 - pre-meiosis; and Stage 4 - post-meiosis) in both female and male flowers.

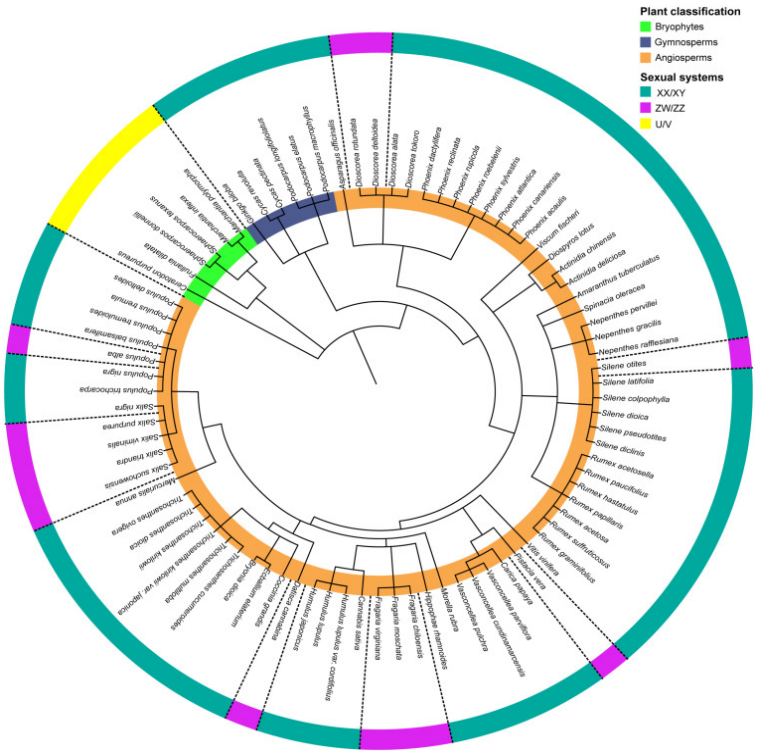



**Figure 2.** Phylogenetic tree of species with known sex determination systems and sex chromosome. Plant classification (inner circle): Bryophytes (green), Gymnosperm (blue) and Angiosperm (orange); Sexual systems (outer circle), male heterogametic system XX/XY (green), female heterogametic system ZW/ZZ (pink) and haploid U/V system (yellow) (Adapted from Leite Montalvão et al., 2021).

Genetic models used to the evolution of dioecy predict that the genetic mutations responsible for female and male sterility have occurred separately and sequentially (Charlesworth and Charlesworth, 1978). Despite male and female floral structures, the seed setting depends on pollination in the end: It indicates compatible and incompatible protein-protein interaction between pollen and stigma (Roulston, 2000). The development of flowering in dioecious plants is not yet understood well. Male and female flower development depends upon different factors, such as proteins and metabolites involved in various pathways. In this current study, in an

attempt to understand the molecular mechanisms behind the developmental conditions of male and female flowers of Putranjiva *roxburghii* Wall. and *Cycas nayagarhensis,* we studied the changes at proteome and metabolome levels and the relevant pathways that regulate sex determination across male and female flowers.

### 1.3. Understanding Dioecy using Proteomics approaches

The protein content of reproductive organs governs their development and maturation, leading to successful pollination (Mondo et al., 2021). A pollen grain is attached to stigma through pollination during reproductive events, and the factors behind this process are not yet understood. The proteomes of flower development in males and females help to understand successful pollen formation and then transfer to the female flower's stigma at appropriate times. Proteomics technologies facilitate identification and quantification of the total protein content of a cell, tissue, or organism (Aslam and Woelders, 2017). Proteomics approaches could help in the identification of novel proteins that are associated with sexual dimorphism. This can help better understand the evolutionary, molecular and developmental mechanisms associated with dioecy. Proteomics methods can also aid in understanding the mechanism of sex determination in plants which is complex involving both genetic and environmental factors (Yang et al., 2015). Here we aim to explore the proteins that simultaneously regulate the pollination cycle in both sexes. This process involves many inducers, signalling pathways, protein interactions, etc. Previous proteomic studies gave new understanding into sex-specific differences where male poplar is more efficient than females at photosynthesis and nutrient usage, which can possibly be due to the male-biased sex determination ratio in nutrient-deficient conditions (Song et al., 2018). Proteomic studies also showed that the shoot height and photosynthetic rate in female plants are more affected by Mn stress conditions than in male plants. Therefore, the variation in protein expression in both sexes under Mn stress clearly showed a stable photosynthetic rate and stable gene expression level for better plant defense (Chen et al., 2013) as reported by (Zapata et al., 2022). Protein-protein interaction network analysis confirmed

*ERL1*, *AG*, *AGL8*, *LFY*, *WUS*, *AP2*, *WRKY*, and *CO genes* as the crucial elements for hormonal response regulation which are required for the formation and development of gametophytes along with anatomical reproductive structures. The different patterns of MADS-box gene expression were shown in the female and male flowers of the dioecious plant's sorrel (Ainsworth et al., 1995). In some species, inappropriate sex organs are retained in their rudimentary form than being aborted (Weatherwax et al., 1916). It has been found that silver compounds such as $AgNO_3$ and $Ag_2S_2O_3$ have a masculinizing effect on monoecious plants for example, *Cucumis sativus* (Golabadi et al., 2017). It also affects female dioecious plants such as *Silene latifolia* and *Cannabis sativa*. The mechanism of molecular regulation, which includes AgNO3-mediated induction of stamen development (Enhances, 2002), remains unknown.

## 1.4. Understanding dioecy using Genomics

Genomics and transcriptomics studies can play a vital role in understanding sex determination mechanisms at the gene level (Vicoso et al., 2013). The RNA-Seq approach was used for the first time to ascertain sex-based gene expressions in *Silene latifolia* and unfold the dosage compensation in plants. These studies also lead to understanding Y-chromosome-linked degeneration and identifying other sex-linked genes (Prentout et al., 2020). Similarly, many studies linked with the de novo and other sequencing approaches have identified regulatory molecules controlling plant reproductive development. For example, *OGI* (*Oppressor of MeGI*), a Y-chromosome-encoded small RNA governs pollen fertility by targeting *MeGI* (a homeodomain transcription factor) (Akagi et al., 2016). Moreover, studies of pollen development in *Asparagus* resulted identified genes controlling the microspore formation and tapetum development which were expressed specifically only in male flowers (Tsugama et al., 2017). The advantage of transcriptomics over genomics has led to a more insightful understanding of differential gene regulations that ascribed to environmental changes or the signals based on the sex of the plant, as evident in dioecious plant species. The only disadvantage of the transcriptome is the inability to determine the expression levels of proteins

in reproductive parts, which ultimately is a key regulator in the genetic information carrying out various structural and functional controls (Kimberlin et al., 2019). The early genetics and floral organ identity pathways in dioecious species like Thalictrum and *Spinacia oleracea* have been widely studied. In *T. dioicum*, the floral organ-identifying genes belonging to B and C classes were found to be differentially expressed between female and male flowers during early development, implying the homeotic genes regulation in gender determination. Comparative Transcriptomics may help to detect the downstream genetic cascades at subcellular processes, but they may not be very useful to detect developmental trigger points. Research into the evolution of sex chromosomes and sex evolution in plants has revealed processes and patterns that are remarkably similar in animal systems, including the suppression of recombination and Y chromosome degeneration (Di Stilio et al., 2005). The complex genetic, epigenetic, and hormonal processes control the reproductive organ abortion by morphological and cellular mechanisms. The discovery of male and female organ arrest at different stages occurs within the same species. GA hormone stimulates stamen development and increases auxin to enhance the female flower percentage (Galun et al., 1965). The discovery of the feminizing F locus, which encodes 1-amnincyclopropane-1-carboxylic acid synthase, resulted in the identification of the functional role of ethylene in unisexual flower development and acetyl-CoA carboxylase (ACC), an ACS1- encoded enzyme responsible for rate-limiting step in ethylene production (Defilippi et al., 2005). CmWIP1 transcription factor, a newly discovered factor from the melon locus, represses CmACS7, inhibiting gynoecium development and staminate flowers (Zhang et al., 2022). As shown from previous study in melon and cucumber, for type I flower development, few sex-differentiating genes play a role far downstream of organ identification and primordium initiation. Sex expression in these is also modulated by environmental factors such as day length and temperature which were shown to affect endogenous hormone levels. Comparative transcriptomics analysis would indicate downstream genetics pathways and subcellular processes (Li et al., 2017). It will incorporate developmental processes with regulatory mechanisms from the plant's existing developmental pathways. In the case of

dioecious *S latifolia*, it was found that SISUP expresses in females but not in males due to premature male flower arrest. Fom the other study, in Zea, six mutated tassel seeds gave perfect flowers in the staminate inflorescence but the gynoecium was aborted in the male flower after initiation by PCD. Exogenous jasmonate application reinstate staminate flowers in ts1 homozygotes. The spAP3 and SpPI B class genes from *S. oleracea* are expressed continuously during the course of stamen development in male flowers, whereas in female flowers, their expression was untraceable (West and Golenberg, 2018).

## 1.5. Pollination network and pollen biology in dioecious plants

Pollination is the transfer of pollen from the stamen to the stigma, an essential event in the plant life cycle (Dowding, 1987). The dioecious condition where individual plants having either male or female flowers limit their reproductive capacities as they suffer from severe pollen limitation because of their inability to self-pollinate and the requirement of pollinator movement from males to females for seed production. Because of this limitation, only 50% of the individuals in Dioecious populations instigate seed production. Despite these constraints, dioecious plants maintain their population growth rates similar to cosexual plants through various ecological and physiological adaptations like precocious flowering, insect pollination, higher seed production in females, animal-dispersed fleshy fruits etc. (Ohya et al., 2017). These ecological traits have been studied and debated in previous studies (Renner and Ricklefs, 1995). However, the physiological and biochemical adaptations of released pollens at the pollination stage have limited reports.

## 1.6. Pollen structure and functions

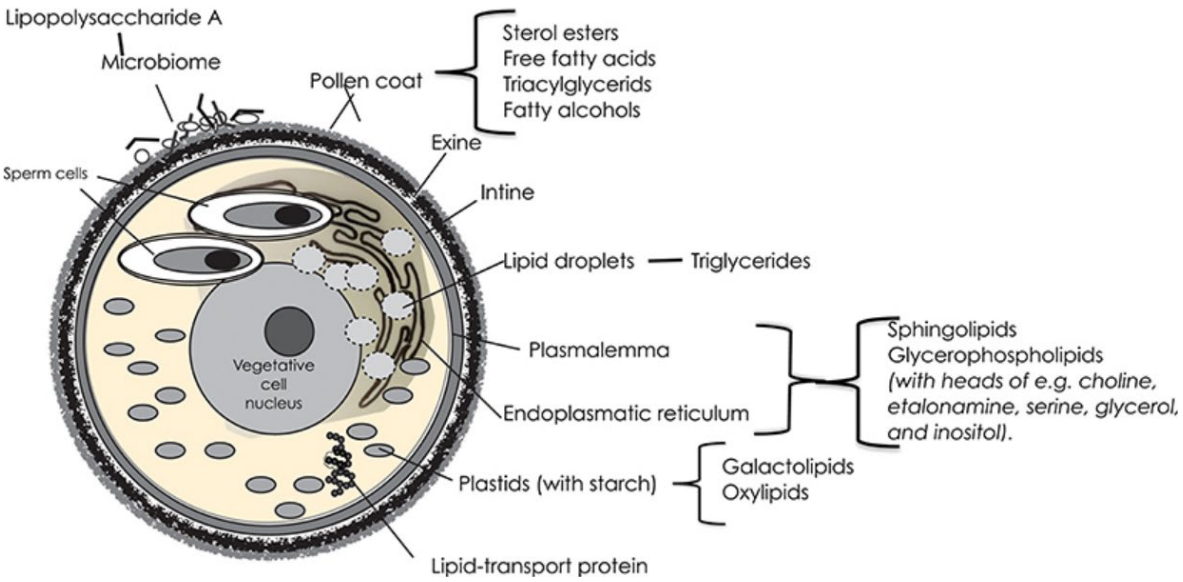



**Figure 3.** The trinucleate angiosperm pollen grain structure showing with different features that intine, exine and microbe interactions (Adapted from Dahl et al., 2018)

A pollen grain is a male gamete with an outer wall coated by sporophytic cells. Seed-bearing plants, during their adaption to terrestrial life, evolved internal fertilization systems similar to animals. In gymnosperm, microsporangia are organized into cone-like structures; in angiosperms, they form four pollen sacs inside an anther. The cell lining towards the inner side of the sporangium nourishes microspores with nutrients. In the case of angiosperm, mitosis results in a generative cell (small) and a vegetative cell (large), and the mitotic division of the generative cell results in two sperm cells with large nuclei (Fig. 3). The sperm cell consists of a plasma lemmae that extends from the plasma membrane to the nuclear envelope and is involved in the synthesis and transport of proteins and lipids. Fully matured pollen contains fatty acids like hexadecenoic acid and octadecanoic acid. This mitotic division is species dependent and happens before and after anther dehiscence and pollen germination. In

gymnosperm, mitosis produces two to five male gametophytes and the generative cell divides after pollen dehiscence, giving rise to two sperm cells (Dahl et al., 2018).

In this current study, to understand more about the pollen biology in dioecious plants during the pollination stage, we studied the metabolic changes across unreleased and released pollens from *Putranjiva roxburghii* wall and *Cycas nayagarhensi. Putranjiva roxburghii* wall. does a dioecious plant belong to the Euphorbiaceae family, primarily found in Asian tropical regions and a wind-pollinated species (Wurdack et al., 2004). During wind pollination, pollens are blown off through the wind to the flower's stigma. The journey of pollen consists of microspore formation, pollen maturation, pollination, pollen stigma interaction, fertilization and seed sets (Mariani, et al., 1990, Heslop-Harrison 1982, Erickson and Markhart, 2002). After maturation, it is believed that most pollen goes to developmental arrest for dispersal under various environmental conditions. The tolerance mechanism of the pollen provides adaptations necessary for survival during dispersal. Once the pollen is released, exposure to extreme temperatures would be its first obstacle for its survival. In general, to mitigate extreme temperatures during their early developmental stages, living bodies develop thermotolerance mechanisms and pathways for their survival (Hallberg, 1985). Earlier, it was reported that when pollen faced long exposure to high temperatures (>40°C) in the cotton plants, there was still successful pollination resulting in grains (Arshad et al., 2021). Therefore, from the above reports, successful pollination is noted under unfavourable environmental temperature stress conditions, suggesting some adaptations might have occurred in pollens for viability beyond the developmental arrest. These molecular, biochemical, cellular and metabolic adaptations during stress are yet to be explored later, *heterotrophic* pollen development requires energy and macromolecules for various metabolic processes. The role of metabolites was highlighted in the involvement of fertilization as well as the development of pollen. Environmental stress interrupts the metabolic pathways of carbohydrates, amino acids, lipids, plant hormones, alkaloids, and flavonoids (Hatami et al., 2016). Therefore, variations in specific metabolites

and biomolecules between released and unreleased pollens would be responsible for pollination and environmental temperature stress related adaptations. Abiotic stress produces a high level of reactive oxygen species (ROS) and cells protect themselves from toxicity through active mechanisms (Choudhury et al., 2017). Proteins are essential macromolecules for various processes involving development, metabolism, reproduction, adaptation, and defence in a living system (Wilkins et al., 1996). A higher temperature in the environment interrupts the protein homeostasis of pollen which is maintained through sub-cellular stress response systems (Ranjan et al., 2017). A metabolomic study revealed that sugar insufficiency is the primary reason for reproductive failure in the rice floral organs, including pollen grains, during drought and heat stress (Li et al., 2015). Starch and sucrose accumulation was more in the tomato pollen at the maturation stage with less glucose and fructose (Sato et al., 2006). Likewise, maize showed more prolonged-term effects on pollen development and fertilization (Begcy et al., 2019). The role of low molecular weight sugars like glucose, sucrose and fructose in pollen grain metabolism is unclear (Qian et al., 2008). Lipids are essential to maintain sporopollenin, pollen coat and exine (Piffanelli et al., 1998) and are mainly stored in the pollen coat or exine part and intine cytoplasmic part (Zhang et al., 2016). The content of fatty acids, phenolic, and flavonoid compounds in citrus pollen decrease during high temperature. Wind-pollinated birch and maize show the presence of polyamines on the pollen surface as well as the majority of plants' pollen surface contains a higher amount of polyamine linked with hydroxyl cinnamic acid (Vogt, 2018). Polyamines (Putrescine, Spermidine and Spermine) in the pollens are essential for pollen development, cell wall deposition and sporopollenin deposition in the pollen tube (Aloisi et al., 2016). Polyamines are a part of the phenylpropanoid pathway linked to exine development and sporopollenins are associated with phenol metabolism (Bassard et al., 2010). Wind pollination exposes pollen to various environmental stresses associated with various metabolic changes and adaptations. For a successful pollination event, pollen must maintain its viability and resistivity. How the pollen manages to be viable after dispersing from the flower anther independently, it’s an adaptive strategy and the molecular and cellular bases of these processes

need to be explored. Here, we studied the changes in pollen structure for the flight during wind pollination, changes in specific metabolites and active biomolecules like free amino acids, proteins, polyamines, hormones and sugars (sucrose, fructose, glucose) in pollen during wind pollination. Metabolite profiling of both released and unreleased pollen was performed during LC-MS along with the biochemical characterization of polyamines, sugars, hormones, lipids, fatty acids, amino acids, flavonoids and phenols involved in the adaptive strategy. These studies on released pollens exposed to temperature and wind stress give the idea of pollen adaptations or behaviour for resistibility, functionality and viability in wind pollination and stress conditions. Furthermore, bacteria and viruses influence the flower phenotype by interacting with floral pollinators. Microbial abundance and composition of multiple scales of microbes in the floral tissues have affected the ecological drive variation. It has been suggested that microbial metabolites might be helpful in floral phenotypic changes and pollinator attraction for reproduction (Vannette et al., 2020). Long-lived growth forms, biotic seed dispersal and fleshy fruits are associated with male-biased flowering in tree species, whereas clonality and abiotic pollen dispersal are associated with female-biased flowering herbaceous species. Mostly female bias has been reported in the species having heteromorphic sex chromosomes (Checchi et al., 2011).

## 1.7. Role of metabolites and hormones in Dioecy

Many plant growth regulators and hormones significantly regulate sex-determining genes as well as developmental pathways in type I and type II unisexual flowers. The multiple roles of hormones in plant development are highly pleiotropic. Hormone levels are controlled through enzymes encoded at loci by allelic composition. Due to external stress, hormone levels are altered as a result the developmental response will also be modified. Thus, environmental factors can affect a range of sexual phenotypes expressed from a particular genotype. Due to the sex-related differences in dioecy plants, the expression of hormonal genes and hormone levels has been varied in the whole plant, affecting vegetative growth. It has also affected the

stress tolerance condition of male and female plants differently (Munné-Bosch, 2015). From the metabolomic study of the staminate and pistillate flower, it has been identified that oxylipin signaling is associated with sex expression of the female flower by enrichment of alpha-linolenic acid pathway and consensus expression of 12-oxo-phytodienoicacid (OPDA). Except along with these phyto cannabinoid-like compounds has also been found to be upregulated in the female flower (Welling et al., 2021). Terpene is the secondary metabolite found in most plant tissues which are known as the toxic repellent of plant enemies. Several primary and secondary metabolites have been synthesized from terpenoids via isoterpenoid biosynthetic pathways (Cheng et al., 2007). Phytohormones such as gibberellic acid, abscisic acid, cytokinin, etc., have been synthesized from the primary metabolism of isoprenoid compounds. These isoprenoid compounds have several functions, sterol acts in membrane stabilization, ubiquinone help in respiration, and chlorophylls and plastoquinone are responsible for photosynthesis. Moreover, phytoalexin is meant for plant defense response (Hammerschmidt et al., 1999). There are about 120 terpenes that have been identified from Cannabis species responsible for the flavor addition in different varieties (Calvin et al., 2018). Similarly, flavonoids are the most important secondary metabolites ubiquitously distributed in most plant species with a functional role in plant biochemistry, physiology, and ecology (Jedinák et al., 2004). Phenolic compounds like metabolite are distributed throughout the plant species that are primarily responsible for the defense mechanism of the plant kingdom (Bennett et al., 1994), along with acting as a growth inhibitor and a dormancy factor (Smýkal et al., 2014). Alkaloids are the primary group of secondary metabolic compounds. These are the essential nitrogenous compounds derived from amino acids. Alkaloids are mostly cytotoxic and anti-inflammatory, with mild analgesic activity and cardiovascular and antineoplastic properties (Barbosa et al., 2006). Metabolomic cross-talk between Phe and Trp biosynthetic pathways controls the flower's color formation (Maeda et al., 2010). The female flower of *Cucurbita pepo* has been studied to understand nectar secretion (Nepi et al., 2001). Proteins and amino acids were found to be involved in nectar secretion and organ development (Nepi et al., 2012). Nectar secretion

in flowers is also controlled by the coregulatory action of auxins, jasmonic acids, gibberellins, trehalose etc., (Mathur et al., 2021). Moreover, female tissues have been guided by the pollen tube toward the ovule by secreting specific metabolites such as arabinogalactans and some small peptides like LURE (Okuda et al., 2009). Protease and invertase enzymes were found to catalyze sugar and amino acid biosynthesis. Transcriptional regulators of flavonoid biosynthesis have been characterized. Similarly, the carotenoid biosynthetic pathway has been identified as a master regulator in the petal development of Mimulus flowers (Sagawa et al., 2016). Anthocyanin biosynthesis controlled R2R3-MYB TF with the co-regulation of the carotenoid biosynthetic pathway in the *Medicago trun-catula* flower (Verdier et al., 2012). Carbohydrates synthesized by sepal helped to develop and expand corolla limb development (Kwak et al., 2007). Sucrose has also been identified from tobacco and arabidopsis (Daloso et al., 2015). Jasmonic acid along with other signaling molecules participates in the development of floral reproductive tissues (Creelman and Mullet, 1995). Comparative phylogenetic analysis was performed to investigate the architecture of floral symmetry transcript levels of homeotic genes that have been described by ABCDE flowers development models such as A (sepal), A+B (petals), B+C (anther), and C (carpel) (Theissen and Melzer, 2007). JA helps in male reproductive organ development, stamen consisting of anther and filament (Cheng et al., 2009). Lateral stamen development has been controlled by the floral homeotic gene AGAMOUS. Among several metabolites, Jasmonic Acid (JA) has been identified as a significant secondary metabolite involved in plant adaption and survival during environmental stress conditions (Avramova, 2017). JA was shown to play a significant role in flowering plants too. The mutants associated with JA biosynthetic pathways were male sterile (Turner et al., 2002). JA mutant coi1-1 has been identified as a male-sterile factor responsible for the male sterility phenotype in the arabidopsis plant (Figueroa et al., 2015). Similarly, overexpression of $JAZ_1$ with Jas domain deletion resulted in male sterility (Oblessuc et al., 2020). MYB21 and MYB24 are the MYB transcription factors that target JAZ protein and are significantly involved in filament elongation and the development of anther (Wang et al., 2019). $MYB_{21}$ has been involved in

stamen development regulated by a combination of MYC5 and other redundant transcription factors (Song et al., 2017).

Previous studies reported that mostly male plants were more susceptible to herbivore damage than female plants, whereas in female plants, Rumex acetosella has more insect damage than male plants. The male and female plants have been identified with different carbohydrate and mineral concentrations. Notably, male willows reserve more phenolic glycosides, tannins and other higher concentrations of secondary metabolites (Yang et al., 2020). Moreover, the phenolic glycosides, salicin, salicortin, and tannins in plants have been influenced by vertebrate and invertebrate herbivores (Foley et al., 2005). Floral scent and color that attracts pollinators have been contributed by several secondary metabolites, which are synthesized in the male and female plants. The metabolites play an essential role in the flower's color and scent and serve as a photo protector against UV radiation of solar energy in the atmosphere. Primary metabolites act as a metabolic precursor and provide a metabolic network for the biosynthesis of secondary metabolites to support flower development conditions (Welling et al., 2021). Hormone cascades imply that sex-determining genes would evolve similarly to cis-regulatory response elements without accompanying chromosomal linkage. Understanding cis-elements evolution at the genome level could provide better insight into how hormonal levels influence the sexual changes in dioecious plants. Hormones regulate various developmental mechanisms during ontogeny and their levels are influence by pleiotropic mutations (Diggle et al., 2011). Hormone levels are controlled by loci that encode specific hormone receptors or enzymes involved in metabolism. However, external stress alters hormone levels thereby affecting development. As hormones influence sex determination, the evolution of functionally linkage genes in dioecious lineages is also affected by hormones. Some physically linked genes were found to be associated with reproductive organ development or suppression (Tanurdzic and Banks, 2004). Studies were conducted in dioecious species like *Thalictrum dioicum* and *Spinacia oleracea* to understand the genetics of early flower commitment and pathways

involved in floral organ identity. In *T. dioicum*, the B and C class floral organ identity genes were differentially expressed in males and females during their early developmental stages, indicating this homeotic gene regulation in the expression of sex determination (Sather et al., 2010).

## 1.8. Model plants selected for this study

### 1.8.1. Cycas

Cycads are commonly known as living fossils because they originated in the mid Permian. Cycads were dominated during Mesozoic era and spread over terrestrial areas. Cycads were diversified in this period. This evolutionary period is called the age of Cycads and dinosaurs (Liu et al., 2022). Ancient Cycads are lineages and modern Cycads are woody plants with frond-like leaves clustered at the stem tips. Living Cycads are dioecious and produce individual male and female cones (Terry et al., 2007). Cycas produce loose clusters of megasporophylls instead of a true female cone. According to the global list, 16 species of Cycas were reported in India out of 118 species (Kamila et al., 2022). The tribal people belong to Bagata, Saora, Konda, Kondha, Valmiki, Jatapu, Gadaba, and Reddy tribes of Odisha and Andhra Pradesh states that use the plants for their living hood. Cycas leaves, seeds, coralloid roots, male cones and stem pith of *C. sphaerica* were used for several purposes such as medicine, food, and insect repellent (Raju and Jonathan, 2010). Seed flour is also frequently utilized by the tribal communities of this particular region to make various delicious food items. Roti, Pittu, Idli, porridge, Dosa, cakes, sweetmeats, etc., are also prepared by the tribals for food consumption (Singh and Radha, 2008). Not only seed but also Pith of this plant is used to cook foods for small babies. Sago flour is very often used as a common food items. Tender leaves are also consumed as leafy vegetables (Reddy et al., 2006). The biogeography and pollination strategy of Cycads is little known before the angiosperm evolution. Among the Cycadales, from the family like Zamiaceae and Cycadaceae, the Zamiaceae are known as insect-pollinated species. However, Cycadaceae are considered wind-pollinated species. There were many doubts regarding the pollination strategy

of Cycas. *C.revoluta* performs both anemophily and entomophile. However, the interesting observation was that anemophily is restricted to only female trees growing within 2-m radius of male trees. The nitidulids feed primarily on Cycad tissue and serve as pollinators during the time of pollen release. *Cycas revoluta* was shown to be pollinated by insects. *Cycas revoluta* cones are pineapple shaped. A strong, unpleasant odor exists in the nearby area (Lu, Y et al., 2011). The chemical composition of floral scents often attracts different pollinators. Moreover, thermogenesis is also another vital feature of cycad pollination. It helps to secret sexual fluid as well as nectar in the gymnosperm flower, which plays a vital role in pollination. Wind-pollinated gymnosperm flowers contain a low concentration of sugar in the pollination drops. So, insects are discouraged from attracting flowers due to low sugar concentration. Insect-pollinated Gnetales groups have specific tissue that secretes sexual fluids in which significant biochemical components are present. These biochemical components act as nectar's signature, which attracts insects for pollination.

**Classification**

**Kingdom:** **Plantae**

**Clade:** **Gymnosperms**

**Division:** **Cycadophyta**

**Class:** **Cycadopsida**

**Order:** **Cycadales**

**Family:** **Cycadaceae**

**Genus:** ***Cycas***

**Species:** ***C. nayagarhensis***

**Figure 1.1.** ***Cycas nayagarhensis***

### 1.8.2. Putranjiva roxburghii Wall.

*Putranjiva roxburghii, the* synonyms of *Drypetes roxburghii* (Wall.). Its common name is 'Putranjiva.' It is a dioecious tree found in tropical regions like Nepal, Bangladesh, India, China, Thailand and Sri Lanka. Putranjiva is a small genus that belongs to the Putranjivaceae family but was previously in the Euphorbiaceae family (Kumar et al., 2015). Putranjivaceae has three species but only one species named as *Putranjiva roxburghii species* found in India (Tripathi, N. N., & Kumar, N. 2007). It is an ornamental tree and also famous as child life tree (Chaudhary et al., 2008). Some terpenoids, flavonoids, triterpenes, and Roxburghonic keto acid are identified from the trunk bark and leaf of *P. roxburghii* (Garg and Mitra, 1971). But after that there are no proteins were identified. Like Cycas, *Putranjiva roxburghii* Wall is traditionally used by tribals to treat various health ailments. In this study, anti-oxidant, analgesic and anti-inflammatory activities of ethanolic extract of leaves and stem of *Putranjiva roxburghii* were evaluated in rats using different evaluation models for each activity.

**Classification**

**Kingdom: Plantae**

**Clade: Angiosperm**

**Phylum: Tracheophyta**

**Class: Magnoliopsida**

**Order: Malphigiales**

**Family: Putranjivacece**

**Genus: *Putranjiva***

**Species: *P. roxburghii* wall.**

**Figure 1.2. *Putranjiva roxburghii* wall.**

**Table 1.** List of previous studies on sex determination in plants.

| S.No. | Plant parts | Techniques used | Species name | Response observed | Reference |
|---|---|---|---|---|---|
| 1 | Flower bud | Proteomic Analysis | *Coccinia grandis* | Sex expressional proteins | Devani et al. (2019) |
| 2 | Leaf samples | Proteomics | *Pistacia chinensis* | Sex determination | Xiong et al. (2013) |
| 3 | Flower bud | Proteomics | *A.Chinensis. var* | Sex determination | Zhang et al. (2021) |
| 4 | Flower bud | Proteomics | *Mercurialis annua* | Sexual dimorphism based on photosynthesis, metabolism energy, biotic and abiotic stress. | Khadka et al. (2019) |
| 5 | Leaves | Proteomics | *Simmondsia chinensis* | Sex determination | AI-Obaidi et al. (2017) |
| 6 | Leaves | Proteomics and Physiology | *Populus Cathayana* | Sex determination | Chen et al. (2013) |
| 8 | Flower bud | Transcriptomics | *Asparagus officinalis* | Sex determination | Harkess et al. (2016) |
| 9 | | Transcriptomics | *G. biloba* | Sex determination | Gong and Filatov (2022) |
| 10 | Leaves | Metabolic study | *Mauritia flexuosa* | Sex determination | dos Santos Freitas et al. (2018) |
| 11 | Flowers, tepal, leaves, roots | Transcriptomics and proteomics | *Amborella trichopoda* | Sex determination | Tornero et al. (2020) |
| 12 | Fruit | Transcriptomics | Kiwi | Sex determination | Kim et al. (2010) |
| 13 | Leaves | Transcriptomics | *Trachycarpus fortunei* | Sex determination | Feng et al. (2020) |
| 14 | Leaves | DNA methylation and transcriptomics | *Carica papaya* | Sex determination | Deputy et al. (2002) |
| 15 | Leaves | Transcriptomics and whole genome sequence | *Caucasian persimmon* | Sex determination | Akagi et al. (2016) |
| 16 | Flower | Metabolomics | Cannabis | Sex determination | Welling et al. (2021) |
| 17 | Flower | Transcriptomics | *Cucumis sativus L.* | Sex determination | Zhou et al. (2018) |

| | | | | | |
|---|---|---|---|---|---|
| 18 | Complete plant parts | Genomics | *Phone dactylifera* | Sex determination | Al-Dous et al. (2011) |
| 19 | Leaves | Transcriptomics | *Neolitsea sericea* | Sex determination | Chung et al. (2000) |
| 20 | Flower bud | Genomics | *Vitis vinifera* | Sex determination | Massonnet et al. (2020) |
| 21 | Flowers | Transcriptomics | *Idesia polycarpa maxima.* | Sex determination | West, (2020) |
| 22 | Leave and stem bark | Metabolomics | *Morus alba L.* | Sex determination | Wu et al. (2022) |
| 23 | Flower buds | Metabolomics | *Herpetospermum pedunculosum* | Sex determination | Liu et al. (2022) |
| 24 | Flowers | Metabolomics | *Salix paraplesia* | Sex determination | Cai et al. (2021) |
| 25 | Leaves | Cytology and Transcriptomics | *Belgica aurea* | Sex determination | Paparella et al. (2022) |
| 26 | Flower | Transcriptomics | *Spinacia oleracea L.* | Sex determination | Liu et al. (2021) |
| 27 | Flower buds | GWAS | *Populus deltoides* | Sex determination | Xue et al. (2020) |
| 28 | Flower | Transcriptomics | *Vernicia fordii* | Sex determination | Mao et al. (2017) |
| 29 | Leaves | GWAS | *Ginkgo biloba* | Sex determination | Liao et al. (2020) |
| 30 | Xylem, phloem, Leaves, shoots | Transcriptomics | *Salix arbutifolia* | Sex determination | He et al. (2022) |
| 31 | Leaves | Metabolome and Transcriptome | *Ginkgo biloba* | Sex determination | Guo et al. (2020) |
| 32 | Floral buds | Transcriptomics | *Carica papaya* | Sex determination | Liu et al. (2021) |
| 33 | Flower bud | Transcriptomics | *Cannabis Sativa* | Sex determination | Adal et al. (2021) |
| 34 | Leaves | AFLP, SCAR | *Eucommia ulmoides* | Sex determination | Wang et al. (2011) |
| 35 | Flowers and shoots | Illumina Hiseq And Miseq | *Silene latifolia* | Sex determination | Papadopulos et al. (2015) |

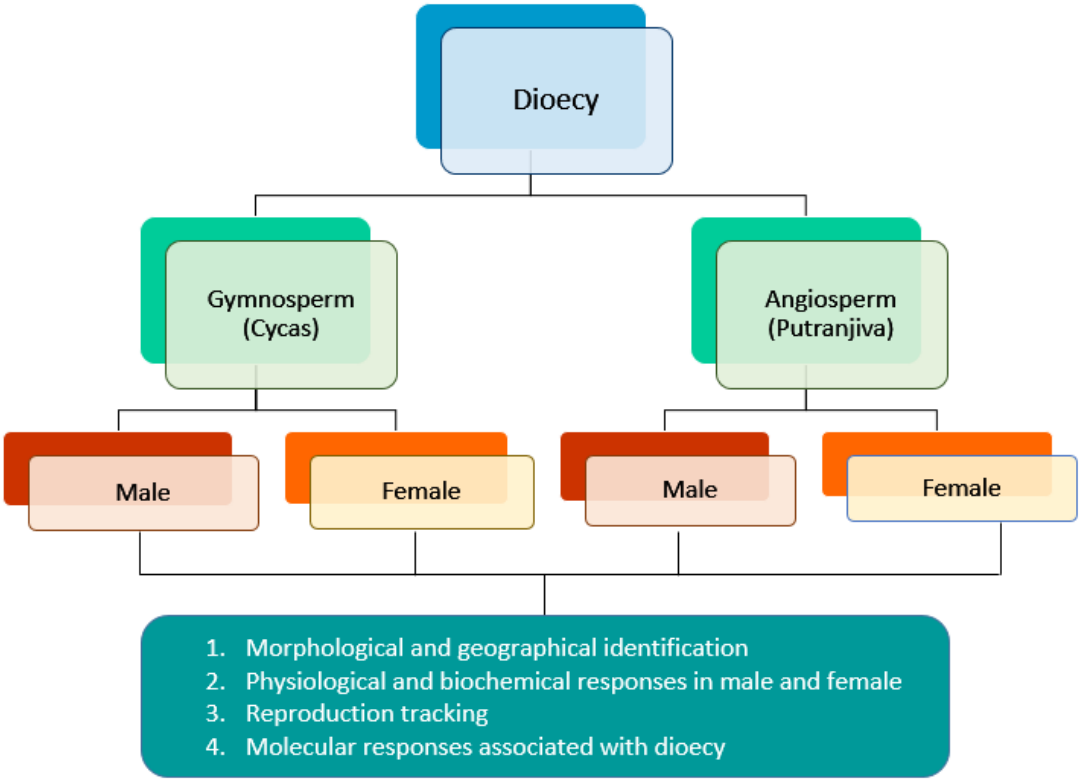



**Figure 4.** Schematic presentation of the complete study

## 1.9. Objectives

Based on the above background information, we designed the following objectives.

1. **Morphological analysis of dioecy and pollination event of Cycas and Putranjiva through field study**

2. **To study pollen biology and metabolite interaction during reproduction of Cycas and Putranjiva**

3. **Proteome analysis of Cycas and Putranjiva during reproduction to understand the protein-protein crosstalk between male and female floral bodies**

# Chapter 2. Materials and Methods

# MATERIALS AND METHODS

## 2.1. Chemicals, materials, and instruments

### 2.1.1. Chemicals and solvents

All the chemicals and the solvents used in this study were procured from HiMedia Laboratories Pvt. Ltd., Sigma Aldrich Chemicals Pvt. Ltd., E-Merck, Qiagen, Invitrogen, Thermoscientific and Merck Millipore.

### 2.1.2. Apparatus and equipments

Apparatus and equipments needed for the research experiments include beakers, test tubes, glass flasks, reagent bottles, screw cap bottles, petri dishes, measuring cylinders, and pipettes used from the Riviera, Borosil, Anubmbra, and Duran brands.

### 2.1.3. pH settings and sterilization

We used the pH meter (Digisun Electronics, DL-707) to adjust the pH. All the Eppendorf tubes, apparatuses, growth media and glass equipments were sterilized with the help of an autoclave (Systec, autoclaves) for 16 min at 15 lbs pressure. The heat-sensitive compounds were sterilized with the help of syringe filters with a membrane pore size of 0.2 µm and 0.4 µm procured from Sigma and PALL, Life Sciences.

### 2.1.4. Milli-Q and deionized water

Milli-Q water and deionized Water were used for all the research experiments, media, chemical and stock solution preparation. The Water was used from the water plant facility unit of the School of Life Sciences at the University of Hyderabad, Hyderabad.

## 2.2. Field study site and sample collection

The survey of the Odisha state was done for the Cycas plant throughout the year during 2015-2020 and the study site was Nayagarh, dist. Odisha. Putranjiva plant sites were the University of Hyderabad campus and Sanjeevaiah Botanical Park, Hyderabad, Telangana State (Lat N 17º 27' 13. 4784", Long E 78º 18' 52.0272"). The sample of both the plant parts like leaves, flowers, cones, pollen, other reproductive organs, pollinator insects from the flower, etc., were collected from the field and brought to the lab for analysis.

## 2.3. Plant material and pollen collection

The ornamentally grown Putranjiva plants from the University of Hyderabad Campus, Hyderabad, India, were used for this study. The sample was collected in April 2019 at ~25°C. The developmental stages of the male flower were monitored along with the releasing period of pollen as described in the method (Shivanna and Rangaswamy, 2012). Time and environmental temperatures during the pollen release were recorded continuously in seven stages (s1-flower of 10 days after budding, s2 - 12 days old flower, s3 - 14 days old flower, s4 - 16 days old flower, s5 - 18 days old flower, s6- 20 days old flower, s7- 22 days old flower). The small flowers were considered to collect the unreleased (UR) pollen, and showy bulky anthers were separated and dissected. The collected anthers were used to harvest the pollen and the stickiness to endothecium was minimized by smashing with a glass rod containing 1 ml of 1% teepol in Milli-Q water. The solution was vortexed thoroughly for 30 min and filtered through a muslin cloth. The filtrate was poured into different vials for counting. Likewise, the Released pollen was collected from the dehiscent anther. The hemocytometer was used for pollen counting (Godini, 1981). Finally, for the comparative analysis, pollen was collected at ~25°C under two conditions, the UR and R pollen states.

## 2.4. Morphological analysis

The morphology of male and female plants of Cycas and Putranjiva were analyzed. The samples

were leaves, flowers, cones of Cycas and pollen collected from the field and their morphological parameters were analyzed. The number of leaves, flowers, cones etc., were counted. The leaf length was measured using the millimeter scale, and the weight was measured in grams. The plant parts were dried entirely in an oven at 50°C.

## 2.5. Phytochemical screening

Various phytochemical tests were performed on the Cycas and Putranjiva male and female methanolic leaves extract to detect Phytoconstituents described by Brain 1975 and Turner (1996).

### 2.5.1. Extraction from plant material for phytochemical tests

The collected leaves of both female and male plants were air-dried for two to three days at room temperature till they dried completely. 100 g of dried samples were ground to fine powder with the help of blender. The extraction was done in different steps. Samples were extracted with n-hexane (500 ml) followed by methanol (500 ml) using the soxhlet apparatus. Then the solvent was evaporated through a rotary evaporator at 37ºC. The left-over residues were collected from 6% dry weight and stored at - 20ºC for further use. In this study, methanol extract was used for phytochemical screening. Finally, the samples were prepared in methanol solution for phytochemical screening. The obtained extracts were subjected to the qualitative test.

### 2.5.2. Detection of Alkaloids

The leaves extract (50 mg) was stirred with a few ml of diluted hydrochloric acid followed by filtration. Wagner reagent was prepared by mixing 1.27 g of iodine and 2 g of potassium iodide in 5 ml of Water and finally the volume was made up to 100 ml using distilled Water. A few drops of Wagner's reagent were added to the few ml of the filtrate by the side of a test tube.

### 2.5.3. Detection of carbohydrates and glycosides

Leaf extract (100 mg leaf) was dissolved in the 5 ml of double distilled water. 1 ml filtrate was boiled in the water bath, adding 1 ml with each 1 ml of Fehling's solution A and B (1:1ratio). Fehling's solution A was prepared by dissolving the 34.66 g of copper sulphate in double distilled water and making up the volume to 500 ml using distilled Water. Fehling's solution B was prepared by dissolving the 50 g of sodium hydroxide and 173 g of potassium sodium tartrate in distilled water and making up the volume to 500 ml. The red precipitate indicates the presence of sugar.

### 2.5.4. Detection of saponins

Leaf extract (50 mg leaf) was diluted with distilled Water and made up the volume to 20 ml. The suspension was shaken in a graduated cylinder for 15 min. A 2 cm layer of foam indicates the presence of saponins.

### 2.5.5. Detection of protein and amino acids

To detect protein and amino acids, extract (100 mg leaf) was dissolved in 10 ml of distilled water and filtered through Whatman filter paper. Two drops of ninhydrin solution were added to 2 ml extract. A characteristic purple colour indicates the presence of amino acids.

### 2.5.6. Detection of phytosterols, fixed oils and fats

The leaf extract (50 mg leaf) was dissolved in 2 ml acetic anhydride. Dropwise, 2 ml of concentrated sulfuric acid was slowly added along the sides of the test tube. The change of colour confirms phytosterols. A small quantity of extract was pressed between the two filter papers. The presence of stain indicates oils.

### 2.5.7. Detection of phenolic compounds and tannins

The methanolic leaf extract (50 mg leaf) was dissolved in 5 ml double distilled water, and 5% ferric chloride solution was mixed. A bluish-black colour indicates the presence of phenolics and a dark green colour indicates the presence of tannins compounds.

## 2.6. Physiological analysis

### 2.6.1. Stomatal count

Stomata of leaves were counted with the help of a scanning electron microscope for the variation.

### 2.6.2. Chlorophyll a and b quantification

The chlorophyll a and b were measured following Arnon, 1949. The leaves were collected from the field and grounded in ice-cold 80% acetone. The fine grounded leaves powder was mixed with 80% acetone and centrifuged at 5000 rpm at 4ºC for 15 minutes. The supernatant OD was taken at 645nm and 663nm.

The concentration of chlorophyll a and b were calculated as follows

Chlorophyll a concentration -12.7 (A663) - 2.69 (A645)

Chlorophyll b concentration - 22.9 (A645) - 6.48 (A663)

### 2.6.3. Photosynthetic leaf gas exchange measurements

Chlorophyll A fluorescence measurement of both Putranjiva and Cycas plants were measured by Handy PEA (HansaTech), according to Singha et al., 2019. Plants were kept in the dark for 30 min, and measurements were taken at nm. Ten different plants from each group were taken from both reproductive and non-reproductive plants for the measurements, and values were represented as triplicates (n=3). Light responsive and photosynthetic curves were measured with intensities of measuring light, saturating pulse, actinic light, and far-red light, respectively.

The saturation pulse and actinic light were set at 0.8 and 30 sec. The light curve was measured for 88 sec with ~190 PAR to ~3000 PAR (photosynthetic active radiation).

### 2.6.4. Elemental analysis

Elements quantification was done with energy-dispersive X-ray spectroscopy coupled with field emission scanning electron microscope (FESEM, Philips XL-30). The same procedure was followed for elemental analysis according to (Kumar and Majeti, 2014) the samples were mounted on aluminum stubs and then kept for gold coating. The voltage was adjusted at 20 Kv with 8.5 mm working distance. The spectral peaks from X ray emission were quantified using INCA software. The spectral peaks intensity was determined with the standards which was inbuilt present with the software.

### 2.6.5. Viability test of pollen

The viability of Putranjiva pollen was assayed as described here with slight modifications (Muhlemann et al., 2018). Briefly, the pollen was resuspended into a solution (1.27 mM Ca $(NO_3)_2$, 290 mM sucrose, 0.16 mM boric acid and 1mM $KNO_3$) (1% wt/vol) comprising 10 µl propidium iodide and 0.001% (wt/vol) fluorescein diacetate. The live and dead pollen were quantified through a flow cytometer (Muse$^{TM}$ Cell Analyzer; Merck Millipore).

## 2.7. Microscopic study and staining

### 2.7.1. Scanning Electron Microscopy (SEM)

The Putranjiva pollens were first fixed using the 2.5% glutaraldehyde, post fixed with 1% osmium tetroxide. At each point, the pellet was washed with the 0.1 M phosphate buffer saline (PBS) 3-4 times. Finally, the pellet was dehydrated in the increasing order of ethanol concentration (50%, 60%, 70%, 80%, 90%, 100%) on the coverslip and left for complete drying. The coverslip was mounted on the Cambridge stubs and sputtering of gold particles was

done. The observation was recorded under the scanning electron microscope (JEOL JSM 5600).

### 2.7.2. Transmission Electron Microscopy (TEM)

Cycas pollen were outsourced to the Sri Sai Histology Center, Rajendra Nagar, Hyderabad, for the ultrathin sections and transmission electron microscopy with the standard method (Bozzola and Russell, 1999). The samples were fixed using the 2% paraformaldehyde, 2.5 % glutaraldehyde, in 0.1M sodium cacodylate (pH 7.2) at 4°C for 24 h. Again, post fixation was done in 1% potassium permanganate or 2% osmium tetroxide for an hour at room temperature with 0.05 M PBS (pH 7.2) buffer. The Samples were dehydrated in the ascending order ethanol series and then embedded in the Spurr's resin. Ultra-thin sections (50-70 nm) and semi-thin sections (200-300 nm) were prepared with the help of ultra-microtome (Leica Ultra cut UCT-GA-D/E-1/100)). These sections were stained with toluidine blue, uranyl acetate, 4% lead citrate and counter-stained with Reynolds lead citrate, then mounted on the copper grids. The grids were analyzed using transmission electron microscopy (Hitachi, H-7500).

### 2.7.3. Safranin staining of pollen

The Putranjiva pollens were stained with the 1% safranin solution in distilled Water (Jones, 2012). Pollens were heat fixed in the slide, and then a few drops of safranin were added. The slides were maintained at room temperature for 5 minutes and then washed with running tap water. Slides were dried completely and observed under the light microscope (Olympus B201).

### 2.7.4. 4', 6-diamidino-2-phenylindole (DAPI) staining of pollen

The Putranjiva pollens were stained with the DAPI solution using the previous method with some modifications (Park et al., 1998). The collected pollens were mixed with the 200 µl DAPI staining solution (1 µg/ml DAPI, 0.1% Triton x-100, 1mM EDTA, 0.1 M sodium phosphate) in 1.5 ml Eppendorf centrifuge tubes. The solution was mixed through short vortex and centrifugation; the pellet was washed with double distilled Water. Pellet was fixed on the

microscope slide and a cover slip was placed on it. The slide was visualized under the laser scanning confocal microscope (ZEISS LSM900).

## 2.8. Biochemical analysis from leaves (Cycas and Putranjiva) and Pollen (Putranjiva)

### 2.8.1. Total protein, reducing sugar and lipid content

Protein extraction was done using the phenol-based method (Wang et al., 2006). The precipitated pellet containing protein was estimated using the Bradford method (Bradford, 1976). Reducing sugars were measured as described earlier (Nelson, 1944) using the Dinitro salicylic acid (DNS) method. The absorbance was observed at 540 nm wavelength. Glucose was used as a standard for carbohydrates and reducing sugars with minor modifications. Lipid estimation was performed using the gravimetric method (Singha et al., 2019).

### 2.8.2. Total carbohydrate estimation

The phenol-sulfuric acid method was used for carbohydrate estimation according to Dubois 1956 as glucose standard. The 500 µl samples were serially diluted and transferred to 15ml screw cap glass tubes, followed by the addition of 1500 µl of concentrated $H_2SO_4$ and 300 µl of 5% (W/V) phenol added solution. The mixture was vortexed and incubated for 5 min at 90ºC followed by cooling at room temperature. The absorbance was read at 490nm. The carbohydrate was estimated using a standard curve between 5 to 50µg with different glucose concentrations.

### 2.8.3. Total Phenolic content

1 mg of pollen was collected from UR and R pollen in separate tubes wherein 500 µl pre-chilled methanol was added and stored at 4ºC for 30 minutes. The mixture was grounded, transferred into 1.5 ml Eppendorf and vortexed for 10 minutes. The total mixture was then centrifuged for 15 minutes at 11000g. Pellet was discarded and the clear supernatant was collected in a new

autoclaved Eppendorf and stored at -80ºC for further experiments. Phenolic content was evaluated using the Folin-Ciocalteu reagent with some modifications (Yang et al., 2007). Absorbance was recorded at 725 nm wavelength using a UV-VIS spectrophotometer (Chemito UV2100). To prepare the standard curve, gallic acid was used and values were noted as mg gallic acid equivalents (GAE) g/DW.

### 2.8.4. Total flavonoid content

For flavonoid evaluation around, 50 µl of the extract was mixed with 60 µl of 10% aluminum chloride and 30 µl of 5% sodium nitrite ($NaNO_2$). The total mixture was incubated at room temperature for 10 minutes, followed by the addition of 350 µl of 1M NaOH. The total volume was made up to 1 ml with Milli-Q and absorbance was noted at 510 nm wavelength against standard quercetin equivalents (mg QE)/g of fresh weight (Gul et al., 2011).

### 2.8.5. Fatty acid methyl ester analysis

To determine fatty acid from unreleased and released pollen, the samples were first methylated and identified through outsourcing to Royal Life Sciences Pvt. Ltd, Secunderabad, India (Erdyneeva et al., 2021).

## 2.9. Antioxidant assay from leaves (Cycas and Putranjiva) and Pollen (Putranjiva)

### 2.9.1. Enzymatic assay

#### 2.9.1.1. Superoxide dismutase (SOD), Catalase, Ascorbate peroxidase (APX) and Guaiacol peroxidase (GPX) enzyme assay

The superoxide dismutase activity (SOD) was assayed according to Beauchamap and Fridovich 1971. The mixture contained with 50µg of protein, 50 mM sodium phosphate buffer (7.8), 13 mM methionine, 75µM nitroblue tetrazolium (NBT), 0.1 mM ethylenediamine-tetra acetic acid (EDTA) added to last 2µM riboflavin. The mixture absorbance was measured at 560nm. A total

reaction mixture lacking protein which incubated under light was taken as a positive control, and protein incubated at dark as blank. The second antioxidant enzyme Catalase was assayed according to Aebi 1984. The essay reaction mixture contained 3ml with 50mM sodium phosphate buffer (pH 7.0), 19mM $H_2O_2$ and finally added 100µg protein. The absorbance was taken at 240nm. The Ascorbate peroxidase enzyme (APX) was measured according to Nakano and Asda 1981. The total mixture contained 50mM sodium phosphate buffer (pH 7.0), 0.5mM ascorbic acid 250mM $H_2O_2$ and 50µg of protein. The decreasing absorbance represents the activity amount of oxidized ascorbate. Guaiacol peroxidase (GPX) was measured according to Putter 1974. The total mixture contained 50mM sodium phosphate buffer (pH 7.0), 20 mm guaiacol solution, 12mM $H_2O_2$ and 50µg of protein. The absorbance was taken at 436 nm.

### 2.9.2. Non enzymatic assay

#### 2.9.2.1. Phosphomolybdenum assay

The total antioxidant activity of compounds was estimated using the phosphomolybdenum-based method with some modifications (Prieto et al., 1999). The stock solution of 1 mg/ml of compounds was prepared and finally, 100 µg/ml was mixed with the 1 ml solution solution (0.6 M sulphuric acid , 28 mM sodium phosphate, and 4 mM ammonium molybdate) in an Eppendorf tube. The tubes were incubated for 10 min at 95°C for completion of the reaction. Tubes were left for cooling at room temperature. The absorbance was recorded using a spectrophotometer at 695 nm wavelength against a blank solution. The standard material was the ascorbic acid ($r^2 = 0.964$) at different concentrations and the total antioxidant capacity of the compounds was expressed in mg of ascorbic acid equivalents (mg AAE)/g of dry weight (dw).

#### 2.9.2.2. DPPH (1,1-diphenyl-2-picyl hydrazyl radical) radical scavenging activity

The methanolic extracts of the samples were measured for their free radical scavenging activity. The protocol was slightly modified according to literature (Ozturk et al., 2007). Plant leaf

extracts were dissolved in methanol (50 mg in 100ml). DPPH solution was prepared in methanol with a concentration of 0.1mM. The samples extracted at different concentrations (50-500µg/mL) were mixed with 1 ml of 0.1mM DPPH solution. The total mixture was incubated at dark for 30 minutes. The OD was taken at 517nm using a UV-VIS spectrophotometer. The BHT standard was used for % inhibition calculation using the following formula.

$\%Inhibition = [(A_{control} - A_{sample})/A_{control}] * 100$

Where $A_{control}$ is the absorption of the control and $A_{sample}$ is the absorption of the test sample.

## 2.10. Metabolomic analysis of leaves from Cycas and pollen from Putranjiva

### 2.10.1. High Performance Liquid Chromatography (HPLC) analysis

#### 2.10.1.1. Quantification of sugars

Total sugars were extracted from pollen and stigma of Putranjiva using the Giannoccaro method through HPLC (LC-20AD Shimadzu, Japan) (Giannoccaro et al., 2008). The fresh 100mg samples were extracted in 1 ml (1:10 w/v) of Milli-Q water for 15 min with vortexing. The extracted samples were centrifuged at 13,000g rpm for 10 min at room temperature, and 500µl of supernatant was collected. 1.5 ml of acetonitrile was added and mixed in a thermomixer for 30 min. The mixture was centrifuged at 13,000g rpm for 10 min at room temperature. The supernatant was collected and kept in the dry bath at 95ºC until complete solvent evaporation, followed by addition of 1 ml Mili Q water. The mixture was filtered through a 0.22µm filter. Sugars were separated using an NH2 column (Shodex-Asahipak NH2P-50-4E) in reverse phase HPLC with acetonitrile: Water (70:30v/v) as solvent. The set flow rate was 0.8 ml per minute. Peaks were detected by photodiode array detector at 190 nm wavelength. fructose, glucose and sucrose were used as standards.

#### 2.10.1.2. Quantification of amino acids, hormones and polyamines

Proline was quantified according to literature (Guo and Trotter, 2004). Extracted samples were derivatized with OPA reagent (1:1:1) (McKerrow, 2000) and subjected to HPLC with a flow rate set at 0.8 ml/min and UV detection at a wavelength of 262 nm. For hormone analysis, the collected pollen was grounded with an extraction buffer (2-propanol: H2O: Concentrated HCl 2:1:0.002 v/v/v) and 1 ml dichloromethane was added to it. After centrifugation (4°C at 13000 rpm for 5 minutes), the lower phase was collected, evaporated using a nitrogen stream and dissolved in methanol (Pan et al., 2010). The mobile phase consisted of solvent A (1% of glacial acetic acid (v/v) and solvent B (100% acetonitrile). The injection volume was 20 µl and the flow rate was set as 1.5 ml/min. For the separation of standard phytohormones and samples, a linear gradient program was set as follows: 0-55% acetonitrile for 25 minutes, then 100% acetonitrile for 3 minutes, followed by 1% acetic acid wash. The separation lasted 30 minutes. Standards used were Indole-3-Acetic acid, Abscisic acid, Gibberellic acids, Salicylic acid, Jasmonic acid, Indole-3-Butyric acid, 1-Naphthaleneacetic acid and Kinetin (procured from Sigma-Aldrich). As reported earlier, polyamines of pollen under different conditions were analyzed using HPLC (Smith and Davies, 1987). A linear gradient program was executed to run for 30 minutes based on the standard's peak area of the known concentration.

### 2.10.2. Gas Chromatography - Tandem Mass Spectrometry (GC-MS/MS) analysis of Putranjiva flowers and Cycas cone

Both primary and secondary metabolites were analyzed as per literature (Singha et al.,2019). 100mg of different developmental stages of flowering of Putranjiva flower and Cycas cones were taken for metabolomics analysis. Samples were grounded with liquid nitrogen, extracted with extraction buffer containing methanol: chloroform: water (7:2:1), incubated for 1 h at 4˚C and then vortex vigorously for 30 min. The total mixture was kept for centrifugation for 15 min at 8000 g. The clear supernatant was transferred to an Eppendorf tube and kept for speed vac

for 3-4 h until complete evaporation. The Eppendorf tube was stored at -80°C till the derivatization. For the derivatization process, 100 µl BSTFA and pyridine (1:1 ratio) were mixed with residues, followed by 90 min at 30°C. The residues were completely derivatized and run GC-MS (LECO-PEGASUS, GCXGC-TOF-MS, Leco Corporation, USA) with 30m Rxi-5ms column (0.25 mm diameter and 0.25 µm thick). The interference temperature, injection and ion were maintained at 225ºC, 250ºC and 200ºC. The parameters were carried out under the following conditions isothermal heating at 70ºC for 5 min, followed by 5ºC min to 290ºC final heating at 290ºC for 5 min. The helium gas was used as carrier gas at 1.5 $mlmin^{-1}$. The peaks were generated through MetAlign 3.0 and followed the scan ranges from 70 to 1000 at two scans/sec.

### 2.10.3. Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis of from leaves (Cycas) and Pollen (Putranjiva)

#### 2.10.3.1. Sample extraction for LC-MS/MS analysis

Around 1 mg of pollen was grounded and mixed with 1 ml of extraction buffer (methanol: chloroform: water 7: 2: 1 v/v/v). Later vortexed for 30 min at 4°C, followed by centrifugation at 13,000 rpm at 4°C for 15 min. The supernatant was moved to a new collection tube and used for LC-MS/MS analysis.

#### 2.10.3.2. LC-MS/MS based metabolomics

Metabolic extracts from UR and R pollen samples were subjected to LC-MS/MS analysis. LC-MS/MS (Tandem) mass spectral analysis was performed on a 6520 accurate mass qualitative time of flight (Q-TOF) LC-MS/MS system (Agilent Technologies, USA) coupled with HPLC 1200 series equipped with a photodiode array of detector. An autosampler injected 3 µl of the sample for chromatographic separation at 25°C. The variety of metabolites was separated using the reverse phase C-18 column (1.7µm, 150X 3.6, Luna) (Agilent), at the rate of 0.4 ml $min^{-1}$ using 0.1% acetic acid both in solvent A (Water (v/v)) and in solvent B (acetonitrile (v/v)); A

and B were used as a mobile phase. Metabolites were separated using a gradient program according to the method with some modifications (Fragallah et al., 2018). Briefly, the initial mobile phase composition with 1% eluent B followed by a linear gradient of 55% within 48 min and then step gradient to 100% within the 55 min of running and held for 5 min. Finally, the column was washed and re-equilibrated with 1% eluent B for 5 min. Detection of metabolites was done with the array of PDA detectors (200-600 nm) with an absorption spectrum read. The separation of compounds was infused into electron spray ionization (ESI) on sources operated under full-spectrum scan mode within a mass range of 40-1000 m/z. Data was acquired in both positive and negative ionization modes in a separate run. A pair of reference compound masses 121.0509 m/z ($C_5H_4N_4$) and 922.0098 m/z ($C_{18}H_{18}O_6N_3F_{24}$) were measured throughout the sample run for mass correction and obtained the accurate mass. Mass spectrometer parameters were set as follows: nitrogen as drying gas, ion source temperature at 300°C, a capillary voltage of 4 kV and fragmentor voltage of 144 V. Data acquisition was made using Mass Hunter workstation (version 6.0 Agilent Technologies).

## 2.11. Metagenomic analysis of Putranjiva pollen

### 2.11.1. Pollen collection for metagenome

The pollen was collected for metagenome analysis during the pollination period of the Putranjiva plant in April (2019) from the University of Hyderabad, Telangana, India. The collection was carried out with proper precautions wearing Rnase-free gloves and tapping flowers kept near the mouth of an autoclaved 50 ml falcon tube with a sterilized needle. For isolation, culturable bacteria from both pollen and stigma were collected. Putranjiva female flowers were collected, and the stigma was separated from the main flower body through a surgical blade. The outer stigmatic surface was washed with 70% methanol 6 to 7 times. This experiment was performed under the laminar hood. Finally, the stigma and pollen were

transferred to culture media. The cultured bacteria were pellet out of centrifugation at 5000 rpm for 20 min. The pellet was stored at -20°C for DNA extraction.

### 2.11.2. Metagenomic DNA isolation and analysis of Cycas and Putranjiva pollen

The metagenomic DNA was extracted using cTAB buffer along with phenol: chloroform extraction method followed by RNase treatment. The DNA was outsourced to AgriGenome Labs Pvt. Ltd., Bangalore for metagenomic analysis. The DNA from pollen was quantified using Nano Drop. 2×300 MiSeq library was prepared using the Nextera XT Index kit. PCR was performed using the bacterial universal primers 27F (5′-GAGTTTGATCCTGGCTCAG-3'), 1525R (5′-AAAGGAGGTGATCCAGCC-3'). PCR clean-up kit was used to purify the PCR product.

## 2.12. Proteomic analysis

### 2.12.1 Extraction and isolation of protein

The flowers (100g) were taken for protein extraction according to the method described by Xiong et al., 2013 with a slightly modified protocol. Both male and female flowers from Cycas and Putranjiva were collected and crushed to a fine powder using liquid nitrogen and the resulting powder sample was washed with 10% TCA-Acetone with 2% β-mercaptoethanol and kept for 2 hours incubation at -20˚C. These mixtures were centrifuged at 8000 rpm for 20 min. The dark-colored supernatant was discarded and washed twice with 10% acetone without TCA. The pellet was kept inside the fume hood removing excess acetone. The dried pellet proceeded to the protein extraction step. The extraction buffer was prepared with 0.1 MDTT, 0.1M Tris HCL (pH 8.8), and 2% SDS in a 1:10 ratio. The pellet was mixed with extraction buffer and kept for vertexing for 30 min. The protein was mixed with extraction buffer, which was separated by adding an equal volume of tris-saturated phenol (pH 8.0). This light pink phenol phase was separated and centrifuged at 8000 rpm for 30 min. 0.1M ammonium acetate

precipitation buffer was prepared. The 1:5 ratio was mixed with supernatant and kept for incubation overnight. The floating white pellet with supernatant was centrifuged again with the previous 8000 rpm for 30 min.

### 2.12.2. Nanoscale liquid chromatography with tandem mass spectrometry (nano LC-MS/MS) analysis

The purified protein pellet was used for trypsin digestion. 100 µg samples were mixed with 100 mM DTT and kept for 1 hour on a heat block (95ºC), followed by the addition 250 mM imino diacetic acid (IDA) and incubation in dark for 45  at the dark condition at room temperatures. The heat block was set at 37ºC. After 45 min, the trypsin was added to the suspension and kept for digestion overnight. The solution was centrifuged at 10,000 g. The resultant supernatant was dried under vacuum. The vacuumed dried sample was solubilized in 20 µl of 0.1% formic acid and incubated at 37°C for 45 min. 10 µl of sample was injected into the C18 column for separation of peptides. ACQUITY UPLC BEH C18 column (Waters, UK) (150 mm 2.1mm 1.7um) was used along with A SYNAPT G2 QTOF for protein mass detection. MS spectra was obtained in positive mode under the following conditions: 3500 capillary voltage per hour, source temperature at 350°C, desolvation temperature at 150°C, protein mass range between 50 k.Da to 150 k.Da, transfer collision and trap energy between 20v to 45v. The flow rate was 300 ml/ min with a 60 min acquisition time. Two solvent systems were used (Solvent A: 0.1% formic acid in H2O, and solvent B (0.1% formic acid in ACN). The linear gradient program was set as follows: 98% solvent A and 2% solvent B for 1 min, changed to 50% solvent A and 50% solvent B for 29 min, followed by 20% solvent A and 80% solvent B for 15 min, and finally set to 98% solvent A and 2% solvent B for 2 min. At the end of each program, washing with blank was done to reduce data error.

### 2.12.3. Protein identification

The generated raw data were extracted and analysed using PLGS software 3.0.2 (Waters, India). The FASTA format of Euphorbiaceae family were downloaded from NCBI and transferred hits to another file. Cycas panzhihuaensis proteomic data was downloaded in FASTA format from https://db.cngb.org/codeplot/datasets/PwRftGHfPs5qG3gE. Each run was processed using the following parameters: fragment tolerance 100 ppm, minimum two fragments for peptide match, minimum five proteins fragment match, and 50 ppm peptide tolerance. For protein identification, the Putranjiva raw data was searched against euphorbiaceous database and Cycas raw data was searched against Cycas_panzhihuaensis proteomic data.

### 2.12.4. Identification of differentially expressed proteins

In order to understand the regulation of protein expression and their mechanism of action over the course of the development of female flower in comparision to male flower, label free quantification (LFQ) was performed to identify the differentially expressed proteins (DEPs). Differential expression analysis was performed on 274 commonly identified proteins using MetaboAnalyst 5.0 sofware. LFQ intensities from technical triplicates across female and male samples were normalized using sum, log2 transformed, autosaled and the relative abundance of each protein (fold change, FC) between female and male flowers was calculated.

First, common proteins across the triplicates were retrieved for both male and female samples. Protein abundance was calculated on the basis of the peak areas (LFQ intensity). Later, the peak area matrix was imported to MetaboAnalyst 5.0 (Pang, 2021) for further statistical analysis. The data were normalized by the medium, log transformed, and auto scaled. Fold change (relative abundance of each protein) between female and male flowers was calculated based on the normalized peak area values. Fold changes are calculated as the ratios between two group means (Female/Male). Features with a minimum fold change of 1.5 (ratio $\geq$ 1.5 or <0.5; p-value $\leq$0.05) were considered to have been changed significantly (differentially expressed) between

female and male flower samples. Hierarchical clustering analysis (HCA) was performed for pairwise comparison (Male and female) and the results are displayed as heatmap.

#### 2.12.5. Functional classification of identified proteins

Proteins were annotated for their GO terms and biological process using PANTHER (v17.0) database (http://www.pantherdb.org/). The PANTHER statistical over-representation analysis was carried out on proteins exclusive to male and female flowers. Fisher's exact test with FDR correction was selected for the analysis. It determines, for each functional category (GO or PANTHER classification), whether the submitted list of proteins are statistically over- or under-representated relative to the reference list. (Mi et al., 2013). Metascape was used to compare the GO based process enrichment analysis between male and female proteins (Zhou et al., 2019).

### 2.13. Functional characterization of uncharacterized proteins

#### 2.13.1. Functional annotation

Uncharacterized proteins from Cycas and Putranjiva that were found to be expressed at the level of translation were subjected to bioinformatics based functional annotation to know their functional roles. The putative functional annotation was based on the functional motifs and domains using different bioinformatics databases and tools. Protein domain architecture was predicted through Simple Modular Architecture (SMART). Proteome information from NCBI, classification of the domain within the structure hierarchy from CATH (Sillitoe et al., 2015). The conserved domain associated with the molecular function of the proteins were determined through the CDD (Conserved Domain Database) (Lu et al., 2020). Hypothetical proteins were classified based on functional families through SUPERFAMILY and InterPro (Finnn, 2014, Gough et al., 2001, Sigrist, 2013). Protein motifs associated with catalytic function were

identified using InterProScan in the form of GO term (Mitchell et al., 2019). Above all databases, default parameters were used.

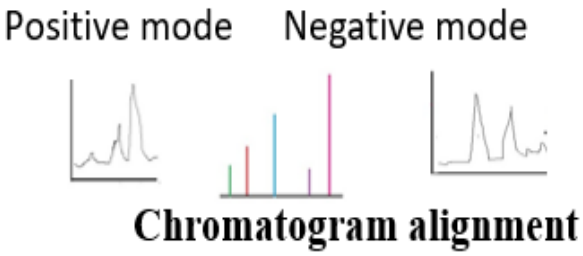
### 2.13.2. Sub-cellular localization

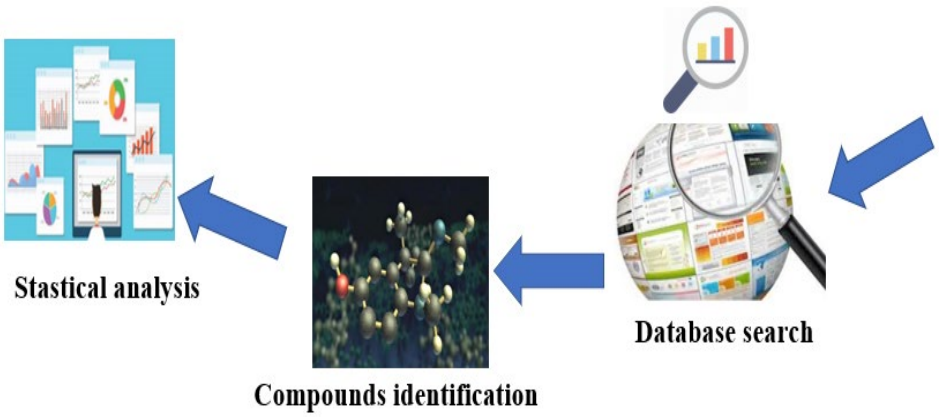
Subcellular localization of hypothetical proteins was provided to estimate protein function at the cellular level. CELLO was used for cellular location information (Yu et al., 2014).

### 2.13.3. Protein-Protein interaction analysis

Protein-protein interaction distribution, interaction number, and key nodes provide a strong understanding of structural and biological properties. String database was used for Ppi analysis which provides a functional association between proteins. The confidence scores above 0.7 were considered and the Euphorbiaceous reference genome was used for network generation. Further, Cytoscape was used to interact with proteins (Shannon et al., 2003).

# Chapter 3. Results



# RESULTS

## 3.1. Morphological study of dioecy and analysis of pollination event of *Cycas nayagarhensis* and *Putranjiva roxburghii* Wall.

### 3.1.1. Field study for Cycas and Putranjiva

The field study was conducted in Nayagarh, Odisha to study Cycas and Sanjeeviah Botanical Garden, Telangana to study Putranjiva. We observed from the field study that both selected areas have a rich diversity of angiosperms and gymnosperms. A total 78 male and 39 female Cycas plants were found in Nayagarh, Odisha. From the same area, a new species called '*Cycas nayagarhensis*, belonging to the cycad genera, was recently documented by Singh et al., 2015. Each location is tabulated with their identification (Table.1). In Sanjeeviah Botanical Garden, a total of 19 Putranjiva plants were spotted, of which 12 were female and 7 were male. Later, the morphological characters of both the model plants were examined and documented.

### 3.1.2. Morphological variation

#### 3.1.2.1. Flowering

In Cycas, male coning starts in February and continues up to April. In May, Cone releases pollen and then dries off. In old plants, coning lasts till August. Female coning starts in June and continues until December (Fig.1). In Putranjiva, male flowering occurs twice a year, and female flowering occurs once a year. Female flowering starts in April. Male flowering starts in February and lasts till the first week of May, and the second flowering is from September to December (Fig.2).

**Fig. A.** July 2016 (F)

**Fig. B.** April 2019 (M)

**Fig. C.** June 2017 (F)

**Fig. E.** May 2018 (M)

**Fig. F.** June 2017 (F)

**Fig. G.** March 2019 (M)

**Fig. H.** April 2017 (M)

**Fig. I**. April 2018 (M)

**Fig. J.** May 2018 (M)

**Figure 1. (A- J).** Morphology of the cones of the male and female Cycas plant at different months throughout the year

**Fig. A.** 24-Jun-2016

**Fig. B.** 6-July-2018

**Fig. C.** 29-March-2018

**Fig. D.** 15-Sept-2017

**Fig. E.** 10-Nov-2019

**Fig. E.** 18-April-2019

**Fig. F.** 29-June-2017

**Fig. G.** 28-June-2019

**Fig. H.** 10-Nov-2019

**Figure 2. (A-H).** Flowering in the male and female Putranjiva plant at different time periods throughout the year

### 3.1.2.2. Flower

The male cone was ovoid, 15-45 cm long, 10-20 cm in diameter, and orange in color. During the reproduction phase, the cone turns dark orange. In the month of May, when the temperature rose, the cone bursts and released abundant pollen with a pungent odor. The female cone is wider, megasporophylls were round and green in colour. The ovuliferous region was 7-13 cm long and glabrous.

Flowers in Putranjiva were greenish. Flower production is more in males than females. Male flowers were smaller compared to females. Flowers from both male and female were penta sepalous and petals were fused. The female flower stalk was more prolonged than the male flower. In Putranjiva, we found larger anther and stigma, unlike other angiosperms. Stigma was covered by the glandular body and did not produce nectar and scent. Anther dispersed abundant pollen (Fig.3).

### 3.1.2.3. Pollination system

We collected weevils from both Cycas male and female cones. A scanning electron microscope (SEM) was performed to confirm whether pollen was attached to the weevil. More pollen was found attached to the dorsiventral region and mouth parts (Fig. 4). We did not find any nectar fluid from the Putranjiva flower but the stigma contained glands (Fig. 5 A, B). we checked whether the glands produced sugars or not, so we collected female flowers under three conditions before pollination (BP), during pollination (DP) and after pollination (AP). DP contents more sucrose(7.051mg/G) than glucose (3.57mg/G) and fructose 3.057 mg/G (Fig. 5 C).

## Detection and identification of visitor in Putranjiva flower

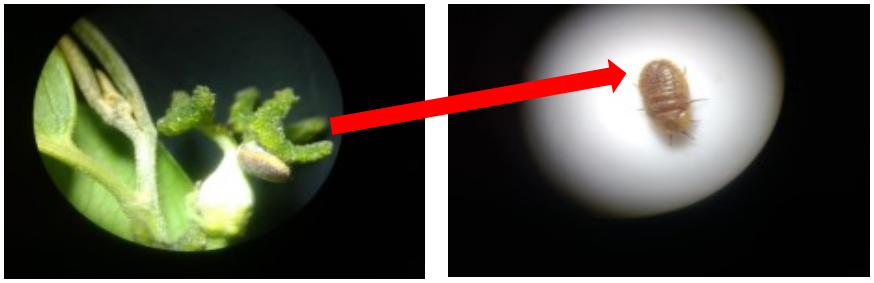


Fig. A Fig. B

**Fig. A & B. Visitor found during flowering of Putranjiva**

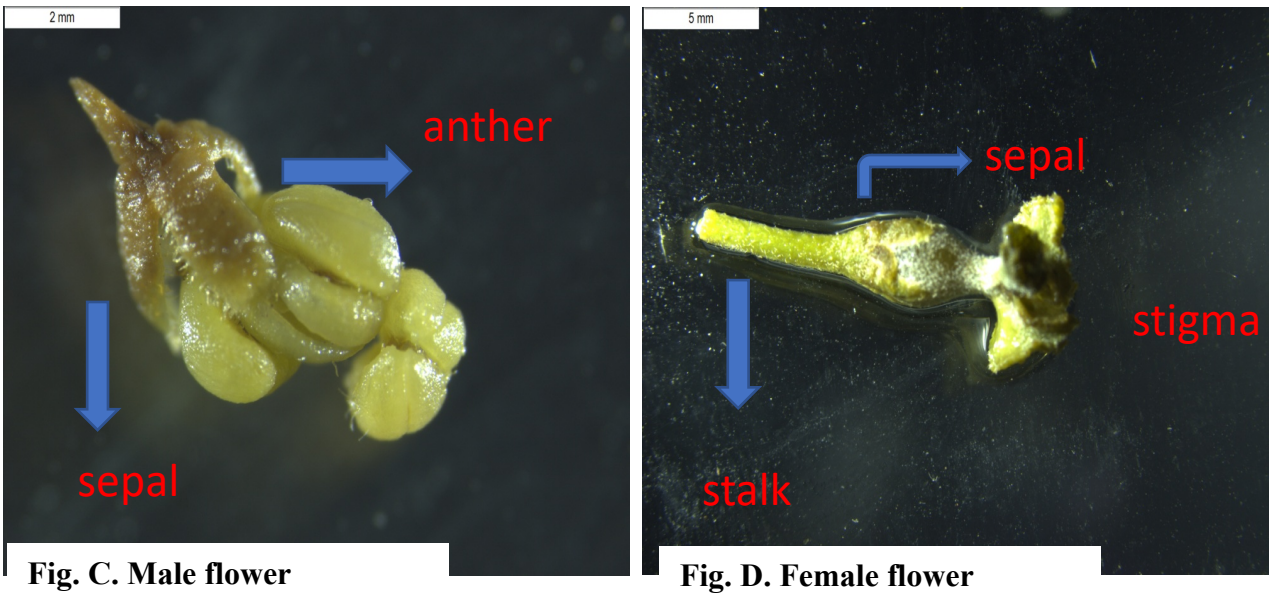



**Fig. C. Male flower** **Fig. D. Female flower**

**Figure 3. A.** A regular visitor in the Putranjiva female flower **B.** Inverted microscope image of the insect **C.** Structure of the male Putranjiva flower with bulky anther **D.** Structure of the female Putranjiva flower with showy bigger stigma

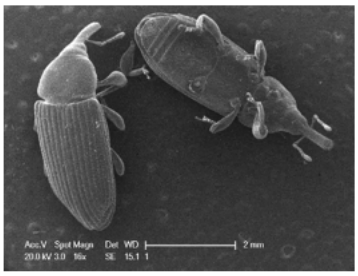
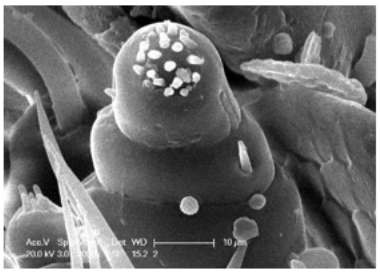


Fig. A



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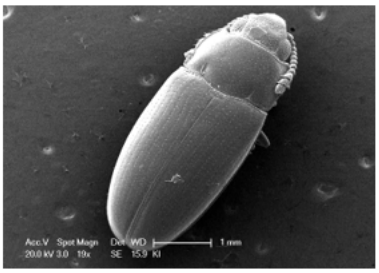


Fig. C

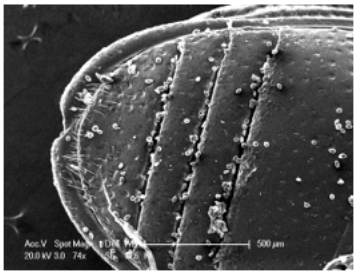


Fig. D

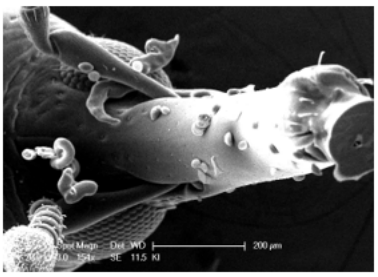


Fig. E

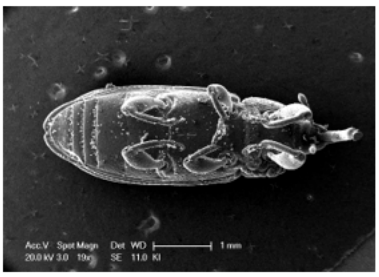


Fig. F

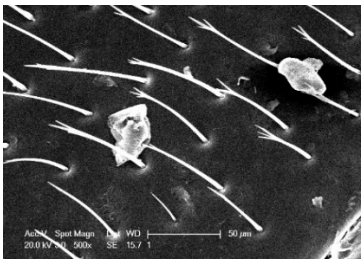


Fig. G

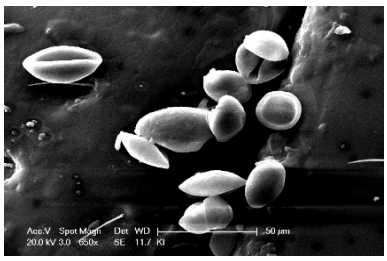


Fig. H

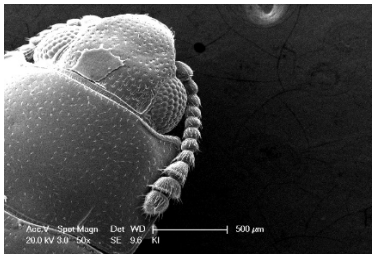


Fig. I

**Figure 4.** Scanning electron microscopy images of the weevils, **A-I.** Images showing the pollen grains on different body part of weevils.

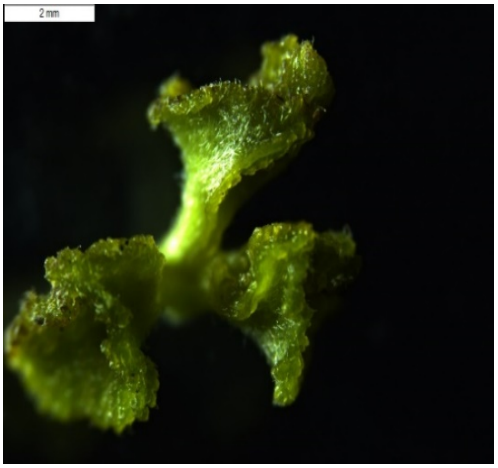



**Fig. A.**

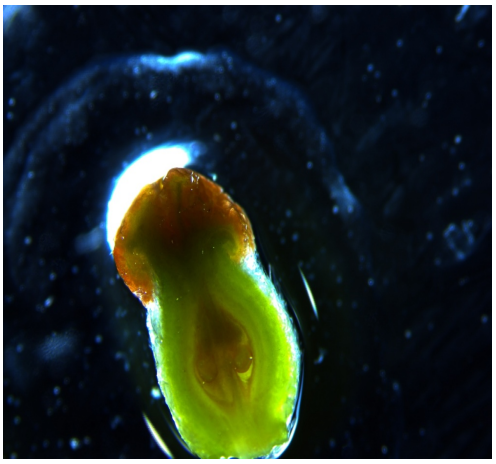


**Fig. B.**

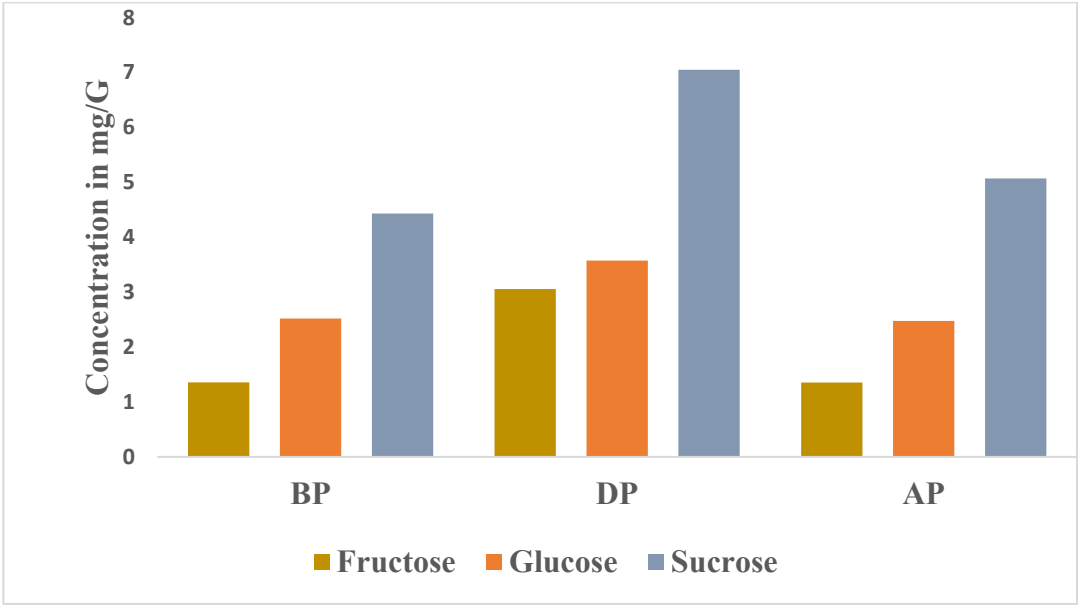



**Fig. C.**

**Figure 5. A.** Putranjiva Stigma during pollination **B.** Putranjiva stigma before pollination **C.** Bar graph representing major 3 sugars in BP (Before pollination), DP (During pollination) and AP (After pollination)

### 3.1.2.4 Leaves

The female Cycas and Putranjiva had significantly larger leaves. Cycas leaves were pinnately compound whereas in case of Putranjiva, analysis of leaf length, breadth, petiole length, internode distance and serrated distance parameters of male and female were done (Fig. 6). We found larger leaf size, length, breadth, petiole and internode but more serrated in the male leaf of Putranjiva (Fig.7). Males have more leave near the branch's end. In contrast, females hold fewer with fewer large leaves (Fig.7).

### 3.1.3. Phytochemical screening

Phytochemicals of the methanolic extracts of Cycas leaf and Putranjiva leaf were quantitatively analyzed. The test showed the presence of carbohydrates, glycoside, protein, amino acid, phytosterol, reducing sugar, alkaloid, tannin, and phenolic in all the extracts. Saponin was absent in Putranjiva (Table. 2).

### 3.1.4. Biochemical analysis

The concentration of total phenolic content for both males and females in Cycas and Putranjiva was quantified. Putranjiva males had a high content of phenol 32.727 mg/g and 18.942 mg/g in females. The Cycas females had 3.28 mg/g and 2.52 mg/g in males (Fig.9 A). In the case of protein, the Cycas females had less content of protein 2.19 mg/g compared to male 2.83 mg/g and in Putranjiva 8.443 mg/g for males and 6.98 mg/g for females (Fig. 8 A). In Putranjiva, the reducing sugar content was more in the female plant 8.041 mg/g than male plant 5.867 mg/g, but in the Cycas plant, the female had less 5.286 mg/g than the male 6.649 mg/g (Fig. 9 B). The total flavonoid content of Cycas female plant was 57.08 mg/g compared with the male plant 26.08 mg/g, whereas Putranjiva female plant content was more 22.213 mg/g

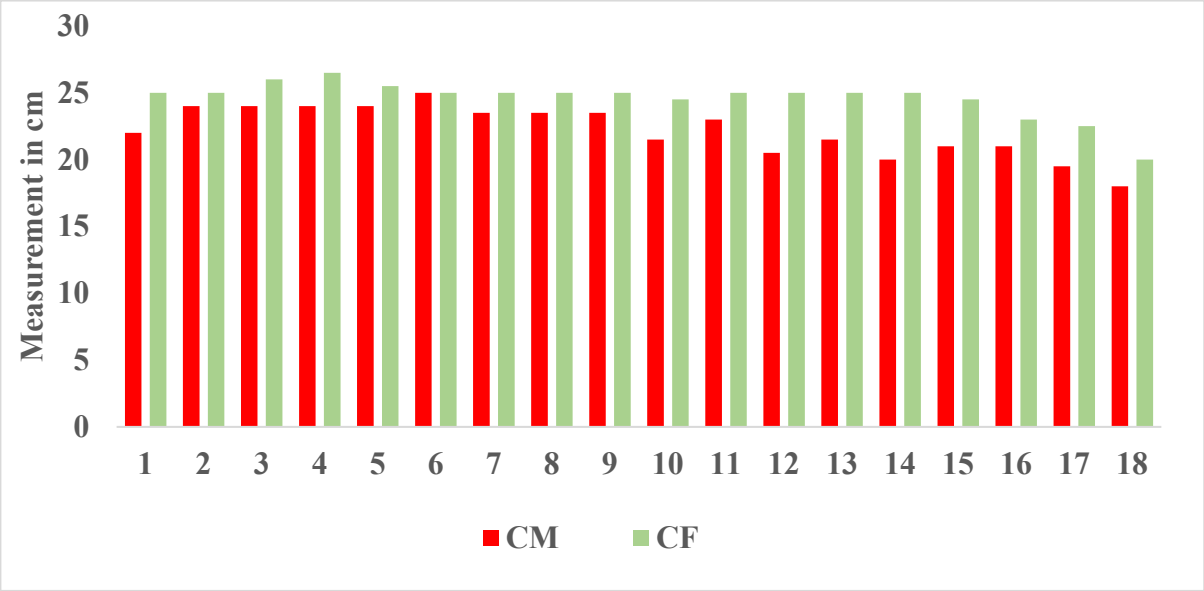



**Fig. A.**

**Fig. B.** Female Cycas leaves

**Fig. C**. Male Cycas leaves

**Figure 6. A.** Structure and morphology of leaves **A.** Comparison of Cycas Leaf length. **B.** Female Cycas leaves **C.** Male Cycas leaves.CM- cone male, CF- cone female. Data represent the means ± SD; (n = 3)

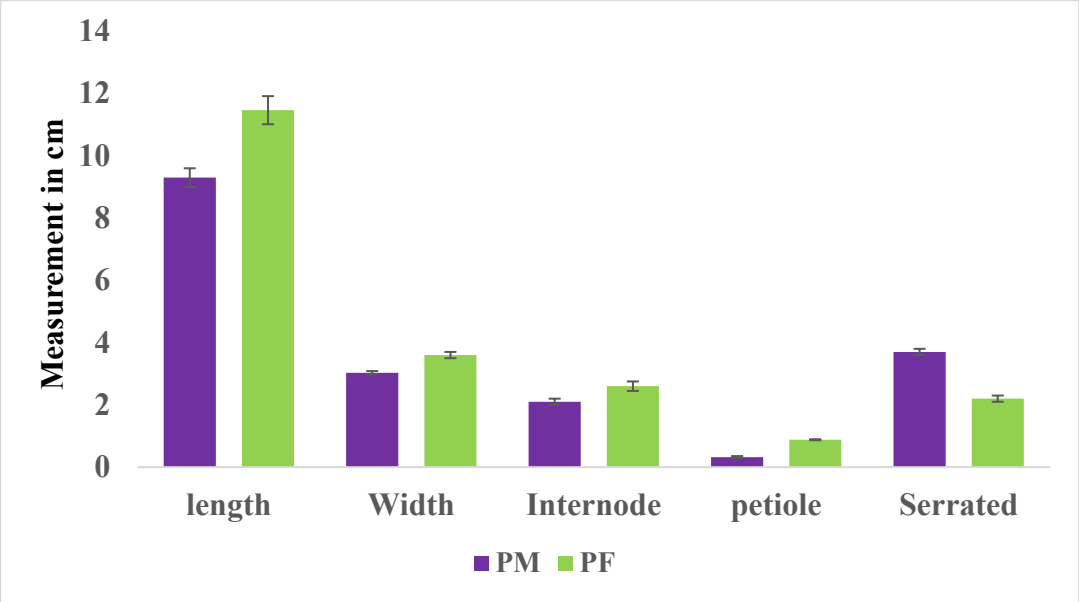



**Fig. A.**

**Fig. B.**

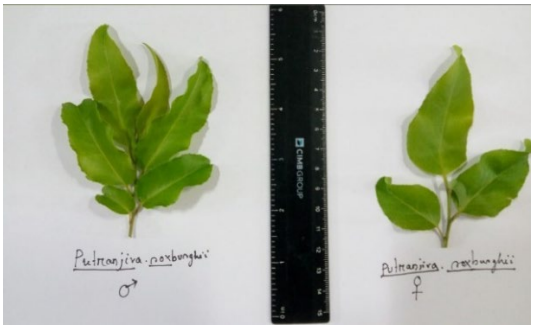



**Fig. C.**

**Figure 7.** Comparison of leaf morphology of Putranjiva plant **A.** measurement of various parameters of Putranjiva male leaves. **B, C.** examination of various parameters of Putranjiva female leaves. Data represent the means± SD; (n = 3)

**Table 2.** Quantitative analysis of phytochemicals of the leaf extract of Cycas and Putranjiva. Values are means of triplicate determination; + Positive; - Negative

| Chemical constituents | Reagents/Test | Putranjiva male | Putranjiva Female | Cycas male | Cycas Female |
|---|---|---|---|---|---|
| Alkaloids Reducing sugars | Wagner reagents | + | + | + | + |
| Carbohydrate Glycosides | Fehlings Test | + | + | + | + |
| Saponins | Foam observation test | + | + | + | + |
| Proteins Aminoacids | Ninhydrin test | + | + | + | + |
| Phytosterols | Sulphuric acid test | _ | _ | + | + |
| Fixed oils | Filter paper test | + | + | + | + |
| Phenolics Tannins | Ferric chloride test | + | + | + | + |

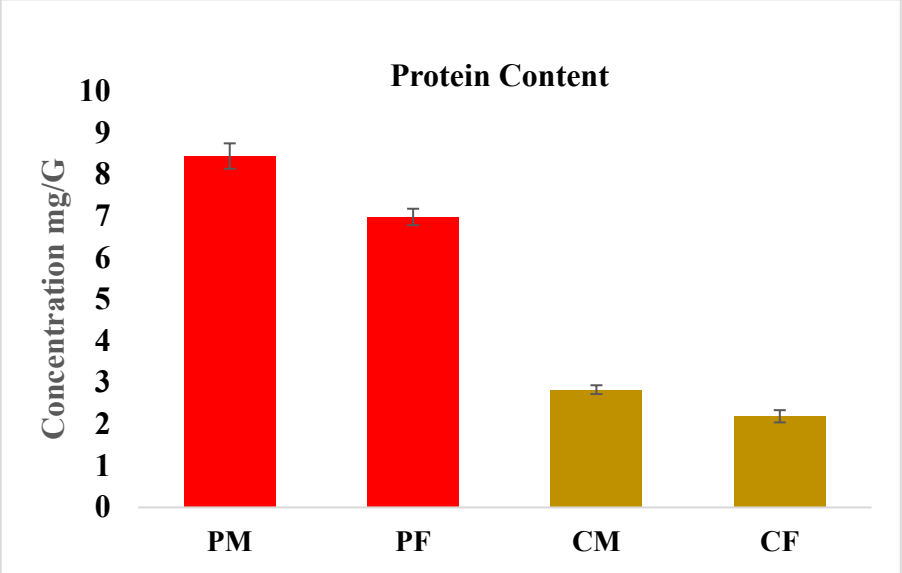



**Fig. A.**

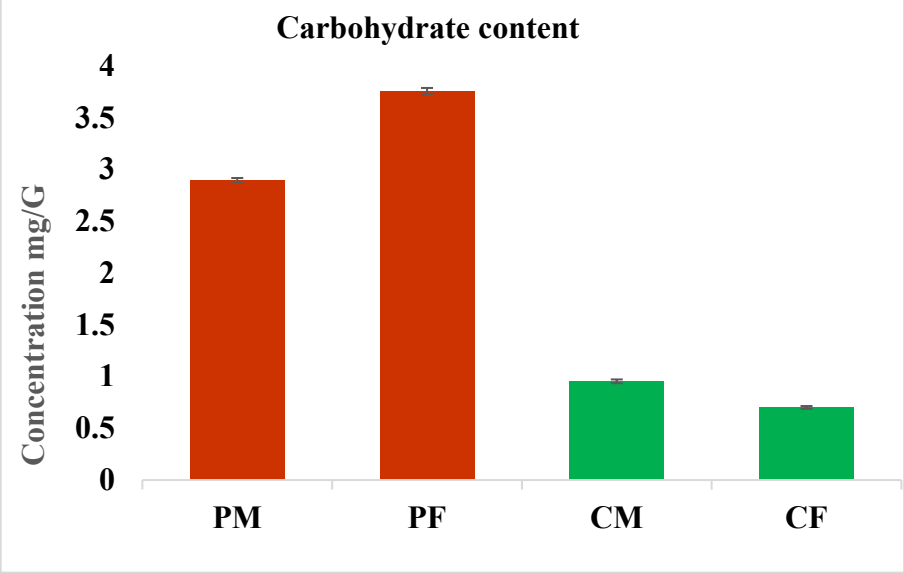



**Fig. B.**

**Figure 8. A.** The total protein content of male and female plants **B.** The total carbohydrate content of male and female plant. PM- Putranjiva male, PF- Putranjiva female, CM- Cycas male, CF- Cycas female. Data represent the means± SD; (n = 3)

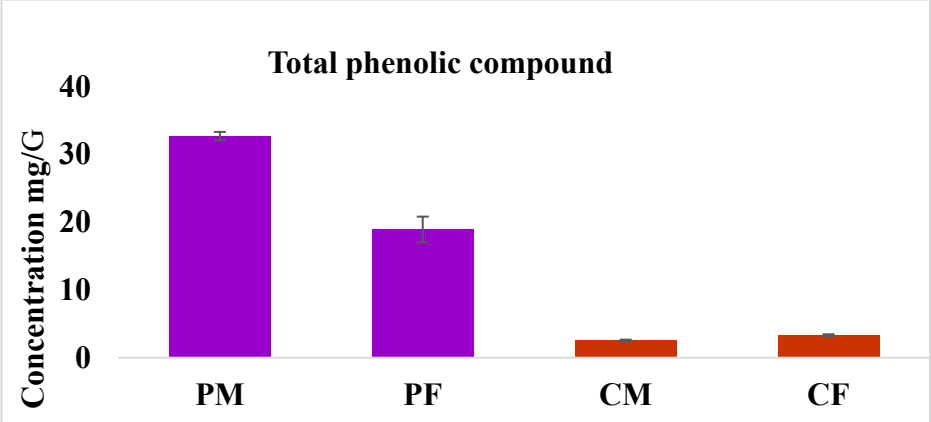



**Fig. A.**

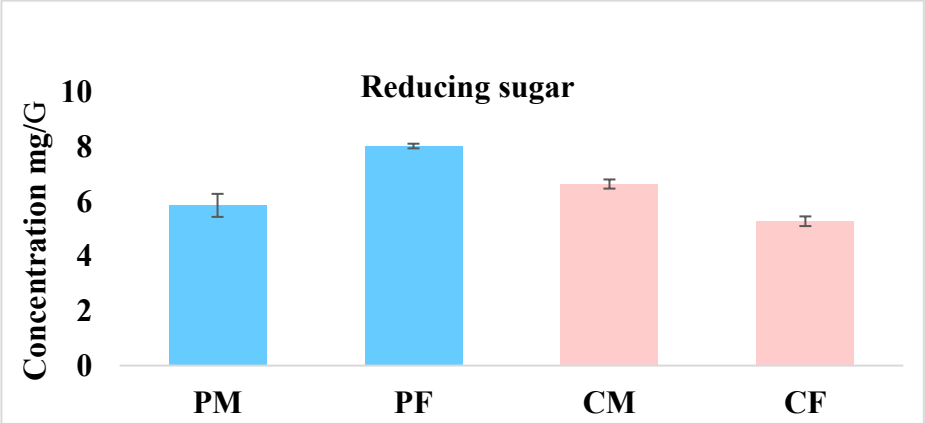



**Fig. B.**

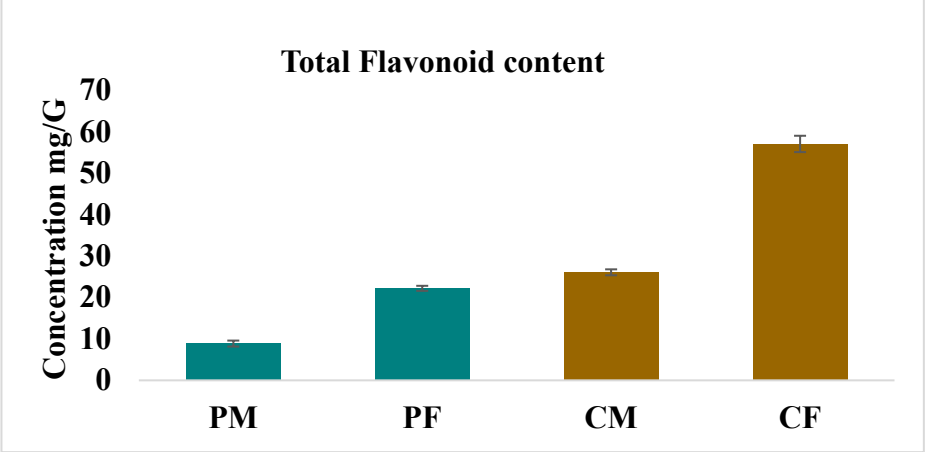



**Fig. C.**

**Figure 9.** Biochemical quantification of male and female plants. **A.** Total phenolic content **B**. Reducing sugar **C**. Total flavonoid content. PM- Putranjiva male, PF- Putranjiva female, CM- Cycas male, CF- Cycas female. Data represent the means + SD; (n = 3)

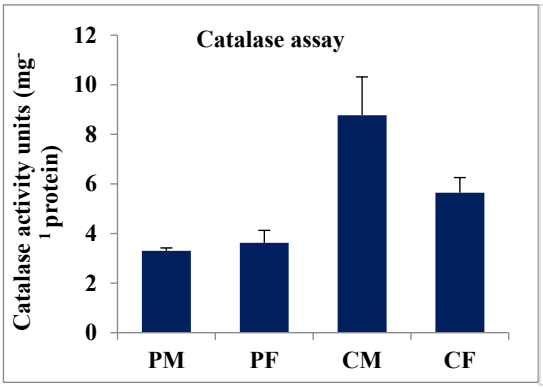



**Fig. A.**

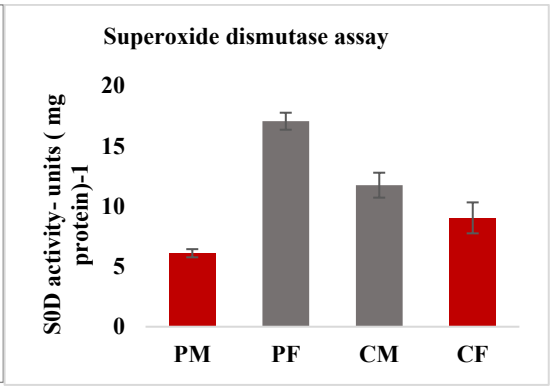



**Fig. B.**

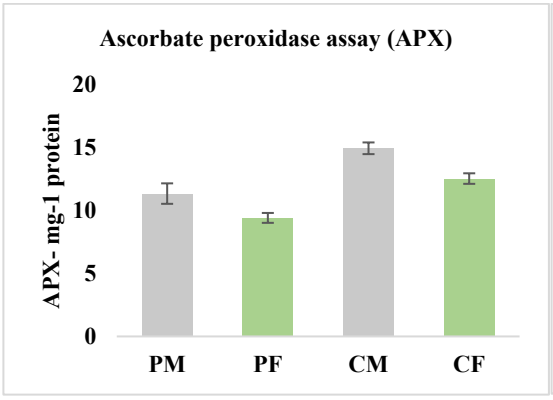



**Fig. C.**

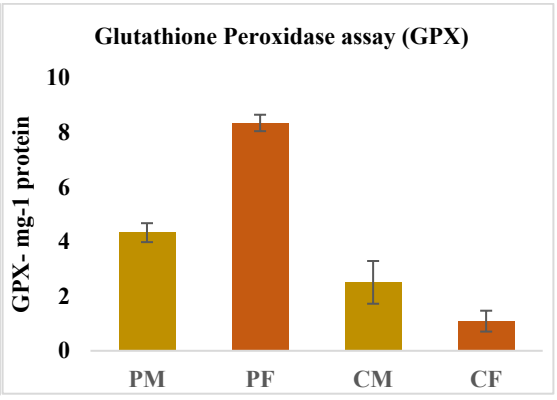



**Fig. D.**

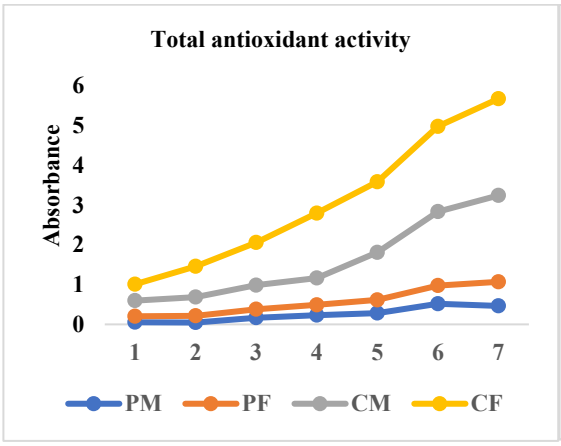



**Fig. E.**

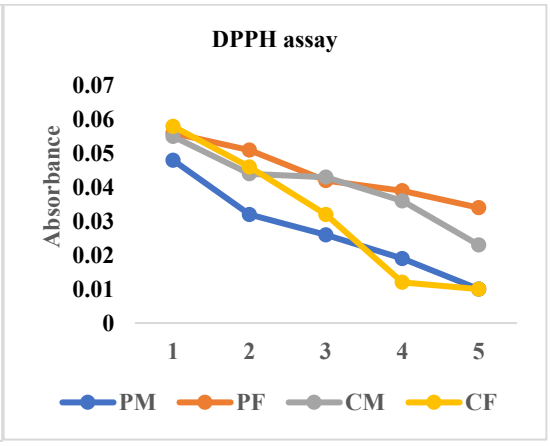



**Fig. F.**

**Figure 10. A.** Antioxidant enzyme assay **A.** Catalase assay (CAT) **B.** Superoxide dismutase assay (SOD) **C.** Ascorbate peroxidase assay (APX) **D**. Glutathione peroxidase assay (GPX) **E.** Total antioxidant activity (TAC) **F.** DPPH assay. PM- Putranjiva male, PF- Putranjiva female, CM- Cycas male, CF Cycas female. Data represent the means + SD; (n = 3)

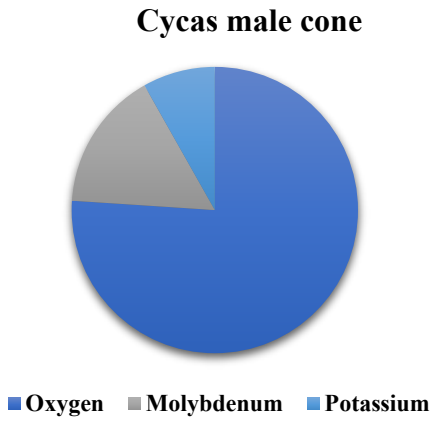



Fig. A.

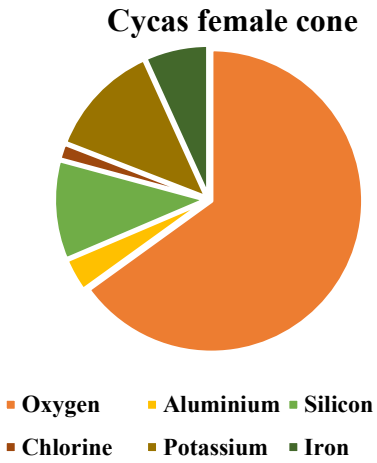



Fig. B.

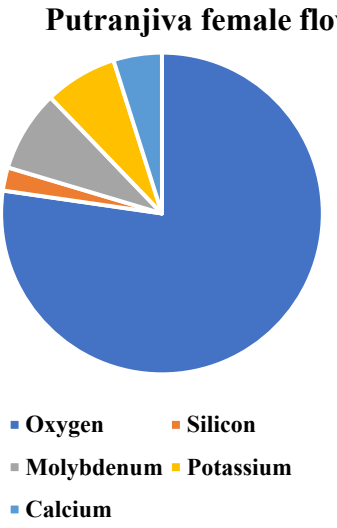



Fig. C.

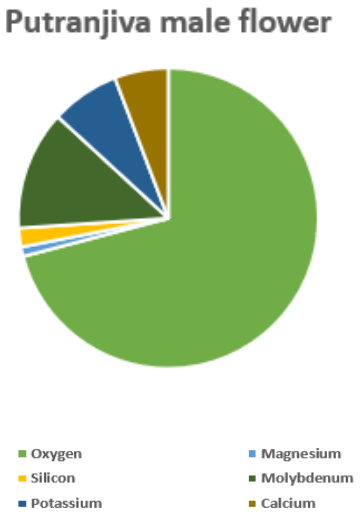



Fig. D.

**Figure 11.** Elemental analysis through EDAX. **A.** Cycas male cone, **B.** Cycas female cone **C.** Putranjiva female flower **D.** Putranjiva male flower

than the male plant 8.88 mg/g (Fig. 9 C). The phenolic content was higher in Putranjiva males and lowered in females. In the case of Cycas, phenolic content was lower in the male plant. Both female plants had a higher flavonoid content. The antioxidant potential was increasing in Putranjiva and Cycas female plants however, antioxidant activity was more in Cycas than Putranjiva (Fig. 10 E). DPPH scavenging activity increased in the Cycas female plant (Fig. 10 F).

### 3.1.5. Antioxidant enzyme assay

The highest SOD value was 17.0621 units mg-1 of protein in the Putranjiva female plant compared to the Putranjiva male SOD value of 6.1012 units mg-1 of protein (Fig. 10 B). Cycas male SOD value was 11.7512 units mg-1 of protein while Cycas female was 9.0375 units mg-1 of protein, respectively (Fig. 10 B). Likewise, the catalase activity in both dioecy leaves showed 3.62 µmol $min^{-1}$ $mg^{-1}$ protein in Putranjiva females, in comaprision to 3.30 µmol $min^{-1}$ $mg^{-1}$ protein in Putranjiva males and 8.76720 µmol $min^{-1}$ $mg^{-1}$protein in Cycas male to 9.0375 µmol $min^{-1}$ $mg^{-1}$ protein in Cycas female (Fig. 10 A). The ascorbate peroxidase (APX) enzyme was increased in both Putranjiva and Cycas males (Fig. 10 C). The increased activity was observed at 11.3514 µmol$min^{-1}$ $mg^{-1}$ protein for Putranjiva male, relative to 9.42 µmol $min^{-1}$ $mg^{-1}$ protein for Putranjiva female and in the case of Cycas male 14.952µmol $min^{-1}$ $mg^{-1}$ protein, in comaprision to 12.54 µmol $min^{-1}$ $mg^{-1}$ protein in female. The GPX was observed 4.3266 µmol $min^{-1}$ $mg^{-1}$protein for Putranjiva males and 8.3452µmol $min^{-1}$ $mg^{-1}$ protein for Putranjiva females similarly, 2.5062 µmol $min^{-1}$ $mg^{-1}$ protein for Cycas male and 1.086 µmol $min^{-1}$ $mg^{-1}$protein for Cycas female (Fig.10 D).

### 3.1.6. Elemental analysis of foliar from Cycas and Putranjiva

The elementals were quantified for both Putranjiva males and females. The male flower contain oxygen 70.13%, calcium 5.73%, molybdenum 12.72%, potassium 7.31%, magnesium 1.01%, and silicon 2% (Fig. 11 D), whereas the female flower contains oxygen 77.29%, calcium 4.88%,

potassium 7.31%, molybdenum 8.19%, silicon 2.34% (Fig.11 C). Cycas male cone contains oxygen 76%, potassium 8.18%, molybdenum 17.78%, whereas female cone contains oxygen 65%, iron 6.78%, potassium 12.33%, chlorine 1.69%, silicon 10.61%, and aluminium 3.54% (Fig. 11 A, B).

### 3.1.7. Physiology parameters

The concentration of Chl 'a' in the leaves of Cycas male and female plants was recorded at 20.295 mg/g and 21.617 mg/g. Chl 'b' concentration was 8.39 mg/g for Cycas male and 9.09 mg/g for Cycas female; simultaneously, the carotenoid concentration was recorded for Cycas male leaves were at 10.241 mg/g and female leaves were 11.314 mg/g (Fig.12 B). There was a reduction of Chl 'a', Chl 'b' and carotenoid in the male Cycas plant. Chlorophyll 'a' was quantified in Putranjiva male and female leaves as 10.99 mg/g and 20.991 mg/g. The concentration of Chl 'b' was quantified in Putranjiva male and female sexual dimorphic leaves were found 8.285 mg/g and 14.9 mg/g. The concentration of total carotenoids in Putranjiva was recorded as 7.92 mg/g in male leaves and 28.413 mg/g in female leaves (Fig. 12 A).

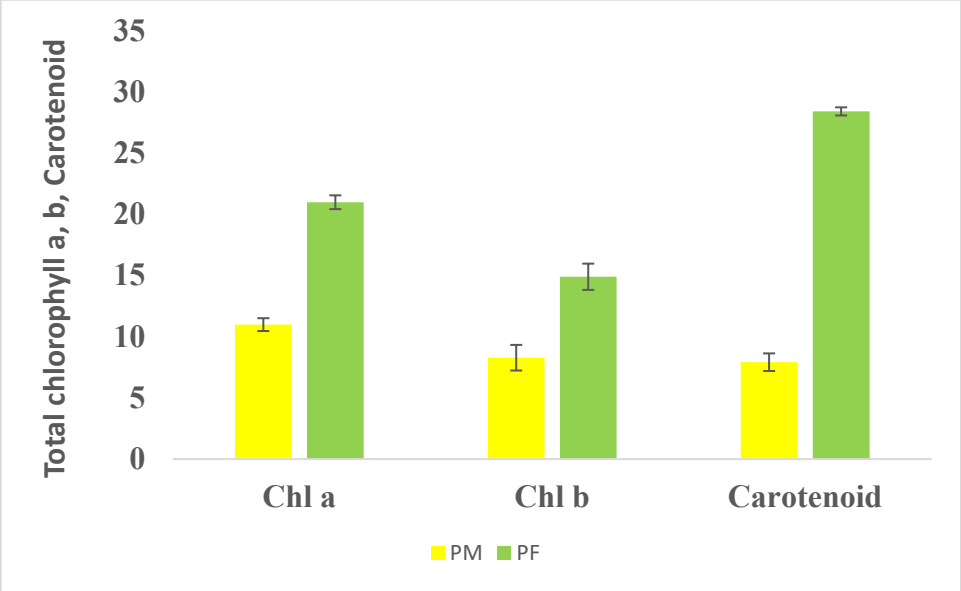



**Fig. A.**

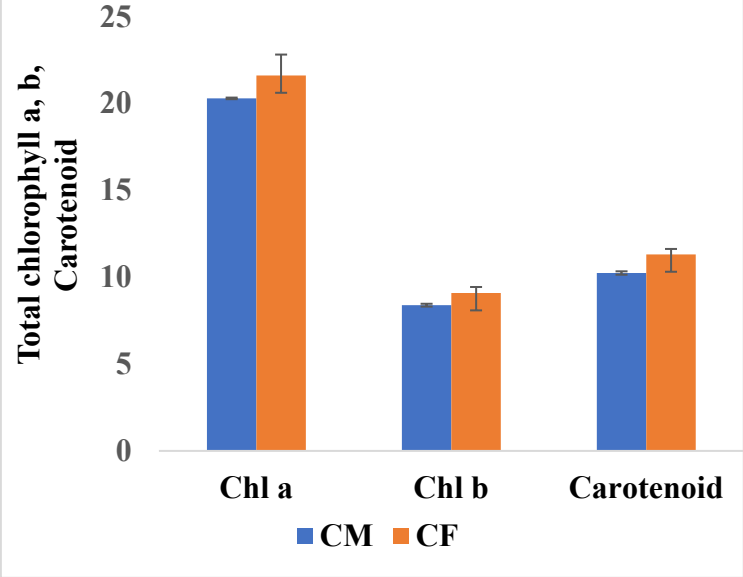



**Fig. B.**

**Figure 12. A.** Quantification of Chlorophyll a, b and carotenoid in Putranjiva leaves **B.** Quantification of chlorophyll a, b and carotenoid in Cycas leaves. Data represent the means ± SD; (n = 3)

**Abaxial side of Cycas (F) leaf**

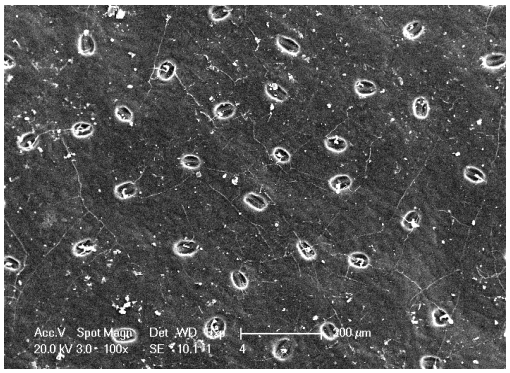



**Fig. A. Abaxial surface of Putranjiva (M) leaf**

**Abaxial side of Cycas (M) leaf**

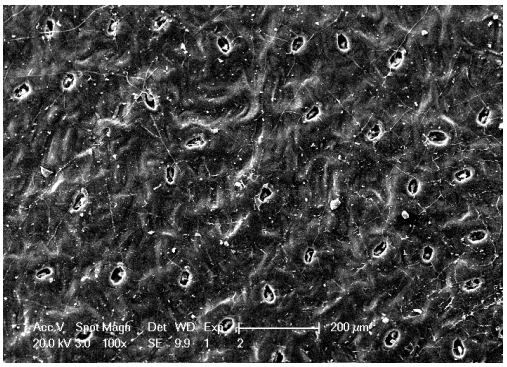



**Fig. B. Abaxial surface of Putranjiva (F) leaf**

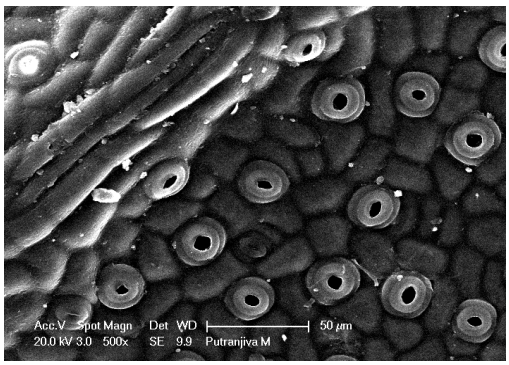



**Fig. C**

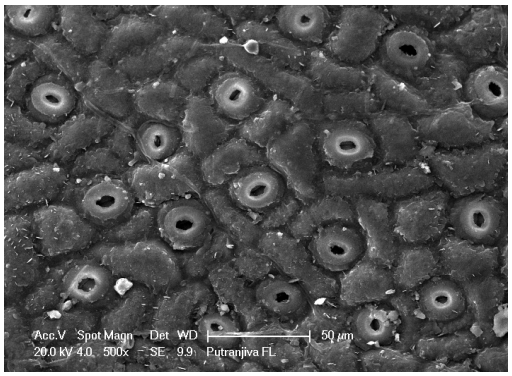



**Fig. D**

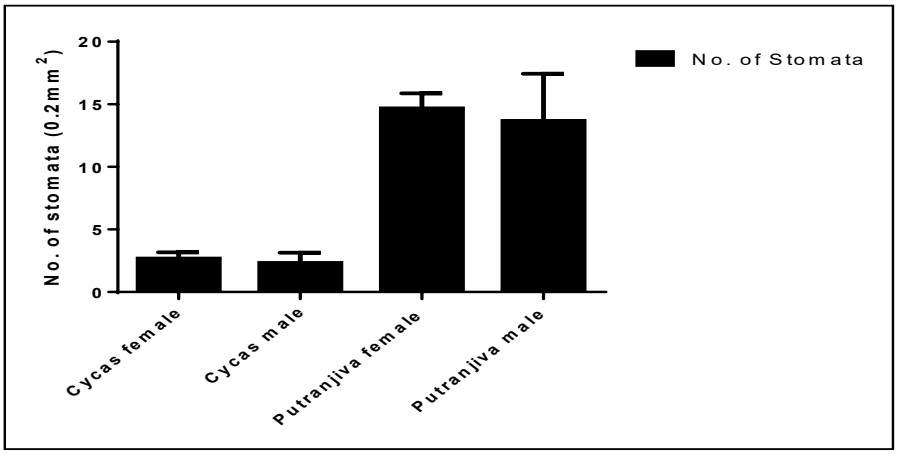



**Fig. E**

**Figure 13.** Stomatal quantification **A.** Cycas male **B.** Cycas female **C.** Putranjiva male **D.** Putranjiva female **E.** stomata quantification represents through Bar graph. Data represent the means ± SD; (n = 3)

### 3.1.8. Stomatal quantification

Scanning electron microscopy was performed to quantify stomata. Analysis show that stomata was present on the abaxial side in Cycas male and female leaves, and was sunken (Fig. 13 A, B). In Putranjiva, stomata were present on both adaxial and abaxial sides (Fig. 13 C, D). Quantification shown that stomata are more in females compared to males, in both Cycas and Putranjiva (Fig. 13 E).

### 3.1.9. PS I and PS II parameters

We compared photosynthetic parameters in Cycas male (CM) and female plant (CF) along with Putranjiva male (PM) and female plant (PF). We found that ETR (I) 's photosynthetic electron transport rate represents the photosynthetic efficiency. The initial yield was reduced in PM and then PF (Fig. 15 D) but in the case of Cycas, the PAR intensity was increased till 400 µmol photons $m^{-2}$ $s^{-1}$ then decreased in Cycas female (Fig. 14 D). Acceptor side Y (NA) of PS I was quantified (Fig. 15 C). The value of the acceptor side increased in PF then PM in contrast till 300 µmol photons $m^{-2}$ $s^{-1}$, whereas in CM it increased later both CM and CF remain unchanged (Fig. 14 C). The donor side of PSI was slightly different like in CM and CF it was unchanged and in case of PM and PF, the initial Y (I) was reduced in PM and CM whereas it increased in PF and CF (Fig. 15, 14 A & D).

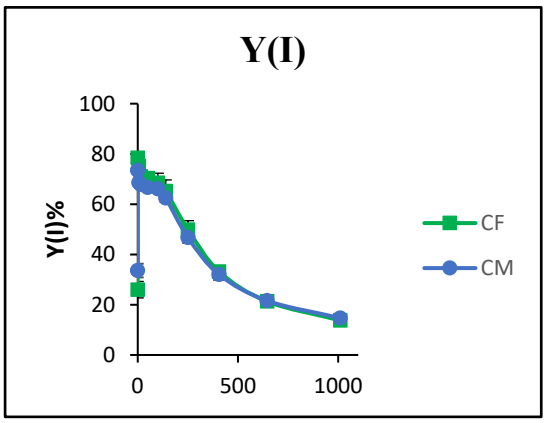



Fig. A.

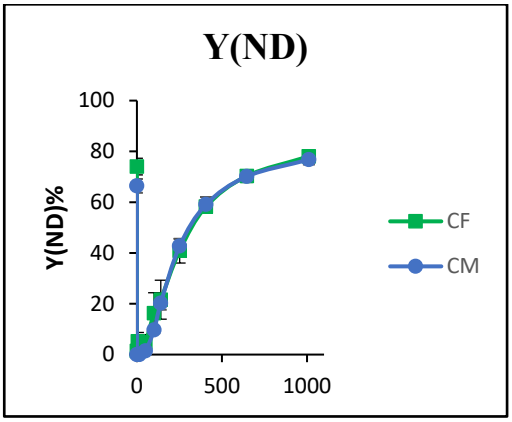



Fig. B.

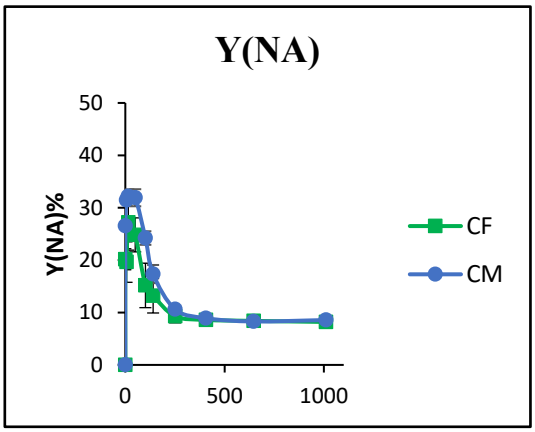



Fig. C.

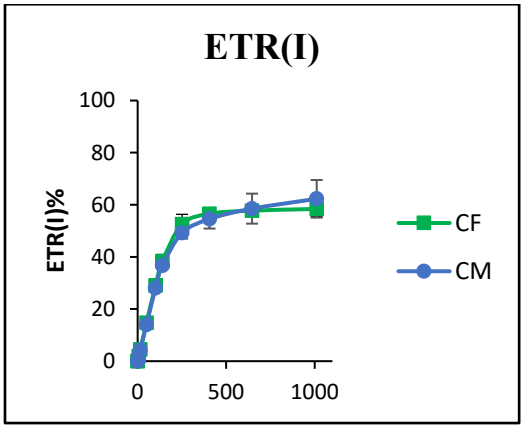



Fig. D.

**Figure 14. A.** Y(ND)-Y(NA) **B.** Y(ND) signifies donor-side regulation of PSI **C.** Y(NA) is the yield of non-regulated energy dissipation of PSII **D.** Electron rate transport of Cycas. Data represents the means ± SD; (n = 3)

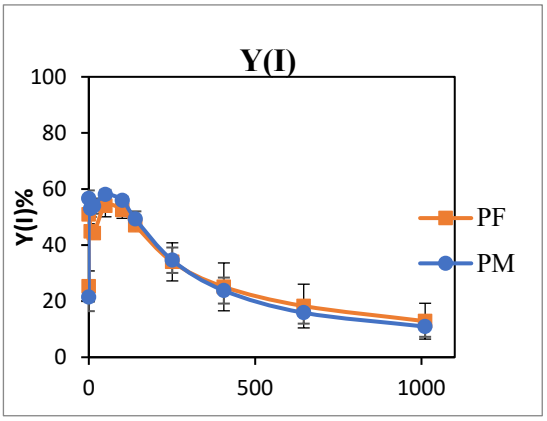



**Fig. A.**

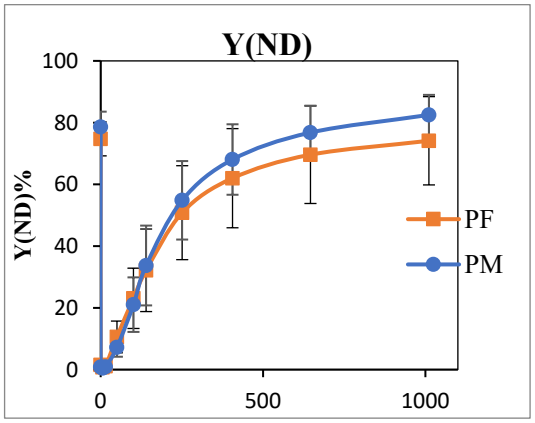



**Fig. B.**

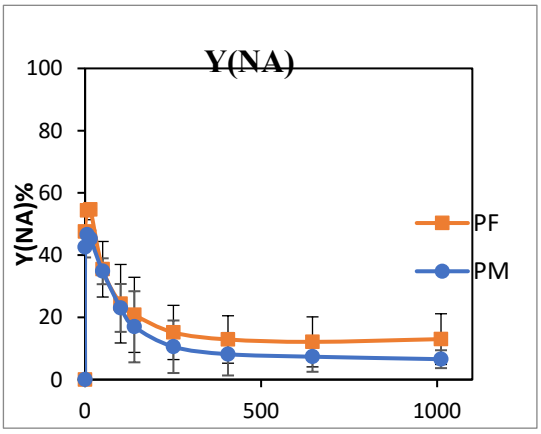



**Fig. C.**

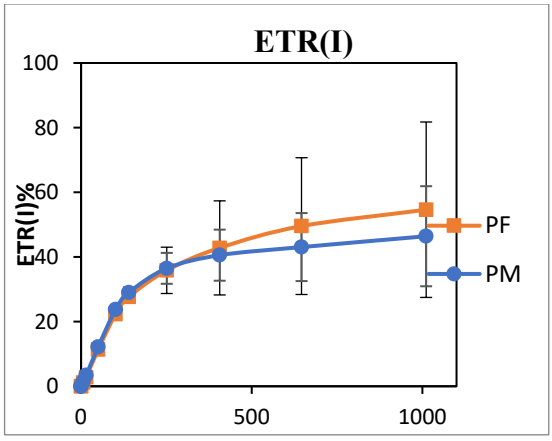



**Fig. D.**

**Figure 15. A.** Y(ND)-Y(NA) B. Y(ND) signifies donor-side regulation of PSI, **C.** Y(NO) is the yield of non-regulated energy dissipation of PSII **D.** Electron rate transport of Putranjiva. Data represents the means ± SD; (n = 3)

## 3.2. Pollen biology

### 3.2.1. Pollen biochemistry study of Putranjiva pollen

#### 3.2.1.1. Variations in pollen number at different flowering stages

To understand the maturation time of the Putranjiva flower, pollen physiology and behaviour, we quantified pollen in seven different developmental stages using a hemocytometer. It was observed that the flower was developed up to the fourth and fifth stages and fully matured, containing the highest number of pollens that busted in the environment (Fig. 17). In the sixth and seventh stages, the maximum number of pollens were released, therefore decreasing the pollen count. This showed that the fifth-stage pollens were fully matured and adapted for wind pollination.

#### 3.2.1.2. Effect of temperature on the pollen viability

We observed unreleased (UR) and released pollens (R) at three different temperatures such as cold (15°C) (R15), moderate (25°C) (R25) and heat (35°C) (R35) conditions. ROS activities were checked for the level of damage caused to the pollens at these temperatures and finally, the viability of pollens was analyzed through fluorescence-activated cell sorting (FACS). Pollen viability was checked by using propidium iodide (PI) and fluorescein diacetate (FDA). The absence of esterase activity with the help of PI staining confirms the dead pollens. This viability assay showed 97.3% viable pollens in unreleased (UR) conditions. Among the released pollen conditions, the viability observed at cold (15°C, R15) was 71%, at moderate (25°C, R25) was 85.6% and at heat (35°C, R35) was 55% viability (Fig. 18). Pollen reduces its viability in cold and heat-stress conditions.

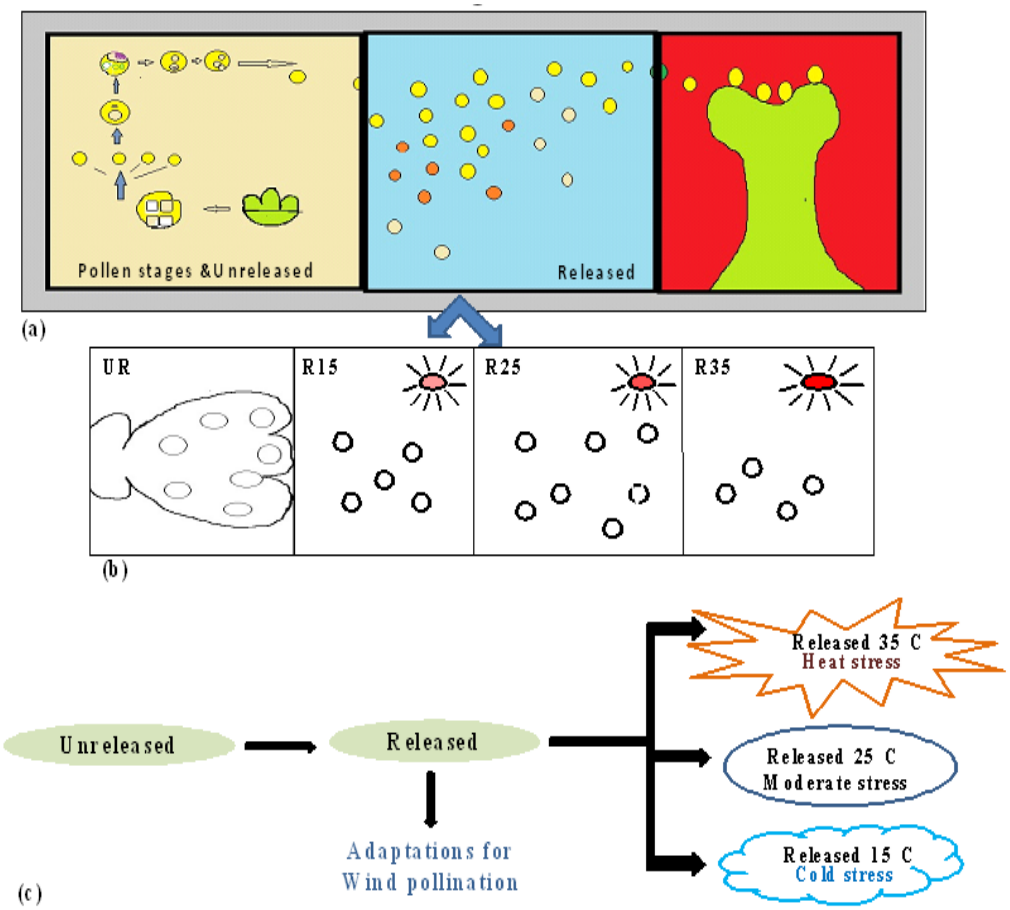
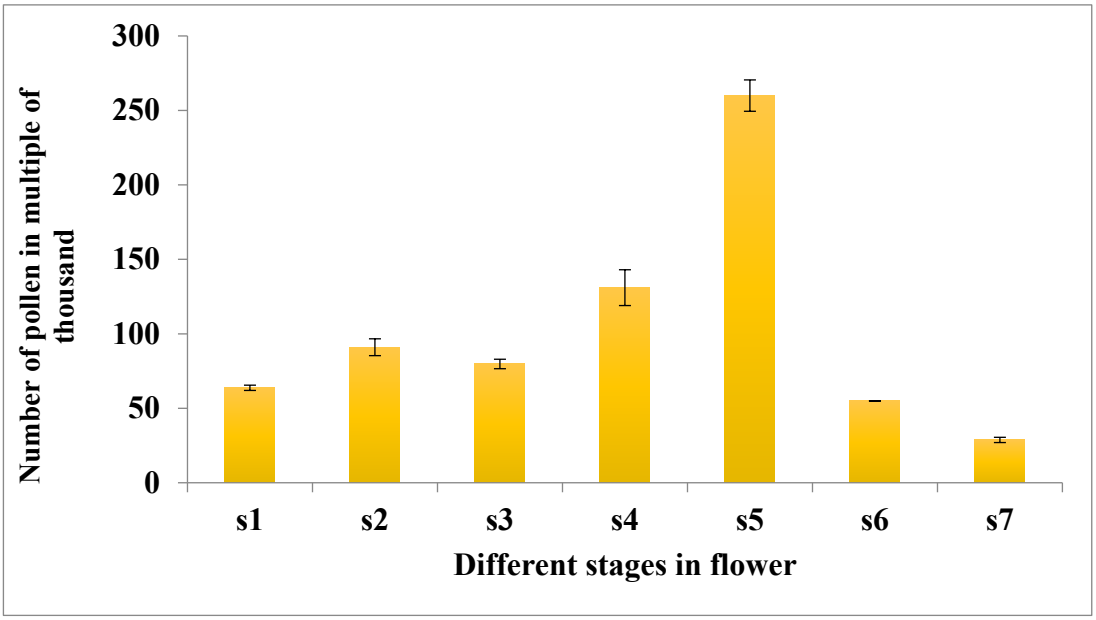



**Figure 16.** Schematic model representation of unreleased (UR) and released (R) Putranjiva pollen



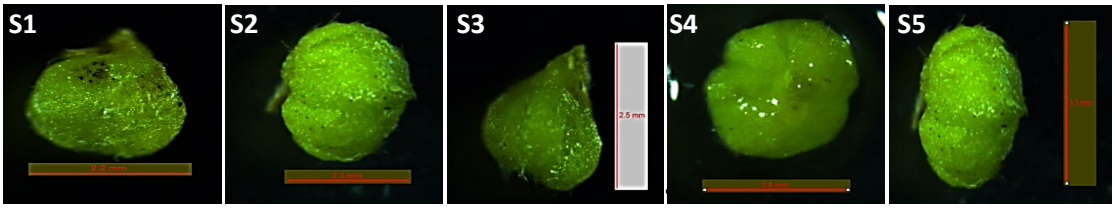




**Fig. A.**

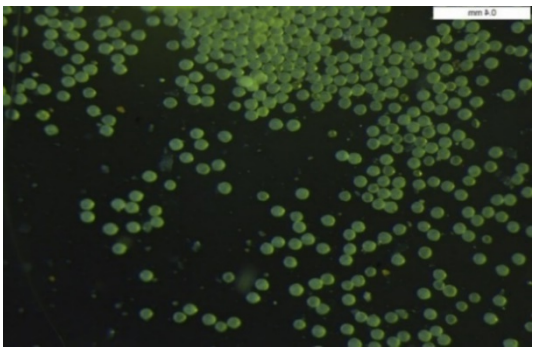


**Fig. B.**

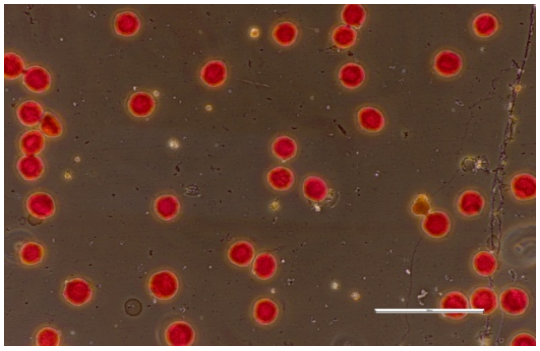


**Fig. C.**

**Figure 17.** Pollen counting in different flowering stages in Putranjiva flower **A.** Pollen content in different developmental stages of *Putranjiva* plant. Data represents the mean ± SD; (n = 10). Here s1-flower of 10 day after budding, s2 - 12 days old flower, s3 - 14 days old flower, s4 - 16 days old flower, s5 - 18 days old flower, s6- 20 days old flower, s7- 22 days old flower **B.** Pollen examined under stereo microscope **C.** Pollen safranine stained pollen examined under stereo microscope1 Data represent the means ± SD; (n = 3)

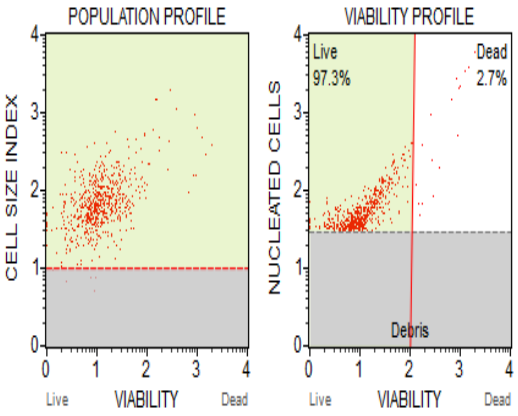



**Fig. A.**

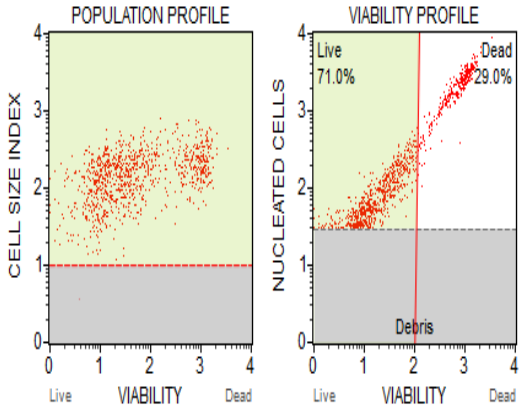



**Fig. B.**

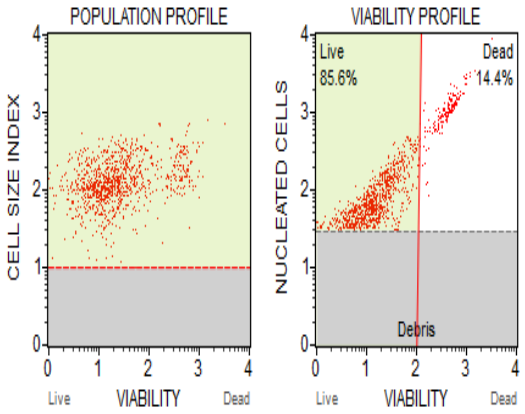



**Fig. C.**

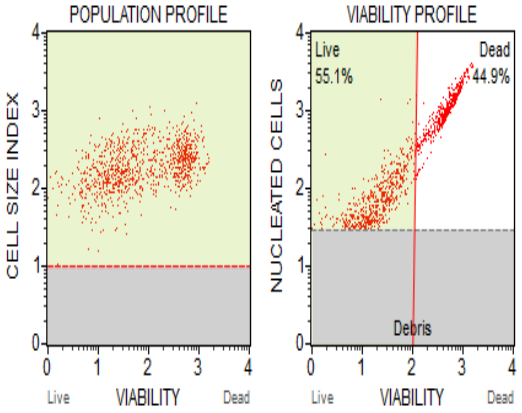



**Fig. D.**

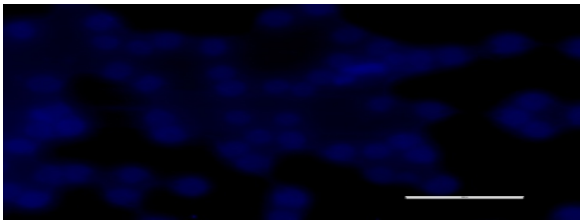


**Fig. E.**

**Figure 18. A**. Pollen viability analysis of Unreleased condition (UR) **B, C** and **D.** Pollen viability analysis of Released condition (R) through flow cytometry E. DAPI stained viable pollen under confocal microscope

### 3.2.1.3. Structural adaptations of released and unreleased pollen

Putranjiva unreleased (UR) pollen was circular to an elliptical shape and radially symmetrical. The apertures were tricolpate and the outer membrane exine was granulate-perforate (Fig. 19 A, B). Early-stage pollens also showed aperture. Surface granulate-perforate structures made the exine layer rough which was more visible in mature pollens. Finally, released Pollen (R, disperse pollen) adapted into an elliptical shape (Fig. 19 C, D) due to the shrinking of ridges from the minor groove points, producing the deep major grooves which would be helpful for a flight of pollen during wind pollination.

### 3.2.1.4. Total phenolic & flavonoid content and antioxidant activity

The total phenolic content of four different extracts was determined, which was higher in unreleased pollen and lower in released polled (R35). Our result showed that phenol quantity decreased with the increasing temperature (Fig. 20 A). The flavonoid content of pollen samples with catechin equivalent was between 2.15±0.09 to 1.81±0.03 mg CEQ/g fresh weight. Here, unreleased pollens exhibit the highest and the quantity decreased in release condition R15, R25 which was nearly equal to R35 (Fig. 20 B). The phosphomolybdenum assay showed effective antioxidant activity in various concentrations of R35 and R15 followed by R25 (Fig. 20 C). The UR had the less activity.

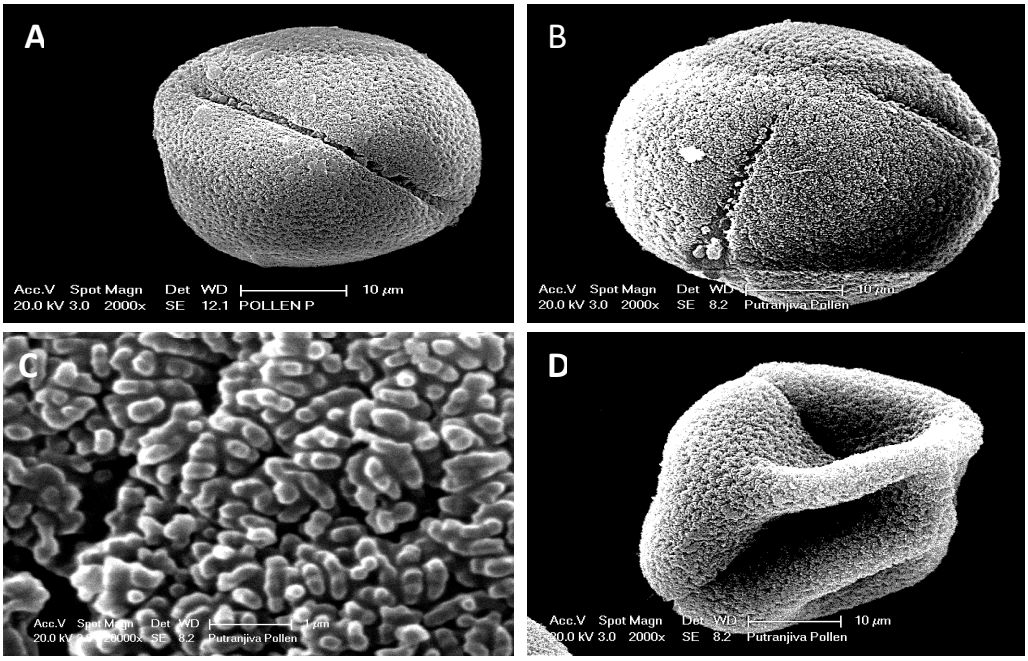



**Figure 19. A, B.** Scanning electron micrograph of Unreleased pollen **C** and **D.** Released pollen

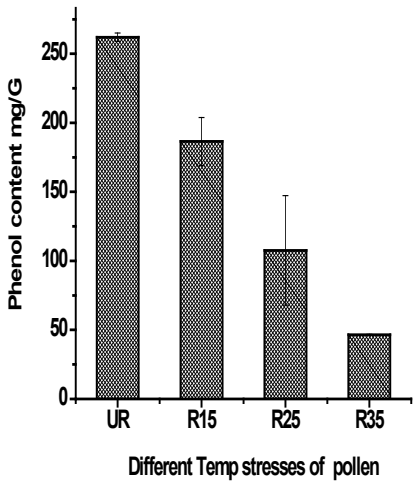



**Fig. A.**

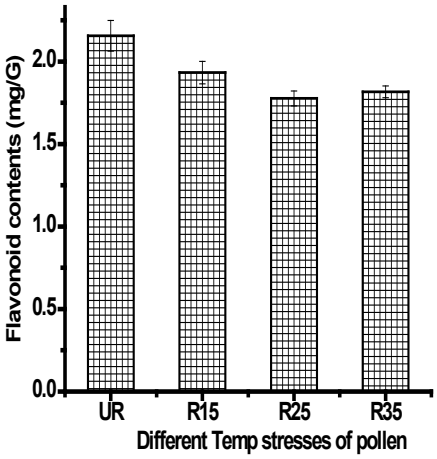



**Fig. B.**

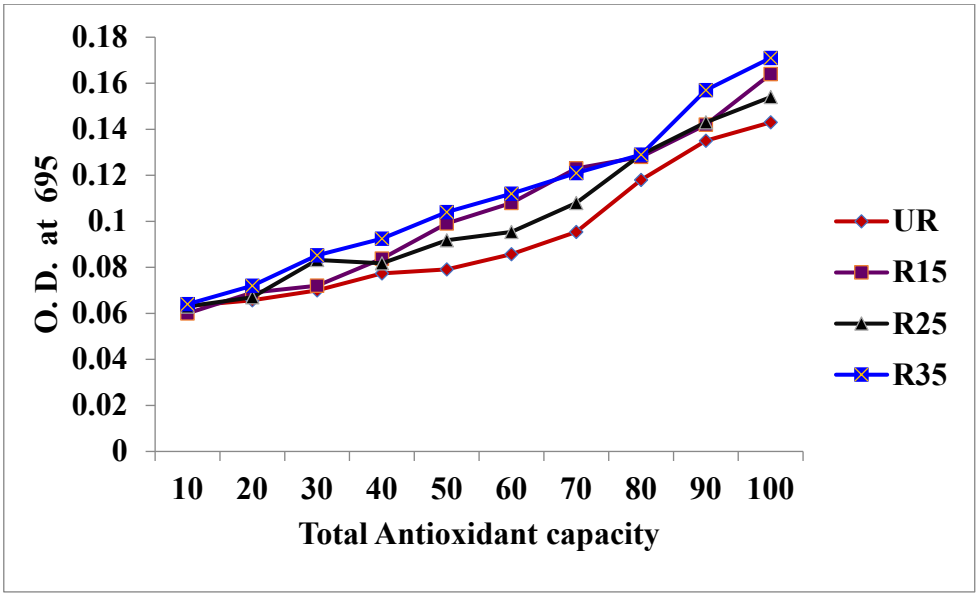



**Fig. C.**

**Figure 20. A, B** and **C.** Total phenol content, total flavonoid content and total antioxidant test Biochemical and antioxidant tests **A.** Total phenol contents at two different stages unreleased (UR) and release (R) **B.** Total flavonoid content at UR and R **C**. Total antioxidant capacity of pollen at UR and R. Data represent the means $\pm$ SD; (n = 3)

### 3.2.1.5. Biochemical tests of pollens

All biomolecules like reducing sugar, lipid, protein, and total sugar were analyzed. Lipid accumulation was more than total protein, carbohydrate, and reducing sugar. Lipid content was 53 mg/g (UR), 65 mg/g (R15), 103 mg/g (R25) and 189 mg/g (R35) at these conditions (Fig. 21 B). Protein content was 13.66 mg/g (UR) then 18 mg/g (R15), 21.90 mg/g (R25) and 32.80 mg/g (R35) at these conditions (Fig. 21 C). Total carbohydrate content was 10.77 mg/g (UR), 16.33 mg/g (R15), 20.11 mg/g (R25) and 32.55 mg/g (R35) (Fig. 21 D). Reducing sugar content was very less in Pollen 0.023 mg/g (UR), 0.017 mg/g (R15), 0.0156 mg/g (R25) and 0.075 mg/g (R35) at these conditions (Fig. 21 A).

### 3.2.1.6. Sucrose accumulation is higher in temperature-facing pollens

The carbohydrates (glucose, fructose and sucrose) were found in all conditions of Putranjiva pollens. Among the four stages, glucose concentration was higher than other sugars. Glucose accumulation in unreleased pollens (UR) was 49.829 mg/g then gradually decreased having 9.705 mg/g (R15), 7.403 mg/g (R25) and 6.78 mg/g (R35) accumulation (Fig. 22 A). Sucrose started accumulating at stress conditions in released pollens. In unreleased pollen, its content was only 5.586 mg/g (UR) then after releasing at different temperatures, it accumulated nearly similar quantity around 10.133 mg/g (R15), 11.936 mg/g (R25) and 10.932 mg/g (R35) at different releasing temperatures (Fig. 22 C). Fructose content was significantly less as compared to other sugars in unreleased pollen. It contained 3.534 mg/g (UR) and then in cold stress (15°C) was 0.534 mg/g (R15), at moderate temperature (25°C) 0.782 mg/g (R25) and heat stress (35°C) contained 0.23 mg/g (R35) (Fig. 22 B).

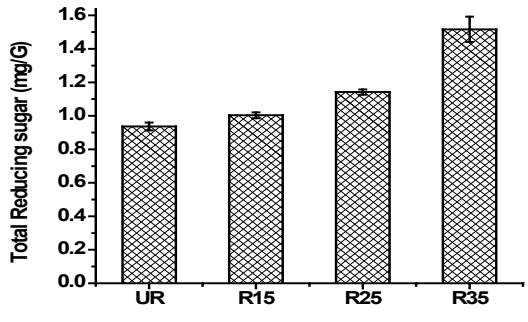



**Fig. A.**

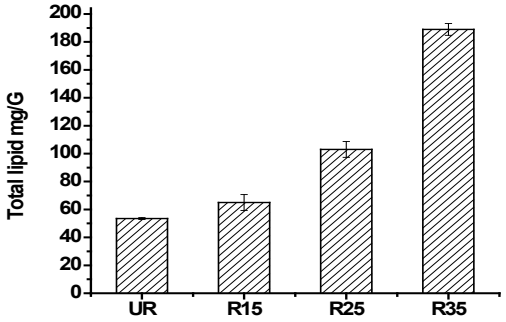



**Fig. B.**

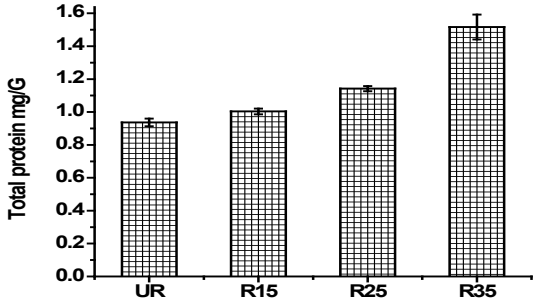



**Fig. C.**

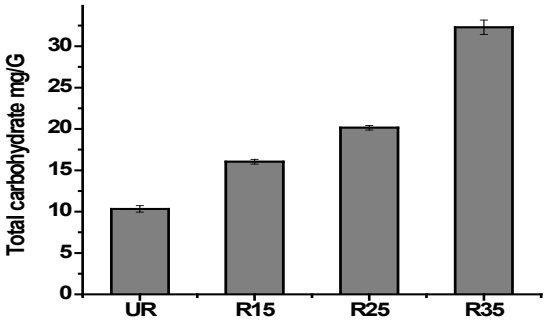



**Fig. D.**

**Figure 21. A-D**. Biochemical quantification at different stages of pollen. Data represent the means ± SD; (n = 3)

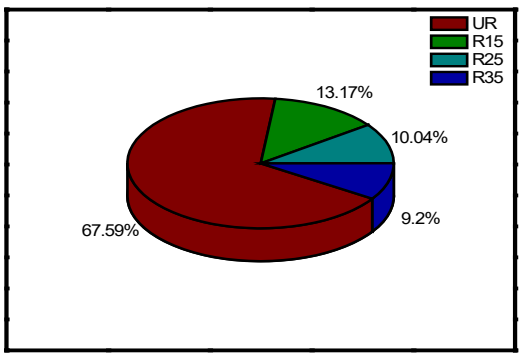



**Fig. A.**

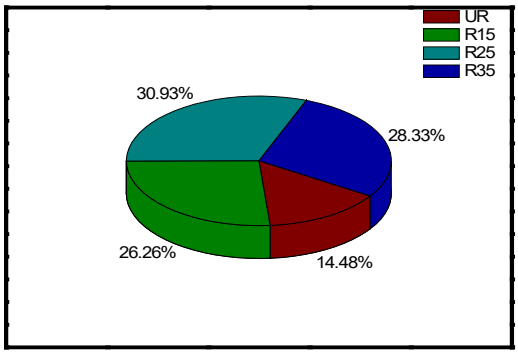



**Fig. B.**

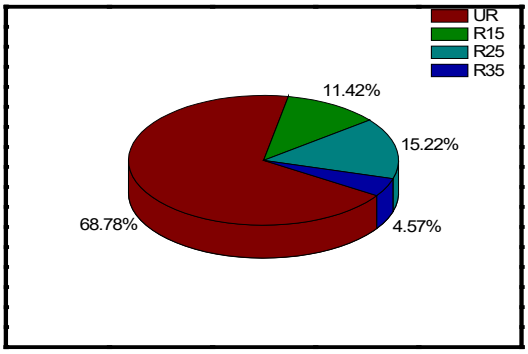



**Fig. C.**

**Figure 22. A-C.** Quantification of sugars through HPLC Quantification of sugars and polyamines in unreleased and released pollen through HPLC **A.** glucose quantification **B.** fructose quantification **C.** sucrose quantification. Polyamine quantification at both stages of pollen (UR, R)

### 3.2.1.7. Higher accumulation of proline in unreleased mature pollens

It was observed that the proline level gradually decreased at the released pollen conditions. Proline content was highest in unreleased conditions (UR) which was 31.7 nmol/ml than in released conditions at cold stress and it was 5.65 nmol/ml (R15), at an average temperature 19.88 nmol/ml (R25) and at heat stress was 2.14 nmol/ml (R35) (Fig. 23).

### 3.2.1.8. Different hormones accosting during unreleased to released pollens

Eight different phytohormones of pollens such as Abscisic acid (ABA), Indole-3-Acetic acid (IAA), 1-Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), Salicylic acid (SA), Kinetin, Gibberellic acid (GA3) and Methyl jasmonate were tested for understanding the role and their function in pollen during pollination at different temperature stresses. All eight hormones have significant changes after temperature treatment in the released condition. The hormonal profile of collected pollen in unreleased condition had a higher accumulation of gibberellic acid (GA3), salicylic acid and kinetin. Their concentration was significantly increased in released pollen at different temperatures compared with the other phytohormones. IAA concentration was found to be 1.56 nmol $g^{-1}$ in unreleased (UR), 1.02 nmol $g^{-1}$ in normal temperature (R25), then 0.476 nmol $g^{-1}$ at cold stress (R15) and 0.722 nmol $g^{-1}$ under heat stress (R35) (Fig. 24 A). Auxin or Indole acetic acid (IAA) decreased in all released conditions compared to unreleased (UR). In the released condition the quantity of IAA at R25 condition was higher than the R15 condition and R35 conditions of pollens. IBA concentration was found to be 0.01 nmol $g^{-1}$ (UR), 0.005 nmol $g^{-1}$ (R15), 0.02 nmol $g^{-1}$ (R25) and 0.015 nmol $g^{-1}$ (R35) (Fig. 24 B). We found more gibberellic acid compare to other hormones. Gibberellic acid in unreleased condition (UR) was 352 nmol $g^{-1}$, in released condition at cold stress (R15) was 479.86 nmol $g^{-1}$, in normal temperature (R25) was 714.14 nmol $g^{-1}$ and in heat stress (R35) was only 358.2 nmol $g^{-1}$ (Fig.24 E). In unreleased condition (UR), the concentration of ABA was 0.07 nmol $g^{-1}$ but when it met the cold stress (R15), it was downregulated having 0.028 nmol $g^{-1}$ concentrations (Fig. 24 G). It was upregulated in the case of pollens released at 25°C with

0.098 nmol g-1, but again it was downregulated at 35°C with 0.047 nmol $g^{-1}$. These results suggest that there was some relation to pollen under temperature stress. Methyl jasmonate was found to be 0.132 nmol $g^{-1}$ in matured unreleased (UR) Pollen (Fig. 24 H). It was not showing any difference between cold (0.009 nmol $g^{-1}$) and heat stress (0.008 nmol $g^{-1}$) but downregulated at normal temperature (0.006 nmol $g^{-1}$). Similarly, NAA also behaved like Methyl jasmonate (Fig. 24 C).

### 3.2.1.9. Polyamine maintains the pollen viability

In unreleased and released conditions, we examined four major polyamines i.e. putresciene, spermine, spermidine and cadaverine. In unreleased pollen, putresciene was observed in significantly higher quantity among four polyamines in pollen, at unreleased it was 223.7 nmol $g^{-1}$ (UR) and for released 178 nmol $g^{-1}$ (R15), 294 nmol $g^{-1}$ (R25), and 148 nmol $g^{-1}$ (R35) (Fig. 25 D). Spermine was the second highest polyamine in pollens which was 119 nmol $g^{-1}$ (UR) followed by 56 nmol $g^{-1}$ (R15), 79 nmol $g^{-1}$ (R25) and 44.6 nmol $g^{-1}$ (R35) (Fig. 25 C). Cadaverine decreased at every condition in released pollens compared to unreleased. It decreased during stress conditions cold R15 and heat stress R35. Cadaverine was 28.3 nmol $g^{-1}$ (UR), 10.61 nmol $g^{-1}$ (R15), 16.47 nmol $g^{-1}$ (R25) and 4.88 nmol $g^{-1}$ (R35) (Fig. 25 A). Spermidine was 2.1 nmol $g^{-1}$ (UR), 5.7 nmol $g^{-1}$ (R15) 5.2 nmol $g^{-1}$ (R25) and 7.62 nmol $g^{-1}$ (R35) (Fig. 25 B). Spermidine showed a gradual increase within released and under temperature stresses. Putrescine was down-regulated under both cold and heat stress.

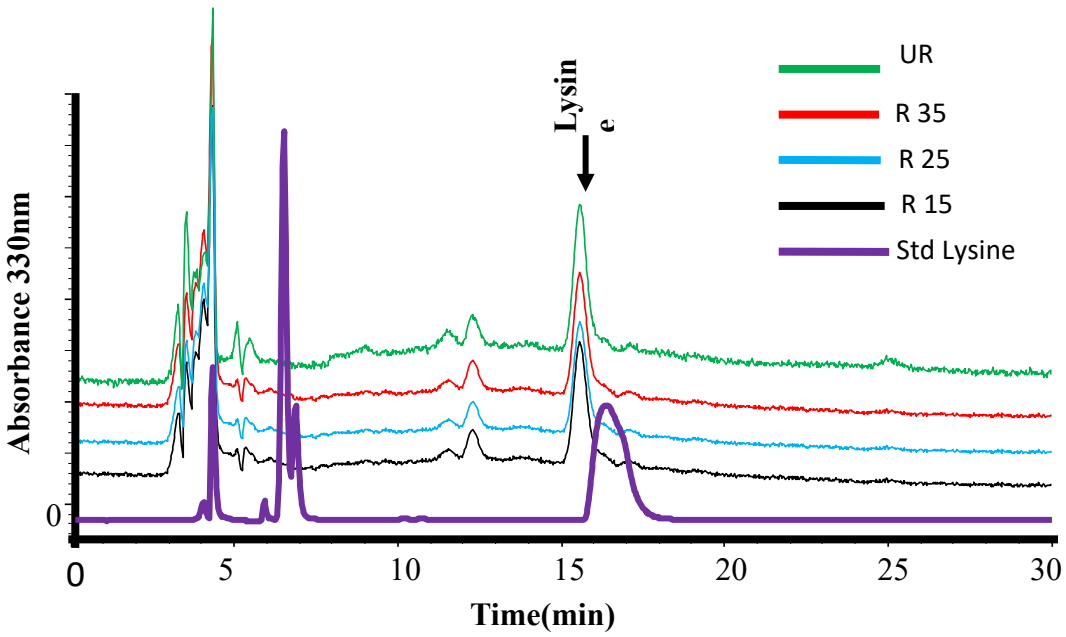



**Fig. A.**

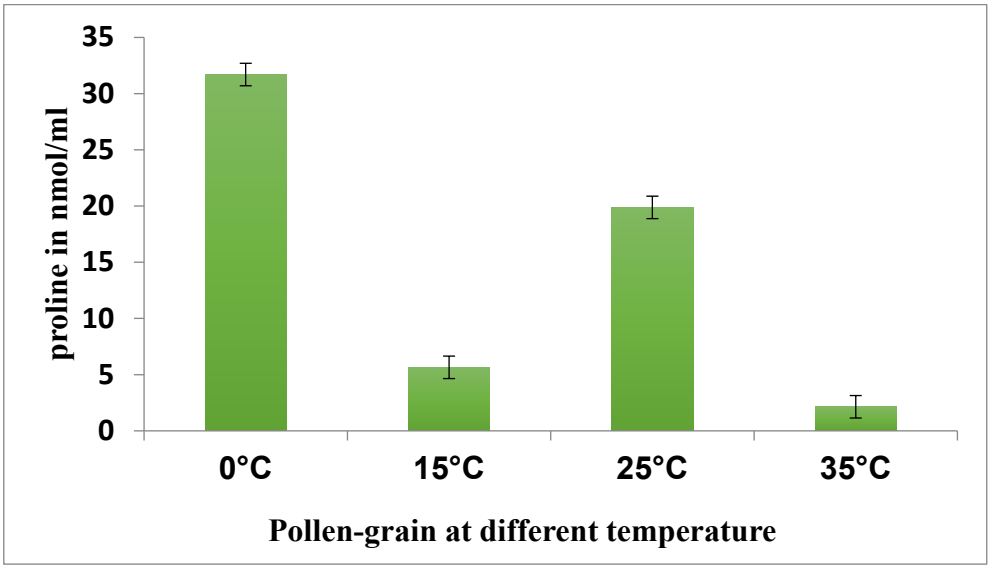



**Fig. B.**

**Figure 23.** Amino acid estimation through HPLC Amino acid estimation through HPLC **A.** Measurement of amino acids content in pollen at both conditions (nmol/ml) **B.** Proline content at both conditions (nmol/ml)

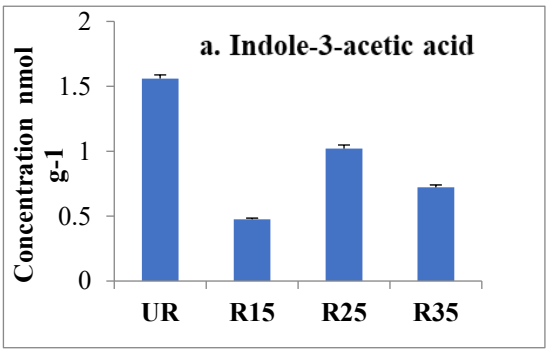



Fig. A.

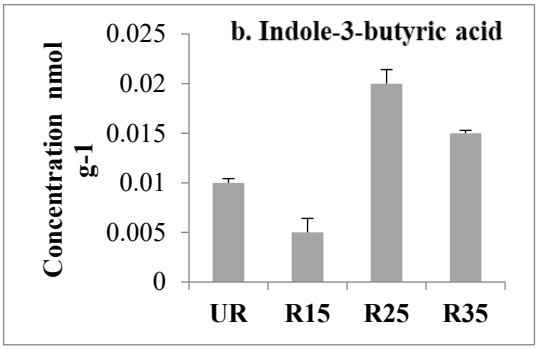



Fig. B.

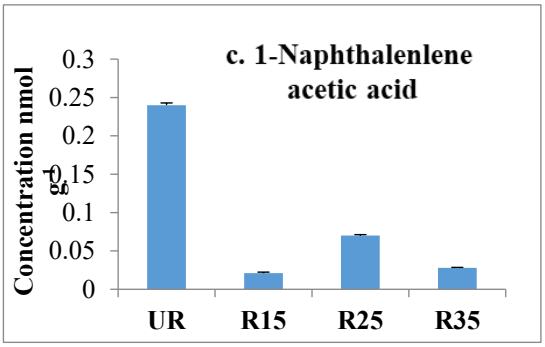



Fig. C.

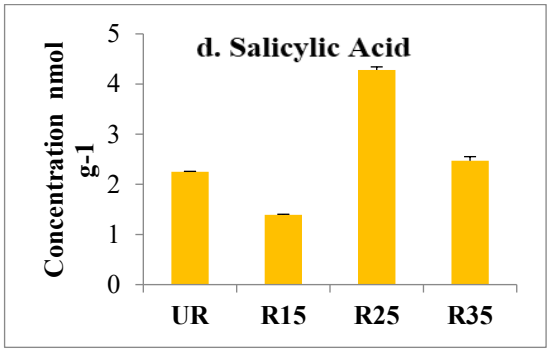



Fig. D.

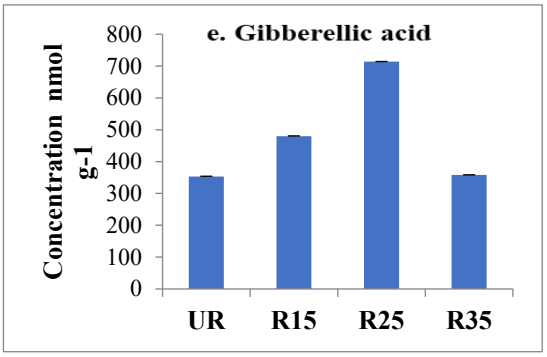



**Fig. E.**

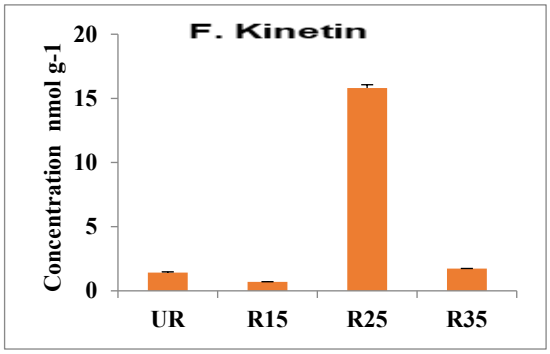



**Fig. F.**

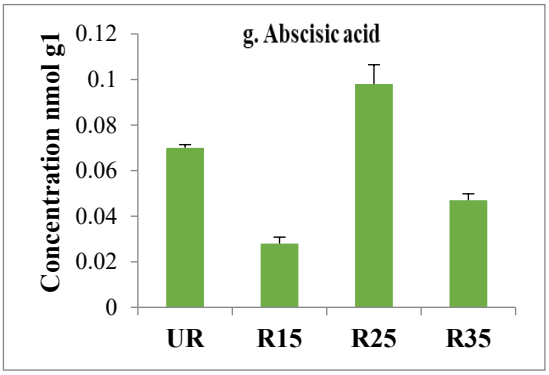



**Fig. G.**

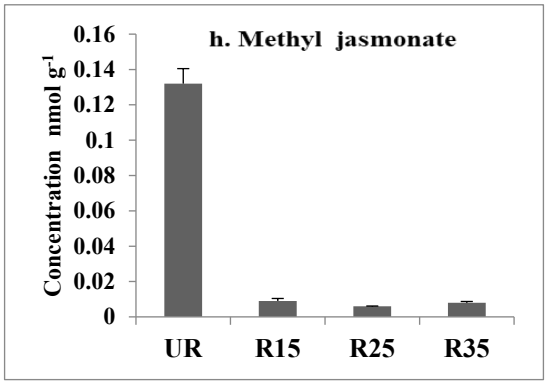



**Fig. H.**

**Figure 24. A-H.** Hormonal quantification at different stages of unreleased to released pollen Hormonal quantification at different stages of unreleased to released pollen. Data represents the means± SD; (n=3) **A.** Indole-3-acetic acid **B.** Indole-3-butyric acid **C.** 1-Napthalene acetic acid **D.** Salicylic acid **E.** Gibberellic acid **F.** Kinetin **G.** Abscisic acid **H.** Methyl jasmonate. Data represent the means ± SD; (n = 3)

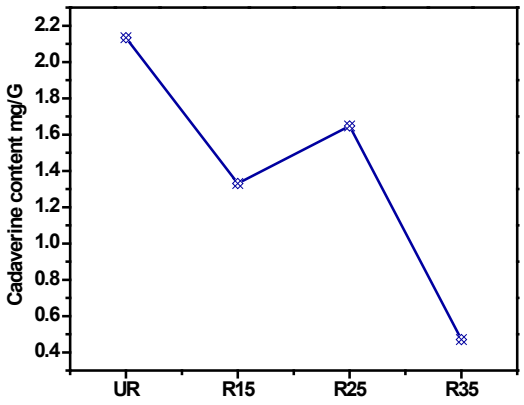



Fig. A.

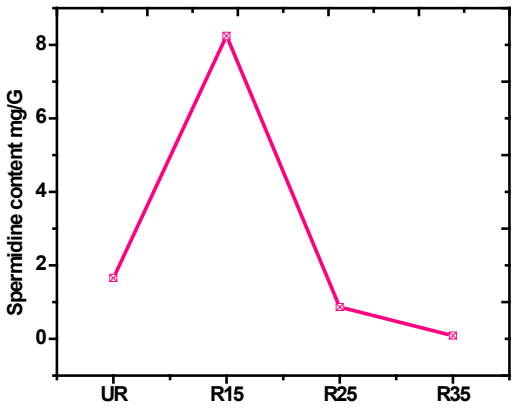



Fig. B.

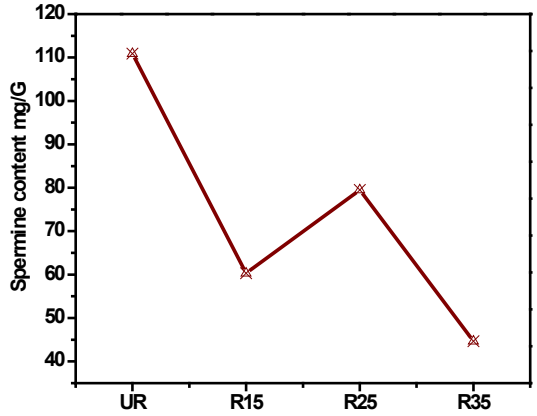



Fig. C.

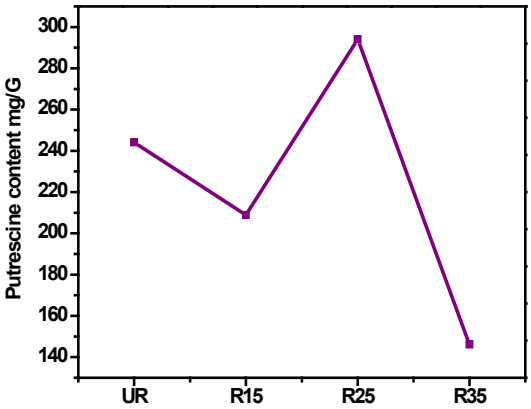



Fig. D.

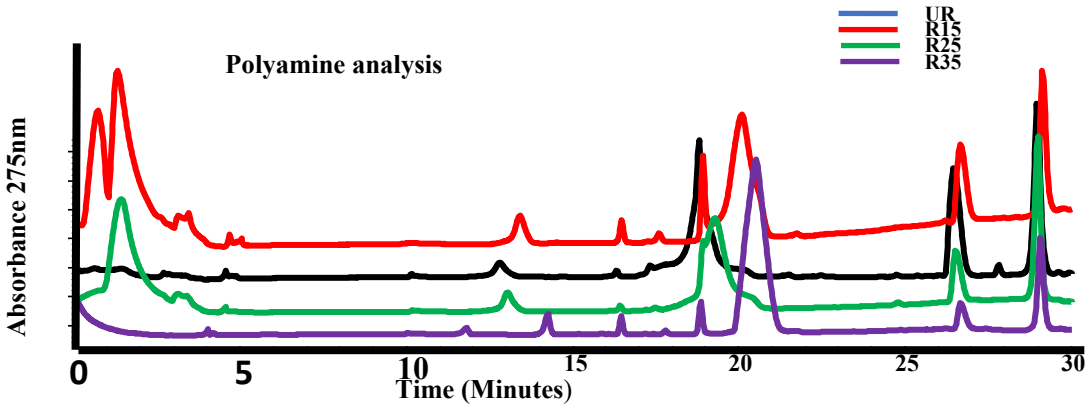



Fig. E.

**Figure 25.** Polyamine quantification at both stages of pollen (UR, R) **A.** Cadaverine quantification **B.** Spermidine quantification **C.** Spermine quantification **D.** Putrescine quantification.

**Table 3.** Fatty acid composition of Unreleased (UR) and Released(R) pollen

| Fatty Acids | Concentration (%) | |
|---|---|---|
| **12:0** | **0.44** | **0.82** |
| **14:0** | **0.49** | **0.82** |
| **15:0** | **0.32** | **0.8** |
| **15:1 w5c** | **1.64** | **1.99** |
| **16:0** | **34.17** | **32.39** |
| **18:2 w6c,9c/18:0** | **47.06** | **45.88** |
| **18:1 w7c** | **3.25** | **2.97** |
| **18:0** | **6.02** | **5.7** |
| **17:0 2OH** | **0.61** | **0.73** |
| **19:0 cyclo w10c/19w6** | **0.74** | **0.7** |
| **18:0 3OH** | **0.6** | **0.65** |
| **20:1 w9c** | **0.48** | **0.64** |

### 3.2.2. Metagenome analysis for both Cycas and Putranjiva pollen

#### 3.2.2.1. 16Sr RNA gene-based metagenome analysis

Genomic DNA was isolated from both samples following the c-TAB method, and proceeded with 16S rRNA gene-based metagenome sequencing of the V3-V4 region. Both the samples could yield good sampling depth as evident from the rarefaction curves. Since the metagenome of both samples was performed separately, we present the results separately.

#### 3.2.2.2. Cycas pollen metagenome

The total number of paired-end reads (68018) for the Cycas samples, which contained 55.86% GC, was acquired. A total of 5395 OTUs were identified from the aforementioned readings after these paired end sequences were processed and grouped in OTUs with the help of QIIME software. 2014 OTUs were chosen for additional analysis after 3291 OTUs with fewer than two readings were eliminated from the original 5395 OTUs. Proteobacteria constituting majority of the OTUs constitute abundant phylum in cypol sample (Fig.26). Other reads mapping to Firmicutes, Actinobacteria, Chloroflexi (~2%), along with unclassified bacteria were present in the Cycas sample.

Varied results were obtained at the class level where clostridia were the most abundant class of bacteria followed by the abundance of OTUs reads of deltaproteobactera, alphaproteobactera, gammaproteobacterial, actinobacteria, acidobacteria, bacteroidia, bacilli, Anaerolineae and unclassified bacteria (Fig. 26 A).

The most abundant phylum in the cycas pollen were Proteobacteria (Fig. 26 C). The most abundant order in the cycas pollen were Clostridiales (Fig. 26 B). The most abundant family in the cycas pollen were Solibacteraceae (Fig. 26 D). Whereas the bacteria where unknown at species level (Fig. 26 F)

The different results were also found at the genus level where most of the reads either belong to unknown bacteria or to unclassified bacteria (Fig.26 E). Of the known bacteria, most of the

OTUs belonged to the uncultured bacteria, comprised of total OTUs. Genera like Geobacter, Lachnospiraceae, Candidatus-solibacter, Bacillus, Anaeromyxobacter, Anaerolinea, and Mycobacterium were also present in the cypol sample. The alpha diversity of the Cycas sample was analyzed by calculating the Shannon, Chao 1 index. Here, Chao 1 measures the species richness while Shannon matric is the measure to estimate observed OUT abundance and accounts for both richness and evenness. The rare fraction curves below represented species richness and evenness.

### 3.2.2.3. Putranjiva pollen metagenome

The total number of paired-end reads (153184) for the pollen samples was acquired. A total of 2087 OTUs were identified after these paired-end sequences were processed and grouped in OTUs with the help of QIIME software.Refraction curve was calculated of species richness of individual sample based on construction (Fig. 27)

In the pollen sample, proteobacteria constituting approximately 34.71% of the OTUs was the most abundant phylum observed in this sample. Other reads mapping to Firmicutes (30.31%), Actinobacteria (~12.35%), Acidobacteria (~5.34%) and several other phyla were present in pollen sample (Fig. 28).

Varied results were obtained at class level as shown in the pie chart where Bacilli (26.04%) was the most abundant class of bacteria followed by the abundance of OTUs reads of alphaproteobacterial (16.57%), gammaproteobacterial (10.19%), actinobacteria (7.43%), acidobacteria (4.08%) and other classes of bacteria (Fig. 29).

The major abundance at order level was Lactobacillales 20.88 %, Rhizobiales 8.98 %, Actinomycetales 6.96 %, Bacillales 5.04 %, Clostridiales 3.98 %, Oceanospirillales 3.49 %, Sphingomonadales 2.94 %, Xanthomonadales 2.25 %, Campylobacterales 2.15 % (Fig. 30).

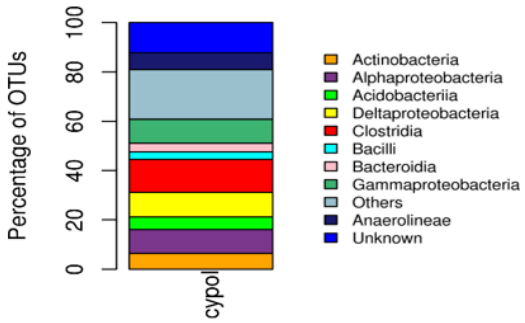



**Fig. A.**

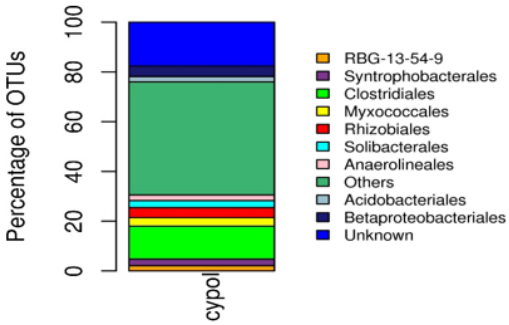



**Fig. B.**

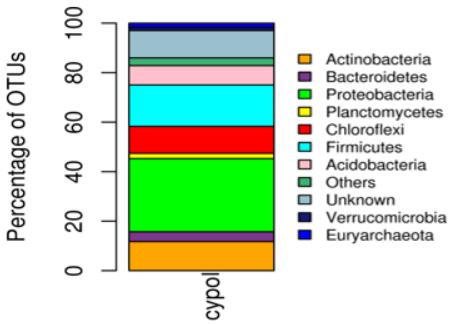



**Fig. C.**

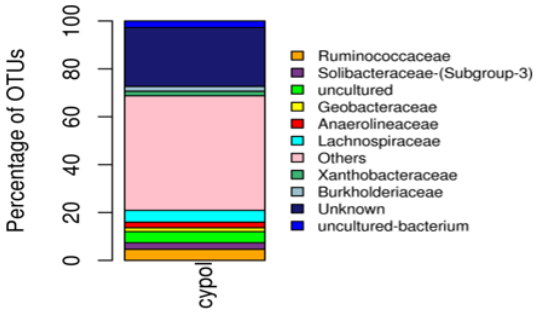



**Fig. D.**

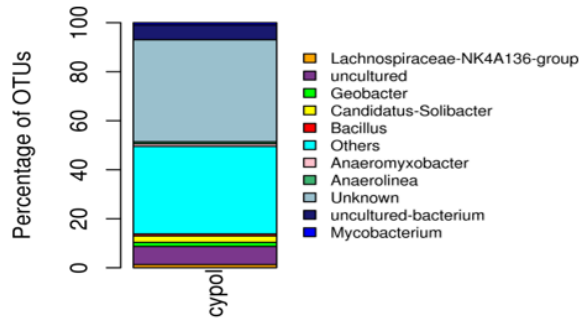



**Fig. E.**

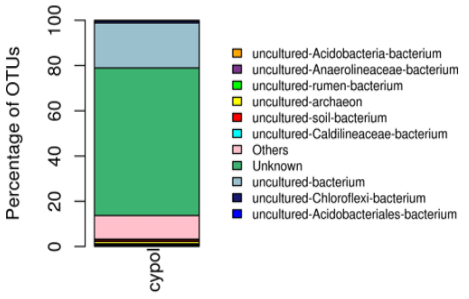



**Fig. F.**

**Figure 26.** Cycas pollen metagenome study. Taxonomic distribution of Cypol sample at Phylum level (A), Class level (B) Order level (C) Family level (D) Species level

The major abundance at family level was Leuconostocaceae 19.74 %, Hyphomicrobiaceae 3.87 %, Oceanospirillaceae 3.48 %, Bacilliaceae 3.12 %, Sphingomonadaceae 2.71 %, Methylobacteriaceae 2.46 %, Campylobacteraceae 2.15 % (Fig. 31)

Different results were also seen at the genus level, where most read either belong to unknown or unclassified bacteria (Fig. 32). Most known bacteria, most of the OTUs, belong to the uncultured bacteria, which comprise 53.33% of total OTUs. Weissella is the most abundant genera (19.67%) among all the known genera in the pollen sample. Genera like Bacillus (2.8%), Rhodoplanes (2.27%), Arcobacter (2.12%), Methylobacterium (1.45%) and several other genera were also present in the pollen sample. The major abundance at species level of unclassified species 19.66 % from Weissella genus (Fig. 33). The alpha diversity of the Pollen sample was analyzed by calculating the Shannon, Chao 1 index. Here, Chao 1 measures the species richness while Shannon matric is the measure to estimate observed OTU abundance and accounts for both richness and evenness. Both the species richness and evenness were represented in the rare fraction curves below.

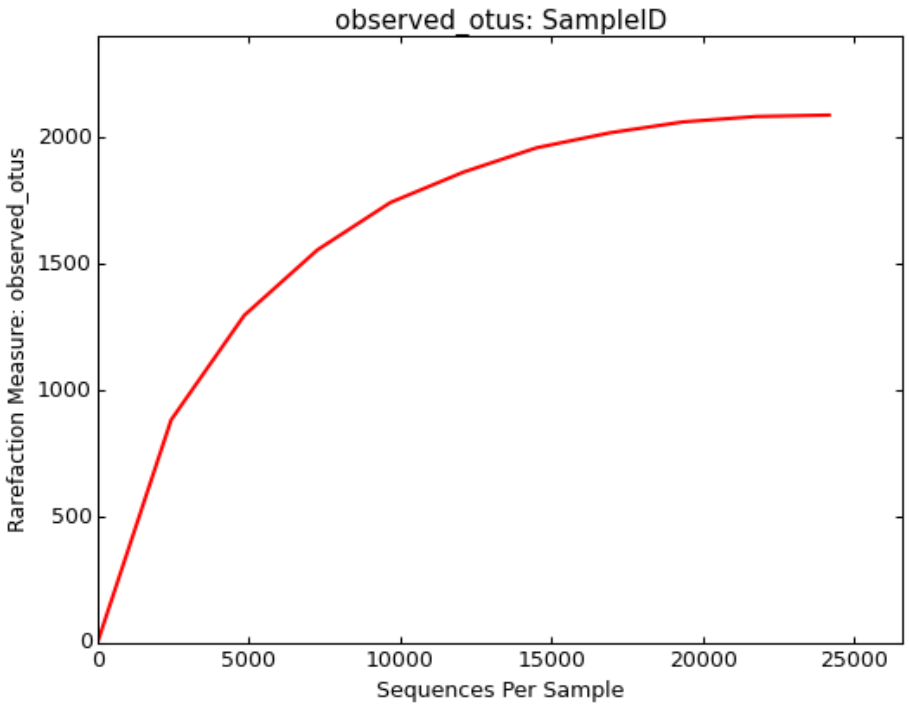



**Figure 27.** Refraction curve of Putranjiva poll

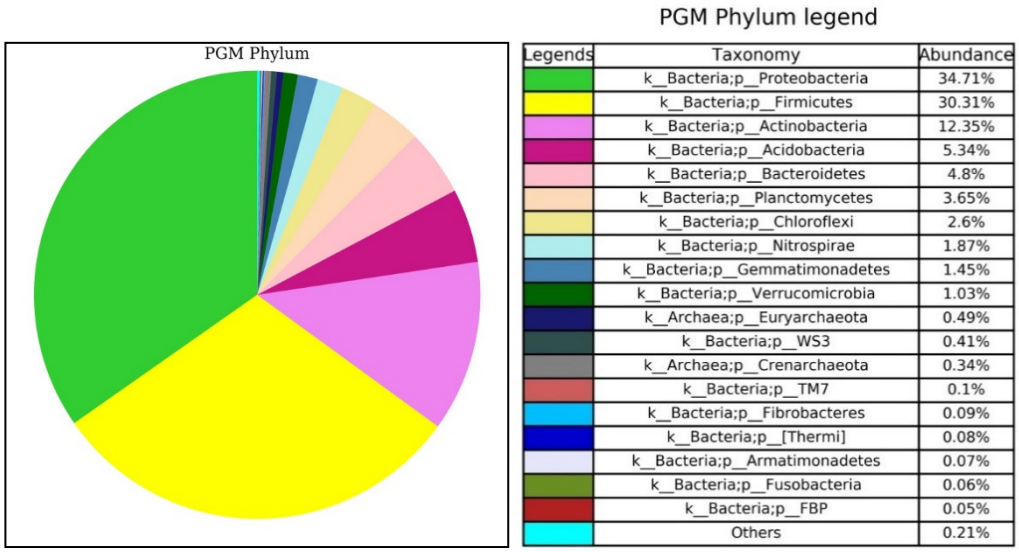
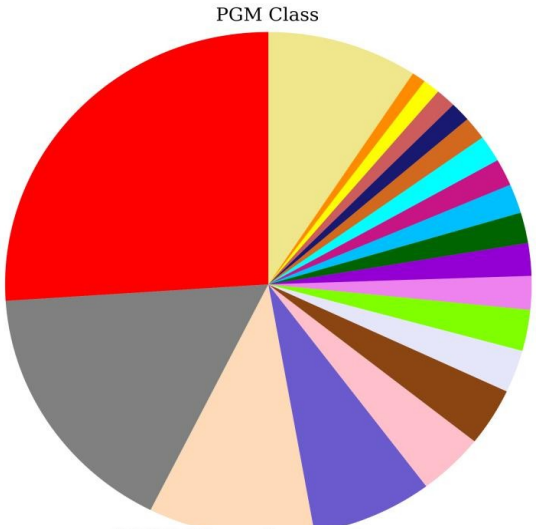


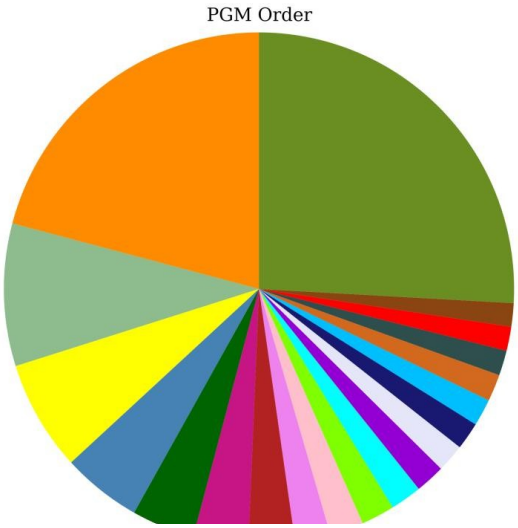

**Figure 28.** Pie chart showing the absolute abundance of each phylum within each bacteria community from the figure, it can be inferred that the most abundant class is Bacilli




PGM Class legend

| Legends | Taxonomy | Abundance |
|---|---|---|
| | k__Bacteria;p__Firmicutes;c__Bacilli | 26.04% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria | 16.57% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria | 10.19% |
| | k__Bacteria;p__Actinobacteria;c__Actinobacteria | 7.43% |
| | k__Bacteria;p__Firmicutes;c__Clostridia | 4.08% |
| | k__Bacteria;p__Proteobacteria;c__Betaproteobacteria | 3.71% |
| | k__Bacteria;p__Planctomycetes;c__Planctomycetia | 2.73% |
| | k__Bacteria;p__Actinobacteria;c__Thermoleophilia | 2.63% |
| | k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria | 2.15% |
| | k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria | 2.09% |
| | k__Bacteria;p__Acidobacteria;c__Acidobacteria-6 | 1.96% |
| | k__Bacteria;p__Nitrospirae;c__Nitrospira | 1.87% |
| | k__Bacteria;p__Bacteroidetes;c__Bacteroidia | 1.73% |
| | k__Bacteria;p__Actinobacteria;c__Acidimicrobiia | 1.7% |
| | k__Bacteria;p__Acidobacteria;c__Solibacteres | 1.47% |
| | k__Bacteria;p__Gemmatimonadetes;c__Gemm-1 | 1.25% |
| | k__Bacteria;p__Bacteroidetes;c__Flavobacteriia | 1.24% |
| | k__Bacteria;p__Bacteroidetes;c__[Saprospirae] | 1.04% |
| | k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria] | 0.86% |
| | Others | 9.25% |

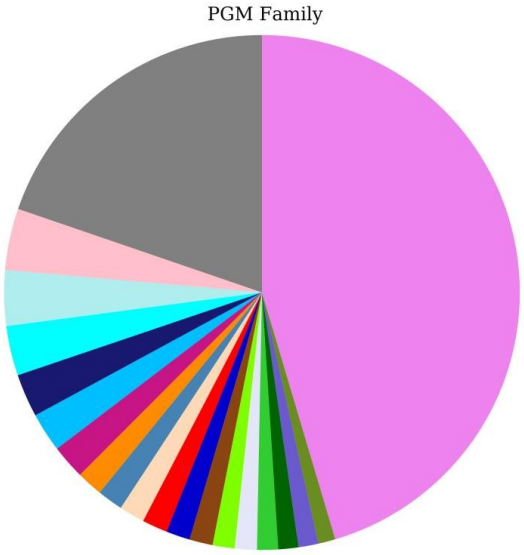
**Figure 29.** Taxonomic distribution of PGM sample at Class level




PGM Order legend

| Legends | Taxonomy | Abundance |
|---|---|---|
| | k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales | 20.88% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales | 8.98% |
| | k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales | 6.96% |
| | k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales | 5.04% |
| | k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales | 3.98% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales | 3.49% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales | 2.94% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales | 2.25% |
| | k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales | 2.15% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales | 2.11% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales | 1.99% |
| | k__Bacteria;p__Nitrospirae;c__Nitrospira;o__Nitrospirales | 1.87% |
| | k__Bacteria;p__Acidobacteria;c__Acidobacteria-6;o__iii1-15 | 1.77% |
| | k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales | 1.73% |
| | k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales | 1.7% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales | 1.7% |
| | k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales | 1.58% |
| | k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Gaiellales | 1.51% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales | 1.48% |
| | Others | 25.88% |

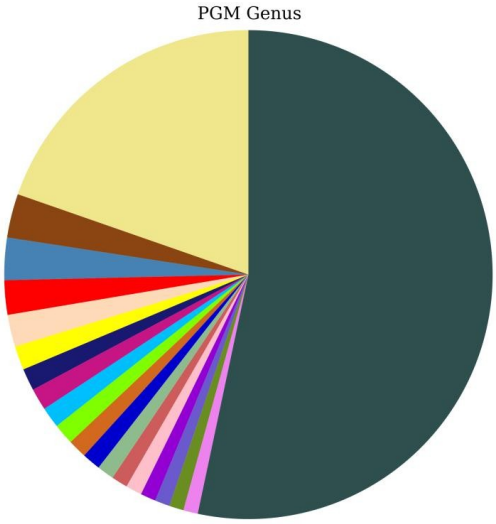
**Figure 30.** Taxonomic distribution of PGM sample at Order level




PGM Family legend

| Legends | Taxonomy | Abundance |
|---|---|---|
| | k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae | 19.74% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae | 3.87% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae | 3.48% |
| | k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae | 3.12% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae | 2.71% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae | 2.46% |
| | k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae | 2.15% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae | 1.64% |
| | k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae | 1.61% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae | 1.6% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae | 1.59% |
| | k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardioidaceae | 1.5% |
| | k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Gaiellales;f__Gaiellaceae | 1.46% |
| | k__Bacteria;p__Acidobacteria;c__Acidobacteria-6;o__iii1-15;f__Unclassified | 1.38% |
| | k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae | 1.38% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae | 1.32% |
| | k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae | 1.26% |
| | k__Bacteria;p__Gemmatimonadetes;c__Gemm-1;o__Unclassified;f__Unclassified | 1.25% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Colwelliaceae | 1.07% |
| | Others | 45.41% |

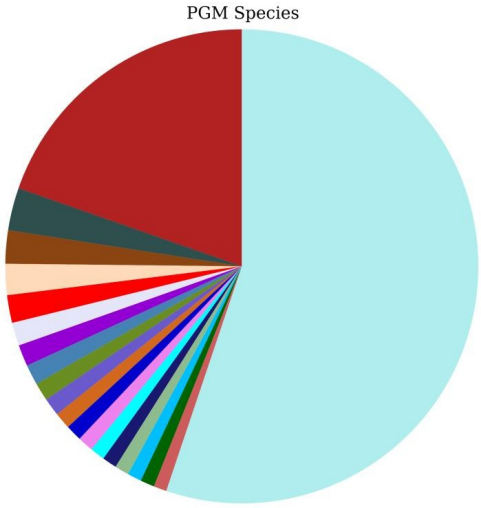
**Figure 31.** Taxonomic distribution of PGM sample at Family level




PGM Genus legend

| Legends | Taxonomy | Abundance |
|---|---|---|
| | k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;g__Weissella | 19.67% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Unclassified | 2.9% |
| | k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus | 2.8% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Rhodoplanes | 2.27% |
| | k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Arcobacter | 2.12% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Unclassified | 1.58% |
| | k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Gaiellales;f__Gaiellaceae;g__Unclassified | 1.46% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium | 1.45% |
| | k__Bacteria;p__Acidobacteria;c__Acidobacteria-6;o__iii1-15;f__Unclassified;g__Unclassified | 1.38% |
| | k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella | 1.38% |
| | k__Bacteria;p__Gemmatimonadetes;c__Gemm-1;o__Unclassified;f__Unclassified;g__Unclassified | 1.25% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Unclassified | 1.22% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae;g__Unclassified | 1.13% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas | 1.07% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Colwelliaceae;g__Thalassomonas | 1.07% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Unclassified | 1.01% |
| | k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Solirubrobacterales;f__Unclassified;g__Unclassified | 0.99% |
| | k__Bacteria;p__Nitrospirae;c__Nitrospira;o__Nitrospirales;f__0319-6A21;g__Unclassified | 0.96% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Kaistobacter | 0.96% |
| | Others | 53.33% |

**Figure 32.** Taxonomic distribution of PGM sample at Genus level




PGM Species legend

| Legends | Taxonomy | Abundance |
|---|---|---|
| | k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;g__Weissella;s__Unclassified | 19.66% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Unclassified;s__Unclassified | 2.9% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Rhodoplanes;s__Unclassified | 2.27% |
| | k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Arcobacter;s__Unclassified | 2.12% |
| | k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__Unclassified | 1.89% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Unclassified;s__Unclassified | 1.58% |
| | k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Gaiellales;f__Gaiellaceae;g__Unclassified;s__Unclassified | 1.46% |
| | k__Bacteria;p__Acidobacteria;c__Acidobacteria-6;o__iii1-15;f__Unclassified;g__Unclassified;s__Unclassified | 1.38% |
| | k__Bacteria;p__Gemmatimonadetes;c__Gemm-1;o__Unclassified;f__Unclassified;g__Unclassified;s__Unclassified | 1.25% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Unclassified;s__Unclassified | 1.22% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae;g__Unclassified;s__Unclassified | 1.13% |
| | k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__copri | 1.11% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Colwelliaceae;g__Thalassomonas;s__Unclassified | 1.07% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Unclassified;s__Unclassified | 1.01% |
| | k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Solirubrobacterales;f__Unclassified;g__Unclassified;s__Unclassified | 0.99% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__Unclassified | 0.98% |
| | k__Bacteria;p__Nitrospirae;c__Nitrospira;o__Nitrospirales;f__0319-6A21;g__Unclassified;s__Unclassified | 0.96% |
| | k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Kaistobacter;s__Unclassified | 0.96% |
| | k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Glaciecola;s__Unclassified | 0.89% |
| | Others | 55.18% |

**Figure 33.** Taxonomic distribution of PGM sample at Species level

### 3.2.3. Metabolic profiling of pollen from Putranjiva and Cycas plants

#### 3.2.3.1. Central metabolism associated with unreleased and released Putranjiva pollen

The Metabolite profiling generated ~276 metabolites from unreleased (UR) and ~271 from released Pollen (R25) through LC/MS analysis (Fig. 36 B). Most compounds were detected in a positive mode compared to a negative mode in both unreleased and released conditions (Fig. 34, 35). Total identified metabolites were classified into 12 groups based on their chemical structure and biological functions like amino acids, alkaloids, flavonoids, vitamins, lipid derivatives, metabolic intermediates, hormones, fatty acids, phenol derivatives, organic acids, carbohydrates and polyamines (Fig. 36 C). The expression of metabolites was characterized by the intensity of peaks at two conditions. These metabolites were mainly lipids and fatty acids. The comparative metabolomic study revealed that there were conventional switches to metabolic pathways. To analyze the changes in secondary metabolites during pollen grain released (R) and unreleased (UR) conditions through the LC– QTOF–MS analysis. The semi-polar metabolites were extracts from pollen grains in both released and unreleased conditions. Based on the detected peak of metabolites, retention time and molecular weight in LC-MS, the chemical structures of peaks were determined and identified in both released and unreleased conditions.

In comparison, total 55 putative lipids were detected in different pollen samples from released and unreleased conditions. Most putatively identified lipid metabolites belonged to the class of phospholipids and phenols. Pollen grain contains lipids such as phosphatidic acids, phosphatidylcholine, diacylglycerol, monoacylglycerol, triacylglycerol, phosphatidylserine, phosphatidylethanolamine, lysophosphatidic acid, sphingosine 1-phosphate, lysophodpholopids, dihydroceramide, ceramides (N-acylsphingosine) those were highly abundant in released conditions as compared to unreleased conditions. However, a decline of the phospholipid under unreleased conditions has shown significant differences. The

predominant phosphatidylinositol and phosphatidyl ethanolamine isomers accumulation was observed in response to all released conditions. Around 27 of the putative metabolites were identified, common for both pollen grain released and unreleased conditions. Similarly, we found differences in phenol-derived metabolites during released and unreleased conditions. Total 19 phenol-derived metabolites were putatively identified from both conditions. Among the total phenols, five metabolites such as phenolpropenoid, catechin-7-glucoside, gallic acid 3-sulfate, gutyllevulinate and caffeic acid were commonly found in both released and unreleased conditions. While some phenol derived metabolites such as benzoic acid, oleuropeic acid, 5-deoxykievitol, kaempferol, limocitrol, Tyrosol-4-sulfate 3, 5-dihydroxybenzoic acid sulfate were found only in a released condition. The concentration level of selected metabolites like Coumaric acid, cinnamic acid, caffeolcoA, glycerol-3-phosphate, phosphatidic acid, diacylglycerol, phenyl propanoids, anthocyanin, isoflavones, benzoic acid derivatives was detected at both conditions. These 10 metabolites showed a significant difference in the secondary metabolite content in both unreleased and released pollen conditions.

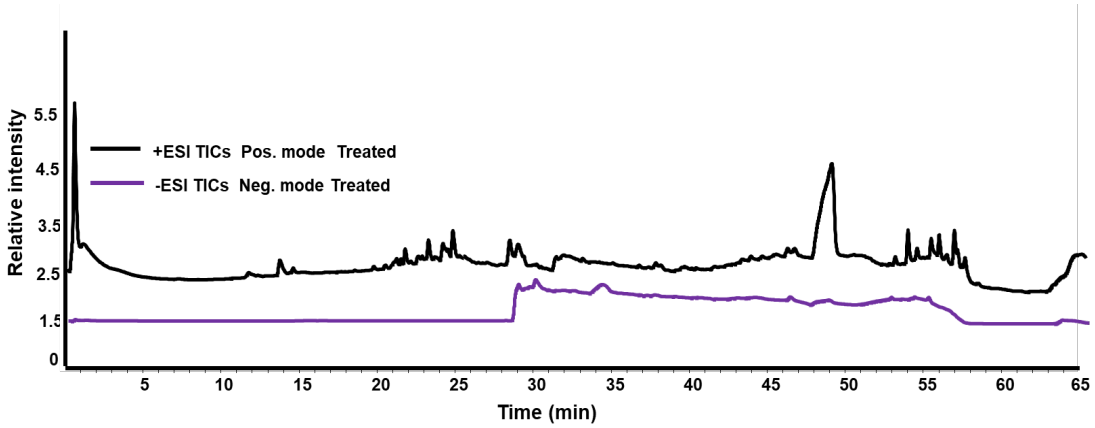



**Fig. A.**

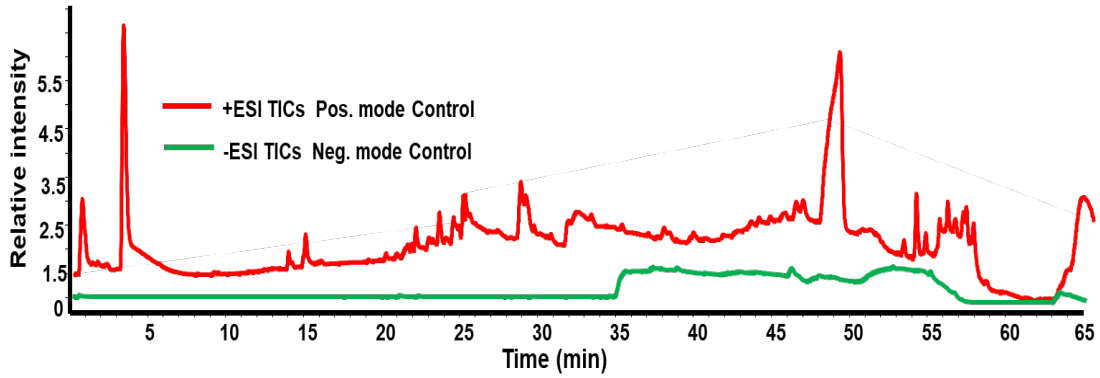



**Fig. B.**

**Figure 34. A.** Chromatogram of Unreleased pollen **B.** Chromatogram of Released pollen.

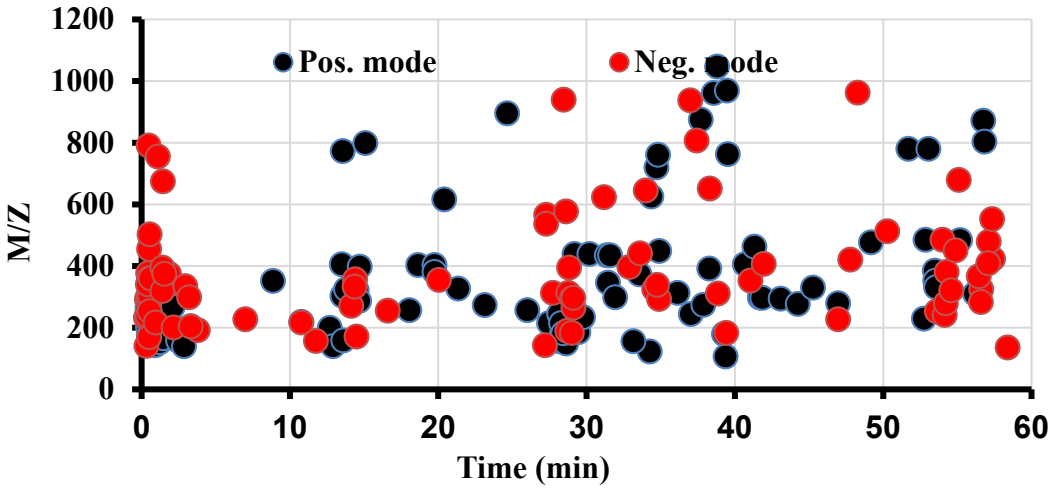



**Fig. A.**

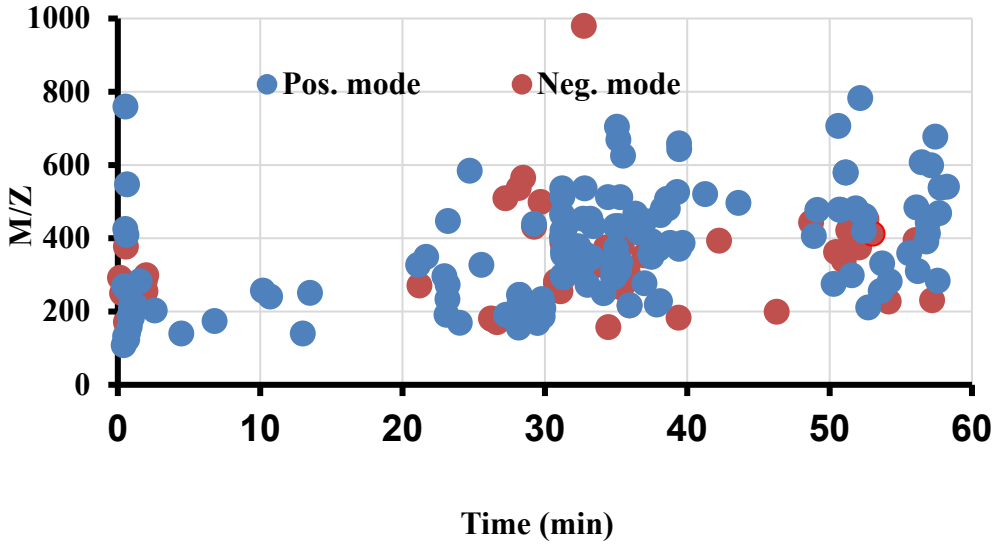



**Fig. B.**

**Figure 35.** Pos-positive ionized mode, Neg-negative ionized mode **A, B.** Both unreleased and released pollen.

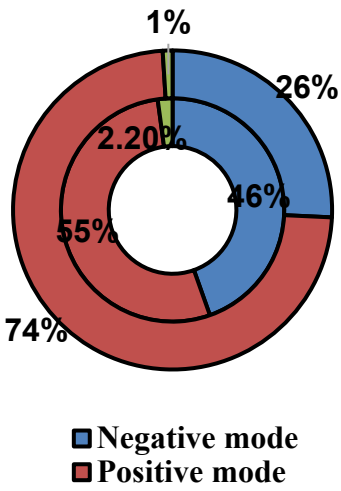



Fig. A.

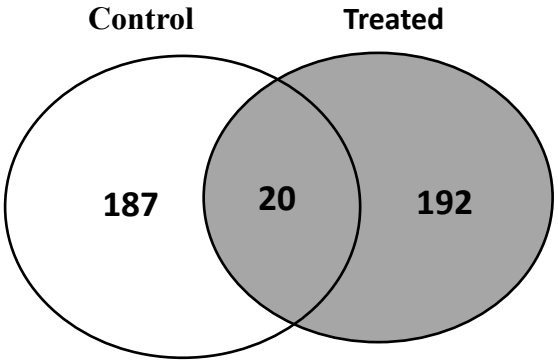



Fig. B.

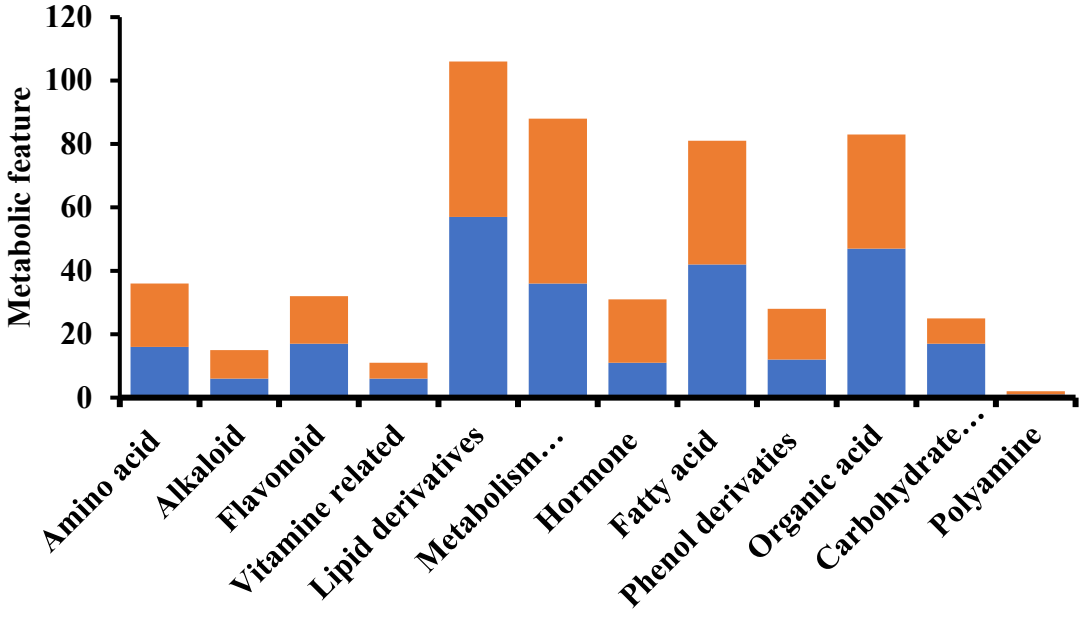
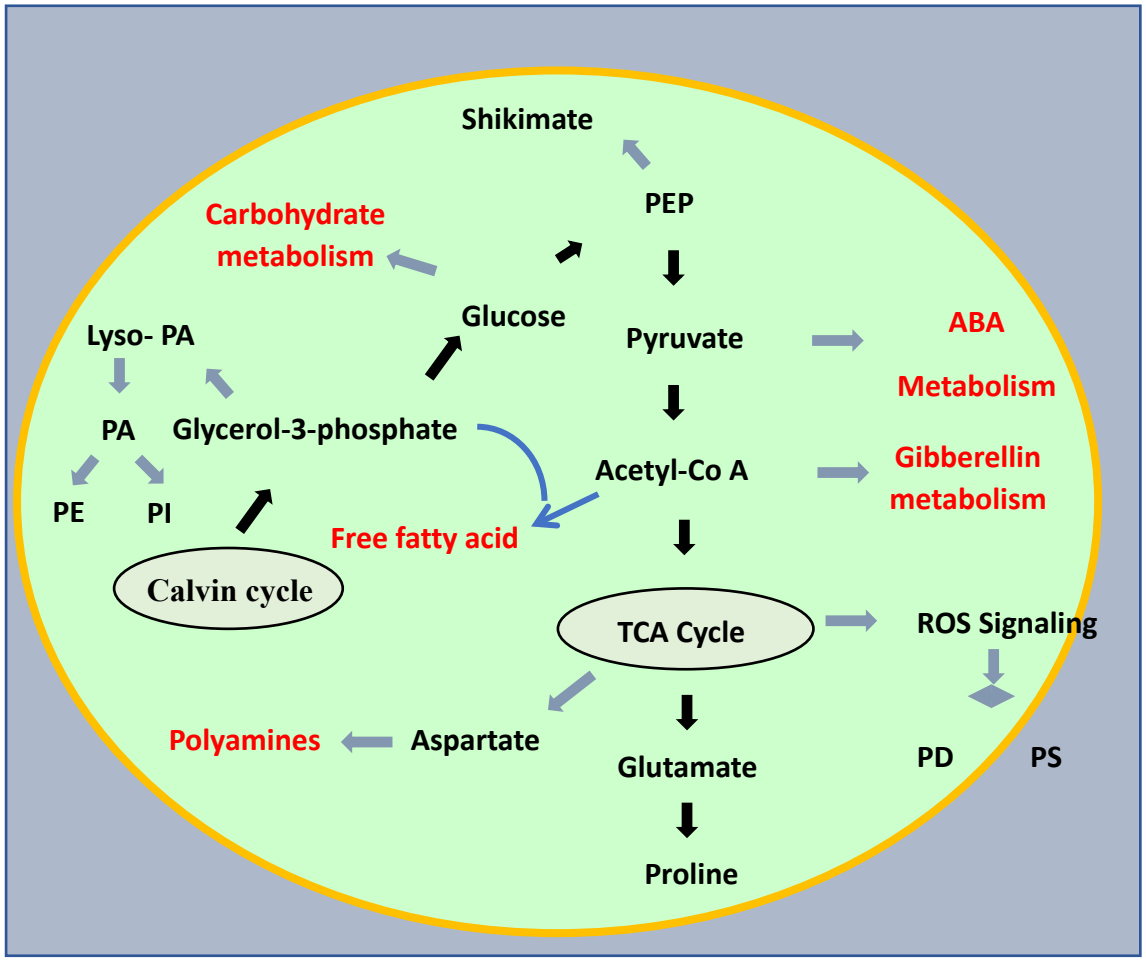



Fig. C.

**Figure 36. A.** Pos-positive ionized mode, Neg-negative ionized mode **B.** Venn diagram showing the unique and common metabolites from released and unreleased pollen. **C.** Bar graph showing the total number of classified metabolites at both unreleased and released pollen.




pollen. Red colour indicates the upregulation of pathways/metabolites. PD - Pollen death, PS - Pollen survival, Lyso- PA- Lysophosphatidic acid, PA- Phosphatidyl acid, PI- phosphatidylinositol, PE-Phophatidylethanolamine and ABA-Abscisic acid.

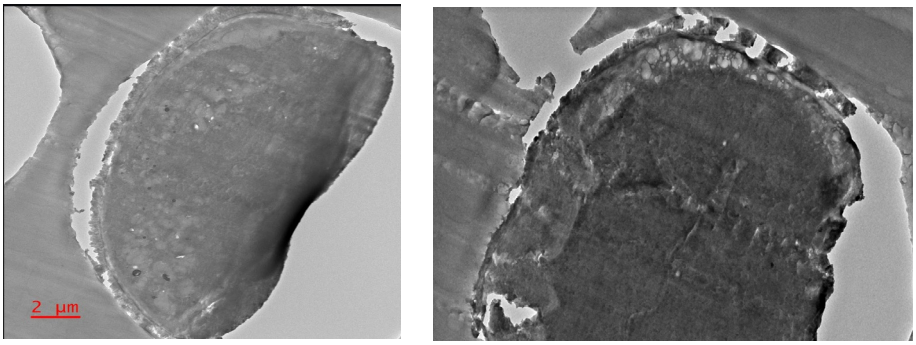



**Fig. A.** **Fig. B.**

**Figure 38.** Transmission electron microscope image of Cycas pollen

### 3.2.3.2. Structure analysis of Cycas pollen through the transmission electron microscope

The structure of pollen was analyzed at two conditions first from the cone which is bagged therefore unpollinated and second one pollen (insect pollinated cone). The pollen appears elliptical, which is having thinner exine, low lipid bodies (Fig. 38 A). The pollen is oval shaped, which has thick spongy exine having the mor lipid oil bodies (Fig. 38 B). The structural comparison showed the difference at morphological, developmental and granular level.

### 3.2.3.3. Understanding mutualism between Weevil and Cycas pollen through metabolomic study

Total metabolites were identified from weevil and pollen bodies by using LC-MS/MS. Metabolites were related to aminoacyl-tRNA biosynthesis, aromatic amino acid biosynthesis, phenylalanine metabolism, isoquinoline alkaloid biosynthesis, biosynthesis of unsaturated fatty acid, biosynthesis of secondary metabolites, tyrosine metabolism, arginine biosynthesis, arginine and proline metabolism, tropane, piperidine and pyridine alkaloid biosynthesis, TCA cycle, carbon fixation in photosynthetic organisms, pyruvate metabolism, terpenoid-quinone biosynthesis in weevil (Fig 39). Whereas in Cycas pollen,aminoacyl-tRNA biosynthesis, aromatic amino acid biosynthesis, arginine biosynthesis, Histidine metabolism, ubiquinone and other terpenoids, quinone, phenyl metabolism, citrate cycle (TCA cycle), valine, leucine and isoleucine biosynthesis, tryptophan metabolism, vitamin B6 metabolism, linoleic metabolism and pentose phosphate pathways were enriched (Fig.41). Most enriched metabolites were found in phenylalanine, tyrosine and tryptophan biosynthesis for both Cycas pollen and Weevil (Fig 40, 42).

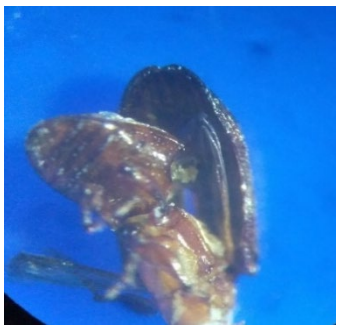


**Fig. A.**

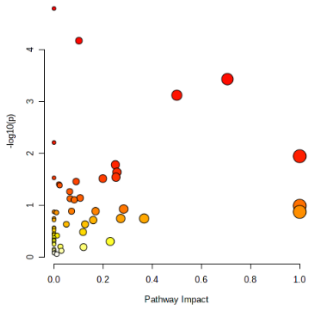



**Fig. B.**

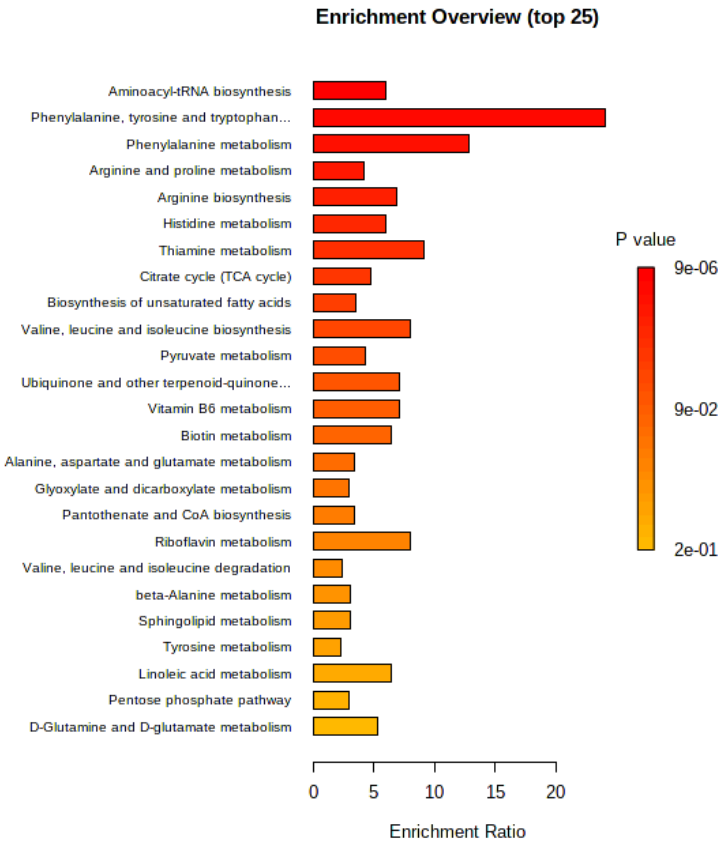



**Fig. C.**

**Figure 39. A.** Weevil collected from *Cycas nayagarhensis* male cone **B & C.** Overview of enrichment analysis of weevil metabolites

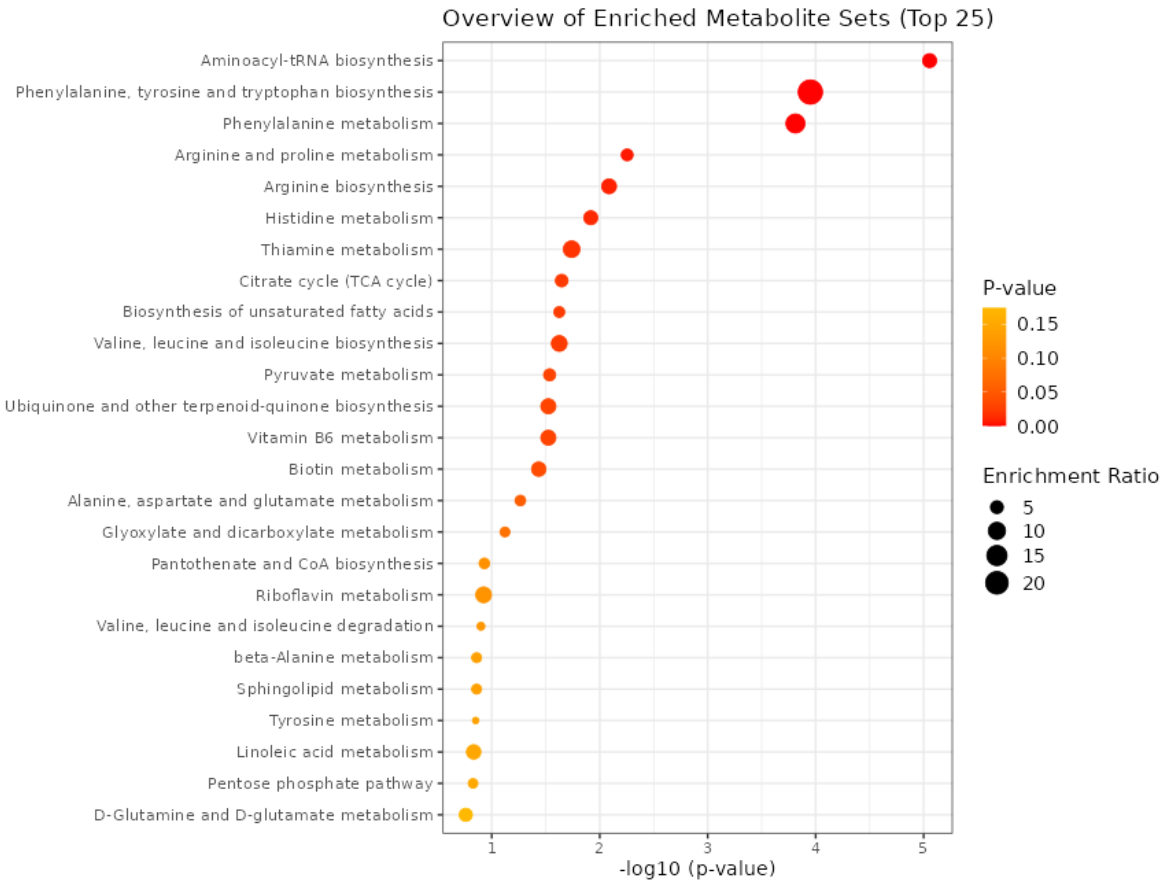



**Figure 40.** Enrichment of weevil metabolites

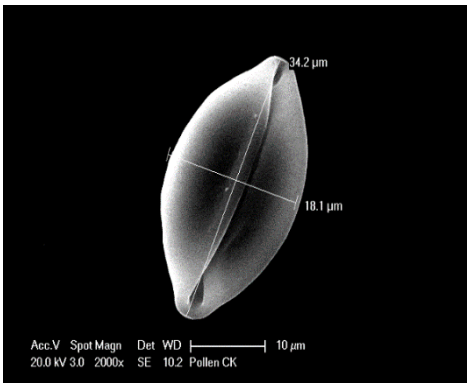



**Fig. A.**

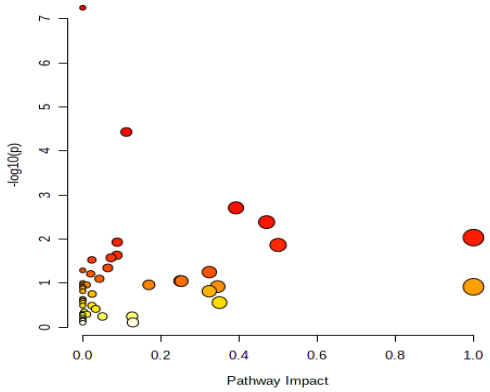



**Fig. B.**

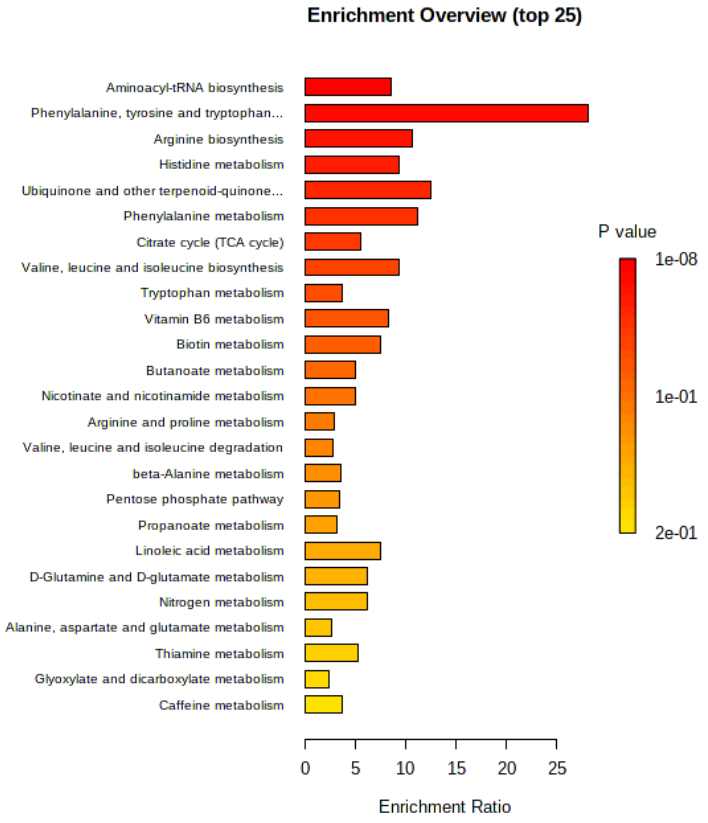



**Fig. C.**

**Figure 41. A**. Pollen collected from weevil body **B.** Overview of enrichment analysis of *Cycas nayagarhensis* pollen metabolites **C.** Enrichment analysis showing top 25 pathways

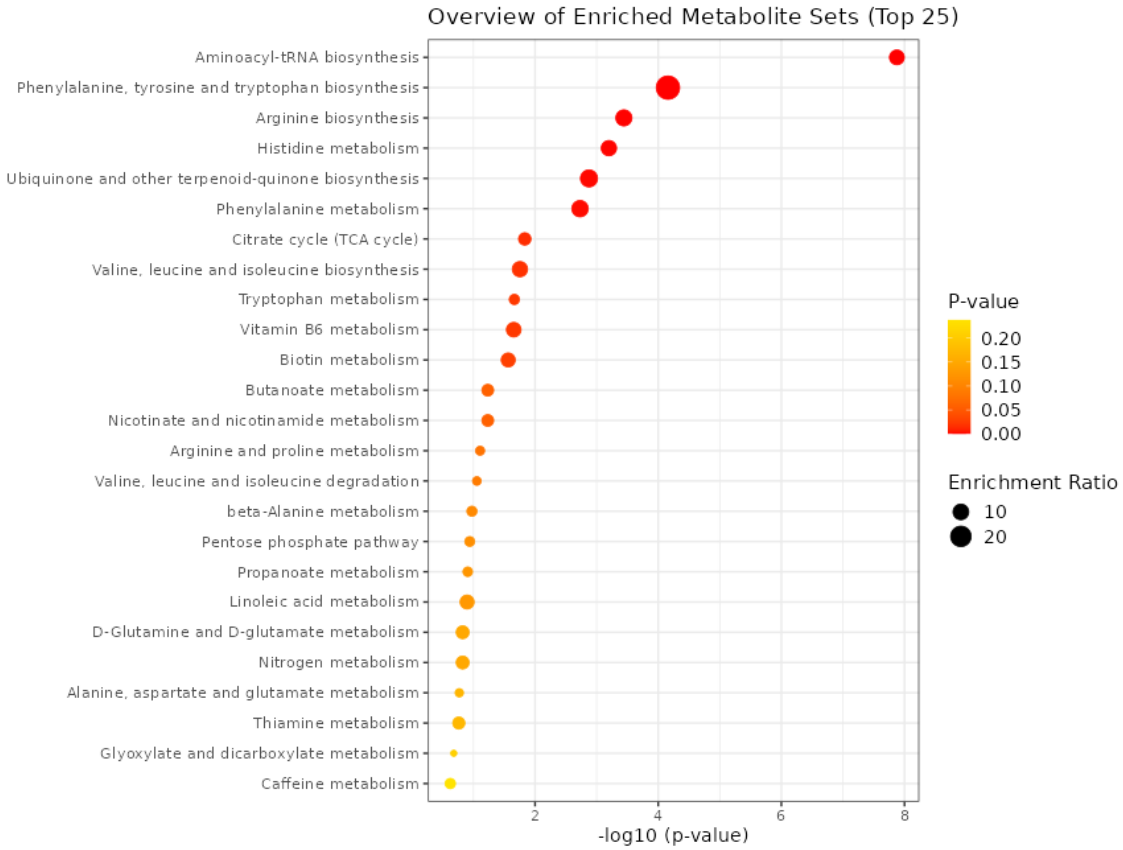



**Figure 42.** Enrichment analysis - *Cycas nayagarhensis* pollen metabolites

## 3.3. Metabolomic screening of different flowering developmental stages in Cycas and Putranjiva

### 3.3.1. Metabolome analysis of Cycas male and female cones

The untargeted metabolic profiling of both Cycas male and female cones was carried out using gas chromatography-mass spectrometry (GC-MS) (Fig. 44 C, D). Female and male cones were collected during their reproductive phase (Fig. 43 C, D). A total of 177 metabolites were identified in both reproductive cones and were found to be related to amino acid metabolism, carbohydrate metabolism, organic acid metabolism, fatty acid metabolism and miscellaneous metabolites. Cycas cone accumulated several metabolites to reproduction functions (Fig. 45 A). PCA was performed for an overall assessment of the difference between the samples. The two sample cohorts were found segregating into separate groups (Fig. 45 B) A total of 19 metabolites were found in both male and female. Both male and female matured flowers accumulate metabolites during reproduction through various pathways of sugar transport, amino acid and fatty acid pathways Correlation heatmap was plotted to check the correlation pattern between the datasets (Fig. 46) The fold change was quantified in males versus females, showing Hexanoic acid, Melibiose, D-Mannopyranoside, Oxadizole, Levoglucosan, Leucine and a significant hexadecenoic acid increase in males. The Succinic acid, Talonic acid, Tetradecanoic acid and aspartic acid showed down-regulation (Fig. 45 C).

### 3.3.2. Metabolite profiling in flowers of Putranjiva

Untargeted metabolic profiling of Putranjiva flowers was performed using GC-MS (Fig. 44 A, B). Female and male flowers were collected during their reproductive phase (Fig. 43 A, B). A total of 131 metabolites were identified in male flowers, whereas 325 metabolites were identified in female flowers. Among these, 41 metabolites we found to be common between male and female flowers. A total of 90 metabolites were found to be unique to males and 284 are unique to females (Fig. 47 A). The number of metabolites identified in male and female

flowers is related to amino acid metabolism, carbohydrate metabolism, organic acid metabolism, fatty acid metabolism other metabolites (Fig. 47 C). PCA was performed for an overall assessment of the difference between the samples. The two sample cohorts were found segregating into separate groups (Fig. 47 B). Correlation heatmap was plotted to check the correlation pattern between the datasets (Fig. 48 B). In response to the reproduction event, some particular metabolite pathways were responded such as phenylalanine-tyrosine tryptophan pathway and valine, leucine and isoleucine metabolism. Phenylalanine and L-Arginine metabolites showed a significant increase in males and in females (Fig. 48 A). This Isopalmitic acid and Indole acetic acid were present in both males and female. Additionally, nitrogen metabolism and butanoate metabolism pathways are related to these many metabolites (Fig. 48 B)

.

**Fig. A.**

**Fig. B.**

**Fig. C.**

**Fig. D.**

**Figure 43.** **A.** PFS2 (Putranjiva female flower during pollination) **B.** PMS2 (Putranjiva male flower during pollination) **C.** CFS2 (Cycas female cone during pollination) **D**. CMS2 (Cycas male cone during pollination)

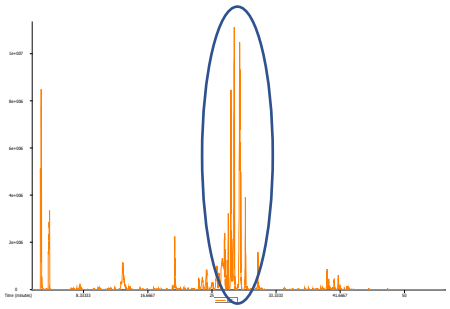


**Fig. A.** Male flower during pollination

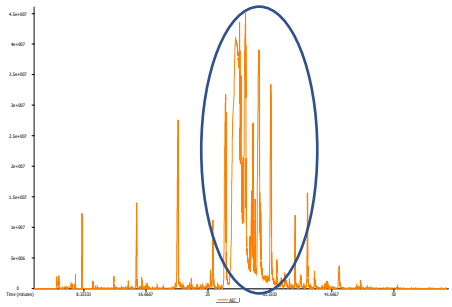


**Fig. B.** female flower during pollination

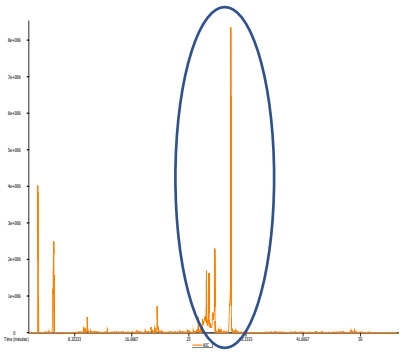


**Fig. C.** Male cone during pollination

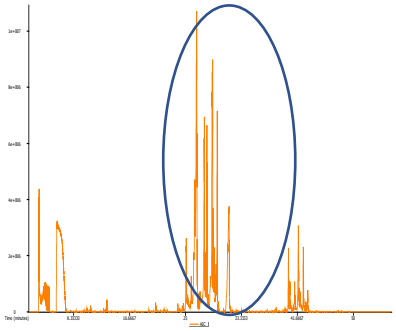
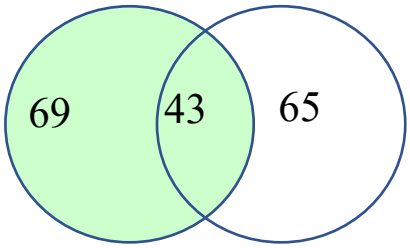


**Fig. D.** Female cone during pollination

**Figure 44. A, B.** GC-MS chromatograms of metabolites from Putranjiva male and female flowers, **C, D.** GC-MS chromatograms of metabolites from Cycas male and female cone (In each chromatogram, X-axis denotes time and Y-axis denotes abundance)

**Fig. A.**



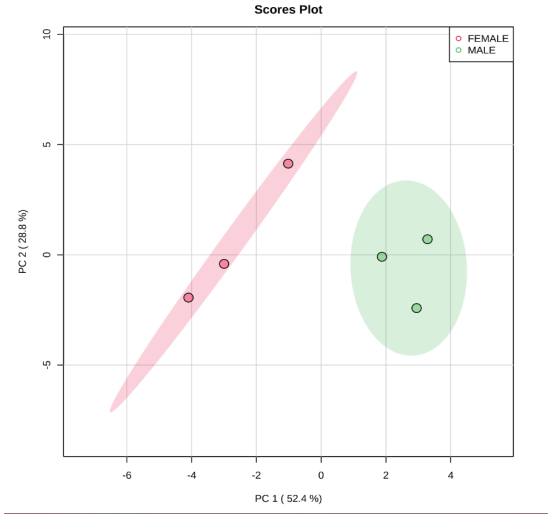




**Fig. B.**

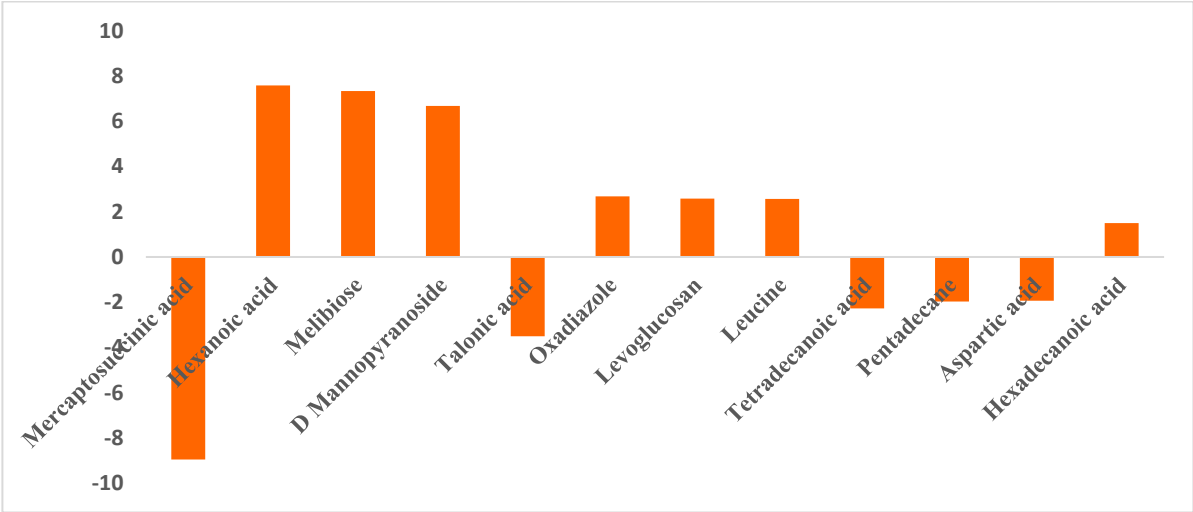



**Fig. C.**

**Figure 45. A.** Venn diagrams of metabolites observed in Cycas male and female flowers. **B.** PCA analysis of metabolites in Cycas male and female flowers. **C.** Fold changes of metabolites in Cycas male and female flowers.

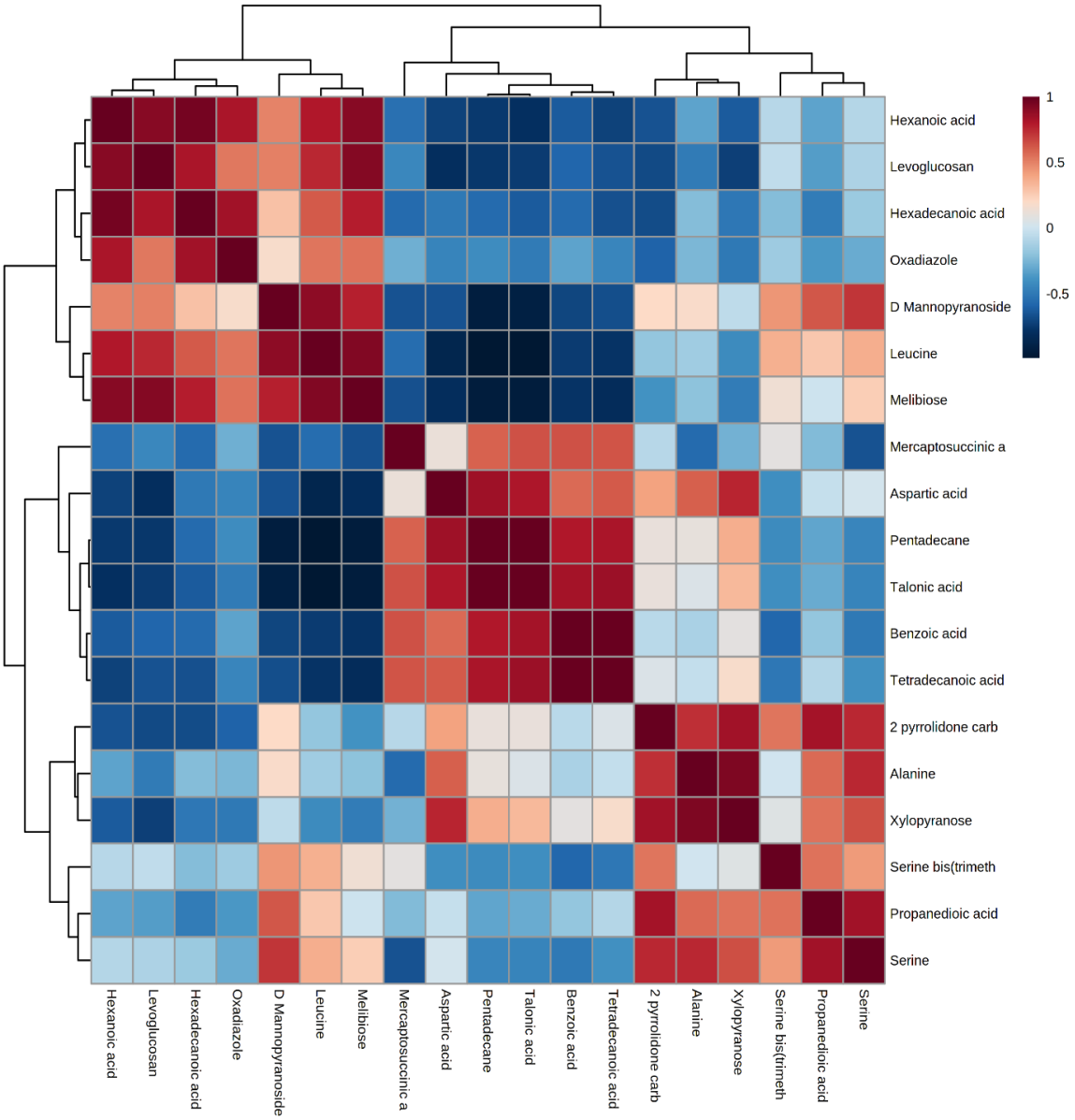



**Figure 46.** Correlation heatmap of metabolites in Cycas male and female cone.

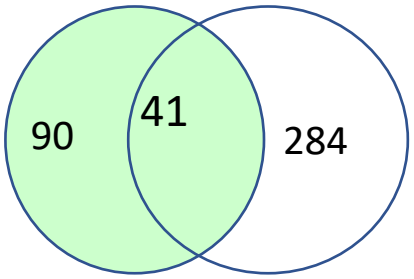



**Fig. A.**

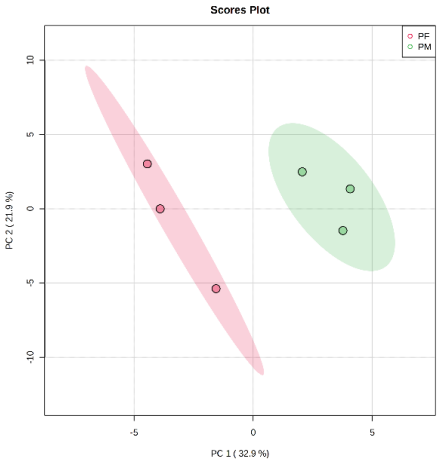



**Fig. B.**

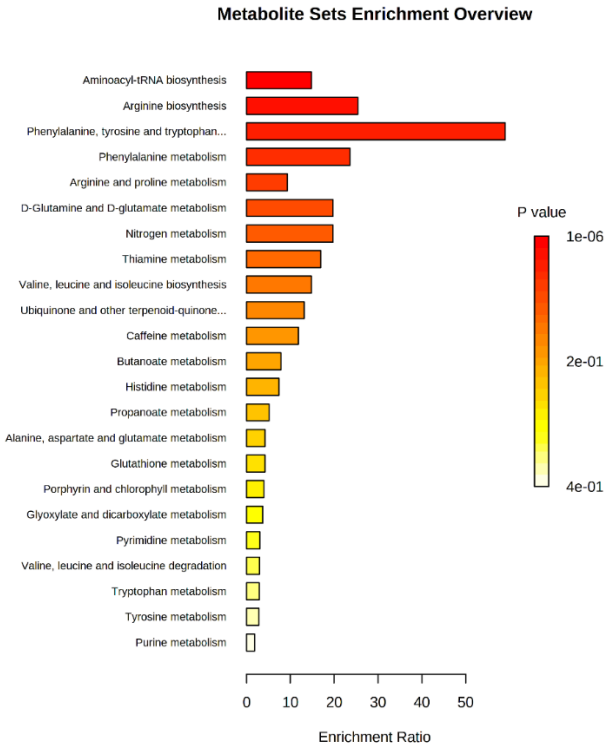



**Fig. C.**

**Figure 47. A.** Venn diagrams of metabolites observed in Putranjiva male and female flowers. **B.** PCA analysis of metabolites in Putranjiva male and female flowers. **C.** Enrichment analysis – metabolites in Putranjiva flowers.

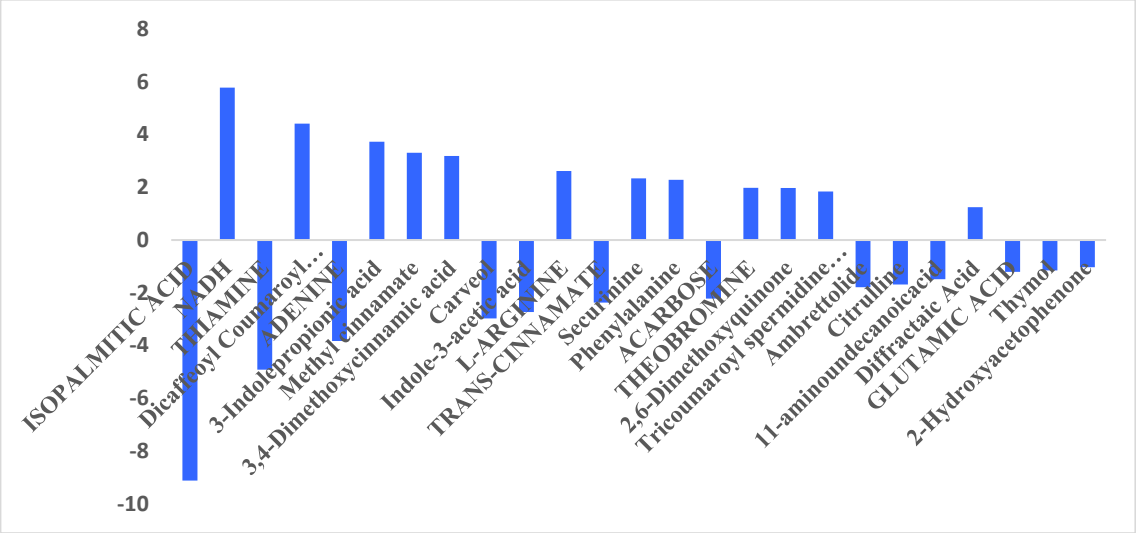



**Fig. A.**

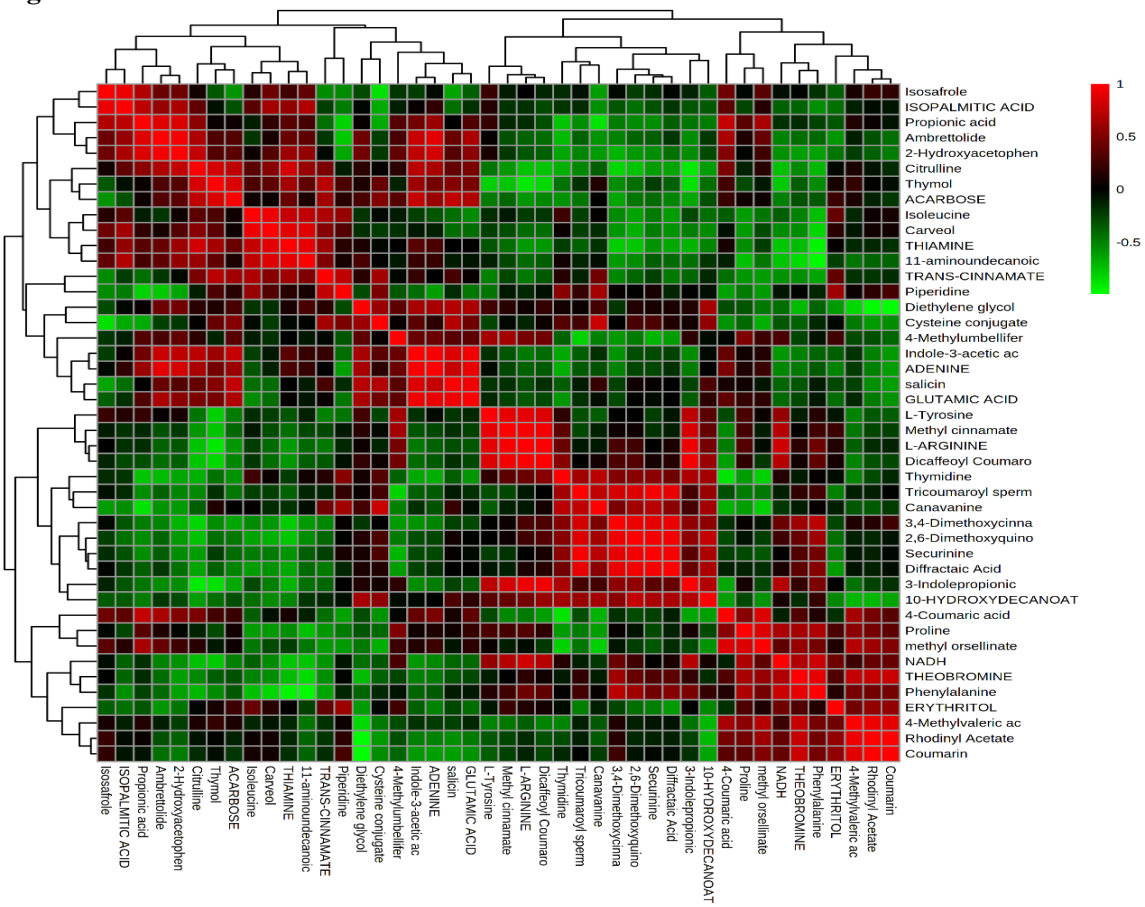



**Fig. B.**

**Figure 48. A.** Fold changes of metabolites in Putranjiva male and female flowers. **B.** Correlation heatmap of metabolites in Putranjiva male and female cone.

## 3.4. Proteomic analysis of Cycas and Putranjiva during reproduction helps to understand the protein-protein crosstalk between male and female floral bodies

### 3.4.1. Proteomics of Cycas male and female cones

A total of 3537 differentially expressed proteins were identified in Cycas males and Cycas females. From the total number of proteins in Cycas, 1285 proteins were found in Cycas male, whereas 1475 proteins were found in Cycas female. There were 773 common proteins detected in the Cycas cone (Fig. 49 B). The total proteins were categorized according to the molecular weight and pH basis. More proteins were found to be within the range of pH 6 and 60kd+ (Fig. 49 C) The lowest pH and molecular weight were coming under pH ten and 1kDa among these Cycas male proteins were more than the female cone (Fig. 49 A). The highly upregulated proteins were found CYCAS_ 000047, CYCAS_010600, CYCAS_017200, CYCAS_000846, CYCAS_001550, CYCAS_002962, CYCAS_004183, CYCAS_005360, CYCAS_006949, CYCAS_009146, CYCAS_011626, CYCAS_012099, CYCAS_012606, CYCAS_017266, CYCAS_019704, CYCAS_020536 from the common proteins. CYCAS_017200 protein was highly upregulated in females, and CYCAS_008089, CYCAS_008738, CYCAS_014726, and CYCAS_030968 proteins were highly upregulated in males. From the unique list of proteins, CYCAS_032110, CYCAS_014252, CYCAS_014342, CYCAS_007031, and CYCAS_003370 were upregulated in female and CYCAS_014252 was highly abundant. CYCAS_008001, CYCAS_008947, CYCAS_012543, CYCAS_013992, CYCAS_014426, CYCAS_017276, CYCAS_018942, CYCAS_022911, CYCAS_026353 proteins were downregulated in female whereas CYCAS_030810, CYCAS_019410, CYCAS_016687, CYCAS_004852, CYCAS_002052 were downregulated in male plant.

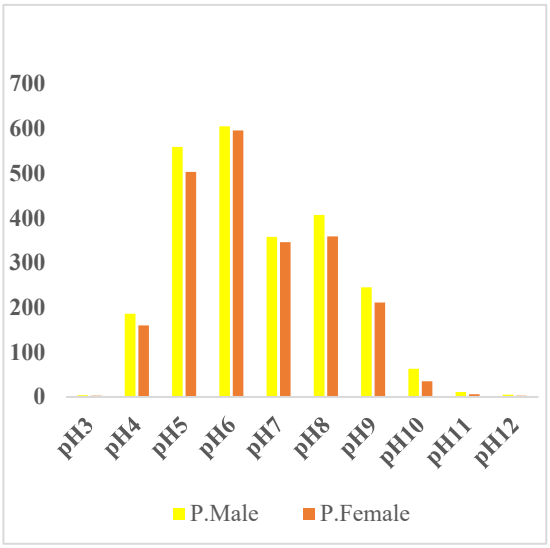



**Fig. A.**

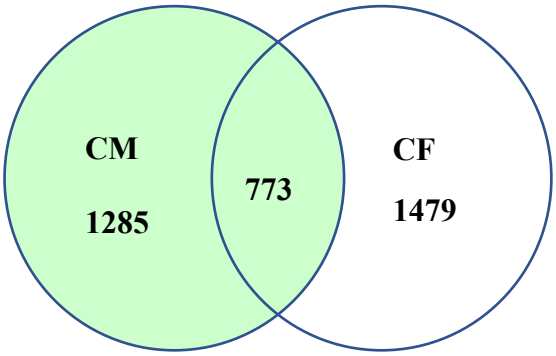



**Fig. B.**

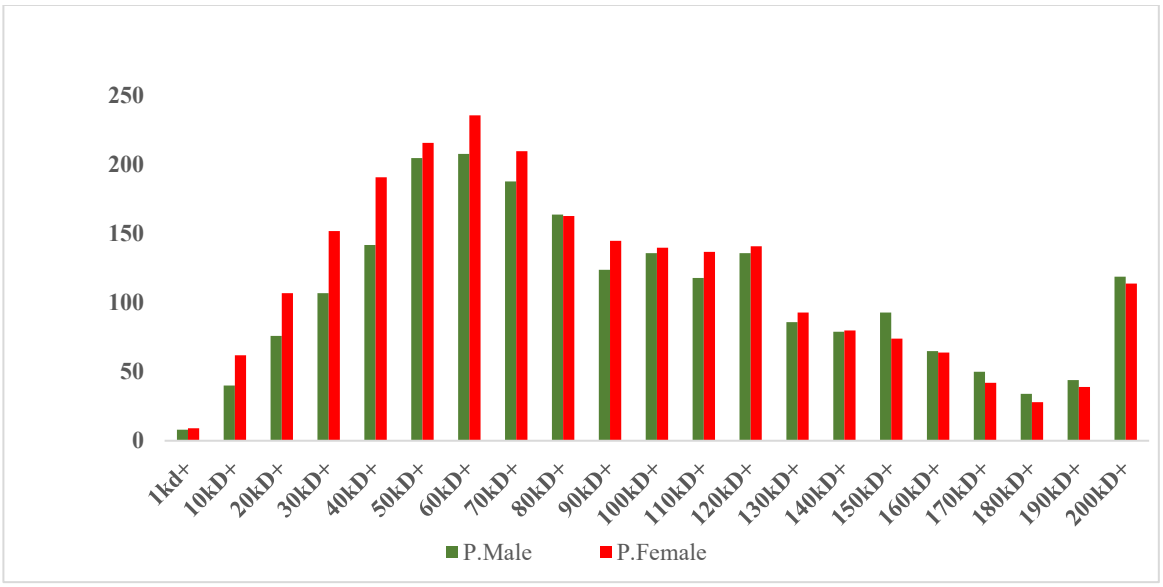



**Fig. C.**

**Figure 49. A.** Classification of proteins based on pH in Cycas male and female flowers. **B.** Venn diagrams showing proteins observed in Cycas male and female flowers. **C.** Classification of proteins based on molecular weight in Cycas male and female flowers.

### 3.4.2. Proteomics of Putranjiva flowers

#### 3.4.2.1. Proteome profiling

Proteome profiling of both male and female samples was performed in triplicates resulting in three sets of protein data for each male and female sample. In male, 777,592 and 780 proteins were identified respectively from each set. Similarly in female, 563,559 and 542 proteins were identified respectively from each set. First, the common proteins were searched across the three data sets in both male and female. This step resulted in final count of 478 proteins from male and 519 proteins from female (Fig. 50 A, B). Among these, 274 proteins were found in both Putranjiva female and male flowers (Table 1), 204 proteins were identified exclusively in male flowers (Table 2), and 245 proteins exclusively in female flowers (Table 3) (Figure 50 C).

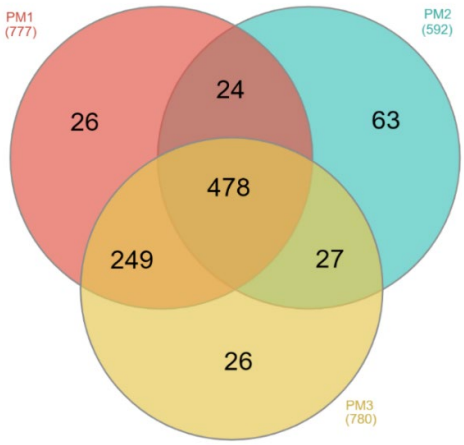



**Fig. A.**

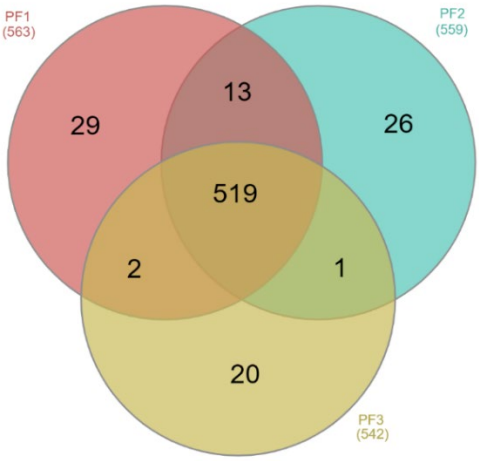



**Fig. B.**

Total proteins from male: 478
Total proteins from female: 519

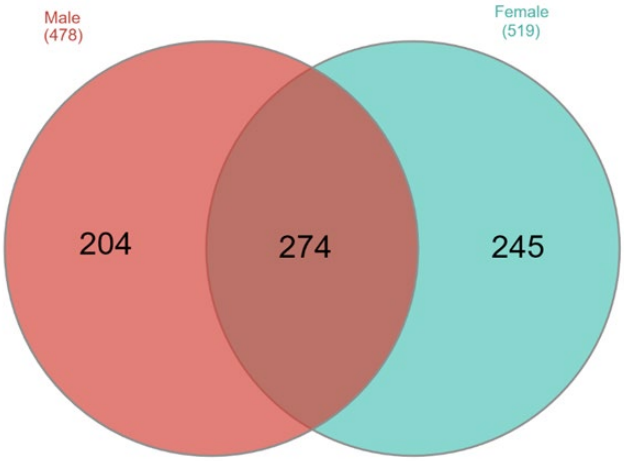



**Fig. C.**

Common: 274
Unique to male: 204
Unique to female: 245

**Figure 50.** Venn diagrams showing the number of proteins identified from Putranjiva flowers through proteome profiling. **A.** Proteins identified from triplicate samples of male flowers **B.** Proteins identified from triplicate samples of female flowers **C.** Comparision between male and female flowers.

**Table 4.** List of 274 common proteins identified across male and female flowers.

| S.No. | Accession | Description |
|---|---|---|
| 1 | A0A067JNG6 | Uncharacterized protein |
| 2 | A0A067JPT3 | Homeobox domain-containing protein |
| 3 | A0A067KPJ2 | Uncharacterized protein |
| 4 | A0A1P8BFJ0 | Leucine-rich repeat protein kinase family protein |
| 5 | A0A251IXZ3 | Plus3 domain-containing protein |
| 6 | A0A251JEZ9 | Uncharacterized protein |
| 7 | A0A251K2W8 | FRIGIDA-like protein |
| 8 | A0A251KMA2 | Uncharacterized protein |
| 9 | A0A2C9UBF8 | LisH domain-containing protein |
| 10 | A0A2C9UFF5 | Uncharacterized protein |
| 11 | A0A2C9UQR6 | FRIGIDA-like protein |
| 12 | A0A2C9UR94 | Fe2 G dioxygenase domain-containing protein |
| 13 | A0A2C9UYS0 | Exportin-T |
| 14 | A0A2C9VBA7 | FRIGIDA-like protein |

| | | |
|---|---|---|
| 15 | A0A2C9VD05 | Uncharacterized protein |
| 16 | A0A2C9W3M2 | Homeobox domain-containing protein |
| 17 | A0A2C9W3T1 | FRIGIDA-like protein |
| 18 | A0A6A6KEK9 | Homeobox domain-containing protein (Fragment) |
| 19 | A0A6A6KFN6 | Uncharacterized protein |
| 20 | A0A6A6KGB5 | Uncharacterized protein |
| 21 | A0A6A6L9I6 | RING-type E3 ubiquitin transferase |
| 22 | A0A6A6LAV2 | Uncharacterized protein |
| 23 | A0A6A6LAY4 | FRIGIDA-like protein |
| 24 | A0A6A6LC41 | Uncharacterized protein |
| 25 | A0A6A6LSQ1 | Uncharacterized protein |
| 26 | A0A6A6M039 | Shikimate_dh_N domain-containing protein |
| 27 | A0A6A6M453 | FRIGIDA-like protein |
| 28 | A0A7G2EKE3 | FRIGIDA-like protein |
| 29 | A0A7G2FDL8 | (thale cress) hypothetical protein |
| 30 | A4GSN8 | Nuclear-pore anchor |
| 31 | B9DFY8 | AT5G52882 protein |
| 32 | B9DGD6 | Acetyl-coenzyme A synthetase, chloroplastic/glyoxysomal |
| 33 | B9DGE3 | AT4G02570 protein |
| 34 | B9DGQ8 | AT3G27570 protein (Fragment) |
| 35 | B9DGT6 | Phosphoenolpyruvate carboxylase |
| 36 | B9DGX7 | AT3G54800 protein |
| 37 | B9DGX8 | AT4G28760 protein |
| 38 | B9DHI8 | AT1G22610 protein (Fragment) |
| 39 | B9DI55 | AT3G09840 protein |
| 40 | B9S3Y9 | Homeobox domain-containing protein |
| 41 | B9SG32 | FRIGIDA-like protein |
| 42 | C0LGQ5 | LRR receptor-like serine/threonine-protein kinase GS |
| 43 | C0Z2K2 | AT1G79050 protein |
| 44 | F4HQG6 | Dicer-like 1 |
| 45 | F4HRS2 | Protein SWEETIE |
| 46 | F4HRT5 | Protein CR WDED NUCLEI 1 |
| 47 | F4HVS0 | Zinc finger protein BRUTUS-like At1g74770 |
| 48 | F4HY56 | Homeobox-DDT domain protein RLT1 |
| 49 | F4I642 | Ubiquitin-specific protease 15 |
| 50 | F4I9A2 | Trans-Golgi network-localized SYP41-interacting protein 1 |
| 51 | F4IDB2 | Protein PHYT |

| | | |
|---|---|---|
| 52 | F4IN78 | Protein HUA2-LIKE 2 |
| 53 | F4J7T2 | Chromatin modification-related protein EAF1 B |
| 54 | F4J7T3 | Chromatin modification-related protein EAF1 A |
| 55 | F4J9M5 | Probable ATP-dependent DNA helicase CHR12 |
| 56 | F4JP36 | Protein HAPLESS 2 |
| 57 | F4JRF5 | Homeobox-DDT domain protein RLT3 |
| 58 | F4JT76 | Vacuolar protein sorting-associated protein 54, chloroplastic |
| 59 | F4JTN2 | Protein LAZ1 |
| 60 | F4JVI3 | Transcription termination factor MTERF5, chloroplastic |
| 61 | F4JWP9 | 109 kDa U5 small nuclear ribonucleoprotein component GFL |
| 62 | F4JY12 | Protein GFS12 |
| 63 | F4JY24 | ISWI chromatin-remodeling complex ATPase CHR17 |
| 64 | F4K1J4 | Histone-lysine N-methyltransferase ATXR7 |
| 65 | F4K2E9 | Pre-mRNA-splicing factor ATP-dependent RNA helicase DEAH7 |
| 66 | F4K3G5 | Protein ENHANCED D |
| 67 | F4K4D6 | PWWP domain-containing protein 1 |
| 68 | F4K6B6 | Heat shock protein 81-2 |
| 69 | F4KDH9 | FIP1[V]-like protein |
| 70 | F4KDN0 | KH domain-containing protein HEN4 |
| 71 | F4KIA8 | Protein RRC1-like |
| 72 | F8TCX5 | DNA-directed RNA polymerase (Fragment) |
| 73 | J9UMU3 | Cell wall invertase |
| 74 | K4KAR9 | Cytochrome b6 (Fragment) |
| 75 | K4KJF6 | ATP synthase subunit alpha, chloroplastic (Fragment) |
| 76 | O22476 | Protein BRASSIN |
| 77 | O22898 | Long chain acyl-CoA synthetase 1 |
| 78 | O23447 | Putative pectinesterase/pectinesterase inhibitor 43 |
| 79 | O23617 | Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 5 |
| 80 | O49500 | E3 ubiquitin-protein ligase MBR2 |
| 81 | O64752 | Lysine-specific demethylase JMJ15 |
| 82 | O64827 | Histone-lysine N-methyltransferase SUVR5 |
| 83 | O80548 | MA3 D |
| 84 | O80786 | DNA replication licensing factor MCM5 |
| 85 | O80925 | ADP-ribosylation factor GTPase-activating protein AGD7 |
| 86 | O81301 | Probable pectinesterase/pectinesterase inhibitor 40 |
| 87 | O81635 | Kinesin-like protein KIN-14G |

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|---|---|---|
| 88 | O82387 | Cell division control protein 6 homolog |
| 89 | O82595 | B3 domain-containing transcription factor NGA4 |
| 90 | O82799 | B3 domain-containing transcription factor NGA1 |
| 91 | P0DO23 | 2-oxoglutarate and iron-dependent oxygenase domain-containing protein CP2 |
| 92 | P29384 | Developmental protein SEPALLATA 2 |
| 93 | P34881 | DNA (cytosine-5)-methyltransferase 1 |
| 94 | P43254 | E3 ubiquitin-protein ligase C |
| 95 | P43299 | DNA replication licensing factor MCM7 |
| 96 | P93024 | Auxin response factor 5 |
| 97 | P93831 | Histone-lysine N-methyltransferase CLF |
| 98 | Q06738 | Low-temperature-induced 78 kDa protein |
| 99 | Q0PCS3 | Protein CHR |
| 100 | Q0WL52 | BTB/P |
| 101 | Q0WVF5 | DNA replication licensing factor MCM4 |
| 102 | Q0WW17 | Protein REDUCED WALL ACETYLATI |
| 103 | Q1PEC0 | Probable pectinesterase/pectinesterase inhibitor 42 |
| 104 | Q1PEP5 | Nucleolin 2 |
| 105 | Q2LAE1 | Histone-lysine N-methyltransferase ASHH2 |
| 106 | Q2V3V8 | AT3G14870 protein |
| 107 | Q38796 | Homeobox protein LUMINIDEPENDENS |
| 108 | Q39086 | Receptor-like serine/threonine-protein kinase SD1-7 |
| 109 | Q39112 | Gibberellin 20 oxidase 3 |
| 110 | Q3E9B4 | Transcription repressor |
| 111 | Q42569 | Cytochrome P450 90A1 |
| 112 | Q564K3 | Condensin complex subunit 2 |
| 113 | Q56WK6 | Patellin-1 |
| 114 | Q56YN8 | Structural maintenance of chromosomes protein 3 |
| 115 | Q570R7 | Transcription factor PIF7 |
| 116 | Q588V7 | Helicase and polymerase-containing protein TEBICHI |
| 117 | Q5KS41 | Wax ester synthase/diacylglycerol acyltransferase 11 |
| 118 | Q5SCM4 | Ribulose bisphosphate carboxylase large chain (Fragment) |
| 119 | Q5XPJ6 | Protein SCAR4 |
| 120 | Q5XPK0 | Scar-like domain-containing protein WAVE 5 |
| 121 | Q5XV31 | FRIGIDA-like protein 5 |
| 122 | Q66GQ5 | Protein REDUCED WALL ACETYLATI |
| 123 | Q66LG9 | Centromere protein C |

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|---|---|---|
| 124 | Q68LV8 | Maturase (Fragment) |
| 125 | Q6EVK6 | ATP-dependent helicase BRM |
| 126 | Q6NKZ8 | Cytochrome P450 714A2 |
| 127 | Q6Q1P4 | Structural maintenance of chromosomes protein 1 |
| 128 | Q6XAT2 | LRR receptor-like serine/threonine-protein kinase ERL2 |
| 129 | Q6XJG8 | 26S proteasome non-ATPase regulatory subunit 2 homolog B |
| 130 | Q700C2 | Squamosa promoter-binding-like protein 16 |
| 131 | Q710E8 | Origin of replication complex subunit 1A |
| 132 | Q7PC88 | ABC transporter G family member 31 |
| 133 | Q7X9V2 | Protein PH |
| 134 | Q7Y1C4 | Protein BREAST CANCER SUSCEPTIBILITY 2 homolog B |
| 135 | Q7Y1C5 | Protein BREAST CANCER SUSCEPTIBILITY 2 homolog A |
| 136 | Q84JG2 | SWI/SNF complex subunit SWI3B |
| 137 | Q84TI7 | Sodium transporter HKT1 |
| 138 | Q84VG7 | Protein FRIGIDA-ESSENTIAL 1 |
| 139 | Q84W56 | Ribonuclease J |
| 140 | Q8GUI6 | Lysine-specific demethylase JMJ14 |
| 141 | Q8GWE0 | Pentatricopeptide repeat-containing protein At4g16390, chloroplastic |
| 142 | Q8GWT4 | Protein arginine N-methyltransferase 1.5 |
| 143 | Q8GX86 | Probable pectinesterase/pectinesterase inhibitor 21 |
| 144 | Q8GYU3 | Transcriptional elongation regulator MINIY |
| 145 | Q8GZA8 | Protein ULTRAPETALA 1 |
| 146 | Q8H0V4 | DUF724 domain-containing protein 7 |
| 147 | Q8H103 | Glucose-6-phosphate isomerase 1, chloroplastic |
| 148 | Q8H1D3 | BTB/P |
| 149 | Q8H1R2 | Probable inactive purple acid phosphatase 24 |
| 150 | Q8L5R3 | Tubulin-folding cofactor D |
| 151 | Q8L6Y4 | Polycomb group protein EMBRY |
| 152 | Q8L785 | Glycine--tRNA ligase, chloroplastic/mitochondrial 2 |
| 153 | Q8L7C8 | Protein REDUCED WALL ACETYLATI |
| 154 | Q8L840 | ATP-dependent DNA helicase Q-like 4A |
| 155 | Q8L8A8 | Growth-regulating factor 2 |
| 156 | Q8LFV3 | Cyclic dof factor 3 |
| 157 | Q8LGH4 | Cullin-4 |
| 158 | Q8LK56 | Transcriptional activator DEMETER |
| 159 | Q8LL04 | RNA polymerase II C-terminal domain phosphatase-like 3 |

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|---|---|---|
| 160 | Q8LNZ2 | Kinesin-like protein KIN-7B |
| 161 | Q8LPQ9 | Flowering time control protein FPA |
| 162 | Q8RWV9 | Uncharacterized protein At5g08450 |
| 163 | Q8RWY3 | ISWI chromatin-remodeling complex ATPase CHR11 |
| 164 | Q8RXA7 | DENN domain and WD repeat-containing protein SCD1 |
| 165 | Q8RXD4 | Protein BREAST CANCER SUSCEPTIBILITY 1 homolog |
| 166 | Q8RXD6 | E3 ubiquitin-protein ligase BRE1-like 1 |
| 167 | Q8RY95 | Squamosa promoter-binding-like protein 14 |
| 168 | Q8S340 | Purple acid phosphatase 22 |
| 169 | Q8S8P5 | Probable WRKY transcription factor 33 |
| 170 | Q8S9K3 | Zinc finger protein VAR3, chloroplastic |
| 171 | Q8VY05 | SWI/SNF complex subunit SWI3D |
| 172 | Q8VYD4 | Cation/H(+) antiporter 23, chloroplastic |
| 173 | Q8VZG7 | Ribonuclease TUD |
| 174 | Q8VZR2 | Alpha-L-arabinofuranosidase 2 |
| 175 | Q8W234 | Transcriptional corepressor SEUSS |
| 176 | Q8W475 | SWI/SNF complex subunit SWI3A |
| 177 | Q8W4D0 | Peptidyl-prolyl cis-trans isomerase CYP71 |
| 178 | Q8W4Q4 | MA3 D |
| 179 | Q93WU7 | Probable WRKY transcription factor 58 |
| 180 | Q93WV0 | Probable WRKY transcription factor 20 |
| 181 | Q93WV7 | Probable WRKY transcription factor 67 |
| 182 | Q93ZE9 | Phosphatidylinositol/phosphatidylcholine transfer protein SFH3 |
| 183 | Q93ZL5 | Cyclic dof factor 2 |
| 184 | Q940U6 | Protein FLU |
| 185 | Q94BR1 | MA3 D |
| 186 | Q94JM3 | Auxin response factor 2 |
| 187 | Q96301 | Probable UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase SPINDLY |
| 188 | Q9AR19 | Histone acetyltransferase GCN5 |
| 189 | Q9ASP6 | Heterogeneous nuclear ribonucleoprotein Q |
| 190 | Q9C5J3 | Protein RRC1 |
| 191 | Q9C5P7 | Protein transport Sec1a |
| 192 | Q9C5U1 | Histidine kinase 3 |
| 193 | Q9C5U2 | Histidine kinase 2 |
| 194 | Q9C5X4 | Histone H3-lysine(4) N-trimethyltransferase ATX1 |
| 195 | Q9C821 | Proline-rich receptor-like protein kinase PERK15 |

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|---|---|---|
| 196 | Q9C895 | E3 ubiquitin-protein ligase BRE1-like 2 |
| 197 | Q9C950 | Protein RTF1 homolog |
| 198 | Q9CA42 | Protein CR |
| 199 | Q9CA61 | Cytochrome P450 98A8 |
| 200 | Q9CAY3 | Glycerol-3-phosphate acyltransferase 5 |
| 201 | Q9FF86 | BAHD acyltransferase DCR |
| 202 | Q9FFH1 | Homeobox-DDT domain protein RLT2 |
| 203 | Q9FG77 | Probable WRKY transcription factor 2 |
| 204 | Q9FGX1 | ATP-citrate synthase beta chain protein 2 |
| 205 | Q9FIZ3 | LRR receptor-like serine/threonine-protein kinase GS |
| 206 | Q9FJ21 | Probable pectinesterase/pectinesterase inhibitor 58 |
| 207 | Q9FKM5 | DNA repair protein XRCC3 homolog |
| 208 | Q9FLH0 | Protein CR |
| 209 | Q9FLT0 | Ribonuclease TUD |
| 210 | Q9FMR9 | RuvB-like protein 1 |
| 211 | Q9FMS5 | PHD finger protein MALE STERILITY 1 |
| 212 | Q9FN05 | Probable glucan 1,3-alpha-glucosidase |
| 213 | Q9FN09 | BTB/P |
| 214 | Q9FNE4 | PWWP domain-containing protein 3 |
| 215 | Q9FNQ0 | Protein LE |
| 216 | Q9FNR3 | Exocyst complex component EX |
| 217 | Q9FT70 | ATP-dependent DNA helicase Q-like 4B |
| 218 | Q9FT72 | ATP-dependent DNA helicase Q-like 3 |
| 219 | Q9FT73 | ATP-dependent DNA helicase Q-like 2 |
| 220 | Q9FXG3 | Protein REDUCED WALL ACETYLATI |
| 221 | Q9FY48 | E3 ubiquitin-protein ligase KEG |
| 222 | Q9FY74 | Calmodulin-binding transcription activator 1 |
| 223 | Q9LEY4 | Protein HUA2-LIKE 1 |
| 224 | Q9LF02 | At5g16260 |
| 225 | Q9LFE0 | SART-1 family protein D |
| 226 | Q9LHS5 | Cell division related protein-like |
| 227 | Q9LIP9 | Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1 |
| 228 | Q9LJD8 | MAP3K epsilon protein kinase 1 |
| 229 | Q9LMG7 | Probable inactive purple acid phosphatase 2 |
| 230 | Q9LMJ4 | Exocyst complex component EXO 70 B2 |
| 231 | Q9LMT8 | Homeobox-leucine zipper protein HDG12 |

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|---|---|---|
| 232 | Q9LNC5 | 110 kDa U5 small nuclear ribonucleoprotein component CL |
| 233 | Q9LNJ9 | Bifunctional fucokinase/fucose pyrophosphorylase |
| 234 | Q9LP46 | Protein SCAR3 |
| 235 | Q9LPD9 | DNA replication licensing factor MCM2 |
| 236 | Q9LPV8 | Eukaryotic peptide chain release factor subunit 1-2 |
| 237 | Q9LPV9 | WD repeat-containing protein LWD1 |
| 238 | Q9LT47 | Polycomb group protein FERTILIZATION INDEPENDENT ENDOSPERM |
| 239 | Q9LV35 | Actin-interacting protein 1-2 |
| 240 | Q9LY17 | Probable pectinesterase 50 |
| 241 | Q9M086 | DDB1- and CUL4-associated factor homolog 1 |
| 242 | Q9M268 | B3 domain-containing transcription factor NGA2 |
| 243 | Q9M2Z1 | Leucine-rich repeat receptor-like serine/threonine-protein kinase BAM2 |
| 244 | Q9M4A1 | Meiotic recombination protein SP |
| 245 | Q9M8Y0 | Probable UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase SEC |
| 246 | Q9MAP7 | Subtilisin-like protease SBT3.5 |
| 247 | Q9SAF6 | Protein CROWDED NUCLEI 2 |
| 248 | Q9SB51 | Ubiquitin carboxyl-terminal hydrolase 16 |
| 249 | Q9SB89 | DEAD-box ATP-dependent RNA helicase 27 |
| 250 | Q9SBJ1 | [Pyruvate dehydrogenase (acetyl-transferring)] kinase, mitochondrial |
| 251 | Q9SDW0 | Trihelix transcription factor GT-3a |
| 252 | Q9SF36 | PWWP domain-containing protein 2 |
| 253 | Q9SFB6 | MAP3K epsilon protein kinase 2 |
| 254 | Q9SGY7 | Putative proline-rich receptor-like protein kinase PERK11 |
| 255 | Q9SIV2 | 26S proteasome non-ATPase regulatory subunit 2 homolog A |
| 256 | Q9SJV6 | AT2G01100 protein |
| 257 | Q9SMX9 | Squamosa promoter-binding-like protein 1 |
| 258 | Q9SQY7 | At3g10570 |
| 259 | Q9SR02 | Mediator of RNA polymerase II transcription subunit 14 |
| 260 | Q9SSE9 | Lysine-specific demethylase JMJ25 |
| 261 | Q9STL9 | MA3 domain-containing translation regulatory factor 4 |
| 262 | Q9SUM4 | VIN3-like protein 2 |
| 263 | Q9SYQ8 | Receptor protein kinase CLAVATA1 |
| 264 | Q9SZH4 | RNA-binding KH domain-containing protein PEPPER |
| 265 | Q9T0I5 | Proline-rich protein 4 |

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|---|---|---|
| 266 | Q9T0J9 | (Z)-gamma-bisabolene synthase 1 |
| 267 | Q9T0K1 | (Z)-gamma-bisabolene synthase 2 |
| 268 | Q9XER9 | ENHANCER OF AG-4 protein 2 |
| 269 | Q9XGW1 | Protein argonaute 10 |
| 270 | Q9XI07 | SWI/SNF complex subunit SWI3C |
| 271 | Q9ZP16 | Cysteine-rich receptor-like protein kinase 11 |
| 272 | Q9ZQ12 | Growth-regulating factor 6 |
| 273 | Q9ZQ94 | UDP-glycosyltransferase 73C5 |
| 274 | Q9ZTX8 | Auxin response factor 6 |

**Table 5.** List of proteins unique to male flowers.

| S.No. | Accession | Description |
|---|---|---|
| 1 | A0A067JHQ1 | Terminal flower 1c |
| 2 | A0A067JI55 | FRIGIDA-like protein |
| 3 | A0A067LHT7 | CCT domain-containing protein |
| 4 | A0A067LKK9 | FRIGIDA-like protein |
| 5 | A0A178UV71 | FRIGIDA-like protein |
| 6 | A0A1P8B7M1 | Sequence-specific DNA binding transcription factor ATNDX |
| 7 | A0A1Z1G631 | Chloroplast oxygen-envolving enhancer protein 1 |
| 8 | A0A2C9U1Q8 | DIOX_N domain - containing protein |
| 9 | A0A2C9U4Z4 | Squamosa promoter-binding-like protein |
| 10 | A0A2C9U8G6 | Squamosa promoter-binding-like protein |
| 11 | A0A2C9UBM3 | Uncharacterized protein |
| 12 | A0A2C9UFR2 | Uncharacterized protein |
| 13 | A0A2C9UMJ9 | Uncharacterized protein |
| 14 | A0A2C9VNK0 | PAS domain-containing protein |
| 15 | A0A2C9VQD6 | FRIGIDA-like protein |
| 16 | A0A2C9VS53 | Fe2 OG dioxygenase domain-containing protein |
| 17 | A0A2C9VZG1 | Uncharacterized protein |
| 18 | A0A5A4WG37 | Maturase K (Fragment) |
| 19 | A0A5S9Y7K1 | Uncharacterized protein |
| 20 | A0A5S9YF32 | (thale cress) hypothetical protein |
| 21 | A0A654EQB2 | FRIGIDA-like protein |
| 22 | A0A654FJ74 | Uncharacterized protein |
| 23 | A0A654G085 | (thale cress) hypothetical protein |
| 24 | A0A6A6K852 | FRIGIDA-like protein |

| | | |
|---|---|---|
| 25 | A0A6A6LTS5 | FRIGIDA-like protein |
| 26 | A0A6A6MD67 | Clp R domain-containing protein |
| 27 | A7UL74 | Protein CHROMOSOME TRANSMISSION FIDELITY 7 |
| 28 | A9XUN2 | NAD(P)H-quinone oxidoreductase subunit 5, chloroplastic (Fragment) |
| 29 | B8XCH5 | Protein QUIRKY |
| 30 | B9DFX0 | AT4G10000 protein |
| 31 | B9DG14 | AT5G41100 protein |
| 32 | B9DG31 | CTP synthase |
| 33 | B9DGB2 | Lactoylglutathione lyase |
| 34 | B9DGF1 | AT2G26970 protein |
| 35 | B9DGJ8 | AT2G30990 protein |
| 36 | B9DGM9 | Auxin response factor |
| 37 | B9DGR4 | AT5G48150 protein |
| 38 | B9DGR6 | AT1G63940 protein |
| 39 | B9DGU1 | AT1G14710 protein |
| 40 | B9DGY7 | AT2G38710 protein |
| 41 | B9DH23 | AT1G68410 protein |
| 42 | B9DH45 | AT3G12570 protein |
| 43 | B9DH51 | AT3G04810 protein |
| 44 | B9DHL6 | Methylenetetrahydrofolate reductase (Fragment) |
| 45 | B9DI26 | Ferredoxin--NADP(+) reductase (Fragment) |
| 46 | B9DI34 | AT5G23575 protein (Fragment) |
| 47 | B9R9Z3 | Polycomb protein embryonic flower, putative |
| 48 | B9RA29 | FRIGIDA-like protein |
| 49 | B9RBB6 | Squamosa promoter-binding-like protein |
| 50 | B9REX4 | FRIGIDA-like protein |
| 51 | B9RS34 | Uncharacterized protein |
| 52 | B9SMS0 | FRIGIDA-like protein |
| 53 | B9T283 | Polycomb protein embryonic flower, putative |
| 54 | C0Z2C1 | Signal recognition particle 54 kDa protein |
| 55 | C0Z2F0 | AT5G53860 protein |
| 56 | C0Z2G3 | AT5G50010 protein |
| 57 | C0Z320 | AT1G31500 protein |
| 58 | C0Z388 | AT5G04280 protein |
| 59 | F4IP06 | SWR1 complex subunit 2 |
| 60 | F4IZM8 | Protein HUA2-LIKE 3 |
| 61 | F4J6A8 | DnaJ and Myb-like DNA-binding domain-containing protein |

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|---|---|---|
| 62 | F4JCB2 | RNA polymerase II C-terminal domain phosphatase-like 5 |
| 63 | F4JIN3 | DnaJ protein ERDJ2B |
| 64 | F4JJR8 | Dehydrogenase/reductase SDR family member FEY |
| 65 | F4JYE9 | Dihydrofolate synthetase |
| 66 | F4K3F4 | DNA topoisomerase I |
| 67 | F4KAN2 | Galactose oxidase/kelch repeat superfamily protein |
| 68 | F4KCE9 | Kinetochore-associated protein KNL-2 homolog |
| 69 | F4KCL7 | Outer envelope protein 64, mitochondrial |
| 70 | K4K5M0 | NdhI (Fragment) |
| 71 | K4K6Q9 | DNA-directed RNA polymerase (Fragment) |
| 72 | K4KA62 | PetD (Fragment) |
| 73 | O04928 | Phosphatidate cytidylyltransferase 1 |
| 74 | O22263 | Protein disulfide-isomerase like 2-1 |
| 75 | O22607 | WD-40 repeat-containing protein MSI4 |
| 76 | O22921 | Probable WRKY transcription factor 25 |
| 77 | O23147 | Polygalacturonase ADPG1 |
| 78 | O23674 | Type III polyketide synthase A |
| 79 | O48847 | Transcriptional corepressor LEUNIG_HOMOLOG |
| 80 | O49545 | Leucine-rich repeat receptor-like serine/threonine-protein kinase BAM1 |
| 81 | O49608 | Transcription factor MYB32 |
| 82 | O49613 | Heavy metal-associated isoprenylated plant protein 25 |
| 83 | O65312 | Histone-lysine N-methyltransferase MEDEA |
| 84 | O65440 | Leucine-rich repeat receptor-like serine/threonine-protein kinase BAM3 |
| 85 | O65590 | Probable WRKY transcription factor 34 |
| 86 | O80437 | Glycerol-3-phosphate 2-O-acyltransferase 6 |
| 87 | O81439 | Probable plastid-lipid-associated protein 1, chloroplastic |
| 88 | O82794 | MADS-box protein AGL24 |
| 89 | P22953 | Heat shock 70 kDa protein 1 |
| 90 | P31414 | Pyrophosphate-energized vacuolar membrane proton pump 1 |
| 91 | P37702 | Myrosinase 1 |
| 92 | P42737 | Beta carbonic anhydrase 2, chloroplastic |
| 93 | P82642 | Defensin-like protein 232 |
| 94 | P93015 | Squamosa promoter-binding-like protein 3 |
| 95 | Q0WML0 | ABC transporter B family member 27 |
| 96 | Q0WP44 | Protein HASTY 1 |
| 97 | Q0WQF4 | Vacuolar protein sorting-associated protein 53 A |
| 98 | Q0WT48 | DnaJ protein ERDJ2A |

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|---|---|---|
| 99 | Q38849 | Potassium channel KAT2 |
| 100 | Q38958 | Pectin lyase-like superfamily protein |
| 101 | Q39021 | Mitogen-activated protein kinase 1 |
| 102 | Q39022 | Mitogen-activated protein kinase 2 |
| 103 | Q39029 | Protein kinase |
| 104 | Q39238 | Serine/threonine-protein kinase TOUSLED |
| 105 | Q3E8Z8 | Putative pectinesterase/pectinesterase inhibitor 28 |
| 106 | Q3ED68 | 2-oxoglutarate and iron-dependent oxygenase domain-containing protein ICU11 |
| 107 | Q42605 | Bifunctional UDP-glucose 4-epimerase and UDP-xylose 4-epimerase 1 |
| 108 | Q43725 | Cysteine synthase, mitochondrial |
| 109 | Q4PSN0 | Probable pectinesterase 29 |
| 110 | Q4VMX4 | ATP synthase subunit beta (Fragment) |
| 111 | Q5HZ36 | GATA transcription factor 21 |
| 112 | Q5SCJ3 | Ribulose bisphosphate carboxylase large chain (Fragment) |
| 113 | Q5XPJ9 | Protein SCAR2 |
| 114 | Q5YDB6 | RNA polymerase II C-terminal domain phosphatase-like 1 |
| 115 | Q6AWX6 | Protein SCAR1 |
| 116 | Q7PC79 | Exportin-T |
| 117 | Q84P23 | 4-coumarate--CoA ligase-like 9 |
| 118 | Q8GSA7 | Calmodulin-binding transcription activator 3 |
| 119 | Q8GUN6 | Chaperone protein dnaJ 50 |
| 120 | Q8GX23 | Proline-rich receptor-like protein kinase PERK5 |
| 121 | Q8GY84 | DEAD-box ATP-dependent RNA helicase 10 |
| 122 | Q8GZ26 | RNA-binding protein BRN2 |
| 123 | Q8LFS6 | RNA-binding protein BRN1 |
| 124 | Q8RWR1 | Lysine-specific demethylase JMJ30 |
| 125 | Q8RXN0 | ABC transporter G family member 11 |
| 126 | Q8S9D1 | Pentatricopeptide repeat-containing protein At5g21222 |
| 127 | Q8VWQ4 | Probable WRKY transcription factor 56 |
| 128 | Q8VWV6 | Probable WRKY transcription factor 61 |
| 129 | Q8W1E3 | Cyclic dof factor 1 |
| 130 | Q93YN9 | Inositol-pentakisphosphate 2-kinase |
| 131 | Q93Z75 | Ureide permease 5 |
| 132 | Q93Z79 | Cytochrome P450 714A1 |
| 133 | Q93ZA9 | AT4G10360 protein |
| 134 | Q940H8 | FRIGIDA-like protein 4b |

| | | |
|---|---|---|
| 135 | Q944A6 | Sucrose nonfermenting 4-like protein |
| 136 | Q948R9 | Protein REGULATOR FATTY ACID COMPOSITION 3, Chloroplastic |
| 137 | Q949Y3 | Bifunctional purple acid phosphatase 26 |
| 138 | Q94A02 | Tryptophan aminotransferase-related protein 2 |
| 139 | Q94BP0 | Probable transcriptional regulator SLK2 |
| 140 | Q94F30 | Ubiquitin-like-specific protease ESD4 |
| 141 | Q94FL9 | Transcription factor MYB3R-4 |
| 142 | Q94JY0 | Protein IN CHLOROPLAST ATPASE BIOGENESIS, Chlroplastic |
| 143 | Q96318 | 12S seed storage protein CRC |
| 144 | Q96524 | Cryptochrome-2 |
| 145 | Q9C5H5 | Mitogen-activated protein kinase kinase kinase 5 |
| 146 | Q9C5X3 | SNARE-interacting protein KEULE |
| 147 | Q9C6B8 | Auxin efflux carrier component 1 |
| 148 | Q9C6W5 | ABC transporter G family member 14 |
| 149 | Q9C8W3 | Alpha-(1,4)-fucosyltransferase |
| 150 | Q9C9W5 | Glycerate dehydrogenase HPR, peroxisomal |
| 151 | Q9CA60 | Cytochrome P450 98A9 |
| 152 | Q9FG68 | Transcription factor RAX1 |
| 153 | Q9FGR9 | KDEL-tailed cysteine endopeptidase CEP1 |
| 154 | Q9FI43 | Calcium-dependent mitochondrial ATP-magnesium/phosphate carrier protein 2 |
| 155 | Q9FIE3 | Protein VERNALIZATION INSENSITIVE 3 |
| 156 | Q9FJX0 | Peptidyl-prolyl cis-trans isomerase CYP65 |
| 157 | Q9FKA0 | NAC domain-containing protein 92 |
| 158 | Q9FL94 | AT5G45030 protein |
| 159 | Q9FMX3 | Sugar transport protein 11 |
| 160 | Q9FT69 | ATP-dependent DNA helicase Q-like SIM |
| 161 | Q9FT74 | ATP-dependent DNA helicase Q-like 1 |
| 162 | Q9FX31 | Homeobox-leucine zipper protein HDG11 |
| 163 | Q9FXF2 | Probable LRR receptor-like serine/threonine-protein kinase RFK1 |
| 164 | Q9FY54 | Probable sucrose-phosphate synthase 2 |
| 165 | Q9FYC2 | Pheophorbide a oxygenase, chloroplastic |
| 166 | Q9FZ22 | Probable glycerol-3-phosphate acyltransferase 2 |
| 167 | Q9LD43 | Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha, chloroplastic |
| 168 | Q9LHF5 | VIN3-like protein 1 |
| 169 | Q9LHS7 | Glycerol-3-phosphate acyltransferase 7 |
| 170 | Q9LMT2 | Nuclear poly(A) polymerase 1 |

| | | |
|---|---|---|
| 171 | Q9LMX4 | Probable inactive purple acid phosphatase 1 |
| 172 | Q9LQT8 | DELLA protein GAI |
| 173 | Q9LT78 | Probable cysteine protease RD21C |
| 174 | Q9LUI3 | CRC domain-containing protein TS1 |
| 175 | Q9LUR0 | Condensin-2 complex subunit H2 |
| 176 | Q9LVH5 | |
| 177 | Q9LY09 | Tapetal oleosin GRP-17 |
| 178 | Q9LYW9 | DnaJ protein P58IPK homolog |
| 179 | Q9LZK5 | DnaJ protein ERDJ3B |
| 180 | Q9M0G4 | Tyrosine decarboxylase 2 |
| 181 | Q9M0M5 | Scarecrow-like protein 13 |
| 182 | Q9M2D9 | Transcription factor MYB17 |
| 183 | Q9M2E8 | Homeobox-leucine zipper protein HDG1 |
| 184 | Q9M304 | Long chain base biosynthesis protein 2b |
| 185 | Q9M8S6 | Potassium channel SKOR R |
| 186 | Q9MA44 | Signal peptide peptidase-like 5 |
| 187 | Q9MAU3 | Transcription initiation factor TFIID subunit 6 |
| 188 | Q9S7G7 | Transcription factor MYB3R-1 |
| 189 | Q9S7P5 | Squamosa promoter-binding-like protein 12 |
| 190 | Q9S840 | Squamosa promoter-binding-like protein 2 |
| 191 | Q9SAA9 | Sterol 14-demethylase |
| 192 | Q9SJ09 | Probable WRKY transcription factor 59 |
| 193 | Q9SLH3 | DELLA protein RGA |
| 194 | Q9SND6 | Mitogen-activated protein kinase kinase kinase 20 |
| 195 | Q9SW80 | BEL1-like homeodomain protein 2 |
| 196 | Q9SX27 | Transcription factor PERIANTHIA |
| 197 | Q9SX48 | Sugar transport protein 9 |
| 198 | Q9SYN5 | AT1G78460 protein |
| 199 | Q9XI62 | Probable apyrase 3 |
| 200 | Q9ZPS7 | Transmembrane 9 superfamily member 3 |
| 201 | Q9ZRF9 | Probable LRR receptor-like serine/threonine-protein kinase RPK1 |
| 202 | Q9ZTW3 | Vesicle-associated membrane protein 721 |
| 203 | Q9ZUU0 | WRKY transcription factor 44 |
| 204 | V5N411 | Alkaline/neutral invertase |

**Table 6.** List of proteins unique to female flowers.

| S.No. | Accession | Description |
|---|---|---|
| 1 | A0A067JLV2 | Squamosa promoter-binding-like protein |
| 2 | A0A067KIC4 | FRIGIDA-like protein |
| 3 | A0A067LBS6 | Uncharacterized protein |
| 4 | A0A178UAP8 | EMF1 |
| 5 | A0A178UMP1 | FRIGIDA-like protein |
| 6 | A0A178UR23 | Uncharacterized protein |
| 7 | A0A178UTB8 | (thale cress) hypothetical protein |
| 8 | A0A178VKX5 | Transcription factor |
| 9 | A0A1P8B7C3 | Protein DETOXIFICATION |
| 10 | A0A251KPG9 | Uncharacterized protein |
| 11 | A0A2C9VCZ5 | FRIGIDA-like protein |
| 12 | A0A2C9VMB5 | Uncharacterized protein |
| 13 | A0A2C9W8J0 | FRIGIDA-like protein |
| 14 | A0A2C9W9J7 | G_PROTEIN_RECP_F2_4 domain containing protein |
| 15 | A0A2I4R2G2 | CONSTANS |
| 16 | A0A410S7G0 | Ribulose bisphosphate carboxylase large chain (Fragment) |
| 17 | A0A5S9Y9Y0 | FRIGIDA-like protein |
| 18 | A0A6A6K650 | FRIGIDA-like protein |
| 19 | A0A6A6K8H7 | FRIGIDA-like protein |
| 20 | A0A6A6KB41 | FRIGIDA-like protein |
| 21 | A0A6A6LF16 | FRIGIDA-like protein |
| 22 | A0A6A6LHH7 | CCT domain-containing protein |
| 23 | A0A6A6LS23 | Homeobox domain-containing protein |
| 24 | A0A6A6LTW0 | Uncharacterized protein |
| 25 | A0A6A6NBP5 | Uncharacterized protein |
| 26 | A0A7G2F0T5 | FRIGIDA-like protein |
| 27 | A2RVQ5 | Agamous-like MADS-box protein AGL16 |
| 28 | A8MQS3 | AT4G36640 protein |
| 29 | A8MS68 | Dihydrolipoyl dehydrogenase 1, chloroplastic |
| 30 | B3DNN5 | Anaphase-promoting complex subunit 6 |
| 31 | B5X0I6 | Protein CTR9 homolog |
| 32 | B9DG09 | AT3G01980 protein |
| 33 | B9DG18 | Catalase |
| 34 | B9DGP1 | AT5G16150 protein |

| | | |
|---|---|---|
| 35 | B9DGR5 | AT1G08550 protein |
| 36 | B9DGU6 | AT2G20370 protein (Fragment) |
| 37 | B9DGW8 | AT2G45380 protein (Fragment) |
| 38 | B9DH26 | AT3G58520 protein |
| 39 | B9DH49 | AT3G56650 protein |
| 40 | B9DHN9 | AT4G36390 protein (Fragment) |
| 41 | B9DHR1 | Carboxypeptidase (Fragment) |
| 42 | B9DHU4 | Pectate lyase (Fragment) |
| 43 | B9DHU6 | AT4G26900 protein (Fragment) |
| 44 | B9DI40 | AT1G36730 protein (Fragment) |
| 45 | B9DI51 | Carbamoyl-phosphate synthase (glutamine-hydrolyzing) (Fragment) |
| 46 | B9DI66 | AT4G04640 protein (Fragment) |
| 47 | B9RDH3 | Gibberellin 20-oxidase, putative |
| 48 | B9REW4 | FRIGIDA-like protein |
| 49 | B9RKT2 | FRIGIDA-like protein |
| 50 | B9RNR3 | Salt-tolerance protein, putative |
| 51 | B9SSS8 | FRIGIDA-like protein |
| 52 | C0LGE4 | Probable LRR receptor-like serine/threonine-protein kinase At1g12460 |
| 53 | C0LGW6 | LRR receptor-like serine/threonine-protein kinase ERL1 |
| 54 | C0Z2C6 | AT1G09310 protein |
| 55 | C0Z2I3 | UDP-glucuronate decarboxylase |
| 56 | C0Z2J2 | AT2G19940 protein |
| 57 | C0Z2J9 | AT5G64240 protein |
| 58 | C0Z2N2 | AT1G57750 protein |
| 59 | C0Z2P9 | AT1G27700 protein |
| 60 | C0Z2X0 | AT4G24220 protein |
| 61 | C0Z338 | AT5G47720 protein |
| 62 | C0Z348 | Glycerophosphodiester phosphodiesterase |
| 63 | C0Z373 | AT3G15095 protein |
| 64 | F4HQA1 | Protein PAF1 homolog |
| 65 | F4ICF4 | RHOMB ID like protein 10, Chloroplastic |
| 66 | F4IMQ0 | Protein FLC EXPRESS OR |
| 67 | F4JLP5 | Dihydrolipoyl dehydrogenase 2, chloroplastic |
| 68 | F4JT98 | Probable transcriptional regulator SLK3 |
| 69 | F4JUD2 | Transcriptional corepressor LEUNIG |
| 70 | F4KD43 | Cyclin family protein |

| | | |
|---|---|---|
| 71 | F4KGW7 | AINTEGUMENTA-like 6 |
| 72 | K4K7I4 | NAD(P)H-quinone oxidoreductase subunit 5, chloroplastic (Fragment) |
| 73 | K4KCR0 | NAD(P)H-quinone oxidoreductase subunit 1 (Fragment) |
| 74 | K4KCX2 | PSI-J (Fragment) |
| 75 | K4KSE8 | DNA-directed RNA polymerase subunit (Fragment) |
| 76 | O04017 | Protein CUP-SHAPED COTYLEDON 2 |
| 77 | O04197 | Coronatine-insensitive protein 1 |
| 78 | O04425 | Flowering time control protein FCA |
| 79 | O22040 | Mitogen-activated protein kinase kinase kinase ANP1 |
| 80 | O22207 | Ubiquitin carboxyl-terminal hydrolase 5 |
| 81 | O22256 | Probable pectinesterase/pectinesterase inhibitor 20 |
| 82 | O23244 | Purple acid phosphatase 25 |
| 83 | O23264 | Selenium-binding protein 1 |
| 84 | O23277 | Protein PUTATIVE RECOMBINATION DEFECT 1 |
| 85 | O49139 | Putative DNA (cytosine-5)-methyltransferase CMT1 |
| 86 | O64470 | Spermidine hydroxycinnamoyl transferase |
| 87 | O65187 | Myrosinase-binding protein 1 |
| 88 | O80588 | Protein END |
| 89 | O80592 | Amino acid permease 8 |
| 90 | O80939 | L-type lectin-domain containing receptor kinase IV.1 |
| 91 | O82239 | E3 ubiquitin-protein ligase RFI2 |
| 92 | O82302 | Protein phosphatase 2C 29 |
| 93 | P23321 | Oxygen-evolving enhancer protein 1-1, chloroplastic |
| 94 | P29385 | Agamous-like MADS-box protein AGL5 |
| 95 | P46309 | Glutamate--cysteine ligase, chloroplastic |
| 96 | P46689 | Gibberellin-regulated protein 1 |
| 97 | P47927 | Floral homeotic protein APETALA 2 |
| 98 | P54873 | Hydroxymethylglutaryl-CoA synthase |
| 99 | P56765 | Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic |
| 100 | P61837 | Aquaporin PIP1-1 |
| 101 | Q03250 | Glycine-rich RNA-binding protein 7 |
| 102 | Q05085 | Protein NRT1/ PTR FAMILY 6.3 |
| 103 | Q05753 | Ankyrin repeat domain-containing protein, chloroplastic |
| 104 | Q05999 | Serine/threonine-protein kinase D6PKL3 |
| 105 | Q08891 | Fatty acyl-CoA reductase 2, chloroplastic |
| 106 | Q0WMY3 | AT4G37190 protein |

| | | |
|---|---|---|
| 107 | Q0WVM7 | Probable transcriptional regulator SLK1 |
| 108 | Q0WW55 | Thymidylate kinase |
| 109 | Q1PEM5 | Proline-rich receptor-like protein kinase PERK3 |
| 110 | Q38833 | Chlorophyll synthase, chloroplastic |
| 111 | Q38847 | Agamous-like MADS-box protein AGL15 |
| 112 | Q38851 | Transcription repressor MYB6 |
| 113 | Q38858 | Calreticulin-2 |
| 114 | Q38895 | Transcriptional regulator SUPERMAN |
| 115 | Q38896 | Cold shock domain-containing protein 4 |
| 116 | Q38914 | AP2-like ethylene-responsive transcription factor ANT |
| 117 | Q38924 | Fe(3+)-Zn(2+) purple acid phosphatase 12 |
| 118 | Q38960 | WD repeat-containing protein LWD2 |
| 119 | Q39072 | Cyclin-B1-5 |
| 120 | Q3EAI1 | Transcription factor bHLH60 |
| 121 | Q42191 | Mitochondrial inner membrane protein OXA1 |
| 122 | Q42371 | LRR receptor-like serine/threonine-protein kinase ERECTA |
| 123 | Q49C22 | Maturase K |
| 124 | Q4VMJ5 | PHYC (Fragment) |
| 125 | Q56Z20 | AT2G20420 protein (Fragment) |
| 126 | Q5MAU8 | Probable inactive purple acid phosphatase 27 |
| 127 | Q5MFV6 | Probable pectinesterase/pectinesterase inhibitor VGDH2 |
| 128 | Q5MFV8 | Pectinesterase 5 |
| 129 | Q5XF07 | DNA-(apurinic or apyrimidinic site) endonuclease |
| 130 | Q680Q4 | E3 SUM |
| 131 | Q6E7H0 | Origin of replication complex subunit 3 |
| 132 | Q6EWX0 | Origin of replication complex subunit 3 |
| 133 | Q7Y201 | Probable pectinesterase/pectinesterase inhibitor 13 |
| 134 | Q84J95 | Gibberellin-regulated protein 5 |
| 135 | Q84JU4 | Protein-tyrosine-phosphatase IBR5 |
| 136 | Q84JX1 | Probable pectinesterase/pectinesterase inhibitor 19 |
| 137 | Q84L32 | Probable ubiquitin receptor RAD23a |
| 138 | Q84L33 | Ubiquitin receptor RAD23b |
| 139 | Q84LR6 | Probable inactive purple acid phosphatase 14 |
| 140 | Q84TF0 | Aldo-keto reductase family 4 member C10 |
| 141 | Q84UU4 | Alpha-humulene/(-)-(E)-beta-caryophyllene synthase |
| 142 | Q84W92 | Probable histone-arginine methyltransferase 1.3 |
| 143 | Q84WW6 | Histone-lysine N-methyltransferase ASHH1 |

| | | |
|---|---|---|
| 144 | Q8GXF0 | DNA repair protein RAD51 homolog 3 |
| 145 | Q8GXW1 | DELLA protein RGL2 |
| 146 | Q8L7G0 | Auxin response factor 1 |
| 147 | Q8L8A7 | Growth-regulating factor 4 |
| 148 | Q8L9T5 | RING-H2 finger protein ATL2 |
| 149 | Q8LAA6 | Probable aquaporin PIP1-5 |
| 150 | Q8LAZ7 | COP9 Signalosome complex subunit 5a |
| 151 | Q8RW96 | Serine/threonine protein phosphatase 2A 59 kDa regulatory subunit B' gamma isoform |
| 152 | Q8RWZ1 | Protein STRUBBELIG |
| 153 | Q8RX28 | Histone deacetylase 5 |
| 154 | Q8RX86 | Alpha-galactosidase 2 |
| 155 | Q8S9L0 | Squamosa promoter-binding-like protein 10 |
| 156 | Q8VYZ2 | Purple acid phosphatase 8 |
| 157 | Q8W4P1 | Cyclin-dependent kinase C-2 |
| 158 | Q93WT0 | Probable WRKY transcription factor 31 |
| 159 | Q93ZQ9 | AT1G07840 protein |
| 160 | Q944Q0 | Serine/threonine-protein kinase WNK8 |
| 161 | Q946Y7 | Syntaxin-61 |
| 162 | Q94BT6 | Adagio protein 1 |
| 163 | Q94CE5 | Gamma-aminobutyrate transaminase POP2, mitochondrial |
| 164 | Q94KD3 | Vacuolar protein sorting-associated protein 52 A |
| 165 | Q9C5G0 | Protein SUPPRESSOR F FRI4 |
| 166 | Q9C5Q8 | Small RNA 2'- |
| 167 | Q9C660 | Proline-rich receptor-like protein kinase PERK10 |
| 168 | Q9C717 | Protein FLX-like 3 |
| 169 | Q9C788 | Cytochrome P450 704B1 |
| 170 | Q9C983 | Probable WRKY transcription factor 57 |
| 171 | Q9C9B4 | AT1G73930 protein |
| 172 | Q9C9K7 | AT-hook motif nuclear-localized protein 29 |
| 173 | Q9CAA4 | Transcription factor BIM2 |
| 174 | Q9FFH0 | Transcription activator GLK2 |
| 175 | Q9FFW5 | Proline-rich receptor-like protein kinase PERK8 |
| 176 | Q9FGV1 | Auxin response factor 8 |
| 177 | Q9FH40 | Transcription initiation factor TFIID subunit 14b |
| 178 | Q9FH76 | Abscisic acid 8'-hydroxylase 3 |
| 179 | Q9FJH5 | Heavy metal-associated isoprenylated plant protein 3 |

| | | |
|---|---|---|
| 180 | Q9FJV0 | Mitogen-activated protein kinase kinase 6 |
| 181 | Q9FL33 | DNA replication licensing factor MCM3 |
| 182 | Q9FL59 | FT-interacting protein 1 |
| 183 | Q9FM79 | Pectinesterase QRT1 |
| 184 | Q9FM96 | Glucosidase 2 subunit beta |
| 185 | Q9FMH8 | Probable cysteine protease RD21B |
| 186 | Q9FMK9 | Probable inactive purple acid phosphatase 29 |
| 187 | Q9FV70 | Transcription factor E2FC |
| 188 | Q9FYG7 | Transcription factor TCP1 |
| 189 | Q9FZK0 | Squamosa promoter-binding-like protein 11 |
| 190 | Q9LJR7 | Xyloglucan endotransglucosylase/hydrolase protein 3 |
| 191 | Q9LJU7 | Purple acid phosphatase 18 |
| 192 | Q9LK03 | Proline-rich receptor-like protein kinase PERK2 |
| 193 | Q9LKL2 | Two-component response regulator-like APRR1 |
| 194 | Q9LMA8 | Protein TIFY 10A |
| 195 | Q9LMM0 | Glycerol-3-phosphate 2-O-acyltransferase 4 |
| 196 | Q9LS97 | AT3G18790 protein |
| 197 | Q9LSF8 | Dimethylnonatriene synthase |
| 198 | Q9LTZ9 | Galactan beta-1,4-galactosyltransferase GALS2 |
| 199 | Q9LV09 | Protein BOBBER 1 |
| 200 | Q9LV27 | Target of rapamycin complex subunit LST8-1 |
| 201 | Q9LV48 | Proline-rich receptor-like protein kinase PERK1 |
| 202 | Q9LVJ1 | Subtilisin-like protease SBT1.4 |
| 203 | Q9LXI7 | Probable purple acid phosphatase 20 |
| 204 | Q9LYN8 | Leucine-rich repeat receptor protein kinase EMS1 |
| 205 | Q9LZK4 | Transcription factor MYB11 |
| 206 | Q9M024 | Thylakoid ADP,ATP carrier protein, chloroplastic |
| 207 | Q9M365 | Probable F-box protein At3g61730 |
| 208 | Q9M7I7 | Chlorophyllase-2 |
| 209 | Q9M8K6 | Transcription factor MUTE |
| 210 | Q9M8Z5 | Guanine nucleotide-binding protein-like NSN1 |
| 211 | Q9M9L3 | Nuclear envelope-associated protein 1 |
| 212 | Q9MAH8 | Transcription factor TCP3 |
| 213 | Q9MAN1 | B3 domain-containing transcription factor NGA3 |
| 214 | Q9S784 | HVA22-like protein c |
| 215 | Q9S7C9 | AT-hook motif nuclear-localized protein 27 |
| 216 | Q9S7T5 | Transcription repressor OFP14 |

| | | |
|---|---|---|
| 217 | Q9SE43 | Homeobox-leucine zipper protein REVOLUTA |
| 218 | Q9SFG0 | Sugar transport protein 6 |
| 219 | Q9SFG6 | Protein FANTASTIC FOUR 4 |
| 220 | Q9SGW3 | 26S proteasome non-ATPase regulatory subunit 8 homolog A |
| 221 | Q9SHJ5 | Glycerol-3-phosphate acyltransferase 1 |
| 222 | Q9SI37 | WRKY transcription factor 1 |
| 223 | Q9SIV9 | Purple acid phosphatase 10 |
| 224 | Q9SJ45 | Transcription repressor OFP15 |
| 225 | Q9SKI4 | Replication protein A 70 kDa DNA-binding subunit A |
| 226 | Q9SL42 | Peptidyl-prolyl cis-trans isomerase Pin1 |
| 227 | Q9SMY6 | Putative pectinesterase/pectinesterase inhibitor 45 |
| 228 | Q9SQI2 | Protein GIGANTEA |
| 229 | Q9SR79 | Probable inactive purple acid phosphatase 16 |
| 230 | Q9STG9 | Amidophosphoribosyltransferase 2, chloroplastic |
| 231 | Q9STP8 | Acyl-CoA-binding domain-containing protein 2 |
| 232 | Q9SU24 | Origin of replication complex subunit 1B |
| 233 | Q9SUR6 | Cystine lyase COR13 |
| 234 | Q9SUS1 | Probable WRKY transcription factor 29 |
| 235 | Q9SVS8 | Gibberellin 3-beta-dioxygenase 3 |
| 236 | Q9SX31 | Proline-rich receptor-like protein kinase PERK9 |
| 237 | Q9SZQ5 | WD repeat-containing protein VIP3 |
| 238 | Q9SZR9 | ABC transporter G family member 9 |
| 239 | Q9XFB1 | Axial regulator YABBY 3 |
| 240 | Q9XGX0 | Protein SHORT INTERNODES |
| 241 | Q9XI19 | PLASTID TRANSCRIPTIONALLYT ACTIVE protein6, Chloroplastic |
| 242 | Q9XIN7 | NAC domain-containing protein 40 |
| 243 | Q9ZPI6 | Peroxisomal fatty acid beta-oxidation multifunctional protein AIM1 |
| 244 | Q9ZU34 | Actin-interacting protein 1-1 |
| 245 | Q9ZU49 | Lipid phosphate phosphatase 1 |

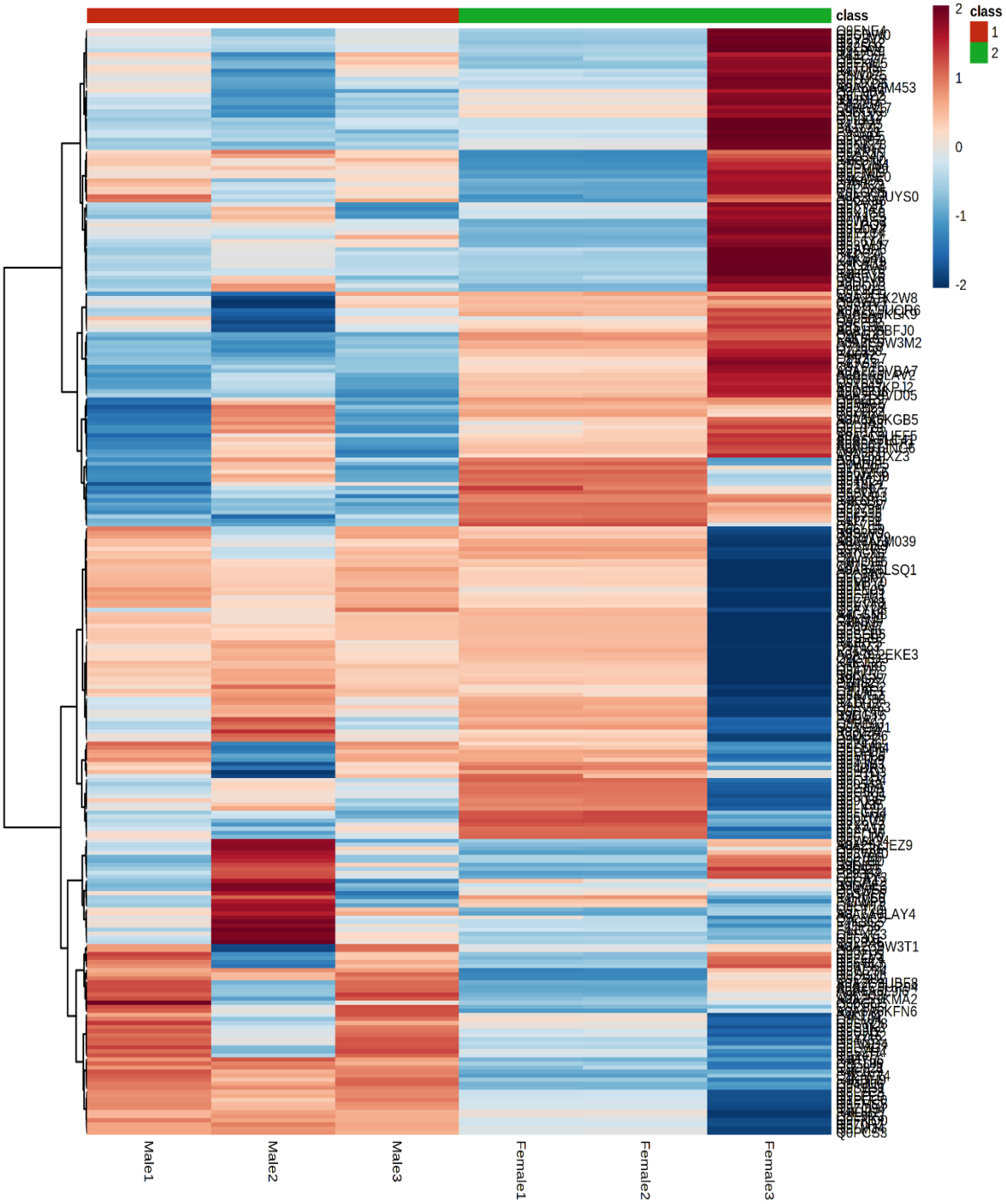


**Figure 51.** Hierarchical Clustering analysis. Heatmap of 274 commonly expressed proteins in Putranjiva male and female flowers providing an intuitive visualization of a data table. Each colored cell on the map corresponds to a concentration value in the data table, with proteins in rows and samples in columns.

### 3.4.2.2. Differential expression analysis

From differential protein expression analysis, 76 proteins out of 274 common proteins were found to be significantly differentially expressed (with fold change ≥1.5 and P ≤ 0.05) between female and male flowers. Of these, 45 proteins were significantly upregulated and 31 proteins were downregulated in female flowers when compared to male flowers (Figure 52).

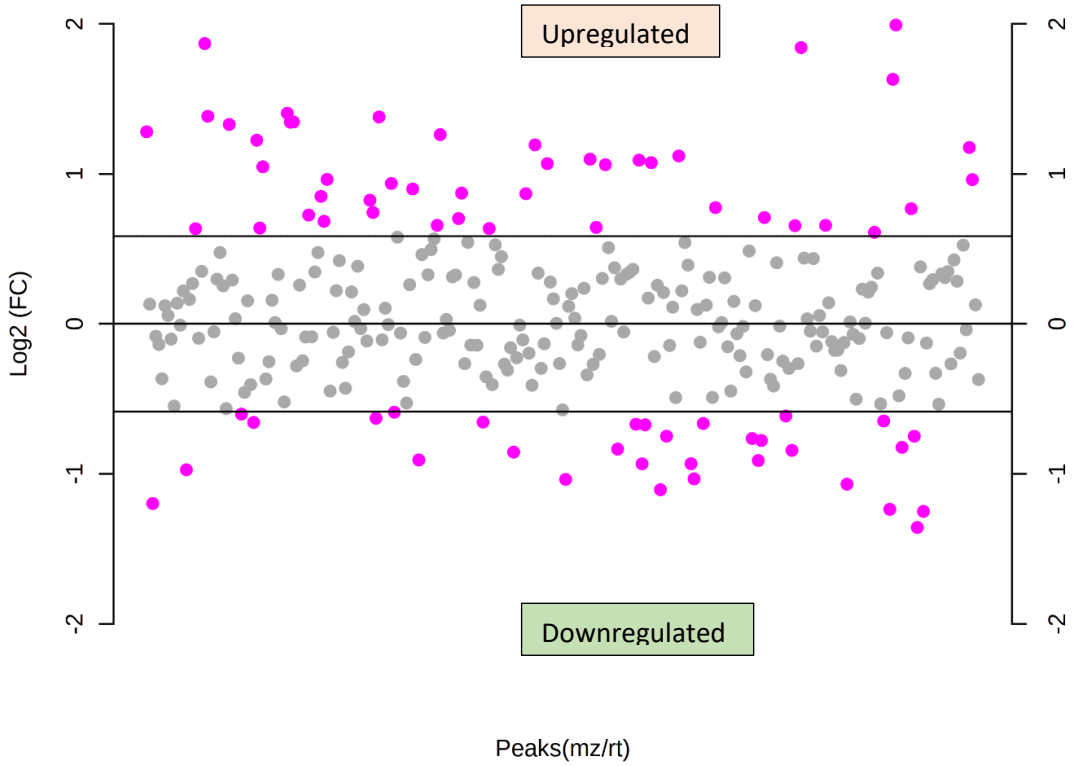


**Figure 52.** Fold Change (FC) Analysis to compare the absolute values of change between two group means (Female and male proteins). From the graph, proteins falling within positive log2 value range were shown upregulated and within negative log2 value range were shown downregulated. Proteins whose FCs are beyond the given FC threshold (fold change ≥1.5 and P ≤ 0.05; either up or down) were considered significant.

**Table 7.** Table showing the list of upregulated and downregulated proteins in female flowers compared to male flowers

| Accession | Protein name | Fold Change | log2(FC) |
|---|---|---|---|
| **Upregulated proteins** | | | |
| F4JVI3 | Transcription termination factor MTERF5, chloroplastic | 3.9788 | 1.9923 |
| Q2V3V8 | AT3G14870 protein | 3.6525 | 1.8689 |
| O23447 | Putative pectinesterase/pectinesterase inhibitor 43 | 3.5852 | 1.8421 |
| Q3E9B4 | Transcription repressor OFP8 | 3.0955 | 1.6302 |
| O82387 | Cell division control protein 6 homolog | 2.6466 | 1.4041 |
| B9DGQ8 | AT3G27570 protein (Fragment) | 2.6098 | 1.384 |
| P43254 | E3 ubiquitin-protein ligase COP1 | 2.6018 | 1.3795 |
| J9UMU3 | Cell wall invertase | 2.5428 | 1.3464 |
| Q9LHS5 | Cell division related protein-like | 2.5396 | 1.3446 |
| Q9FGX1 | ATP-citrate synthase beta chain protein 2 | 2.5131 | 1.3295 |
| Q9T0J9 | (Z)-gamma-bisabolene synthase 1 | 2.4294 | 1.2806 |
| Q39112 | Gibberellin 20 oxidase 3 | 2.3977 | 1.2617 |
| O82799 | B3 domain-containing transcription factor NGA1 | 2.3364 | 1.2243 |
| Q8LNZ2 | Kinesin-like protein KIN-7B | 2.287 | 1.1934 |
| Q5KS41 | Wax ester synthase/diacylglycerol acyltransferase 11 | 2.2597 | 1.1761 |
| Q8S8P5 | Probable WRKY transcription factor 33 | 2.1726 | 1.1194 |
| Q68LV8 | Maturase (Fragment) | 2.1408 | 1.0981 |
| F4J9M5 | Probable ATP-dependent DNA helicase CHR12 | 2.1311 | 1.0916 |
| Q9LY17 | Probable pectinesterase 50 | 2.1061 | 1.0746 |
| O22898 | Long chain acyl-CoA synthetase 1 | 2.0968 | 1.0682 |
| Q710E8 | Origin of replication complex subunit 1A | 2.0866 | 1.0612 |
| O82595 | B3 domain-containing transcription factor NGA4 | 2.0669 | 1.0475 |
| Q42569 | Cytochrome P450 90A1 | 1.9497 | 0.96329 |
| Q9LPV9 | WD repeat-containing protein LWD1 | 1.9478 | 0.96181 |
| Q9LPV8 | Eukaryotic peptide chain release factor subunit 1-2 | 1.9137 | 0.93634 |

| | | | |
|---|---|---|---|
| Q5XV31 | FRIGIDA-like protein 5 | 1.8657 | 0.89974 |
| F4K6B6 | Heat shock protein 81-2 | 1.8303 | 0.87209 |
| F4JY24 | ISWI chromatin-remodeling complex ATPase CHR17 | 1.825 | 0.86791 |
| K4KAR9 | Cytochrome b6 (Fragment) | 1.8029 | 0.85032 |
| Q8H0V4 | DUF724 domain-containing protein 7 | 1.7711 | 0.82462 |
| F4HRT5 | Protein CROWDED NUCLEI 1 | 1.7114 | 0.77518 |
| Q9SDW0 | Trihelix transcription factor GT-3a | 1.7025 | 0.76764 |
| Q8RXD6 | E3 ubiquitin-protein ligase BRE1-like 1 | 1.6734 | 0.74275 |
| Q8LGH4 | Cullin-4 | 1.6535 | 0.72556 |
| Q0WW17 | Protein REDUCED WALL ACETYLATION 2 | 1.6347 | 0.709 |
| Q9ZQ12 | Growth-regulating factor 6 | 1.627 | 0.70221 |
| Q6NKZ8 | Cytochrome P450 714A2 | 1.6066 | 0.68397 |
| B9SG32 | FRIGIDA-like protein | 1.5771 | 0.65728 |
| Q8VZG7 | Ribonuclease TUDOR 1 | 1.5767 | 0.65692 |
| Q8GZA8 | Protein ULTRAPETALA 1 | 1.5745 | 0.65486 |
| Q9M4A1 | Meiotic recombination protein SPO11-2 | 1.562 | 0.64335 |
| Q9M268 | B3 domain-containing transcription factor NGA2 | 1.557 | 0.63879 |
| P93831 | Histone-lysine N-methyltransferase CLF | 1.5535 | 0.63557 |
| Q9SJV6 | AT2G01100 protein | 1.5527 | 0.63477 |
| Q9MAP7 | Subtilisin-like protease SBT3.5 | 1.5267 | 0.6104 |
| **Downregulated proteins** | | | |
| Q9LMJ4 | Exocyst complex component EXO70B2 | 0.66495 | -0.58867 |
| Q9FT70 | ATP-dependent DNA helicase Q-like 4B | 0.6591 | -0.60143 |
| Q5XPJ6 | Protein SCAR4 | 0.65347 | -0.6138 |
| Q9C895 | E3 ubiquitin-protein ligase BRE1-like 2 | 0.64658 | -0.6291 |
| Q9XI07 | SWI/SNF complex subunit SWI3C | 0.63847 | -0.64732 |
| Q2LAE1 | Histone-lysine N-methyltransferase ASHH2 | 0.63524 | -0.65462 |
| Q9ZTX8 | Auxin response factor 6 | 0.63419 | -0.65702 |
| Q8RXD4 | Protein BREAST CANCER SUSCEPTIBILITY 1 homolog | 0.6311 | -0.66405 |
| F4K2E9 | Pre-mRNA-splicing factor ATP-dependent RNA helicase DEAH7 | 0.62899 | -0.66888 |
| Q9LMG7 | Probable inactive purple acid phosphatase 2 | 0.62737 | -0.67262 |

| | | | |
|---|---|---|---|
| Q9M8Y0 | Probable UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase SEC | 0.59518 | -0.74861 |
| Q8L5R3 | Tubulin-folding cofactor D | 0.59498 | -0.74908 |
| Q9FNQ0 | Protein LEO1 homolog | 0.58883 | -0.76407 |
| Q8L7C8 | Protein REDUCED WALL ACETYLATION 1 | 0.58343 | -0.77738 |
| Q8W234 | Transcriptional corepressor SEUSS | 0.56526 | -0.82301 |
| Q9FMS5 | PHD finger protein MALE STERILITY 1 | 0.56076 | -0.83456 |
| Q9C5P7 | Protein transport Sec1a | 0.55754 | -0.84286 |
| Q9FFH1 | Homeobox-DDT domain protein RLT2 | 0.55293 | -0.85483 |
| A0A6A6LAY4 | FRIGIDA-like protein | 0.53336 | -0.90681 |
| F4IDB2 | Protein phytochrome-dependent late-flowering | 0.53187 | -0.91086 |
| Q9C821 | Proline-rich receptor-like protein kinase PERK15 | 0.52414 | -0.93199 |
| Q9FN05 | Probable glucan 1,3-alpha-glucosidase | 0.52391 | -0.93262 |
| Q8VZR2 | Alpha-L-arabinofuranosidase 2 | 0.50958 | -0.97262 |
| Q8GWT4 | Protein arginine N-methyltransferase 1.5 | 0.48872 | -1.0329 |
| O64752 | Lysine-specific demethylase JMJ15 | 0.48743 | -1.0367 |
| Q9LFE0 | SART-1 family protein DOT2 | 0.47686 | -1.0684 |
| Q1PEC0 | Probable pectinesterase/pectinesterase inhibitor 42 | 0.46479 | -1.1054 |
| Q9SBJ1 | Pyruvate dehydrogenase (acetyl-transferring)] kinase, (mito) | 0.43606 | -1.1974 |
| Q570R7 | Transcription factor PIF7 | 0.42448 | -1.2362 |
| Q9ZQ94 | UDP-glycosyltransferase 73C5 | 0.42042 | -1.2501 |
| Q9SB51 | Ubiquitin carboxyl-terminal hydrolase 16 | 0.39046 | -1.3568 |

### 3.4.2.3. Functional classification of upregulated proteins

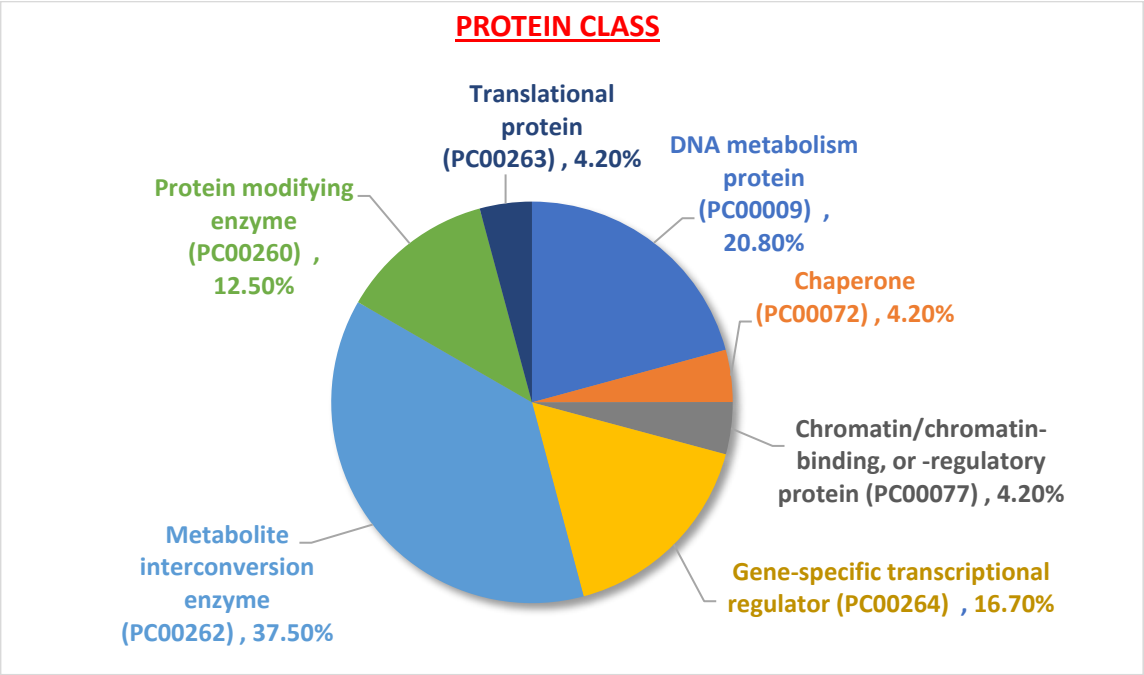


**Figure 53.** Pie chart showing the classification of upregulated proteins into different PANTHER protein classes (The input percentage was calculated on the basis of the number of proteins mapped to the protein class divided by the submitted list of upregulated proteins mapped to Arabidopsis thaliana genes from a whole genome)

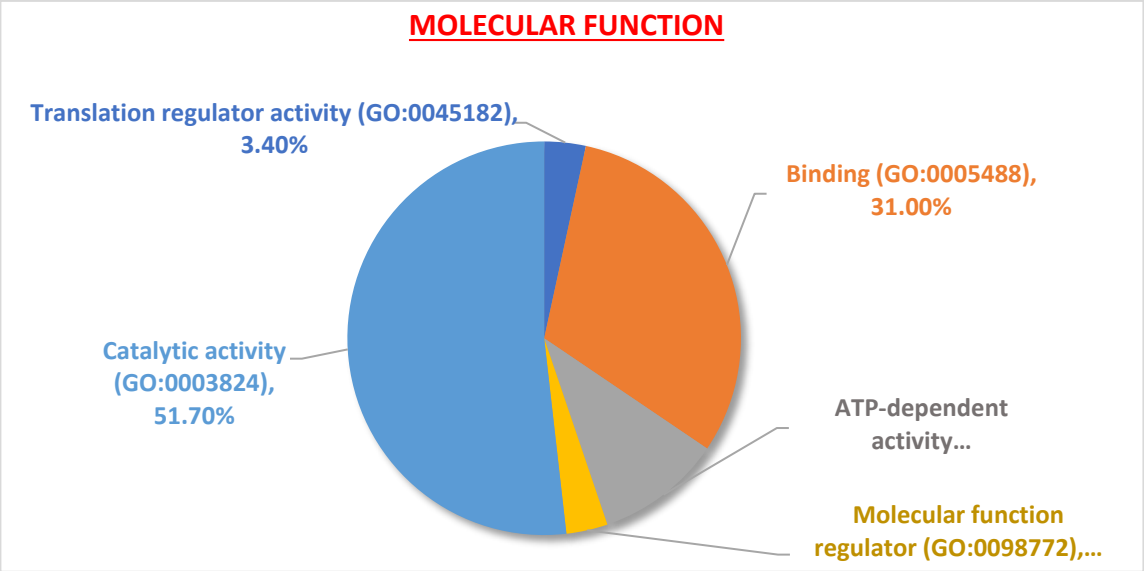
MOLECULAR FUNCTION
Translation regulator activity (GO:0045182), 3.40%
Binding (GO:0005488), 31.00%
Catalytic activity (GO:0003824), 51.70%
ATP-dependent activity...
Molecular function regulator (GO:0098772),...

**Fig. A.**

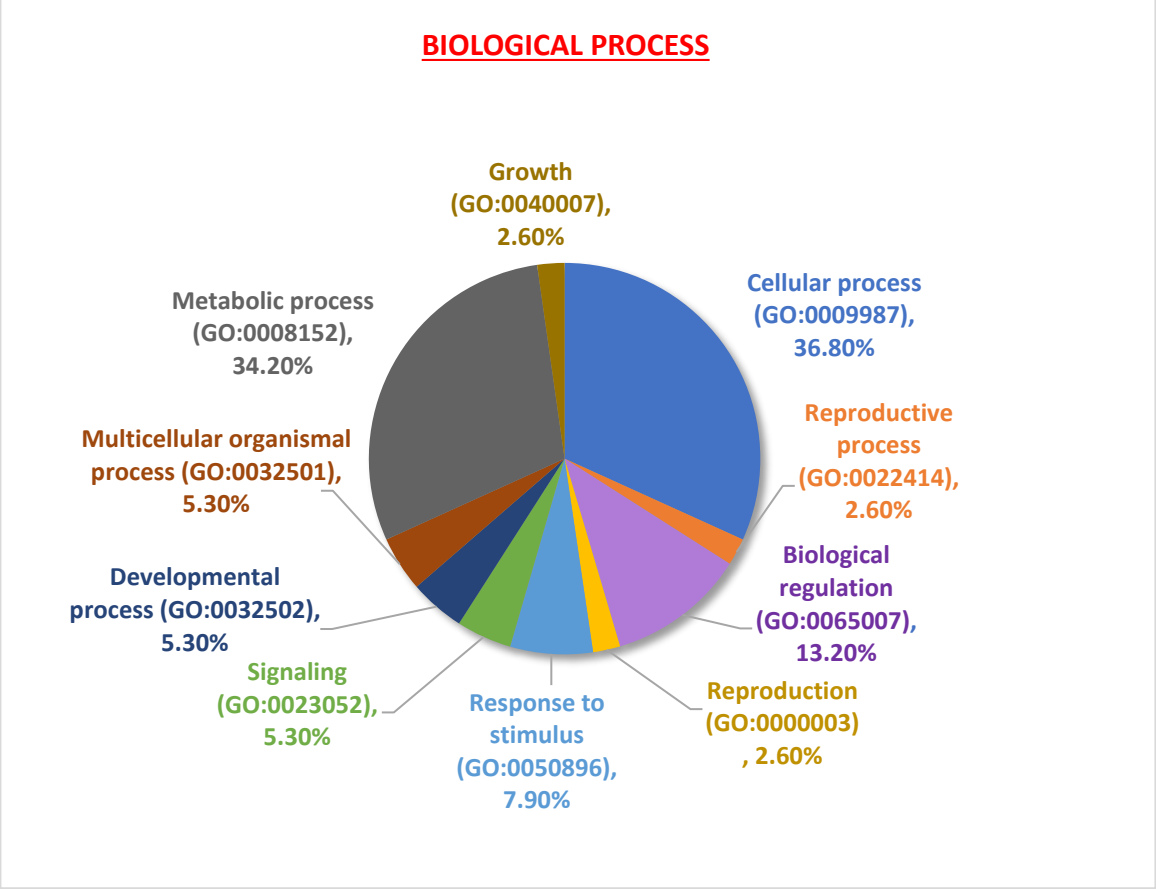
BIOLOGICAL PROCESS
Growth (GO:0040007), 2.60%
Cellular process (GO:0009987), 36.80%
Metabolic process (GO:0008152), 34.20%
Reproductive process (GO:0022414), 2.60%
Multicellular organismal process (GO:0032501), 5.30%
Biological regulation (GO:0065007), 13.20%
Developmental process (GO:0032502), 5.30%
Reproduction (GO:0000003), 2.60%
Signaling (GO:0023052), 5.30%
Response to stimulus (GO:0050896), 7.90%

**Fig. B.**

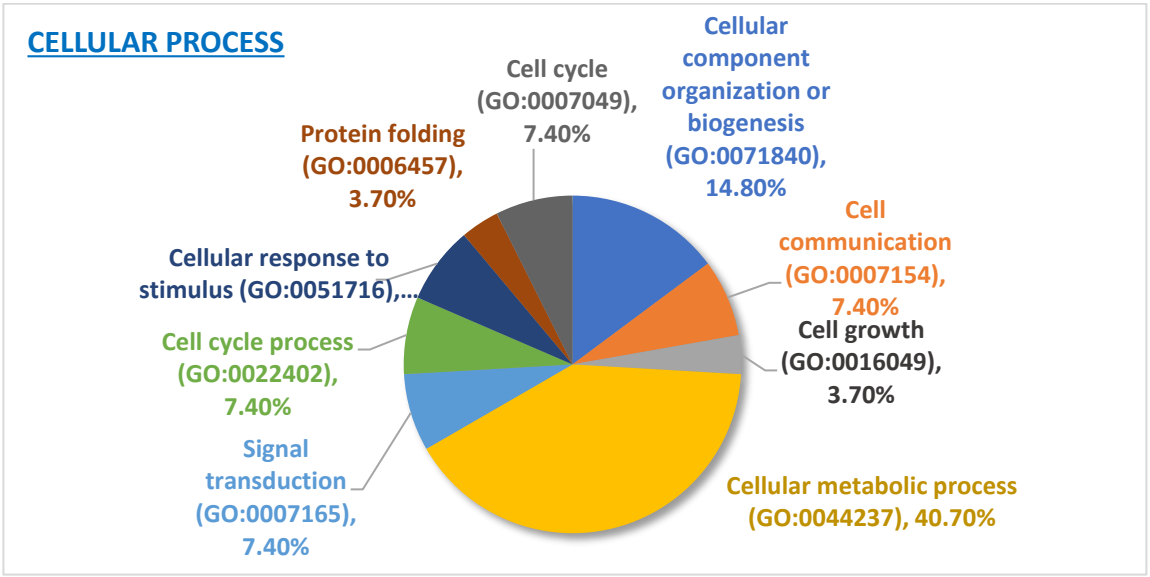
CELLULAR PROCESS
Cell cycle (GO:0007049), 7.40%
Cellular component organization or biogenesis (GO:0071840), 14.80%
Protein folding (GO:0006457), 3.70%
Cell communication (GO:0007154), 7.40%
Cellular response to stimulus (GO:0051716),...
Cell growth (GO:0016049), 3.70%
Cell cycle process (GO:0022402), 7.40%
Signal transduction (GO:0007165), 7.40%
Cellular metabolic process (GO:0044237), 40.70%

**Fig. C.**

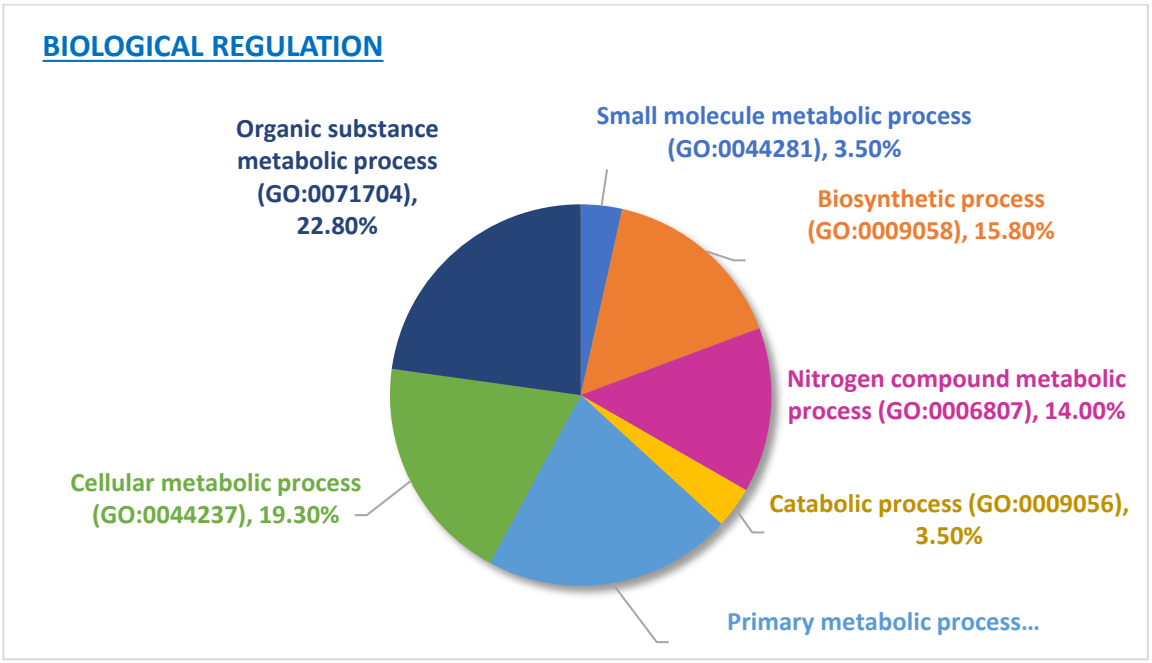
BIOLOGICAL REGULATION
Organic substance metabolic process (GO:0071704), 22.80%
Small molecule metabolic process (GO:0044281), 3.50%
Biosynthetic process (GO:0009058), 15.80%
Nitrogen compound metabolic process (GO:0006807), 14.00%
Cellular metabolic process (GO:0044237), 19.30%
Catabolic process (GO:0009056), 3.50%
Primary metabolic process...

**Fig. D.**

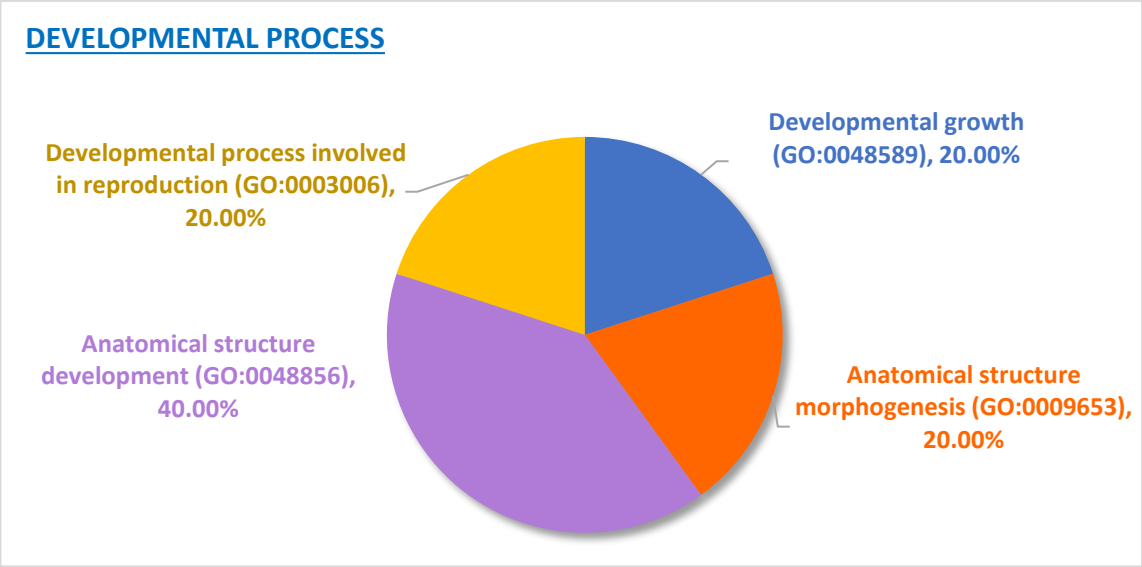


**Fig. E.**

**Figure 54.** Gene ontology (GO) analysis of the upregulated proteins using PANTHER classification system. GO terms were assigned to the 38 mapped proteins, with 6 of these being unclassified **A.** Molecular Function, **B.** Biological process. Sub-categories of biological process like **C.** Cellular process, **D.** Biological regulation, and **E.** Developmental process. (The input percentage was calculated on the basis of the number of proteins mapped to the GO term divided by the submitted list of upregulated proteins mapped to Arabidopsis thaliana genes from a whole genome).

Among upregulated proteins, metabolic interconversion enzymes (9 proteins) were the major class of proteins. The most common enzymes were hydrolases, ligases, oxidoreductases and transferases. Also, 5 proteins associated with DNA metabolism (DNA helicases, replication origin binding proteins and endodeoxyribonuclease) and 4 proteins with gene-specific transcription regulator activity (transcription factors) were detected. Protein modifying enzymes (3), chaperones (2), and chromatin binding proteins (2) were the other classes of proteins identified (Figure 53).

29 proteins could be assigned a molecular function. Molecular function analysis shows that the most of the upregulated proteins are involved in catalytic activity (specifically with hydrolase and transferase activity) and binding (nucleic acid binding, protein binding), as well as proteins involved in ATP dependent activity (ATP hydrolysis activity, ATP-dependent activity acting on DNA, long-chain fatty acid-CoA ligase activity), translation regulator activity (Fig. 54 A).

GO biological process ontology defines majority of the proteins to be involved in cellular process (14 proteins) and metabolic process (13 proteins). 5 proteins were found to be related to biological regulation. Interestingly, we could detect 4 proteins related to developmental process and multicellular organismal process that are associated with flower development, reproductive structure development, post-embryonic development, cell morphogenesis and shoot system development. Proteins annotated with reproduction, reproductive process, growth, and response to stimuli were the other classes of proteins identified (Fig. 54 B, C, D, E).

### 3.4.2.4. Functional classification of down regulated proteins

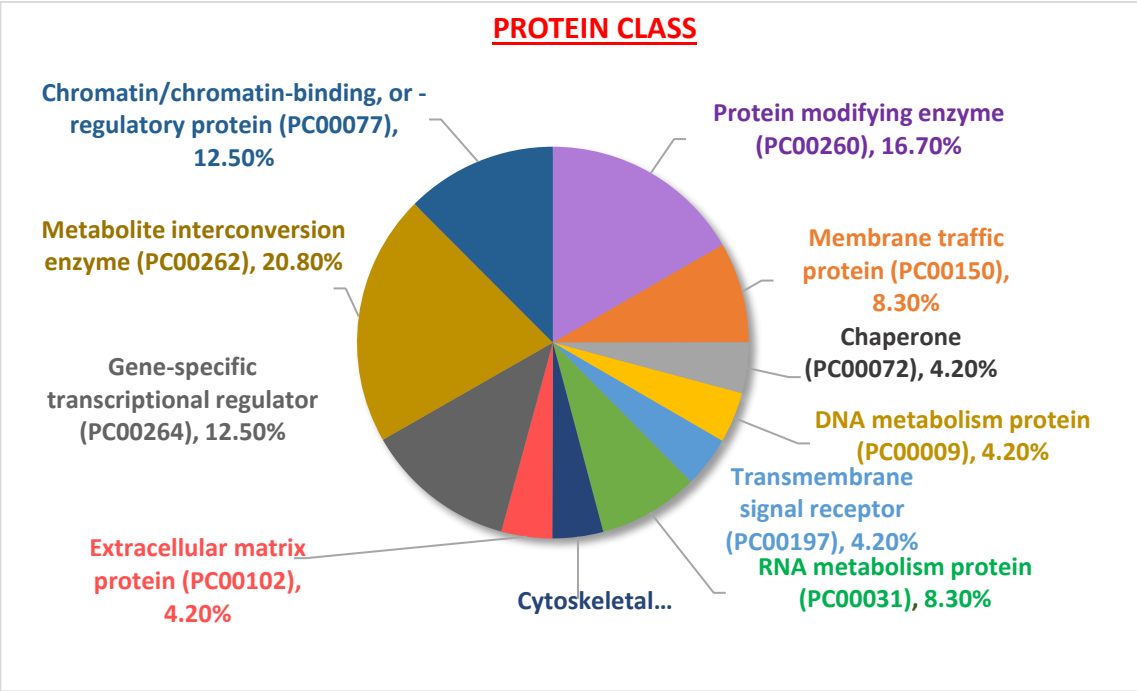


**Figure 55.** Pie chart showing the classification of upregulated proteins into different PANTHER protein classes (The input percentage was calculated on the basis of the number of proteins mapped to the protein class divided by the submitted list of upregulated proteins mapped to Arabidopsis thaliana genes from a whole genome).

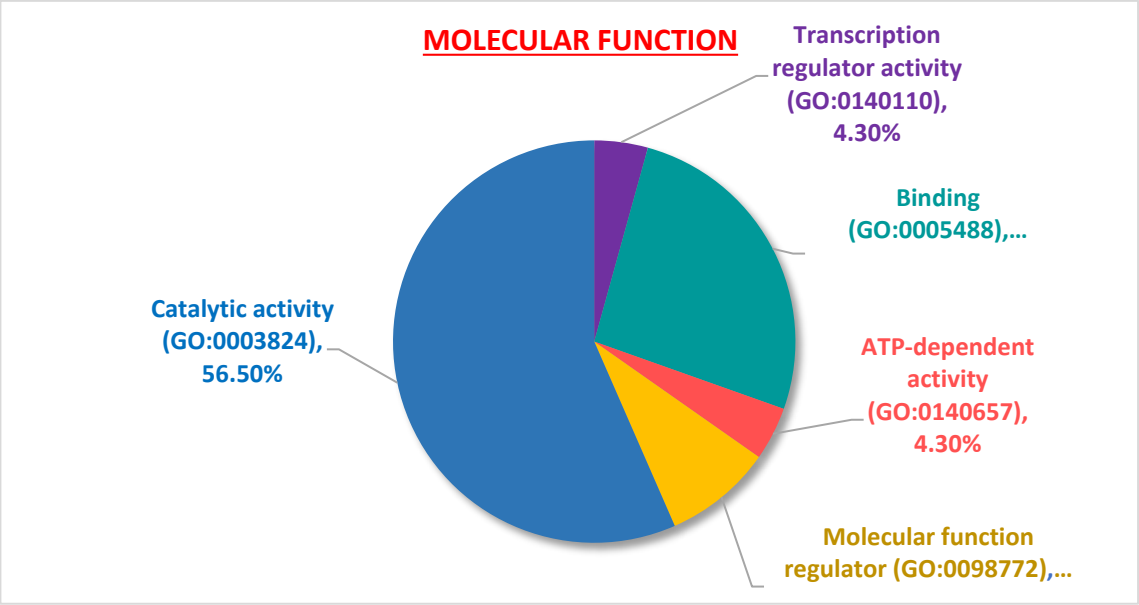
MOLECULAR FUNCTION
Transcription regulator activity (GO:0140110), 4.30%
Binding (GO:0005488),...
ATP-dependent activity (GO:0140657), 4.30%
Molecular function regulator (GO:0098772),...
Catalytic activity (GO:0003824), 56.50%

**Fig. A**

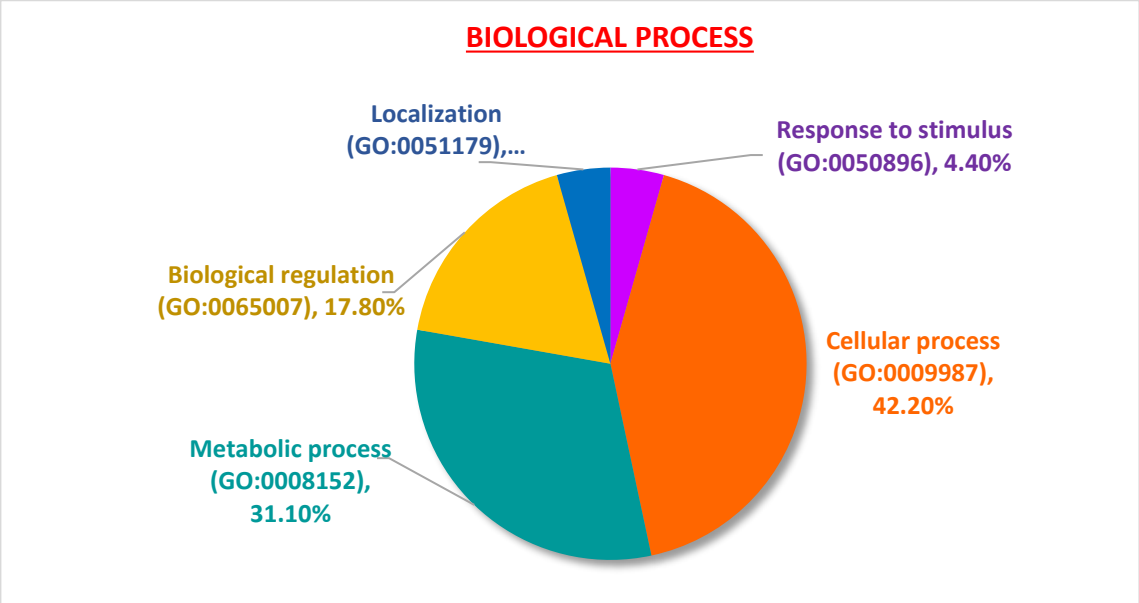
BIOLOGICAL PROCESS
Localization (GO:0051179),...
Response to stimulus (GO:0050896), 4.40%
Biological regulation (GO:0065007), 17.80%
Cellular process (GO:0009987), 42.20%
Metabolic process (GO:0008152), 31.10%

**Fig. B**

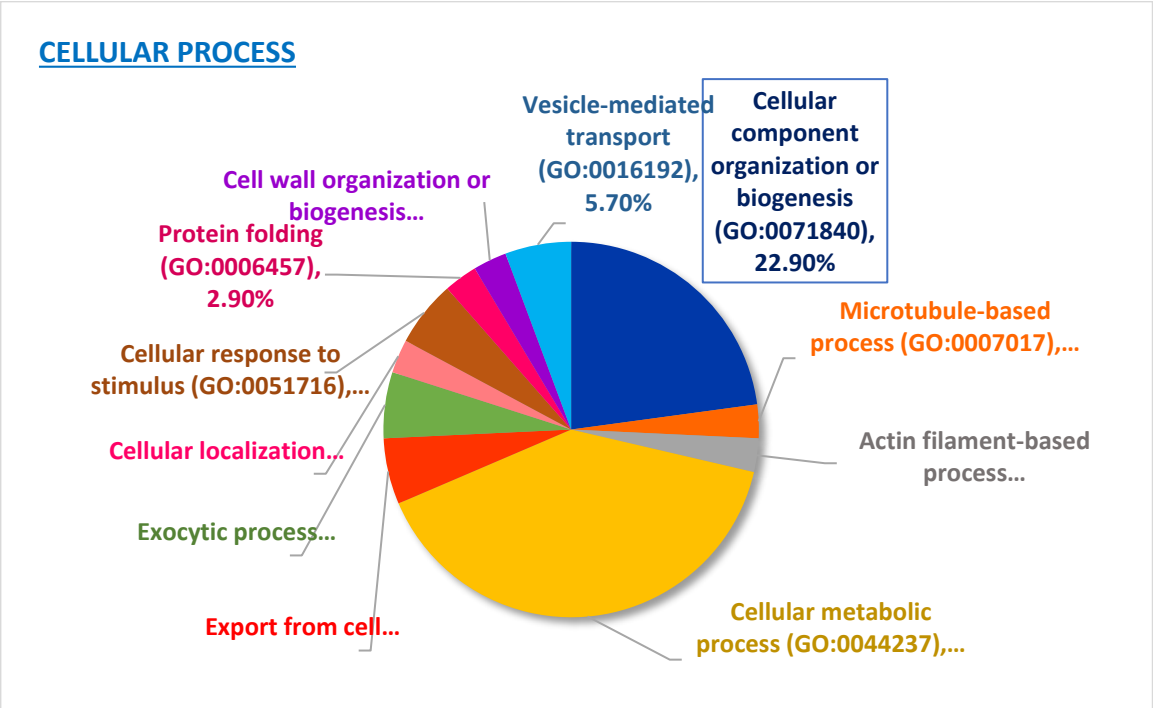
CELLULAR PROCESS
Vesicle-mediated transport (GO:0016192), 5.70%
Cellular component organization or biogenesis (GO:0071840), 22.90%
Cell wall organization or biogenesis...
Protein folding (GO:0006457), 2.90%
Microtubule-based process (GO:0007017),...
Cellular response to stimulus (GO:0051716),...
Actin filament-based process...
Cellular localization...
Exocytic process...
Export from cell...
Cellular metabolic process (GO:0044237),...

**Fig. C**

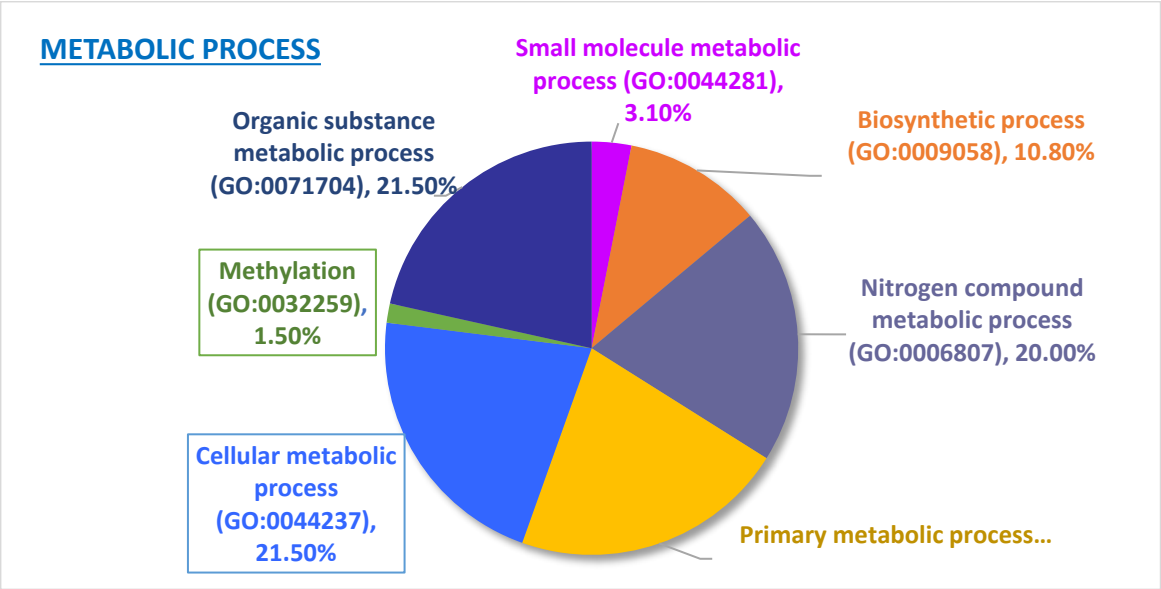
METABOLIC PROCESS
Small molecule metabolic process (GO:0044281), 3.10%
Organic substance metabolic process (GO:0071704), 21.50%
Biosynthetic process (GO:0009058), 10.80%
Methylation (GO:0032259), 1.50%
Nitrogen compound metabolic process (GO:0006807), 20.00%
Cellular metabolic process (GO:0044237), 21.50%
Primary metabolic process...

**Fig. D**

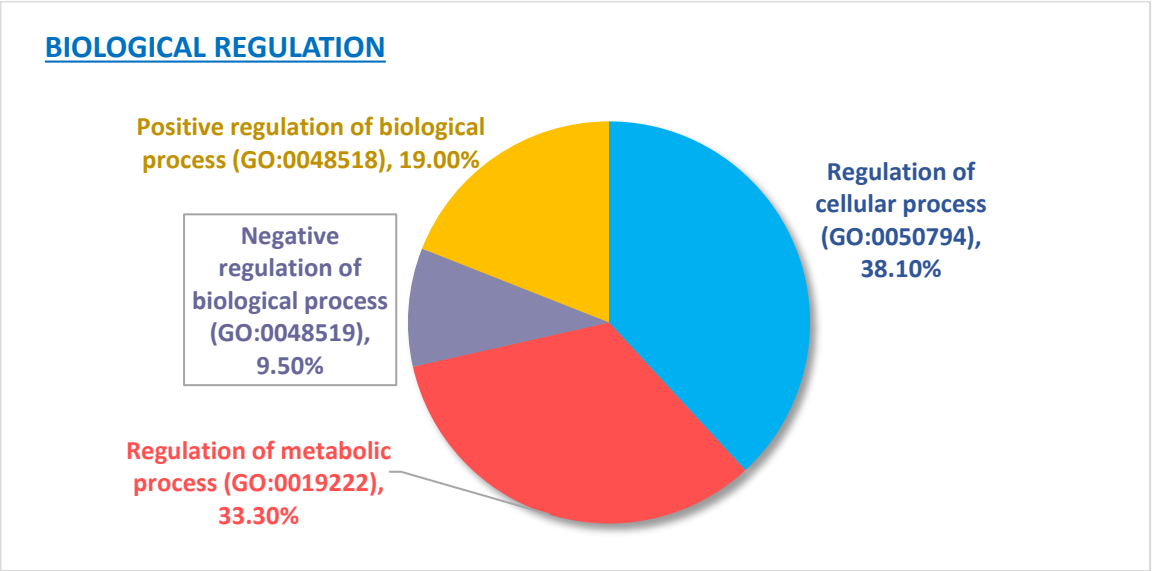


**Fig. E**

**Figure 56.** Gene ontology (GO) analysis of the upregulated proteins using PANTHER classification system. GO terms were assigned to the 38 mapped proteins, with 6 of these being unclassified **A.** Molecular Function, **B.** Biological process. Sub-categories of biological process like **C**. Cellular process, **D.** Biological regulation, and **E.** Developmental process. (The input percentage was calculated on the basis of the number of proteins mapped to the GO term divided by the submitted list of upregulated proteins mapped to Arabidopsis thaliana genes from a whole genome).

Out of 31 downregulated proteins, 24 proteins were classified under different protein classes. Most of these proteins were associated with enzymatic activity (5 metabolic interconversion enzymes and 4 proteins modifying enzymes) like hydrolases, transferases, kinase, protease and ligase. 3 proteins were found to be associated with gene-specific transcription regulator activity (transcription factors) and 3 proteins were chromatin binding or regulatory proteins (transcription factors). Membrane traffic protein (2), RNA metabolism protein (2), cytoskeleton

protein (1), extracellular matrix protein (1), chaperone (1), transmembrane signal receptor (1) and DNA metabolism (1) were the other classes of proteins identified. (Fig. 55)

Molecular function analysis shows that majority of the down regulated proteins include binding proteins (sytaxin binding, RNA polymerase II transcription machinery binding, cytoskeletal protein binding, enzyme binding) and involved in catalytic activity (hydrolase activity, oxidoreductase activity, transferase activity, demethylase activity). Other classes include ATP dependent activity (ATP hydrolysis, helicase activity), molecular function regulator (enzyme regulation) and transcription regulator activity. (Fig. 56 A)

The GO analysis for biological processes shows that most of the down regulated proteins were associated with cellular process (19) and metabolic process (14). 8 proteins were found to be related to biological regulation (negative regulation of transcription and positive regulation of cytoskeleton organization, protein modification). Proteins annotated with response to stimulus and localization were the other classes of proteins that are most lowly expressed (Fig. 56 B, C, D, and E).

Classification of up and downregulated proteins into different protein classes showed similar trends, with minor differences. Proteins with kinase and protease activity are downregulated. Similarly, proteins associated with membrane trafficking, RNA metabolism, cytoskeleton, extracellular matrix and transmembrane signalling are downregulated. Whereas, protein associated with translation process was upregulated. GO based molecular function analysis also showed similar trends, except downregulation of transcription regulator activity. Comparing GO based biological process, major differences were observed in biological regulation and cellular process.

Upregulated proteins are mostly involved in regulating organic substance metabolic process, nitrogen compound metabolism, cellular metabolic process, primary metabolic process and biosynthetic process. Among the downregulated proteins, there are no proteins involved in

these processes. Whereas, negative regulation of transcription, positive regulation of cytoskeleton organization and protein modification are downregulated. Among cellular process, processes related to cell cycle, cell communication, and cell growth were upregulated. Whereas cellular process like vesicular mediated transport, cellular export, cellular localization, and microtubule-based mechanisms are downregulated.

### 3.4.2.5. Functional annotation of proteins unique to male and female flowers

PANTHER GO-Slim Biological Process was chosen as annotation data type for statistical overrepresentation analysis. This analysis assigned proteins exclusive to male and female flowers to different biological process. Few biological processes were found to be common between male and female (Fig. 57) and few were exclusive to male (Fig. 58) and female (Fig. 59).

Compound biosynthesis, regulation of biosynthetic process, regulation of transcription are the common biological processes corresponding to representative GO terms in both male and female flowers (Fig. 57).

**GO enrichment analysis of proteins exclusive to male flowers**

Biological processes like multicellular organismal reproductive process and multicellular organism reproduction are the representative GO terms, both related to reproduction. These were followed by negative and positive regulation of various biological processes like positive regulation of cytoskeleton organization and negative regulation of cell signalling, communication and reposnse to stimuli. Among the other functions, hormone-mediated signaling pathway and cellular response to hormone stimulus are highly representative. (Fig. 58)

**GO enrichment analysis of proteins exclusive to female flowers**

Morphogenesis of a branching structure and Histone H3-K4 methylation are the representative GO terms specific to female flowers, indicating their role in flower development and gene

regulation. Anatomical structure formation involved in morphogenesis is the next representative GO term, also related to flower development. This was followed by DNA replication initiation. Reproductive system development, Developmental process involved in reproduction, post-embryonic development, Regulation of developmental process, Positive regulation of transcription, DNA-templated, Reproductive process, Aromatic compound biosynthetic process, Metabolic process were the other representative GO terms that are specific to female fowers (Fig. 59).

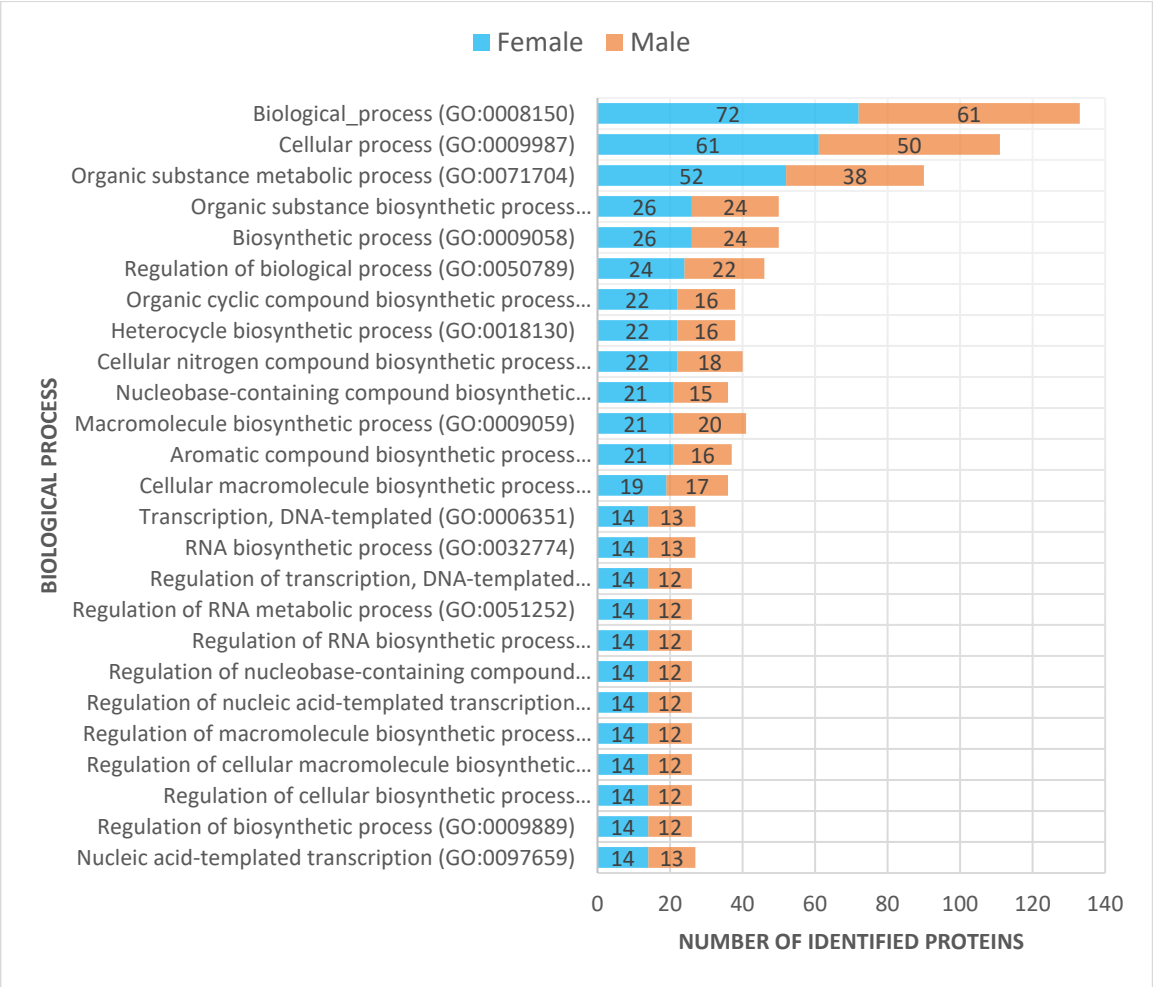


**Figure 57.** Biological process overrepresented in both male and female flowers. Numbers in the bars represent number of proteins in the submitted list for a particular PANTHER GO-Slim Biological Process category, based on the reference list.

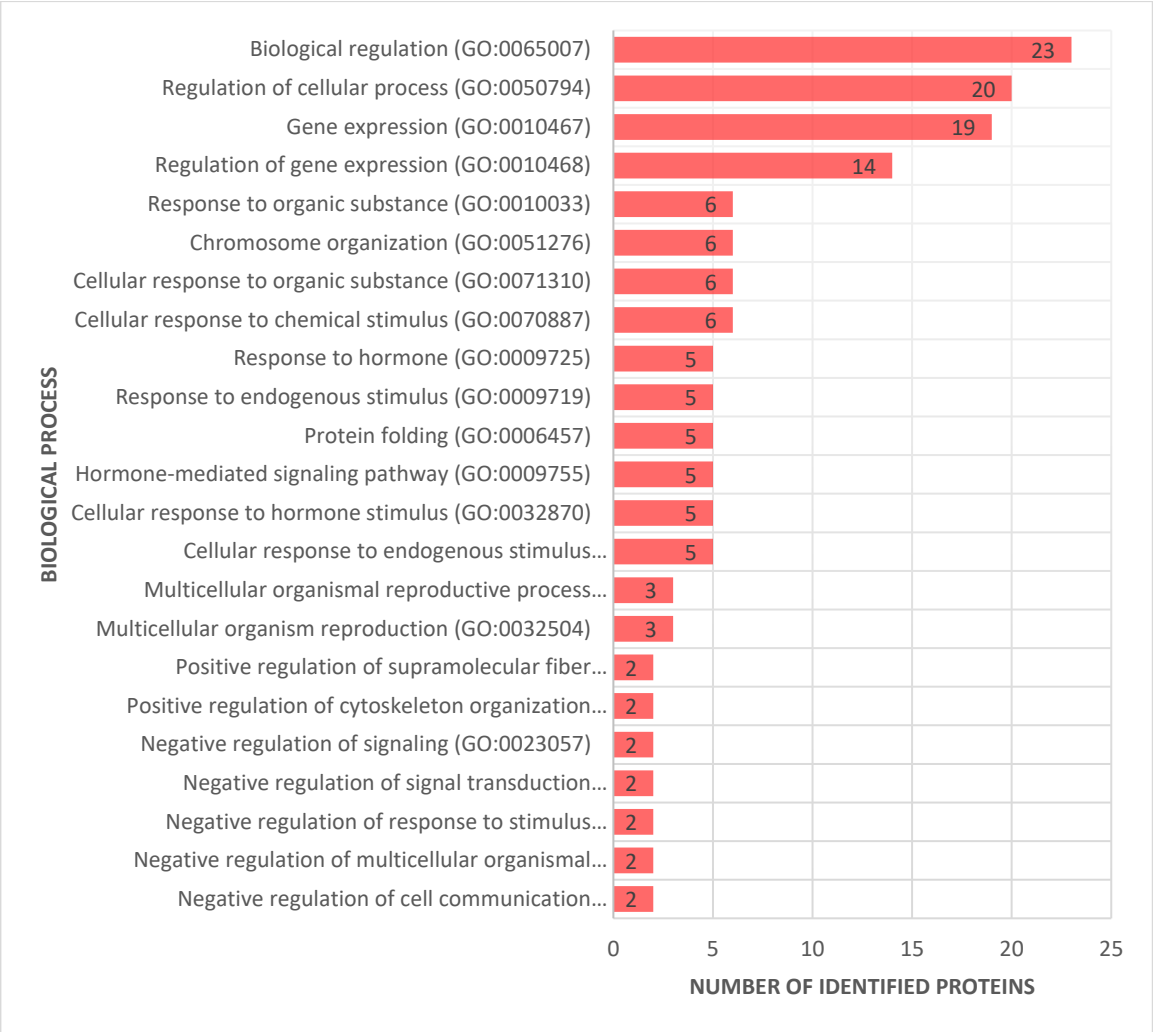


**Figure 58**. Biological process overrepresented exclusively in male. Numbers in the bars represent number of proteins in the submitted list for a particular PANTHER GO-Slim Biological Process category, based on the reference list.

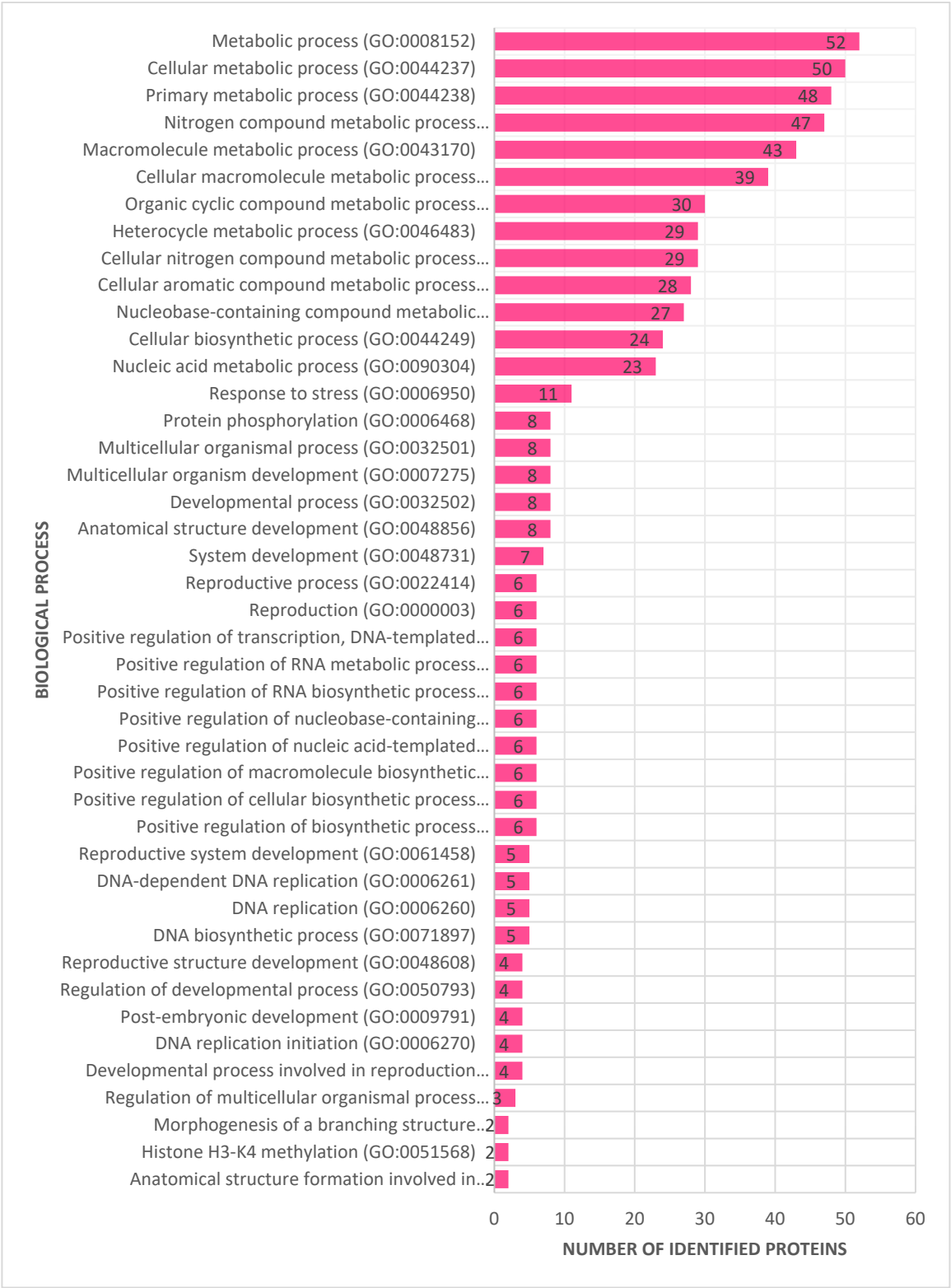


**Figure 59**. Biological process overrepresented exclusively in female. Numbers in the bars represent number of proteins in the submitted list for a particular PANTHER GO-Slim Biological Process category, based on the reference list.

### 3.4.2.6. Functional annotation of uncharacterized proteins

From the proteomic profiling, 24 proteins that are assigned as 'uncharacterized' were identified. These were annotated as 'uncharacterized' in UniProt without any evidence of translation (UniProt, release 2022_04). But current study identified their expression at protein level from our study. Annotation of these sequences could help in reaching consensus about their functional significance. We selected 4 uncharacterized proteins that are highly expressed in both male and female, one each from Cycas and Putranjiva for functional annotation using bioinformatics tools and databases (Fig. 60). Four features correlating to protein function, *viz.*, orthology, domain and motif, subcellular localization and protein-protein interactions were selected to estimate whether these sequences are functional. This annotation based on feature selection predicted these proteins to be associated with gene regulation, transport, gametic cell fate determination and flowering.3

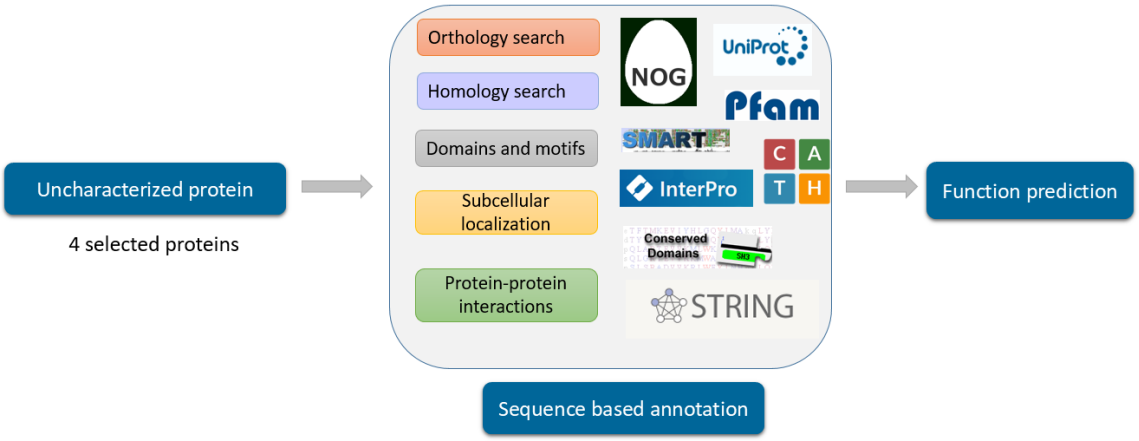


**Figure 60.** Workflow used for the functional annotation of uncharacterized proteins.

The amino acid sequences of the 4 selected uncharacterized proteins (CYCAS_030968 (Cycas Male); CYCAS_014252 (Cycas Female); A0A2C9UMJ9 (Putranjiva Male) and A0A178UR23 (Putranjiva Female) were submitted to the workflow and 4 proteins could be successfully

assigned a function. From the analysis, it was predicted that most of these proteins were predicted to be involved in flowering related processes such as chromatin mediated gene regulation, gametic cell fate determination, and regulation of flowering (Fig, 61, 62). This analysis emphasizes the importance of attending to uncharacterized or hypothetical proteins for better understanding of the mechanisms in the cell. Also, this method provided the evaluation of combining proteomics methods with different bioinformatics-based annotation methodology to characterize the uncharacterized protein data.

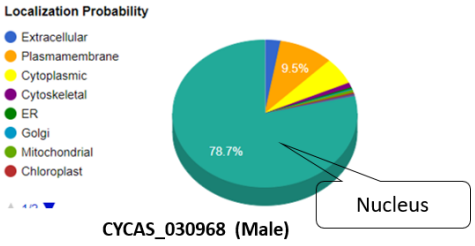


**Fig. A.**

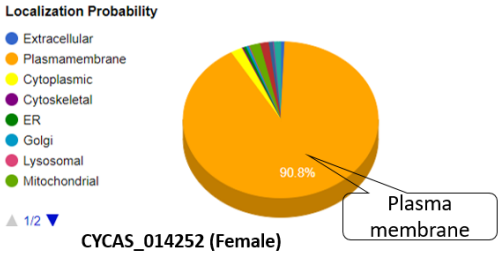


**Fig. B.**

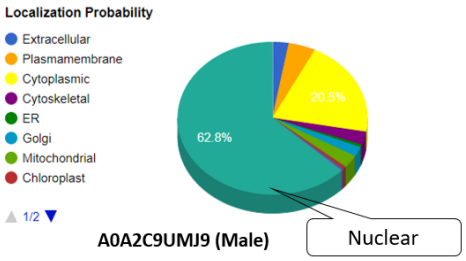


**Fig. C**

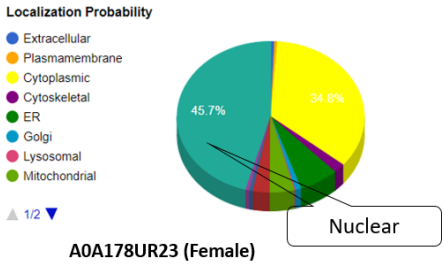


**Fig. D**

**Figure 61.** Subcellular localization analysis using CELLO2GO.

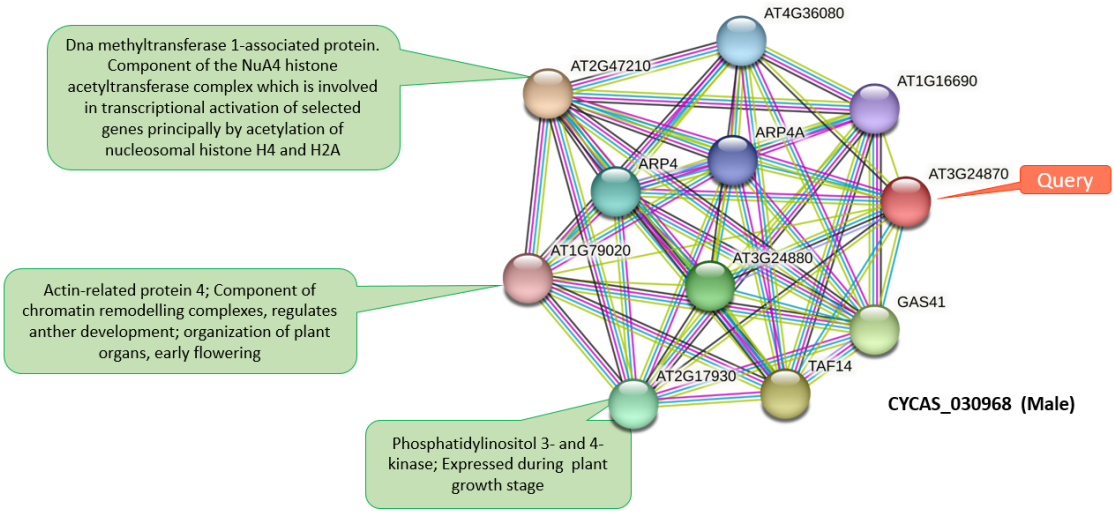
Dna methyltransferase 1-associated protein. Component of the NuA4 histone acetyltransferase complex which is involved in transcriptional activation of selected genes principally by acetylation of nucleosomal histone H4 and H2A
Actin-related protein 4; Component of chromatin remodelling complexes, regulates anther development; organization of plant organs, early flowering
Phosphatidylinositol 3- and 4-kinase; Expressed during plant growth stage
AT4G36080
AT2G47210
AT1G16690
ARP4A
ARP4
AT3G24870
Query
AT1G79020
AT3G24880
GAS41
TAF14
AT2G17930
CYCAS_030968 (Male)

**Fig. A**

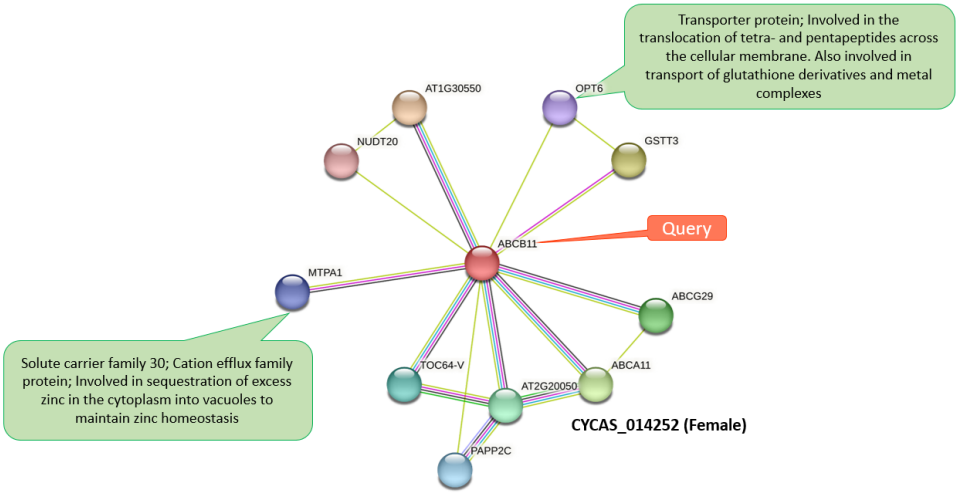
Transporter protein; Involved in the translocation of tetra- and pentapeptides across the cellular membrane. Also involved in transport of glutathione derivatives and metal complexes
Solute carrier family 30; Cation efflux family protein; Involved in sequestration of excess zinc in the cytoplasm into vacuoles to maintain zinc homeostasis
AT1G30550
OPT6
NUDT20
GSTT3
Query
ABCB11
MTPA1
ABCG29
TOC64-V
ABCA11
AT2G20050
CYCAS_014252 (Female)
PAPP2C

**Fig. B**

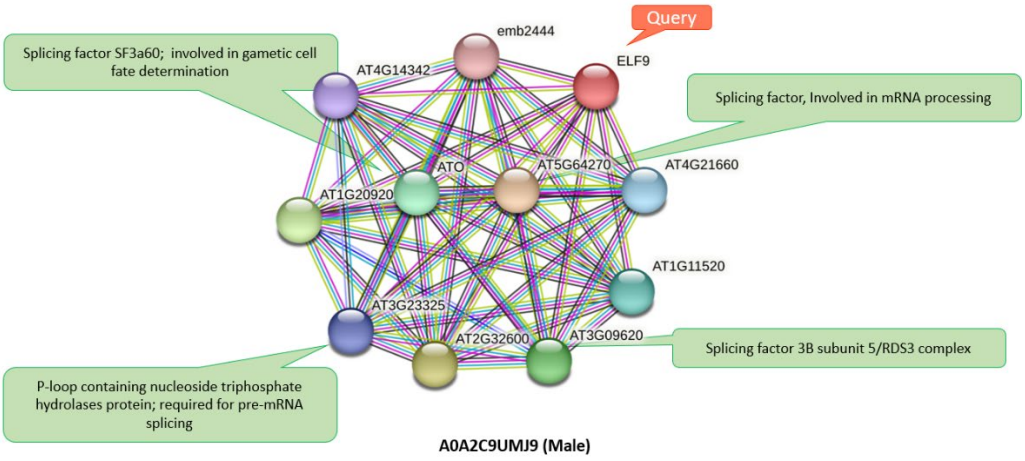


**Fig. C**

**Figure 62.** Protein-protein interactions identified using STRING database.

(A). Cycas_030968 (Male) (B). Cycas_014252 (Female) (C). A0A2C9UMJ9 (Putranjiva male)

# Chapter 4. Discussion

# DISCUSSION

The dioecy was a virtual event that took place during the course of evolution. The theory of ecological causation explains the adaption of males and females in different ecological niches having differentiation in secondary characteristics between sexes (Shine, 1989). The theory of sex allocation states that sexual functions are maintained with greater marginality due to the partition of female and male plant characters (Charnov, 1978). The morphological and biochemical study of *Cycas nayagarhensis* and *Putranjiva roxburghii* Wall. Male and female plants showed the difference in carbohydrate, glycoside, protein, amino acid, phytosterol, reducing sugar, alkaloid, flavonoid, tannin, and phenolic compounds content, variation in leaf, antioxidant enzymes, elements, chlorophyll, stomata and carotenoid content in male and female plant, along with structural differences in the plant organs. Therefore, variation and adaptation in the *Cycas nayagarhensis* and *Putranjiva roxburghii* Wall exist, and separation of sexes in the form of male and female plants as supported by the theory of ecological causation and sex allocation was observed. Adaptations found in the male and female plants were further discussed here.

Foliage is the form of collective leaves, a lateral growth of plant stem that helps in photosynthesis. It is also responsible for plant appearance, apart from the photosynthesis mechanism and the detached leaves from deciduous plants assess as a plant biomass (Orsat and Routray, 2017). We found that leaf size, distribution and diversity were more in female plants than in males for both angiosperm and gymnosperm. Our finding is supported by the previous literature, where leaf size was larger in the female plant compared to the male plant to fulfill the high energy demand for axillary fruit and seed development through higher photosynthesis (Scott and Aarssen, 2013). The dioecious plant jojoba leaves contain mostly ATP synthase and Ribulose-1,5-bisphosphate carboxylase (Rubisco) abundantly in both males and females, which were involved in energy metabolism, photosynthesis and response to abiotic stress and biotic

stress. These two proteins were upregulated in the male plant compared to the female taken as a biomarker in Jojoba plant (AI-Obeidi et al., 2017). Male plants receive more herbivore damage than females. To minimize the leaf tissue damage from herbivores, male plants adopted various mechanisms like high leafing intensity and increased production of buds that may result in more flowering and branching (Jing and Coley, 1990). We observed similar results where the male Putranjiva plant has more leaves than female. This increase in leaf number of dioecious male plant and decrease in their mass along with the higher branching intensity, spreads out the leaves more diffusely, possibly thus constraining the foraging efficacy of some herbivores (Brown and Lawton, 1991). Therefore, the herbivore pressure may be responsible for smaller leaf size selection in males. Whereas, in female plants, the slow growth and chemically defended leaves are comparatively less preferred by herbivores. Also, having large leaves may be energetically more favorable for fruit production. Thus, in female plants, selection for larger leave is more potent than for smaller leaves which could reduce herbivore pressure (Jing and Coley, 1990). The resources utilized to grow stems, leaves and roots are sifted to the development of flowers, fruits, and seeds (Dawson and Geber, 1999). The sexually dimorphic leaf size was found in Cycas and Putranjiva but not in all the species. The production of flowers in dioecious plants differs from each another with respect to their life cycles which involves differences in plant morphology, photosynthesis, water uses and tolerance to herbivores (Delph, 1999, Cornelissen and stiling, 2005). In general, more flowers are produced in males and flowering emerges early in the male plant (Lloyd and Webb, 1977). Similarly, the Putranjiva male plants have more flowers compared to female plant. Reproductive success of many dioecious species and the sex ratio was changed by the resource availability and allocation pattern.

Our results of the biochemical analysis of dioecy Cycas and Putranjiva provides more insights into sex determination among male and female plant. But antioxidant enzymatic activity increased in males than in females which may be due to scavenging of free radicals (Zhang et al., 2011). In water deficit stress conditions, chlorophyll and carotenoids decreases; however,

they are less sensitive. Carotenoids act as less sensitive, suppressing lipid peroxidation by ROS scavenging (Gill and Tuteja, 2010). In Populus, chlorophyll a and carotenoids are less than chlorophyll b in male than in female plants. However, in a previous study, male plants contained more chlorophylls and carotenoids in drought conditions (Chen et al., 2010).

Carbohydrate transport in leaves is influenced by venation organization (Roth-Nebelsick et al., 2001). Carbohydrate export follows the source-sink theory from leaves for the accretion and maintenance of non-photosynthetic tissue (Rennie and turgeon, 2009). In the case of Putranjiva leaves, it is broad and therefore contain more veins in leaves having more sugar content but in the case of Cycas leaves, it has more wax coating and thicker. Carbohydrate like fructans incorporates carbon source in the polymeric form, which helps in stomatal function in onion leaf (Darbyshire and Allaway, 1981). The soluble carbohydrate sorbitol was the important final product of photosynthesis (Moing et al.,1992). More drought and stress-tolerant plants accumulate more sugars (Adams et al., 2013). Not only in drought stress but in case of water stress carbohydrate accumulates high in wheat plants (Kameli and Lösel, 1996). From this it was evident that carbohydrate accumulate during stress.

Phenolic compounds come under the class of secondary metabolites used in the pharmaceutical, food, and nutraceutical industries for their biological activities (Giacometti et al., 2018). The phenolic content was more in Putranjiva males than the females. In the case of Cycas, phenolic content was lower in the male plant than in the female. The variations in our studied dioecious plants could be due to the evolutionary variations, particularly structural related and flavonoid content related across ancient versus advanced. We found some previous report where these secondary metabolites, total flavonoids, and total phenolics, accumulates more in wheat leaves during abiotic stress condition (Treutter, 2006).

The presence of various classes of flavonoids in flowers helps to understand the phylogeny of angiosperm by genetic differences. We found that the female Cycas and Putranjiva plant's flavonoid content was more than the male plant. These compounds arise independently in plants

with genetic selection pressure. For example, anthocyanidins content from flowers distributed in relation to their pollinators (Crawford, 1978).

Antioxidant enzymes protect plants from biotic and abiotic stress by osmoregulation. These antioxidant enzymes remove reactive oxygen species which varies from species to species. These enzymes are present in stroma, cytosol, peroxisomes, and mitochondria. The enzymatic antioxidant systems superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) help the reduction in efficiency of rubisco through a biochemical mechanism by the accumulation of stress metabolites in *populus balsamifera* (Sanderson et al., 2019). These SOD and CAT homomeric enzymes encode the nucleus where nuclear DMI plays a significant role in physiological responses (Prokic et al., 2018). The metalloenzyme SOD (Superoxide dismutase) provides a defensive mechanism against ROS oxidative stress. The heme-containing enzyme CAT removes $H_2O_2$ which oxidases in the peroxisome. The GPX enzyme consumes $H_2O_2$. Our result showed a higher accumulation of SOD in both females than males, supporting more scavenge free radicals. Among all antioxidant enzymes, SOD showed more activity in *Poulus cathayana* could be the reason for its presence, whereas APX, CAT and GPX showed less activity because of less involvement (Xu et al., 2008). The Ascorbate peroxidase (APX) enzyme was increased in both Putranjiva and Cycas male plants in our studies similarly, APX was found more in the stem of male *P. chinensis* which displayed the sex-related difference in a tissue-specific way (Xiong et al., 2013). The microscopic structure of stomata carries two guard cells as a pair that surround a pore Stomata function by regulating guard cells through turgor pressure. It moderates the gas exchange rates between the leaf and the atmosphere (Zeiger et al., 1987). The movement is regulated by many environmental factors, like soil moisture, light, humidity, temperature and $CO_2$ concentration. The adaptation of stomatal morphology is diversified across species to specific environments like small-sized stomata facilitate faster aperture response by a reduction in whole leaf area (Chen et al., 2017). The stomatal densities and sizes vary due to different

environmental conditions simultaneously with genetic factors during evolution adaptions like in dioecious (Dittberner et al., 2018). Similarly, we found variation in the stomata of Cycas and Putranjiva plants. Our current study indicates that stomata's number, density, and size are co-related with water use efficiency.

Development and metabolic profiling were performed in both Putranjiva unreleased (UR) and released (R) pollen to understand the metabolic changes associated with pollen maturity. Metabolites were identified using LC-MS/MS and biomolecules like amino acids, sugars, phytohormones, and polyamines were validated and quantified through HPLC. Quantification of biomolecules showed the variation of their expression between the UR and R pollen. Previous studies showed that pollen exposure to abiotic stress is associated with changes in the protein structure, ROS production and metabolic stability (Del pino et al., 2019). Higher ROS acts as a signalling molecule that triggers cytotoxicity in the cells, producing various antioxidants to decrease toxicity (Møller et al., 2007). In the present study, no ROS was found in UR pollen but in the R pollen with increased antioxidant activity. This increase in the antioxidant activity in R pollen would be to limit nutrient oxidation. These findings suggest that UR pollen is unstressed due to the internal environment of the flower, whereas after release, they are stressed due to external environmental factors. Flavonoids are reported to promote pollen tube growth and pollen germination in vitro (Grunewald et al., 2020). Flavonol is an essential factor for intine development regulated by flavonoid metabolism and the intine layer is important for pollen maturation. Its underdevelopment leads to the sterility of pollen. In our study, the flavonoid content was found to be adequate in both UR and R conditions. These results were consistent with the previous findings (Muhlemann et al., 2018), suggesting that the flavonoid would be essential for maturation in UR pollen and germination and tube growth in R pollen of the Putranjiva plant.

Carbohydrate is the major energy source that maintains osmotic balance, provides membrane protection and participates in molecular signalling (Kandpal et al., 2020). From our findings,

the higher contents of glucose and fructose in UR pollen when compared to the R pollen, indicate their role as significant energy sources in pollen maturity. Sucrose accumulation was much higher in the R pollen than the UR pollen indicating its role as a stored energy source for later successful fertilization, including pollen germination and tube growth. IAA is an essential hormone for maintaining fertility in flower tissue and stimulating pollen and floral organ formation (Zhang et al., 2019). In our study, IAA was detected in UR pollen and was found to be decreased in R pollen. Previous studies have shown that IAA biosynthesis reduces heat stress (Sakata et al., 2010). In this study, though the pollen was not subjected to heat stress, exposure to the external environment may stress the pollen. Proline is known for pollen viability and potency (Kiran et al., 2021). Higher proline content in the UR pollen than in R pollen supports its role in pollen viability. As Putranjiva pollinates through wind, stress from the external environment might be responsible for the decreased proline content after release. Gibberellic acid was also reported to regulate pollen viability (Li et al., 2021, Cecchetti et al., 2017) and our findings support this with a more significant accumulation of gibberellic acid along with kinetin in R pollen. Salicylic acid protects from abiotic stress (Hayat et al., 2010). Salicylic acid and abscisic acid were more in R pollen than UR, indicating a significant role in maintaining viability during stress at R pollen. Jasmonic acid was shown to have a role in anther dehiscence in Arabidopsis (Jibran et al., 2017) and similar results were found in this study. The jasmonic acid level was higher in the UR pollen at the dehiscence state, which later decreased after release demonstrating a similar role of anther dehiscence in the Putranjiva plant.

Polyamines (PAs) are important in the pollen for microsporogenesis, rehydration, tube growth, quiescence, viability and the protection of the DNA from the reactive oxygen species (ROS) through the free radicals scavenging (Falasca et al., 2010). In this study, it was found that all the major four PAs were present at adequate amounts in the pollen of Putranjiva. Polyamine like putrescine, was found to be essential for pollen maturity, integrity, viability, and pollination (Aloisi et al.,2016). Often molecules like polyamines have a pleiotropic effect according to their

concentration. The previous study has shown that the spermine was involved in cell wall construction and cytoskeleton organization during pollen tube growth. It was evident that its concentration during pollen germination and tube elongation is finely regulated with an excess of spermine (100 µm) blocking the tube growth whereas physiological concentrations favor the pollen tube growth and germination (Aloisi et al., 2017). In this study, the spermine and cadaverine content gradually decreased from the UR pollen to the R pollen, suggesting their role in maturation. Arginine is the precursor molecule for all the polyamine biosynthetic pathways, which later forms the first central polyamine putrescine which then forms the spermidine and spermine (Chen et al., 2019). Therefore, arginine a vital amino acid was found in Putranjiva pollen during maturation. Previous research in kiwifruit pollen has shown that a deficiency of spermidine content causes functional loss or degeneration (Falasca et al., 2010). It has been reported that spermine and spermidine predominate in the early stages of developing pollen for the embryogenetic process (Ninković et al., 1995). In the present study, the spermidine was found to be higher in UR pollen which may be involved in functional development.

Similarly, both these PAs and cadaverine were present at a higher concentration at UR pollen suggesting their critical role in maturation. In this study, we noted that incomplete development arrest in R pollen is associated with metabolic activity. The pollen vitality and stability were maintained in R pollen due to the accumulation of the essential metabolite. For example, the metabolites related to phenolic pathways are down-regulated, which correlates with the phenolic content estimation in R pollen. From previous studies, abiotic stress impacted the fatty acid metabolism in Arabidopsis, where a decrease in polyunsaturated fatty acid was found in the leaf cell membrane (Higashi et al., 2015). According to a study, three fatty acids, *viz.,* stearic acid, linolenic acid, and palmitic acid, are essential for pollen coat formation, and the deficiency of those leads to pollen dehydration thereby leading to sterility (Xue et al., 2018). The higher accumulation of palmitic acid (C16:0), stearic acid (C18:0) along with 12-Octadecadienoic acid

(C18:2 w6c) is essential for pollen coat formation to protect the pollen from the environmental stress after pollen dispersal.

In our study, we identified metabolites like kinetin, gibberellic acid, salicylic acid, sucrose, 12-Octadecadienoic acid (C18:2 w6c), lysophatidylinositol, phosphatidylglycerol, linoleic acid and its derivatives, lysophosphatidic acid, phosphatidylcholine, monoacylglycerol, sphingosine-1-phosphate and oleacein factors which are upregulated in the R pollen. Some of these factors were previously reported (Hernándezet al., 2020) to be involved in pollen tube growth and germination. The factors like auxin family hormones indole-3-acetic acid and 1-naphthalene acetic acid, proline, glucose, fructose, cadaverine, spermine and spermidine were upregulated in the UR pollen which would be essential for the maturation of pollen. We hypothesized from this study, that pathways involved in the metabolism of amino acids, carbohydrates, lipids, phenolic compounds, hormones, and polyamines are interconnected and carry out the metabolic homeostasis required for the development, growth, and viability of pollen and pollination adaptations. Our research has identified and quantified the metabolites and their putative metabolic pathways.

Research on wind pollinated Putranjiva pollen and insect-pollinated Cycas pollen on microbes improves the understanding of pollination strategy, protection and ecological aspects of pollen-microbial interaction. The pollinator's job is to bring pollen to the stigma and visit multiple plant species (Vecherskii et al., 2021). Pollen associate’s bacteria microbiota which influences the pollination type and shows the diversity and species specificity. The association between pollen and bacteria is poorly understood. Very interestingly pollen provides a specific and unique microhabitat. In our study the abundance, diversity and community of bacteria of Putranjiva and Cycas pollen were done. The bacterial community was carried by all plant organs, including pollen (Junker and Keller, 2015).

From the metabolomic profiling and pathway enrichment analysis of Weevil and Cycas pollen, phenylpropanoid pathway was found to be enriched in both Weevil and Cycas pollen. From the

previous studies (Battat et al., 2019; Verne et al., 2011) it was shown that pollen flavonols emit floral scent (ODO1) which attracts insect for pollination.

Sex determination mechanism in dioecious plants is puzzling. Sex chromosomes, sex related genes, their regulation, and transcription factors are the key factors that influence sex determination and differentiation. However, as the function encoded by genes is executed through proteins, the factors that affect the development of separate male and female individuals or organs can be better understood at protein level. In general, plant sex is determined at the reproductive growth stage. Hence, to understand the mechanism of sex determination at protein level, we studied the protein profiles of female and male flowers of *Putranjiva roxburghii* Wall. Ours is the first proteomic report focused on identification of proteins expressed in female and male flowers of *Putranjiva roxburghii* Wall.

Mass spectrometry-based label-free quantitative proteomics identified 76 differentially expressed proteins across female and male flowers. GO based functional classification was used to understand the functional significance of these differentially expressed proteins in female flowers in comparision with male flowers. Analyses show that 37.5% of the upregulated proteins are associated with metabolite interconversion enzymatic activity. Oxygenases (Cytochrome P450 90A1, Cytochrome P450 714A2, Gibberellin 20 oxidase 3), acyltransferases (Wax ester synthase/diacylglycerol acyltransferase 11, Protein REDUCED WALL ACETYLATION 2), transferase (ATP-citrate synthase beta chain protein 2), ligase (Long chain acyl-CoA synthetase 1) and esterase (Putative pectinesterase/pectinesterase inhibitor 43) constitute this class of proteins, inferring their role in metabolic processes. These are followed by proteins associated with DNA metabolism (20.8%). These proteins can be categorized into DNA helicases (Probable ATP-dependent DNA helicase CHR12, ISWI chromatin-remodeling complex ATPase CHR17), replication origin binding proteins (Origin of replication complex subunit 1A, Cell division control protein 6 homolog), and endodeoxyribonuclease (Meiotic recombination protein SPO11-2). Organogenesis in plants is is a postembryonic process and is

continuos (Gutierrez, 2005). Cell proliferation is essential to initiate differentiation and organ formation. Hence, proteins associated with DNA replication and regulation in dividing plant cells has an essential role in growth, differentiation and organogenesis (Sanchez et al., 2012). In addition, 4 transcription factors (16.7%) associated with the regulation of growth and stress tolerance were found to be upregulated. B3 domain-containing transcription factors NGA1 (O82799), NGA2 (Q9M268) and NGA4 (O82595) have been shown to regulate lateral organ growth (Alvarez et al., 2006). Probable WRKY transcription factor 33 (Q8S8P5) was found to be associated with positive regulation of salt stress response and abscisic acid (ABA) signalling (Jiang and Deyholos, 2009) along with plant thermotolerance (Li, et al., 2011). Few protein modifying enzymes like Subtilisin-like protease SBT3.5 (Q9MAP7), Cullin-4 (Q8LGH4), E3 ubiquitin-protein ligase COP1 (P43254) were upregulated. Cullin-4 has been shown to regulate photomorphogenesis and flowering time (Chen et al., 2010), and was found to be essential for plant embryogenesis (Zhang e al., 2008). E3 ubiquitin-protein ligase COP1 regulates various plant developmental processes by modulating the expression of flower-specific transcription factor like AtMYB21 (Shin et al., 2002). Histone-lysine N-methyltransferase CLF (P93831) known to regulate flowering (Saleh et al., 2007) was found to be upregulated.

Downregulated proteins are significantly involved in metabolite interconversion enzymatic activity, protein modification and chromatin regulation. While few of the downregulated proteins are also associated with membrane trafficking, cytoskeleton organization and extracellular matrix. In contrast to upregulated proteins, downregulated proteins constitute glucosidase (Probable glucan 1,3-alpha-glucosidase), phosphatase (Probable inactive purple acid phosphatase 2), glycosyltransferase (UDP-glycosyltransferase 73C5), acetyltransferase (Protein REDUCED WALL ACETYLATION 1) and esterase (Probable pectinesterase/pectinesterase inhibitor 42). Protein REDUCED WALL ACETYLATION 1 is associated with secondary cell wall biosynthesis (Manabe et al., 2013). Under chromatin regulating proteins, protein PHYTOCHROME-DEPENDENT LATE-FLOWERING

(F4IDB2) is involved in the regulation of flowering (Endo et al., 2013). Lysine-specific demethylase JMJ15 (O64752) has been shown to control flowering time (Yang et al., 2012). SWI/SNF complex subunit SWI3C (Q9XI07) is a chromatin remodelling complex that plays an essential role in vegetative as well as reproductive phases of plant development (Sarnowski et al., 2005). 3 proteins belonging to gene-specific transcription regulator class were downregulated. Transcriptional corepressor SEUSS (Q8W234) controls floral organ specification/polarity by repressing the transcription of the floral organ identity gene AGAMOUS. SEUSS also play an essential role in the development of female floral reproductive tissues (Bao et al., 2010). Homeobox-DDT domain protein RLT2 (Q9FFH1) maintain vegetative phase in flowering plants and prevent early transition from vegetative to reproductive phase (Li et al., 2012). Two memrane traffic proteins known to be associated with defense (Exocyst complex component EXO70B2) and vesicle-mediated transport (Protein transport Sec1a) were downregulated., Two poteins related to RNA metabolism were downregulated. These proteins include pre-mRNA-splicing factor ATP-dependent RNA helicase DEAH7 (F4K2E9) that enables auxin-mediated development processes (Tsugeki et al., 2015) and protein LEO1 homolog (Q9FNQ0) that regulated flowering time (Zhang et al., 2002). Additional proteins downregulated in female relative to male include Protein SCAR4 (Q5XPJ6) and SART-1 family protein DOT2 (Q9LFE0). Protein SCAR4 is involved in the regulation of actin nucleation, and microtubule organization, and thus regulating plant cell morphogenesis and development (Brembu et al., 2004). SART-1 family protein DOT2 is essential to control embryo patterning and for maintaining correct meristematic patterning in the root and vegetative shoot, thus playing an important role in root and shoot development (Casson et al., 2009).

Of additional interest were proteins exclusively expressed in the respective proteomes of male and female flo0wers. Among dioecious plants, female plants are more reproductively functional than male plants (Zhang et al, 2021). In female flowers, proteins corresponding to first ten

overrepresented biological processes mostly found to be associated with reproduction, morphogenesis, growth and development. There were 2 proteins (Transcription factor TCP1, Transcription factor TCP3) relevant to morphogenesis of a branching structure and 2 proteins relavant to Histone H3-K4 methylation (Protein CTR9 homolog, WD repeat-containing protein VIP3) that were exclusively expressed in female flowers and are overrepresented. Histone H3-K4 methylation has central role in transcription regulation and has been shown to be have an important role in flowering (He Y, 2009), flowering-time variation (Yang et al, 2012; Jiang et al., 2009), chromatin reprogramming to establish cell specification or new cell fates during sexual reproduction in plants (Fang et al., 2021). These were followed by proteins associated with DNA replication initiation (Origin of replication complex subunit 1B, Origin of replication complex subunit 5, DNA replication licensing factor MCM3, Origin of replication complex subunit 3) and may contribute to plant growth, differentiation and organogenesis (Sanchez, et al., 2012). Few proteins related to reproductive system and reproductive structure development were overrepresented. These proteins include DELLA protein RGL2, Probable F-box protein At3g61730, Axial regulator YABBY 3, AT-hook motif nuclear-localized protein 27 (AHL27) and AT-hook motif nuclear-localized protein 29 (AHL29). DELLA proteins are a family of growth repressors. RGL2 represses gibberellin (GA) signalling pathway. GA deficiency blocks anther development, thus leading to male sterility (Cheng et al., 2004). F-box protein At3g61730, designated as RMF (reduced male fertility) functions to degenerate tapetum, thus preventing anther formation in female flowers (Kim et al.,2010). Axial regulator YABBY 3 plays an important role in embryogenesis and organogenesis by specifying abaxial cell fate in lateral organs (Siegfried et al., 1999). AHL27 and AHL29 peroteinshas been shown to prevent early flowering (Xiao et al., 2009).

In male flowers, top ten overrepresented GO terms were related to different biological processes like reproduction, regulation and hormone mediated signalling. A number of proteins related to multicellular organismal reproduction and eproductive process were overrepresented, such as

DELLA protein RGA, DELLA protein GAI and Polygalacturonase ADPG1. RGA and GAI has been shown to regulate apical dominance, stem elongation, seed dormancy and germination (Dill and Sun, 2001, Cao et al., 2005). Also, it has been demonstrated that RGA and GAI proteins transcriptionally repress floral homeotic genes like AGAMOUS (AG) APETALA3 (AP3) and PISTILLATA (PI), and and retard floral organs growth (Yu et al., 2005). ADPG1 promotes cell separation through pectin degradation, thus contributing to anther dehiscence, silique dehiscence and seed abscission (Ogawa et al., 2009). Couple of proteins overrepresented in male flowers, associate with positive regulation of cytoskeleton organization (Protein SCAR2 and Protein SCAR1), suggesting these proteins may play a role in regulating epidermal cell expansion (Zhang et al., 2005). We also identified hormone-mediated signalling pathways, such as serine/threonine-protein kinases, BAM1 and BAM3, that are exclusively expressed on male flowers and contribute to anther and male gametophyte development (DeYoung et al., 2006).

Hence, the GO based overrepresentation analysis of the identified proteins exclusive to female and male flowers suggest female and male specific function, respectively, indicating the sex differentiation at the proteome level.

Functional annotation of uncharacterized proteins from Putranjiva and Cycas has shown that these protein sequences annotated as ‘unchracaterized’ from genomics and proteomics experiments cannot be ignored, and functional annotation using available bioinformatics tools and databases could assign them to new functional pathways, and thus providing more insights into biological process at molecular and cellular level.

# Chapter 6. Summary and Major findings

# SUMMARY AND FINDINGS

- Secondary sexual organ trait like flower size: in angiosperm, male flower size was smaller than the female flower and in gymnosperm female cone was more significant than the male cone.
- In female plants, selection favored a relatively large leaf, as this promotes the greater capacity for localized photosynthate production.
- Antioxidant scavenging capacity was more in females than males, in both Cycas and Putranjiva
- Female Putranjiva had significantly larger leaves, whereas males tended to have more leaves, like Cycas.
- Chlorophyll content was more in females when compared to males, in both Cycas and Putranjiva.
- Physiologically, males and females were not significantly different as evident from the stomatal count.
- Gradually pollen viability decreased after releasing from the flower.
- SEM analysis structure showed the aerodynamic nature of Pollen.
- Unreleased Pollen contained more glucose and fructose, whereas released Pollen contained more sucrose.
- Proline, Kinetin, and salicylic acid were essential for pollen development, maturity and viability.
- Spermine, cadaverine, and spermidine were found in higher concentrations in unreleased Pollen, whereas putrescine was found in higher concentrations in released Pollen.
- Accumulated 12-Octadecadienoic acid, together with palmitic acid and stearic acid had been identified to be crucial component for Pollen.

- From this study, we found lipid and phenolic compounds playing a major role in dioecious pollination events.
- Lipid metabolism was upregulated in dispersed conditions whereas the phenolic pathway was downregulated in dispersed conditions.
- From proteomics study, 146 out of 274 common proteins were found to be significantly differentially expressed (p-value < 0.05 and fold change of 2) in females compared to males from differential expression analysis.
- Upregulated proteins were found to be related to flower development, vegetative to reproductive phase transition, photoperiodism, regulation of metabolism whereas down regulated proteins were associated with negative regulation of flower development, reproductive shoot system, polysaccharide metabolic process.
- Proteins unique to females were found to be related to ovule development, vegetative to reproductive phase transition, photoperiodism, specification of floral organ identity, response to jasmonic acid etc.
- Proteins unique to males were found to related to anther development, stamen morphogenesis, abscisic acid-activated signalling pathway, circadian rhythm, response to ethylene etc.
- The proteins common to both male and female proteins were related to flower development, regulation of flower development, response to gibberellin, regulation of gene expression, regulation of transcription by RNA polymerase II, response to osmotic stress, response to oxidative stress etc.

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# Comparative metabolomic analysis of unreleased and released pollen from *Putranjiva roxburghii* Wall.

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ABSTRACT

Elucidating the biochemical and metabolic adaptations of unreleased and released pollen provides more insights into pollen development, maturation, and vitality. Hence, in the present investigation, *Putranjiva roxburghii* Wall. was selected to critically examine the biochemical and metabolite profiling of released and unreleased pollen states. Metabolomic profiling revealed 276 metabolites from the unreleased pollen and 271 metabolites from the released pollen, falling into 12 different categories. The levels of auxin family hormones like indole-3-acetic acid and 1-naphthalene acetic acid, along with other biomolecules like glucose, fructose, proline, 12-Octadecadienoic acid (C18:2 w6c), cadaverine, spermine and spermidine were higher in the unreleased pollen suggesting their functional significance in the pollen maturation. Whereas, the levels of kinetin, gibberellic acid, salicylic acid, sucrose, lipid, 12-Octadecadienoic acid were higher in the released pollen compared to unreleased pollen. Furthermore, the metabolites like lysophosphatidylinositol, phosphatidylglycerol, lysophosphatidic acid, linoleic acid and its derivatives, phosphatidylcholine, monoacylglycerol, sphingosine-1-phosphate and oleacein were found unique in released pollen, indicating these metabolites may participate to complete the pollen development. Among the carbohydrates, sucrose accumulation was higher in released pollen, signifying it to be a potent source of energy reservoir for future tube growth and germination processes. Additionally, increased fatty acid levels in the released pollen may act as a structural component preventing pollen from adverse environmental conditions for their viability and fertility. Thus, the current study unraveled the metabolic pathways associated with released pollen revealing the metabolic activities. This is the first study done on the wind pollination pollen in *Putranjiva roxburghii* Wall.



## 1. Introduction

*Putranjiva roxburghii* Wall. (Euphorbiaceae), is a dioecious, flowering plant species native to India, usually grown in evergreen forests of Asian tropical regions (Wurdack et al., 2004). It is an important medicinal and ornamental tree. Its fruits, seeds, and leaves are used to treat various ailments like fever, inflammation, female infertility and rheumatism (Reanmongkol et al., 2009; Supriya et al., 2017). Putranjiva is a wind-pollinated species (Matthews et al., 2013). Pollen development requires energy and involves different macromolecules associated with various metabolic activities. Pollen fertilization, development and maturation are associated with differential regulation of certain metabolites like carbohydrates, amino acids, lipids, plant hormones, alkaloids, flavonoids, and other organic compounds (Paupière et al., 2014, 2017).

Proteomics studies associated with pollen development identified proteins involved in carbohydrate metabolism, cell elongation, cell expansion, cell wall formation and cytoskeleton development, energy metabolism and protein synthesis (Zhang et al., 2021). Accumulation of starch and sucrose are essential for pollen maturation (Ko et al., 2022). Lipids and phospholipids like phosphoinositides (PIs) are an essential part of sporopollenin and pollen coat (Heilmann and Ischebeck, 2016; Piffanelli et al., 1998). Phenolic compounds possess an antioxidant property that reduces the oxidation level in the cell (Singh et al., 2021). Flavonoids are responsible for pollen maturation, tube growth, pollen viability and maintaining the homeostasis of reactive oxygen species (ROS) (Muhlemann et al., 2018). Polyamines (putrescine, spermidine and spermine) present on the pollen surface were found to be essential for pollen development, cell wall deposition and sporopollenin deposition in the pollen tube (Vogt., 2018; Mandrone et al., 2019).

The metabolic changes impact on pollen performance and development, resulting in structural deformities, immaturities and infertility. A metabolomic study revealed that during abiotic stress, sugar insufficiency is the major source of reproductive failure in pollen grains (Li et al., 2015). Metabolite profiling in chickpea showed that

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# Understanding dioecy in Cycas nayagarhensis and Putranjiva roxburghii Wall.: A Morphological, Biochemical and Omics approach

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