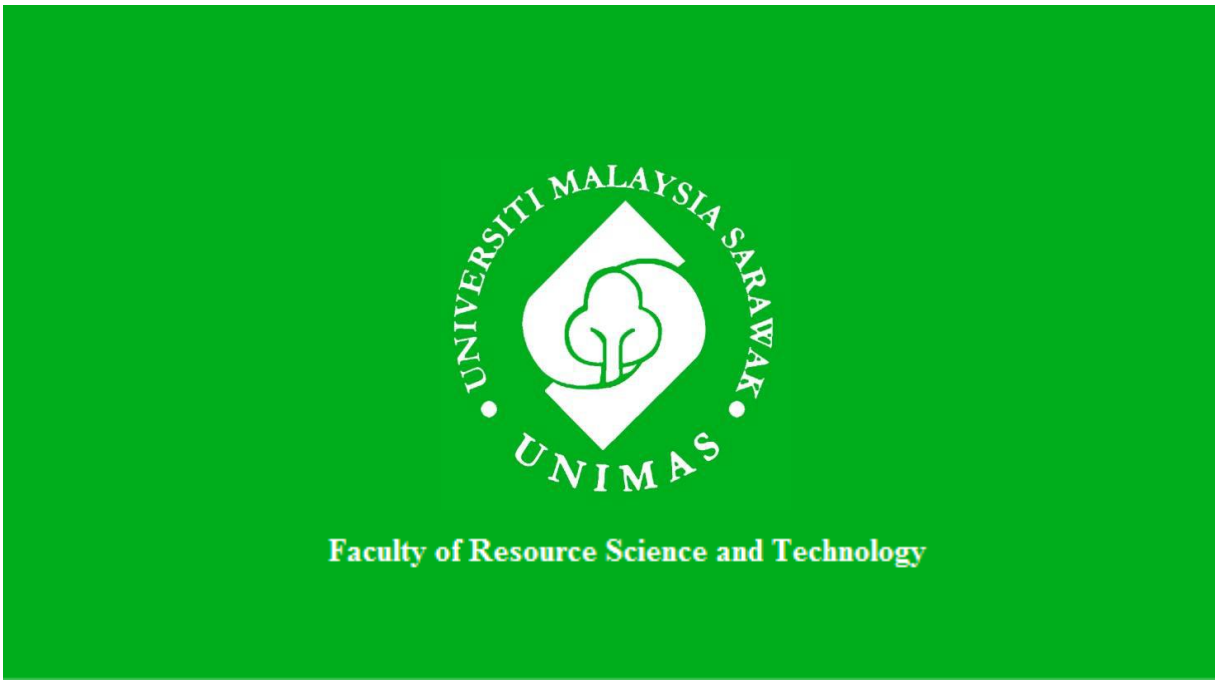

Breeding System and Phytochemical Analysis of *Orthosiphon aristatus* (Blume) Miq.

NUR KARIMAH BINTI MOHAMAD

Bachelor of Science with Honours
(Plant Resource Science and Management)
2015

Breeding System and Phytochemical Analysis of *Orthosiphon aristatus* (Blume) Miq.

Nur Karimah binti Mohamad
(37988)

This dissertation is submitted in partial fulfilment of the requirements for
The Degree of Bachelor of Science with Honours in
Plant Resource Science and Management

Department of Plant Science and Environmental Ecology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
94300 Kota Samarahan
Sarawak

APPROVAL SHEET

Name of candidate : Nur Karimah Binti Mohamad

Title of dissertation : Breeding system and phytochemical analysis of *Orthosiphon aristatus (*Blume) Miq.

.....

(Dr Freddy Yeo Kuok San)

Supervisor

.....

(Dr Zinnirah Shabdin)

Co-supervisor

.....

(Dr Rebicca Edward)

Coordinator of Final Year Project

Department of Plant Science and Environmental Ecology

DECLARATION

I hereby declare that no portion of the work referred to this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher education.

.....

(Nur Karimah Binti Mohamad)

Program of Plant Resource Science and Management

Department of Plant Science and Environmental Ecology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

ACKNOWLEGDEMENTS

Alhamdulillah, my deepest gratitude to the Almighty for granting me strength and patience through the ups and downs to complete this work. I would like to thank my supervisor, Dr Freddy Yeo Kuok San who had been the best guider, who had given full support and unwavering commitment, who kept on pushing me hard to do my best in order to accomplish this project. Thank you for your endless trust and encouragement even when I had done a lot of mistakes. Thank you for being the best lecturer ever. Not to forget, my co-supervisor, Dr Zinnirah Shabdin who had provide useful suggestions and comment especially in the phytochemical analysis part.

Furthermore, I would also like to extend my appreciation to my family, especially my parents; Mohamad bin Ismail and Zalika Khuzaimah binti Ngah, who had been my key motivator to keep on going and never giving up. To my precious friends, Nur Syauqina Syasya and Nurul Hafizah who painted my life with their adorable antics and laughter, thank you for being there for me and for your limitless support. To my FYP mates, Nor Ain, Siti Noridayu, Flora Eliza, Lee Kien and Daniel Chia who had been with me through this tough time completing this project, thank you for sharing your strength and lending me your helping hands when I needed them the most.

Lastly, thank you to all my friends and classmates in Plant Science and Management program. My appreciation also goes to all lecturers and staffs of Department of Plant Science and Environmental Ecology who have guided me and equipped me with necessities needed to accomplish my final year project. My deepest gratitude to all of you.

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List of Abbreviations

BAW	n-Butanol: Acetic Acid: Water
C	Carbon
GLC	Gas Liquid Chromatography
HPLC	High Performance Liquid Chromatography
H_3BO_3	Boric Acid
N	Nitrogen
NH_3	Ammonia
NPK	Nitrogen, Phosphorus, Potassium
OAV-1	*O. aristatus* variety (white)
OAV-2	*O. aristatus* variety (purple)
PC	Paper Chromatography
R_f	Retention Factor
ROS	Reactive Oxygen Species
UV	Ultraviolet
2D	Two Dimensional

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Nur Karimah Binti Mohamad

Program of Plant Resource Science and Management
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Faculty of Resource Science and Technology
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ABSTRACT

Orthosiphon aristatus (Blume) Miq. is a perennial herb under the mint family, Lamiaceae. The study on *O. aristatus* was conducted with several aims to accomplish which include (1) to study the breeding system of *O. aristatus* through artificial pollination, (2) to determine the flower developmental at which the pollen is most viable, (3) to investigate the potential of flavonoids as phytochemical markers, and (4) to determine whether there is any significant effect of different drying conditions on the flavonoid profiles of *O. aristatus*. In artificial pollination tests, two tests conducted which were self-pollination and cross pollination. All artificial pollination tests were considered as successful if seeds were developed. Pollen viability test were conducted using *in vitro* germination method and the pollens were collected from three different developmental stages; pre-anthesis, anthesis and post-anthesis. Whilst, for the phytochemical marker analysis of flavonoids, two dimensional paper chromatography (PC) was utilised as the separation technique. Retention factor (R_f) value was calculated for each compound observed and the flavonoid profiles of OAV-1 and OAV-2 were compared. Lastly, the different drying conditions were imposed on the *O. aristatus* samples to see if there is any significant effect on the flavonoids content of the leaf samples. From the study conducted, *O. aristatus* was suggested to be a pre-dominantly self-pollinated species with the support of pollen viability test that indicated viable pollen can be observed during pre-anthesis stage. The flavonoid profiles of OAV-1 and OAV-2 showed polymorphism which can be used as marker in breeding program. Drying quality test revealed that temperature plays important factor in the flavonoid contents of *O. aristatus* leaf samples as exposure to high temperature (60℃) caused degradation of flavonoid contents

Keywords: *Orthosiphon aristatus*, breeding system, artificial pollination, pollen viability, *in vitro* germination, flavonoids, two dimensional paper chromatography, drying quality.

ABSTRAK

Orthosiphon aristatus (Blume) Miq) adalah tumbuhan saka di bawah kelurga pudina, Lamiaceae. Kajian ke atas O. aristatus dijalankan dengan beberapa tujuan untuk dicapai termasuk (1) untuk mengkaji sistem pembiakan O. aristatus melalui pendebungaan artifisi, (2) untuk menentukan peringkat pertumbuhan bunga yang menghasilkan debunga yang paling subur, (3) untuk menyiasat potensi flavonoids sebagai penanda phytokimia, dan (4) untuk menentukan jika keadaan pengeringan yang berbeza terdapat kesan signifikasi ke atas profil flavonoid O. aristatus. Dalam ujian pendebungaan artifisi, dua jenis ujian telah dilakukan iaitu ujian pendebungaan sendiri dan pendebungaan menyilang. Semua ujian artifisi dianggap berjaya apabila biji berjaya dihasilkan. Ujian kesuburan debunga telah dijalankan menggunakan kaedah percambahan in vitro dan debunga-debunga telah dikutip daripada tiga peringkat pertumbuhan yang berbeza; pre-anthesis, anthesis dan post-anthesis. Manakala, untuk analisis penanda phytokimia flavonoids, kaedah kertas kromatom dua dimensi digunakan. Nilai faktor retensi (R_f) dikira dan profil flavonoid OAV-1 dan OAV-2 dibandingkan. Akhir sekali, keadaan pengeringan yang berbeza dideahkan kepada sampel O. aristatus untuk melihat kesannya ke atas kandungan flavonoids. Daripada kajian yang dilakukan, O. aristatus dicadangkan sebagai species yang melakukan pendebungaan sendiri, yang disokong oleh ujian kesuburan debunga yang menunjukkan debunga subur dapat diteliti ketika peringkat pre-anthesis. Profil flavonoid OAV-1 dan OAV-2 menunjukkan polimof yang boleh digunakan sebagai penanda dalam program pembiakan. Ujian kualiti pengeringan menunjukkan haba memainkan faktor penting dalam kandungan flavonoid dalam sampel daun O. aristatus yang menunjukkan pendedahan kepada suhu tinggi (60℃) menyebabkan degradasi kepada kandungan flavonoid.

Kata kunci: *Orthosiphon aristatus, sistem pembiakan, pendebungaan artifisi, kesuburan debunga, percambahan in vitro, flavonoids, kaedah kertas kromatom dua dimensi, kualiti pengeringan.*

1.0 BACKGROUND OF STUDY

1.1 Introduction

Orthosiphon aristatus (Blume) Miq. is a perennial herb under the mint family, Lamiaceae which is widely distributed in Southeast Asia and Australia. The word *Orthosiphon* is derived from latin words, *orthos* (straight) and *siphon* (cylindrical) based on its morphological features (Ameer et. al, 2011). Meanwhile, the word 'aristatus' means bearded. *O. aristatus* has several synonyms which includes *O. stamineus* Benth., *O. longiflorum* Ham., *O. spiralis* Merr., and *O. grandiflorus* Bold. Various vernacular names were given to *O. aristatus* such as Misai Kucing (Malaysia), Cat's whiskers, Java Tea (Europe), mao xu cao (China) and kabling gubat (Philippines) (FRIM, 2009). The prominent floral feature of this herb is the extension of pistil and stamens beyond the corolla that give the "cat's whiskers" visual (Andyanna, Setiawan & Insanu, 2013). There are two varieties of *O. aristatus* in Malaysia based on its floral and calyx colour, which are *O. aristatus* white variety (OAV-1) and *O. aristatus* purple variety (OAV-2) (Lee, 2004).

O. aristatus is widely known for its medicinal value and it has been vastly used in traditional remedies to treat diseases such as jaundice, hypertension, diabetes, rheumatoid, oedema, and gall-stone (Andyanna, Setiawan & Insanu, 2013). It also has potent antioxidant and anti-inflammation properties (Ameer et. al, 2011). In addition, this herb has synergistic bio-enhancing ability for tamoxifen against breast cancer (Ahamed Basheer & Abdul Majid, 2010). The demand for *O. aristatus*-related products increases with the changing lifestyle of modern generation that prioritize the healthy lifestyle. The products are usually marketed in the form of herbal tea or in capsules as supplementary diet.

Very few studies on *O. aristatus* are focusing on the breeding system and floral development of *O. aristatus*. Study by See (2014) suggested that the anthesis occur approximately 24 days after the emergence of the flower buds. He also pointed out that *O.*

aristatus is a predominantly self- pollinating species. See (2014) used a limited numbers of flowers in his artificial pollination study. Hence, this study would like to add on more information to the findings of See (2014) by performing artificial pollination test to add on the number of flowers of See (2014) and determining the flower developmental stage which has viable pollens for pollination.

This study would perform flavonoids profiling using the leaf samples of *O. aristatus*. Flavonoids are potential phytochemical markers for breeding of in *O. aristatus* cultivation. Flavonoids are naturally codominant inherited that enables the distinction between homozygosity and heterozygosity which can be applied in plant breeding practice to compare the parents and progenies (Bohm, 1998). Flavonoids are the secondary metabolites that present ubiquitously in plants and have been garnering interest from researchers due to diverse important roles in plant growth and development. It is known to enhance tolerance to biotic stressors and employed as defence agents against herbivores and pathogens. Flavonoids also form the basis for allelopathic interaction among other plants species (Gould & Lister, 2006).

This study is focusing on the breeding system of *O. aristatus* and the pollen viability of the species. It also aims to analyse of flavonoids as potential chemical markers and investigate the effect of different drying conditions on the flavonoid profiles. This study also aspired to help better understanding on the biology of this herb and to provide necessary data for the development of potential markers which can be used in modern breeding.

1.2 Problem Statement

The artificial pollination study by See (2014) had used limited number of flower samples of *O. aristatus* for part of his artificial pollination study. In addition to that, the flower developmental stage at which the viable pollen is produced is not known for this species. Besides that, there is limited marker information which can be applied in breeding program using flavonoids. The effect of different drying conditions (using air-drying, sin-drying and oven-drying methods) on flavonoids of this species has not been determined.

1.3 Objectives

1. To study the breeding system of *O. aristatus* through artificial pollination.
2. To determine the flower development stage which the pollen is most viable.
3. To investigate the potential of flavonoids as phytochemical markers.
4. To determine whether there is any significant effect of different drying conditions on the flavonoid profiles of *O. aristatus*.

2.0 LITERATURE REVIEW

2.1 Classification and Description of *Orthosiphon aristatus*

Orthosiphon aristatus Blume (Miq.) is from the medicinal herb belongs to the mint family, Lamiaceae under the order Lamiales. *Orthosiphon* comes from the Latin words *orthos* and *siphon* which mean straight and cylindrical respectively (Ameer et al., 2012). Leaves of this plant are used commonly in Southeast Asia and European countries for herbal tea, well known as "Java tea" (Indu Bala & Ng, 2003). It is also known as the cat's whiskers due to its elongated pistils and stamens beyond the corolla of the flower that resemble the cat's whiskers (Andyanna, Setiawan & Insanu, 2013). The taxonomic classification of *O. aristatus* is in Table 1.

Table 1: Taxonomic classification of *O. aristatus.*

Taxonomic Rank	**Classification**
Kingdom	Plantae
Class	Magnoliopsida
Phylum	Magnoliophyta
Order	Lamiales
Family	Lamiaceae
Genus	*Orthosiphon*
Species	*Orthosiphon aristatus* Blume (Miq.)

(Source: Encyclopedia of Life, www.eol.com)

O. aristatus is a perennial herb that can grow up to 25 to 200 cm tall, with quadrangular, poorly ramified, ascending stem. It has opposite leaves with ovate or rhombic shape. The leaf is cuneate at base, acute or acuminate at apex, has serrate margin

and has glabrous texture (Figure 1). The stipule is absent. Inflorescence is an opposed cyme arranged in terminal racemes ranging from 7-29 cm long. Its flowers are bilabiate, pedicellate with gland-dotted calyx and white or pale lilac tubular corolla with four long protruding stamens from the corolla tube (Figure 2). The ovary is superior with long protruding slender style and enlarged, club-shaped and shallowly cleft stigma. The fruit splits into 4 oblong-ovoid nutlets with brownish colour and rugose texture (Dzulkarnain, Widowati, Isnawati & Thijssen, 1999).

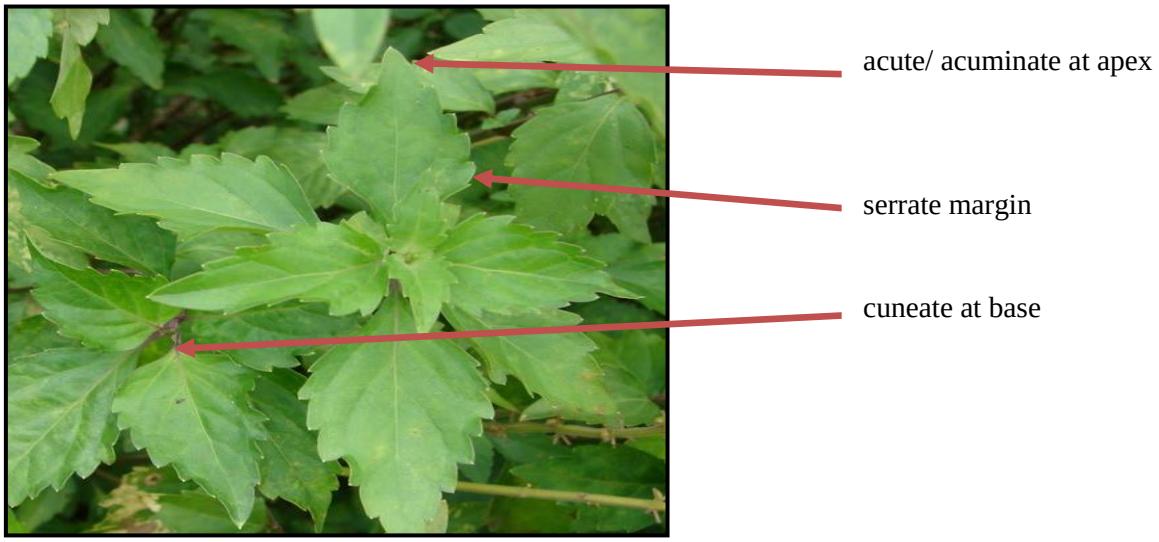

Figure 1: The leaf structures of *O. aristatus*

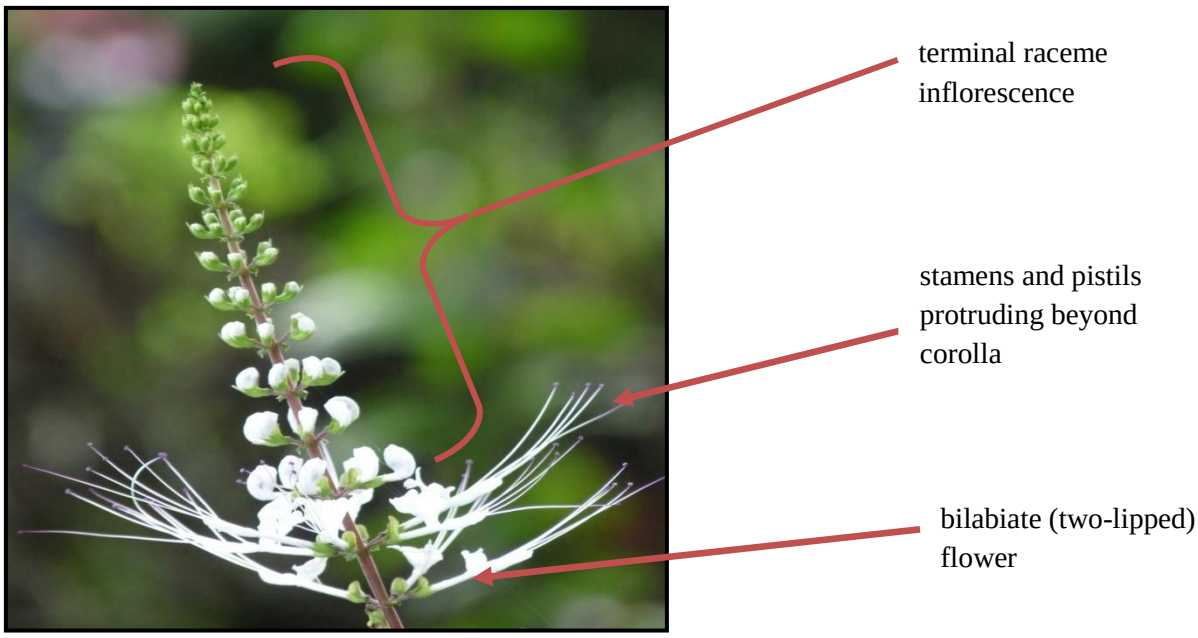

Figure 2: The inflorescence of *O. aristatus*

2.2 Comparisons between Varieties of *O. aristatus*

2.2.1 Morphological Comparisons

There are two varieties of *O. aristatus* known in Malaysia which are the white-flowered (OAV-1) and the purple-flowered (OAV-2) varieties. Basically, both varieties have similar morphological characteristics aside from their colour. The similar physical features include the root system, vascular system, leaf arrangement, structures and characteristics, reproduction structure and pollination system. The similarities are further described in Table 2.

Table 2: The morphological similarities between OAV-1 and OAV-2 (Chan & Loo, 2006).

Physical Features	OAV-1 and OAV-2
Leaves	- Arrangement: opposite - Petioled - Colour: dark green
Stem shape	- Quadrangular
Inflorescence	- Racemose verticilaster type - With 6 flowers in whorls along floral axis
Flowers	- Lower flowers are older and and bloom earlier than lower flowers - Bibiliate (two-lipped) flowers - Each flower possessed irregular gamosepalous calyx that was made up of five sepals - Have 4 stamens, 2 long and 2 short attached to the dorsal part of corolla tube
Stigma	- 2 lobed stigma with rough surface
Anther	- Consists of 2 lobes, each lobes has 2 pollen sacs (loculli)
Pollen grains	- Spherical, 6 furrow-like colpus.
Seeds	- Oval-shaped - Hard rough testa - Contain 4 seeds in each fruit pod.

There are also several differences OAV-1 and OAV-2 that enable us to differentiate them that include the leaf shapes, colour of leaf venation, colour of corolla and calyx, colour of fruit and presence of yellow spots on the leaves surface (Table 3).

Table 3: The morphological differences between OAV-1 and OAV-2 (Chan & Loo, 2006).

Physical Features	**OAV-1**	**OAV-2**
Leaf shapes	Rhomboid shape with acuminate apex and obtuse base	Ovate shape with acute apex and truncate base
Colour of corolla	White without purple tint	With purple tint at the lobes of corolla
Colour of calyx	Green	Maroon
Colour of fruit	Greenish red when mature	Purplish red when mature
Colour of leaf venation	Green	Purple
Presence of yellow spots	Absent	Present on abaxial and adaxial leaf surface

Although Chan & Loo (2006) reported that the yellow spots present on surfaces of OAV-2 leaves, the finding does not acquaint with See (2014). In the observation by See (2014), he did not found any presence of yellow spots on leaf surfaces of OAV-2 that are able to be used to distinguish between the two varieties of *O. aristatus*.

2.2.2 Chemical comparisons

Through chemical aspect, there is no significant difference between OAV-1 and OAV-2 of *O. aristatus* (Chew, Hamdan, Ismail & Ahmad, 2004). They are basically made up of the same chemical constituents (Table 4). However, Lee (2004) reported that OAV-2 contains higher bioactive compound compared to OAV-1.

Table 4: The chemical components of *O. aristatus* (Ameer et al., 2012).

Compound	Class
Sinensetin	Flavonoids
Eupatorin	Flavonoids
3'-Hydroxy-5,6,7,4'- tetramethoxyflavone	Flavonoids
Tetramethylseutellarein	Flavonoids
Salvegenin	Flavonoids
Ladanein, vomifoliolm	Flavonoids
Pillion	Flavonoids
Caffeic acid	Polyphenol
Rosmarinic acid	Polyphenol
Cichoric acid	Polyphenol
Orthosipol A - Z	Diterpene
Staminol A - D	Diterpene
Ursolic acid, oleanolic acid, betulinic aid	Triterpene
Naphtelene	Aromatic hydrocarbon

2.3 Condition for Cultivation

O. aristatus is propagated using the stem cuttings at about 15-20 cm long. The cuttings are planted in shade and often placed three to four cuttings in a hole (Dzulkarnain, Widowati, Isnawati & Thijssen, 1999). According to Affendy et al. (2010), 50% of relative light intensity is needed for good plant growth for *O. aristatus.* In plantation, planting in a nursery for 45 days by positioning the cutting vertically with only one bud present is preferred (Rashid et al., 2012). The livestock manure such as chicken dung can be used as the organic fertilizers for *O. aristatus*. The inorganic NPK fertilizer (10: 10: 10) can also be used to enhance growth of the plants.

O. aristatus is almost a carefree plant without any serious pests or diseases and it can easily adapt to its surrounding (Affendy et al., 2010). However, there are several problems that need to be noted in its early juvenile stage. Weeds can distort the growth of *O. aristatus*. The weeds can be prevented by attentive removal from the area. We can also use the plastic cover to prevent the infestation of weeds.

Fungal diseases and pests of *O. aristatus* can be caused by *Botrytis cinerea*, *Corticium rolfsii*, *Moniliopsis aderholdii* and *Phytium debaryanum* that cause losses in *O. aristatus* cultivation (Dzulkarnain, Widowati, Isnawati & Thijssen, 1999). Nematodes can also cause problem in which they may cause galls to develop on the roots that disturb the root system (Dzulkarnain, Widowati, Isnawati & Thijssen, 1999).

2.4 Nature of Pollination

Plant pollination process describes the vital method of sexual reproduction in plants. It enables a plant to bear fruit and seeds. The pollination process involve the transfer of pollen from a male part (stamen) to a stigma of a female part (carpel). The pollen contains the male sperm or gametes that will be received by the ovary of a female part and fused together to form an embryo.

There are two type of pollination which are self-pollination and cross-pollination. Self pollination involves the transfer of pollen within one flower or between flowers of the same plants. On the other hand, cross-pollination occurs between flowers of different plants or varieties or cultivars within the same species.

2.4.1 Artificial Pollination

Artificial pollination is the human intervention in the pollination process of plants when natural pollination is unreliable or undesirable. Artificial pollination is preferred in breeding because the breeders can choose whether to pollinate the plant with its pollen (selfing) or with pollen from other plants (crossing) or from other closely related species (hybridising) (de Jong, 2002). Through artificial pollination, breeders do not have to wait for specific seasons to pollinate flowers.

There are several types of artificial pollination for examples hand pollination and liquid spray pollination which usually done after an emasculation to remove any unwanted flowers or flower parts. Even though artificial pollination is preferred, it has its own limitations. Artificial pollination has restricted effective period and the success depends on the environmental influences such wind and rain (Sakamoto et al., 2009; Zeraatkar, Karimi, Shamshiri & Tajabadipur, 2013). Hand pollination involves tedious work that leads to high input cost. However, it can be solved by using the liquid spray technique as

an alternative that reduce the time, labour and input cost. Liquid spraying needs less than half of the time required from hand pollination (Zeraatkar, Karimi, Shamshiri & Tajabadipur, 2013) and it was reported that only one third of pollen grains needed for hand pollination was required in the study of Japanese pear by Sakamoto et al. (2009).

Artificial pollination is used in a lot of studies such as to evaluate the pollination methods suitable for specific plants (Zeraatkar, Karimi, Shamshiri & Tajabadipur, 2013), to analyse effects of pollination methods on pollen distribution on stigma and pollen tube growth (Kimura, Okamoto & Hirano, 1998), and to evaluate the effects of types of pollination on fruit and offspring quality (Chautá-Mellizo et al., 2012).

2.5 Pollen Viability

Pollen is an exceptional plant tissue that can be manipulated to the advantages of the breeders. The aspects related to pollen quality include the pollen viability and its morphological homogeneity which are important in pant pollination and fertilization. In relation to the artificial pollination mentioned before (Section 2.4.1), the determination of pollen viability is vital as the success of the tedious hand pollination in plants is dependent to the viability of pollen (Pline et al., 2002). There are three types of tests that can be performed to evaluate the pollen viability of a species which are based upon i) pollen staining or fluorescence, ii) fertilization or seed set, and iii) pollen germination (Hecker & McClintock, 1988; Bolat & Pirlak, 1999; Soares, de Jesus, Dos Santos-Serejo & de Oliveira, 2013).

Pollen viability test- pollen staining

Pollen staining test is done by observing the colour of the pollen under microscope after being stained by chemical stains such as acetocarmin, prepione, carmin, anilin blue and Alexander's stain. The coloured pollen is considered viable while the non-coloured pollen is considered as dead or not viable (Figure 3). We can also use the fluorochromatic procedure in which the pollens are dipped into fluorescien diacetate. The viable pollen will fluoresce under UV light, whilst the non-viable pollen will produce faint or no fluorescing (Cavusoglu & Sulusoglu, 2013). The staining/ fluorescing test is the easiest and fastest way to determine the viability of pollen. However, it does not provide precise information and considered useful when there is an adequate estimation of viability beforehand. In fluorescing test, the immature pollen will also fluoresce and counted as viable therefore providing inaccurate estimation of the viable pollens present. Staining/ fluorescing test tends to overestimate the numbers of viable pollen (Soares, de Jesus, Dos Santos-Serejo & de Oliveira, 2013).

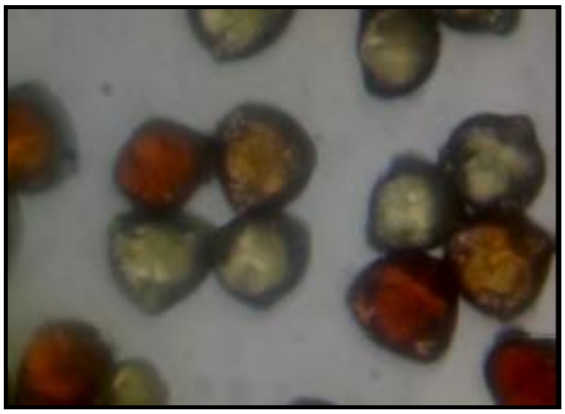

Figure 3: Pollen staining test using TTC (2,3,5- Triphenyl tetrazolium chloride). Viable pollens are dyed light red while the non-viable pollen are dyed with faint yellow or appear with no colour (Cavusoglu & Sulusoglu, 2013).

Pollen viability test- fertilization or seed set

Fertilization or seed set test examines the capability of the pollen to fertilize given plant. Unpollinated, receptive stigmas are pollinated lightly with the selected pollen. Some species require emasculation of the male parts in order to prevent the pollination from occurring with pollen from other male inflorescence besides the test pollen. The pollen application must be handled with care as too much pollen may cause dehydration whilst too little pollen may decrease the chance of success (Heslop-Harrison, Heslop-Harrison & Shivanna, 1985). The fertilization or seed set test provide the true measure of the success of the pollination with the condition that there is no incompatibilities or embryo abortion (Pline et al., 2002). However it requires too much time to obtain the data needed (Hecker & McClintock, 1988). In addition, the pollen viability is not the sole factor that affect fertilization, the receptivity of the stigma and pollen disposition also play essential aspects in the success of the fertilization of the egg cell (Pline et al. 2002).

Pollen viability test- pollen germination

The pollen germination test assumes that if the pollen is able to germinate the pollen tube, then it is likely to be able to fertilize the egg cell (Heslop-Harrison, Heslop-