THE REPRODUCTIVE BIOLOGY AND CYTOTOXIC ACTIVITY OF *PERSICARIA CHINENSIS* (L.) H. GROSS VAR. *CHINENSIS* (POLYGONACEAE)

WONG MIN MAY

FACULTY OF SCIENCE
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WONG MIN MAY

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FACULTY OF SCIENCE
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ABSTRACT

Persicaria chinensis var. *chinensis* is a native variety in Malaysia and found in the tropics growing in lowland and lower montane forests. Growth habit and morphological differences were observed between the highland and lowland varieties. The anthesis of the highland wild population was at 0600–0730 hours whereas that of the lowland variety was at 0745–0930 hours.

The anther is tetrasporangiate and the wall development conforms to the Monocotyledonous type. Cytokinesis in the microspore mother cells is simultaneous forming tetrahedral and decussate tetrads in the highland variety and mostly tetrahedral tetrads in the lowland variety. The mature pollen grains are shed at the three-cell stage. The ovule is orthotropous, bitegmic and crassinucellate and the micropyle is formed by both the outer and inner integuments. The development of the embryo sac conforms to the monosporic *Polygonum* type. An occurrence of twin eggs within an ovule was observed in the lowland variety. In the highland variety, the development of the endosperm is *ab initio* Nuclear type.

The highland variety is heterostylous; pin flowers have taller style, shorter filaments, less thick and less wide stigma than the thrum flower. The number of pollen grains in a pin flower is higher than that in a thrum flower. The pollen grains of the thrum flowers are bigger than those of the pin flowers. The pin and thrum pollen grains are 3-colpate, medium-sized, suboblate to oblate and have reticulate exine with thick, narrow muri and free clavae in the deep lumina.

The lowland variety is observed to have only thrum flower. They have shorter style, longer filaments, thicker and wider stigma than those of the highland thrum flowers. The pollen grains of the lowland variety are bigger than those of the highland thrum flowers. Their lumina were less wide and the muri were thicker, less narrow and shorter than those of the highland thrum pollen with free clavae.

In the highland variety, the optimum sucrose and PEG concentration for pollen germination was 15% and 30% respectively. Bursting of pollen grain occurred in the medium without PEG. Pollen grains reached maximum germination (50%) in the modified medium with an average pollen tube length of 140 µm.

The highland variety of *P. chinensis* var. *chinensis* was self-compatible. Open pollination produced three times more fruits than self pollination and they are cross-compatible and show outbreeding. The pollen-ovule ratio suggested the breeding system was facultative xenogamy. The most common insect visitors are from the Order of Hymenoptera. Seed germination is epigeal showing 77% viability. The average percentage of germination for the fresh seeds was 47% in the natural highland environment and 34% in the lowland laboratory environment.

In Malaysia, *P. chinensis* var. *chinensis* has been used traditionally as one of the main ingredients in Chinese traditional medicine to treat various lung ailments including cancer. The *in vitro* neutral red cytotoxicity assay was successfully applied to screen the cytotoxic activity against 18 crude methanol, dichloromethane and water extracts from the highland and lowland varieties of *P. chinensis* var. *chinensis* derived from the leaf, stem and whole plant. Overall results showed that the methanol extracts from the leaf samples of the highland and lowland varieties possessed greater cytotoxicity effect against cancer cell lines. Two active extracts obtained from the methanol leaf extracts of the highland and lowland varieties were more effective in suppressing the expression of CaSki and SKOV-3 cells. The dichloromethane extract from the stem sample of the highland variety also showed significant cytotoxic effect against HT-29 cells. Since both varieties showed signs of the selectivity for CaSki, SKOV-3 and HT-29 cells, these findings suggest that *P. chinensis* var. *chinensis* should be further researched to isolate and identify the active compound(s) from the active extracts which demonstrated significant cytotoxic activities.

ABSTRAK

Persicaria chinensis var. *chinensis* ialah varieti tempatan yang terdapat di tropik dan tumbuh di tanah pamah dan hutan hujan gunung bawah. Perbezaan dalam sifat pertumbuhan dan morfologi dapat diperhatikan di antara varieti tanah tinggi dan pamah. Masa antesis populasi varieti tanah tinggi ialah 0600–0730 sementara varieti tanah rendah ialah 0745–0930. Dehis anter dan stigma reseptiviti adalah serentak.

Anter adalah tetrasporangiat dan pembangunan dinding anter mematuhi jenis monokot. Sitokinesis serentak berlaku dalam sel-sel ibu mikrospora dan membentuk kedua-dua jenis tetrad 'tetrahedral' dan 'decussate' dalam varieti tanah tinggi dan kebanyakannya tetrad 'tetrahedral' dalam varieti pamah. Debunga matang terbentuk di peringkat tiga sel. Ovul ialah ortotropus, bitegmik dan krasinuselat dan mikropil terbentuk daripada kedua-dua integumen luar dan dalam. Perkembangan pundi embrio mematuhi jenis yang monosporik *Polygonum*. Kejadian telur kembar dalam ovul diperhatikan dalam varieti tanah rendah. Dalam varieti tanah tinggi, pembangunan endosperma mengikuti jenis *ab initio* Nukleus.

Bunga-bunga varieti tanah tinggi ialah heterostil; bunga pin mempunyai stil yang lebih tinggi, filamen yang lebih pendek, stigma yang kurang tebal dan kurang luas dibanding dengan bunga thrum. Bilangan butir debunga pada bunga pin lebih banyak daripada bunga thrum. Debunga bunga thrum lebih besar daripada bunga pin. Butir debunga pin dan thrum adalah 3-kolpat, ukuran sederhana, daripada suboblat kepada oblat dan mempunyai eksin retikulat dengan muri yang tebal, sempit dan klavae yang bebas di antara lumina.

Bunga thrum tanah pamah mempunyai stil yang lebih pendek, filamen yang lebih panjang, stigma yang lebih tebal dan lebih luas daripada bunga thrum varieti tanah tinggi. Ukuran debunga varieti tanah pamah adalah lebih besar daripada debunga thrum varieti tanah tinggi. Lumina debunga thrum tanah rendah adalah kurang luas

daripada debunga thrum tanah tinggi dan Muri debunga thrum varieti tanah pamah adalah lebih pendek daripada debunga thrum varieti tanah tinggi.

Dalam varieti tanah tinggi, sukrosa optima dan kepekatan PEG untuk percambahan masing-masing adalah 15% dan 30%. Debunga terpecah berlaku dalam medium tanpa PEG. Debunga mencapai percambahan maksimum (50%) dalam medium yang diubah suai dengan tiub debunga mempunyai purata panjang 140 μm.

Varieti tanah tinggi *P. chinensis* var. *chinensis* berupaya berkacuk sendiri. Pendebungaan terbuka yang dihasilkan adalah tiga kali ganda lebih banyak buahnya daripada pendebungaan sendiri dan mereka berupaya berkacuk luar dan perkacukan asing telah ditunjukkan. Nisbah debunga-ovul mencadangkan sistem pembiakan adalah xenogami fakultatif. Pelawat serangga yang paling biasa adalah terdiri daripada Order Hymenoptera. Percambahan biji adalah epigeal dan menunjukkan viabiliti bijinya sebanyak 77%. Purata peratusan percambahan benih segar adalah 47% di bawah percambahan di persekitaran tanah tinggi semulajadi dan 34% di bawah persekitaran makmal di tanah rendah.

Di Malaysia, *P. chinensis* var. *chinensis* telah digunakan secara tradisional sebagai salah satu bahan utama dalam perubatan tradisional Cina untuk merawat pelbagai jenis penyakit peparu termasuk kanser. *In vitro* sitotoksiti neutral merah berjaya digunakan untuk menyaring aktiviti sitotoksik terhadap 18 metanol mentah, diklorometana dan ekstrak air dari varieti tanah tinggi dan tanah pamah *P. chinensis* var. *chinensis* yang diperolehi daripada daun, batang dan keseluruhan tumbuhan (daun dan batang). Keputusan keseluruhan menunjukkan bahawa ekstrak metanol daripada sampel daun varieti tanah tinggi dan tanah pamah mempunyai kesan sitotoksik yang lebih tinggi terhadap sel kanser. Dua ekstrak aktif yang diperolehi daripada ekstrak metanol daun varieti tanah tinggi dan tanah rendah adalah lebih berkesan dalam ungkapan menekan sel CaSki dan sel SKOV-3. Ekstrak diklorometana dari sampel

batang varieti tanah tinggi juga menunjukkan kesan sitotoksik yang ketara ke atas sel HT-29. Memandangkan kedua-dua varieti tanah tinggi dan tanah pamah menunjukkan tanda-tanda selektiviti terhadap CaSki, SKOV-3 dan sel-sel HT-29, dengan itu penemuan ini menunjukkan bahawa *P. chinensis* var. *chinensis* perlu dikaji lebih lanjut untuk mengasingkan dan mengenalpasti sebatian aktif yang khusus dari ekstrak aktif yang menunjukkan aktiviti sitotoksik yang ketara.

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LIST OF SYMBOLS AND ABBREVIATIONS

i.e.	id est (that is)
ºC	degree Celsius
et al.	et alia (and other)
var.	variety (botany)
syn	synonym
sp.	species
FRIM	Forest Research Institute Malaysia
KLU	The University of Malaya Herbarium
SING	The Singapore Herbarium
BKF	The Forest Herbarium, Bangkok
P/E	polar length over equatorial diameter
g	gram
%	percentage
PEG	polyethylene glycol
H_3CO_3	boric acid
$Ca(NO_3)_2.4H_2O$	calcium nitrate tetrahydrate
$MnSO_4.1H_2O$	manganese (II) sulphate monohydrate
KNO	potassium nitrate
TZ	tetrazolium
m	meter
mm	millimeter
cm	centimeter
µg	microgram
µl	microlitre
ml	milliliter
mg	milligram
SEM	scanning electron microscope
LM	light microscope
rpm	rotation per minute
cont.	continued
n	number of specimens studied
=	equals to
–	to (long hyphene)
n.a.	not available
ATCC	American Tissue Culture Collection
CO_2	carbon dioxide
DMSO	dimethyl sulfoxide
IC_{50}	half maximal inhibitory concentration
ELISA	enzyme-linked immunosorbent assay
OD	optical density
NR	neutral red
kg	kilogram
PBS	phosphate buffer saline
MeOH	Methanol
DCM	dichloromethane
≤	less than equal
≥	greater than equal
RPMI	Rosewell Park Memorial Institute
GPS	Global Positioning Systems

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

The Southeast Asian tropical rain forests, also known as Indo-Malayan or Malesian rain forests, are one of the most extensive and species-rich terrestrial habitats of the world (Richards, 1952; Whitmore, 1984). The flora of Malesia is estimated to support 25,000 species of flowering plants (van Steenis, 1971) which is about ten percent of the world's flora, with the Southeast Asian tropical rain forests estimated to comprise 6,500 medicinal plant species (Samy *et al*., 2005).

In Malaysia, 19.12 million hectares of rain forest area occupying some 58.1 percent of the land area in this country is estimated to have 15,000 plant species (Saw, 2010), with ten percent of them reported to have medicinal value (Samy *et al*., 2005). This suggests that, Malaysia is exceedingly rich in the natural resources used in traditional medicine in Southeast Asia.

Polygonaceae, the buckwheat or knotweed family (Henderson, 1959) is a cosmopolitan family of perennial herbs, some shrubs, small trees, climbers or twining lianas characterized by alternate simple leaves with sheathing ocrea stipules, unilocular ovary and endospermic seeds (Brandbyge, 1993; Brummitt, 1992; Heywood *et al*., 2007; Hutchinson & Dalziel, 1954). This family generally includes 43 (Brandbyge, 1993) to 55 (Qaiser, 2001) genera, embracing about 1200 species, and occupying a wide range of habitats (Brandbyge, 1993; Brummitt, 1992; Heywood *et al.*, 2007; Mabberley, 2008). Most genera are restricted to the temperate Northern regions while the remaining are tropical or subtropical (Heywood *et al*., 2007; Mabberley, 2008).

The genus *Persicaria* (L.) Mill. was first established in 1754 by Miller without typification but later lectotypified by Britton and Brown (1913) based on *Polygonum persicaria* L. (Kantachot *et al*., 2010). This genus currently consists of about 150

species worldwide. *Persicaria* is a cosmopolitan genus found mainly in temperate regions but some species occur in the tropical and subtropical regions from lowland to higher montane elevations (Heywood *et al.*, 2007; Mabberley, 2008). This taxon commonly perennials, with some of the species being edible and widely cultivated, for example *Persicaria minor* (Huds) Opiz (syn = *Polygonum minus* Huds) or usually known as *daun kesum* in the Malay language.

The exact circumscription and infrageneric classification of the *Persicaria* and *Polygonum* L. are still open to debate (Heywood *et al.*, 2007; Ronse Decraene & Akeroyd, 1988; Steward, 1930; Wilson, 1990). Some authors categorize *Persicaria* as merely a section of *Polygonum sensu lato* (Kuo *et al*., 1996; Li *et al.*, 2003; Ridley, 1924; Steward, 1930). However, many authors have proposed recognizing *Persicaria* as a separate genus based on the palynological and anatomical data (Haraldson, 1978; Hedberg, 1946), floral character and fruit morphology (Ronse Decraene & Akeroyd, 1988; Ronse Decraene *et al*., 2000, 2004) and phylogenetic analyses (Frye & Kron, 2003; Kantachot *et al*., 2010; Kim & Donoghue, 2008). Freeman and Reveal (2005), Hara (1982), Qaiser (2001) and Wilson (1990) have separated *Persicaria* as a distinct genus in their revision of the family Polygonaceae. *Persicaria chinensis* (L.) H. Gross var. *chinensis* is classified into the tribe *Persicarieae* under the subfamily Polygonoideae, which comprises three genera that are native to the temperate regions of Eurasia. They are commonly perennial and annual herbaceous species often with succulent stems, two whorls of five tepals and not usually winged or keeled (Heywood *et al.*, 2007).

The family Polygonaceae has not been revised in Peninsular Malaysia until Ridley (1924), who reported more than 600 species worldwide and recognized the genus *Polygonum* with nine species in the Malay Peninsula while Turner (1995) reported 14 species in this family and documented the genus *Persicaria* with 11 species

in peninsular Malaysia and Singapore. In Thailand, Kantachot *et al.* (2010) has reported 21 species of *Persicaria*, and three varieties from the species *P. chinensis* have been recorded. In Malaysia, two varieties have been identified based on the differences in the leaf shape and size, namely *P. chinensis* var. *chinensis* and *P. chinensis* (L.) H. Gross var. *ovalifolia* (Meisn.) H. Hara (syn *Polygonum malaicum* Danser & *Polygonum auriculatum* Meisn.) (Turner, 1995). The *ovalifolia* variety is found growing side by side with the *chinensis* variety in the wild population.

The medicinal plants from the family Polygonaceae, namely *Persicaria chinensis* (L.) H.Gross var. *chinensis* (syn *Polygonum chinense* L.), has been chosen as the target plant as this plant is being used as folk medicine in the Malaysian Chinese community (Henderson, 1959). *P. chinensis* var. *chinensis*, a perennial or scrambling herb, is native to tropical and subtropical eastern Asia (Wagner *et al.*, 1990). Ridley (1924) reports that this species is commonly found in hilly areas at around 1300 m in Perak, Pahang and Kedah. *P. chinensis* var. *chinensis* is found growing abundantly in the wet valleys and on grassy slopes in China from sea level up to altitude of 2400 m (Li *et al.*, 2003) and widely distributed in Taiwan, Japan, Philippines, India, Sri Lanka, Himalaya mountain region and Southeast Asian countries (Li *et al.*, 2003; Qaiser, 2001). This plant has capitate inflorescence, glabrous ocrea and persistent perianth present even in the fleshy fruit. The leaf shape varies from ovate to elliptic or lanceolate. The morphology of *P. chinensis* var. *chinensis* is highly variable, especially the leaf shape and size. This species has four varieties (Kanthachot *et al.*, 2007, 2010) and they differ in the leaf shape, size, texture and stem indumentums.

In Malaysia, the wild variety of *P. chinensis* var. *chinensis* is commonly found in the montane forests up to 1200 m in the Main Range in Perak and Pahang, such as in Cameron Highlands and Gunung Ulu Kali. This plant is also found cultivated in the lowlands especially among the Chinese community as it is one of the main ingredients

used to treat lung ailments in traditional medicine. However, the wild variety of this plant is rarely used in traditional medicine because the morphology of the wild variety, especially the growth habit, leaf shape and size are generally different from the cultivated variety. According to Turner (1995), the wild variety of this scrambling herb is normally found in damp waste ground in lowlands and mountains and is a native species in Malaya (Henderson, 1959). The wild variety can be propagated by seeds and cuttings but the cultivated variety hardly produce any mature fruits and it is commonly propagated by cuttings.

Persicaria chinensis var. *chinensis*, also known as Mountain knotweed, Chinese knotweed, or hill buckwheat is used medicinally in China where it is known as *huo tan mu, ch'ih ti li,* and *shan ch'iao mai* (Chinese) (Wiart, 2006). In China, the whole plant is used traditionally to clear heat and eliminate toxins, to treat dysentery, inflammatory skin disease, eczema and corneal nebula (Wan *et al*., 2009). The roots of *Polygonum chinense* are used to treat fluxes, to remove intestinal worms, and to counteract scorpion poisoning (Wiart, 2006). This plant is found to contain 25-R-spirost-4-ene-3,12-dione, stigmast-4-ene-3,6-ione, stigmastane-3,6-dione, hecogenin, and aurantiamide, which are anti-inflammatory and anti-allergic (Tsai *et al*., 1998; Wang *et al*., 2005; Xie *et al*., 2007). In Taiwan, *Polygonum chinense* is used in its entirety as a folk medicine to treat many infectious diseases (Jiangsu, 1986). Recently reported by Galloway & Lepper (2010), *Persicaria chinensis* has been introduced to New Zealand, possibly as a Chinese medicine ingredient but this plant has been found growing rampantly along the boundary of several properties in Auckland and its invasiveness has been investigated.

The pharmacological property of this herb is still unexplored (Wiart, 2006). Hence, this project is undertaken to evaluate the cytotoxic activity of the crude methanol, dichloromethane and water extracts of the leaves, stems and whole plant of

both the cultivated and wild variety of *P. chinensis* var. *chinensis* by an *in vitro* growth inhibition assay system against selected cancer cell lines of CaSki, SKOV3, HT29, A549, MCF-7 and normal human lung fibroblasts cell MRC5.

As knowledge on the reproductive biology of both the cultivated and wild varieties is extremely lacking, this project also aims to elucidate and enhance the basic knowledge pertaining to the reproductive biology of this variety including its phenology, embryology, pollen morphology, pollination and fruit formation. This information which is generally lacking and incomplete, particularly in Malaysia, will therefore help in establishing the breeding system of this medicinal plant.

CHAPTER 2

LITERATURE REVIEW

2.1 The family Polygonaceae, genus *Persicaria*

2.1.1 Taxonomic history

The Polygonaceae is a large cosmopolitan family consisting mostly of herbs, some shrubs, trees (Tribe Triplareae including *Triplaris* Loefl., *Ruprechtia* C.A.Mey), or clambering, climbing (*Coccoloba* L., *Brunnichia* Banks ex Gaertn.), twining lianas (tribe Coccolobeae including *Muehlenbeckia* Meisn., *Fallopia* Adans.) (Brandbyge, 1993; Freeman & Reveal, 2005; Heywood *et al*., 2007; Mabberley, 2008; Qaiser, 2001). Members of the Polygonaceae are greatly varied, of diverse morphological clades and has been described to range from 43 (Brandbyge, 1993; Kuo *et al*., 1996) to 55 genera (Heywood *et al*., 2007; Qaiser, 2001), and comprises 1100 species (Brandbyge, 1993; Kuo *et al*., 1996; Li *et al*., 2003) to 1200 species (Heywood *et al*., 2007; Mabberley, 2008; Qaiser, 2001).

Since this family was recognized in 1789 by Jussieu, the taxonomy of Polygonaceae has been continuously revised, depending on the morphological characters chosen to describe the subgroups with respect to the circumscription of subfamilies, tribes and genera (Brandbyge, 1993; Freeman & Reveal, 2005; Hara, 1982; Haraldson, 1978; Kim & Donoghue, 2008; Kuo *et al*., 1996; Li *et al*., 2003; Qaiser, 2001; Ronse Decraene & Akeroyd, 1988; Sanchez & Kron, 2008; Sanchez *et al*., 2011; Van Leeuwen *et al*., 1988; Wodehouse, 1931; Wood & Graham, 1965).

The family Polygonaceae has been classified into two subfamilies, Eriogonoideae and Polygonoideae (Brandbyge, 1993; Freeman & Reveal, 2005; Haraldson, 1978; Heywood *et al*., 2007; Mabberley, 2008). These subfamilies can be readily distinguished because members of the subfamily Eriogonoideae are often

woody, the leaves are whorled or opposite and the nodes are without ocrea; the inflorescences are sympodially branched with cymose and trimerous flowers (Brandbyge, 1993; Heywood *et al.*, 2007; Mabberley, 2008). Reveal (1989) divided the subfamily Eriogonoideae into two tribes, i.e. Eriogoneae and Pterostegieae, which consist of 15 genera and 2 genera respectively. On the other hand, members of the subfamily Polygonoideae have alternate leaves with sheathing ocrea stipules; they grow monopodially and the inflorescences are racemose with cymose branches. The subfamily Polygonoideae has five tribes, i.e. Triplareae, Coccolobeae, Rumiceae, Polygoneae and Persicarieae (Brandbyge, 1993; Heywood *et al.*, 2007; Mabberley, 2008). Between these two subfamilies, Eriogonoideae is rather well delimited as justified by some studies (Ronse Decraene & Akeroyd, 1988; Ronse Decraene *et al*., 2000, 2004; Ronse Decraene & Smets, 1991). The delimitation of the tribes Polygoneae and Persicarieae also seems to be justified but some of the genera still need to be further investigated (Brandbyge, 1993).

Since Linnaeus established the genus *Polygonum* at 1754, it has posed a great challenge to taxonomists who have tried to subdivide the genus into more natural units at generic rank, subgeneric and sectional. As it is, there is still a large number of controversies that need to be resolved before a convincing conclusion could be reached (Ronse Decraene & Akeroyd, 1988). Furthermore, individual authors are using different treatments and characters in different orders of importance resulting in the overlapping of numerous synonyms (Freeman & Reveal, 2005; Li *et al.*, 2003; Qaiser, 2001). In 1930, Steward had opined that, among the genera in Polygonaceae, the genus *Polygonum* was of outstanding difficulty and was one of the most troublesome groups to the systematic botanist. Wilson (1990) also states that the genus *Persicaria* is a segregate from *Polygonum sensu lato* and the debate continues as to its exact circumscription and infrageneric divisions. Therefore, the classification of

Polygonaceae has always been a point of disagreement among botanists, particularly in the genus *Polygonum*. *Polygonum* has been divided into as many as nine sections or 15 genera (Haraldson, 1978; Hedberg, 1946; Ronse Decraene & Akeroyd, 1988; Wood & Graham, 1965).

Steward (1930) has divided the genus *Polygonum* into eight sections: *Avicularia*, *Bistorta*, *Persicaria*, *Cephalophilon*, *Echinocaulon*, *Tiniaria*, *Aconogonon* and *Fagopyrum*. The species *Polygonum chinense* has been placed under section *Cephalophilon*. The genera *Polygonum sensu lato* have a number of different pollen types prompting Hedberg (1946) to propose the segregation of some sections into a number of genera and the genus *Persicaria* comprises the sections *Cephalophilon*, *Persicaria*, *Amblygonon*, *Tovara*, and *Echinocaulon*. Haraldson (1978) believes that the genus *Persicaria* is highly advanced in tribe Persicarieae and has recognized the genus *Persicaria* with four sections: *Persicaria*, *Cephalophilon*, *Echinocaulon*, and *Tovara*. The above conclusions are based mainly on her studies of the trichome structure, pollen morphology as described by Hedberg (1946), pollination behaviour and floral morphology. *Persicaria chinensis* is placed under the section *Cephalophilon*. Since then, Ronse Decraene and Akeroyd (1988) have also disagreed with uniting *Persicaria* with *Polygonum sensu stricto* and suggest maintaining *Persicaria* as a distinct genus (same as Haraldson, 1978) based on the significant floral characters (the morphology of the tepals, the structure of the nectaries and the morphology of the filaments) and classified genus *Persicaria* with four sections: *Persicaria*, *Bistorta*, *Tovara* and *Aconogonon*. The genus *Persicaria* section *Persicaria* (Ronse Decraene & Akeroyd, 1988) including section *Cephalophilon*, section *Echinocaulon* and section *Amblygonon* and *Persicaria chinensis* have been recognized in this section. Most recently, phylogenetic work has proposed recognizing *Persicaria* as a separate genus derived from the tribe Persicarieae and subfamily Polygonoideae (Frye & Kron, 2003; Kim &

Donoghue, 2008; Sanchez *et al.*, 2009, 2011) and *Persicaria chinensis* has been categorized in the section *Cephalophilon*.

However, some authors (Kuo *et al.*, 1996; Li *et al.*, 2003) still include *Persicaria* as one of the sections in the genus *Polygonum*. In the Flora of China, Li *et al.* (2003) have recognized the genus *Polygonum* with six sections: *Polygonum*, *Persicaria*, *Bistorta*, *Cephalophilon*, *Aconogonon*, and *Echinocaulon*. *Polygonum* section *Polygonum* represents *Polygonum* in the narrow sense (*Polygonum sensu stricto*) and the remaining five sections of *Polygonum* in the broad sense (*Polygonum sensu lato*) with wider circumscription (Li *et al.*, 2003). *Polygonum chinense* has been included in the section *Cephalophilon* and four varieties have been recorded.

This study adopts the classification of the most recent findings based on the phylogenetic works done by several researchers as mentioned above and earlier proposed classifications (mostly by Haraldson, 1978) and some ideas of Ronse Decraene and Akeroyd (1988). Hence, *Persicaria chinensis* has been adopted as the name of this species.

2.1.2 Distribution and ecology

This family has a worldwide distribution and is adapted to a wide range of different habitats ranging from tropical to arctic regions. The subfamily Polygonoideae is mainly found in the temperate regions of North America (Freeman & Reveal, 2005) and the tribe Persicarieae is north circumpolar with an occasional representation in the southern hemisphere. The genus *Persicaria* with about 100–150 (Freeman & Reveal, 2005; Qaiser, 2001) species, are widely distributed in both hemispheres but are mainly found in the north temperate regions (Qaiser, 2001). The genus *Polygonum* presents 4 species native to Peninsular Malaysia and Singapore (Henderson, 1959) and later Turner (1995) reports 11 species for *Persicaria* in Peninsular Malaysia and Singapore.

Persicaria chinensis var. *chinensis* is distributed in east Asian countries such as India, Bhutan, Myanmar, Japan, Philippines, Peninsular Malaysia, Singapore, Thailand, Vietnam, Sri Lanka and is introduced into Pakistan. This variety also grows in thickets or shaded places throughout Taiwan and is found abundantly in wet valleys or grassy slopes in China (Kuo *et al.*, 1996; Li *et al.*, 2003; Qaiser, 2001). In Peninsular Malaysia, this species is found growing naturally in Bukit Fraser, Pahang; Gunung Berimbun near Telom, Perak; Tapah, Perak and Gunung Kerbau, Kedah (Ridley, 1924). Turner (1995) describes *Persicaria chinensis* var *chinensis* as a widespread scrambling herb found at damp waste sites in lowlands and mountains.

2.1.3 Embryology and anatomy

Embryological information of the family Polygonaceae is very scanty and restricted only to a few species while the systematic position of the family is controversial (Maheswari Devi & Manorama, 1985). A few studies have been attempted to discuss the systematic position of the family using embryological evidences (Maheswari Devi & Manorama, 1984, 1985).

In this family, the embryological studies are characterized by the presence of 3-celled pollen grains; orthotropus, bitegmic and crassinucellate ovule on basal placentation with nucellar beak; periclinal divisions of the nucellar epidermis, hypotase; nuclear type of endosperm development with a small endosperm pouch and Asterad type of embryogeny (Agoram & Krishnamurthy, 1980; Doida, 1960; Dudgeon, 1918; Fink, 1899; Hofmeister, 1849; Lonay, 1922; Maheswari Devi & Manorama, 1984, 1985; Mahony, 1935, 1936; Mukherjee, 1972; Neubauer, 1971; Pausheva, 1977; Periasamy, 1964; Rao & Mukherjee, 1973; Rao, 1936; Soueges, 1919a, 1919b, 1920a, 1920b; Stevens, 1912; Strasburger, 1879; Woodcock, 1914) .

Maheswari Devi and Manorama (1984, 1985) reported that the anther wall development of *Polygonum chinense*, *P. alatum* Dulac, *P. capitatum* Buch.-Ham. *ex* D. Don, *P. molle* Wight, *P. flaccidum* Roxb. and *P. strigosum* R.Br. follow the Monocotyledonous type, with secretory tapetum. The dicotyledonous type of anther wall development has been observed in *Antigonon leptopus* Hook. & Arn. (Agoram and Krishnamurty, 1980), a herbaceous and perennial vine climbing by tendrils. Simultaneous cytokinesis during microsporogenesis results in tetrahedral tetrads in *Polygonum chinense*, *P. alatum*, *P. capitatum*, *P. molle*, *P. strigosum* and both tetrahedral and decussate tetrads in *P. flaccidum* (Maheswari Devi & Manorama, 1984, 1985). In *P. strigosum*, different sizes of tetrads and mature pollen grains are observed even within the same anther locule. Pollen polymorphism is quite common in this species (Maheswari Devi & Manorama, 1984).

The development of the female gametophyte is of the *Polygonum* type (Johri *et al.*, 1992). The first clear account of the *Polygonum* type of embryo sac development was reported in *Polygonum divaricatum* L. (Strasburger, 1879). In addition, fertilization is porogamous, and the pollen tube is persistent and unbranched. The synergids remain intact during the entry of the pollen tube (Mahony, 1935). Wall formation is initiated at the micropylar end of the globular proembryo in *Polygonum pensylvanicum* L. (Neubauer, 1971). A meristematic layer differentiates at the periphery in the cellular endosperm. The cell in the central region becomes multinucleate. After 10 days of its formation, the meristematic layer develops into an aleurone layer. The nucellus persists as a thin covering. The chalazal region of the endosperm remains free-nuclear in *Fagopyrum esculentum* Moench (Davis, 1966) and this aspect requires further study (Johri *et al.*, 1992).

The fruit anatomy of the Polygonaceae is relatively simple and basically similar (Brandbyge, 1993; Dammer, 1893; Graham & Wood, 1965) where two or three carpels

enclose an orthotropous ovule with three dorsal traces running into the styles which sometimes are interrupted by a non-lignified zone at the level of the hypotase. The ovule will develop into a seed with the carpel wall differentiating into the pericarp, a mostly heavily sclerified exocarp or epidermis with several layers of parenchymatous mesocarp cells, and an endocarp. The seed develops an outer layer of rectangular cells (exotesta) surrounding a mealy or horny endosperm and embryo which the embryo is mostly straight with incumbent cotyledons and is positioned eccentrically in one corner of the seed (Ronse Decraene *et al.*, 2000). However, seed and fruit development are little known in Polygonaceae (Corner, 1976) expect for detailed studies on *Persicaria pennsylvanica* (L.) M.Gómez (Neubauer, 1971) and *Polygonum aviculare* L. (Lonay, 1922).

Heterostyly is almost always associated with reciprocal anther stigma positions. Although it had been discovered earlier, heterostyly achieved prominence only after (Darwin, 1864, 1865, 1892) described its functional significance as a mechanism facilitating the transfer of cross-fertilizing pollen between anthers and stigmas of the same height. This floral polymorphism is usually accompanied firstly, by a sporophytically controlled, diallelic self-incompatibility system that prevents self, intramorph fertilizations, and secondly by several other supporting floral features, particularly stigmas and pollen floral morphs (Barrett, 1990; Ganders, 1979).

Heterostyly occurs in some 24 families and over 124 genera of flowering plants (Ganders, 1979). In Polygonaceae, heterostylous flowers were first described in *Fagopyrum esculentum* over a century ago (Darwin, 1892). This species was known as one of the best studied heterostylous species from a genetic view point (Morris, 1951; Samborska-Ciania *et al*., 1989). Subsequently, heterostyly was also reported for the genus, *Oxygonum* Burch. (Graham, 1957; Hong, 1999), *Polygonum* (Reddy *et al*., 1977;

Chen & Zhang, 2010), *Aconogonon* (Meisn.) Rchb. (Hong, 1991), and *Persicaria* (Nishihiro & Washitani, 1998a).

2.1.4 Pollen studies

The acetolysis method introduced by Erdtman (1954) half a century ago is still a very popular and highly successful technique in palynology (Hesse & Waha, 1989). It is widely considered as the common basis for the comparison of pollen grains (Coetzee & Van der Schijff, 1979). Acetolysis is very useful technique for pollen grains with thick and stable exines; resulting in very clean pollen surfaces and thus gives excellent topographic information. However this method has numerous disadvantages (Hesse & Waha, 1989). The acetolysis procedure is known to cause collapse, damage, shrinking, contortion, twisting or warping (Coetzee & Van der Schijff, 1979; Halbritter, 1998) to the pollen grains with thin and/or fragile exines. It also often makes bilaterally symmetrical grains appear radially symmetrical and may result in incorrect measurements being taken for the polar and equatorial axis. Consequently, pollen grains might be classified in wrong symmetry and size classes (Smith & Tiedt, 1991).

Modern palynology with its many morphological and functional applications must consider all pollen characteristics, especially sporoderm characters and non-sporopollenin features (Halbritter, 1998). However, the non-sporopollenin parts of the pollen cannot be observed in acetolysis samples because the mixture of sulfuric acid and anhydrous acetic acid destroys all non-sporopollenin substances in the acetolysis process (Hesse & Waha, 1989). This problem can be overcome by using the scanning electron microscope and transmission electron microscope technique (Halbritter, 1998; Heslop-Harrison *et al*., 1986; Rowley & Skvarla, 1987).

Polygonaceae is considered a eurypalynous (multipalynous) family by various workers (Hedberg, 1946; Nowicke & Skvarla, 1977; Wodehouse, 1931). Pollen

morphology characters of *Polygonum* section *Persicaria* were previously studied by several researchers (Hedberg, 1946; Wang & Feng, 1994; Zhang & Zhou, 1998; Zhou *et al*., 1999). However, the *Persicaria* as a separated genus was studied by Wodehouse (1931), Hong and Hedberg (1990) and Yasmin *et al*. (2010).

An extensive study on the pollen morphology of the Polygonaceae was published by Wodehouse (1931). He described several species from a range of North American genera with an explanation of "developmental tendencies" and also provided a key to the species. In his studies, four species from the genus *Polygonum* have been identified, including *Polygonum chinense*. However, most of the species, described, do not occur in the tropical and subtropical regions of eastern Asia. Hedberg (1946), in his classic paper on the pollen morphology of the genus *Polygonum*, described ten main pollen types and a number of aberrant types. He proposed a new taxonomic classification based on pollen morphology and constructed a key to the pollen type. Yasmin *et al.* (2010) summarized the pollen morphological characters of the genus *Persicaria* into two groups; tricolpate pollen (Capitata type pollen) and pantaporate pollen (Persicaria type pollen). Persicaria type pollen were observed in most of the *Persicaria* species while the other 5 species of *Persicaria*, i.e. *P. capitata* (Buch.-Ham. *ex* D.Don) H. Gross, *P. nepalensis* (Meisn.) H. Gross, *P. chinensis*, *P. posumbu* (Buch.-Ham. *ex* D.Don) H. Gross and *P. tenella* (Blume) H. Hara, fall into the Capitata type (comparable to Cephalophilon type suggested by Wang & Feng, 1994; Zhang & Zhou, 1998).

2.1.5 Pollination biology

Corlett (2004) provided an overview of flower visitors and pollination in the entire Oriental (or Indo-Malayan) Region. Burkill (1919) observed the pollination of some flowers in the Singapore Botanic Gardens and other parts of the Malay Peninsula. The ecology of insect pollination of some Malaysian dipterocarps and understorey trees further adds valuable information to the pollination ecology in tropical forests (Appanah, 1981, 1985, 1990; Appanah & Chan, 1981; Bawa, 1990; Chan & Appanah, 1980).

The pollination studies of the *Persicaria* in Malaysia have not been investigated so far and foreign researchers have studied some other species including *Polygonum thunbergii* Siebold & Zucc. (Momose & Inoue, 1993), *Antigonon leptopus* (Raju *et al*., 2001), and *Persicaria japonica* (Meisn.) Nakai (Nishihiro & Washitani, 1998b).

2.2 *In vitro* cytotoxic activity of the family Polygonaceae against human cancer cell lines

Nature has been a source of medical treatment as our ancestors had used plants as a source of medicine thousands of years ago. Currently, plant based systems are providing many effective anticancer agents and will continue to play an essential role in the primary health care of 80% of the world's population (Das & Yadav, 1998; Nakanishi, 1999). As summarized recently, a total of 187 plant species belonging to 102 genera and 61 families have been identified as an active or promising source of phytochemicals with antitumor properties, corresponding to a 41% increase during the last five years. Among them, only 15 species belonging to ten genera and nine families have been utilized in cancer chemotherapy at the clinical level, whereas the rest of the identified species are either active against cancer cell lines or exhibit chemotherapeutic properties in tumor-bearing animals under experimental conditions (Kintzios, 2006).

Plant metabolites with antitumor and anticancer properties are primarily cytotoxic; probably due to their evolution-driven development as natural pesticides for the self-defence of plant organisms (Kintzios, 2006). However, some plant metabolites exert cytotoxic effects in a less direct way. For example, flavanoids can inhibit cancer cell proliferation by modulating the activity of cyclin-dependent kinases (Chang *et al*., 2004; Dai & Grant, 2003), but also demonstrate a cytotoxic estrogen-like activity in high concentrations (Nair *et al*., 2004; Oh & Chung, 2004; Woo *et al*., 2005). In other words, some plant metabolites can act as chemotherapeutic agents due to their growth-regulatory properties (Kintzios, 2006).

Preliminary *in vitro* test is an initial phytochemical screening for the discovery of novel anticancer drugs. The first step is the selection of starting materials, mainly based on ethnobotanical information and followed by the second step which is the identification of the biological activity (in the case of cancer chemotherapy and

certainly includes selective cytotoxicity tests) of the extracts derived from the plant materials (Kintzios, 2006). A cytotoxicity test determines whether a product or compound will have any toxic effect on living cells, and is also generally used as a screening tool for raw materials or component products before they are put into the design of a medical device.

Subsequently, extracts are prefractionated by means of chromatography and these fractions are then screened for biological activity *in vitro* (Constant & Beecher, 1995). Thus, preclinical tests usually evaluate the cytotoxicity of a candidate antitumor agent *in vitro*, that is, on cells cultured on a specific nutrient medium under controlled conditions (Kintzios, 2006).

The *Polygonum* genus is well known for producing a variety of secondary metabolites including flavonoids (López *et al*., 2006; Peng *et al*., 2003; Yagi *et al*., 1994), triterpenoids (Duwiejua *et al*., 1999), anthraquinones (Beerling *et al*., 1994; Matsuda *et al*., 2001; Yim *et al*., 1998), coumarins (Sun & Sneden, 1999), phenylpropanoids (Murai *et al*., 2001; Takasaki *et al*., 2001), lignans (Kim *et al*., 1994), stilbenoids (Nonaka *et al*., 1982), tannins (Wang *et al*., 2005), and drimane-type sesquiterpene dialdehyde polygodial (Alves *et al*., 2001; Asakawa & Aratani, 1976; Derita *et al*., 2008; Fukuyama *et al*., 1980; Hagendoorn *et al*., 1994).

Five chemical constituents have been derived from *Polygonum chinense* from Taiwan (Tsai *et al*., 1998) included one new compound, 25R-spirost-4-ene-3,12-dione, and four known compounds, i.e. stigmast-4-ene-3,6-dione, stigmastane-3,6-dione, hecogenin and aurantiamide acetate. Tsai *et al*. (1998) also recorded that these five compounds exhibited anti-inflammatory and anti-allergic activities. In addtition, twelve chemical constituents have been reported from *Polygonum chinense* from China. They were identified as syringic acid, apigenin, 3,3'-di-O-methylellagic acid, gallic acid, protocatechuic acid, isorhamnetin, caffeic acid, quercetin, luteolin, gallicin, avicularin

(quercetin-3-O-α-L-arabinofuranoside) and 3,4,8,9,10-pentahydroxy-dibenzo [b,d] pyran-6-one. All these compounds except gallic acid and quercetin, were reported for the first time in this species (Xie *et al*., 2007).

CHAPTER 3

REPRODUCTIVE BIOLOGY

3.1 General introduction

In the Polygonaceae, studies on the reproductive biology are very scanty and limited to very few genera only (Bailey, 1994; Bowlin *et al*., 1993; Diggle *et al*., 2002; Forman & Kesseli, 2003; Grimsby *et al*., 2007; Kawano *et al*., 1990; Maun, 1974). The present study was carried out in an attempt to provide additional morphological data and enhance the basic knowledge pertaining to the reproductive biology and the breeding system of the highland and lowland varieties of *Persicaria chinensis* var. *chinensis* in Peninsular Malaysia.

3.2 Materials and methods

3.2.1 Study sites and voucher specimens

The lowland variety of *Persicaria chinensis* var. *chinensis* is planted in Sagil, Tangkak, Johor (Table 3.1 & Figure 3.1) while the highland wild population of *P. chinensis* var. *chinensis* is located at Gunung Ulu Kali, Bentong, Pahang. Four different study sites in Gunung Ulu Kali were identified for study (Table 3.1); the altitude and the GPS readings are listed in Table 3.1.

Voucher specimens of *Persicaria chinensis* var. *chinensis*, both dried and fixed samples, were deposited at the Herbarium, University of Malaya (KLU). The accession numbers of the respective lowland and highland varieties are listed in Table 3.1.

Table 3.1: Location of study sites and voucher accessions.

Species	Population named	Site of collection (GPS reading)	Number of inflorescence tagged	Altitude of the locality (meter)	Herbarium voucher accession number
Lowland population of *P. chinensis* var. chinensis	LC	N 2°18'26'' E 102°36'40''	31	33	KLU 47716
Highland population of *P. chinensis* var. chinensis	H2	N 03º24'49.7" E 101º47'02.4"	25	1350	KLU 47714 & KLU 47715
	H3	N 03º24'54.9" E 101º47'02.3"	25	1390	
	H4	N 03º25'54.6" E 101º47'05.4"	24	1620	
	H5	N 03º24'44.7" E 101º47'21.9"	14	1300	

In addition, field collections of the other 10 taxa of genus *Persicaria* occur in Peninsular Malaysia have been carried out in several places of Peninsular Malaysia, including Tasik Bera, Cameron Highlands, Taiping, Pulau Langkawi and the Kinta Valley.

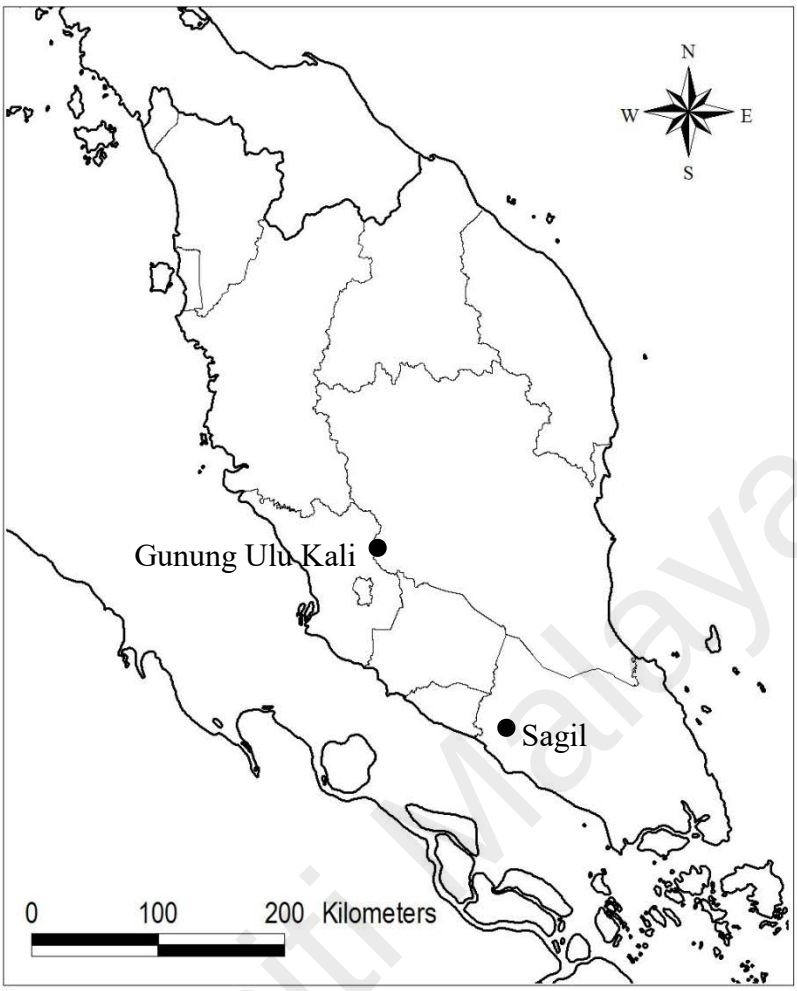


Figure 3.1: Study sites of *Persicaria chinensis* var. *chinensis* in Peninsular Malaysia.

3.2.2 Plant morphology

The morphology of the plants collected was examined and the habitat of the highland population was noted during field work. The buds, flowers and fruits at different stages of development were collected, dissected and examined under the binocular microscope (Leica Zoom 2000) to study the gross morphology. Plant samples were pressed, dried, labelled and deposited in the KLU herbarium. The mature flowers at anthesis were fixed, dehydrated, critical point dried, coated with gold and scanned (Appendix A) using a JEOL JSM-6400 scanning electron microscope.

3.2.3 Flower and fruit development

The plants were tagged when the inflorescences emerged from the terminal of the stem. The formation of the buds was closely monitored. As soon as these buds appeared, they were measured and counted. For the highland populations, observation of the flower buds was carried out at weekly intervals at Gunung Ulu Kali, Bentong, Pahang, whereas that of the lowland population was observed at 2-day intervals during the first 3 weeks and at 7-day intervals from week 4 until week 6. The development of the flower and the fruit was continuously monitored. The number of flower buds and successful fruit set were recorded until the fruits reached maturity. In addition, the time of anthesis, anther dehiscence, stigma receptivity, possible pollinating agents and pests of the plants were recorded. In the development of the fruits, the length and diameter of the fruits were measured at weekly intervals. The average increase in length and diameter was calculated.

The lowland plants were watered regularly, especially when rainfall was irregular, and organic fertilizer was applied once a month to ensure that the plants were supplied with sufficient nutrients.

3.2.4 Embryological studies

Buds, flowers and fruits at different stages of development were collected in the morning from 0800–1000 hours and immediately fixed in Craf III solution (Appendix B). All specimens were fixed in the field and suction at 25–30 atmospheric pressure was applied for 10–15 minutes in the laboratory using a Hotpack vacuum oven (model 273700). This is to remove trapped air and facilitate rapid penetration of fixative into the tissue. The specimens were left in the fixative at room temperature for at least 48 hours.

After fixation, the specimens were dehydrated through an ascending series of alcohol by increasing the concentration of tertiary butyl alcohol (Apendix B). When dehydration was completed, the specimens were gradually infiltrated with different grades of wax and finally embedded in Paraplast Plus Tissue Embedding Medium (McCormik Scientific, U.S.A.). Microtome sections of buds and flowers were cut at 6 μm thickness while the fruits at 8 μm.

The sections were mounted using egg albumen and distilled water was used to aid in the stretching of the tissue by warming the sections on an electrothermal slide drying bench (model MH6616).

The mounted slides were dried at 40ºC for at least 48 hours before being stained in a combination of safranin-fast green solution (Appendix B). Photomicrographs were taken using a Leica 2400 compound microscope attached with a Leica DFC290 camera.

3.2.5 Pollen morphology

Immediately after anthesis, the pollen grains were collected and dried at 40ºC. They were then acetolysed, stained and mounted in glycerine jelly (Appendix C). The equatorial diameter and polar length of the pollen grains were measured using a Leica DM1000 compound microscope with computer programme LAS 3.8 and attached with a Leica DFC290 camera. Forty measurements were recorded and the ratio of the polar length and equatorial diameter (P/E) was determined.

Pollen grains were acetolysed, critical point dried, mounted on stubs, coated with gold and observed under a JEOL JSM-6400 scanning electron microscope (Appendix A).

3.2.6 Pollen viability and pollen germination test

Pollen viability was first tested by using 0.1% 2,3,5-triphenyl tetrazolium chloride solution. Pollen grains that stained pink were considered viable and those colourless were considered non-viable. As tetrazolium salt is easily reduced by light, the solution and pollen slides were kept in the dark (the presence of light will inhibit reduction of tetrazolium salt). The pollen grains were dusted onto a drop of the freshly prepared solution on a concave slide and the slide was kept in a petri dish lined with moist filter paper and kept in the dark for at least 30 minutes.

For the pollen germination test, mature inflorescences were collected, placed in a flask with water and taken back to the laboratory. Pollen grains from the flowers which had just reached anthesis and dehisced were used for the germination studies in sucrose solutions. The inflorescences from the wild populations were kept at 22ºC whereas the cultivated lowland population was kept at room temperature. All the inflorescences were put in flasks filled with water.

The pollen grains were germinated using the following media:

a) Sucrose solution at various concentrations : 4, 6, 8, 10, 12, 15, 18%

b) 100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.01 g boric acid.

c) 100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.01 g boric acid, H_3CO_3 and 0.03 g calcium nitrate tetrahydrate, $Ca(NO_3)_2.4H_2O$

d) 100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.04 g boric acid, H_3CO_3; 0.2 g calcium nitrate tetrahydrate, $Ca(NO_3)_2.4H_2O$ and 0.2 g manganese (II) sulphate monohydrate, $MnSO_4.1H_2O$

e) 100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.04 g boric acid, H_3CO_3; 0.2 g calcium nitrate tetrahydrate, $Ca(NO_3)_2.4H_2O$; 0.2 g

manganese (II) sulphate monohydrate , $MnSO_4.1H_2O$, and 0.2 g potassium nitrate, KNO_3.

f) 100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.04 g boric acid, H_3CO_3; 0.2 g calcium nitrate tetrahydrate, $Ca(NO_3)_2.4H_2O$; 0.2 g manganese (II) sulphate monohydrate, $MnSO_4.1H_2O$; 30 g polyethylene glycol (PEG) with molecular weight 20000 (Merck) and 0.2 g potassium nitrate, KNO_3.

g) 100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.04 g boric acid, H_3CO_3 and 30 g polyethylene glycol (PEG) with molecular weight 20000 (Merck).

Media from c–g were modified from Adhikari and Campbell (1998). All concave slides with pollen grains for the above experiment were kept at room temperature (28ºC) in a petri dish lined with moist filter paper. The pollen grains were examined under the 10 × 10 compound microscope at hourly intervals and the length of the pollen tubes was measured after 24 hours. Germinated pollen grains were counted under the 10 × 10 magnification using a simple telecounter. Pollen grains with tube lengths equal to, or longer than the diameter of the pollen grains were considered as germinated.

After 12 hours, a sample of the pollen grains from the same flower was cultured in the most suitable medium. This germination procedure was repeated until all the pollen grains failed to germinate.

For the *in vivo* germination study, the stigmas together with the style from the open flowers, were removed and placed on a microscope slide with a drop of lactophenol (Merck). The specimens were gently pressed with cover slip and then observed under the compound microscope. Photographs of the germinated pollen grains were taken using a Leica 2400 compound microscope.

3.2.7 Pollination experiments and pollen-ovule ratio

Pollination experiments were conducted in the highland population in the morning as anther dehiscence and stigma receptivity occur at 0600 hours. Pollen compatibility was tested using the following method (After Bawa, 1974).

a) Open pollination

The inflorescences were simply tagged to estimate the efficacy of natural pollination (control experiment).

b) Bagging experiment

The inflorescences with mature flower buds were bagged using a fine porous nylon cloth.

The pollen-ovule ratio was determined by dividing the estimated number of pollen grains per flower by the number of ovules per flower (Cruden, 1977). If the number of pollen grains per anther was 2000 or less, all the pollen grains in an anther were counted by using a simple telecounter under the dissecting microscope and then multiplied by the number of anthers in a flower. If the number of pollen grains per anther was greater than 2000, then the pollen grains were suspended in a solution of aniline-blue in lactophenol and the number of pollen grains in a known volume of suspension was counted by using a haemacytometer (Cruden, 1977).

For *Persicaria chinensis* var. *chinensis*, the number of pollen grains per anther was less than 2000 (in examination under a microscope). A mature flower bud was picked from the sample and an anther was squashed (using needles) into a few drops of distilled water (mixed with a few drops of detergent) in a concave slide under the dissecting microscope. Detergent was used to help separate the pollen grains, to detach

the pollen grains from the anther wall, and to reduce the surface tension. Subsequently, twenty replicates from different flowers were chosen randomly and the number of pollen grains were counted and recorded.

Cruden (1977) established that the relationship between the number of pollen grains and ovules (the P:O ratio) reflects the breeding system as follows:

Types of breeding system	P:O range
Cleistogamy	2.7–5.4
Obligate autogamy	18.1–39.0
Facultative autogamy	31.9–396.0
Facultative xenogamy	244.7–2588.0
Obligate xenogamy	2108.0–195525.0

3.2.8 Possible pollinating agents and pests

Soon after anthesis, pollinating agents were seen to visit the flowers. These visitors were photographed using a Nikkor VR macro lens 105 mm F3.8. These possible pollinating agents were then recorded and identified by an entomologist and pests were also observed and photographed.

3.2.9 Seed viability, seed germination and seedling morphology

2,3,5-triphenyl tetrazolium chloride (TTC) is used to stain and determine the viability of the seeds of the highland populationn of *P. chinensis* var. *chinensis*. The basic principle of the tetrazolium testing technique is that, when presoaked and incubated seed is treated with tetrazolium salt solution, the dehydrogenase enzyme in actively respiring areas of living tissues in the embryo and endosperm produce hygrogen ions, which in turn react with the colourless tetrazolium salt to form a stable, red-coloured, triphenylformazan which is insoluble in water (Yu & Wang, 1994).

A 1% tetrazolium (TZ) salt solution (MERCK) was prepared by dissolving 1 g of 2,3,5-triphenyltetrazolium chloride in 100 ml of distilled water with a pH between 6.5 and 7.5. The TZ solution was stored in a dark bottle and kept in a refrigerator to retain its strength for several months. In this study, a freshly prepared TZ solution was used and the remaining was kept in the refrigerator.

Seeds of the highland wild population of *P. chinensis* var. *chinensis* were tested and three replicates of 70-seed samples from the field were collected randomly. The small seeds were soaked in distilled water for 24 hours at 40ºC to soften the seed coat. After 1 day, the seeds were cut longitudinally into half and immersed in 1% TZ salt solution and incubated in darkness at 35ºC in an oven for 12 hours. After the staining process, the treated seeds were washed and placed on moist filter paper in petri dishes and their viability was evaluated by using a dissecting microscope (Leica EZ4). The

stained embryos were carefully dissected and removed from the seed coats and endosperms. Seeds with radical, hypocotyl and cotyledons fully stained red were recorded as viable and seeds with radical, hypocotyl and cotyledons all unstained or slightly stained were considered as non-viable.

The seeds from mature fruits were planted in highland soil in the highland environment and sand-garden soil (mixed in the ratio of 2:1) in the lowland environment to determine the percentage of germination. The time taken for the seeds to germinate and the morphology of seedlings were noted.

The photos were taken using Leica MZ95 stereomicroscope computer programme LAS 3.8 attached with Leica DFC290 camera.

3.3 Results

3.3.1 Plant morphology

Persicaria chinensis var. *chinensis* is a perennial scrambling or ascending herb with branched stem that reaches up to 3.5m growing in lower montane forest. The lower stem is normally woody, circular, hollow, glabrous and reddish with brown reddish bark. The upper stem is sparsely pubescent and greenish and the young stem has reddish glandular punctate pubescence. The leaves are simple, entire with reddish margin and alternate, measuring 6–17 cm long and 2–6 mm wide. The leaves are usually ovate to oblong-lanceolate with both surfaces nearly glabrous to short pilose on the veins, sometimes pubescence will also appear along the vein abaxially and often observed as minutely reddish punctate under the microscope. The bases of the leaves are mostly symmetric but sometime asymmetric, with unequal truncate to weakly cordate leaf bases and the leaves apices are acute or acuminate. The petiole measuring 3–7 mm long with 1.5–2.5 mm thick and usually has 1–2-auricles at the base. The cauline leaves are asymmetric with basal lobes overlapping the petiole. The point of attachment of leaf and stem is the ocrea (4–11 mm long), foliaceous, tubular and sometimes with pilose at its apex.

The compound corymb inflorescences are mostly terminal or sometimes subterminal with 2–5 clusters, measuring 4–10 cm in length. The flowers are actinomorphic, bisexual, heterostylous with 5 perianth segments fused up to ½ or more of their length. The perianths are lanceolate to elliptic, acute, entire, whitish, accrescent (become larger with age), fleshy and fragrant. The pedicles are 1–2 cm long and the peduncles are 2–5.5 cm long with glandular pubescence along the entire length. The bracts are sagitate when small, oblong-lanceolate when larger, asymmetrical, and the basal lobes sometimes overlap the pedicel. The bracts are 10–33 mm long and 5–12

mm wide. The number of bracteoles are 3–7, oblong-cordate shaped measuring 2–5 mm long and 1–2 mm wide.

The flowers have eight stamens (very rarely 12–13 stamens, Figure 3.6 O) with 5 epipetalous stamens and 3 distinct, free stamens. The anthers are whitish purple, dorsifixed and they turn dark purplish after dehiscence. The anthers are bilobed and dehisce along a longitudinal line to release a large number of creamy white pollen grains.

The flowers are heterostylous (Figures 3.6 C&D); one with long style (pin flower) and the other with short style (thrum flower), and the flowers are found on different plants. The stigmas are capitate, papillose and when receptive become mucilagenous with stigmatic exudate. The ovary is white, superior with basal placentation, trigonous with 3 filiform styles, free above the middle, connate below with a capitate stigma.

The fruit is baccate, 3.5–5.5 × 4–5.5 mm with a persistent fleshly perianth attached, initially whitish green before turning into bluish black when the fruit ripen. The seeds are black, trigonous and smooth, measuring 2–3.5 × 1.5–2.2 mm.

Table 3.2 shows the comparative morphological characters of the lowland and highland varieties of *Persicaria chinensis* var. *chinensis*. Figures 3.8 and 3.9 show the botanical illustration of the lowland and highland varieties of *Persicaria chinensis* var. *chinensis*.

Table 3.2: Comparative morphological characters of the lowland and highland *Persicaria chinensis* var. *chinensis*

Species character	Lowland population of *P. chinensis* var. *chinensis*	Highland populations of *P. chinensis* var. *chinensis*
HABIT	erect, suberect herb	scrambling herb
Height (m)	about 0.5	up to 1–2
STEM		
Bark surface	woody, smooth	woody, smooth or sandpaper like
Bark colour	brownish red, greenish when young.	brownish red
Young stem indumentum	smooth, punctate	smooth
OCREA		
Length (mm)	(10.9–) 13.5–19.5 (–20.5)	(4–) 5–8 (–10)
Structure	tubular, foliaceous and punctate	tubular, foliaceous and punctate
LEAVES		
Texture (abaxial and adaxial)	chartaceous	chartaceous
Shape	ovate	oblong-lanceolate
Symmetry	equilateral	equilateral
Length (cm)	(4.5–) 5.5–7 (–8)	(7.5–) 8–12 (–15)
Width (cm)	(7.5–) 8–12 (–15)	(3.5–) 4–5.5 (–6)
Base	asymmetrical truncate	asymmetrical truncate
Margin	entire with red margin	entire with red margin
Apex	acuminate	acuminate
PETIOLES		
Length (mm)	(4–) 5–7 (–7.3)	(3–) 3.5–5 (–6)
Thickness (mm)	(1.5–) 1.7–2 (–2.3)	(1.5–) 1.6–2 (–2.2)
Base	n.a.	1–2 auricled
INFLORESCENCES		
Position	terminal or subterminal	terminal or subterminal
Clustering	in clusters of 2–3	in clusters of 4–5
Type	compound corymb	compound corymb
Length (mm)	(15–) 20–50 (–57)	(50–) 65–90 (–100)
Pedicel (mm)	(3–) 3.5–6 (–11)	(10–) 13.5–17.5 (–20)
Peduncle (mm)	(17.5–) 20.5–26.5 (–32)	(35–) 42.5–55 (–63.0)
Indumendent	glandular hair	glandular hair

Sexuality	bisexual	bisexual
BRACTS		
Shape	oblong-cordate	sagitate when young, oblong-lanceolate
Point of attachment (Base)	asymmetrical, basal lobes not overlapping at the peduncle	asymmetrical, basal lobes sometimes overlapping the peduncle
Apex	acute	acute
Length (mm)	(11–) 14–20 (–22.5)	(10–) 20–30 (–33)
Width (mm)	(8–) 9–14 (–15.5)	(5–) 8–11 (–12)
BRACTEOLES		
Number	3–8	3–8
Shape	cordate	oblong-cordate
Apex	acute	acute
Length (mm)	2.5–6.5	2–5
Width (mm)	2.5–4.5	1–2
FLOWERS		
BUDS		
Shape	elliptic	elliptic
Length (mm)	(1.5–) 2–3.3 (–3.7)	(1.5–) 2–4 (–4.5)
Diameter (mm)	(1.2–) 1.6–2.4 (–2.6)	(1–) 1.2–2 (–2.5)
Indumentum	smooth	smooth
PERIANTH		
Number	5	5
Length (mm)	(3.8–) 4–4.5 (–4.7)	(3.5–) 3.8–5 (–5.5)
Width (mm)	(2–) 2.3–2.7 (–3)	(2.5–) 2.8–3.3 (–4)
Apex	acute	acute
Margin	entire	entire
Colour	white	white or whitish pink
Structure	fleshy, fragrant	fleshy, fragrant
ANDROECIUM		
Stamen arrangement	epipetalous	epipetalous
Number of stamen	8	8 rarely 12–13
Stamen type	dorsifixed	dorsifixed
Pin flower - filament length (mm), distinct stamen	n.a.	(1.56–) 1.57–1.63 (–1.68)
Pin flower - filament length (mm) ,	n.a.	(0.97–) 0.99–1.7 (–1.12)

epipetalous stamen		
Thrum flower - filament length (mm), distinct stamen	(2.41–) 2.5–2.74 (–2.77)	(1.99–) 2.04–2.21 (–2.28)
Thrum flower - filament length (mm) , epipetalous stamen	(1.91–) 1.98–2.16 (–2.22)	(1.48–) 1.52–1.64 (–1.72)
Anthers colour	pinkish	purplish blue
GYNOECIUM		
Number of locule	1	1
Number of Ovule	1	1
Ovary diameter (mm)	0.5–1	0.5–0.75
Number of style	3	3
Type of style	filiform	filiform
Pin flower - length of style (mm)	n.a.	(2.17–) 2.23–2.58 (–2.67)
Thrum flower - length of style (mm)	(0.828–) 0.85–0.91 (–0.926)	(0.99–) 1–1.16 (–1.18)
Stigma	capitate	capitate
Placentation	basal	basal
FRUITS	Very rarely formed	
Shape	n.a.	baccate
Length (mm)	n.a.	(3.5–) 4–5 (–5.5)
Diameter (mm)	n.a.	(4–) 4.5–5.2 (–5.5)
Indumentum when mature	n.a.	smooth and fleshy perianth
When ripen colour changes	n.a.	white to whitish-green to dark blue
SEEDS		
Colour	n.a.	black
Indumentum	n.a.	smooth
Length (mm)	n.a.	(1.8–) 2–3 (–3.5)
Diameter (mm)	n.a.	(1.3–) 1.5–2 (–2.3)
Shape	n.a.	trigonous
Type of germination	n.a.	hypogeal

3.3.2 Flower and fruit development

3.3.2.1 Flower development

Four highland populations of *P. chinensis* var. *chinensis* at different altitudes along the way to Gunung Ulu Kali, Bentong, Pahang, were identified for this study (Table 3.1). 88 inflorescences were tagged at the stage of newly emerged very young buds (16–20 mm) and 64 inflorescences successfully produced at least one mature fruit (*c.*72.7%) while the rest were attacked by insects or aborted soon after the buds were formed. The inflorescences were tagged before they reached 20 mm long. The flower buds appeared 7–9 days later and the first flower opened after 12–14 days (Table 3.3).

The development pattern of the compound corymb of the highland populations *P.chinensis* var *chinensis* is in the form of a sigmoid growth curve (Figures 3.2 & 3.4). The length of the inflorescence is between 30–90 mm with most measuring between 45–70 mm. At week 3, most of the inflorescences reached maturity and flowering began when the inflorescences were about 45–55 mm long and fruiting began at week 4. However, when the flowers were about to wilt, the length of the inflorescences decreased by 2–5 mm.

The time taken from the appearance of the flower buds to anthesis is approximately 5–7 days; the very young buds reached anthesis in approximately 12–14 days (Table 3.3). The flower buds opened basipetally but not synchronously. In addition, new flower buds continued to appear on the inflorescence even when it began to degenerate or wilt; a few of the flower buds could still be seen and this happened in the lowland cultivated variety as well. However, not all the flower buds would bloom after the fruit matured (30–46 days), some buds were still growing and they degenerated after the fruit ripened.

Out of the 88 tagged inflorescences, 64 inflorescences (*c*. 72.7%) produced fruit and this could be influenced by the altitude, temperature and humidity of the environment and this will be discussed later.

For the lowland cultivated population, 10 different individuals were planted in one clump. Thirty-one newly emerged inflorescences measuring 6.45–12.75 mm long were tagged. Thirty inflorescences successfully reached maturity and one inflorescence was attacked by pests when young. Flower buds appeared 5–7 days after tagging, when the inflorescences measured 20.21–29.12 mm. On day 8–11, the flowers on the inflorescences (inflorescences measuring 41.07–44.54 mm) started to bloom. The inflorescences reached maximum length (51.26–53.27 mm) on day 15–18 (Table 3.4).

The development pattern of the cultivated population of *P. chinensis* var. *chinensis* was also in the form of a sigmoid growth curve (Figure 3.3). The lengths of the mature inflorescences range from 30–68 mm with a mean of 53.28 mm with most of them measuring between 40–55 mm. On day 9–12, most of the inflorescences were mature and flowering began when the inflorescences were about 41.07–49.63 mm long. However, some of the inflorescences produced the first flower only after day 15–20 and the flowering rate was low, less than 20%, and fruit set was extremely rare in this cultivated population, i.e. less than one percent.

The time taken from the appearance of the flower buds to anthesis was approximately 5–15 days with the first flower opening on day 7–9 after tagging; for very young buds, flower anthesis was approximately 9–18 days with a mean of 13 days after tagging.

3.3.2.2 Anthesis and stigma receptivity

The anthesis time of the highland wild populations was at 0600–0730 hours whereas the lowland cultivated population was at 0745–0930 hours. At 0600 hours, the flowers of the highland populations started to bloom and by 0730 hours, the flowers were fully opened and the anthers had dehisced. On the other hand, flowers of the lowland cultivated population started to open at 0745 hours and were fully opened by 0930 hours. The average temperature and relative humidity measured from 0630–0830 hours in the highland populations ranged from 16.4–19.0ºC and 66–91% respectively whereas the average temperature and relative humidity of the lowland populations were 28.5ºC and 62.8% respectively. During anthesis, the anthers of the highland wild population turned purplish blue and after the flowers were fully-opened, the anthers would slowly turn purplish black after they had dehisced. The anthers of the lowland cultivated variety were mostly pinkish in colour. For both the lowland and highland populations, anther dehiscence occured along the longitudinal slits on the microsporangia which released the mature pollen grains. Anthesis and stigma receptivity occur simultaneously (tested by cutting the stigma soon after anthesis and when placed in 3% hydrogen peroxide, oxygen bubbles were noted).

During anthesis, the stigmas of the highland wild population were sticky and shiny. At the same time, the flowers were fragrant and they produced abundant nectar which attracted possible insect pollinators such as bees, hoverflies, wasps, ants and moths. Most of the insects appeared during 0730–0830 hours and the peak hours of their visits were from 0800–1000 hours. The lowland cultivated population was planted in a housing area; therefore not many pollinators were seen to visit the flowers. Even though the pots of cultivated lowland population were moved to a more natural habitat such as in the garden of the Institute of Biological Sciences, University Malaya and pollinators were seen to visit the flowers, there were still no successful fruit set. The

perianth of the open flowers of the lowland cultivated variety closed in the evening on the day of anthesis, at 1800–1900 hours. The persistent perianth segments of the highland flowers did not drop off after anthesis and in the next 2–8 days they slowly developed into the bluish black fleshy perianth of the fruit.

3.3.2.3 Fruit development

The highland wild population of *P.chinensis* var. *chinensis* took 23–35 days to form a young fruit which then developed into a mature fruit after 30 days (Table 3.3). The fruit enlarged, elongated and increased in size after pollination (Figure 3.5). Therefore, the fruit development was rapid and occurred one week after pollination.

The fruit development was in the form of a sigmoid curve pattern (Figure 3.5). From a very young bud until the young fruit was formed, the average length of the fruit during development was always more than the average diameter of the fruit. However, as the fruit matured, the increase in the diameter of the fruit was faster than that in the length of the fruit. The average fruit size was 4.43 × 5.53 mm. Twenty-one days after tagging, the average diameter of the closed flower was 2.66 mm which was naturally less than that of the blooming flower.

During fruit development, the colour of the perianth changed from whitish to white greenish after twenty-three days. It then gradually turned bluish black when mature after thirty days. The colour change in the fruit began from white to whitish green then bluish green and finally bluish black (Figures 3.6 L & 3.6 Q). On ripening, the colour of the fruit was violet black and the fruit wall turned soft and fleshy; purplish black watery juice was observed when the fruit was pressed. Generally, all the fruits within an inflorescence reached maturity successively.

The number of fruits developed per inflorescence depended on the size of the compound corymb and the number of flowers per inflorescence. The fruit tasted sweet

and slightly sour; was not poisonous and birds were seen to eat the mature fruit. A well-developed *P. chinensis* var. *chinensis* fruit contained one black seed (1.6–2.2 × 2.8–3.5 mm) with a non shiny surface.

The lowland population only produced less than 1% fruit set (Tables 3.5 & 3.6); therefore the fruit development could hardly be observed and only 3 fruits had been seen and the persistent perianth did not turn blackish.

3.3.2.4 Flower and fruit degeneration

The percentages of flower abortion and fruit set in the different populations of the highland wild variety at Gunung Ulu Kali, Bentong, Pahang and the lowland cultivated variety at Sagil, Tangkak, Johor were tabulated in Tables 3.5 & 3.6. For each population of the highland variety, the data was obtained based on 45–250 flower buds/inflorescence randomly selected from the different populations and different altitudes; for the lowland variety, 12–70 flowers buds/inflorescence were used.

Tables 3.5 & 3.6 showed that the average percentage of flower abortion in the highland wild population was 50% (ranged from 41%–65%) while that of the lowland population was 68%. The fruit degeneration of the highland populations ranged from 48%–98% while the lowland population had completely failed to produce mature fruit. However, the wild population 4 (H4), was found to be unusual when compared with the other three populations. H4 had a very high rate of fruit degeneration and also a relatively high percentage of flower abortion with only 2% fruit set and 65% flower abortion. Environmental conditions such as relative humidity, temperature, altitude, percentage of oxygen in the atmosphere, wind and cool days could cause the flower buds and pollinated flowers to drop off. In addition, wind and cool temperatures would also reduce possible insect-pollinator activities which in turn could cause the high rate of flowers being shed prematurely. Furthermore, overproduction of flowers exceeding

its “maximum holding capacity” could also cause the flowers to degenerate resulting in a low percentage of fruit set. All these factors could affect the final number of successful fruit set.

The lowland cultivated population had an extremely low percentage of fruit set, i.e. less than 1% and even though a few fruits had been found, they were immature fruits because the fleshly persistent perianth had not become blackish but remained whitish green (Figure 3.7 K). Furthermore, this lowland cultivated population had a high percentage of flower abortion (Figure 3.7 N), i.e. 68%, and the ovary of most flowers degenerated after anthesis (Figure 3.7 M).

This present study shows that the lowland cultivated population tend to spread by vegetative reproduction. From the slide preparation for embryological studies, degeneration of the egg cells and zygotes were very common. Pollen-ovule ratio showed that, out of eight anthers, 2 anthers were very abnormal producing less than 25 pollen grains and some were staminodes. The pollen grains were observed as irregular in size without protoplasm, and appeared translucent under the microscope. Pollen germination test also gave a negative result with no pollen grains germinating under optimum conditions. The lack of viable pollen grains could have contributed to the low percentage of fruit set.

Table 3.3: Summary of flower and fruit development in the highland populations of *P. chinensis* var. *chinensis* at Gunung Ulu Kali, Bentong, Pahang.

Date of observation	flower/fruit	Duration	Notes
15 September 2009	very young buds (1.59 × 1.33 mm)	found all the time when the inflorescence started to develop	
24 September 2009	young flower buds (young flower buds measuring 2.56 × 1.62 mm; flower buds before anthesis measuring 3.48 × 2.41 mm)	12–35 days	first flower open after 12–14 days
29 September 2009–1 October 2009	both open flowers and flowers after anthesis have been observed (open flowers measuring 3.87 × 3.05 mm; closed flowers measuring 4.39 × 2.66 mm)		
8–20 October 2009	young fruits (4.69 × 3.48 mm)	23–35 days	young fruits (greenish white in colour)
15–31 October 2009	mature fruits (4.43 × 5.53 mm)	30–46 days	mature fruits (bluish black in colour)
1–6 November 2009	whole inflorescences degenerated	45–52 days	some inflorescences wilted earlier or later depending on their size

Table 3.4: Summary of flower and fruit development in the lowland population of *P. chinensis* var. *chinensis* at Sagil, Tangkak, Johor.

Date of observation	Flower/fruit	Duration	Notes
3–6 February 2010	very young buds	found all the time when the inflorescence started to develop	
9 February 2010–13 March 2010	young flower buds (young flower buds measuring 2.56 × 1.62 mm; flower buds before anthesis measuring 3.82 × 2.55 mm)	5–33 days	
12 February 2010–3 March 2010	both open flowers and flowers after anthesis were observed (open flowers measuring 3.87 × 3.05 mm; closed flowers measuring 4.39 × 2.66 mm)	9–20 days	first flower opened after 9–12 days
3 March 2010	fruit rarely formed, only 3 young fruits were observed (4.75 × 2.63 mm)	7–9 days	young fruits (greenish white in colour)
12 March 2010	degenerated/wilt		some inflorescences wilted earlier or later depending on their size.

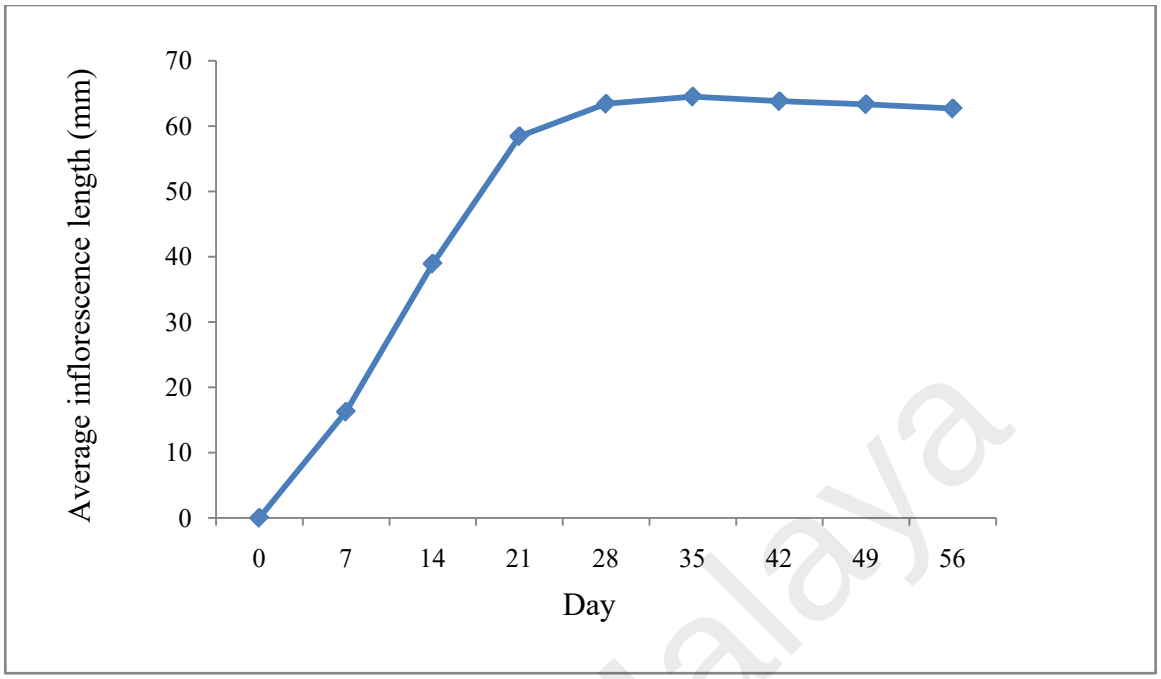


Figure 3.2: Average increase in inflorescence length of *Persicaria chinensis* var *chinensis* in the highland populations, Gunung Ulu Kali, Bentong, Pahang (based on 88 inflorescences).

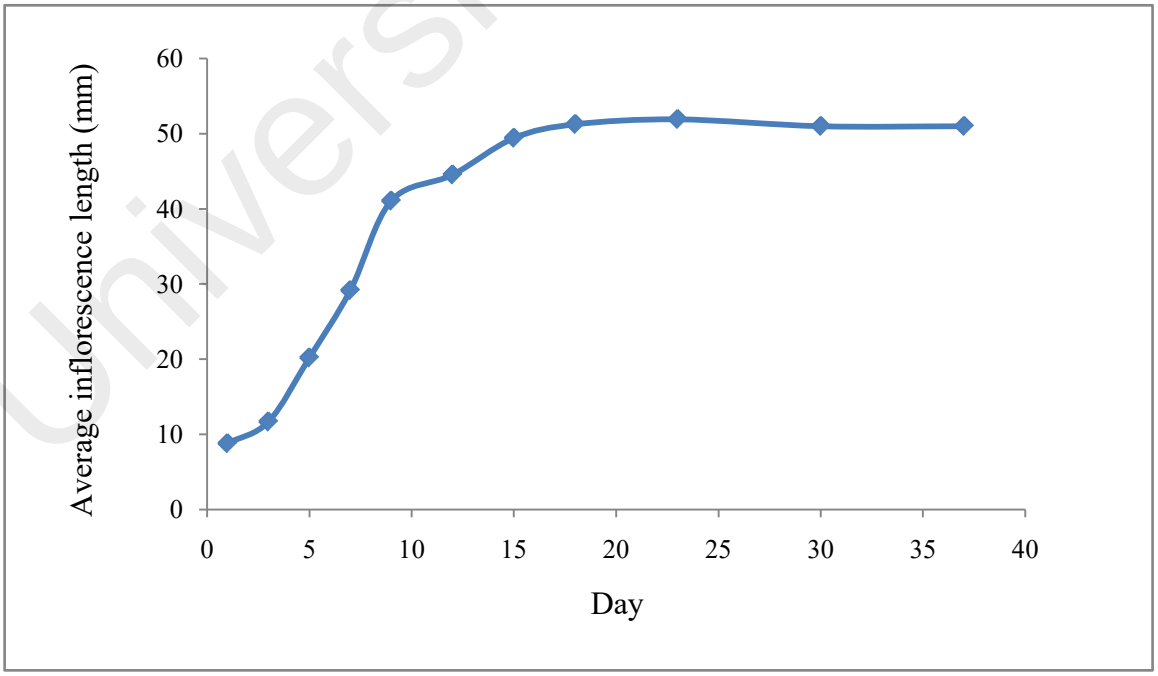


Figure 3.3: Average increase in inflorescence length of *Persicaria chinensis* var *chinensis* in the lowland population, Sagil, Tangkak, Johor (based on 30 inflorescences).

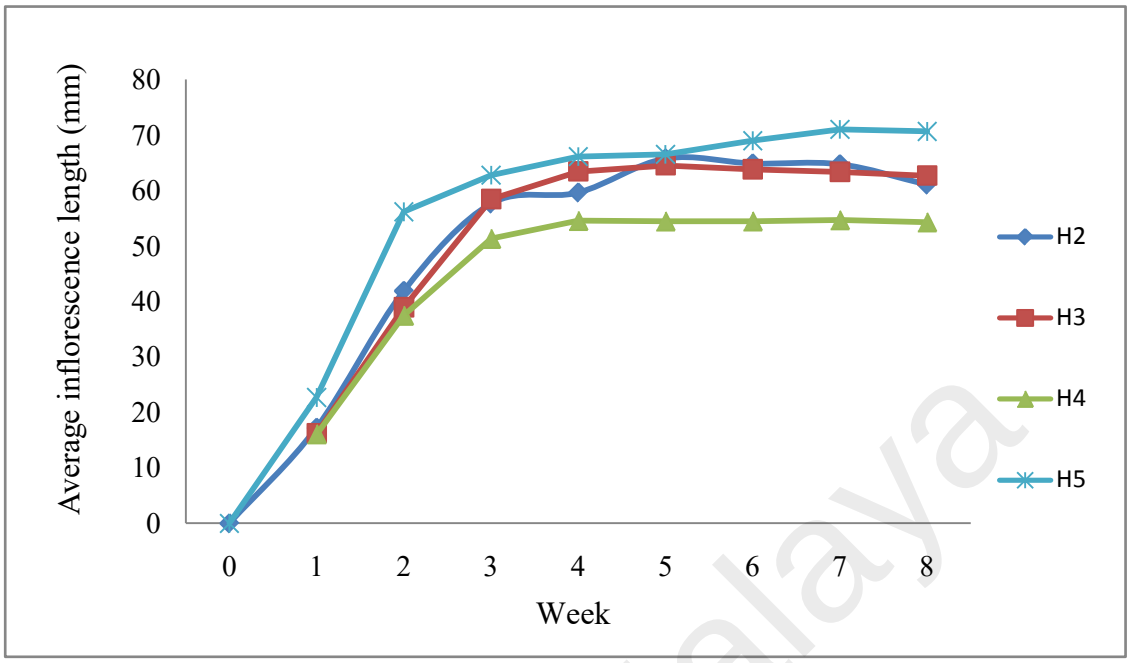


Figure 3.4: Average increase in inflorescence length in the four highland populations of *Persicaria chinensis* var. *chinensis* at Gunung Ulu Kali, Bentong, Pahang.

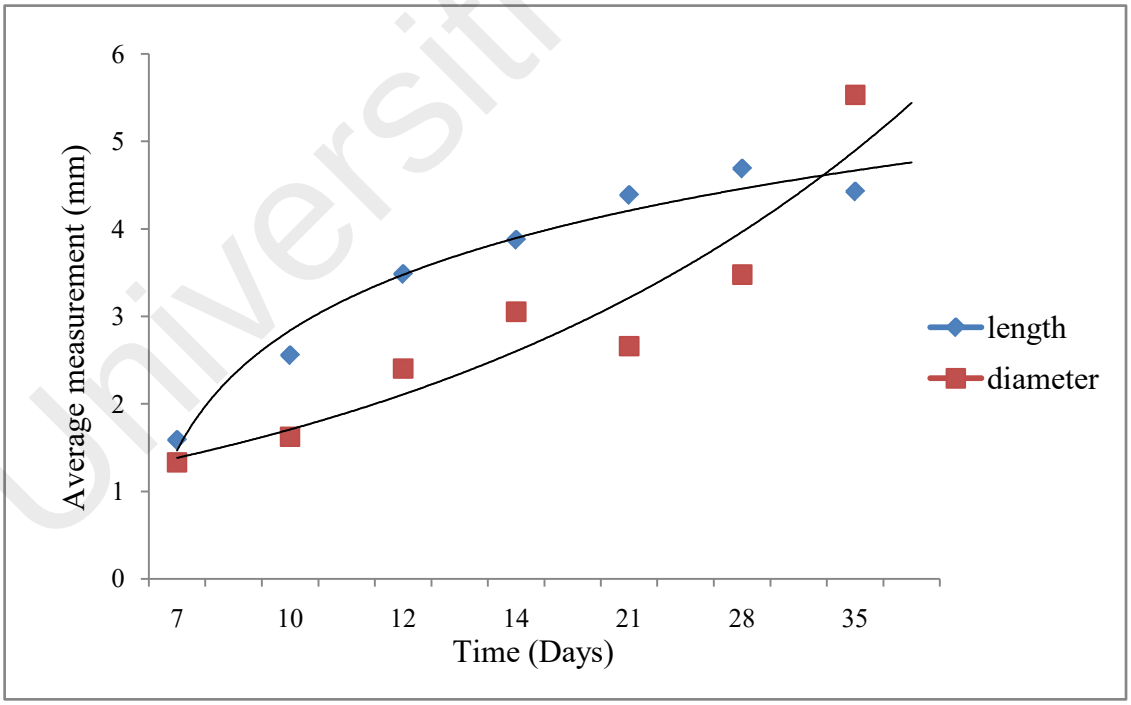


Figure 3.5: Fruit development in the four highland populations of *Persicaria chinensis* var. *chinensis* at Gunung Ulu Kali, Bentong, Pahang (based on 100 fruits).

Table 3.5: Flower abortion and fruit set in the highland and lowland populations of *P. chinensis* var. *chinensis*.

Inflorescence	Highland wild populations of *P. chinensis* var. *chinensis*												Lowland cultivated population of *P. chinensis* var. chinensis		
	H2 (1350 m)			H3 (1390 m)			H4 (1620 m)			H5 (1300 m)			LC (33 m)		
	Flower buds	Open flowers	Mature fruits	Flower buds	Open flowers	Mature fruits	Flower buds	Open flowers	Mature fruits	Flower buds	Open flowers	Mature fruits	Flower buds	Open flowers	Mature fruits
1	131	91	52	182	106	52	164	81	3	46	28	14	67	20	0
2	79	58	41	124	70	5	158	73	1	128	98	55	58	20	0
3	96	24	14	75	46	34	195	86	0	118	71	52	58	12	0
4	134	110	34	93	53	11	169	85	0	178	133	83	45	12	0
5	150	104	31	122	74	27	178	80	2	57	39	5	52	13	0
6	172	71	12	200	117	53	154	48	0	163	83	42	54	16	0
7	115	91	19	186	112	74	164	68	0	91	47	3	40	6	0
8	195	110	69	98	39	8	133	64	3	103	50	31	59	18	1
9	200	138	66	230	140	78	142	62	5	197	69	30	40	20	0
10	135	75	39	179	79	35	90	34	0	204	131	65	42	20	1
11	137	92	26	125	44	24	98	29	0	178	109	56	36	11	0
12	110	78	36	263	130	75	79	14	1	98	44	29	35	8	0
13	133	79	61	143	73	35	98	14	0	83	66	31	40	14	0
14	78	24	12	126	62	44	79	16	0	228	143	86	50	11	0
15	104	34	23	157	113	38	36	20	1				40	9	0
16	79	41	24	106	89	37	150	23	1				33	8	0
17	214	136	68				198	74	4				32	18	0
18	92	38	21				82	33	2				36	12	0
19	173	93	50				137	54	0				12	2	0
20	149	82	45				129	39	0				39	22	0
21	127	46	11				136	26	0				58	20	1
22	105	15	10				118	31	0				29	14	0
23	89	13	4				143	19	1				27	6	0
24	178	102	18				104	18	0				34	11	0
25													31	11	0
26													17	5	0
27													21	6	0
28													35	6	0
29													25	12	0
30													29	13	0
Total	3175	1745	786	2409	1347	630	3134	1091	24	1872	1111	582	1174	376	3
% flower abortion		45			44			65			41			68	
% fruit set			45			47			2			52			0.8
% fruit degeneration			55			53			98			48			99.2

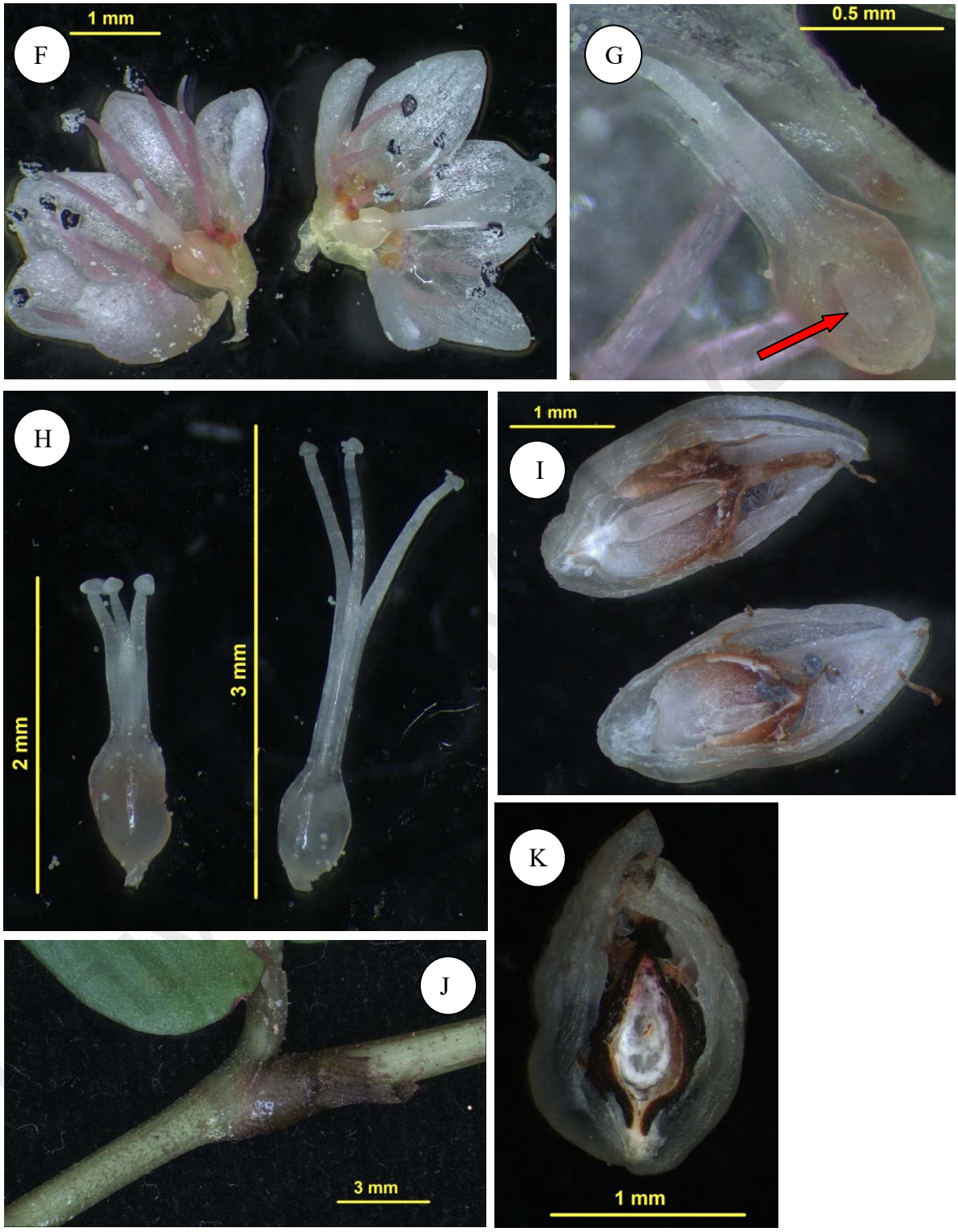
Table 3.6: Summary of the flower abortion and fruit set in the highland and lowland populations of *P. chinensis* var *chinensis*.

Population	Altitude (m)	Average temperature, from 0630–0830 hours (ºC)	Average % humidity from 0630–0830 hours	No. of inflorescence that produced ≥ 1 fruit	No. of flower buds	No. of open flowers	No. of mature fruit	% flower abortion	% fruit set
H2	1350	18.6	66.0	24	3175	1745	786	45	45
H3	1390	17.6	78.5	16	2409	1347	630	44	47
H4	1620	16.4	91.4	11	3134	1091	24	65	2
H5	1300	19.0	85.6	14	1872	1111	582	41	52
LC	33	28.5	62.8	3	1174	376	3	68	0.8

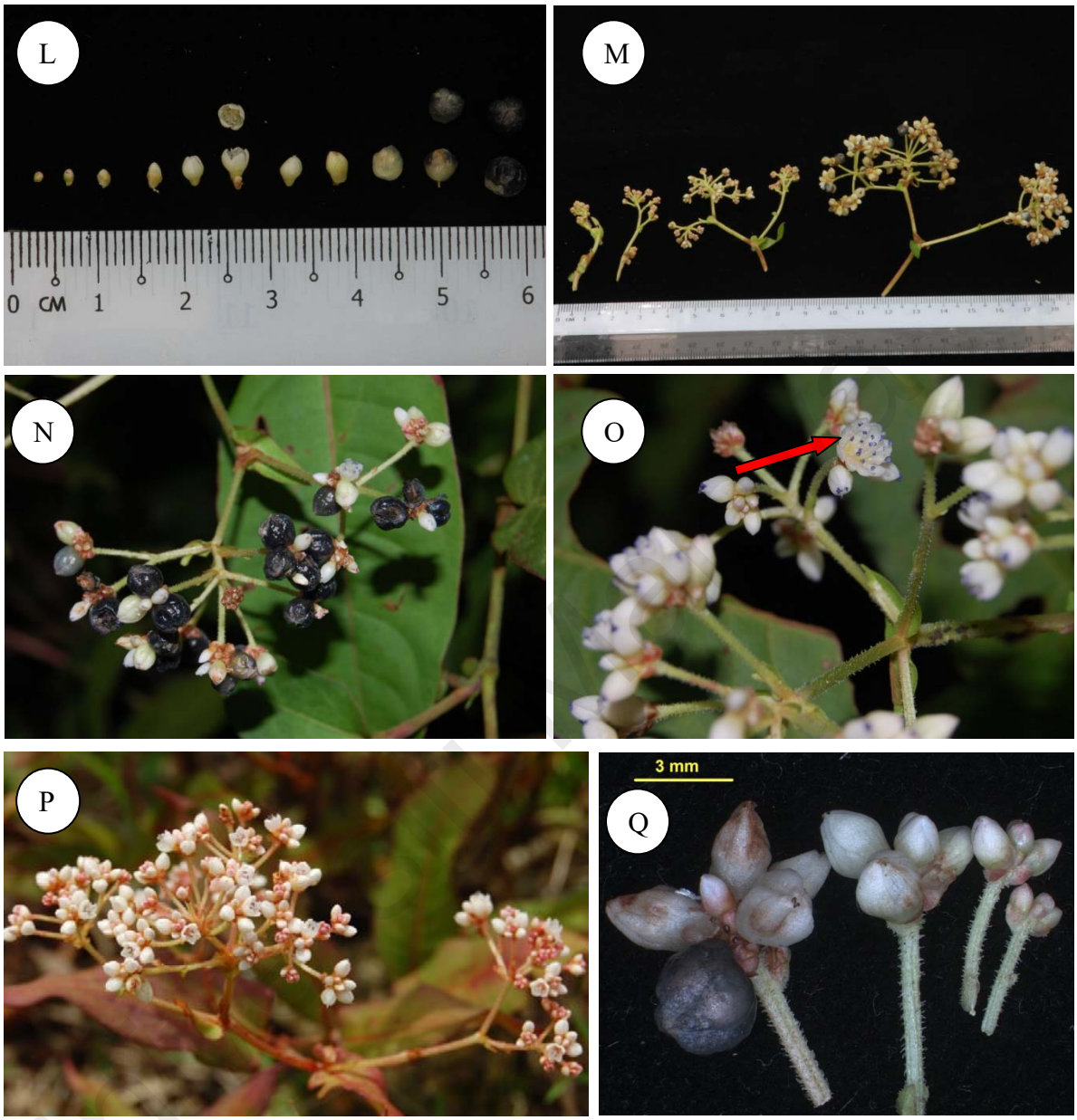
Table 3.7: Comparative flower and fruit development of the highland and lowland populations of *P. chinensis* var. *chinensis*.

CHARACTERS	Highland variety of *P. chinensis* var. *chinensis*	Lowland variety of *P. chinensis* var. *chinensis*
Flower development		
1. Initiation of very young flowers buds in one inflorescence to appearance of flower buds (days)	8–11	5–8
2.Inflorescences length at tagging (mm)	16–20	6.84–12.75
3. Panicle length (mm)	33–90 (majority 45–70)	30–68 (majority 40–55)
4.Panicle length (mm) when flowering began	45–55	41.07–49.63
5. Appearance of the very young flower buds to first anthesised flowers (days)	12–14	9–12
6. Average number of flowers per panicle	13–(68)–143	2–(13)–22
7. Flowering sequence	acropetal, asynchronous	acropetal, asynchronous
Anthesis		
7. Time of flower anthesis and anther dehiscence (hours)	0600–0730	0745–0930
8. Receptiviy of the stigma	anthesis and stigma receptivity occur simultaneously	anthesis and stigma receptivity occur simultaneously
Fruit development		
9. Young flower buds to mature fruits (days)	30–45	n.a.
10. Size of mature fruit (mm)	4.43 × 5.53	n.a.
Flower and fruit degeneration		
11. Flower abortion (%)	41–65	68
12. Fruit set (%)	2–52	0.8

Figure 3.6: Highland wild populations (blue arrows) of *Persicaria chinensis* var. *chinensis* in Gunung Ulu Kali, Bentong, Pahang (A–Q). A: Habit. B: An inflorescence of very young buds (Left) compared to a mature compound corymb inflorescence (Right). C: Thrum-eyed flower. D: Pin-eyed flower. E: Comparative flower morphology of the pin-eyed (Right) and thrum-eyed (Left) flowers.



Cont. Figure 3.6: F: Pin-eyed (Right) and thrum-eyed (Left) flowers, dissected with 5-epipetalous and 3-free anthers. G: Superior ovary with basal placentation (Arrow pointed). H: Pin-eyed (Right) and thrum-eyed (Left) carpels with 3 filiform styles and free above the middle. I: Dissected elongate-ovary after fertilization. J: The ocrea and petiole. K: Longitudinal section of a fruit and seed.



Cont. Figure 3.6: L: Developmental stages of flower bud to fruit. M: Development of an inflorescence. N: Mature fruits, with flower buds and open flowers found in one inflorescence, growing basipetally and new flower buds continuing to appear at the base of the inflorescence. O: A very rare flower with 13 whitish purple anthers (Arrow pointed). P: A compound corymb inflorescence. Q: Inflorescence development from young buds to mature fruit. Pictures C–I, K & Q were taken using a dissecting microscope attached with a camera and computer system.

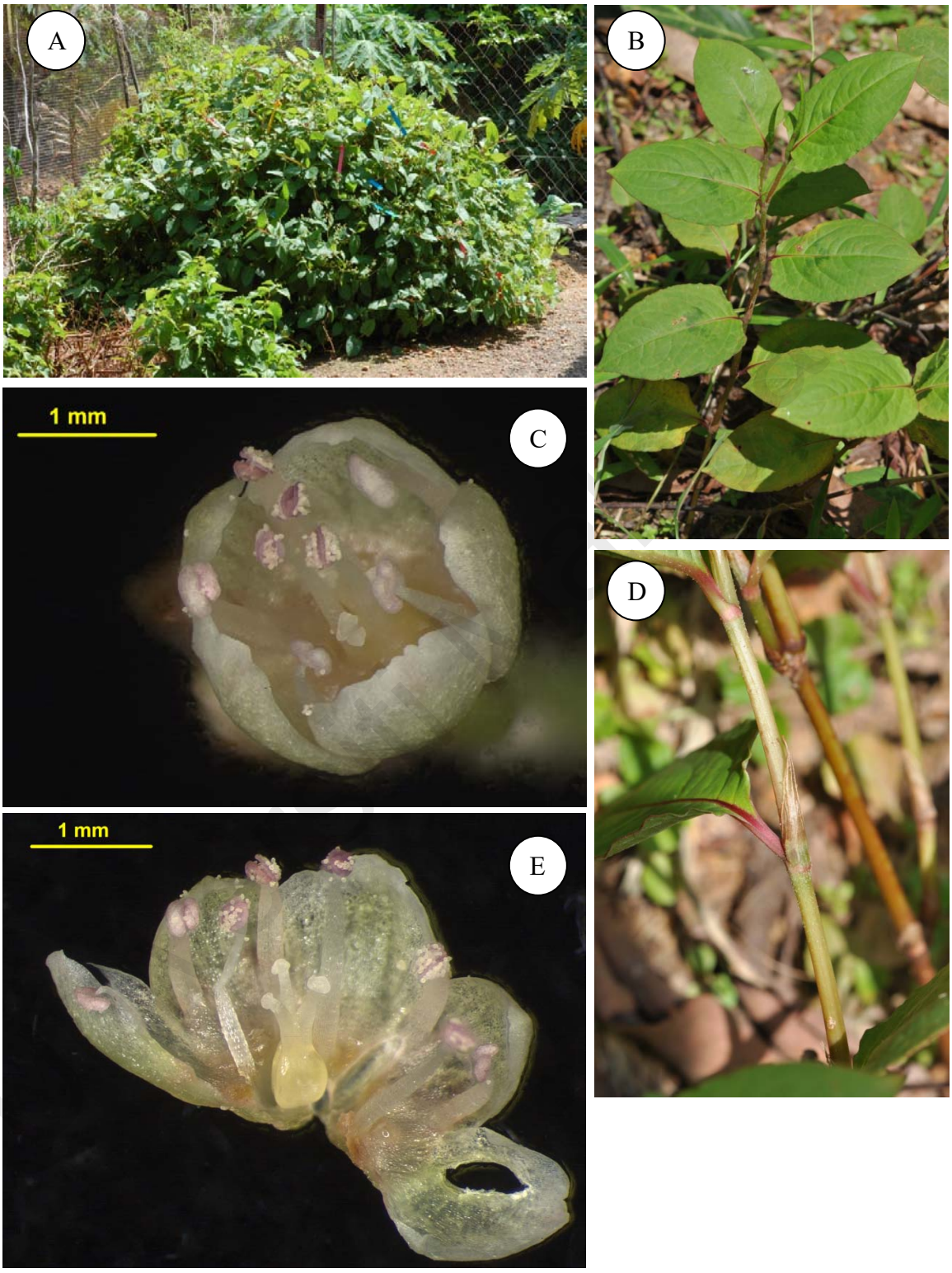
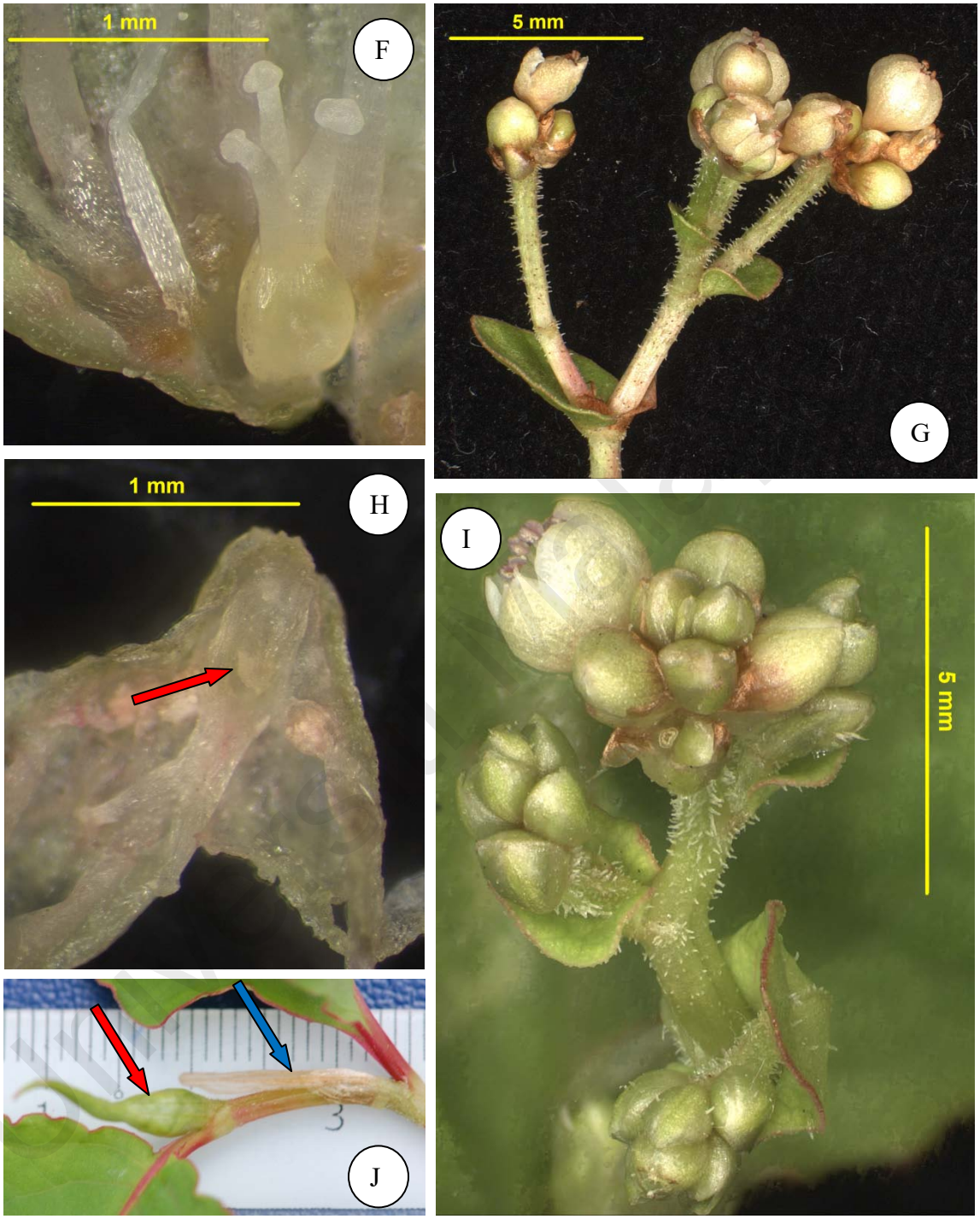
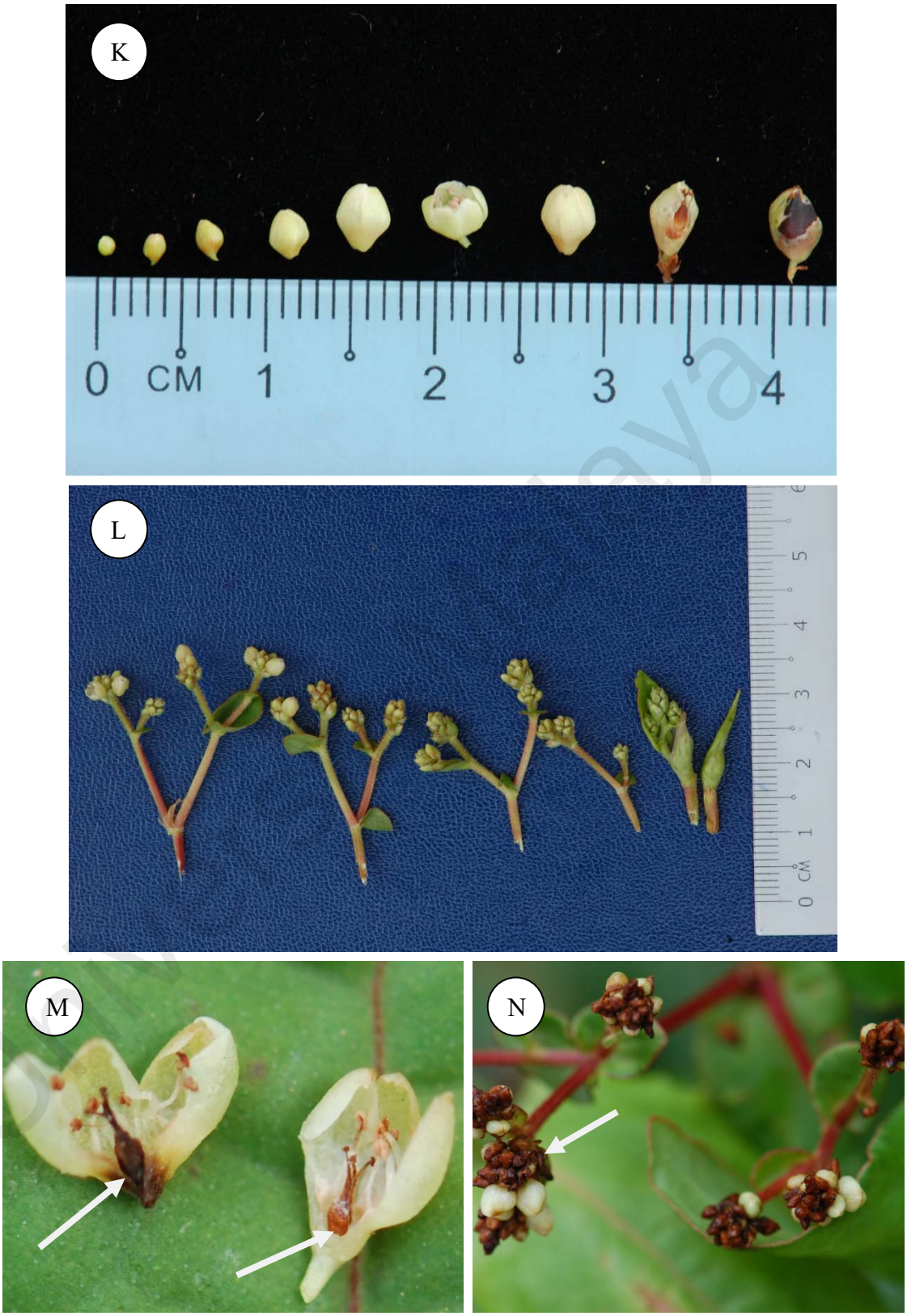


Figure 3.7: Lowland cultivated population of *Persicaria chinensis* var. *chinensis* in Sagil, Tangkak, Johor (A–N). A: Growth habit, shrub (Blue arrows). B: Growing erect and upright. C: Dehisced anthers in a thrum-eyed flower. D: Ocrea and petiole. E: Dissected thrum-eyed flower with 5-epipetalous and 3-free anthers.



Cont. Figure 3.7: F: Superior ovary with 3 filiform styles of thrum-eyed flower. G: Flower buds and closed flowers after anthesis found in one inflorescence, growing basipetally and new flower buds continuing to appear on the base of the inflorescence. H: Basal placentation (Arrow pointed). I: Young inflorescence with the first flower opened, and hairy peduncle. J: Very young bud (Arrow pointed) in an inflorescence and the length of an ocrea (Blue pointed).



Cont. Figure 3.7: K: Development from a very young bud to a fruit (premature fruit without blackish flesh. L: Development of an inflorescence. M: Degenerated ovaries (Arrow pointed). N: Flower abortion in an inflorescence.

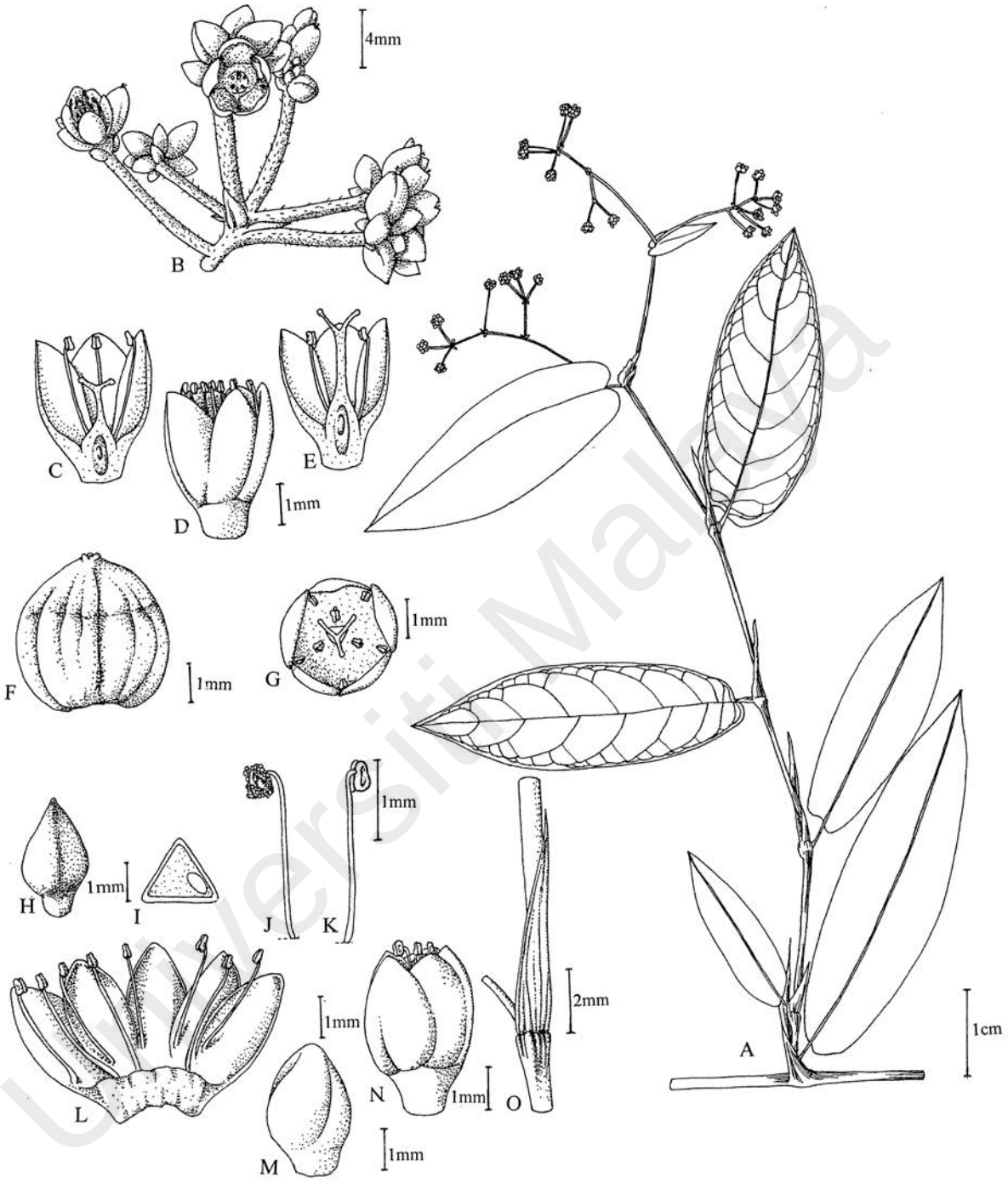


Figure 3.8: Highland variety of *Persicaria chinensis* var. *chinensis*. A: habit; B: inflorescence; C: Longitudinal section of thrum-eyed flower; D: open flower after anthesis; E: Longitudinal section of pin-eyed flower; F: fruit; G: plan view of a flower; H: seed; I: cross section of a seed; J: stamen with dehisced anther; K: stamen; L: perianth opened to show stamens (5 epipetalous and 3 distinct and free filaments); M: flower bud; N: closed flower after anthesis; O: ocrea. (A–O from fresh material).

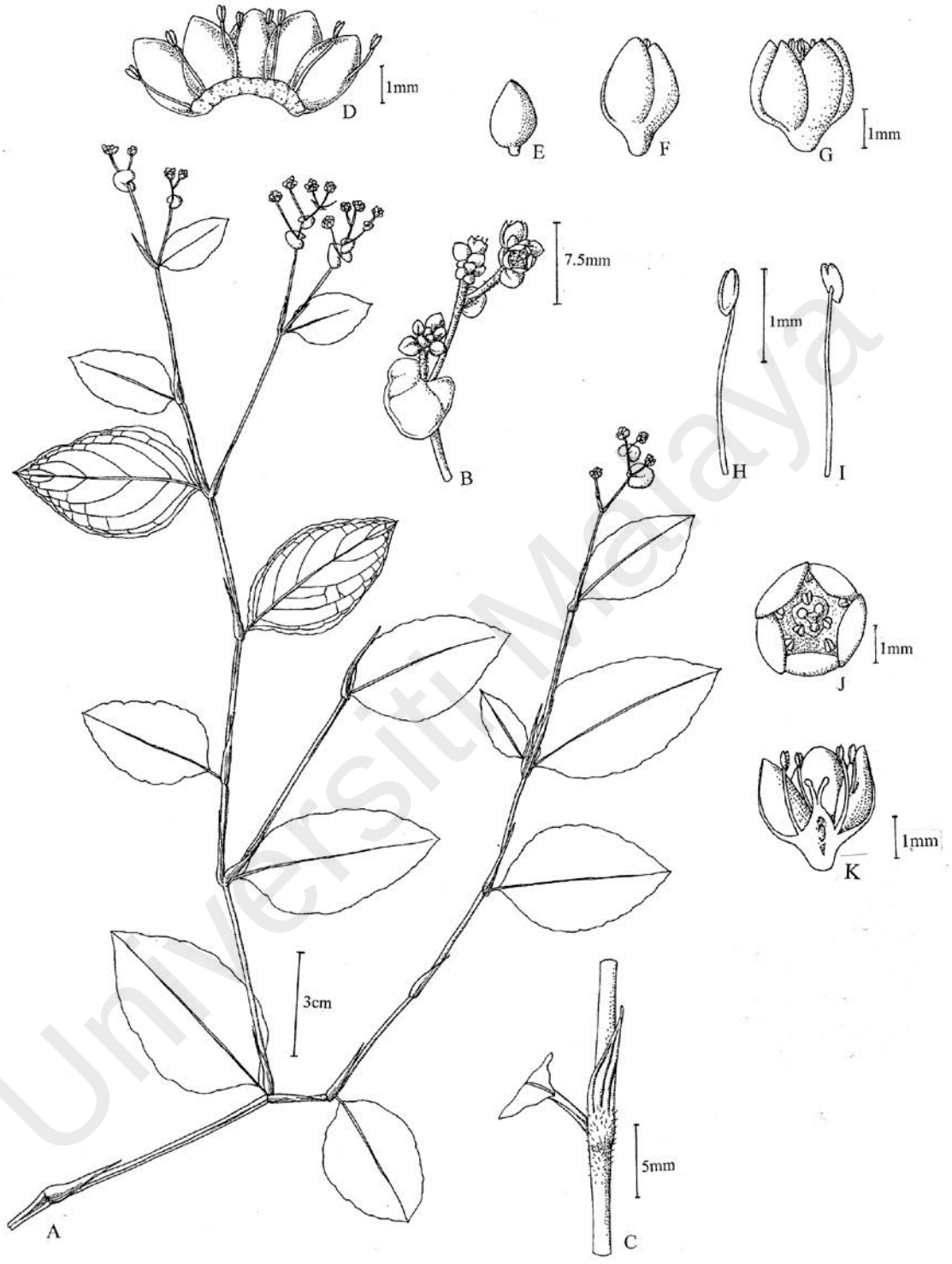


Figure 3.9: Lowland variety of *Persicaria chinensis* var. *chinensis*. A: habit; B: inflorescence; C: ocrea; D: perianth opened to show stamens (5 epipetalous and 3 distinct and free filaments); E: young flower bud; F: flower bud; G: closed flower after anthesis; H: front view of a stamen; I: back view of a stamen; J: plan view of a flower; K: longitudinal section of a thrum-eyed flower.

3.3.3 Embryological studies

3.3.3.1 Microsporangium, microsporogenesis and male gametophyte

Both the highland and lowland varieties of *P. chinensis* var. *chinensis* have eight stamens in the bisexual flower with five epipetalous stamens and three distinct and free stamens. Anther development is identical in the highland and lowland varieties of *P. chinensis* var. *chinensis*.

The anther is tetrasporangiate (Figure 3.11 A) and it is slightly different size. The archesporium is multicellular (Figures 3.10 A–B) and more than one archesporia can be observed in the sporangium and the divisions are not synchronous. The archesporium divides periclinally to produce an outer primary parietal and an inner sporogenous cell (Figure 3.11 B). Periclinal division of the primary parietal cells (Figures 3.10 C & 3.11 C–D) produces the secondary parietal cells. The inner secondary parietal further divides periclinally to form a middle layer and a tapetum while the outer secondary parietal develops directly into the endothecium (Figures 3.10 D–E & 3.11 E). Thus, the anther wall development conforms to the Monocotyledonous type of Davis (1966). Just before meiosis of the microspore mother cells, the anther wall consists of an epidermis, an endothecium, one middle layer and a tapetum (Figures 3.10 E–F & 3.11 F). At this stage, the epidermal cells accumulate a large quantity of tanniniferous materials and were stained red in the slides (Figures 3.10 F & 3.11 F). Simultaneously, the glandular tapetal cells reach their maximum development in which the initially uninucleate tapetal cells divide mitotically (Figures 3.10 F & 3.11 F–G) to become multinucleate, 3- to 4- nucleate just before meiosis of the microspore mother cells (Figures 3.10 F–G & 3.11 H–J). At this stage, the tapetum layer is pushed towards the microspore mother cells (Figure 3.10 F) and the ephemeral middle layer begins to degenerate before the microspore mother cells undergo meiosis (Figures 3.10 G & 3.11 I–J). Before meiosis, the microspore mother cells enlarge and show dense cytoplasm

(Figure 3.11 F). Callose surrounds the microspore mother cells and the microspore tetrads (Figures 3.10 H–J & 3.11 I–N). During microsporogenesis, cytokinesis is simultaneous (Figures 3.10 I & 3.11 K–L) producing tetrahedral and decussate tetrads (Figures 3.10 H–J & 3.11 M–N). For the lowland cultivated *P. chinensis* var. *chinensis,* the microspore tetrads are mostly tetrahedral. The tapetal cells begin to degenerate after the formation of the microspore tetrads and they are completely resorbed when fibrous thickenings develop on the endothecium at the three-celled pollen grain stage (Figures 3.10 F, M–O & 3.11 K–P). The mature pollen grains are shed at the three-celled stage (Figures 3.10 N & 3.11 P). Within the same flower, the anthers showed asynchronous development; for example, one anther was at anaphase I of microsporogenesis while the other locule was at the microspore tetrad stage (Figures 3.10 G–H & 3.11 I).

The nucleus of the one-celled pollen grain divides through mitotic division to form a small generative cell and a large vegetative cell (Figures 3.10 L–M). The generative cell undergoes further division, mitotically, to produce two smaller male gametes (Figures 3.10 N & 3.11 P). The tapetum completely degenerates and the endothecium develops fibrous thickenings when the anther is at the one-celled pollen grain stage. A mature pollen grain is tricolporate and shed at the three-celled stage (Figures 3.10 N & 3.11 P). During the time of dehiscence, the anther wall is extrorse and comprises of only the fibrous endothecium and epidermis (Figures 3.10 O & 3.11 P).

Figure 3.10 A–O Highland wild variety of *P.chinensis* var. *chinensis*, microsporogenesis and microgametogenesis

(ar, archesporium; cyt, cytokinesis; ed, endothecium; ep, epidermis; ft, fibrous thickening; isp, inner secondary parietal; mic, microspore mother cell; ml, middle layer; osp, outer secondary parietal; pg, pollen grain; pp, primary parietal cell; sp, sporogenous; tp, tapetum)

A–O transverse sections of anther

A young anther at archesporium stage

B primary parietal cell and sporogenous cell

C primary parietal cell divides periclinally to produce an inner and outer secondary parietal cells

D inner secondary parietal divides periclinally to produce a middle layer and tapetum

E at the young microspore mother cell stage, showing the anther wall; epidermis, endothecium, middle layer and tapetum

F nucleus of tapetum divides to form a multi-nucleate tapetum

G microspore mother cell divides meiotically

H asynchronous development in two different locules of the same anther

I simultaneous cytokinesis and tetrahedral tetrad

J decussate tetrad

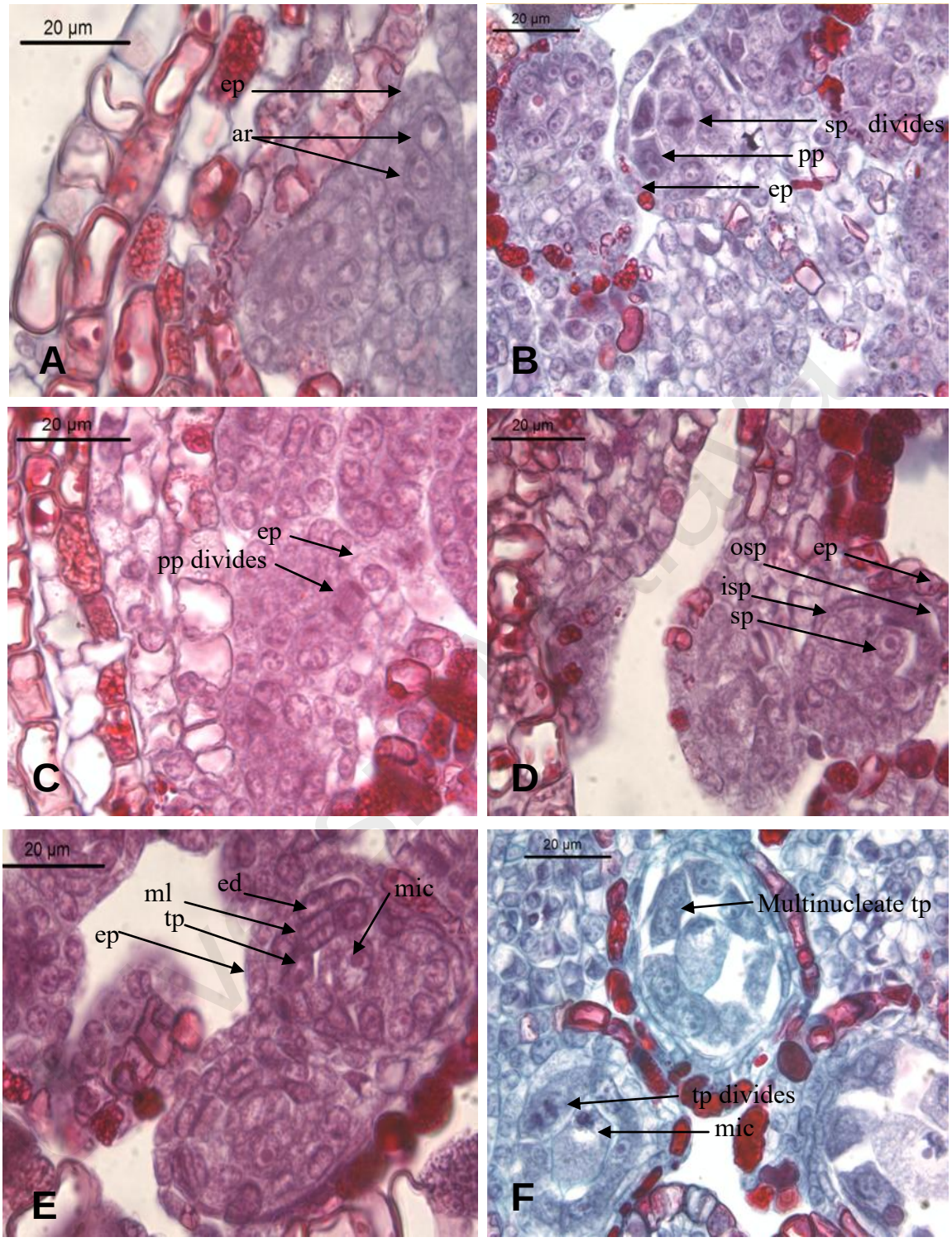
K one-celled pollen grain

L pollen grain divides to form a vegetative and a generative cell

M two-celled pollen grain

N three-celled pollen grain

O mature anther showing fibrous thickening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Multinucleate tp
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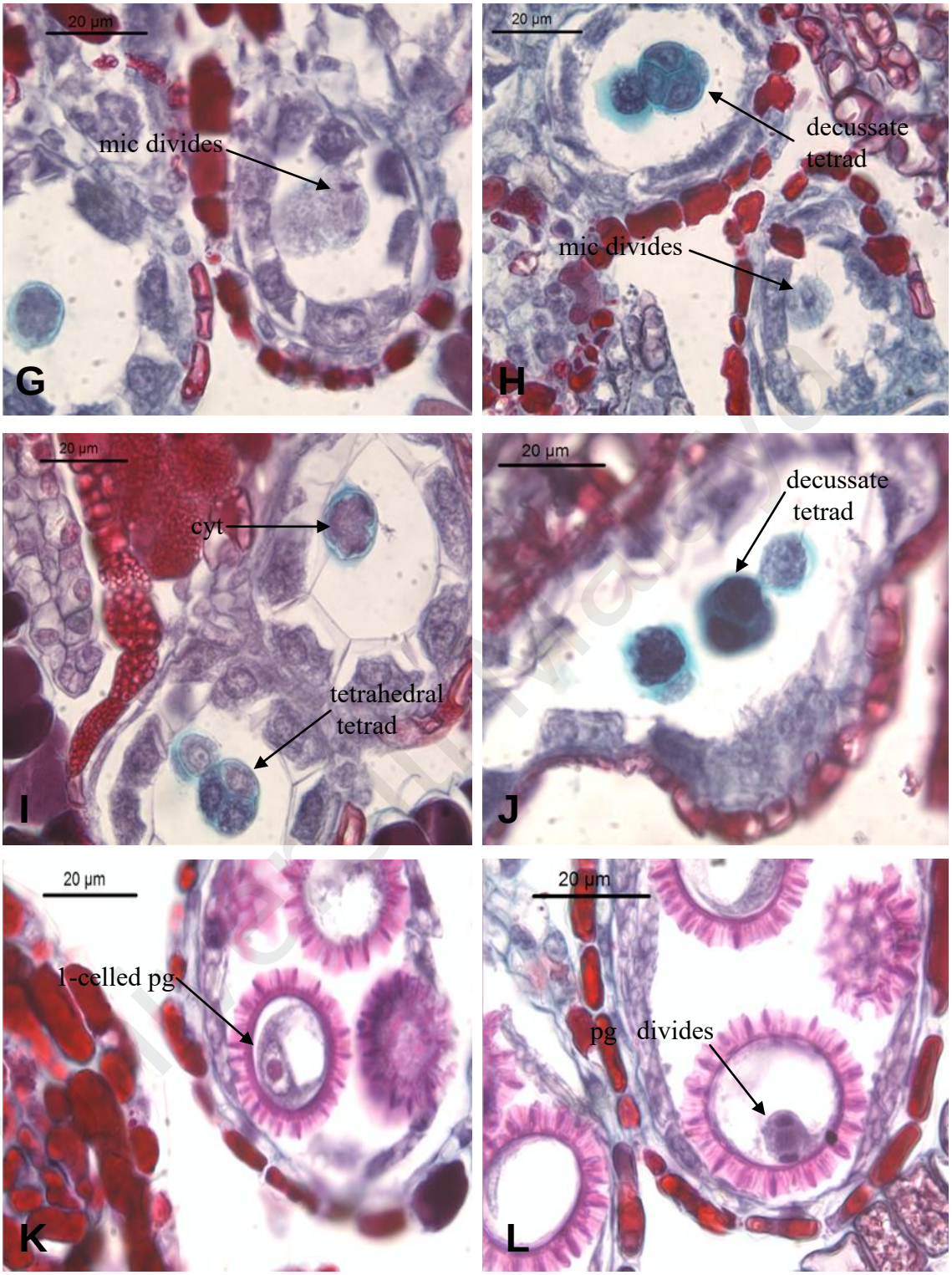
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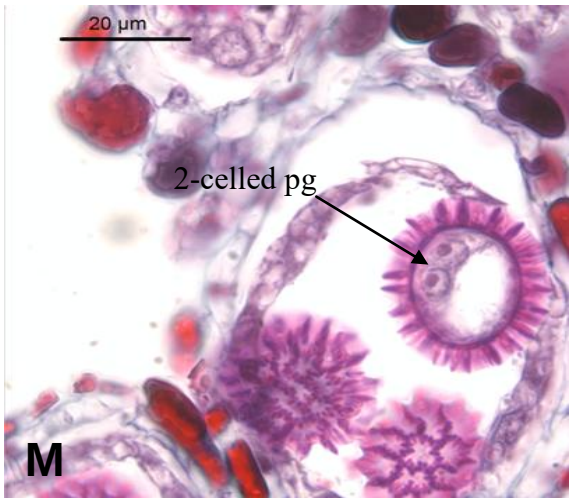
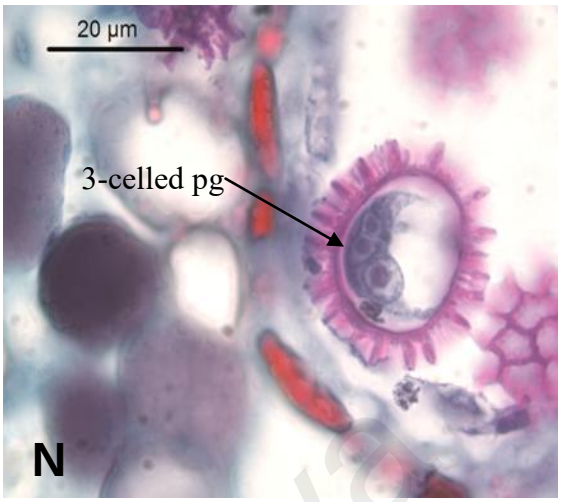




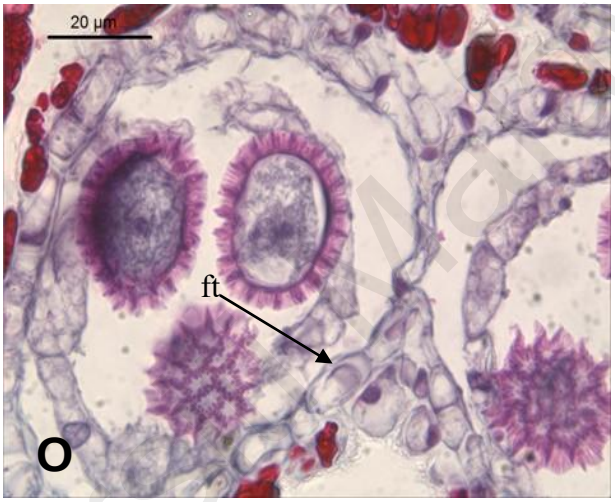


Figure 3.11 A–P Lowland cultivated variety of *P. chinensis* var. *chinensis*, microsporogenesis and microgametogenesis

(ar, archesporium; cyt, cytokinesis; ed, endothecium; ep, epidermis; ft, fibrous thickening; isp, inner secondary parietal; mic; microspore mother cell; ml, middle layer; osp, outer secondary parietal; pg, pollen grain; pp, primary parietal cell; sp, sporogenous; tp, tapetum;)

A–H, O & P transverse sections

I–N longitudinal sections of anther

A tetrasporangiate anther

B young anther showing primary parietal cell and sporogenous cell

C–D primary parietal cell divides periclinally to produce an inner and outer secondary parietal cell

E inner secondary parietal divides periclinally to produce a middle layer and tapetum

F anther wall at late prophase of microspore mother cell showing the endothecium, middle layer and tapetum cells

G multinucleate tapetum

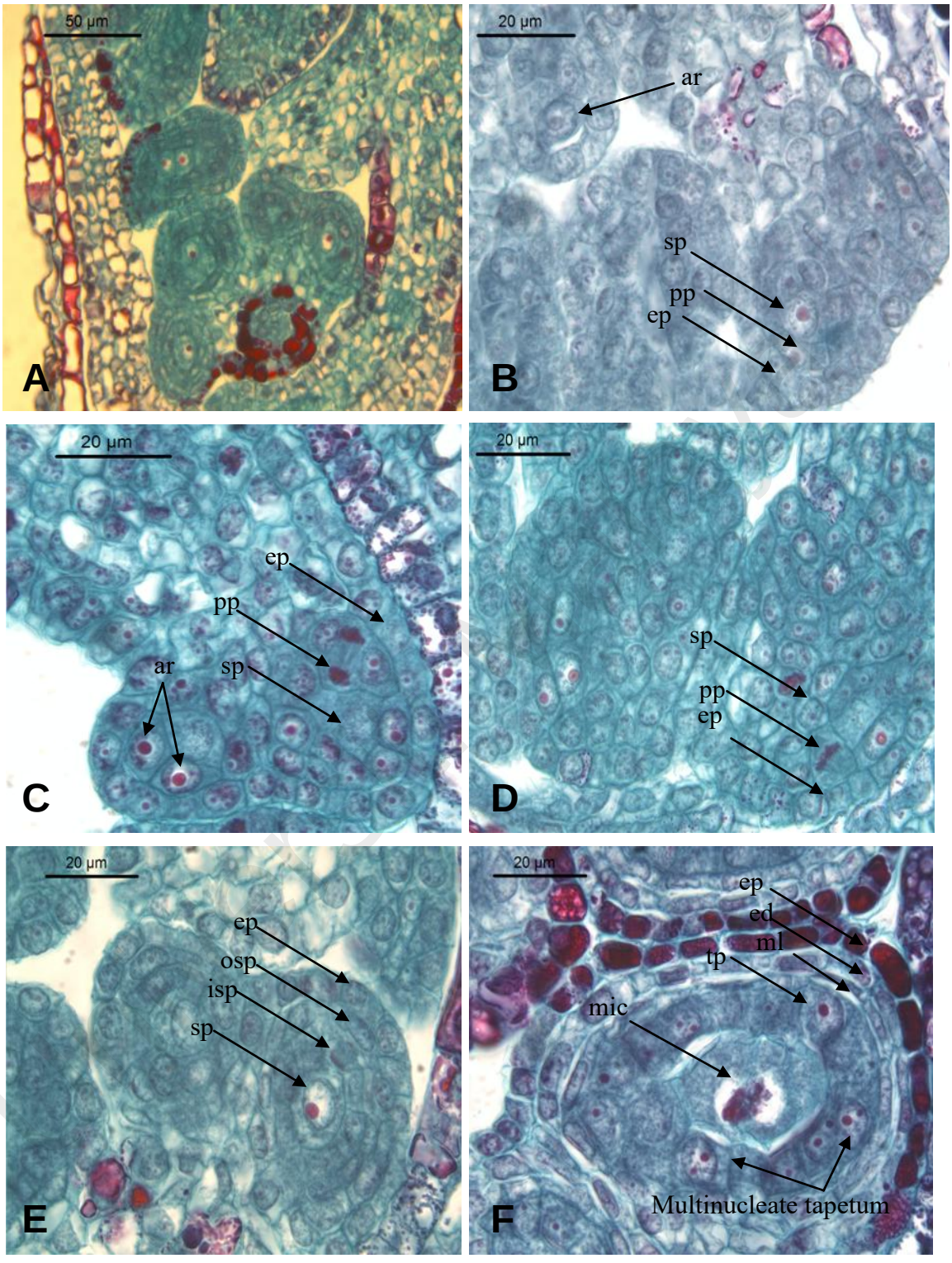
H–J microspore mother cell divides meiotically, with callose surrounding the microspore mother cells

K–L simultaneous cytokinesis

M–N tetrahedral and decussate tetrads

O one-celled pollen grains

P three-celled pollen grain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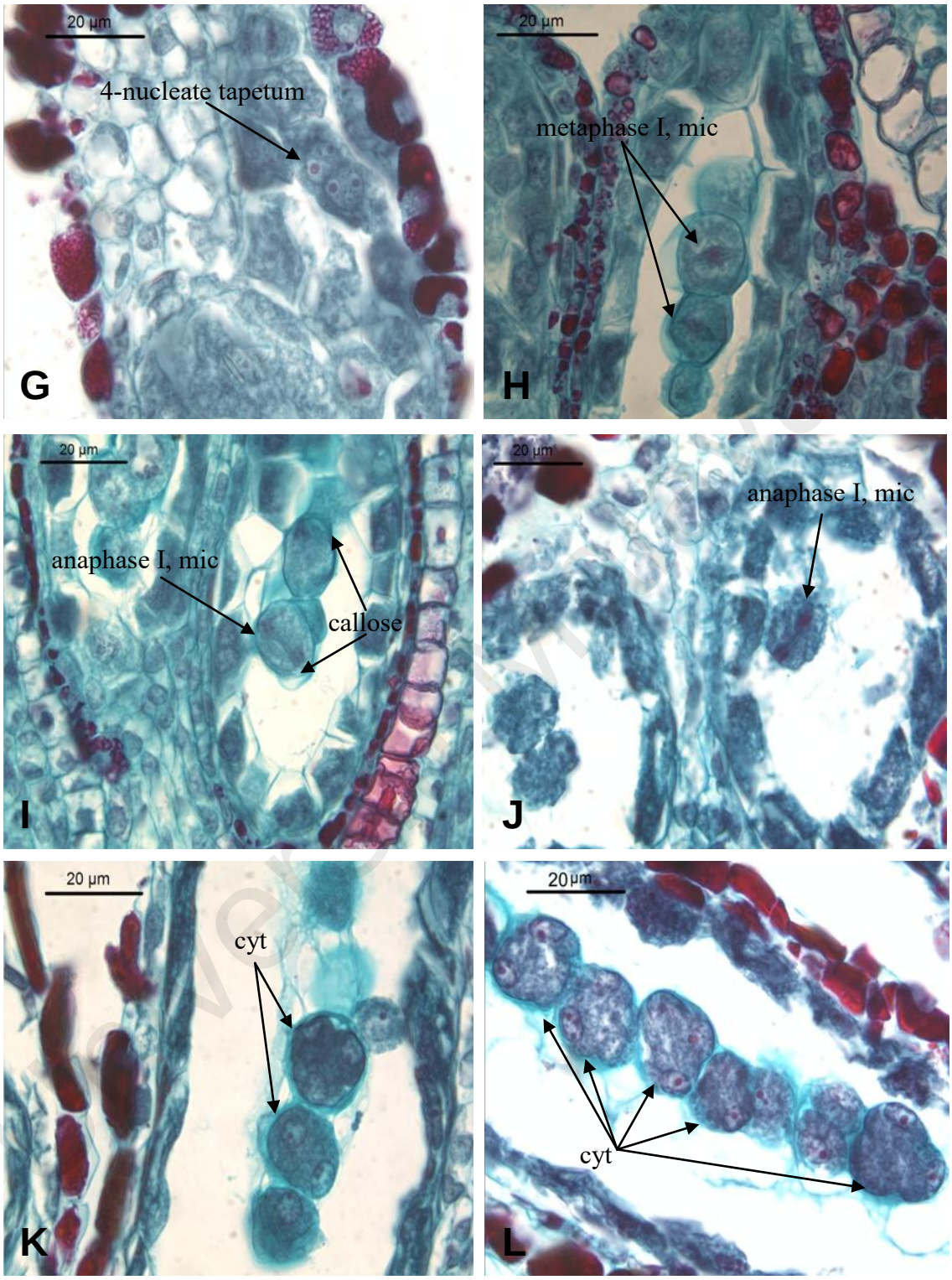
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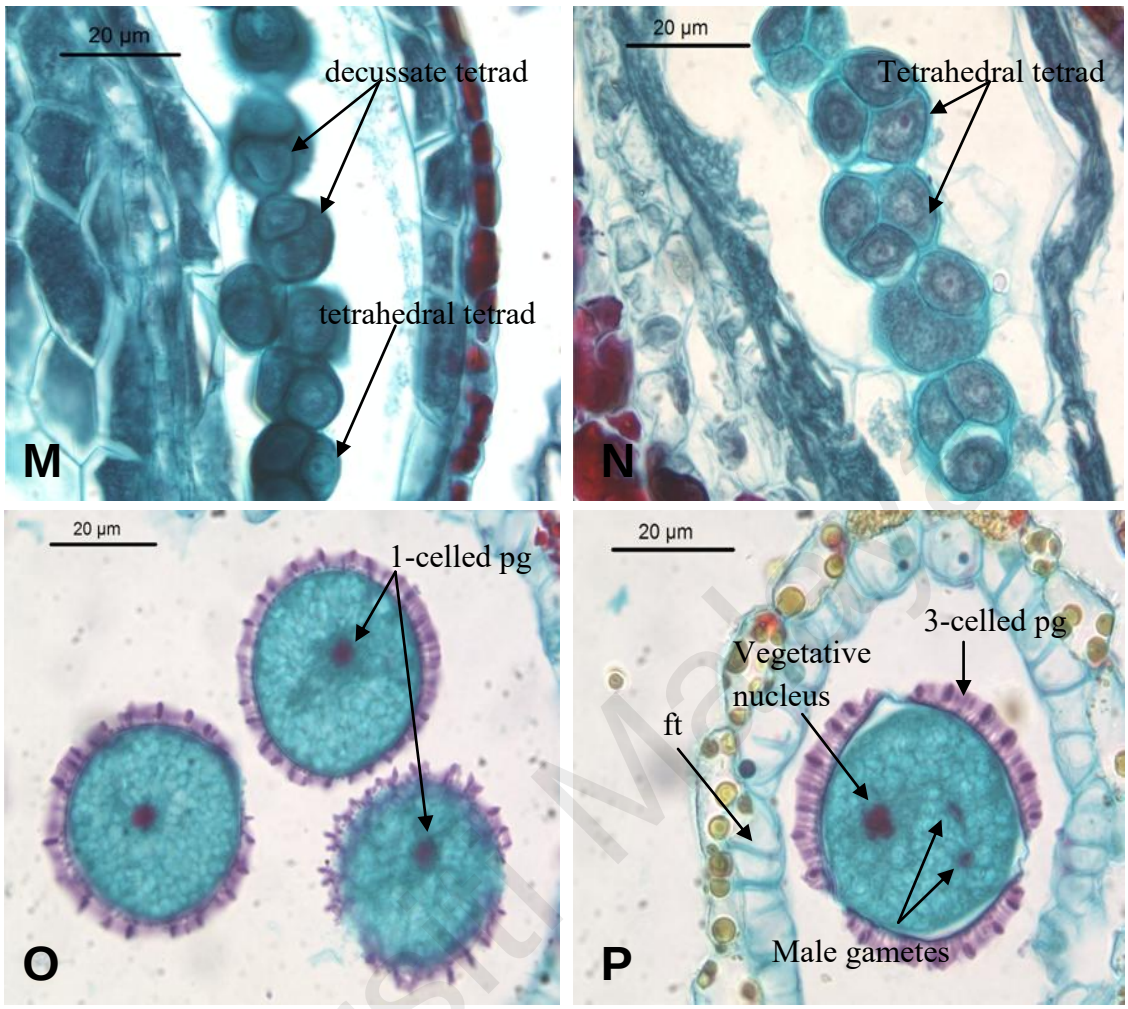


3.3.3.2 Megasporangium, megasporogenesis and female gametophyte

The gynoecium of *P. chinensis* var. *chinensis* is superior, tricarpellary, syncarpous, and uniovulate with a single orthotropous, bitegmic and crassinucellate ovule on basal placentation. The micropyle is formed by both the inner and outer integuments (Figures 3.12 A–B & 3.13 A).

The ovular primodium consists of a mass of homogenous tissue (Figure 3.13 A) and the female hypodermal archesporium is multicellular (Figure 3.13 B), but only one develops into the megaspore mother cell with two layers of nucellus at the micropylar end (Figures 3.12 A–B & 3.13 C–F). By the time the megaspore mother cell is differentiated, the inner integument and the outer integument have been initiated (Figures 3.12 A–E & 3.13 C–D). At this stage, the nucellar beak is also differentiated into two layers (Figure 3.12 B). Both the outer and inner integuments are made up of two layers of cells (Figures 3.12 A–E & 3.13 C–D). When the ovule is at the megaspore mother cell stage, the anther undergoes simultaneous cytokinesis resulting in tetrahedral or decussate tetrads developing into one-celled pollen grains.

The megaspore mother cell undergoes meiosis to form a dyad (Figure 3.13 F), followed by a linear tetrad (Figures 3.12 C–D & 3.13 F–H). This division is synchronous (Figure 3.12.C). At this stage, the anther has produced pollen grains mostly two-celled pollen grains and some are still one-celled pollen grains. The division of the dyad to form tetrad is rapid and this is followed by degeneration of the three micropylar megaspores (Figures 3.12 D & 3.13 G–H) while the chalazal megaspore is functional (Figures 3.12 D & 3.13 G, I). It divides mitotically to produce a two- (Figure 3.12 E), four- (Figure 3.12 F) and finally eight-nucleate embryo sac (Figures 3.12 G–J & 3.13 J–L). At the eight-nucleate embryo sac (Figures 3.12 G–H), a few nucellar cells at the chalazal end of the ovule accumulate abundant tannin and begin to differentiate into the saucer-shaped hypostase (Figure 3.12 O), which is a

plate-like structure ranging in height from a few cells at the periphery to 6–7 cells in the central region (Figure 3.12 Q).

The mature embryo sac consists of two synergids, an egg cell, two polar nuclei (fused to form a secondary nucleus) and three ephemeral antipodals (Figures 3.12 K–O & 3.13 M–P). Hence, the development of the embryo sac conforms to the monosporic *Polygonum* type (Maheswari, 1950). The mature embryo is spindle-shaped and enlarges from 150 µm to 500 µm (Figures 3.12 O–V). Three antipodals start to degenerate before fertilization (Figure 3.12 P) and they have completely degenerated after the formation of primary endosperm cell.

Before fertilization, the embryo sac elongates dramatically until 500 µm. The polar nuclei of the female gametophyte usually fuse prior to fertilization of a secondary nucleus so that the mature gametophyte comprises seven nuclei (Figures 3.12 K–N & 3.13 M–P). The secondary nucleus would shift to the micropylar end of the gametophyte, near to the egg cell or adjacent to the egg cell before fertilization (Figures 3.12 Q–V). An occurrence of twin eggs within an ovule was observed (Figures 3.13 Q–R) in the lowland cultivated *P. chinensis* var. *chinensis*. Furthermore, in the lowland cultivated variety at the mature embryo sac stage, the egg cell had been seen to degenerate in most of the slides (Figures 3.13 X–Y) and this could have been one of the causes of a very low percentage of fruit set (from the study of flower and fruit development).

By the time the mature embryo sac is formed, the nucellar beak is well-developed (Figure 3.12 O) but after fertilization, the nucellar beak begins to degenerate. Pollen tube penetrating the stigma has been observed (Figure 3.13 V) but actual pollen tube growing and penetrating into the embryo sac and fertilising the egg cell have not been observed in the slides. However, fusion of the polar nuclei (measuring 24–30 µm),

degeneration of the synergids, increase in size of the egg nucleus, and elongation of the embryo sac indicate that fertilization has taken place.

Figure 3.12 A–V Highland wild variety of *P. chinensis* var. *chinensis*, megasporangium, megasporogenesis and megagametogenesis.

(b, nucellar beak; e, egg cell; h, hypostase; ii, inner integument; mmc, megaspore mother cell; oi, outer integument; sy, synergid; sn, secondary nucleus)

A–V longitudinal sections of ovule

G–H, I–J, K–N serial sections of one sample

A ovule at megaspore mother cell stage with a two-layered inner integument and outer integument

B ovule with nucellar beak and megaspore mother cell at prophase

C dyad dividing synchronously to form a tetrad during megasporogenesis

D functional megaspore at the chalazal end with three degenerated megaspores

E two-nucleate embryo sac

F four-nucleate embryo sac (Arrows pointed)

G–J formation of an eight-nucleate embryo sac

K two polar nuclei fusing

L the egg cell

M three antipodals

N two synergids

O ovule showing well-developed nucellar beak and two synergids in the embryo sac

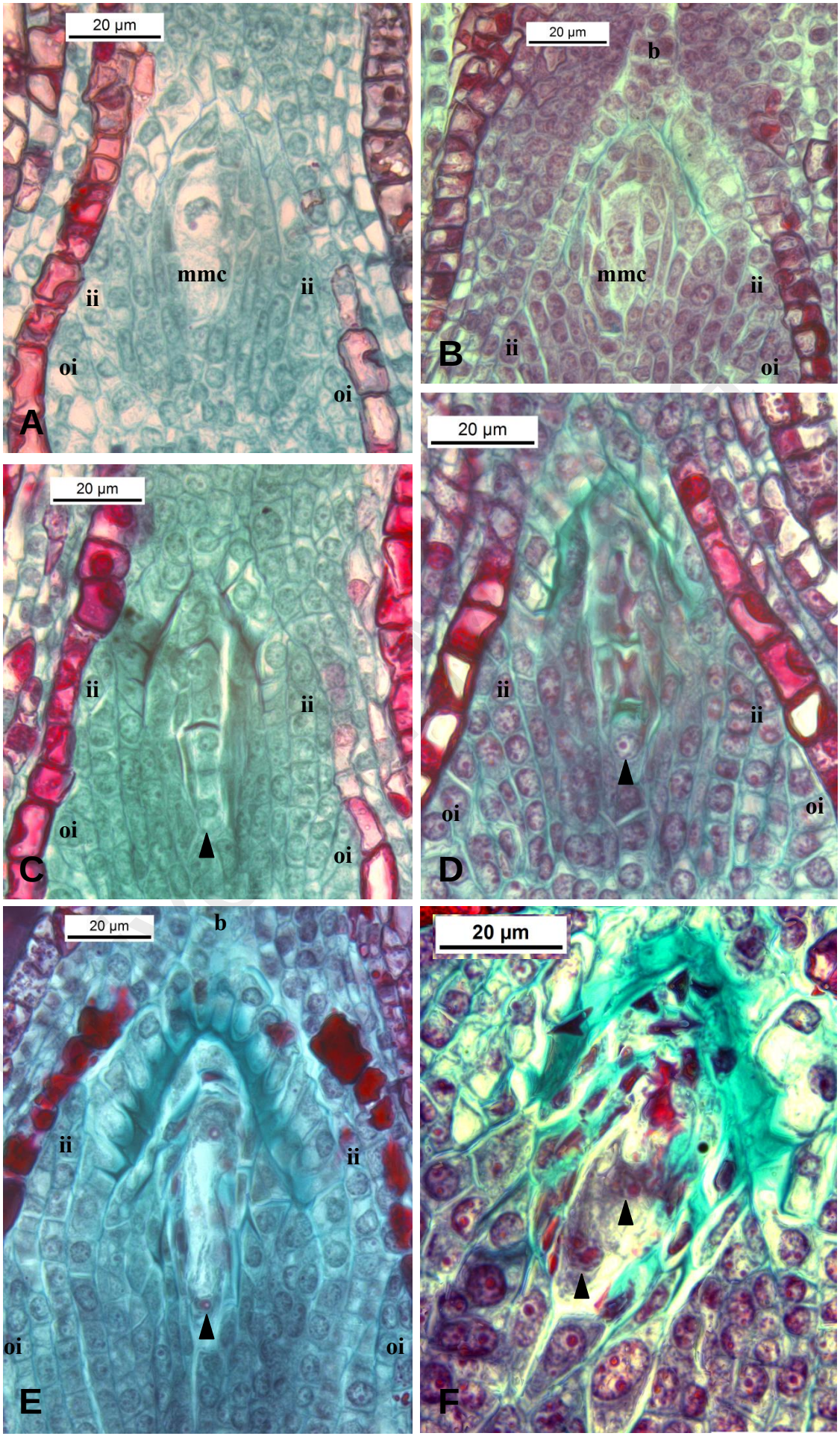
P three degenerated antipodals in embryo sac

Q & S secondary nucleus shown at two different magnifications

R & S the egg cell shown at two different magnifications

U degenerated synergids and the secondary nucleus

V the enlarging embryo sac measuring 500 µm and the large secondary nucleus with a diameter of about 30 µm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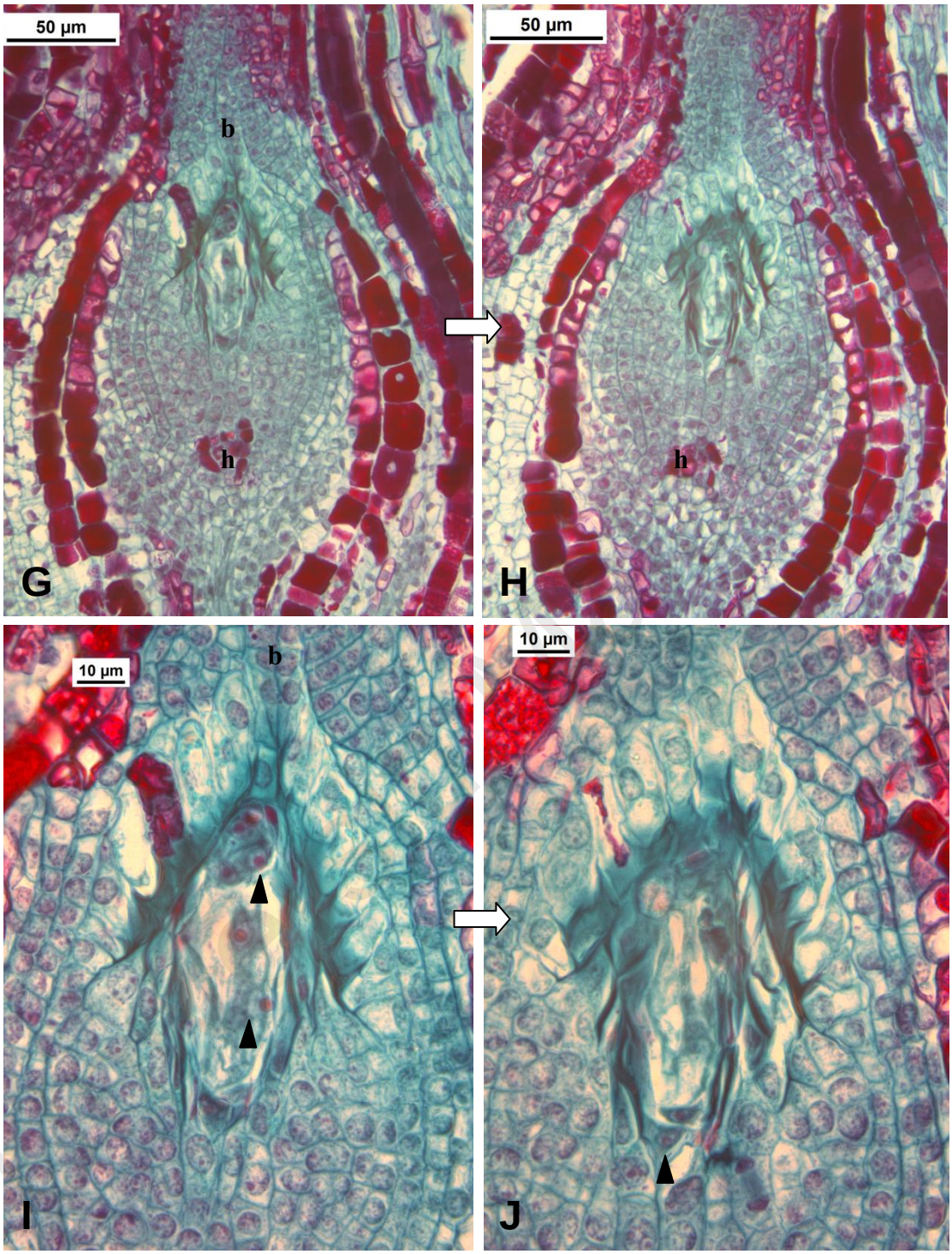
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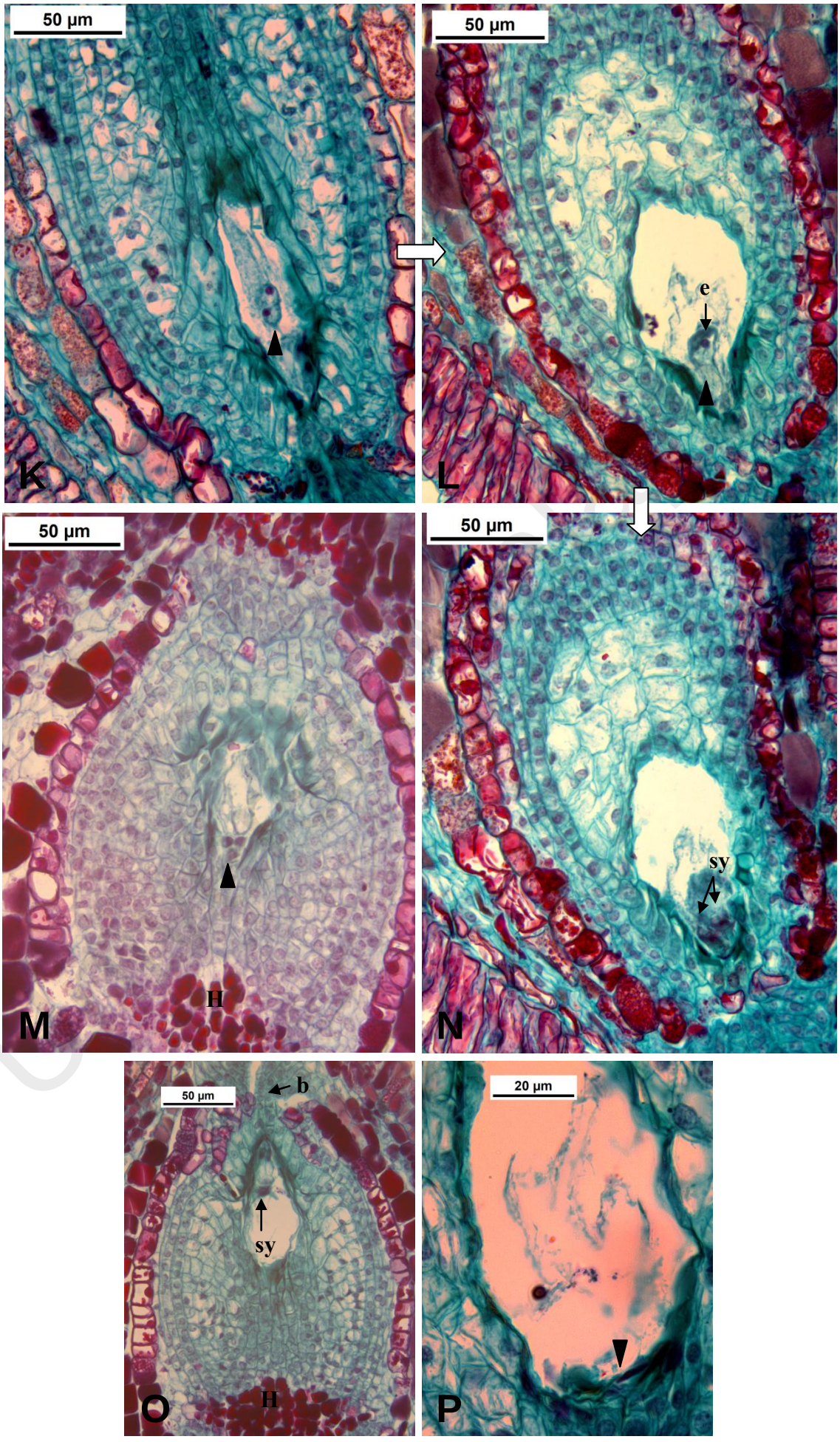
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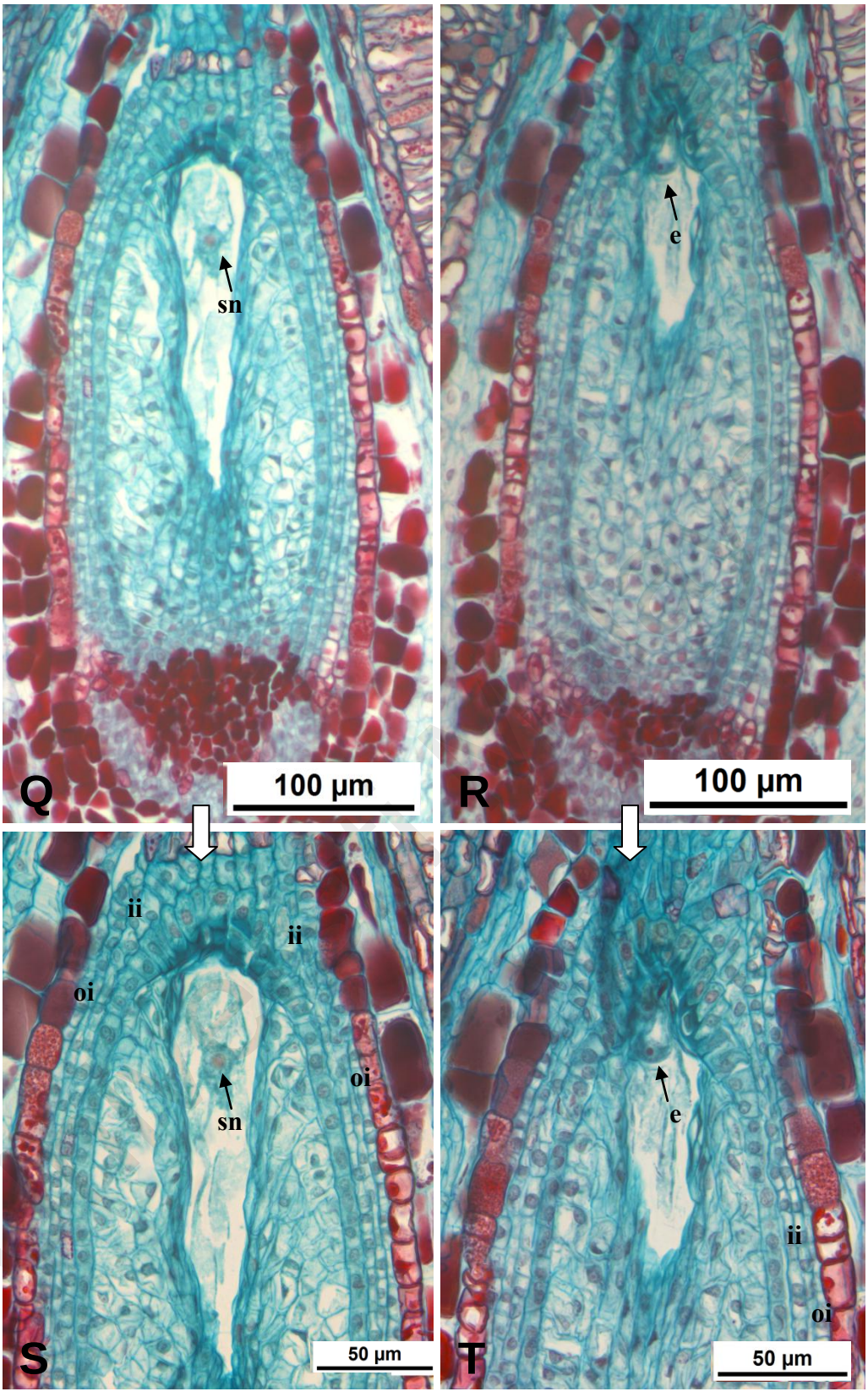
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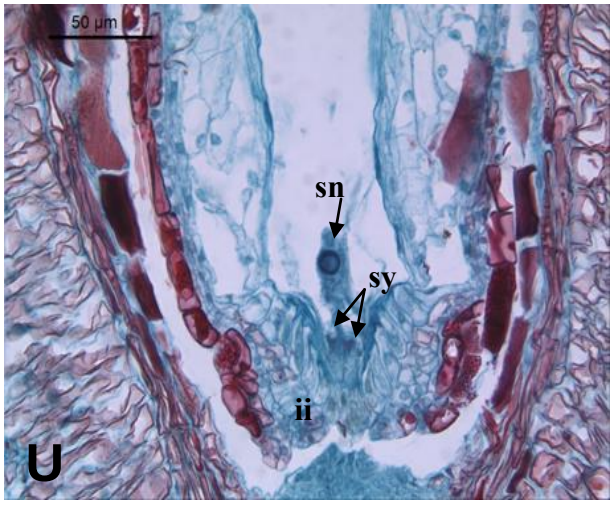


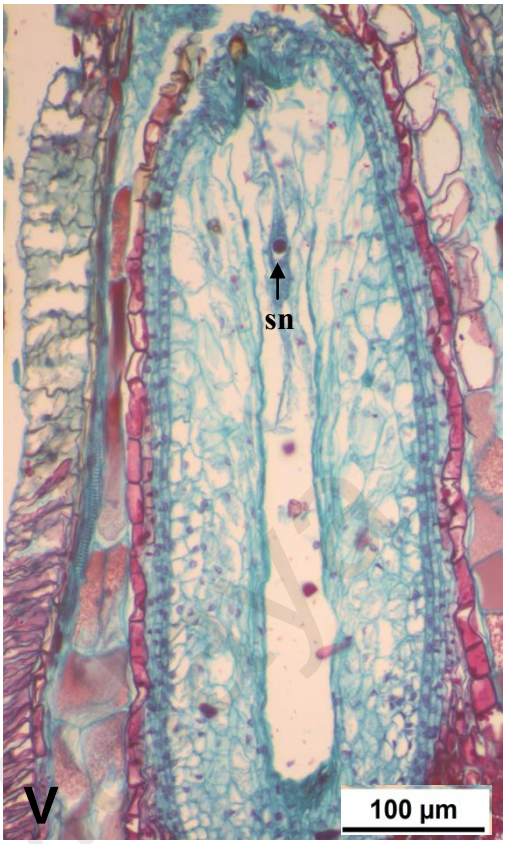


Figure 3.13 A–Y Lowland cultivated variety of *P. chinensis* var. *chinensis*, megasporangium, megasporogenesis and megagametogenesis.

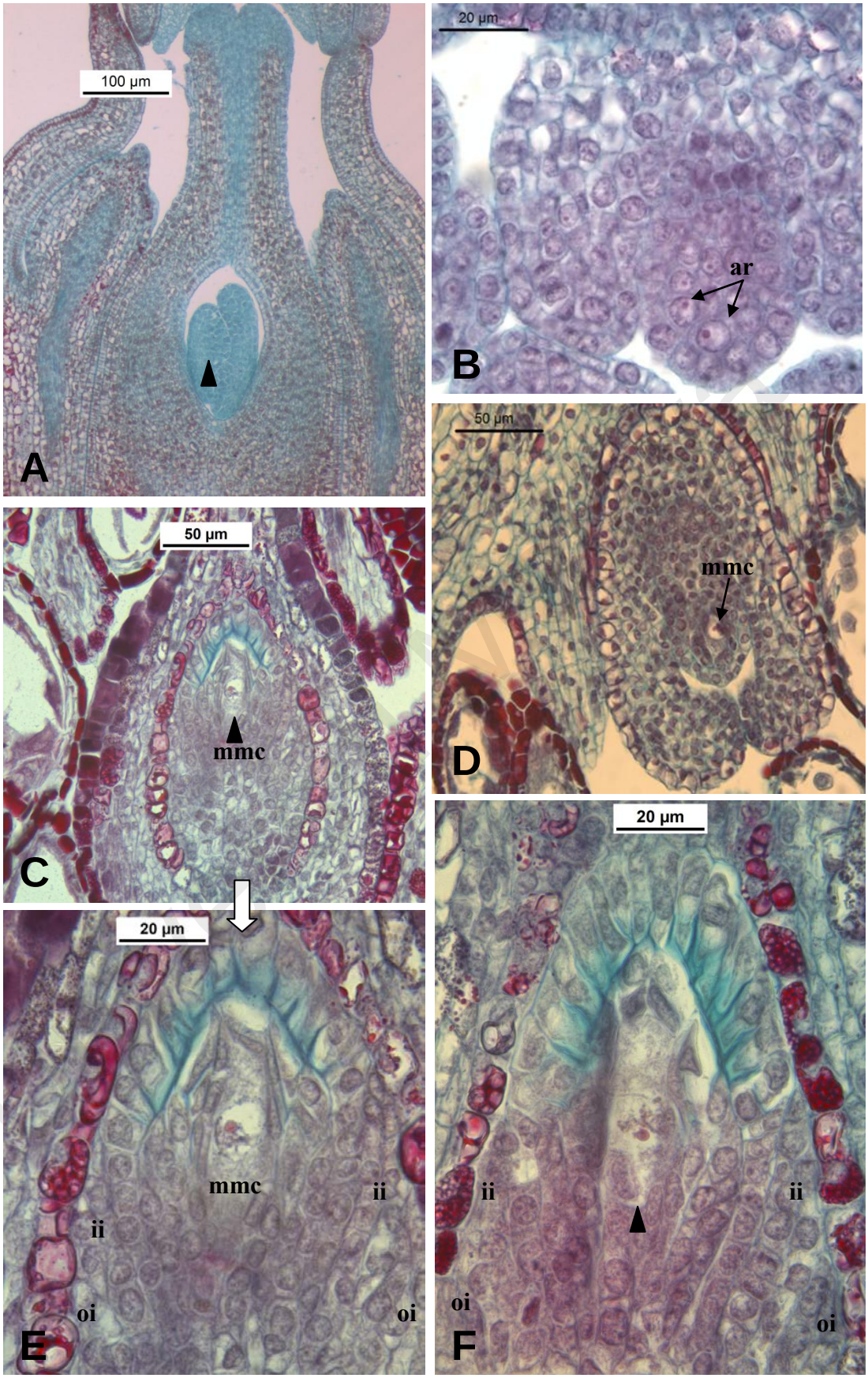
(ap, antipodal; ar, archesporium; e, egg cell; h, hypostase; ii, inner integument; mmc, megaspore mother cell; oi, outer integument; pec, primary endosperm cell; pg, pollen grain; pt, pollen tube; sy, synergid; sn, secondary nucleus; z, zygote;)

A–Y longitudinal sections

G–H, J–L, M–O serial sections in one sample

K & L magnified sections from same slide

A	Ovular primodium with a mass of homogenous cells
B	multicellular archesporium
C–F	Megaspore mother cell, with a two-layered of inner integument and outer integument
G–H	linear tetrad
I	three degenerated megaspores and one functional megaspore at the chalazal
J–L	formation of an eight-nucleate embryo sac
M	three antipodals
N	secondary nucleus
O	two synergids
P	the egg cell
Q–R	two egg cells
S–T	the egg cell and secondary nucleus
U	zygote with dense cytoplasm
V	pollen grain with the pollen tube penetrating the stigma
W	primary endosperm nucleus at the micropylar end and the zygote
X	degenerated egg and secondary nucleus
Y	degenerated egg cell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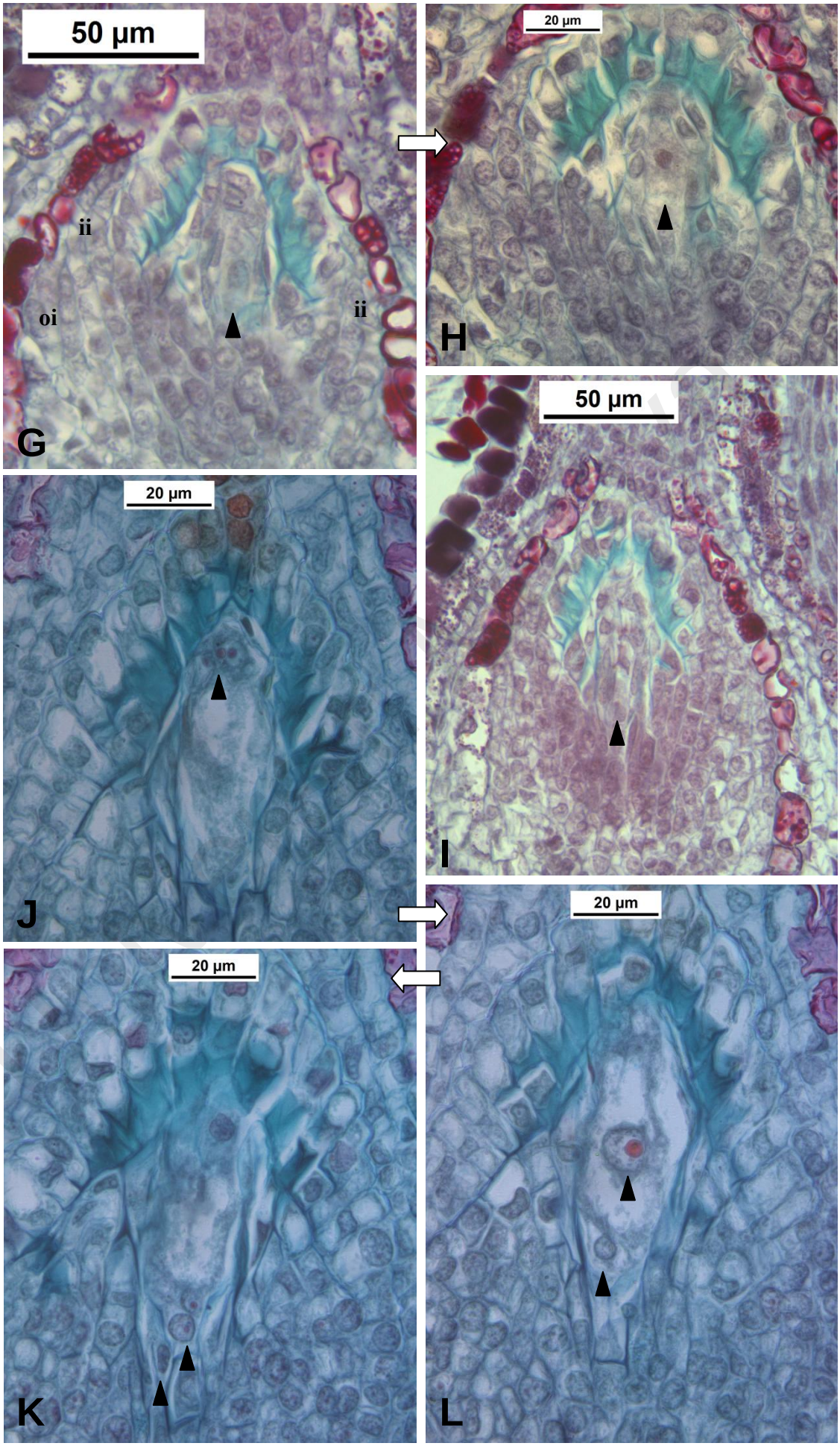
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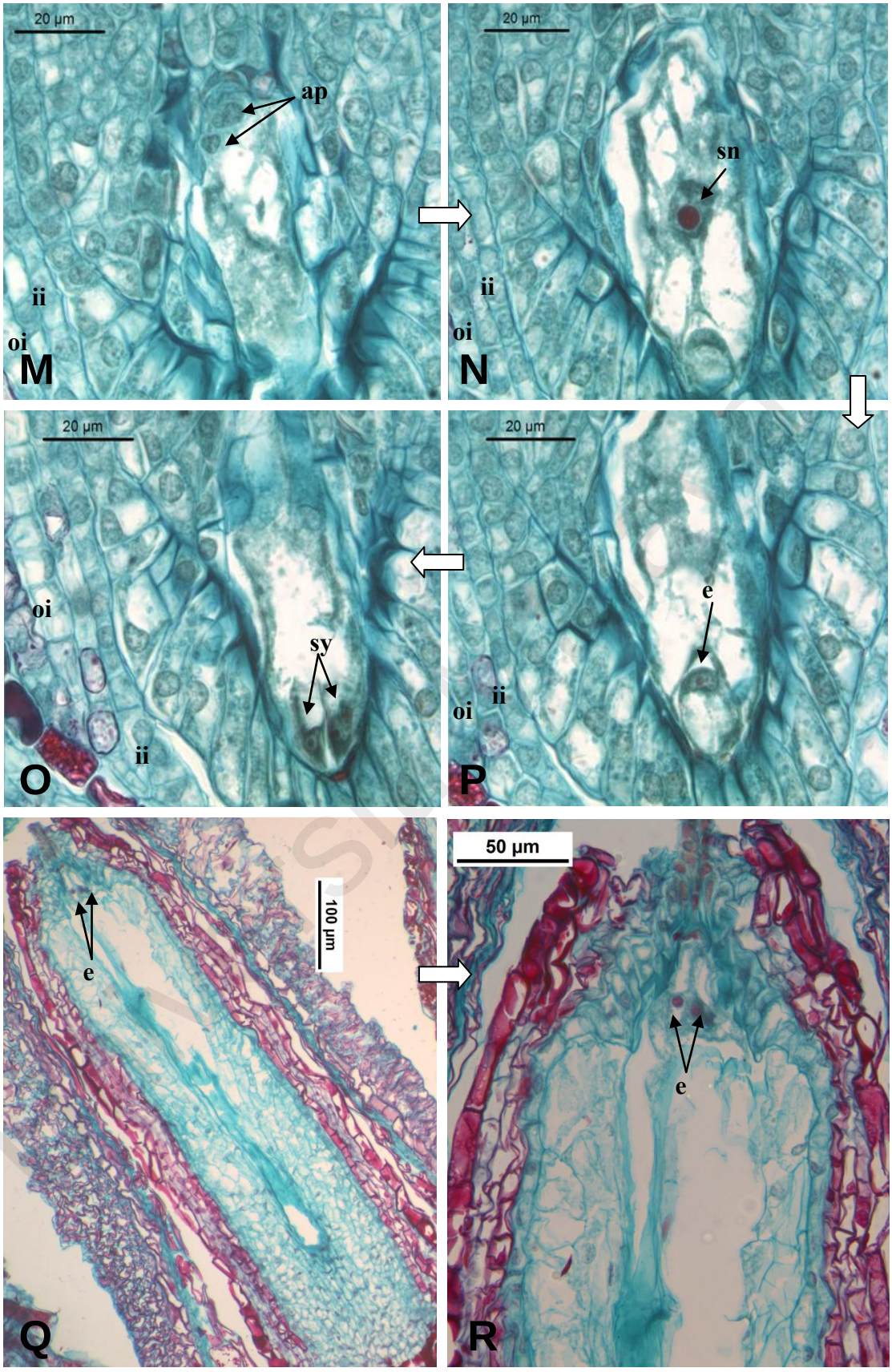
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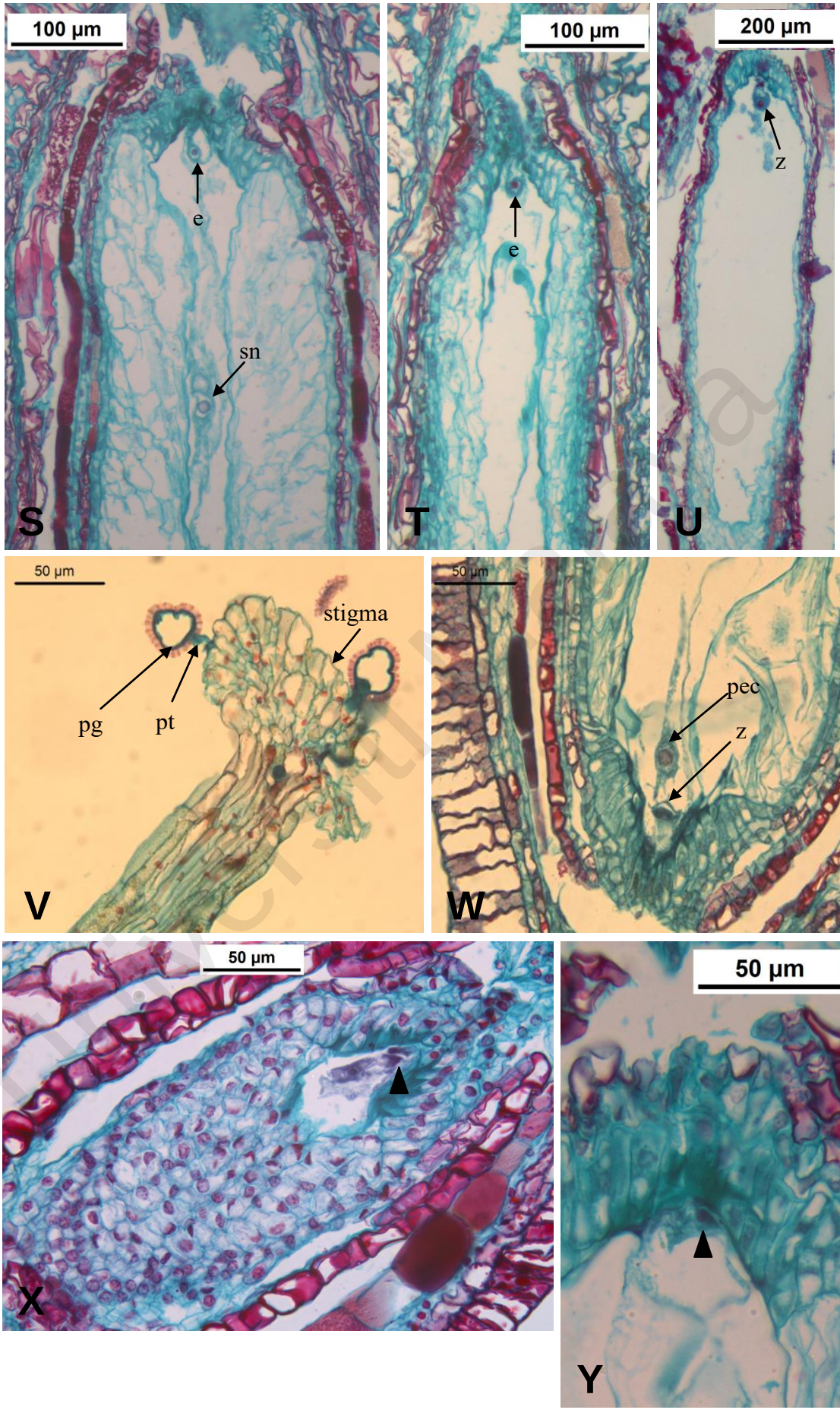
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3.3.3.3 Endosperm and embryo

Soon after fertilization, the primary endosperm nucleus divides to form free nuclei endosperm and subsequently the nuclei divide repeatedly to produce more free nuclei to fill the enlarging embryo sac (Figures 3.14 C, E–I). The development of the endosperm follows the *ab initio* Nuclear type (Davis, 1966). As the number of endosperm nuclei increases, the free nuclei are found in the periphery of the embryo sac enclosing a central vacuole (Figure 3.14 H); the nuclei also tend to concentrate around the micropylar and chalazal region (Figure 3.14 O).

The zygote is clavate and undergoes a short resting period while the primary endosperm nucleus has started to divide. The division of the zygote is transverse giving rise to a basal cell (cb) and a terminal cell (ca) (Figure 3.14 D). Subsequently, the dyad undergoes further divisions to form a proembryo (Figure 3.14 G) followed by a young globular embryo (Figures 3.14 I–J & L) with a 2–3 celled suspensor. Later, the young globular embryo continues to divide forming a large globular embryo (Figures 3.14 K & M) which further develops into a heart-shaped embryo (Figures 3.14 P–Q). The heart-shaped embryo gives rise to a mature, slightly curved dicotyledonous embryo (Figures 3.14 R & T). The endosperm is a food storage tissue which persists until the seed germinates.

At the globular embryo stage, the nuclear endosperm turns cellular from the micropylar region towards the chalazal end, finally filling three quarters of the embryo sac at the late heart-shaped stage with cellular tissue, leaving a small region, less than 10% at the chalazal end with some free nuclei (Figures 3.14 J–K & O). These cells become homogenous only after the embryo reaches the heart-shaped embryo stage (Figures 3.14 P–Q). The seeds are endospermous and starchy (Figure 3.14 R).

Initially, both the integuments are two layered (Figures 3.12 C–E). As the primary endosperm cell develops, the one-layered cells of the inner integument divide anticlinally to form the ruminate endosperm (Figure 3.14 N).

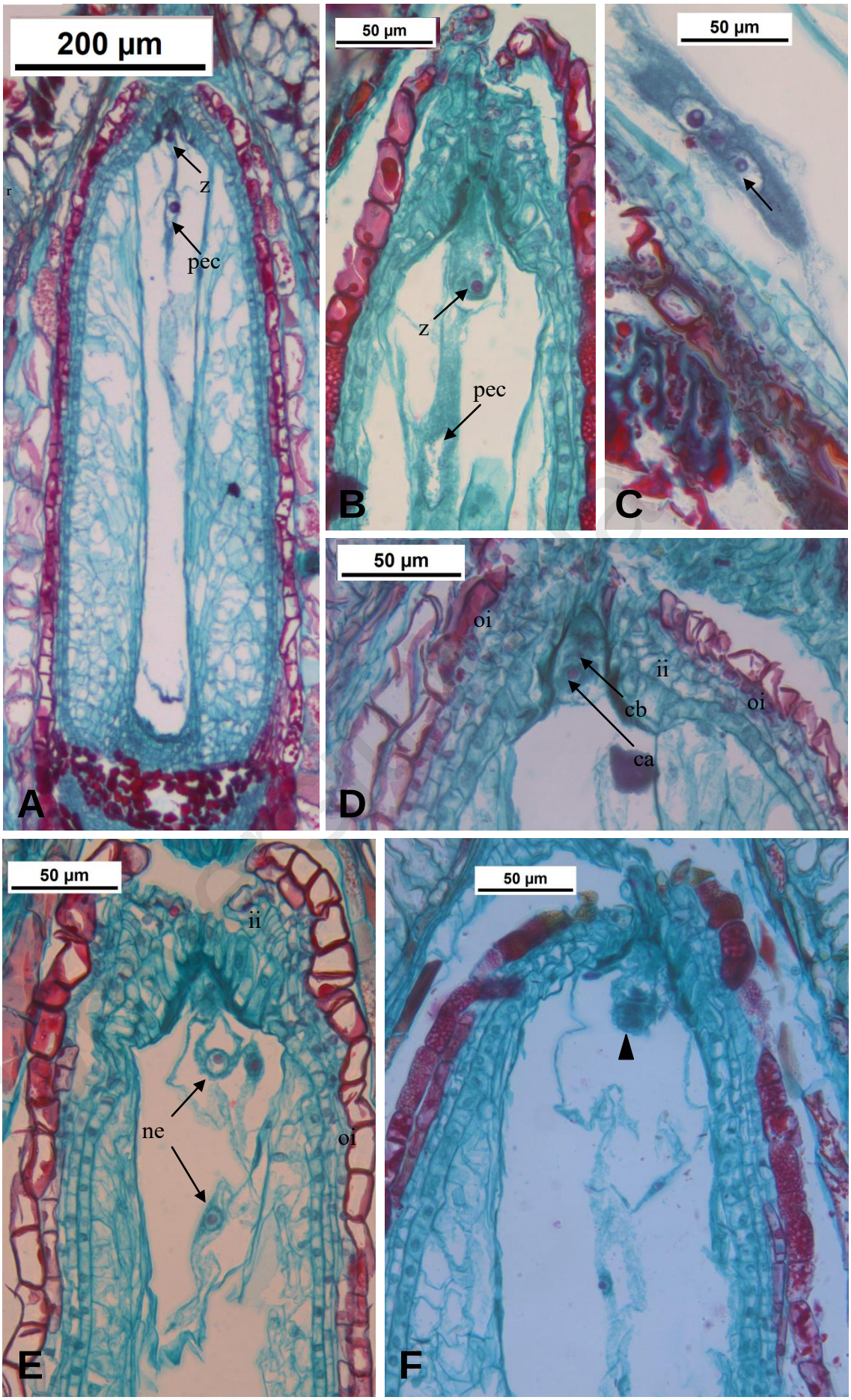
The lowland cultivated variety has a very low percentage of fruit set and a high percentage of flower abortion. Hence, the endosperm and embryogeny could not be studied because no samples had been collected. The sections also showed a very high frequency of embryo sac degeneration (Figures 3.15 A–B) and egg degeneration (Figures 3.13 X–Y).

Figure 3.14 A–T highland wild variety of *P. chinensis* var. *chinensis*, endosperm and embryo

(ca, terminal cell; cb, basal cell; ce, cellular endosperm; ii, inner integument; pec, primary endosperm cell; oi, outer integument; ne, nuclear endosperm; re, ruminate endosperm; z, zygote;)

A–S longitudinal sections of seeds

A	embryo sac with the zygote and primary endosperm cell
B	zygote with dense cytoplasm
C	nuclear endosperm
D	dyad showing a basal cell (cb) and a terminal cell (ca)
E	endosperm nuclei
F	5-celled proembryo
G	2–3 celled suspensor of embryo
H–I	young globular embryo with the nuclear endosperm (at different magnifications)
J	globular embryo with nuclear endosperm moving toward chalazal end
K	late globular embryo with nuclear endosperm developing into cellular endosperm
L	young globular embryo
M	late globular embryo
N	slightly ruminate endosperm
O	cellular endosperm filling the periphery, micropylar and chalazal end of the embryo sac
P	heart-shaped embryo and cellular endosperm
Q	close-up of the heart-shaped embryo
R	dicotyledonous embryo
S	degenerated embryo sac
T	slightly curved, dicotyledonous embryo (from fresh specimen taken under dissecting microscope)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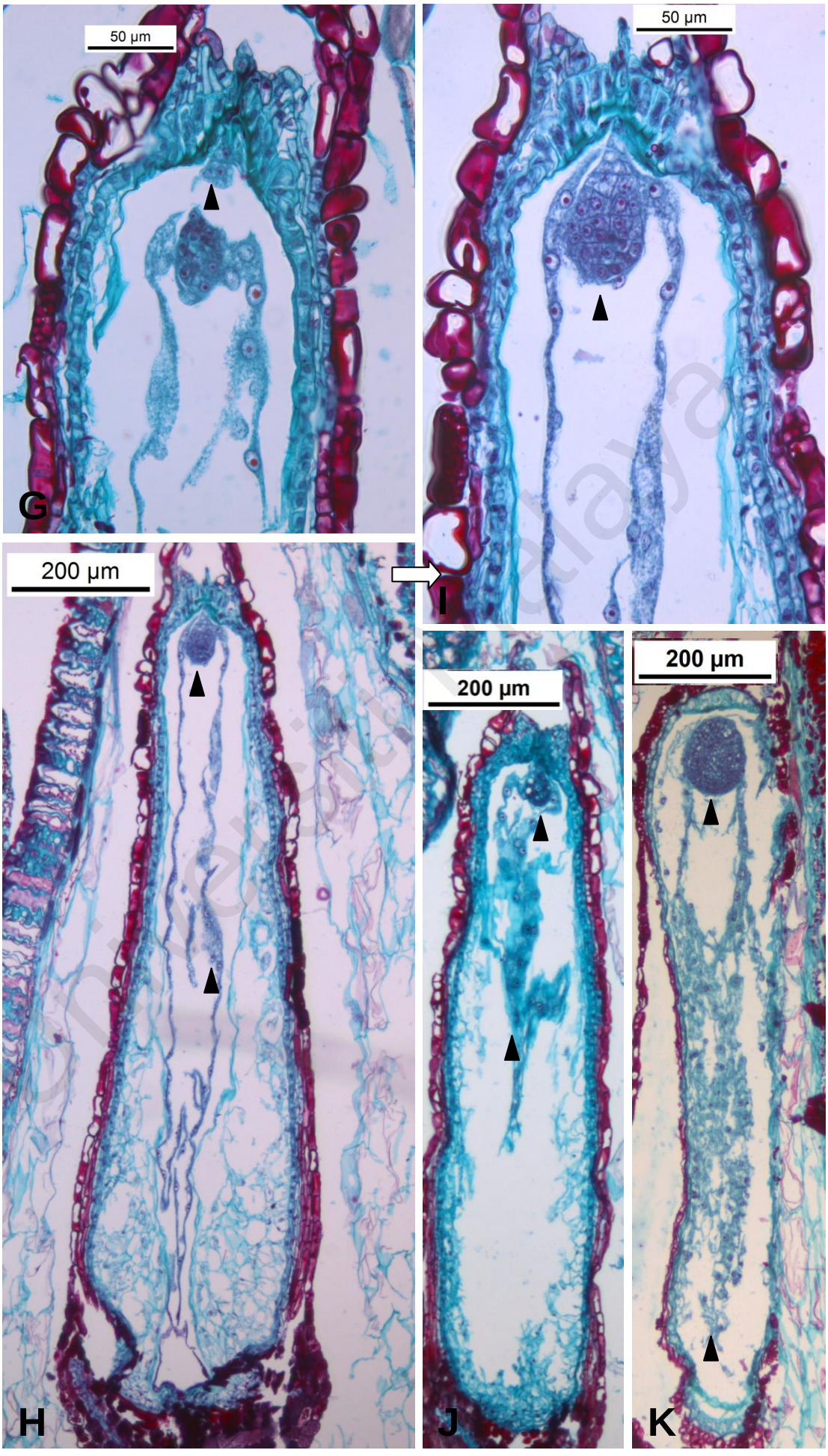
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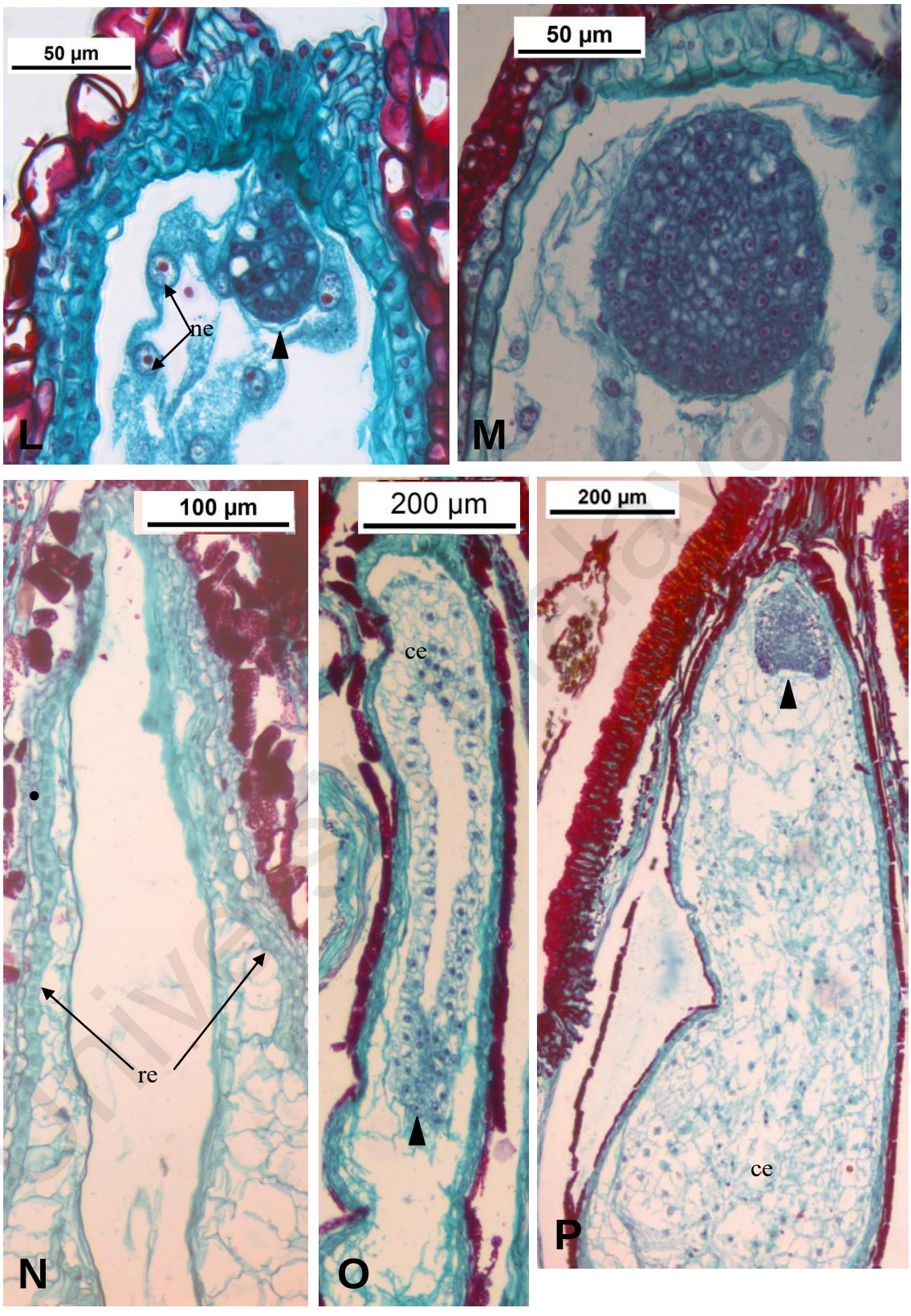
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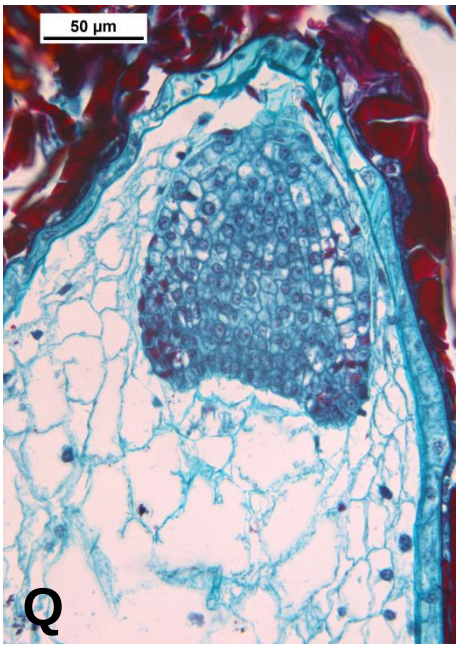
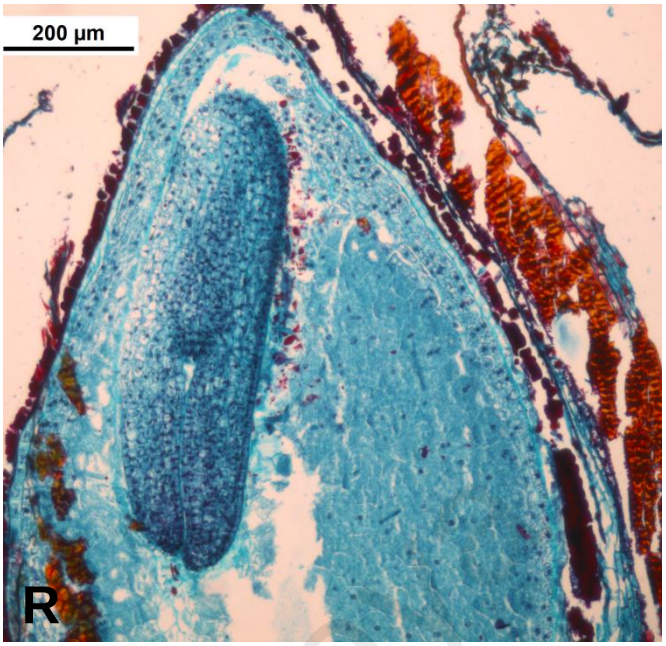
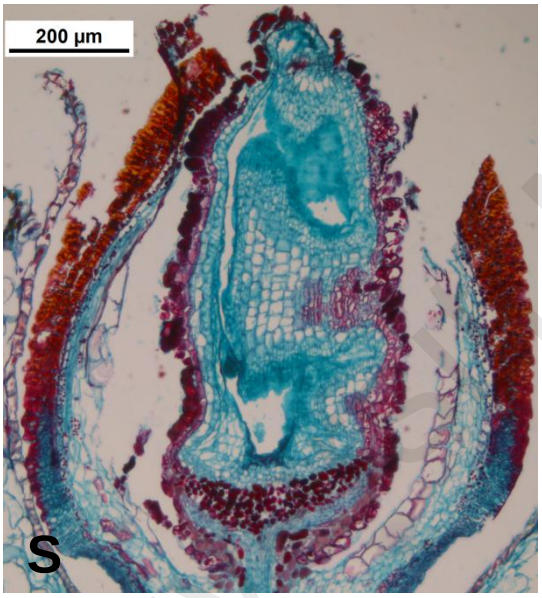






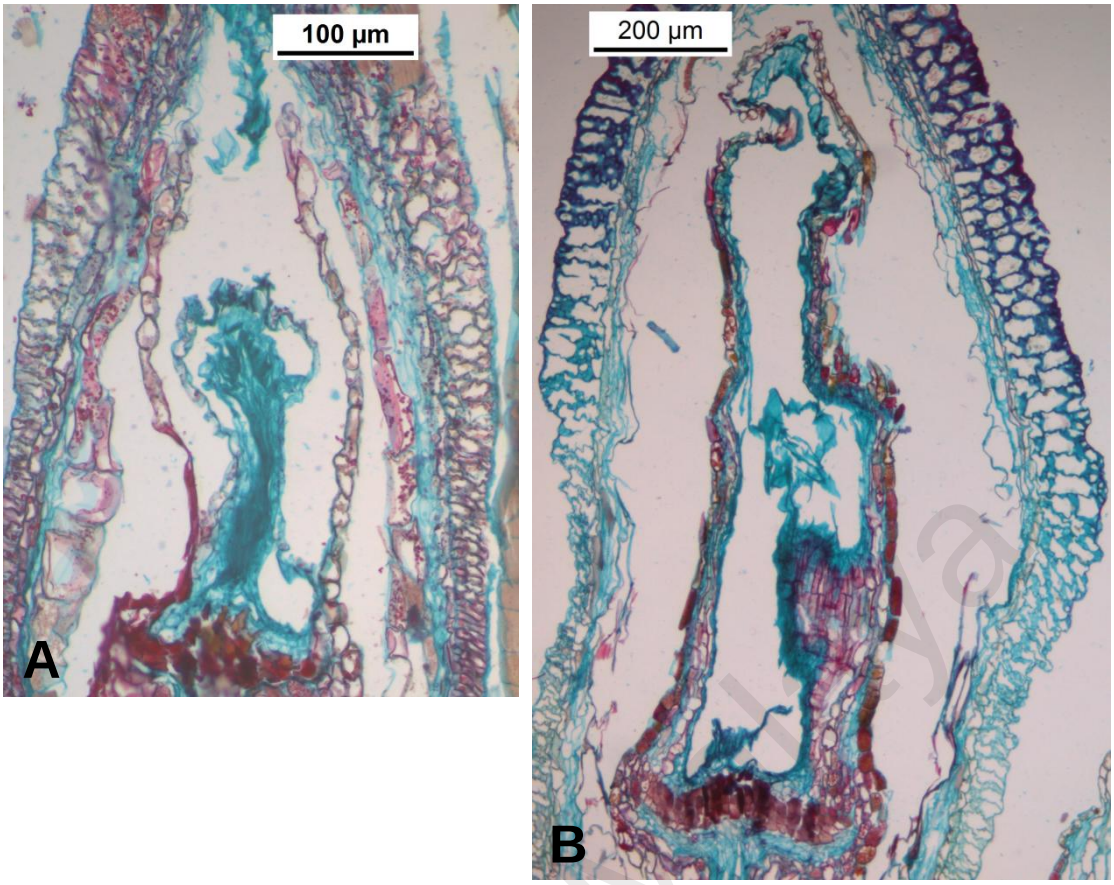


Figure 3.15 A–B Lowland cultivated variety of *P. chinensis* var. *chinensis*, endosperm and embryo

A–B longitudinal sections of seeds

A degenerated embryo sac

B degenerated endosperm

3.3.4 Pollen morphology in heterostylous variety of *P. chinensis* var. *chinensis*

Heterostyly has been clearly observed in the highland populations of *Persicaria chinensis* var. *chinensis*. However, in the lowland population, only thrum flowers have been observed and pin flowers have not been detected in any of the samples examined. As pin flowers were not seen in the lowland population, student's t-test using R statistical software was used to compare the thrum and pin flowers of the highland populations and between the thrum flowers of the highland and lowland populations.

Anther and stigma are reciprocally positioned in the pin and thrum flowers. The average length of style, size of stigma, length of distinct stamen and epipetalous stamen as well as the average number of the pollen grains in an anther is listed in Tables 3.8 & 3.9 (also see Figure 3.16) (Appendix E). All the data are normally distributed (except the data for the number of pollen grains per anther in the lowland population) showing significant differences at $P < 0.001$ level. In the highland populations, the pin flowers have taller style (2.41 mm±0.12) compared to the thrum flowers (1.08 mm±0.06), but the pin stigma were less thick (0.140 mm±0.011) and less wide (0.236 mm±0.018) than those of the thrum flowers (thickness of stigma was 0.172 mm±0.015, and the width of stigma was 0.262 mm±0.015). The average lengths of the filaments of the pin flowers were also shorter (length of filament from distinct stamen was 1.60 mm±0.03, epipetalous stamen was 1.03 mm±0.04) than the thrum-eyed flowers (length of filament from distinct stamen was 2.13 mm±0.08, epipetalous stamen was 1.59 mm±0.07). The number of pollen grains estimated in 8 anthers of a pin flower was 1212±91 (the number of pollen grains in one anther = 151±11) whereas the number of pollen grains in a thrum flower was lower than that of the pin flower (793±54, the number of pollen grains in an anther = 99±7). Between the lowland and highland thrum flowers, the lowland population had the shorter style (0.88 mm±0.02) but wider and thicker stigmas (0.262 mm±0.015 and 0.172 mm±0.015 respectively) with taller stamens (length of

filament, distinct stamen was 2.63 mm±0.10, epipetalous stamen was 2.10 mm±0.09). As the number of pollen grains per anther in the lowland thrum flower did not show a normal distribution, no comparison could be made between the thrum flowers in the highland and lowland population.

The pollen grains were radially symmetrical. Capitata type pollen grains were observed in the highland and lowland varieties (Yasmin *et al*., 2010). Pollen grains of the two morphs of *P. chinensis* var. *chinensis* and the lowland variety differed significantly only in size but similar in shape based on the classification of Erdtman (Erdtman, 1954) and Hesse *et al*. classification (Hesse *et al*., 2009), i.e. the pollen grains were oblate, suboblate to oblate speroidal, with reticulate tectal surface, thick, narrow muri and free clavae in the lumina. Pin-eyed flowers produced significantly smaller pollen grains in significantly larger quantities (average number of pollen grains in one flower = 1212±91) than that of the thrum-eyed flowers (average number of pollen grains in one flower = 793±54).

Pollen grains of *P.chinensis* var. *chinensis*, both cultivated and wild varieties, are small and tricolpate with reticulate exine. They are oblate spheroidal (thrum flowers of both the highland and lowland varieties) to suboblate (pin flowers of the highland variety) according to Erdtman's classification (Erdtman, 1954). However, all the pollen grains in these three samples are described as oblate according to Hesse *et al*. (2009), in which the polar axis is shorter than the equatorial diameter. The average pollen P/E ratio was 0.87 in the pin-eyed flower and 0.88 in the thrum-eyed flowers of the highland variety. The average pollen P/E ratio in the cultivated lowland variety was the highest among these three samples, i.e. 0.93, with the polar length and equatorial diameter measuring 44.76 µm ± 1.96, 41.00 µm (44.76) 49.20 µm and 48.18 ± 2.10, 43.64 µm (48.18) 51.75 µm respectively.

In the highland variety, the polar length of the pin flower pollen grains was 36.23 µm ± 1.02, 34.23 µm (36.23) 37.93 µm, while that of the thrum flower was 40.87 µm ± 1.40, 36.04 µm (40.87) 42.86 µm; the equatorial diameter of the pin flower pollen grains was 41.44 µm ± 0.85, 39.33 µm (41.44) 44.00 µm, while that of the thrum flower was 46.37 µm ± 0.95, 44.73 µm (46.37) 48.98 µm.

Under the light microscope, current study shows no significant differences in the pollen tectal surface; all the pollen grains appeared reticulate (Figures 3.17, 3.18 & 3.19). When observed under the SEM, the exine ornamentation clearly showed the network like pattern formed by the exine elements and the lumina between the muri were wider than 1 µm in these three samples (Figures 3.17 G, 3.18 G & 3.19 G). The exine ornamentation of the pollen between the two morphs of the highland varieties was similar. However in the lowland thrum pollen, the lumina were less wide than the highland thrum pollen and the muri of the lowland thrum pollen were thicker, shorter than the highland thrum pollen (compare Figure 3.18.G and Figure 3.19.G).

When observed under the SEM, the morphology of stigmatic papillae in both pin and thrum flowers are similar in *P. chinensis* var. chinensis, showing no significant differences (Figure 3.20). The papillate stigmas of the thrum flower of the highland and lowland varieties were also quite similar. The stigmatic papillae of the lowland variety were slightly flattened than the highland variety but they looked quite similar (Figure 3.20).

Table 3.8: Comparative morphology of the heterostylous flowers and pollen in the highland variety of *P. chinensis* var. *chinensis*.

Floral characteristics	Highland *P. chinensis*		n	P
	pin flower	thrum flower		
mean length of style (mm)	2.41±0.12	1.08±0.06	30	***
mean thickness of stigma (mm)	0.140±0.011	0.172±0.015	20	***
mean width of stigma (mm)	0.236±0.018	0.262±0.015	20	***
mean length of filament, distinct stamen (mm)	1.60±0.03	2.13±0.08	20	***
mean length of filament, epipetalous stamen (mm)	1.03±0.04	1.59±0.07	20	***
mean number of pollen grains in one anther	151±11	99±7	20	***
mean pollen polar length (P) (µm)	36.23±1.02	40.87±1.40	40	***
mean pollen equatorial diameter (E) (µm)	41.44±0.85	46.37±0.95	40	***
P/E ratio	0.87	0.88	-	-
pollen aperture type	3-colpate	3-colpate	-	-
pollen tectal surface	reticulate	reticulate	-	-
pollen ornamentation	thick, narrow muri & free clavae in the deep lumina	thick, narrow muri & free clavae in the deep lumina	-	-
pollen shape (Erdtman's classification)	suboblate	oblate spheroidal	-	-
pollen shape (Hesse *et al*. classification)	oblate	oblate	-	-

Difference between means were analyzed by Students' t-test (two tails)
*P< 0.05, ** P< 0.01, ***P< 0.001

Table 3.9: Comparative morphology of the thrum flowers and their pollen in the highland and lowland varieties of *P. chinensis* var. *chinensis*.

Floral characteristics	Highland *P. chinensis* thrum flower	Lowland *P.chinensis* thrum flower	n	P
mean length of style (mm)	1.08±0.06	0.88±0.02	30	***
mean thickness of stigma (mm)	0.172±0.015	0.180±0.013	20	***
mean width of stigma (mm)	0.262±0.015	0.283±0.015	20	***
mean length of filament, distinct stamen (mm)	2.13±0.08	2.63±0.10	20	***
mean length of filament, epipetalous stamen (mm)	1.59±0.07	2.10±0.09	20	***
mean number of pollen grains in one anther	99±7	64±33	20	-
mean pollen polar length (P) (µm)	40.87±1.40	44.76±1.96	40	***
mean pollen equatorial diameter (E) (µm)	46.37±0.95	48.18±2.10	40	***
P/E ratio	0.88	0.93	-	-
pollen aperture type	3-colpate	3-colpate	-	-
pollen tectal surface	reticulate	reticulate	-	-
pollen ornamentation	thick, narrow muri & free clavae in the deep lumina	Thicker, less narrow and shorter muri & less wider lumina with free clavea	-	-
pollen shape, (Erdtman's classification)	oblate spheroidal	oblate spheroidal	-	-
pollen shape, (Hesse *et al*. classification)	oblate	oblate	-	-

Difference between means were analyzed by Students' t-test (two tails)

* P< 0.05, ** P< 0.01, ***P< 0.001

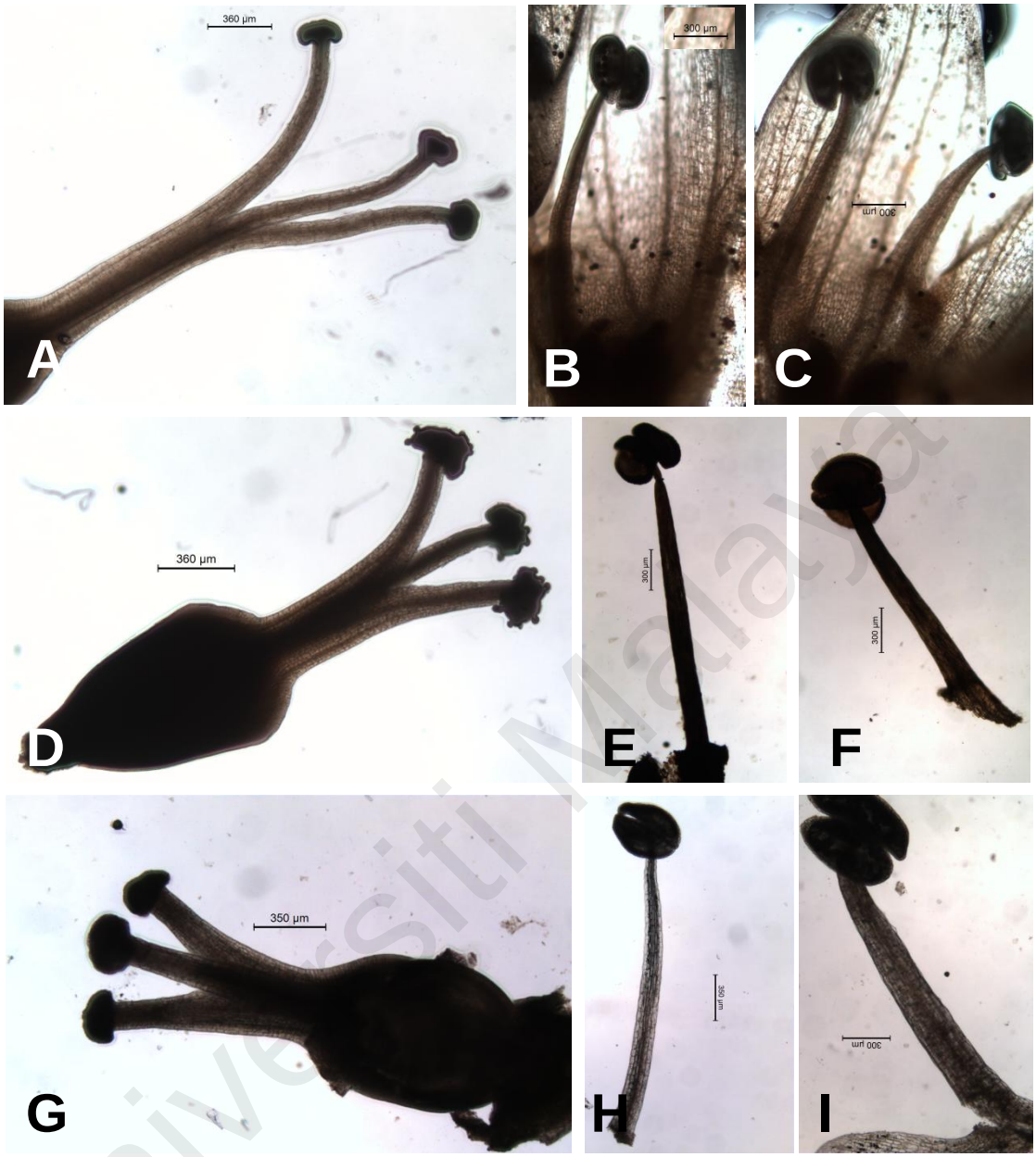


Figure 3.16: Stigmas, style and stamens of the pin flowers (A–C), thrum flowers (D–F) of the highland variety and the thrum flowers of the lowland variety (G–I) of *P. chinensis* var. *chinensis*. A, D & G: stigmas and styles; B, E & H: free and distinct anthers; C, F & I: epipetalous anthers.

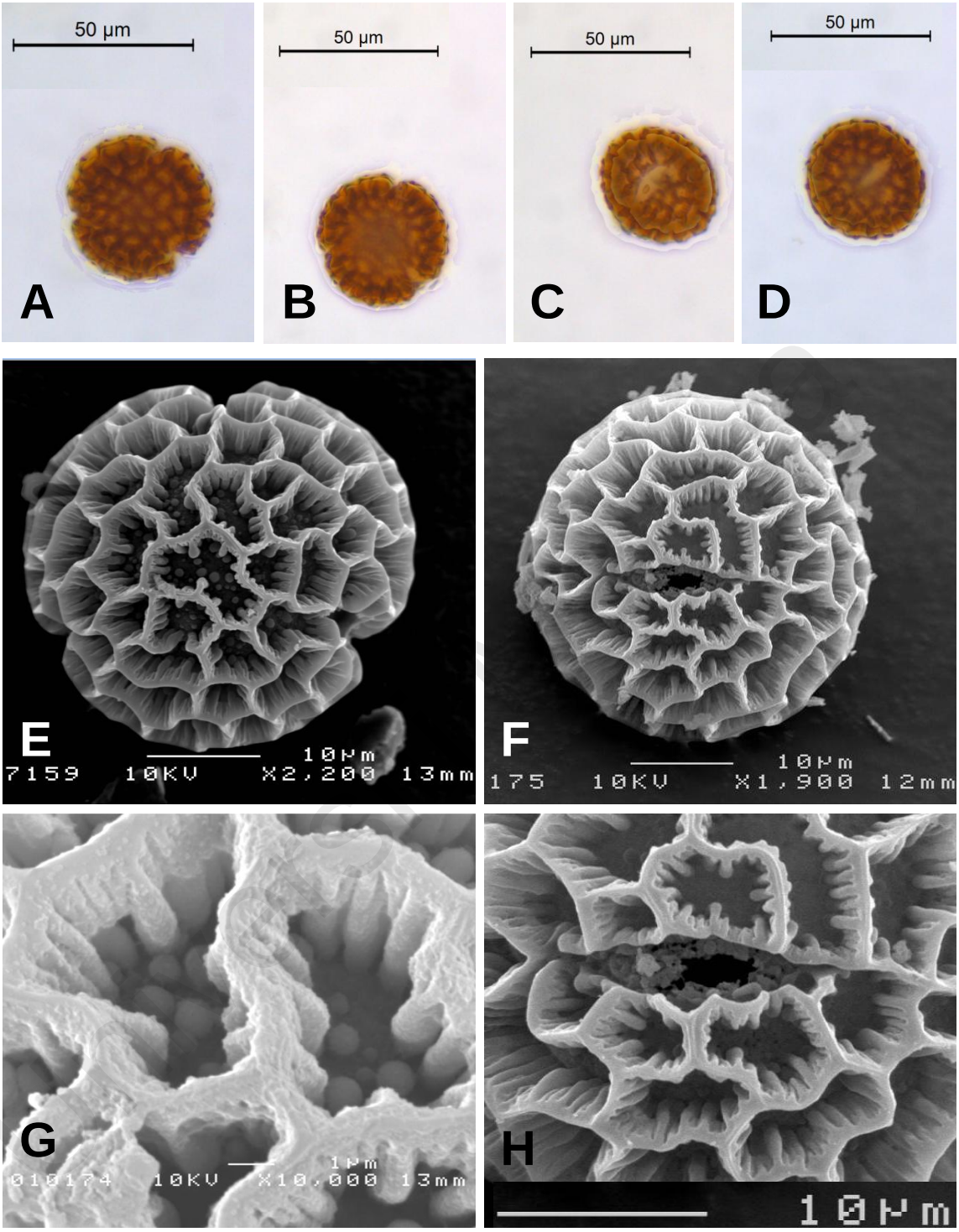


Figure 3.17: Photomicrographs of pollen grains of pin flowers in the highland variety of *P. chinensis* var. *chinensis* from Gunung Ulu Kali, Bentong, Pahang. A–D: Photomicrographs taken under the light microscope. A & B: polar view; C & D: equatorial view; E–H: Photomicrographs taken under SEM; E: polar view; F: equatorial view; G: reticulate exine with thick walls, narrow muri and deep lumina with free clavea; H: colpus of pollen grain

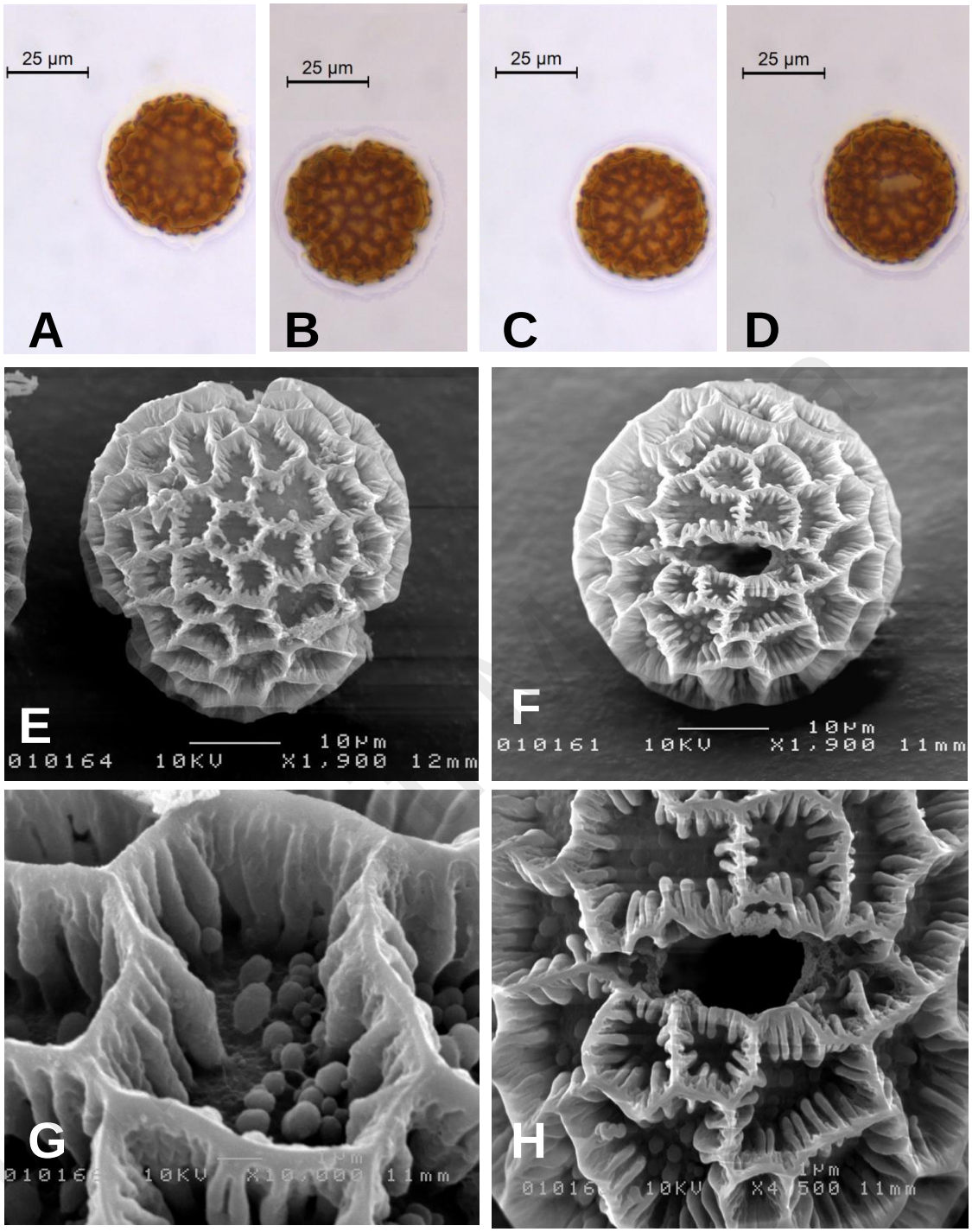


Figure 3.18: Photomicrographs of pollen grains of thrum flowers in the highland variety of *P. chinensis* var. *chinensis* from Gunung Ulu Kali, Bentong, Pahang. A–D: Photomicrographs taken under the light microscope. A & B: polar view; C & D: equatorial view; E–H: Photomicrographs taken under SEM; E: polar view; F: equatorial view; G: reticulate exine with thick walls, narrow muri and deep lumina with free clavea; H: colpus of pollen grain

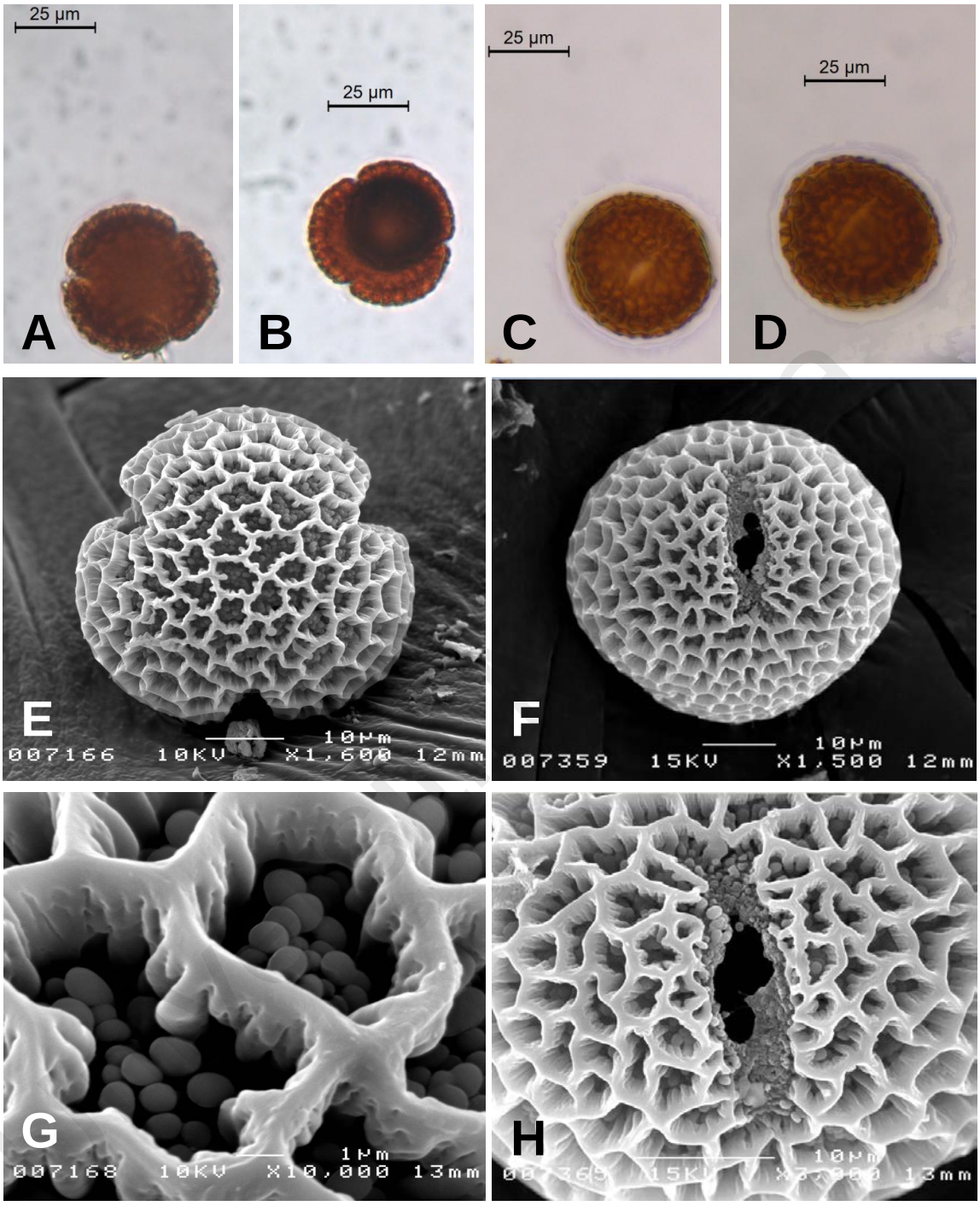


Figure 3.19: Photomicrographs of pollen grains of thrum flowers in the lowland variety of *P. chinensis* var. *chinensis* from Sagil, Tangkak, Johor. A–D: Photomicrographs taken under the light microscope. A & B: polar view; C & D: equatorial view; E–H: Photomicrographs taken under SEM; E: polar view; F: equatorial view; G: reticulate exine with thicker, less narrow and shorter muri & less wider lumina (Compare to highland thrum variety) with free clavea; H: colpus of pollen grain

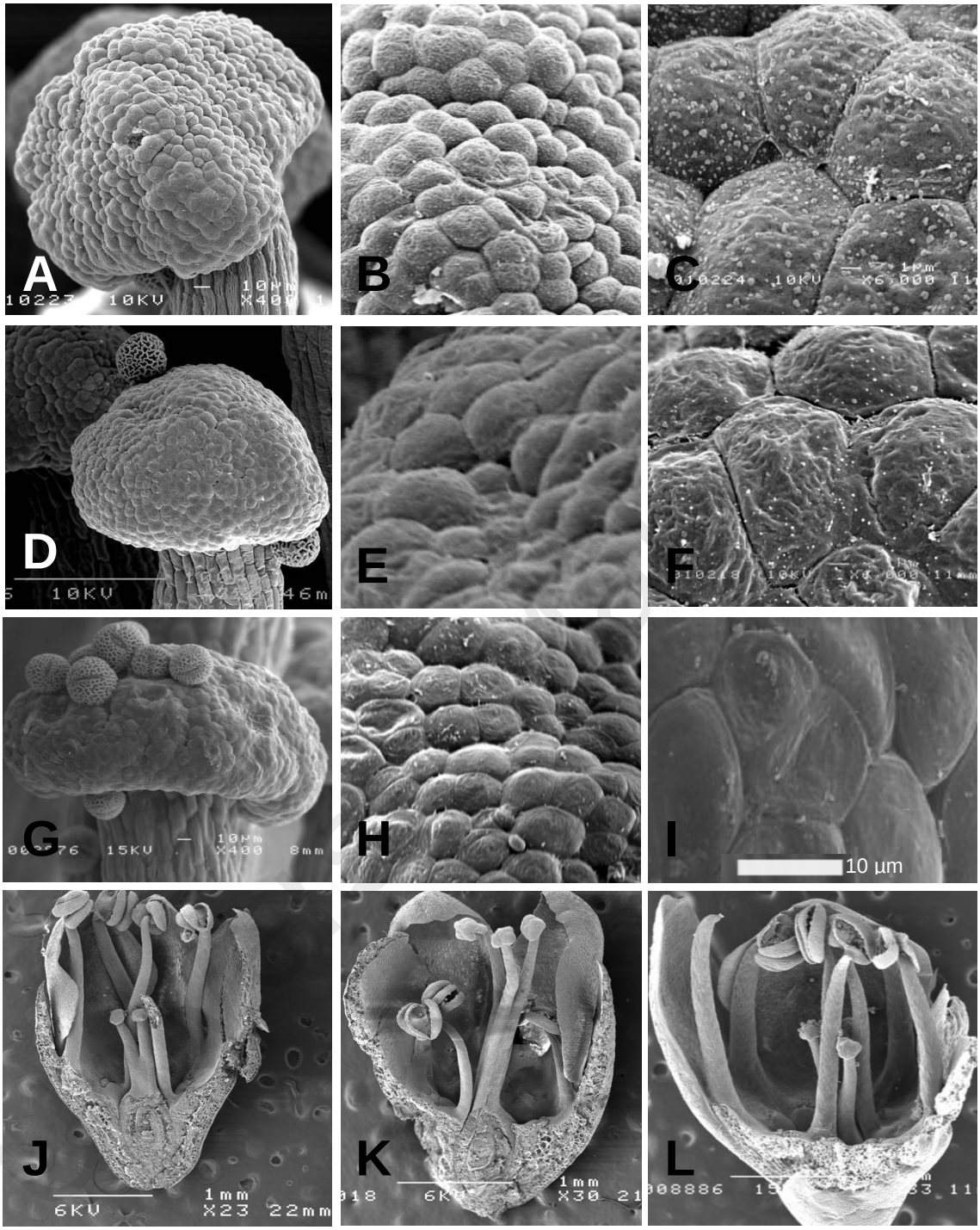


Figure 3.20: Stigma morphology of the pin flowers (A–C), thrum flowers (D–F) of the highland variety and thrum flowers of the lowland variety (G–I) of *P. chinensis* var. *chinensis*. A, D & G: Stigmas; B, E & H: stigmatic papillae; C, F & I: close up of the stigmatic receptive surface; J: thrum flower of the highland variety; K: Pin flower of the highland variety; L: thrum flower of the lowland variety.

3.3.5 Pollen viability and pollen germination test

Pollen viability and germination tests were carried out prior to pollination experiments in order to determine pollen fertility. Pollen viability tests showed that more than 80% of the pollen grains of the highland populations of *P. chinensis* var. *chinensis* were viable; they stained pink in 0.1% 2,3,5-triphenyl tetrazolium chloride solution (TTZ) (Figure 3.21 A1). However, only less than 10% of the pollen grains of the lowland variety stained pink in TTZ solution (Figure 3.21 A2).

Table 3.10 shows pollen germination in different media. Table 3.11 shows the pollen germination of highland populations of *P. chinensis* var. *chinensis* in various sucrose solutions with 'complex medium' (After Adhikari and Campbell, 1998) and 'modified medium' after 5 hours of incubation. Figure 3.24 shows the percentage of pollen germination of the highland variety of *P. chinensis* var. *chinensis* in various sucrose concentrations in the presence of the 'complex medium' and 'modified medium'.

Preliminary *in vitro* germination experiments in different sucrose concentrations, i.e. 4, 6, 8, 10, 12, 15 and 18% revealed that all the pollen grains burst and their cytoplasm exuded through the pores (Figure 3.21 B). Subsequently, 0.01g boric acid and 0.01 g boric acid together with 0.03 g calcium nitrate tetrahydrate respectively, were added to different sucrose solutions, i.e. 4, 6, 8, 10, 12, 15 and 18%, to repeat the pollen germination experiments. Again, all the pollen grains failed to germinate and bursting of pollen grains were observed. The *in vitro* pollen germination experiments failed even with the addition of 0.04 g boric acid, 0.2 g calcium nitrate, 0.2 g manganese sulphate and 0.2 g potassium nitrate to the complex media (i.e. different sucrose concentrations).

By following the method from Adhikari and Campbell (1998), a 'complex medium' with 0.2 g each of $MnSO_4.1H_2O$, $Ca(NO_3)_2.4H_2O$, and KNO_3 dissolved in

100 ml of distilled water with the addition of 0.04g H_3CO, 15 g sucrose and 30 g polyethylene glycol (molecular weight 20000) was prepared. Approximately 21% of the pollen grains germinated at 15% sucrose concentration and 19% of the pollen grains germinated in 12% sucrose and only 12% germinated in 18% sucrose solution. Although the percentage of germination was not as high as in the 15% sucrose with 30% of PEG in 100ml of 0.04g boric acid, but the pollen tubes grew as long as 525 µm (Figure 3.21 D). Some pollen grains produced double tubes of approximately 250 µm in length (Figure 3.21 E) and occasionally, branching of the pollen tubes was also observed (Figure 3.21 F). Experiments using lower sucrose concentrations (i.e. 6, 8, 10%) with the addition of 30% PEG, 'complex medium' and 0.04 g H_3CO were also tested, but the percentage of germination was extremely low (Table 3.11).

A 'modified method' from Adhikari and Campbell (1998), using 100 ml sucrose solution at different concentrations (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.04 g boric acid, and 30 g polyethylene glycol (PEG with molecular weight 20000) successfully germinated the pollen grains after 1 hour (Figure 3.21 C). This satisfactory medium was developed with different concentrations of sucrose (10 to 18%) and polyethylene glycol (20% to 35%) (Figures 3.22 & 3.23). 15% sucrose with 30% of PEG dissolved in 100 ml distilled water with additional of 0.04 g boric acid resulted in the highest percentage of pollen germination; approximately 50% of the pollen grains germinated in this medium solution. 42% of the pollen grains germinated in 18% sucrose with 30% of PEG dissolved in 100 ml of distilled water with additional of 0.04 g boric acid and only 23% of the pollen grains germinated in 12% sucrose with 30% of PEG dissolved in 100 ml distilled water with additional of 0.04 g boric acid (Figure 3.22). Similarly, the effect of PEG concentration was also demonstrated in pollen grain germination experiments. 20% PEG with optimum sucrose solution in 100 ml with 0.04 g boric acid showed only 5% germination. 13% of the pollen grains germinated in 25%

of PEG with 15% of sucrose dissolved in 100 ml distilled water with additional of 0.04 g boric acid and only 18% of the pollen grains germinated in 35% of PEG with optimum sucrose (15%) dissolved in 100 ml distilled water with additional of 0.04 g boric acid (Figure 3.23). However, the pollen tubes in this satisfactory medium (15% sucrose with 30% of PEG in 100 ml of 0.04 g boric acid) did not exceed 280 μm and the average length of the pollen tube was 140 μm.

The maximum pollen viability and germination were observed from the pollen grains of freshly opened flowers within 3 hours of anthesis. After 24 hours, less than 8% of the pollen grains remained viable from the inflorescences placed in water flasks and stored in air-conditioned room at 19ºC. Almost all the pollen lost their viability after 40 hours under the same storage conditions. However, after 24 hours, pollen grains from inflorescences placed in water flasks and kept at room temperature (about 28ºC), completely lost their viability.

The satisfactory media was also tested on the lowland cultivated population, but no pollen grains germinated under this *in vitro* pollen germination method.

The *in vivo* pollen germination test was done by removing the stigmas together with the style from the open flowers and placed on a microscope slide with a drop of lactophenol (Merck). Pollen grains were found to stain blue and germinated pollen tubes were observed in the highland populations of *P. chinensis* var. *chinensis* (Figure 3.25). In the lowland population, some pollen grains stained blue but some pollen grains only stained slightly blue. The size of the pollen grains was not uniform in the lowland population; some were bigger sized than the others (Figure 3.26). However, no pollen grain of the lowland population germinated in the *in vivo* pollen germination test.

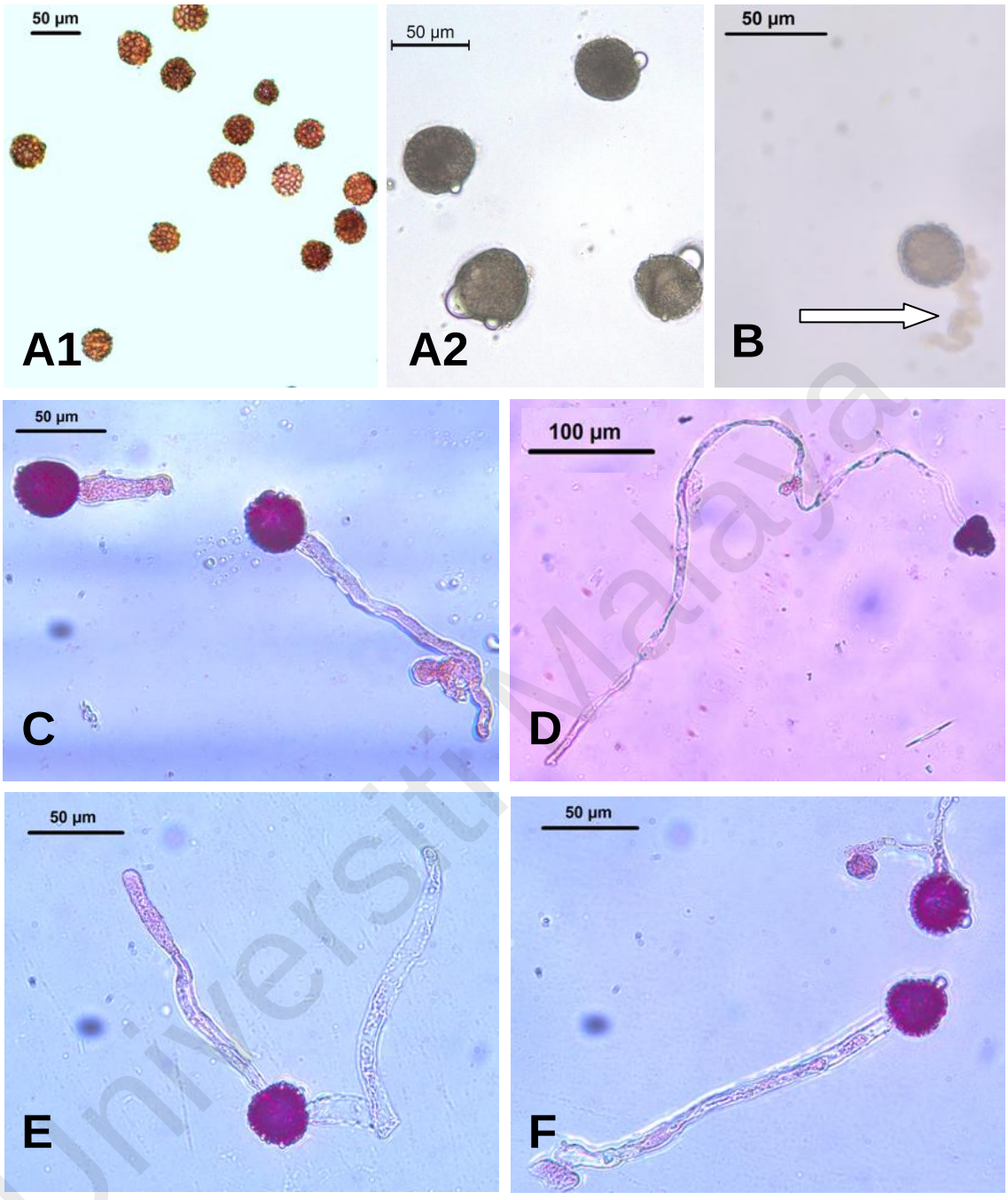


Figure 3.21: *In vitro* pollen viability and pollen germination of highland and lowland varieties of *P. chinensis* var. *chinensis*. A1: Pollen grains of the highland variety stained pink in 0.1% TTZ chloride solution; A2: Pollen grains of the lowland variety did not stain pink in TTZ; B: Bursting of pollen grain, arrow showing the exudation of starch and cytoplasm; C: Pollen grains germinated in the medium of 15% sucrose and 30% of PEG dissolved in 100ml distilled water with additional of 0.04g boric acid; D: Pollen grain in the 'complex medium', with the pollen tube growing as long as 525 µm; E: Pollen grain producing double tubes of approximately 250 µm; F: Pollen grain producing branched pollen tube

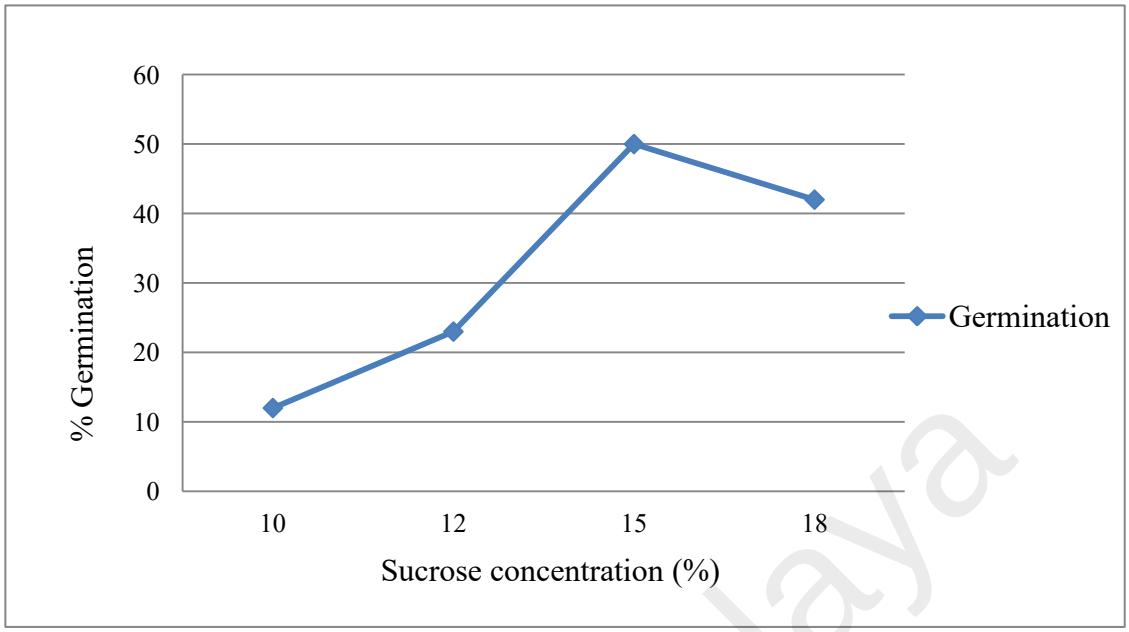


Figure 3.22: Pollen germination of the highland variety of *P. chinensis* var. *chinensis* in different sucrose concentrations supplemented with 30% of PEG (20,000) and 0.04% of boric acid.

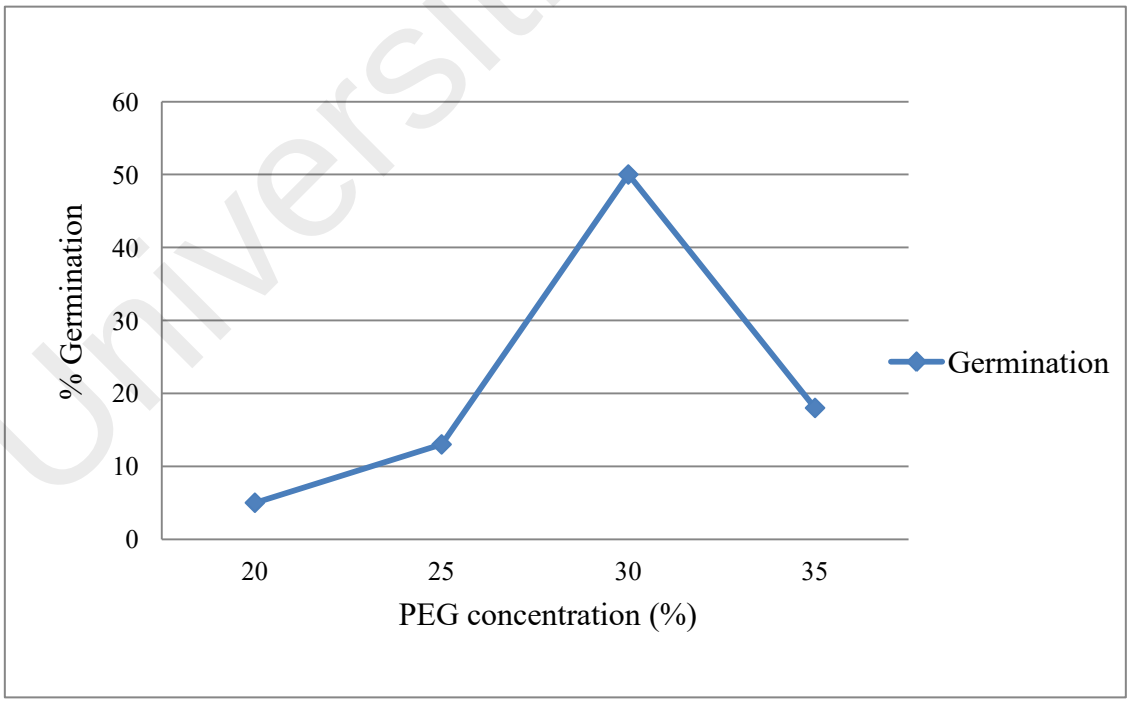


Figure 3.23: Pollen germination of the highland variety of *P. chinensis* var. *chinensis* in different concentrations of PEG (20,000) supplemented with 15% of sucrose and 0.04% of boric acid.

Table 3.10: Pollen germination test in different media.

Medium	Results
sucrose solution at different concentrations (i.e. 4, 6, 8, 10, 12, 15 and 18%)	no germination
100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.01 g boric acid	no germination
100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.01 g H_3CO_3 and 0.03 g $Ca(NO_3)_2.4H_2O$	no germination
100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.04 g H_3CO_3; 0.2 g $Ca(NO_3)_2.4H_2O$ and 0.2 g $MnSO_4.1H_2O$	no germination
100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.04 g H_3CO_3; 0.2 g $Ca(NO_3)_2.4H_2O$ and 0.2 g $MnSO_4.1H_2O$; 0.2 g potassium nitrate, KNO_3.	no germination
100 ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.04 g boric acid; 0.2 g $Ca(NO_3)_2.4H_2O$; 0.2 g $MnSO_4.1H_2O$; 0.2 g KNO_3 and 30 g polyethylene glycol (PEG) with molecular weight 20000 (complex medium, after Adhikari and Campbell, 1998)	Pollen grains germinated and reached maximum germination with 21% germinated in 15% sucrose. Pollen tubes grew as long as 525 µm, produced double tubes and branching of the pollen tubes was also observed. The average length of the pollen tube was 350 µm.
100ml sucrose solution (i.e. 4, 6, 8, 10, 12, 15 and 18%) with 0.04g H_3CO_3 and 30 g polyethylene glycol with molecular weight 20000, PEG (modified medium)	Pollen grains germinated and reached maximum germination with 50% germinated in 15% sucrose. The average length of the pollen tube was 140 µm.

Table 3.11: Pollen germination of highland variety of *P. chinensis* var. *chinensis* in various sucrose solutions with 'complex medium' (After Adhikari and Campbell, 1998) and 'modified medium' (After 5 hours of germination).

Sucrose concent-ration	Added chemical/nutrient	Total number of pollen grains	number of pollen grains germinated	number of pollen grains not germinated	Germi-nation (%)	Average Germina-tion (%)	Average length of pollen tubes
Distilled water	Complex Medium	108	0	108	0	0	-
Distilled water	Modified Medium	120	0	120	0	0	
4%	Complex Medium	123	0	123	0	0	-
	Modified Medium	108	0	108	0	0	
6%	Complex Medium	93	0	93	0	0	-
	Modified Medium	124	0	124	0	0	
8%	Complex Medium	142	1	141	1	1	-
	Modified Medium	137	4	133	3	3	
10%	Complex Medium	102	4	98	4	4	-
	Modified Medium	130	15	115	12	12	
12%	Complex Medium	129	25	104	19	19	280 µm
		85	15	70	18		
		107	23	84	21		
12%	Modified Medium	94	23	71	24	23	120 µm
		77	17	60	22		
		103	24	79	23		
15%	Complex Medium	143	27	116	19	21	350 µm, can reach 525 µm
		75	15	60	20		
		84	19	65	23		
15%	Modified Medium	113	55	58	49	50	140 µm
		107	58	49	54		
		127	59	68	46		
18%	Complex Medium	170	19	151	11	12	320 µm
		134	17	117	13		
		125	14	111	11		
18%	Modified Medium	148	61	87	41	42	100 µm
		101	40	61	40		
		86	38	48	44		

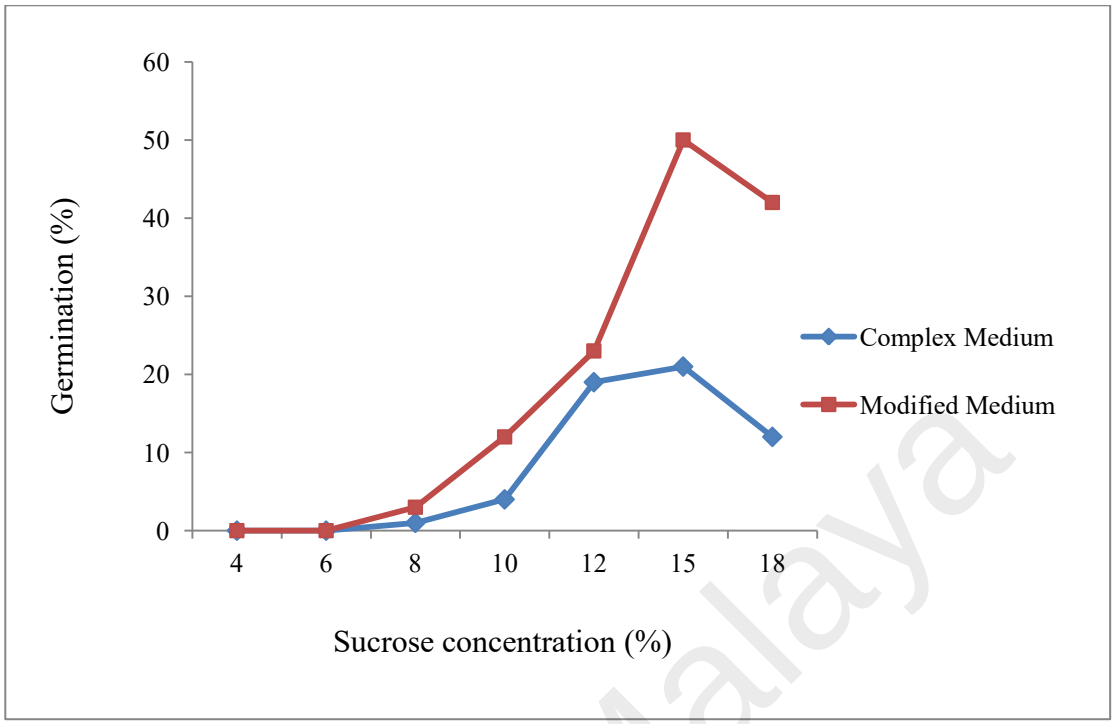


Figure 3.24: Percentage of pollen germination of the highland variety of *P. chinensis* var. *chinensis* in various sucrose concentrations in the presence of the 'complex medium' (After Adhikari and Campbell, 1998) and 'modified medium'

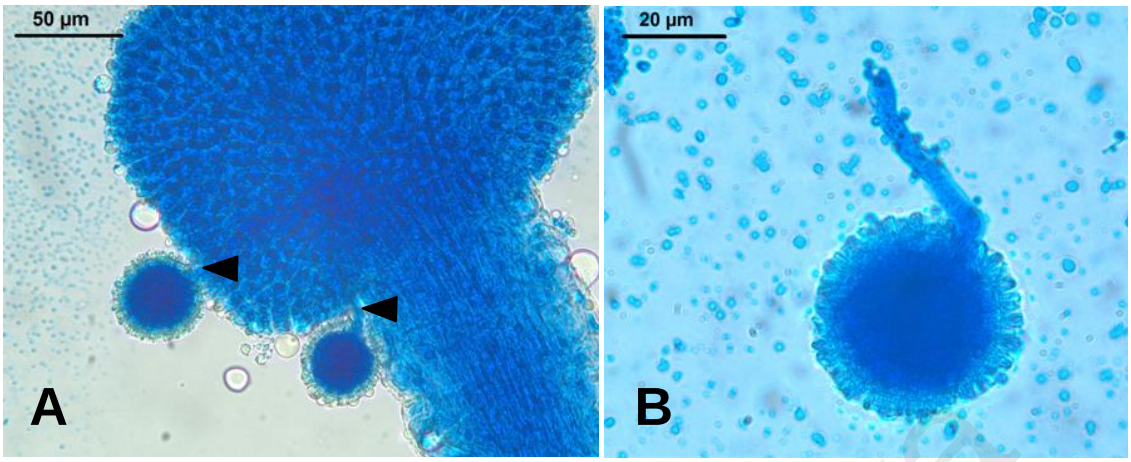


Figure 3.25: *In vivo* pollen germination of the highland variety of *P. chinensis* var. *chinensis*. A: Stigma and pollen grains stained blue by lactophenol and arrows show pollen tubes penetrating the stigma. B: Germinated pollen grain.

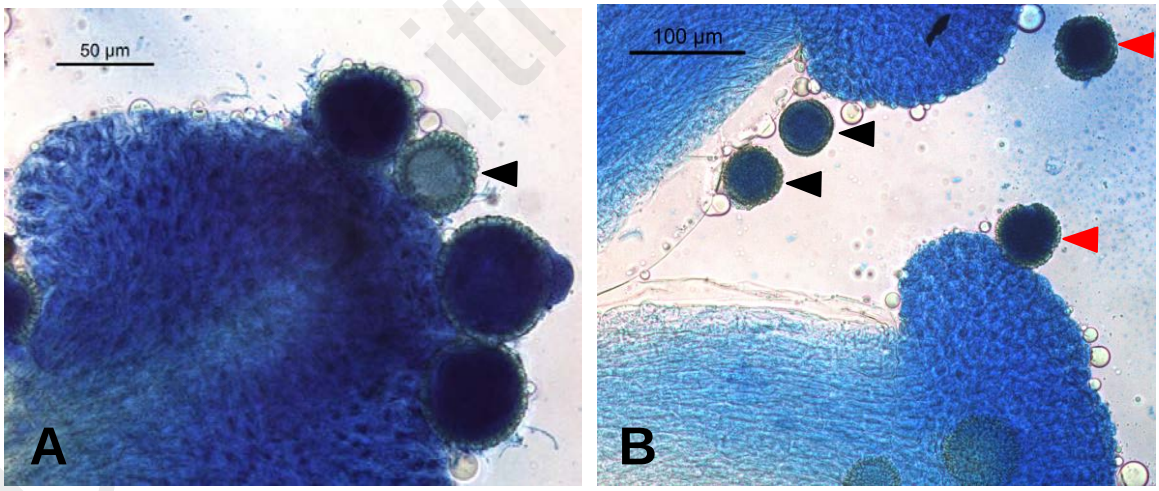


Figure 3.26: *In vivo* pollen germination of the lowland variety of *P. chinensis* var. *chinensis*. A: Stigma and pollen grains of different sizes stained blue by lactophenol and one of the pollen grains slightly stained blue and has translucent cytoplasm. B: black arrows show the pollen grains slightly stained blue and red arrows shows the pollen grains completely stained blue. A&B: No pollen grains germinated.

3.3.6 Pollination experiments and pollen-ovule ratio

3.3.6.1 Open pollination and bagging experiment

The percentage of fruit set of open pollination and bagging experiments (self pollination) of the different populations of *P. chinensis* var. *chinensis* is tabulated in Table 3.12 (Appendix F).

Pollination experiments showed that the highland variety had a higher percentage of fruit set from open pollination as compared to the bagging experiment. The average fruit sets of open pollination and bagging experiment were 47.5% and 16.9% respectively. Fruits were obtained in bagging experiment indicating that *P. chinensis* was self-compatible even though the percentage of fruit set was low, i.e. 16.9%.

Open pollination was more successful as compared to self pollination and it produced three times more fruits than self pollination. Thus, open pollination shows a xenogamous breeding system for this highland wild population; indicating cross-compatibility and outbreeding in this species. The pollen-ovule ratio obtained from the experiment (3.3.6.2: pollen-ovule ratio) also suggested that the breeding system was facultative xenogamy. Moreover, pollinating agents were observed during the data collection period and this will be further discussed in 3.3.7.

Table 3.12: Result of pollination experiments in the highland variety of *P. chinensis* var. *chinensis*

Test	Treatment	Population	No. of inflorescences tagged	No. of open flowers	No. of flowers set fruit		Fruit set (%)
Open pollination	Untreated, not bagged	H2	24	1745	786		45.0
		H3	16	1347	630		46.8
		H5	14	1111	582		52.4
		Total	54	4203	1998	Mean	47.5
bagging experiment (self pollination)	Untreated, bagged	H2	6	305	46		15.1
		H3	6	269	45		16.7
		H5	13	755	142		18.8
		Total	25	1329	233	Mean	16.9

3.3.6.2 Pollen-ovule ratio

In the highland populations of *P. chinensis* var. *chinensis*, 24 anthers were randomly selected from different flowers and examined under a dissecting microscope (Leica Zoom 2000). Each anther was carefully dissected and placed in a concave slide with a drop of distilled water mixed with detergent. An anther had a mean of 151±12 pollen grains (n anther = 8); therefore a flower with eight anthers was estimated to have a total of 1212 pollen grains. Since this flower has only one orthotropous ovule, the pollen-ovule ratio was estimated to be 1212±91. According to Cruden's (1977) classification, based on the pollen-ovule ratio, the breeding system was facultative xenogamy.

On the other hand, in the lowland population, 15 anthers were randomly selected from different inflorescences. Three anthers had been found to have no pollen grains and these were staminodes. Subsequently, all the 8 anthers in one flower were checked (all the anthers in three flowers from different inflorescences were examined). No staminode anthers were found but the pollen grains in the 8 anthers of a single flower did not show normal distribution i.e. two to three anthers in one flower had 12–35 pollen grains and the rest of the anthers had 66–101 pollen grains (number of anther = 8). The pollen grains in all the anthers of a flower did not show normal distribution and the result obtained would show a high variation. An anther has a mean of 64±33 pollen grains (n anther = 39); therefore, the pollen-ovule ratio was approximately 510±263. However, the standard deviation showed a high variation from the mean owing to an abnormal distribution of the pollen grains in every anther that had been examined. According to Cruden's (1977) classification, the breeding system was also facultative xenogamy in the lowland cultivated variety.

3.3.7 Possible pollinating agents and pests

Flower visitors to the highland populations of *P*. *chinensis* var. *chinensis* are diverse, consisting of a wide array of Hymenoptera and Diptera along with a lesser number of Lepidoptera and Coleoptera (Table 3.13; Figure 3.27). The most common visitors are from the order Hymenoptera and Diptera, including the family of Formicidae, Apidae, and Syrphidae. Ants were the most abundant visitors followed by the bees of the Apidae and Syrphidae families. Besides ants and bees, tiny thrips (thunderbugs) from the Thysanoptera were also found visiting the flowers and they were very common inside the flowers. Thrips are unlikely effective pollinators and are more commonly known as pest to the plant as they feed on the pollen grains.

Apis cerana and *Trigona* sp. are well known pollinators. *A*. *cerana* and *Trigona* sp. were seen to land on the flowers and insert their heads and forelegs into the flowers (Figures 3.27 A–D). Both species were always seen to visit the flowers when the flowers were blooming. They remained for about 5–10 seconds in a flower. *Trigona* is observed to be scavengers of pollen grains from the dehisced anthers (Figure 3.27 D). *A*. *cerana* collects pollen grains through adherence to its body while it collects nectar. However, abundant ants were seen to visit the flowers before and after flower anthesis. Ants from the family Formicidae are very common flower visitors but the ants are always considered as villains in floral interaction.

The peak hours of activity of these insects were from 0700–1000 hours wtih some insects from the family Pyralidae appearing right after flower anthesis (normally from 0600–0700 hours). Moths from the Lepidoptera family which function as pollinators usually land on the inflorescence and insert their proboscis repeatedly into the base of the flower to suck nectar. While feeding, its proboscis, thorax, abdomen and legs come into close contact with the flower, thus effecting pollination. Bees come out after flower anthesis and only visit fresh blooming flowers. The production of abundant nectar and strong fragrance emitting from the flowers attracted insect pollinators and

flower visitors or nectar thieves such as ants and thrips. Stick insects (Figure 3.27 K) have also been seen and it is believed that they are not pollinators.

Bees have been observed to land on the flowers during anthesis. The foraging behaviour of the bees was observed. The landing position of the bees on the flower varies with the bee's size. The stingless bee, *Trigona* sp. has a smaller body size and it alights on top of a flower. Most of its ventral body surface comes into contact with the mouth of the flower even though the flower is quite small (Figure 3.27 D). Other larger species like *Apis cerana* land on the compact inflorescence and feed on the open flowers while the rest of their body and legs are in contact with other flowers (Figures 3.27 A–C). The bees most probably gather nectar as food and they were never seen to selectively forage for pollen alone. While the bee is foraging for nectar in the flowers, pollen adhere onto its body and the bee begins to comb its body with its legs to gather the trapped pollen and pack them into a small yellowish pellet in the corbiculae (the pollen basket). When the bee lands on another flower, its mouth, leg, thorax and abdomen come into close contact with the surface of the stigma, enabling the pollen grains to be transferred from its body to the papillate stigmatic surface.

The highland population has also attracted larvae of many insects (Table 3.14; Figure 3.28). Pests are from the order Lepidoptera, mainly from the family Lymantriidae, Pyralidae and Geometridae. Lymantriidae has some serious pest species (Figures 3.28 E–G), which are usually hairy in appearance and they feed on the flower buds. This pest would eventually destroy the whole inflorescence by feeding on them (Figure 3.28 E). The pyralid moth from the family Pyralidae was observed to eat the leaves of the highland populations of *P. chinensis* var. *chinensis* (Figures 3.28 D & J). This is also a common pest. The caterpillar of the looper moth has a characteristic looping movement (Figure 3.28 B) and was observed to feed on the inflorescence.

Table 3.13: List of insect visitors to the highland variety of *P. chinensis* var. *chinensis*.

Order: Hymenoptera

Family: Apidae (bees family)

Species: *Apis cerana* Fabr., *Apis* sp., & *Trigona* sp.

Family: Vespidae (wasp family)

Family: Formicidae (ant family)

Order: Diptera

Family: Syrphidae (hover fly family)

Order: Lepidoptera

Family: Pyralidae (moth family)

Order: Coleoptera

Family: Lycidae (beetle family)

Order: Hemiptera

Family: Pyrrhocoridae (bug family)

Order: Thysanoptera

Family: Thripidae

Genus: *Thrips* sp.

Order: Phasmatodea

Family: Heteronemiidae (stick insect family)

Table 3.14: List of pests to the highland variety of *P. chinensis* var. *chinensis*.

Order: Lepidoptera

Family: Geometridae, Pyralidae & Lymantriidae

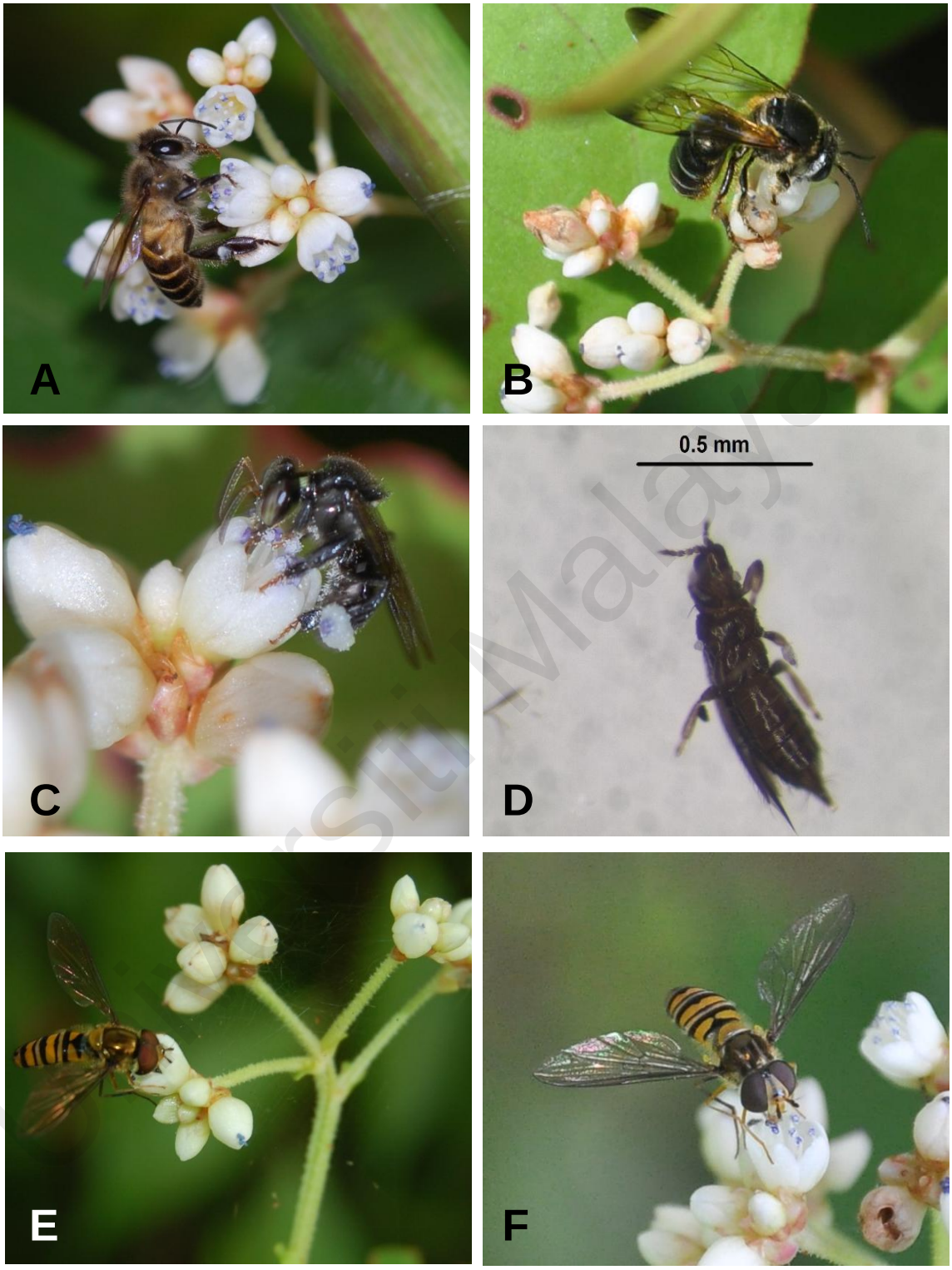
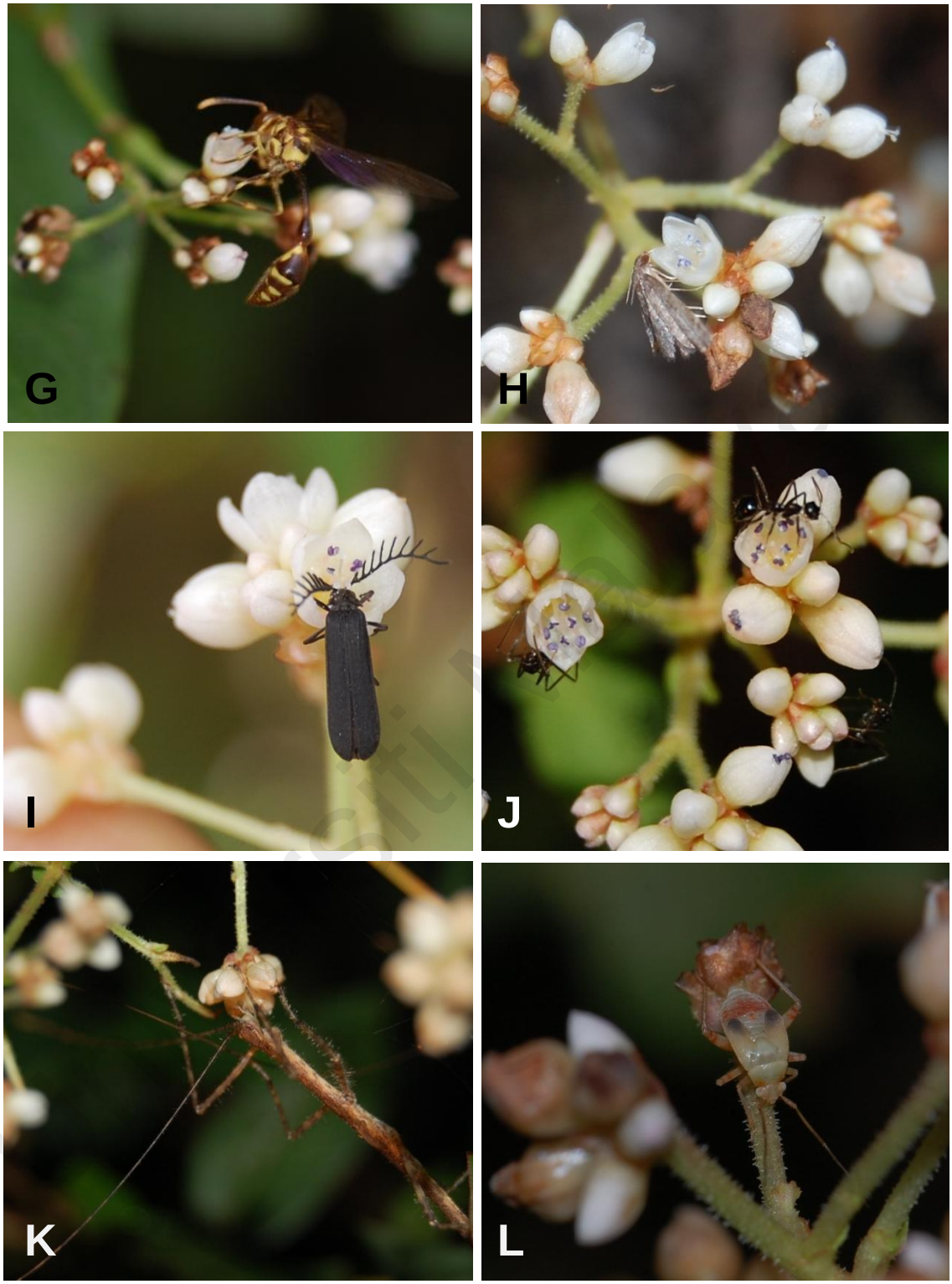


Figure 3.27: Flower visitors of the highland variety of *P. chinensis* var. *chinensis*. A: *Apis cerana* (Apidae); B: Bee from family Apidae; C: *Trigona* sp. (Apidae); D: *Thrips* sp. (Thripidae); E & F: hover fly from family Syrphidae.



Cont. Figure 3.27: Flower visitors of the highland variety of *P. chinensis* var. *chinensis*. G: Wasp from family Vespidae; H: Moth from family Pyralidae; I: Beetle from family Lycidae; J: Ants from family Formicidae; K: Stick insect from family Heteronemiidae; L: Nymph of a bug from family Pyrrhocoridae.

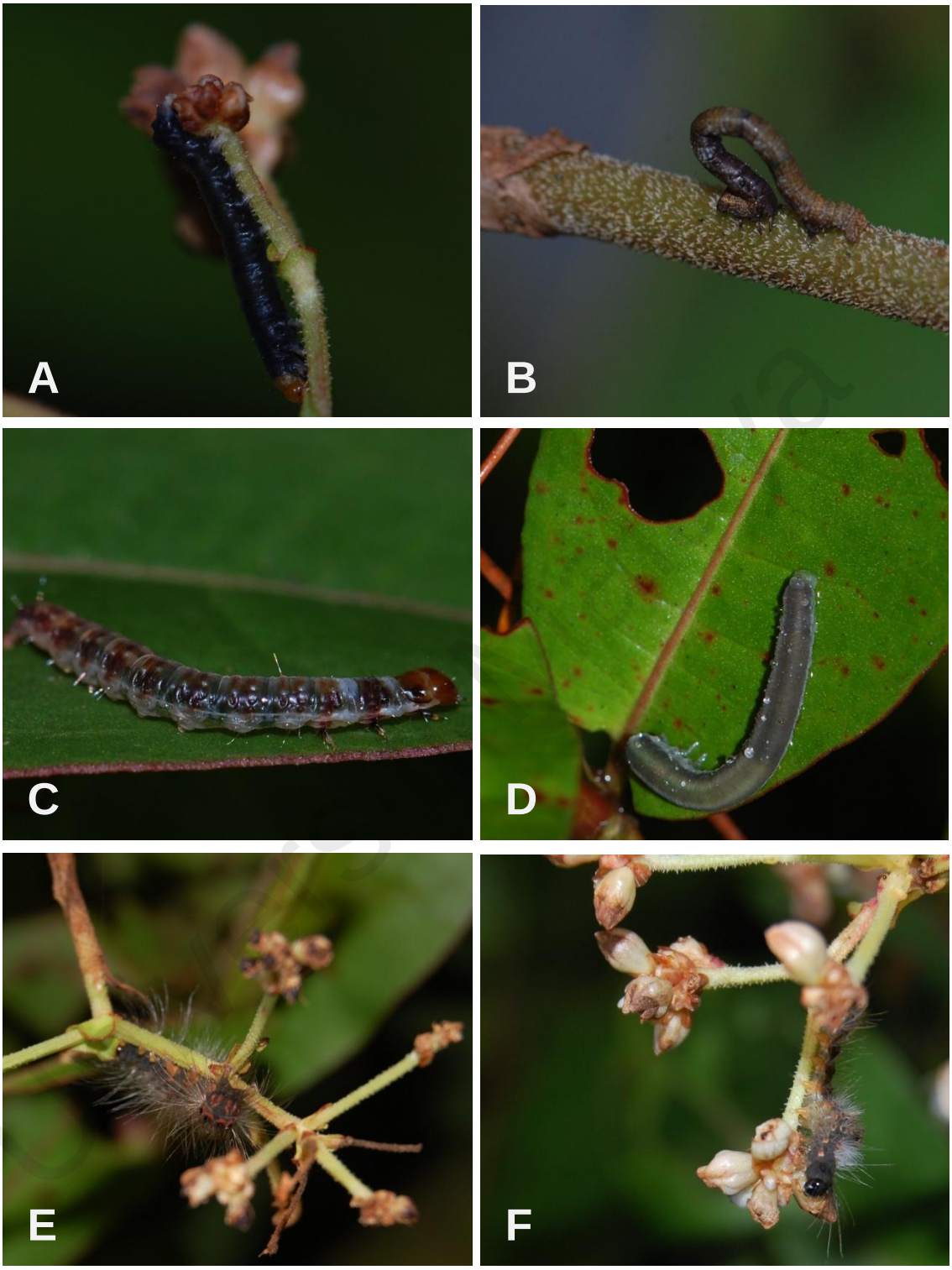


Figure 3.28: Larvae as pests of the highland variety of *P. chinensis* var. *chinensis*. A: Larva of Geometridae Family; B: Larva of looper moth from the family of Geometridae; C & D: Larvae of Pyralidae family; E &F: Larvae of Lymantriidae family, inflorescence as a food source for the larvae.

Cont. Figure 3.28: Larvae as pests of the highland variety of *P. chinensis* var. *chinensis*. G: Larva of Lymantriidae family; H & I: Larvae from the order of Lepidoptera; J: Larva of Pyralidae family ate *P. chinensis* leaves in the population of H3 in Gunung Ulu Kali.

3.3.8 Seed viability, seed germination and seedling morphology

Seeds were considered as viable and not viable based on their stainability in 1% tetrazolium salt solution. Viable seeds were those with the embryos completely stained red and nonviable seeds were those with the embryos slightly stained (Figure 3.29). The seeds showed a quite high percentage of viability, i.e. 77% (Table 3.15).

Fresh seeds showed a moderate germination rate of 47% in highland soil and highland environment, i.e. at 19.0℃ and average humidity of 85.0% (average readings taken from 0630–0830 hours at the altitude of 1315 m at the location of H5 in Gunung Ulu Kali). Under the highland germination conditions, seeds were left in their natural environment in pots (without watering), as the experimental location was the montane forest habitat and field work could only be conducted once a week. Seed germinated under lowland environment, at room temperature in the laboratory, showed a lower percentage of germination, i.e. 34%. In the highland environment, the first seedling appeared after 1 week, while in the lowland laboratory environment, the first seed germinated after 12 days. Under the highland germination conditions, the seeds can still germinate at the sixth to seventh week (the germination period); likewise for the lowland green house conditions.

The highland variety of *P. chinensis* var. *chinensis* shows epigeal germination (Figures 3.30 A & B). Three days after the appearance of the radicle, the hypocotyl completely emerges from the soil and it takes another one to two days for the cotyledons to separate and spread out from the seed coat (Figures 3.30 A & B). The height of the seedling varies from 0.5–1.0 cm. One week after the expansion of cotyledons, the first leaf is formed.

Table 3.15: Seed viability and seed germination of the highland variety of *P. chinensis* var. *chinensis*.

seed viability and seed germination	highland variety of *P. chinensis* var. *chinensis*		
	Test 1	Test 2	Test 3
70-seed samples in 1% tetrazolium test	70	70	70
number of viable seeds	51	54	57
number of nonviable seeds	19	16	13
percentage of seed viability	72.9	77.1	81.4
average seed viability	77.10%		

	highland environment	lowland laboratory environment
total number of seed samples	100	100
number of germinated seeds	47	34
number of not geminated seeds	53	66
Percentage of seed germination	47.0	34.0
Appearance of first seedling (number of days after sowing)	7 days	12 days
height of seedling (cm)	0.5–1.0	0.5–1.0
Germination period (weeks)	6–7	6–7
Type of germination	epigeal	epigeal

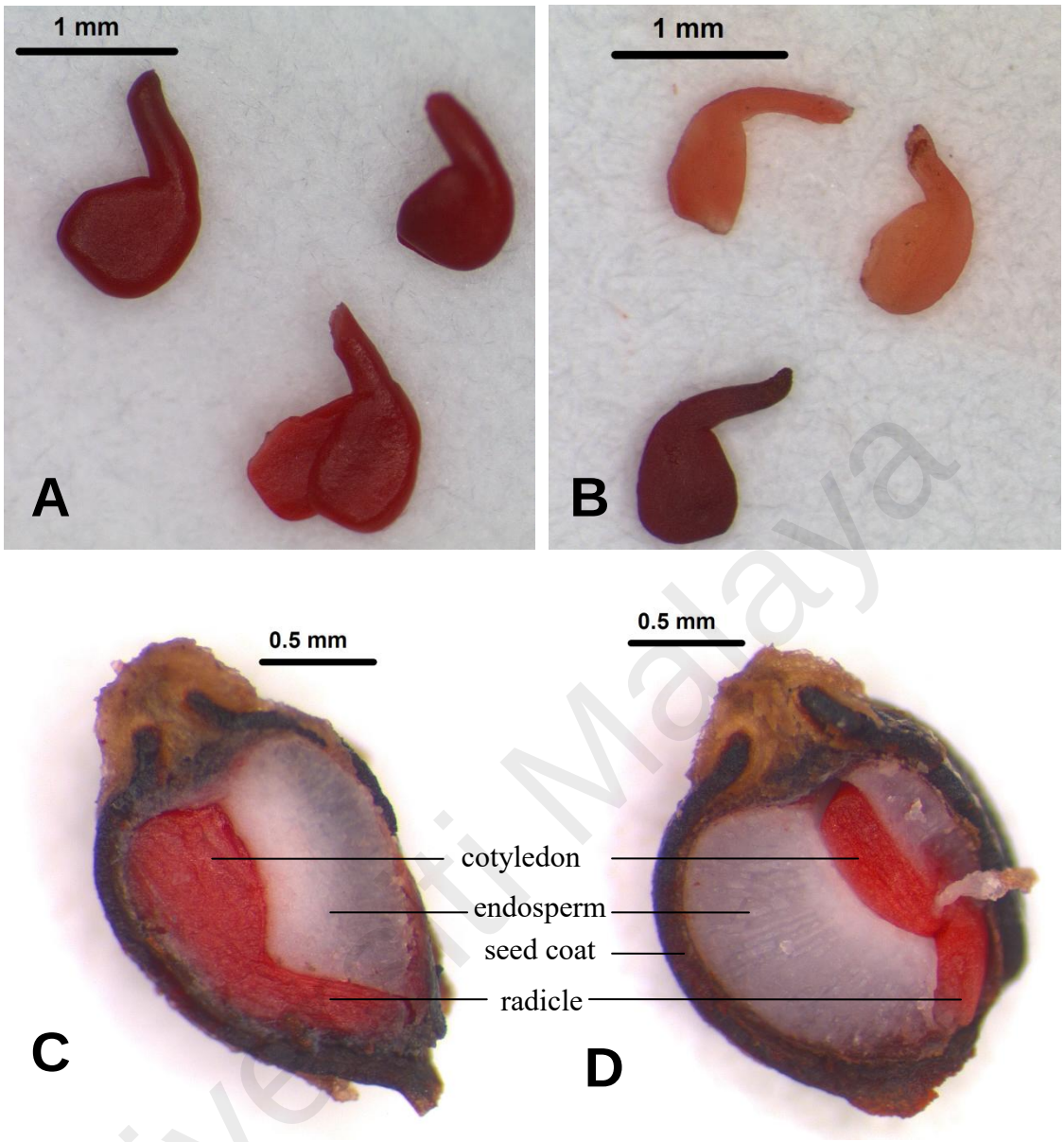


Figure 3.29: Seed viability test in 1% tetrazolium salt solution. A: Embryo completely stained in red, viable seed; B: two embryos slightly stained in red, nonviable seeds, compared to the one completely stained in red below, viable seed; C & D: The endosperm and embryo stained in 1% tetrazolium salt solution, with the endosperm not stained but the embryo completely stained in red. (Photo taken with a Leica MZ95 stereomicroscope).

Figure 3.30: Seedling morphology of the highland variety of *P. chinensis* var. *chinensis*. A: A radicle grows out first followed by the emergence of the cotyledons; B: Epigeal germination; C: The height of the seedling 3 days after the first emergence of cotyledons; D: Appearance of the first leaf, a week after the cotyledons have expanded. (Photo taken with a Nikon D90 with 105 mm macro lens).

3.4 Discussion

3.4.1 Flower and fruit development

The time taken for the appearance of the flower buds to the development of the first mature open flowers was 12–14 days for the highland variety and 9–12 days for the lowland cultivated variety. Fertilized flowers developed into mature fruits in about 16–20 days for the highland variety while less than 1% fruit set was observed in the lowland variety. Fruit development was rapid in the highland variety of *P. chinensis* var. *chinensis* and the growth of fruit corresponded to colour changes in the highland variety, from white to greenish white and when mature, bluish black colour. That was a high percentage of flower and fruit abortion during development, possibly due to competition or inadequate nutrition.

In the highland population H4 at Gunung Ulu Kali, the percentage of flower abortion and fruit degeneration were relatively high. H4 is the highest altitude at Gunung Ulu Kali. The environmental conditions are similar to the upper montane forest with relatively high relative humidity, low temperature during flower anthesis time, low percentage of oxygen in the atmosphere and windy environment. These factors could directly influence the percentage of flower and fruit abortion. Moreover, lack of successful insect pollination was also as one of the main factors causing abortion (Gross & Werner, 1983; Petersen *et al*., 1982).

The flower and fruit development of *P. chinensis* var. *chinensis* has not been reported so far. Thus, the observations and results obtained from this study would provide useful information for the future researcher of this variety.

3.4.2 Embryological studies

3.4.2.1 Microsporangium, microsporogenesis and male gametophyte

The anther of *Persicaria* (Synonym *Polygonum*) is tetrasporangiate and the early stages of the anther wall development in *Persicaria chinensis* var. *chinensis* are similar to other members of Polygonaceae (Agoram & Krishnamurthy, 1980; Davis, 1966; Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985; Mukherjee, 1972; Rao & Mukherjee, 1973; Soueges, 1919a), with secretory type of tapetum, made up of two to four- nucleate tapetal cells. Cytokinesis in the microspore mother cells is simultaneous resulting in tetrahedral and decussate tetrads, its endothecium develops fibrous thickening and pollen grains are finally shed at three-celled stage.

The development of the anther wall is of the Monocotyledonous type in Polygonaceae, as observed in *Polygonum alatum*, *Polygonum capitatum*, *Polygonum molle*, *Polygonum flaccidum*, *Polygonum strigosum*, *Polygonum barbatum*, *Polygonum glabrum*, *Rumex crispus*, *Rheum palmatum*, and *Persicaria chinensis* (Current study; Chen & Wang, 1993; Dudgeon, 1918; Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985; Mukherjee, 1972; Rao & Mukherjee, 1973). However, the Dicotyledonous type of anther wall development was stated in *Antigonon leptopus* (Agoram & Krishnamurthy, 1980) though the paper did not describe the type of anther wall development and the illustration did not show whether it was the Dicotyledonous or Monocotyledonous type. Probably owing to this, Johri *et al*. (1992) has reported the anther wall development of *Antigonon leptopus* as the reduced type. In *Antigonon leptopus*, anther development takes place simultaneously in all the six stamens (Agoram & Krishnamurthy, 1980) but the eight anthers in *P. chinensis* var. *chinensis* show asynchronous development in this study. In *Rheum palmatum*, it was reported that the development in the four locules of an anther is asynchronous (Chen & Wang, 1993).

The hypodermal archesporium is stated as single celled in six *Polygonum* species (*Polygonum alatum*, *Polygonum capitatum*, *Polygonum molle*, *Polygonum flaccidum*, *Polygonum strigosum* and *Polygonum chinense*) (Maheswari Devi & Manorama, 1984, 1985). However, in *Persicaria chinensis* (Current study) it is multicellular and more than one archesporia can be observed in the sporangium and the divisions are not synchronous in the four locules of an anther. The single celled archesporium in the sporangium stated by Maheswari Devi & Manorama (1984, 1985) probably is incorrectly reported as the figures on the longitudinal sections of the anther showed a whole row of microspore mother cells.

The anther wall of the Polygonaceae comprises four layers, i.e. an epidermis, endothecium, middle layer and tapetum (Current study). In Polygonaceae, the uniseriate tapetal cells are derived from the inner secondary parietal cells. However, in *Antigonon leptopus*, the tapetal layer is derived from two sources, one from the inner parietal tissue and the other from the connective tissue (Agoram & Krishnamurthy, 1980). Most of the tapetal cells in *Polygonum* species divide mitotically to become binucleate cells, and these include *Polygonum alatum*, *Polygonum capitatum*, *Polygonum molle*, *Polygonum flaccidum* and *Polygonum chinense* (Now *Persicaria chinensis*) as described in Maheswari Devi & Manorama (1984, 1985), and bi- or trinucleate in *Polygonum strigosum* (Maheswari Devi & Manorama, 1984) while in this present study, the tapetal cells of *Persicaria chinensis* var. *chinensis* become multinucleate, with 3–4 nuclei just before meiosis of the microspore mother cells. The tapetum in *Antigonon leptopus* is reported to be 2-, to 4-nucleate (Agoram & Krishnamurthy, 1980). In a few abnormal anthers of *Rheum palmatum*, the microspore mother cells were observed to be located directly below the epidermis of the anther. No other wall layers were observed (Chen & Wang, 1993).

Cytokinesis in microspore mother cells is simultaneous and the tetrads formed are both tetrahedral and decussate in *Persicaria chinensis* var. *chinensis* and *Polygonum flaccidum* (Present study; Maheswari Devi & Manorama, 1984) whereas in *Polygonum alatum*, *Polygonum capitatum*, *Polygonum molle*, *Polygonum strigosum*, *Polygonum chinense*, *Rheum palmatum* and *Antigonon leptopus* the microspore tetrads have been reported to be tetrahedral (Agoram & Krishnamurthy, 1980; Chen & Wang, 1993; Maheswari Devi & Manorama, 1984, 1985). Microspore tetrads are also isobilateral in Polygonaceae (Johri *et al*., 1992).

In *P. chinensis* var. *chinensis*, the mature pollen grains are shed at the three-celled stage. Similar observations have been reported in *Polygonum flaccidum*, *Polygonum alatum*, *Polygonum capitatum*, *Polygonum molle*, *Polygonum strigosum*, *Polygonum chinense*, *Rheum palmatum* (Chen & Wang, 1993; Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985) but two-celled pollen grains are reported in *Antigonon leptopus* (Agoram & Krishnamurthy, 1980), *Rumex uesicarius* (Johri *et al*., 1992) and *Rumex crispus* (Dudgeon, 1918).

3.4.2.2 Megasporangium, megasporogenesis and female gametophyte

Beginning with the embryological studies of Strasburger (1879) on the development of the female gametophyte and megasporogensis in *Polygonum divaricatum*, the members of this family have become classic examples for ovule development (Dudgeon, 1918). The ovule development of *Persicaria chinensis* is similar and conforms to that of the other species of *Polygonum flaccidum*, *Polygonum alatum*, *Polygonum capitatum*, *Polygonum molle*, *Polygonum strigosum*, *Polygonum chinense* (Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985), *Polygonum glabrum* (Rao & Mukherjee, 1973), *Polygonum barbatum* (Mukherjee, 1972), *Polygonum pensylvanicum* (Neubauer, 1971), *Polygonum viviparum* and *Polygonum*

bistortoides (Diggle *et al*., 2002), *Antigonon leptopus* (Agoram & Krishnamurthy, 1980), *Fagopyrum esculentum* (Campbell, 1997; Mahony, 1936; Obendorf *et al*., 1993), *Rheum palmatum* (Chen & Wang, 1993) and *Rumex crispus* (Davis, 1966; Dudgeon, 1918).

In the *Persicaria* species studied, the ovule is orthotropous, bitegmic and crassinucellar with basal placentation. Similar description has been reported in this family (Davis, 1966; Johri *et al*., 1992), and the members are *Polygonum flaccidum*, *Polygonum alatum*, *Polygonum capitatum*, *Polygonum molle*, *Polygonum strigosum*, *Polygonum chinense* (Maheswari Devi & Manorama, 1984, 1985), *Polygonum viviparum* and *Polygonum bistortoides* (Diggle *et al*., 2002), *Polygonum pensylvanicum* (Neubauer, 1971), *Fagopyrum esculentum* (Campbell, 1997; Mahony, 1935, 1936; Obendorf *et al*., 1993) and *Rumex crispus* (Dudgeon, 1918). Interestingly, the ovule in *Antigonon leptopus* is curved by 180º (Hemi-anatropous) at the functional megaspore stage and after fertilization; the seed becomes straight to reach a typical orthotropus configuration (Agoram & Krishnamurthy, 1980). In *Rheum palmatum*, the ovule is bitegmic and crassinucellate (Chen & Wang, 1993).

Multicellular archesporia have been observed and these are common in the family though usually only one cell functions as the megaspore mother cell (Current study; Chen & Wang, 1993; Davis, 1966; Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985). Occasionally, multiple megaspore mother cells have been observed in both *P. viviparum* and *P. bistortoides* (Diggle *et al*., 2002), and *Rumex crispus* (Dudgeon, 1918).

Development of nucellar beak and hypostase is prominent in this family (Diggle *et al*., 2002; Johri *et al*., 1992). A nucellar beak projects beyond the micropyle and generally degenerated after fertilization has taken place (Current study; Maheswari Devi & Manorama, 1984, 1985). The hypostase, located at the chalazal end of the

nucellus, is a well-defined group of tanninferous cells, formed during the eight-nucleate embryo sac stage and continue to develop into a saucer-shaped or plate-like structure. Both the nucellar beak and hypostase are observed in *Rumex*, *Fagopyrum* and all the *Polygonum* species studied (Current study; Davis, 1966; Diggle *et al*., 2002; Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985; Neubauer, 1971; Obendorf *et al*., 1993) but these prominent structures are not present in *Antigonon leptopus* (Agoram & Krishnamurthy, 1980).

Members of *Polygonum* investigated so far show that megasporogenesis results in the formation of a linear tetrad of megaspores (Current study; Diggle *et al*., 2002; Maheswari Devi & Manorama 1984, 1985; Rao & Mukherjee, 1973). In additional, *Fagopyrum* and *Antigonon* (Davis, 1966; Mahony, 1935; Rao, 1936) also show a linear tetrad of megaspores. However, according to Davis (1966), *Rheum* and *Rumex* produce T-shaped tetrads (Chen & Wang, 1993; Dudgeon, 1918) and the functional chalazal megaspore in all the members of Polygonaceae gives rise to the *Polygonum* type of embryo sac (Current study; Chen & Wang, 1993; Davis, 1966; Diggle *et al*., 2002; Dudgeon, 1918; Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985; Neubauer, 1971; Obendorf *et al*., 1993).

The two polar nuclei fuse to form a secondary nucleus in most of the species of Polygonaceae before fertilization occurs i.e. in *Polygonum flaccidum*, *Polygonum alatum*, *Polygonum capitatum*, *Polygonum molle*, *Polygonum strigosum*, *Polygonum chinense, Polygonum viviparum*, *Polygonum bistortoides*, *Polygonum pensylvanicum*, *Antigonon leptopus*, *Rheum palmatum* and *Rumex crispus* (Current study; Chen & Wang, 1993; Davis, 1966; Diggle *et al*., 2002; Dudgeon, 1918; Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985; Neubauer, 1971; Obendorf *et al*., 1993). In *Polygonum (P. flaccidum*, *P. alatum*, *P. capitatum*, *P. molle*, *P. strigosum*, *P. chinense, P. viviparum*, *P. bistortoides*, *P. pensylvanicum*), and *Fagopyrum*

esculentum, the synergids exhibit the filiform apparatus (Diggle *et al*., 2002; Maheswari Devi & Manorama, 1984, 1985; Mahony, 1935) whereas *Rumex dentatus* and *Antigonon leptopus* produce hooked synergids (Agoram & Krishnamurthy, 1980; Davis, 1966). The three antipodal cells are often ephemeral but in *Rumex* they are persistent and become multi-nucleate (Davis, 1966). Maheswari Devi and Manorama (1985) revealed that the number of antipodals increases up to five in *Polygonum chinense* but in current study, *Persicaria chinensis* var. *chinensis* exhibits three antipodals in both the highland and lowland populations. The antipodals usually degenerate but in some rare instances of *Polygonum chinense*, they remain intact up to the globular embryo stage (Maheswari Devi & Manorama, 1985).

In *P. chinensis* var. *chinensis*, the outer and inner integuments are initiated by the time the megaspore mother cell is differentiated (Current study). In *P. bistortoides* and *P. viviparum*, Diggle *et al*. (2002) revealed that the outer integument of the ovule is initiated at, or just prior to meiosis of the megaspore mother cell. However, Maheswari Devi and Manorama (1985) showed that at the tetrad stage of *P. alatum*, *P. capitatum*, *P. molle* and *P. chinense*, the initials of the outer integument are differentiated. The inner integument contributes to the formation of the micropyle in all the members of Polygonaceae and usually both the outer and inner integuments are two layered (Agoram & Krishnamurthy, 1980; Diggle *et al*., 2002; Neubauer, 1971; Obendorf *et al*., 1993). *Polygonum chinense* is reported to have a four-layered outer integument (Maheswari Devi & Manorama, 1985) whereas only a two-layered outer integument has been observed in this study. In *P. chinense*, it was observed that after fertilization the outer integument becomes meristematic and overgrows the inner integument but never contributes to the micropyle (Maheswari Devi & Manorama, 1985).

In a rare case, the lowland variety of *P. chinensis* var. *chinensis* has been observed to have two egg cells. This has not been reported so far. If further

development occurs, the two eggs after fertilization could give rise to polyembryony. However, the frequency is extremely low. Abortive ovules have been reported in *P. bistortoides* and *P. viviparum*. These ovules ceased development at no specific stage; in some both the outer and inner integuments have developed but there is no evidence of megaspore mother cell formation; in others, the ovules appear nearly mature but yet have collapsed (Diggle *et al*., 2002). In the lowland cultivated variety of *P. chinensis* var. *chinensis*, the embryo sac tends to degenerate before or after the formation of the eight-nucleate stage, or even after the formation of endosperm. Although 32% of the flowers reached anthesis in the field (From the study of flower degeneration), we neither observed a mature embryo in sectioned materials nor mature fruit in the field. Clearly, during post-fertilization development, the possible fertilized ovule aborted and no mature fruit was observed. In addition to a negligible percentage of fruit set compared with the highland wild variety of *P. chinensis* var. *chinensis*, abortion of megaspore mother cells was also particularly common in the lowland variety.

3.4.2.2 Endosperm and Embryo

The endosperm formation in the highland variety of *Persicaria chinensis* var. *chinensis* belongs to the *ab initio* Nuclear type. The members in this family, *Polygonum flaccidum*, *Polygonum alatum*, *Polygonum capitatum*, *Polygonum molle*, *Polygonum strigosum*, *Polygonum chinense* (Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985), *Polygonum barbatum* (Mukherjee, 1972), *Polygonum pensylvanicum* (Neubauer, 1971), *Antigonon leptopus* (Agoram & Krishnamurthy, 1980), and *Rheum palmatum* (Chen & Wang, 1993), have also shown *ab initio* Nuclear type of endosperm formation and the endosperm functions as the chief food reserve in the mature seed (Agoram & Krishnamurthy, 1980; Chen & Wang, 1993; Johri *et al*., 1992; Maheswari Devi & Manorama, 1984, 1985; Mukherjee, 1972; Neubauer, 1971). The endosperm wall formation is initiated from the micropylar toward the chalazal end, as also seen in

other *Polygonum* species (*P. flaccidum*, *P. alatum*, *P. capitatum*, *P. molle*, *P. strigosum*, *P. chinense* and *P. pensylvanicum*) (Maheswari Devi & Manorama, 1984, 1985; Neubauer, 1971). In this study, ruminate endosperm is formed in the highland wild variety and this has never been reported in this genus though *Antigonon leptopus* (Agoram & Krishnamurthy, 1980) and *Coccolaba uvifera* (Periasamy, 1964) were reported to have the Coccoloba type of ruminate endosperm and it was caused by the invagination of the seed coat of the pachychalaza.

The development of embryo in the family Polygonaceae is of the *Asterad* type (Davis, 1966; Johri *et al*., 1992). This has been reported in *P. flaccidum*, *P. alatum*, *P. capitatum*, *P. molle*, *P. strigosum*, *P. chinense* (Maheswari Devi & Manorama, 1984, 1985), *Polygonum pensylvanicum* (Neubauer, 1971), *Polygonum glabrum* (Rao & Mukherjee, 1973) and *Rheum palmatum* (Chen & Wang, 1993). However, the actual type of embryo development could not be determined in the present study as there was a high incidence of seed abortion and the crucial stages could not be obtained.

In the lowland cultivated variety, the embryogeny and endosperm development could not be traced owing to a lack of seed specimens. The virtual absence of sexual reproduction in the lowland cultivated variety of *P. chinensis* var. *chinensis* appears to be due mainly to a low rate of fertilization and to flower/embryo/fruit abortion, since the development in the embryogeny and endosperm is unsuccessful; as neither a mature embryo in sectioned materials nor a completely mature fruit in the field was observed.

Many species including both gymnosperms and angiosperms, regularly abort both flowers and immature fruits during the development of fruits and seeds and commonly produce mature fruits from only a small number of their flowers (Lee, 1988; Stephenson, 1981). They occur in a wide range of latitudes and habitats under both cultivated and natural conditions (Stephenson, 1981).

A variety of factors are attributed to the failure of the development of fruits and seeds, and these include competition among the seeds and fruits for nutrients (Diggle, 1995; Lee, 1988; Stephenson, 1981), weather conditions (Lee, 1988; Stephenson, 1981), low pollen viability (Current study; Diggle *et al*., 2002), poor genetic quality in the maternal selection among embryos (Akhalkatsi *et al*., 1999; Bawa & Webb, 1984; Briggs *et al*., 1987; Donohue, 1999; Guth & Weller, 1986; Marshall & Folsom, 1991; Roach & Wulff, 1987; Stephenson, 1981; Wilson & Burley, 1983) and genetic load or genetic abnormalities (Andersson, 1993; Brink & Cooper, 1940, 1941, 1947; Charlesworth & Charlesworth, 1987; Cooper & Brink, 1940; Wiens, 1984; Wiens *et al*., 1987). In addition, differences in fertilization could be possible due to self-incompatibility; pollen donors could be genetically identical to the recipients (Bawa *et al*., 1985; Bolstad & Bawa, 1982; Hedegart, 1973; Tangmitcharoen & Owens, 1997a, 1997b; Weis & Hermanutz, 1993). Self-incompatibility in pollination would not lead to successful fertilization but compatible genotypes should be normally available within its population. Thus, low pollen viability in the cultivated variety (Current study) leading to limited pollination (Diggle *et al*., 2002) and high percentage of aborted ovules are more likely to explain the low fruits and seeds production in the lowland cultivated populations.

3.4.3 Pollen morphology in heterostylous variety of *P. chinensis* var. *chinensis*

Heterostyly in the family Polygonaceae has been first reported by Hildebrand in *Fagopyrum esculentum* Moench over a century ago (Darwin 1892, quoted by Chen & Zhang, 2010; Reddy *et al*., 1977; Samborska-Ciania *et al*., 1989). Other heterostylous taxa studied in this family included *Oxygonum* (Graham, 1957; Hong, 1999), *Aconogonon* (Hong, 1991) and *Polygonum* (Chen & Zhang, 2010; Liza *et al*., 2010; Reddy *et al*., 1977). *Polygonum chinense* was first presented by Reddy *et al*., (1977), with details such as different style length, stamen height, pollen size, and stigmatic papillae size between the pin and thrum flowers, and also the frequencies of the natural populations of the two flower forms. In addition, present study has provided more detailed information on the floral polymorphism based on the LM and SEM observations.

Heterostyly in flowering plants is related to other physiological and morphological differences among morphs, either in distylous or tristylous plants (Dulberger, 1992). In most of the distylous species studied, the results obtained showed pollen heteromorphism. They offen differ in pollen grain size (Chen, 2009; Chen & Zhang, 2010; Hong, 1991, 1999; Reddy *et al*., 1977), pollen production (Chen, 2009; Ganders, 1979), exine sculpturing (Chen & Zhang, 2010; Dulberger, 1992) and stigmatic papillae morphology (Hong, 1991). Present study reveals that the two flower morphs in *P. chinensis* var. *chinensis* show significant differences in pollen size and pollen production in an anther but they have similar pollen exine sculpturing and stigmatic papillae morphology.

Heterostylous species often show polymorphism in pollen size and number of pollen grains produced per flower (Ganders, 1979). Thrum flowers always have larger pollen grains than pin flowers (Ganders, 1979; Hong, 1991, 1999; Reddy *et al*., 1977) and the number of pollen grains produced per pin flower is also greater than that of the

thrum flower (Chen, 2009; Chen & Zhang, 2010; Ganders, 1979). Present study also shows that the pollen grains in the two flower morphs in *P. chinensis* var. *chinensis* are significantly different in size, i.e. pollen grains of the thrum flowers are significantly larger than that those of the pin flowers. The number of pollen grains produced in a thrum flower is also significantly lower than that in a pin flower. This finding supports Ganders' (1979) study in 15 distylous species, i.e. there is inverse correlation between pollen size and pollen production of the morphs. This inverse correlation between pollen size and pollen production of the morphs (Ganders, 1979; Dulberger, 1992) suggest that polymorphism in pollen production has adaptive value in regulating disassortative pollen flow during cross pollination. Hence, heterostylous plants promote legitimate (Intermorph) pollination, a hypothesis mentioned by Darwin since 1877 and supported by many studies, amongst them Ganders (1974), Barrett and Glover (1985) and Dulberger (1992).

Similar findings in pollen dimorphism have also been reported in *Polygonum jucundum* (Chen & Zhang, 2010), eight species of *Oxygonum* (Hong, 1999), and *Aconogonon campanulatum* (Hong, 1991). However, in some distylous species, no significant size difference in pollen size dimorphism is observed, including *Linus pubescens* (Dulberger, 1973), *Amsinckia spectabilis* var. *spectabilis* (Ray & Chisaki, 1957), *Byrsocarpus coccineus* (Baker, 1962) and a few species of *Cordia*, *Nesaea* and nine rubiaceous species (Darwin, 1877 quoted by Dulberger, 1992).

Darwin (1877) has hypothesized that, the bigger pollen grains in thrum flower is related to the necessity for larger energy reserves in pollen from high-level anthers, thus the bigger pollen grains in thrum flower might be correlated with more substance and can produce longer pollen tubes needed in longer styles. However, Cruden and Lyon (1985) had reported that, there was no significant correlation between pollen grains size and style length. Chen (2009) also reported that pollen size was not correlated with

pistil length in *Primula merrilliana* and *Primula cicutariifolia*. This strongly indicates that it is not necessary for thrum pollen grains to be larger in order to grow successfully down long pin styles. It is, however, possible that the asymmetry of pollen flow noticeable in most heterostylous plants (Less pin pollen going to thrum stigmas) has opted for the production of more but smaller pin grains, leading to dimorphic grain size found in the pollen reserves (Richards, 1997). This difference in carbohydrate reserves between pin and thrum pollen might secondarily augment the heterostyly in some cases; for example, *Lythrum* and *Jepsonia* thrum pollen had detectable starch reserves, which are apparently absent from pin flowers (Ganders, 1979).

Cruden and Lyon (1985) found a strong correlation between pollen grain size and thickness of stigma when comparing 10 unrelated facultative xenogamic species with starchy pollen. This finding is consistent with Amici's (1830) (Quoted by Cruden, 1985) hypothesis which suggested that pollen tubes obtain nutrients from the transmission tissue in the pistil but not from pollen grain resources. Dulberger (1992) summarizes the evidences that strongly suggest that the morphs-specific mode of growth and length of the style, the organization of the transmitting tract, the substances present in the interstices, and the differential sites of inhibition are all interrelated in one way or another in the efficient mechanical promotion of pollination.

The morphology of stigmatic papillae in both pin and thrum flowers are similar in *P. chinensis* var. *chinensis*, showing no significant differences (Figure 3.20). In *Aconogonon campanulatum* (Polygonaceae), the shape of the stigmatic surface is reported to be different in the two morphs, i.e. "cob-like" in pin flowers and "papillate" in thrum flowers (Hong, 1991). *Fagopyrum* and *Rheum* were reported to have dry non-papillate stigmatic surface in contrast with dry papillate stigmatic surface in *Polygonum* and *Oxyria* (Heslop-Harrison, 1981; Heslop-Harrison & Shivanna, 1977). In members of other families, an inverse correlation between stigma width, thickness and style

length are reported in *Jepsonia parryyi*, *Plumbago capensis* and a few *Linum* species. In these species, the stigmatic papillae (Receptive surface) of pin-eyed morph are typically larger than those of the thrum-eyed flowers (Dulberger, 1973, 1992). However, Dulberger (1992) also reported that thrum stigmas are larger than pin stigmas in *Gregoria vitaliana*, *Amsinckia grandiflora*, *Primula malacoides*, *Hedyotis caerulea*, *Paliacourea lasiorrachis*, *Gelsemium sempervirens* and *Neanotis montholoni*. Larger papillae of pin stigmas have been reported to accommodate the larger pollen grains of thrum flowers (Dulberger, 1992). Shivanna *et al*. (1981) suggests that the slight structural differences in the papillae between the two morphs of *Primula vulgaris* may be involved in the control of pollen hydration, chemotropic guidance of emerging tubes and tube penetration. This suggestion is supported by Dulberger (1992) in the distylous *Linum* species. In *P. chinensis* var. *chinensis*, the similarity in the structure, morphology and size of the stigmatic papillae do not seem to be crucial factors in the penetration of pollen tubes among the papillae of distylous flowers, since self-compatible pollen grains have successfully led to fruit set in both the distylous flowers in bagging experiment.

Persicaria chinensis var. *chinensis* has two types of stamens which have so far not been reported, i.e. three free and distinct stamens and five epipetalous stamens. Furthermore, these stamen lengths are significantly different between the thrum and pin flowers of the highland populations and the thrum flower of the lowland populations (See table 3.8). Stamen-length polymorphism is directly correlated with the size of pollen in distylous flowers and inversely correlated with polymorphism of pollen production, taller stamen-length with bigger pollen-size but lesser pollen production in thrum flower and shorter stamen-length with smaller pollen-size but more pollen production in pin flower. There is no evidence to indicate a direct involvement of stamen length in pollination in incompatibility studies (Dulberger, 1992).

Pollen dimorphism in *P. chinensis* var. *chinensis* was first reported by Reddy *et al*. (1977). They reported that pollen size dimorphism was associated with heterostyly, i.e. thrum pollen were larger (44.38±0.62 µm) than pin pollen (34.83±0.27 µm). However, it is not clear whether the measurements reported were equatorial diameter or pollen length, as it was not clearly stated (Present study, refer to table 3.8). Bahadur *et al*. (1985) reported pollen dimorphism in a different aspect, i.e. differences in exine sculpturing. The pollen colpus in a pin flower was comparatively smaller than that of a thrum flower and the reticulate surface of pin pollen was coarser than that of the thrum pollen. Differences in the depth of the lumina, i.e. pin pollen had deep lumina in various sizes and shapes as compared to the larger lumina in penta-hexoganol shape observed in thrum pollen grains. Thick muri were found in pin pollen but thrum pollen showed straight muri with several lumina bacules. Current study shows no significant differences in the pollen tectal surface of pin and thrum pollen of the highland variety; they have similar reticulate exine with similar sizes and shapes of the lumina. The highland thrum pollen grains have larger lumina, thicker and longer muri when compared to the lowland thrum pollen grains. This result shows differences in exine sculpturing between the thrum variety of the highland and lowland pollen grains.

Bahadur *et al*. (1985) summarized that, the differences between the pollen size and exine in *Polygonum chinense* sculpturing were determined early in the ontogeny of floral development and the sporopollenin (Exine material) deposition that characterizes the distylous pollen were under sporophytic control, a feature that characterizes the heteromorphic sporophytic incompatibility system.

Incidentally, in *P. chinensis* var. *chinensis*, the stamen height of the thrum flower is greater than its petal length while the stigma height of the pin flower is greater than its petal length and thus protrudes beyond the petals (Figure 3.6 E). Similar morphology has also been observed in the distylous *Polygonum jucundum* (Chen &

Zhang, 2010). This feature could directly increase success rate of pollination which is aided by non-spesific insect pollinators as a large variety of insect species visit *P. chinensis* var. *chinensis* in its natural environment (Refer to 3.3.7). Besides, wind pollination could also occur in the montane forest environment. Moreover, pollen to ovule ratios (Refer to 3.3.6) suggested that the breeding system of the highland pin and thrum varieties are facultative xenogamy. Hence, it can be considered that *P. chinensis* var. *chinensis* is not an obligate cross pollination plant species, as self- or intramorph pollination could happen and this observation is supported by the bagging experiment in Chapter 3.3.6 (Self pollination gives 16.9% fruit set).

The adaptive significance of floral diversity has been largely interpreted as resulting from the promotion of cross pollination to avoid self-fertilization and to increase fitness of the offsprings (Barrett, 2002a, 2002b). According to Darwin's interpretation, the morphological features of heterostyly and sterility in self- and intramorph cross-pollinations are two distinct outbreeding mechanisms. Dulberger (1992) reports that heterostyly is a significant feature to endorse cross pollination because heterostyly is typically a sporophytically controlled, diallelic incompatibility system which prevents or reduces self- and intramorph fertilization. Barrett's review (2002a) reports that distylous population with two floral morphs provides precise pollen transfer in animal-pollinated plants without costs that are associated with sexual interference and self-pollination.

Persicaria chinensis var. *chinensis* has distylous morphology. However, the pin and thrum flowers are different in terms of the stigma thickness and width, anther height of both the epipetalous and free anther, pollen grain size and total number of pollen grains produced, but they are similar in exine sculpturing. It is generally accepted that the floral and pollen structures are involved in the efficiency of pollen transfer in the heterostylous species (Dulberger & Ornduff, 2000; Ganders, 1979).

However, the molecules involved in sexual interactions connected with the functions of pollen and stigma remain to be further investigated. This includes the protein-protein interactions during the process of pollen adhesion, pollen hydration, cytological studies of pollen activation and germination and enzyme activities of the stigma invasion (Edlund *et al*., 2004).

3.4.4 Pollen viability and pollen germination test

The viability of the highland variety of the *P. chinensis* var. *chinensis* was tested three hours after anthesis (Time taken to bring specimens from Gunung Ulu Kali back to the laboratory). Within 24 hours after anthesis, the viability of the pollen grains (From the inflorescences placed in water flasks and stored in an air conditioned room at 19ºC) of the highland variety of *P. chinensis* var. *chinensis* decreased significantly to almost six-fold (From 50% to 8%). After 40 hours, these pollen grains completely lost their viability.

Further, pollen grains from inflorescences placed in the water flasks and stored at room temperature (28ºC) completely lost their viability after 24 hours. Even after 12 hours, less than 3% of the pollen grains germinated in the modified medium. It has also been reported that high relative humidity is also required to retain the viability of tri-nucleate pollen grains (Shivanna & Johri, 1985). Usually, pollen grains of most plants retain their viability for some time under their natural conditions, and they soon lose their viability after dehiscence of the anthers especially when temperatures are unusually high and humidity is low (Johri & Vasil, 1961). In wheat, it has been reported that pollen grains completely lost their viability within 70 minutes, and 120 minutes in triticale (A hybrid of wheat and rye) (Fritz & Lukaszewski, 1989).

The anthers of *P. chinensis* var. *chinensis* dehisced soon after the flower is opened at about 0600–0730 hours and they lose their viability very rapidly, practically

within the first 12 hours (Present study, Morris, 1947). In *Fagopyrum esculentum*, the highest percentage of seed set was observed when pollen grains from dehisced anthers were collected and applied immediately on the stigma (Namai, 1991). This study demonstrates that there is a short time period, within 12 hours, in which the viable pollen grains of *P. chinensis* var. *chinensis* may be used for pollination and *in vitro* plant breeding studies.

The nature of the osmotica, sucrose and/or polyethylene glycol (PEG) had a significant influence on pollen germination and pollen tube growth (Adhikari & Campbell, 1998; Dumont-BéBoux *et al*., 1999; Shivanna & Sawhney, 1995). PEG was introduced as a medium for pollen germination and pollen tube growth. Generally, PEG with molecular weight less than 1450 is strong osmotica while the larger PEGs have little influence on the osmotic potential of media, unless the media are of high concentrations (Dumont-BéBoux *et al*., 1999). Janes (1974) reported that absorption of PEG by plant cells is inversely proportionate to its molecular size, high molecular weight PEG will probably not be absorbed by the pollen, but play the role as an osmoticum in the medium. When the osmotic pressure of a medium is lower than that of the pollen grain, water from the medium is forced into the pollen grain. As a result, the cell wall at the pores of the pollen grain cannot sustain the additional pressure and bursting occurs (Adhikari & Campbell, 1998). Bursting had been observed in pollen grains incubated in sucrose solutions without PEG in this study, which means osmotic pressure of the sucrose solution is lower than that of the pollen grains, therefore, bursting could occur. As shown in this study, when PEG 20000 and sucrose had been used, the pollen tube showed development and double tubes as well as branched pollen tubes were observed. The effects of sucrose and PEG and their combined influence appear to be very complex and they have drastic, sometimes antagonistic, effects on pollen germination and tube growth (Dumont-BéBoux *et al*., 1999). In Douglas fir

pollen, *Pseudotsuga menziesii*, high molecular-weight PEG slowed pollen tube development and diminished the number of tubes but greatly improved tube morphology (Dumont-BéBoux & von Aderkas, 1997). Similar observations are also obtained in present study. The sucrose/PEG ratio of the medium has been shown to govern the number of tubes, rate of appearance and the morphology of the pollen tubes (Present study; Dumont-BéBoux *et al*., 1999).

The pollen tube growth showed better development in the media containing calcium, manganese and potassium (Current study, see table 3.10). The calcium ion is required for pollen germination, regulation of pollen tube growth and elongation (Brewbaker & Kwack, 1963; Feijó *et al*., 1995; Taylor & Hepler, 1997). The other requirements for the *in vitro* growth of pollen tubes, besides the calcium gradients, also involved various ions, including potassium and manganese (Derksen *et al*., 1995). In some plant species, calcium ions act as a chemotropic agent (Steer & Steer, 1989). Calcium ion is known as a second messenger capable of influencing almost all events in cells and plays a dominant role in pollen tube growth (Derksen *et al*., 1995). In present study, fresh pollen grains failed to germinate in the various concentrations of sucrose. Even with the addition of calcium nitrate, boric acid, manganese sulphate and potassium nitrate, the pollen grains consistently failed to germinate even though the pollen grains were tested to be viable in tetrazolium chloride. Hence, PEG is the most important ingredient to induce pollen germination and regulate pollen tube growth and elongation.

3.4.5 Pollination experiments and pollen-ovule ratio

The occurrence of heterostyly in the Polygonaceae was first described and suggested in *Fagopyrum esculentum* by Hildebrand (Darwin, 1892 quoted by Chen & Zhang, 2010; Reddy *et al*., 1977; Samborska-Ciania *et al*., 1989). However, the

breeding system associated with this floral bi-morphism had been worked on in only a few species in the Polygonaceae family, i.e. *Fagopyrum esculentum* (Namai & Fujita, 1995; Woo *et al*., 1999), *Persicaria japonica* (Nishihiro & Washitani, 1998a, 1998b) and *Polygonum* (Hassan & Khan, 1996, quoted by Liza *et al*., 2010).

Fagopyrum esculentum is the only member in Polygonaceae that has been commercialized as a popular food source and is widely planted and utilized all over the world. This common buckwheat has high nutritional value but its productivity has declined mainly due to self-incompatibility caused by dimorphic heterostylism (Adachi, 1990). *F. esculentum* has been reported to be an insect-pollinated plant, mainly by honey bees (Apidae) (Björkman, 1995; Björkman & Pearson, 1995).

A pilot study on the breeding system of seven species of *Polygonum* has been carried out in China and the results indicated that the breeding system of these species was mainly out-crossing and partly mixed selfing (Fang *et al*., 2009). In this study, it has been suggested that the P/O ratio was positively correlated to the length of inflorescence and the diameter of the flower. The P/O ratio of the species with homostylous flower was significantly lower than that of the species with heterostylous flower (Fang *et al*., 2009).

In *Persicaria japonica*, intraflower and intra-inflorescence pollination were suggested to be negligible in pin and thrum flowers (Nishihiro & Washitani, 1998a). These results contrast markedly with some other studies which suggest that intraflower self-pollination were observed especially in the pin flowers of *Linum perenne* (Ganders, 1974; Nicholls, 1986). In *P. japonica*, Nishihiro & Washitani (1998b) also reported that, herkogamy can sufficiently reduce both intraflower and intra-inflorescence self-pollination.

In one of the trioecious species in the family of Polygonaceae, *Coccoloba cereifera*, the female individuals produce pistillate flowers and set fruits, indicating that

C. *cereifera* is agamospermic with no possibility of pseudogamy (Silva *et al*., 2008). These hermaphrodite individuals of *C. cereifera* also produced fruits in both control treatment and selfing treatment, but the rate of fruiting was lower than the fruits produced by pistillate flowers (Silva *et al*., 2008). Agamospermy has also been reported in a few other species of Polygonaceae such as *Acetosa arifolia*, *A*. *thyrsiflora* (Nygren, 1954) and *Atraphaxis frutescens* (Asker & Jerling, 1992).

Polygonum persicaria and *Polygonum lapathifolium* were probably entomophilous (Insect pollination) and automatic self-pollination (Cleistogamy) has also been reported owing to the incurving of certain stamens on to the stigma (Simmonds, 1945). Practically, plants that can produce cleistogamous flowers will also produce chasmogamous flowers; therefore some self-pollinated flowers could also involve be cross-pollinated. Simmonds (1945) also reported that *Polygonum minus* (Synonym *Persicaria minor*, common local name is daun kesum) was more often self-pollinated than *P*. *persicaria* and *P*. *lapathifolium* while *P*. *aviculare* was habitually self-pollinated.

The breeding system in *Antigonon leptopus* is remarkably unique. The flowers are functionally unisexual at any given time, i.e. the flowers are in the staminate phase on the first day and the pistillate phase on the next day. This sexual system prevents autogamy, minimizes geitonogamy and maximizes xenogamy. Without pollinators, *A*. *leptopus* would not be able to set fruits, hence this plant is obligately dependent on pollinator activity (Raju *et al*., 2001). *Polygonum thunbergii* has been reported to have both cleistogamous and chasmogamous flowers (Momose & Inoue, 1993). *P*. *thunbergii* was cross-pollinated by several types of insects in the chasmogamous flowers. However, chasmogamous flowers of *P*. *thunbergii* could also produce seeds by self-pollination in the complete absence of pollinators. In *P*. *thunbergii*, the fruit set in

bagging experiments was 47%, showing strong self-compatibility in this species (Momose & Inoue, 1993) .

This present study provides further evidence that *Persicaria chinensis* var. *chinensis* is self-compatible. There was 17% of fruit set when the bagging experiment was carried out. In open pollination, 47% of the flowers received pollen grains and set fruits. However, heterostylous plants are found to be strongly cross-pollinated due to its floral characteristics (Reciprocal herkogamy). P/O ratio of *P. chinensis* var. *chinensis* suggests facultative xenogamy, i.e. preferably cross-pollinated during the period when pollinator activity is reliable (Present observation & refer to section 3.3.7) and self-pollinate in the absence of pollinators. Hence, the open pollination experiment showed a higher success rate of fruit set compared to the bagging experiment. This homogamous plant exhibits a number of adaptations to induce cross-pollination, such as nectar production, having showy inflorescences and producing scent to attract the pollinators. However, emasculation experiments need to be carried out to test the possibility of reproduction by apomixis.

3.4.6 Possible pollinating agents

Results from the present study showed that *P. chinensis* var. *chinensis* is visited by a variety of insects from eight families, mainly from the family Formicidae (Ants), Apidae (Honey bees and stingless bees) and Syrphidae (Hoverflies). Pollination of the flowers is not restricted to any particular species of insects. *Persicaria* flowers are borne in showy inflorescences. The hermaphrodite flowers are white with tinges of pink; they produce nectar and ample pollen grains during anthesis. Flowering is non-seasonal and the peak of pollen production is well-synchronized with the highest level of insects foraging activity, i.e. between 0700–1000 hours.

Chen & Zhang (2010) reported twelve families with at least fifteen species of possible pollinators visiting *Polygonum jucundum* and six families of visitors that carried the pollen grains of *P. jucundum* on their bodies. The hover fly family (Syrphidae) was the most frequent visitor of this plant and all of the five species in the family of Syrphidae carried the pollen grains of *P. jucundum* on their bodies.

Various species of insects were observed to visit the flowers of the *Persicaria japonica* (Nishihiro & Washitani, 1998b). The most frequent visitors were *Eristalomyia tenax* (Family Syrphidae) and *Polistes chinensis antennalis* (Family Vespidae). Syrphids collected nectar and pollen, while the wasps collected only nectar. When visiting, the insects moved along the axis of the inflorescence acropetally or basipetally and foraged on almost all the flowers within the inflorescences (Nishihiro & Washitani, 1998b).

Hymenopterans are the most important pollinators worldwide (Beattie, 2006). In Southeast Asia rainforests, social bees (Mostly *Trigona* and *Apis*) are highly important pollinators, followed probably by Lepidoptera, herbivorous beetles and, possibly, *Thrips*. *Apis* bees (But not *Trigona* species) are one of the important species in montane, subtropical and non-forest habitats (Corlett, 2004). In *P. chinensis var. chinensis*, *Apis* and *Trigona* species were the second most common visitors and probably the most effective pollinators, helping to produce fruit set of 47.5% in the open pollination experiment. Since most of the heterostylous plants are self-incompatible, insect pollinators are required to ensure cross-fertilization (Marshall, 1969).

Honey bees, *Apis mellifera*, are the most important pollinator of buckwheat, *Fagopyrum esculentum* in New York (Björkman, 1995; Björkman & Pearson, 1995). However, honey bees are not effective in delivering large pollen loads over a short time, yet large pollen loads are vital for ensuring fertilization by superior pollen through

pollen competition (Björkman, 1995). *Apis cerana* and *Trigona laeviceps* visit a large range of flowers and collect pollen and nectar not only for themselves but also for their broods. This makes it necessary for bees to visit flowers of different or the same trees frequently. Thus, inter-species cross pollination could happen easily during the flowering period (Free, 1977; Soepadmo, 1979, 1989). *Trigona* bees appear to be one of the important pollinators of lowland tropical rain forest (Appanah, 1979; Corlett, 2004).

In *Antigonon leptopus*, bees and thrips were the most consistent foragers in terms of number of visits (Raju *et al*., 2001). *Apis cerana* and *Apis florea* were among the numerous honey bee visitors and they paid a large number of visits to the flowers. Thrips fed on nectars and pollen. They were found moving within and between flowers of the same individual by staying on *A*. *leptopus* all the time. In *P*. *chinensis* var. *chinensis*, thrips were very common visitors and can be found abundantly in an inflorescence. Their foraging behavior was similar as that reported in *A*. *leptopus*, mostly moving within the flower and occasionally moving to the next flowers of the same inflorescence on the same plant. In tropical lowland rain forest, pollination by thrips has been reported in some species of the family Myristicaceae (Bawa *et al*., 1985) and Dipterocapaceae (Appanah & Chan, 1981).

Flowers of *Polygonum thunbergii* were visited by various insect groups, e.g., flies, wasps and bees (Momose & Inoue, 1993). These pollinators were unspecialized and their visits are not restricted to a specific plant group. In a rare buckwheat species, *Eriogonum pelinophilum*, similar observation has been reported (Tepedino *et al*., 2011). *E*. *pelinophilum* attracted a large assortment of non-specialized insects, i.e. wasps, flies, bees and ants. In *Polygonum bistorta*, Simmonds (1945) reported pollination by insects and probably entomophily also occurred in *P*. *persicaria* and *P*. *lapathifolium*.

Hickman (1974) suggests that, ants are one of the pollinators that interact with *Polygonum cascadence*, a small and inconspicuous plant. This annual plant usually grows in a dense population, has sessile flowers that bloom synchronously with readily accessible nectaries, and apparently self-incompatible. The growth habit and floral characters suggested by Hickman (1974) were positively promoting ant-pollinated mechanism in *P. cascadence*. Ant-pollination interactions are characterized by low expenditure of energy by both ant and plant, since walking may reasonably be expected to require less energy per unit distance than flying. Further, *Formica argentea* did not engage in grooming behaviour (Clean and brush movement) when foraging in a *Polygonum* population and all *F. argentea* workers that were collected carried pollen grains.

Ants from the family Formicidae, are the most frequent of the insect visitors in *P. chinensis* var. *chinensis*. *P. chinensis* var. *chinensis* is a scrambling herb that shared some common floral characters with *P. cascadence* such as small flowers, accessible nectaries, dense population and synchronous blooming. In addition, *P. chinensis* var. *chinensis* flowers are produced in terminal inflorescence with long peduncles, the flowers release strong odour when blooming and produce large quantities of nectar and pollen grains. Flowers with these characters have the tendency to be exposed to air-borne pollinators.

Ants are common flower visitors, but ants are considered villains in floral interaction (Faegri & Van Der Pijil, 1979; Herrera *et al*., 1984). Ants, *Formica neorufibarbus*, showed a significant preference for sweet flowers and these ants destroy the ovaries of the flowers during nectar foraging and have been reported as nectar thieves of *Polemonium viscosum* (Galen, 1983). Ants generally are small, wingless, showing frequent grooming and there is the presence of antibiotic secretions that reduce pollen viability (Corlett, 2004; Proctor *et al*., 1996). Pollination by ants are very rare,

although ants appear in great abundance (Hölldobler & Wilson, 1990). Commonly, ant has been considered as poor or ineffective pollinator due to its small size since it will prevent effective contact with anthers and stigmas (Armstrong, 1979; Herrera *et al*., 1984; Inouye, 1980). Some researchers reported that the nest-building and brood-rearing habits of ants need to secrete large amounts of antibiotics to combat pathogenic microorganisms. These secretions would disrupt pollen function, reduce pollen viability, reduce percentage of pollen germination and retard pollen tube growth (Beattie, 2006; Beattie *et al*., 1984; Nakamura *et al*., 1982).

However, ants are very frequent visitors to flowers with accessible nectar, from the ground layer to the upper canopy of the forest (Present study; Corlett, 2004). Ants have been shown to be important pollinators for low-growing plants in open and dry habitats, arid lands and high mountainous regions (Gómez & Zamora, 1992; Gómez *et al*., 1996). In dioecious *Borderea pyrenaica* (Dioscoreaceae), ants were the most effective pollinators although ants were less abundant on flowers than other visitors (Garcia *et al*., 1995). Ants had been shown to effect self-pollination in *Blandfordia grandiflora* (Liliaceae) and produce seed set of 17% but they did not effect cross-pollination (Ramsey, 1995).

In conclusion, the appearance of the visitors in *P. chinensis* var. *chinensis* is mostly concentrated during 0700–1000 hours when nectar and pollen grains are available abundantly. The highland variety of *P. chinensis* var. *chinensis* is mostly found at the elevation from 1200 m–1600 m, hence at the lower montane forest, the activity of bees is rather limited and this may explain why this variety is pollinated by generalist insects, such as ants, thrips, bees, flies, moths and beetles. However, whether the ants and thrips are reliable pollinators need to be further studied to validate the effectiveness of these insects.

3.4.7 Seed viability, seed germination and seedling morphology

Seed viability of highland variety of *P. chinensis* var. *chinensis* tested in 1% tetrazolium test was 77%. Seed germination of *P. chinensis* var. *chinensis* is epigeal. The average percentage of germination for unstored fresh seeds enclosed in perianth segments ranged from 47% germinated under natural highland conditions to 34% under lowland laboratory conditions. Under natural conditions, the seeds often germinate while they are still attached to the perianth segments. These seeds take one week to germinate in their highland environment and 12 days in lowland laboratory environment for the emergence of the first seedling. Three days after the appearance of the radicle, the hypocotyl elongates and the cotyledons emerge two days later. About one week after the appearance of the hypocotyl, the cotyledons start to grow. In the highland environment, without regular watering, seedlings were shorter than those growing in the lowland laboratory (With regular watering). The first leaf was formed one week after the emergence of the cotyledon in the highland environment and after 10 days in the lowland growing environment. Some seeds from the highland and lowland growing environment germinated after 7 weeks.

Seeds with the perianth segments removed in *Rumex crispus* and *R. obtusifolius* showed 95% germination within one week whereas seeds enclosed within perianth segments showed only 60% germination over a three month period (Cavers & Harper, 1966). It is further reported that, the germination behavior of the seeds which are still enclosed in perianth segments is erratic, slow and incomplete (Cavers & Harper, 1966). However, under natural conditions, seeds often germinate while they are still encased in perianth parts.

In *Fallopia japonica* (Synonym *Polygonum cuspidatum*), the germination percentages varied from 9.2±10.5% to 64.4±24.5% depending on seed lots and treatment (Tiébré, *et al*., 2007). Some researchers reported that, the dormant period was

shorter in seeds from a higher altitude than those from a lower altitude in *Polygonum cuspidatum* (Nishitani & Masuzawa, 1996; Shibata & Arai, 1970).

According to Chin (1980) and Chin *et al*. (1977), germination behaviour was classified as 'rapid' if all viable seeds germinated within 12 weeks. Therefore, the germination behaviour of *Persicaria chinensis* var. *chinensis* is considered as rapid and a higher germination percentage and earlier appearance of the first seedling is observed under higher altitude environment (Present study).

CHAPTER 4

CYTOTOXIC ACTIVITY

4.1 General introduction

Persicaria chinensis var. *chinensis* is used traditionally to clear heat and eliminate toxins as well as to treat dysentery, inflammatory skin disease, eczema and corneal nebula (Wan *et al*., 2009; Xie *et al*., 2007; Yang *et al*., 2009). Clinical and pharmacognostic studies show that, this plant can be used to treat inflammation, injury such as contusion, sprain and flu infection (Lin *et al*., 2001; Lin *et al*., 2006).

Polygonum chinense also shows *in vitro* antibacterial properties against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Li *et al*., 2004). Research from China demonstrated the *in vitro* antitumour activity against human hepatocellular carcinoma cell line, Bel-7404, of *Polygonum chinense* using MTT assay (Wei *et al*., 2003). However, the constituents of antitumour properties of *Polygonum chinense* still require further investigation and confirmation (Xie *et al*., 2007).

This project is undertaken to evaluate the cytotoxic activity of the crude methanol, dichloromethane and water extracts of the leaves, stems and whole plant of both the lowland cultivated and highland wild varieties of *P. chinensis* var. *chinensis* by an *in vitro* growth inhibition assay system against selected cancer cell lines of human epithelial carcinoma of cervix cell line (CaSki), human epithelial adenocarcinoma of ovary cell line (SKOV-3), human epithelial adenocarcinoma of colon cell line (HT-29), human epithelial carcinoma of lung cell line (A549), human epithelial adenocarcinoma of mammary gland (breast) cell line (MCF7) and normal human lung fibroblasts cell (MRC-5).

4.2 Materials and Methods

4.2.1 Samples of *Persicaria chinensis* var. *chinensis*

Approximately 2 kg each of the leaves, stem and the whole plant (leaves and stem) of lowland cultivated *Persicaria chinensis* var. *chinensis* were collected from the lowland study site at Sagil, Tangkak, Johor. The highland wild samples of *P. chinensis* var. *chinensis* were collected from Gunung Ulu Kali, Bentong, Pahang, Malaysia (Figure 4.1).

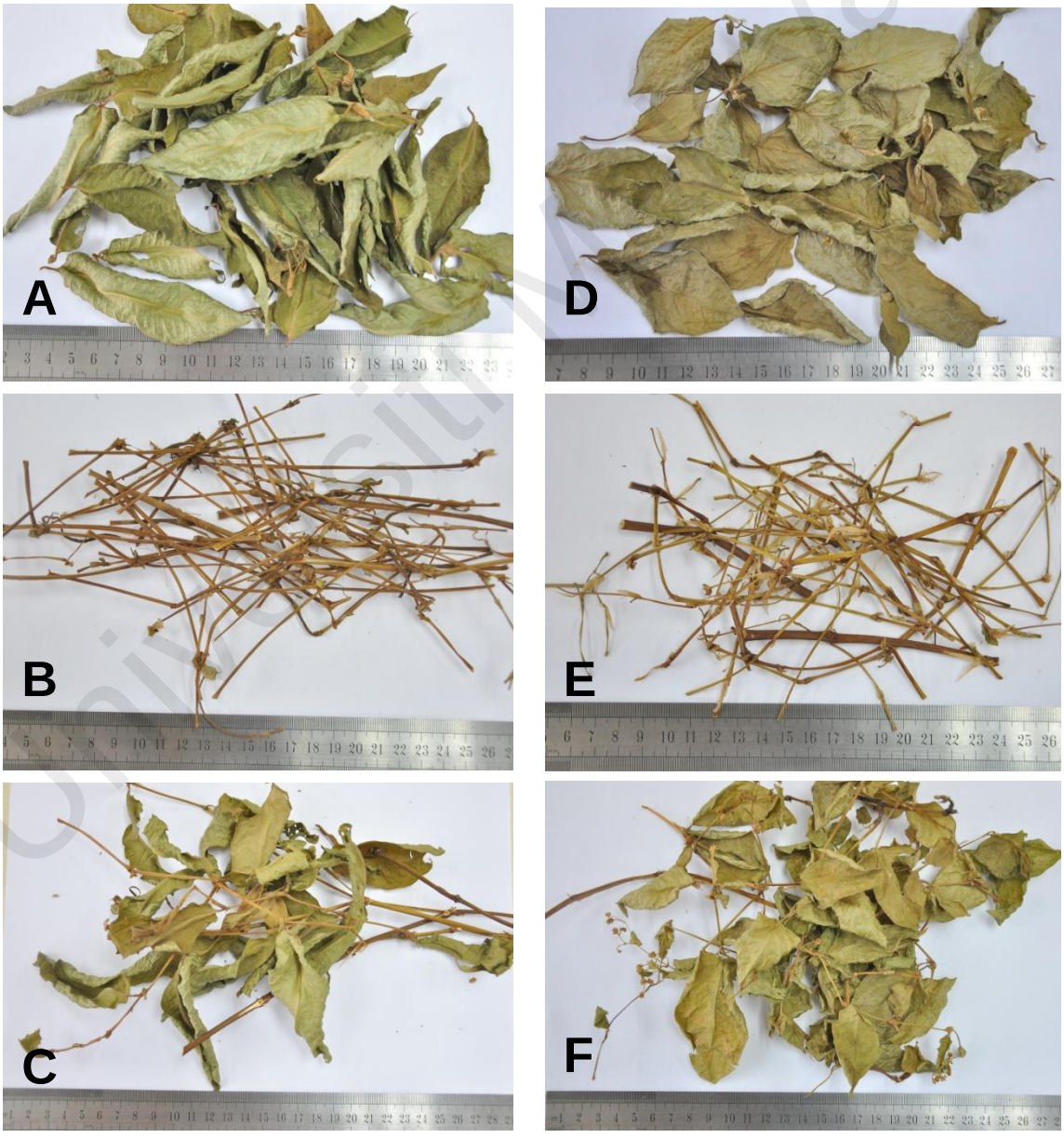


Figure 4.1: The samples of *P. chinensis* var. *chinensis* after drying. A–C: highland variety. D–F: lowland variety. A & D: leaves. B & E: stems. C & F: whole plants.

4.2.2 Preparation of crude extracts

The different plant parts from fresh samples collected were immediately separated from each other, i.e. whole plant, leaf and stem. They were subsequently washed with tap water, cleaned and dried with towels and tissue paper before being placed into an oven (Memmet) at 40ºC. After 72 hours of oven-drying, the dried samples were grounded into fine powder in a blender. 40 g of the powder from each sample was successively extracted with 400ml of dichloromethane, 400 ml of methanol and 400 ml of distilled water for 3 days with constant shaking (200 rpm) on a Series 25D incubator shaker (New Brunswick Scientific) at room temperature (28ºC).

Crude dichloromethane and methanol extracts were obtained after the solvent was removed using a rotary evaporator (Bruchi) whereas the crude water extract was obtained after freeze-drying. 20 mg crude dichloromethane and methanol extracts were then redissolved in 1 ml dimethylsulfoxide (DMSO) (Sigma) in a 1.5 ml provial to obtain a stock solution of 20 mg/ml. 20 mg crude water extract was redissolved in 1 ml sterile distilled water to obtain a stock solution of 20 mg/ml. The stocks were stored at -20ºC (Freezer, Acson) until use. The concentration of the samples was prepared according to the requirement for the neutral red cytotoxicity assay.

4.2.3 Glassware preparation and sterilization technique

Pipettes, beakers, bottles (Schott Duran), conical flasks and other required glasswares were soaked overnight in 7x detergent (Flowlab), and later washed with tap water. All the glasswares were rinsed with distilled water before drying in an oven (Memmert) at 60ºC. Beakers and conical flasks were covered with aluminum foil; all the tips and provials were sealed in plastic bags.

Schott bottles with plastic caps were loosely screwed to allow penetration and escape of steam during autoclave sterilization. All glasswares, tips and provials were

autoclaved for 20 minutes at 120ºC and 1.1 kg/cm^2 (15 lb) pressure. After cooling, the loose caps were tightened immediately and the tips and provials were dried in an oven (Memmert) at 60ºC. Pipettes were placed in an aluminum pipette canister with the tips at the closed end of the canister. The canister was closed tightly and heat sterilized at 180ºC for 2 hours in a dry heat sterilization oven (Memmert).

4.2.4 Cell Lines

Human epithelial adenocarcinoma of ovary cell line (SKOV-3) (ATCC No: HTB-77), human epithelial carcinoma of cervix cell line (CaSki) (ATCC No: CRL-1550), human epithelial adenocarcinoma of mammary gland (breast) cell line (MCF7) (ATCC No: HTB-22), human epithelial carcinoma of lung cell line (A549) (ATCC No: CCL-185), human epithelial adenocarcinoma of colon cell line (HT-29) (ATCC No**:** HTB-38) and normal human lung fibroblasts cell MRC-5 (ATCC No: CCL-171) were purchased from the American Tissue Culture Collection (ATCC, USA). The viability of the cells was checked before and after treatment by the tryphan blue exclusion dye method (section 4.3.2). Cell stocks were stored frozen in liquid nitrogen (-196ºC) until use.

4.2.5 Revival of Cells

Prior to thawing of each frozen cell line, 20% of supplemented Dulbecco's Modified Eagle's Medium (Sigma) or RPMI 1640 medium (Sigma) was thawed at 37℃ (Appendix D). One millilitre of the culture medium was then pipetted into a sterile centrifuge tube ready to be used. Vials containing frozen cells were removed from the liquid nitrogen tank before plunging into a beaker of ice and then transferred to a 37ºC water bath (Grand Instruments) for quick thawing using gentle hand agitation. The cells in the cryovial were then transferred into a pre-prepared sterile centrifuge tube (Orange

Scientific) containing 1 ml of 20% culture medium and spun at 1000 rpm (Clements 2000) for 5 minutes at room temperature. The supernatant was discarded and the pellet resuspended and mixed in the 1 ml of 20% culture media. 9 ml of 20% culture medium was transferred into a 25 ml tissue culture flask (Nunc) and 1 ml of the resuspended cells were added into the culture flask containing 9 ml of culture medium. The suspended cells were observed for any contamination under the inverted microscope (Leica). The cells were subsequently incubated at 37ºC in a 5% CO_2 incubator (Jouan).

4.2.6 Maintenance of Cells

CaSki, MCF7, HT-29 and A549 cells were cultured and maintained in RPMI 1640, SKOV-3 cells in DMEM medium (Dulbecco's Modified Eagle's Medium) and MRC-5 in Eagle's Minimum Essential Medium (MEM) respectively, in 25 ml tissue culture flasks. These cultures were incubated in a humidified atmosphere at 37ºC in a 5% CO_2 incubator. These cultures were subcultured every 2 or 3 days and the 10% culture medium (Appendix D) was changed every 2 days or alternatively from time to time based on the colour changes. The cells were routinely checked under an inverted microscope (Leica) for any contamination. When the cells were fully confluent, they were subcultivated.

4.2.7 Subcultivation of Cells

When adherent cells attached to form a single layer in a tissue culture flask at the confluent stage, they were then ready for subcultivation. 10% culture medium was thawed in a 37ºC water bath and accutase (ICT) was also thawed at room temperature. Confluent cells were washed twice using phosphate buffer saline (PBS) at pH 7.2. The cells were first detached from the flask by incubating in 1 ml accutase and 3 ml PBS solution for 10 minutes at 37ºC. The flask was gently tapped to help detach the cells

still attached to the bottom of the flask. The cell suspension was then transferred into a sterile centrifuge tube and centrifuged for 5 minutes at 1000 rpm at room temperature. The supernatant was discarded and 3 ml of 10% supplemented culture medium was added to resuspend the pellet. The cells were then distributed into 3 flasks (2 new flasks and 1 current flask) each containing 9 ml of culture medium. The flasks were then further incubated in a humidified atmosphere at 37ºC in a 5% CO_2 incubator.

4.2.8 Cryopreservation of cells

The monolayer confluent cells were fed with fresh 10% supplemented medium 24 hours before freezing. The medium in the tissue culture flask was discarded and the cells were washed with 10 ml of sterile PBS pH 7.2 (Appendix D). The cells were detached from the tissue culture flask as described in section 4.2.7. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 3 ml of freezing medium. The cell suspension was aliquoted in 1 ml amounts into 3 sterile cyro-freezing vials (Nalgene). The vials were left to stand in a polystyrene cup which was kept in the vapour phase in a liquid nitrogen tank at -70ºC overnight. The vials were then plunged into the liquid nitrogen tank and stored at -196 ºC.

4.3 Analysis for cytotoxic activity of *P. chinensis* var. *chinensis* extracts

The leaves, stems and whole plants crude extracts derived from the highland and lowland variety of *P. chinensis* were evaluated for their cytotoxic activities against selected cancer and normal cell lines using the neutral red assay.

4.3.1 Serial dilution of *P. chinensis* var. *chinensis* stock solutions

Aliquots of 10 µl from the plant crude methanol and dichloromethane extract stock solutions (20 mg/ml) were diluted in 90 µl of 10% DMSO to produce a concentration of 2000 µg/ml (tube A). It was then further diluted by adding 10 µl from tube A into 90 µl of 10% DMSO to provide a concentration of 200 µg/ml (tube B). 10 µl from tube B was added into another 90 µl of 10% DMSO to provide a final concentration of 20 µg/ml (tube C). (Same method was applied to plant crude water extracts but by replacing DMSO with sterile distilled water)

4.3.2 Incubation of cell lines with *P. chinensis* var. *chinensis* extracts

The confluent cells were detached from the tissue culture flask as described in section 4.2.7. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1 ml of supplemented medium to produce a stock cell suspension. For cell enumeration, 100 µl of the stock cell suspension was transferred into a vial with 900 µl of 0.4% tryphan blue (Appendix D) and mixed. 20 µl of the suspension with dye was loaded at the two edges of the cover slip of a haemacytometer (Scherf). The haemacytometer was then examined under a microscope using objective lens 20x (Leica). The unstained viable cells were counted. The cell suspension at a concentration of 3×10^4 cells/ml was prepared according to the formula below:

$$P_1 \times 10^5 \times V_1 = P_2 \times V_2$$

P_1 : Average number of viable cells counted from the haemacytometer

10^5 : Counting chamber conversion factor & dilution factor with dye

V_1 : Volume of stock cell suspension needed

P_2 : The desired cell concentration in the cell suspension

V_2 : Volume of 10% supplemented medium used for seeding

200 µl of the cell suspension with known cell density was transferred into the wells of a 96-wells plate (Nunc) and incubated for at least 4 hours in a humidified atmosphere at 37ºC in a 5% CO_2 incubator to allow the cells to adhere and achieve 60–70% confluence before the test agents were added. The cells in the 96-wells plate were then treated with fresh plant stock solution serially diluted at varying concentrations (1, 10, 25, 50, 75 and 100 µg/ml) and subsequently incubated for 72 hours in a humidified atmosphere at 37ºC in a 5% CO_2 incubator. All tests were performed in triplicates and untreated cells with growth medium only served as negative controls.

4.3.3 Neutral Red Cytotoxicity Assay

The neutral red cytotoxicity assay was carried out based on the initial protocol described by Borenfreund and Puerner (1985). It quantifies the accumulation of the neutral red dye in the lysosomes of viable and uninjured cells.

At the end of the 72 hours incubation period, the medium in each well with or without the plant extracts was discarded followed by the addition with 200 µl of neutral red (NR) medium with the concentration of 50 µg/ml. The plate was further incubated in a 5% CO_2 incubator at 37ºC for another 3 hours to allow maximum uptake of the neutral red dye into the lysosomes of viable and uninjured cells. After the incubation

period, the medium was discarded and the cells in each well were washed with 200 µl of Neutral Red washing solution (Appendix D). The dye was eluted from the cells by incubation with 200 µl of Neutral Red resorb solution for 30 minutes at room temperature with rapid agitation on a microtiter plate shaker (LT BioMax 500). Dye absorbance was measured at 540 nm using ELISA reader (Titertek Multiskan MCC/340).

The average data from the triplicates for each plant extract concentration was expressed in terms of percentage of inhibition relative to the negative controls. The percentage of inhibition of each test sample was calculated according to the following formula:

$$\% \text{ of inhibition} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\ \%$$

Percentage of inhibition values were then used to plot dose-response curves. The IC_{50} is the concentration of a drug required for 50% inhibition *in vitro* (Cheng & Prusoff, 1973; Neubig *et al*., 2003) and was estimated from the plotted dose-response graphs according to the following formula:

$$\frac{\%_b - 50\%}{[\ ]_b - X} = \frac{50\% - \%_a}{X - [\ ]_a}$$

$[\ ]_a$ = concentration A ; $\%_a$ = % of inhibition caused by $[\ ]_a$
$[\ ]_b$ = concentration b ; $\%_b$ = % of inhibition caused by $[\ ]_b$
X = concentration of the extract when 50% of inhibition occurred.

*From the calculated percentage of inhibition, we use mathematical calculation to estimate that the concentration that can inhibit 50% of cell death actually falls in between the [concentration] $_a$ with its % of inhibition and [concentration] $_b$ with its % of inhibition.

According to the criteria of the American National Cancer Institute, the extract that gave IC_{50} being lower than 30 µg/ml was considered a crude extract promising for further purification (Suffness & Pezzuto, 1990), or considered cytotoxically active.

4.4 Results

4.4.1 Yield of crude extracts

The leaves, stems and whole plants of highland and lowland varieties of *P. chinensis* var. *chinensis* were extracted using three solvents namely methanol, dichloromethane and water. The yield was calculated according to the formula below:

$$\text{Yield (\%)} = \frac{\text{Weight of concentrated extract}}{\text{Weight of sample (40g)}} \times 100\ \%$$

The yield calculated for all 18 extracts are shown in Table 4.1. Total yield percentages ranged from 9.08% to 23.48%. Overall, the lowland variety showed higher percentages of total yield as compared to the highland variety. Leaf extracts were found to yield higher percentages as compared to the stem and whole plant extracts. Generally, the yield percentages from the methanol extracts (ranged from 5.69% to 15.23%) were found to be higher as compared to the water (ranged from 2.73% to 6.20%) and dichloromethane (ranged from 0.62% to 2.05%) extracts.

Table 4.1: Yield percentages from the highland and lowland variety of *P. chinensis* var. *chinensis*.

Plant	Part of the plant	Extraction solvent	Weight (g)	Yield (%)	Total yield (%)
Highland wild variety	Leaves	methanol	2.5819	6.45	11.70
		DCM	0.5874	1.47	
		water	1.5113	3.78	
	Stems	methanol	2.2779	5.69	9.08
		DCM	0.2638	0.66	
		water	1.0908	2.73	
	Whole plants	methanol	2.4079	6.02	10.86
		DCM	0.4422	1.11	
		water	1.4942	3.74	
Lowland cultivated variety	Leaves	methanol	6.0928	15.23	23.48
		DCM	0.8195	2.05	
		water	2.4799	6.20	
	Stems	methanol	4.8529	12.13	17.00
		DCM	0.2493	0.62	
		water	1.6985	4.25	
	Whole plants	methanol	4.3950	10.99	17.20
		DCM	0.6693	1.67	
		water	1.8174	4.54	

4.4.2 Cytotoxic activity of *P. chinensis* var. *chinensis*

The crude methanol, dichloromethane and water extracts of the leaves, stems and whole plants of the lowland and highland varieties of *P. chinensis* var. *chinensis* were evaluated for their cytotoxic potential against various human cancer cell lines and normal cell lines. The extracts were tested at varying concentrations of 1, 10, 25, 50, 75 and 100 µg/ml for 72 hours in a humidified atmosphere at 37ºC in a 5% CO_2 incubator. The negative control consisted of cells not treated with plant extracts. Figure 4.3 shows various untreated human cancer cell lines and normal cell lines. These controls exhibited normal proliferation rates and showed no signs of death after the incubation time of 72 hours.

The average percentage of triplicates and standard deviations of the 18 crude *P. chinensis* extracts against CasKi, SKOV-3, HT-29, A549, MCF7 and normal human lung fibroblasts cell, MRC-5 cells are shown in Appendix G. The cytotoxicity data obtained were expressed as inhibition percentage relative to negative controls and the IC_{50} values were estimated from the plotted dose-response graphs according to the formula mentioned in the materials and methods (graphs in Figures 4.4–4.6). IC_{50} value is the concentration of extract which results in 50% killing of the cells. Extracts with IC_{50} value $\leq$ 30 µg/ml are considered active for the cytotoxicity assay (Suffness & Pezzuto, 1990).

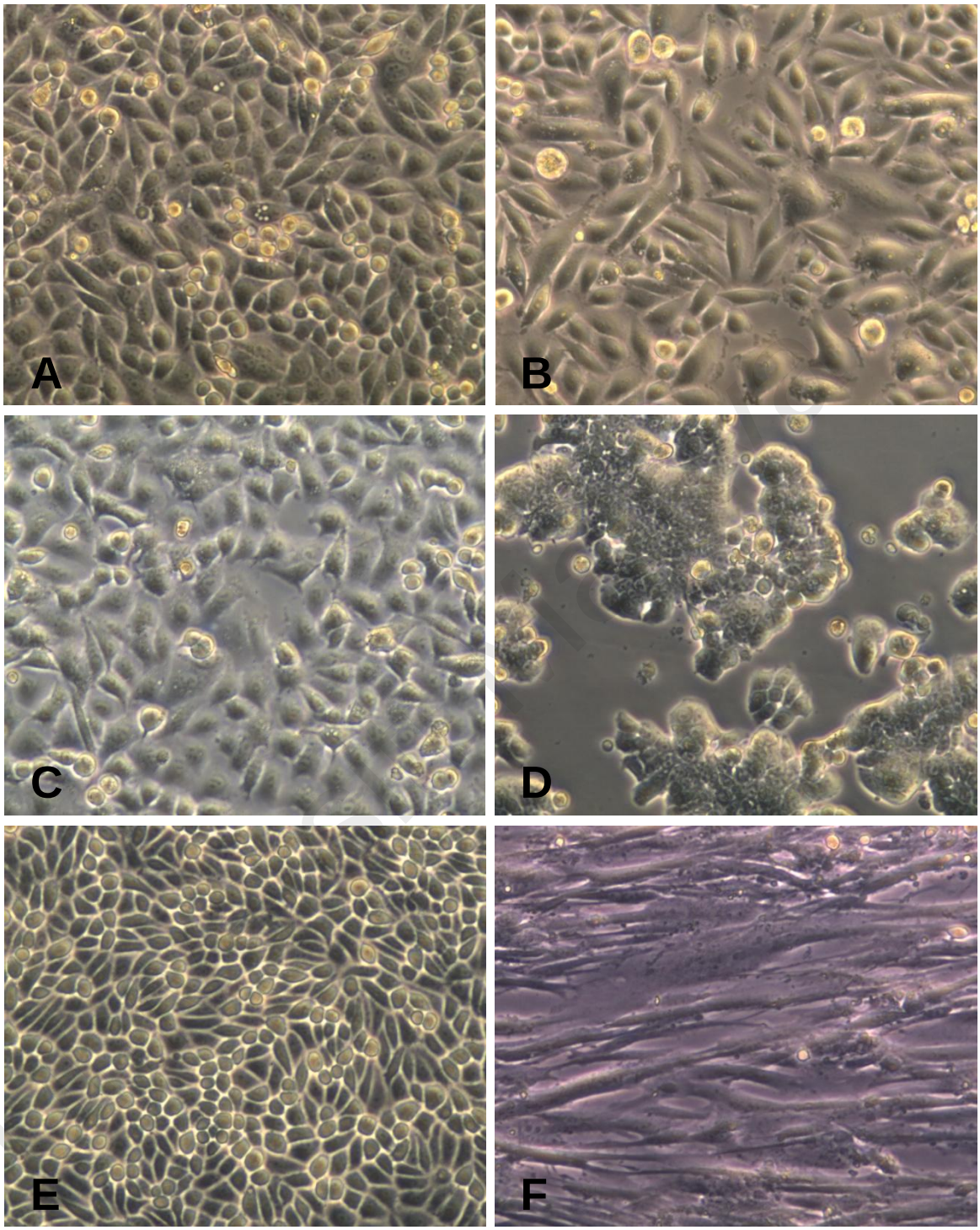


Figure 4.2: Photomicrographs (40× magnification) of various human cancer cell lines (A–E) and normal human cell lines (F). (A): CaSki cells incubated in RPMI 1640 medium. (B): MCF7 cells incubated in RPMI 1640 medium. (C): A549 cells incubated in RPMI 1640 medium. (D): HT-29 cells incubated in RPMI 1640 medium. (E): SKOV-3 cells incubated in DMEM medium. (F): normal human lung fibroblasts cell MRC-5 incubated in MEM medium.

4.4.2. (a) Cytotoxic activity of the methanol extracts of *P. chinensis* var. *chinensis*

As illustrated in Figures 4.4 A–L, all the cancer cell lines evaluated showed different cytotoxic profiles when treated with methanol crude extracts of different parts of the plant derived from lowland and highland varieties. The IC_{50} value for the methanol crude extract was determined and summarized in Tables 4.2 and 4.3.The increased inhibition activity against the cancer cells with increasing concentrations of the methanol extracts were observed after 72 hours of treatment. The results revealed that the leaf crude methanol extracts of both lowland and highland varieties of *P. chinensis* var. *chinensis* exhibited higher inhibition against all cancer cells tested as compared to dichloromethane and water extracts.

The results showed that the leaf crude methanol extracts of both the lowland and highland varieties of *P. chinensis* var. *chinensis* exhibited higher inhibition against all cancer cells tested as compared to the stem and whole plant methanol extracts. The IC_{50} values of the lowland and highland leaf crude methanol extracts ranged from 11 µg/ml–53 µg/ml and 19 µg/ml–62 µg/ml, respectively. The whole plant crude methanol extracts of the highland and lowland varieties exhibited higher inhibition than the stem crude methanol extracts. The IC_{50} values of the lowland and highland whole plant crude methanol extracts ranged from 46 µg/ml–63 µg/ml and 22 µg/ml–73 µg/ml, respectively.

i) Human epithelial carcinoma of cervix cell line, CaSki

Leaf methanol extract from the lowland variety inhibited the proliferation of CaSki with inhibition percentage of 49% at 10 µg/ml, 60% at 25 µg/ml and 86% at 100 µg/ml [Figure 4.4 (B)]. The resulting IC_{50} value of 11.37 µg/ml showed that the extract was actively cytotoxic against CaSki. However, the leaf methanol extracts from the highland variety did not show active cytotoxic activity against CaSki resulting IC_{50}

value of 53.45 µg/ml. Other parts of the plants (stem and whole plant) from the lowland and highland variety were also not active against CaSki and showed IC_{50} values of 54 µg/ml to 85 µg/ml (Tables 4.2 & 4.3).

i) Human epithelial adenocarcinoma of ovary cell line, SKOV-3

The leaf methanol crude extract of the highland variety showed inhibition to the proliferation of the SKOV-3 with the killing percentage of 34% at 10 µg/ml, 60% at 25 µg/ml and 91% at 100 µg/ml. The resulting IC_{50} value of 19.38 µg/ml indicated that the extract is actively cytotoxic against SKOV-3. The inhibition percentages against SKOV-3 cells by the whole plant methanol extract of the highland variety showed 35% at 10 µg/ml, 54% at 25 µg/ml and 90% at 100 µg/ml with a resulting IC_{50} value of 21.71 µg/ml indicated that the extract is actively cytotoxic against SKOV-3. All the other extracts derived from different plant parts of the highland and lowland varieties were not active against SKOV-3 with IC_{50} values ranging from 39 µg/ml to 69 µg/ml (Tables 4.2 & 4.3).

ii) Human epithelial adenocarcinoma of colon cell line, HT-29

All of the methanol crude extracts (leaf, stem and whole plant) derived from the lowland and highland varieties were not active against HT-29. The resulting IC_{50} values ranged from 40 µg/ml to 82 µg/ml (Tables 4.2 & 4.3).

iii) Human epithelial carcinoma of lung cell line, A549

Similar to the HT-29, all the methanol crude extracts from the leaf, stem and whole plant derived from the highland and lowland varieties were not actively cytotoxic against A549.The resulting IC_{50} values ranged from 48 µg/ml to 97 µg/ml (Tables 4.2 & 4.3).

iv) Human epithelial adenocarcinoma of mammary gland (breast) cell line, MCF7

The inhibition percentages of the MCF7 cells for the crude methanol extract of the leaves of the lowland variety were 46% at 25 µg/ml, 69% at 50 µg/ml and 77% at 100 µg/ml. This crude methanol extract of the leaf derived from the lowland variety attained IC_{50} values of 29.27 µg/ml indicated that the extract is actively cytotoxic against MCF7. Crude methanol extracts of the stem and whole plant of the lowland variety and the leaf, stem and whole plant were not actively cytotoxic against MCF7; showed IC_{50} values ranging from 49 µg/ml to 69 µg/ml (Tables 4.2 & 4.3).

v) Normal human lung fibroblasts cell, MRC-5

All of the methanol crude extracts (leaf, stem and whole plant) derived from the lowland and highland varieties were not actively cytotoxic against normal human lung fibroblasts cell, MRC-5. The IC_{50} values ranged from 85 µg/ml and above. This indicated that the methanol crude extracts did not inhibit the growth of normal human fibroblast, MRC-5 (Tablse 4.2 & 4.3).

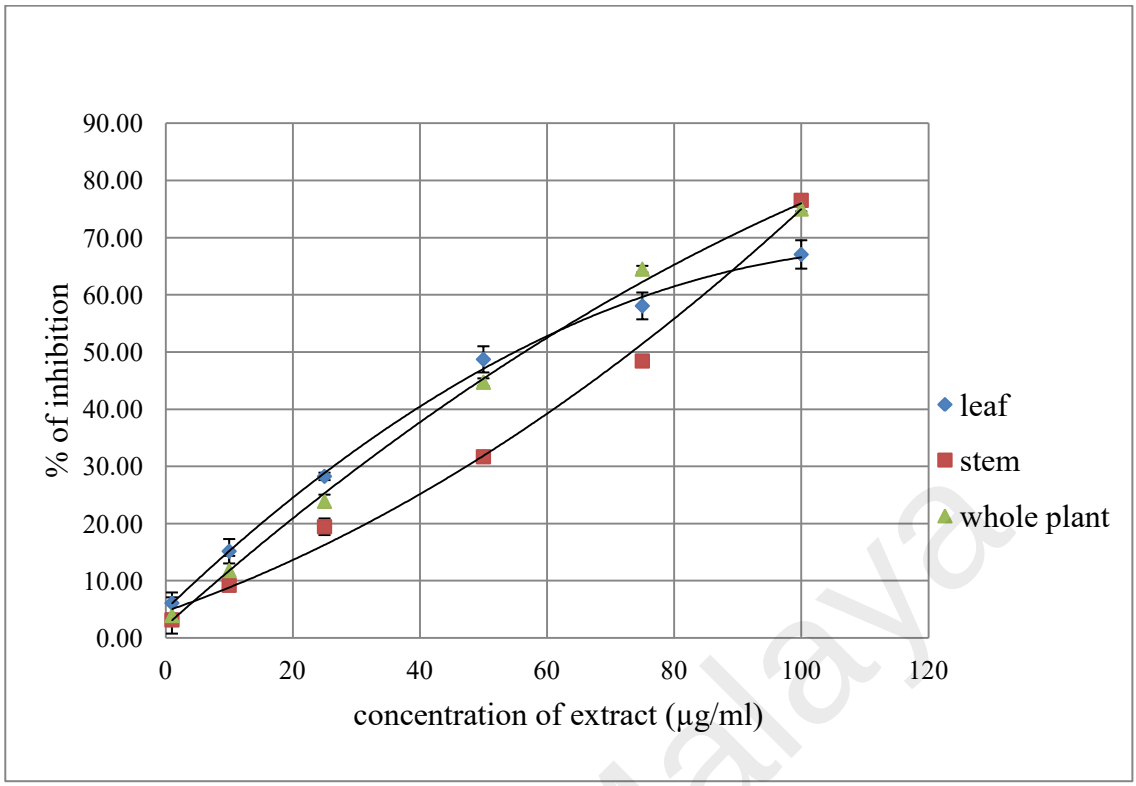


Figure 4.3 (A): The *in vitro* growth inhibition of CaSki cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

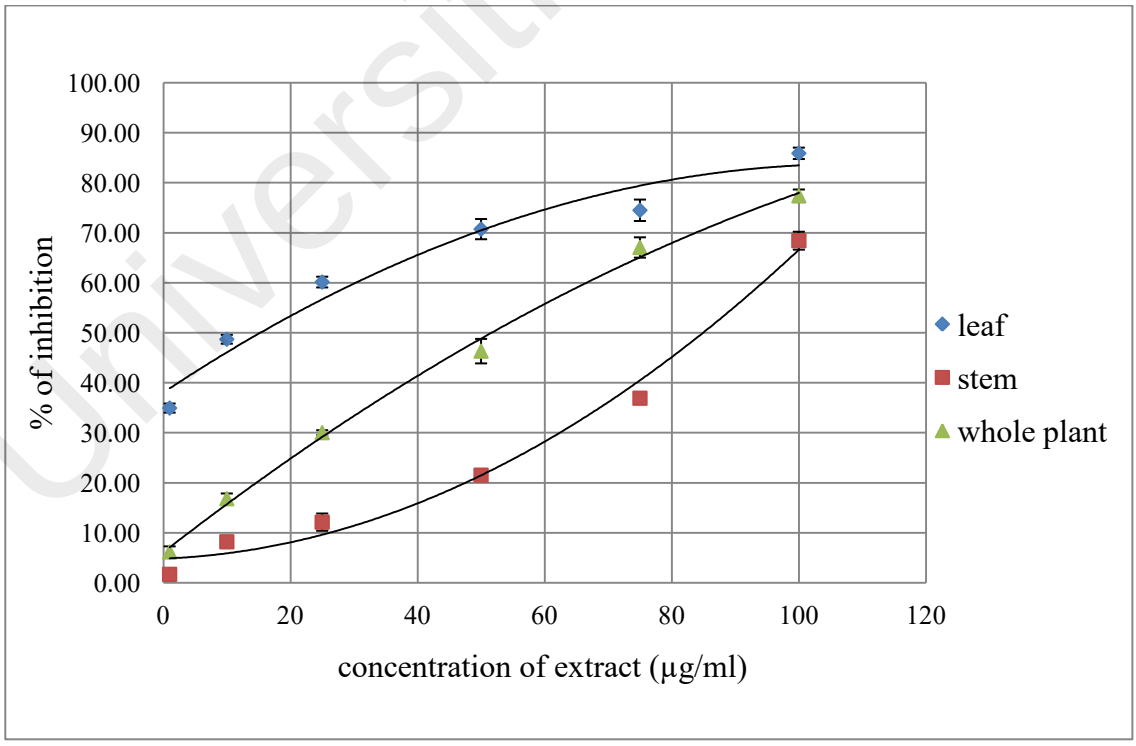


Figure 4.3 (B): The *in vitro* growth inhibition of CaSki cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

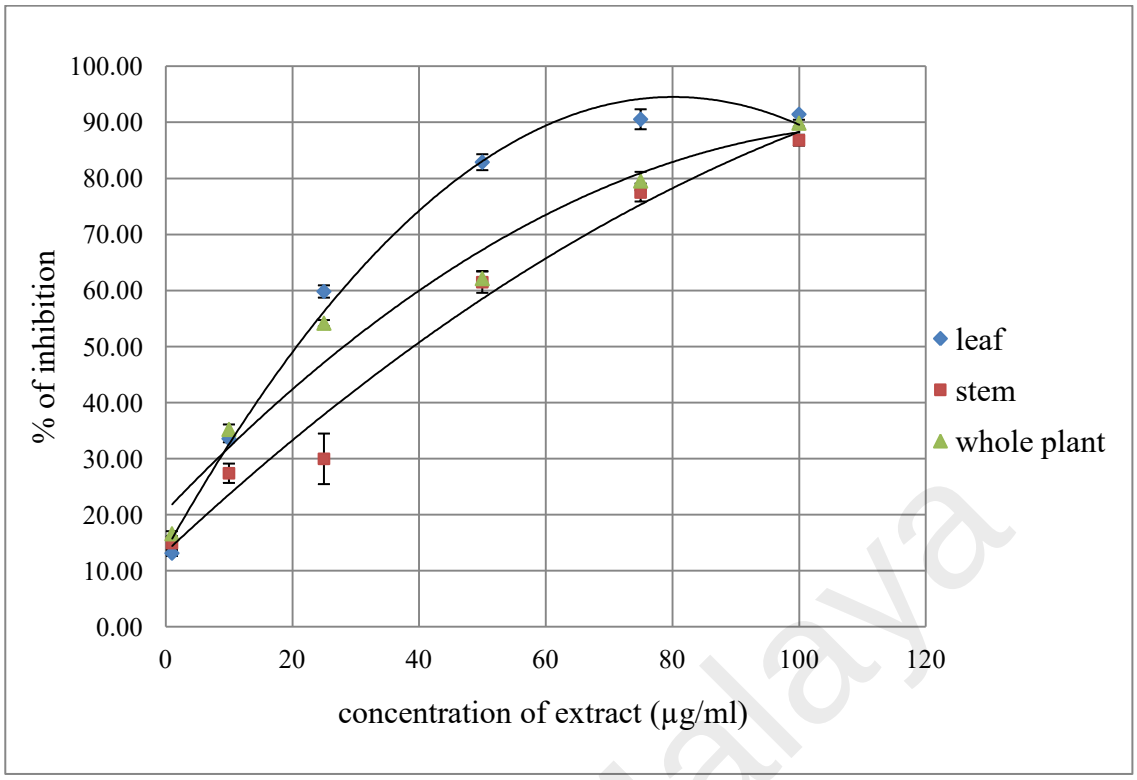


Figure 4.3 (C): The *in vitro* growth inhibition of SKOV-3 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

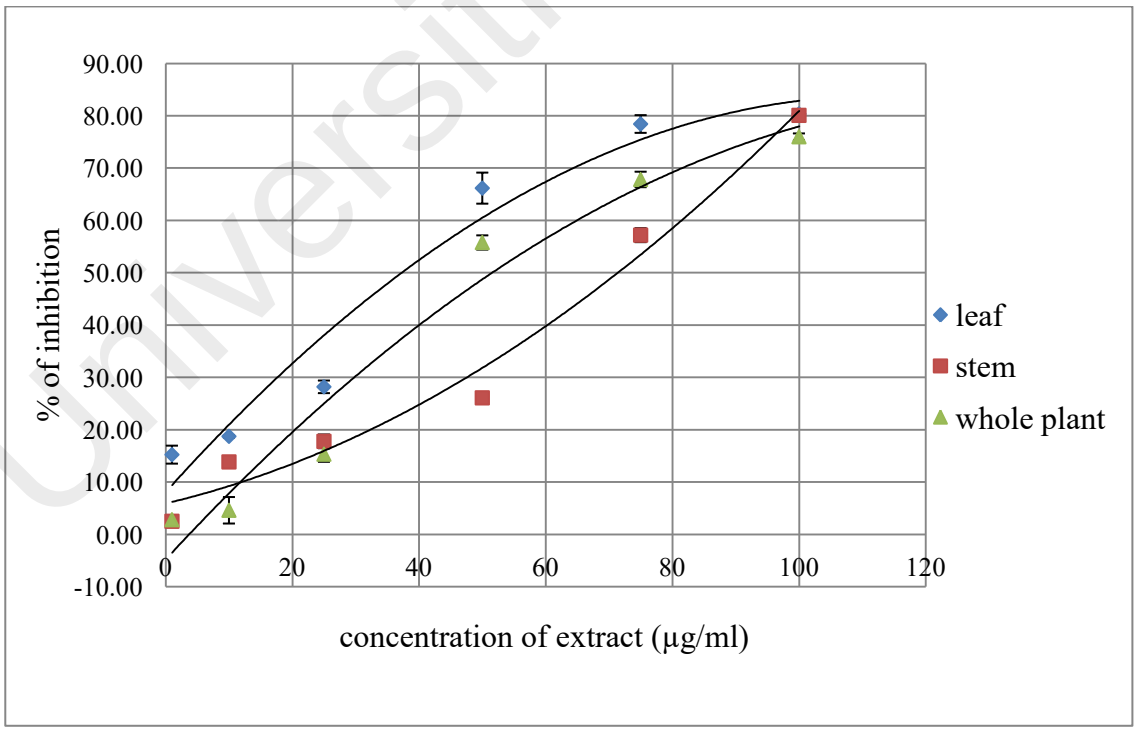


Figure 4.3 (D): The *in vitro* growth inhibition of SKOV-3 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

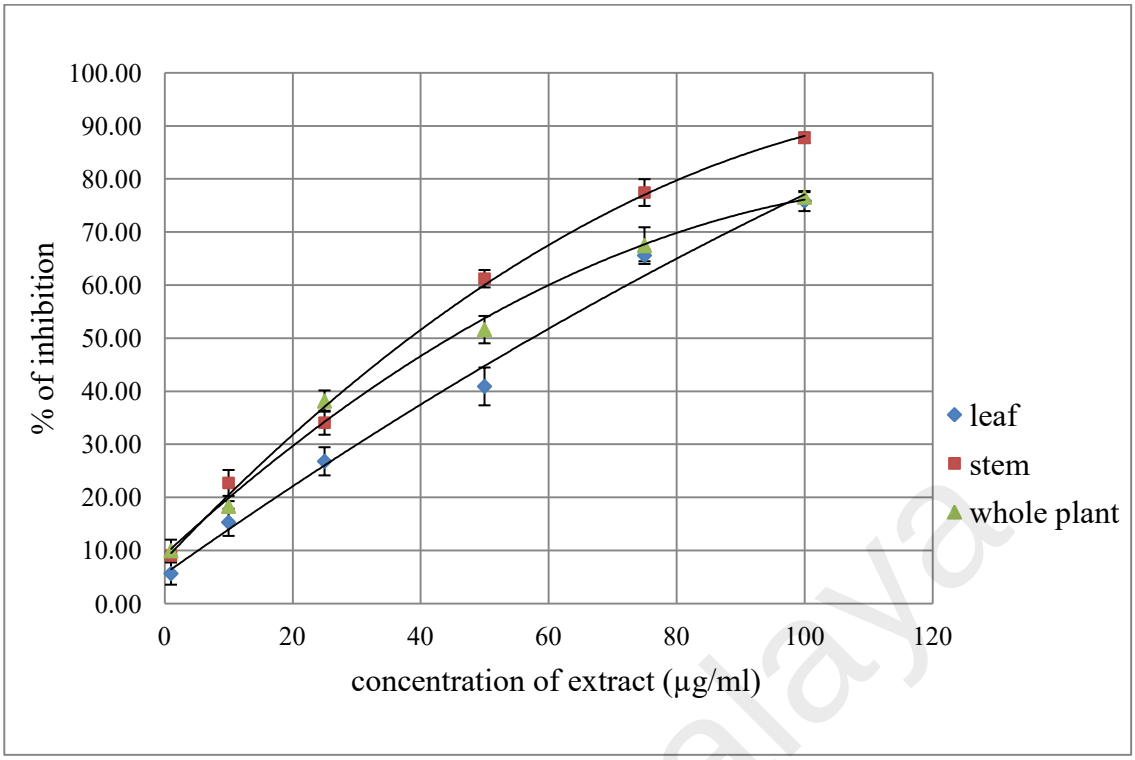


Figure 4.3 (E): The *in vitro* growth inhibition of HT-29 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

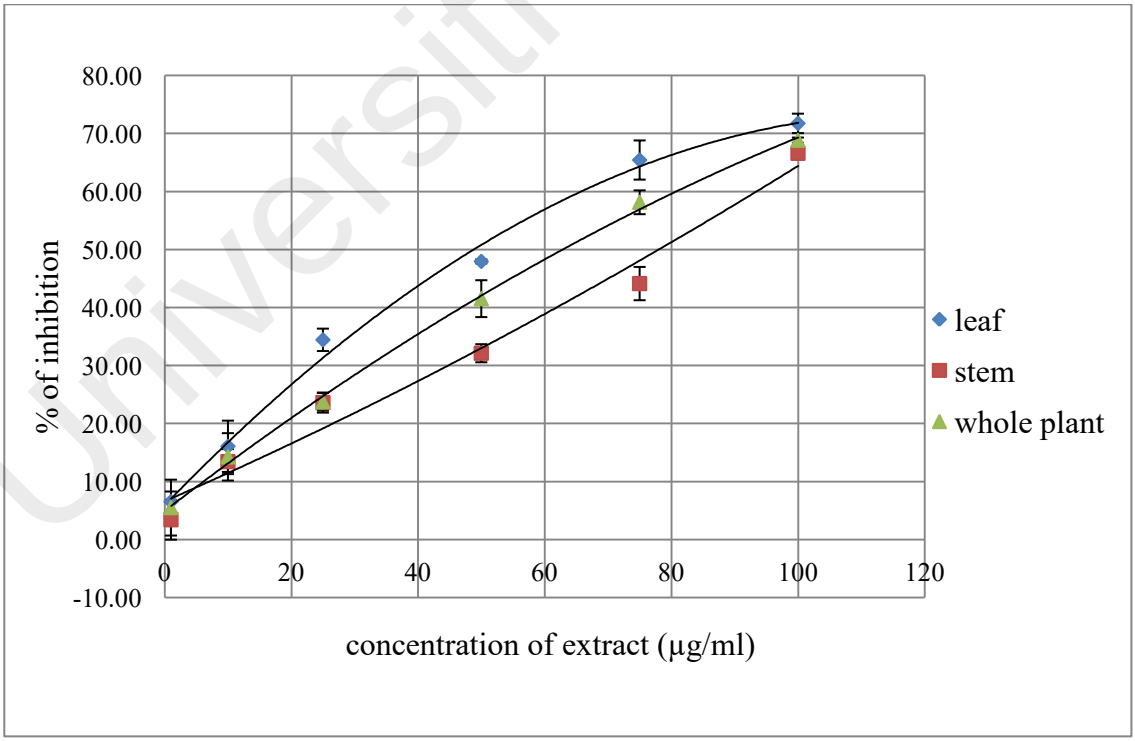


Figure 4.3 (F): The *in vitro* growth inhibition of HT-29 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

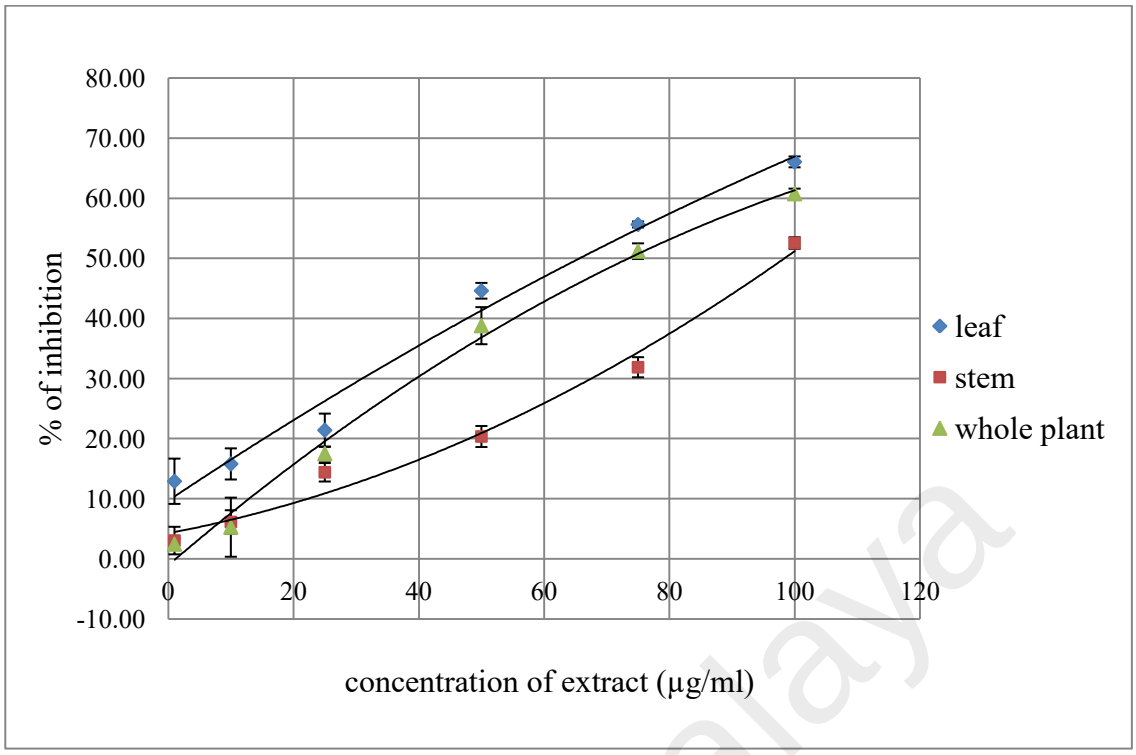


Figure 4.3 (G): The *in vitro* growth inhibition of A549 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

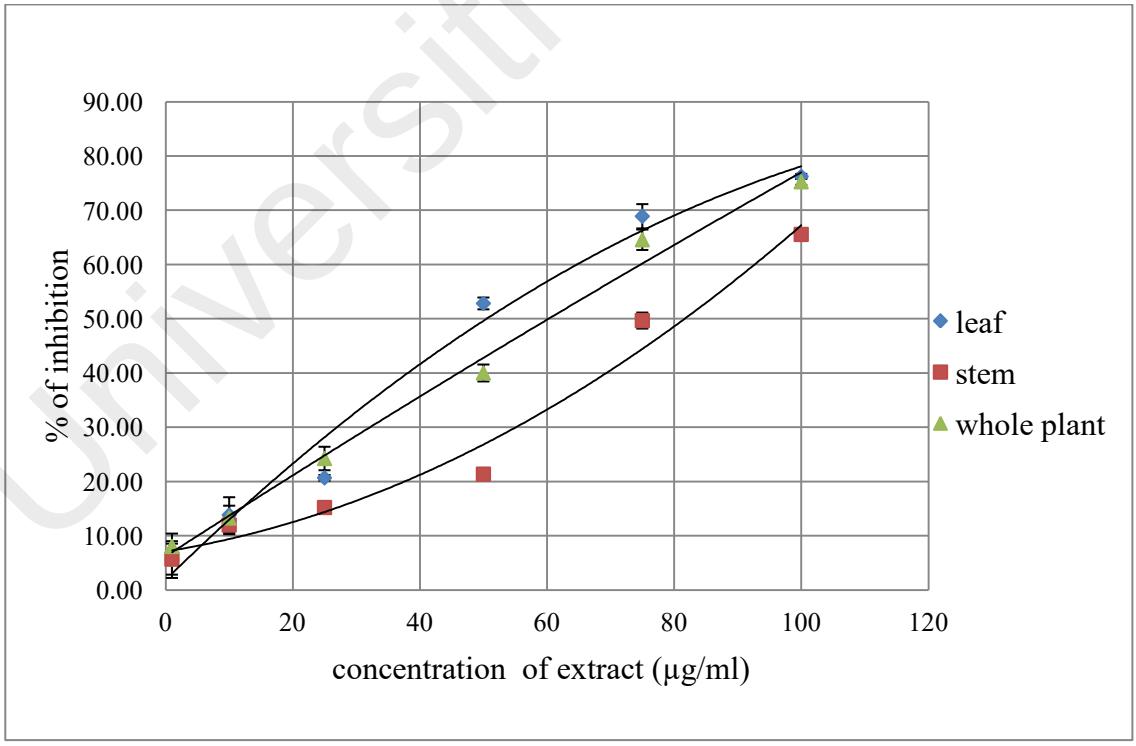


Figure 4.3 (H): The *in vitro* growth inhibition of A549 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

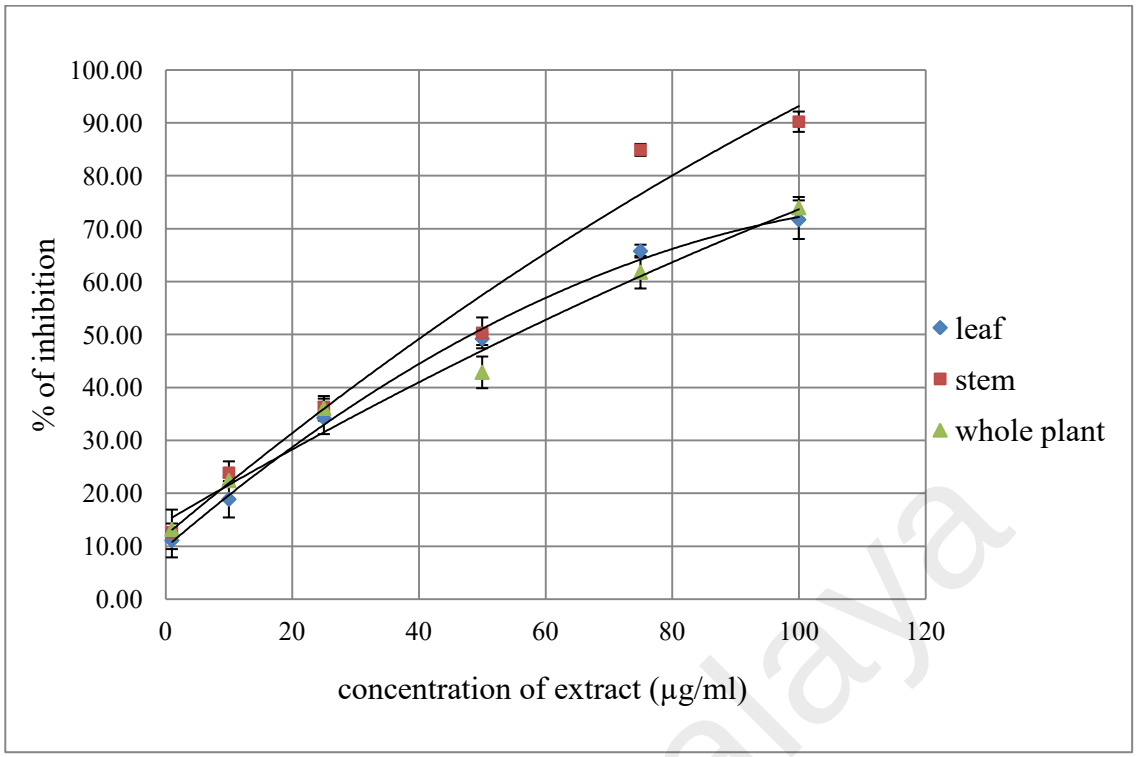


Figure 4.3 (I): The *in vitro* growth inhibition of MCF7 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

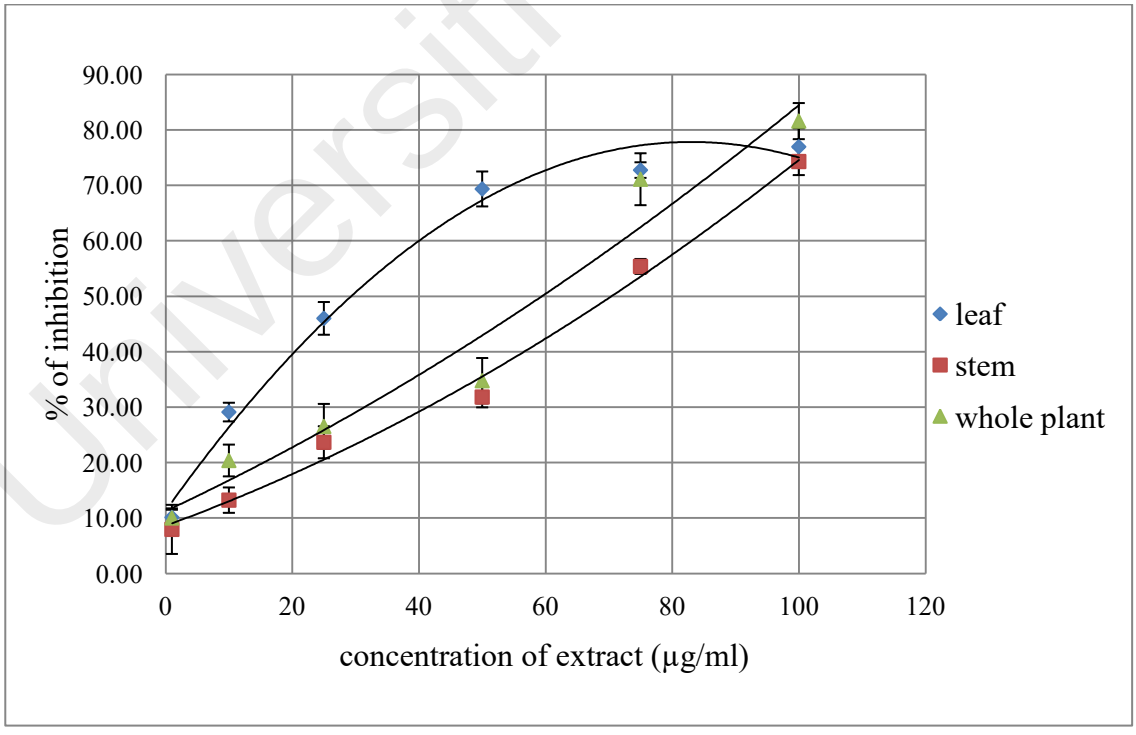


Figure 4.3 (J): The *in vitro* growth inhibition of MCF7 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

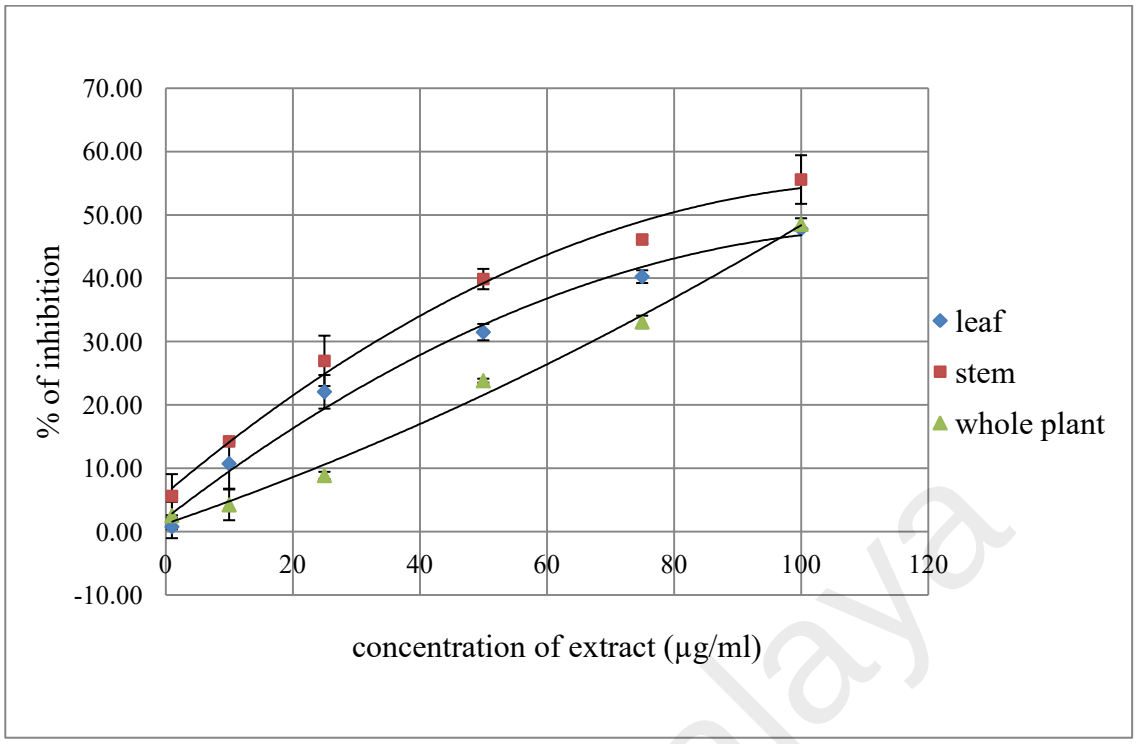


Figure 4.3 (K): The *in vitro* growth inhibition of MRC-5 cells by crude MeOH extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

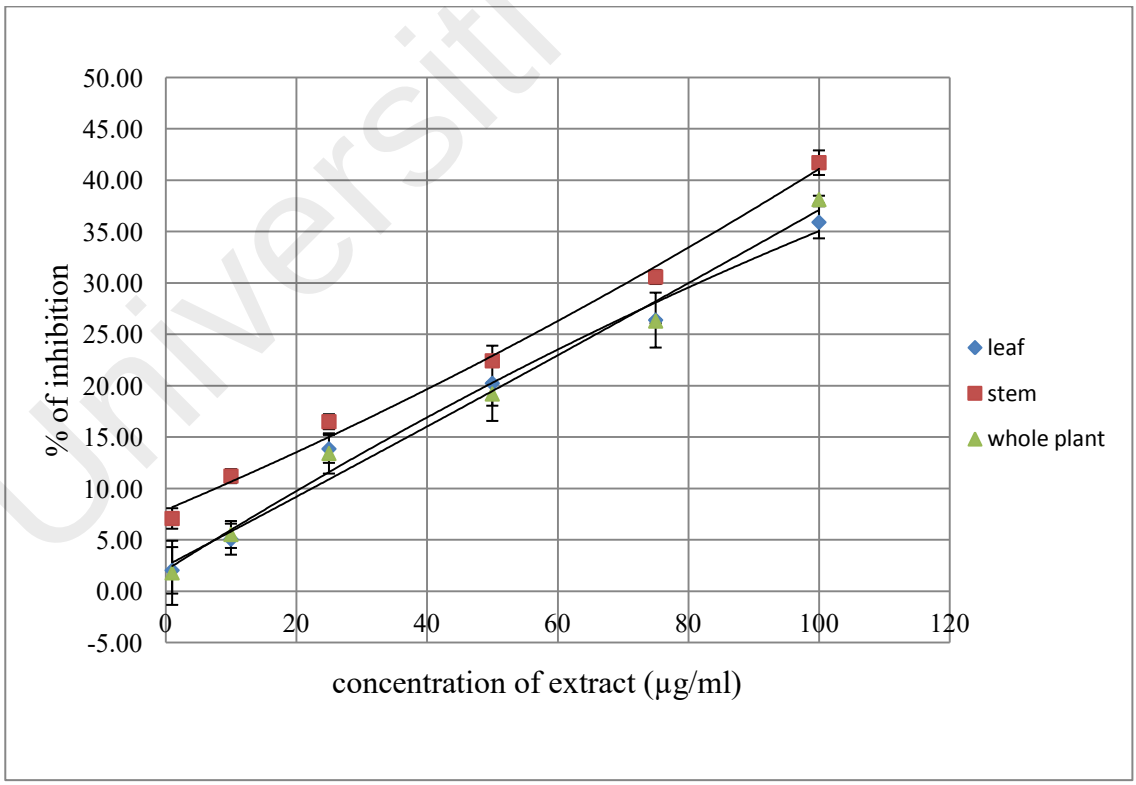


Figure 4.3 (L): The *in vitro* growth inhibition of MRC-5 cells by crude MeOH extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

4.4.2.(b) Cytotoxic activity of the dichloromethane extracts of *P. chinensis* var. *chinensis*

As illustrated from Figures 4.5 (A–L), all cancer cell lines evaluated showed different cytotoxic profiles when treated with dichloromethane crude extracts from different plant parts of the lowland and highland varieties. The IC_{50} values for the dichloromethane crude extract were determined and summarized in Tables 4.2 and 4.3.

The present study shows that most of the dichloromethane crude extracts from *P. chinensis* var. *chinensis* were not actively cytotoxic against all cancer cell lines tested with IC_{50} values of more than 30 µg/ml except the stem and whole plant crude dichloromethane extract of the highland variety when tested against HT-29 which showed IC_{50} value of 13.17 µg/ml and 21.86 µg/ml, respectively. All of the dichloromethane crude extracts from the lowland variety showed very low ability to retard the growth of MCF-7 with the IC_{50} values of more than 100 µg/ml.

i) Human epithelial carcinoma of cervix cell line, CaSki

The leaf, stem and whole plant crude dichloromethane extracts of the highland and lowland varieties showed IC_{50} values ranging from 55 µg/ml to more than 100 µg/ml (Tables 4.2 & 4.3).These results showed that all of the dichloromethane crude extracts were not actively cytotoxic against the CaSki cells.

ii) Human epithelial adenocarcinoma of ovary cell line, SKOV-3

The cytotoxic activity of all the crude dichloromethane extracts of the highland and lowland varieties were not actively cytotoxic against the SKOV-3 cells as the resulting IC50 values ranged from 77 µg/ml to 91 µg/ml (Tables 4.2 & 4.3).

iii) Human epithelial adenocarcinoma of colon cell line, HT-29

All the leaf, stem and whole plant crude dichloromethane extracts from the highland variety exhibited better growth inhibition compared to the lowland variety when tested against HT-29 cells, with IC_{50} values less than 56 µg/ml. The stem crude dichloromethane extracts of the highland variety against HT-29 exhibited an IC_{50} value of 13.17 µg/ml (Tables 4.2 and 4.3). The inhibition percentages against HT-29 cells by the stem dichloromethane extract of the highland variety were 35% at 10 µg/ml, 54% at 25 µg/ml and 90% at 100 µg/ml. The crude dichloromethane extract of the whole plant of the highland variety exhibited an IC_{50} value of 21.86 µg/ml against HT-29 cells. The growth inhibition percentages were 31% at 10 µg/ml, 55% at 25 µg/ml and 77% at 100 µg/ml. These stem and whole plant dichloromethane crude extracts were actively cytotoxic against HT-29 cell lines.

iv) Human epithelial carcinoma of lung cell line, A549

Similar to the SKOV-3 and CaSki cells, all the dichloromethane crude extracts from the leaf, stem and whole plant of the highland and lowland varieties were not actively cytotoxic against A549 cell lines. The resulting IC_{50} values ranged from 52 µg/ml to more than 100 µg/ml (Tables 4.2 & 4.3).

v) Human epithelial adenocarcinoma of mammary gland (breast) cell line, MCF7

Crude dichloromethane extracts of the leaf, stem and whole plant of the highland and lowland varieties were not actively cytotoxic against MCF7 with IC_{50} values ranging from 64 µg/ml to more than 100 µg/ml (Tables 4.2 & 4.3). All the dichloromethane crude extracts from the lowland variety showed very high IC_{50} values,

i.e. more than 100 µg/ml. These results showed a low ability to inhibit the growth of MCF-7 with the IC_{50} values of more than 100 µg/ml.

vi) Normal human lung fibroblasts cell, MRC-5

All of the dichloromethane crude extracts from the lowland and highland varieties were not actively cytotoxic against normal human lung fibroblasts cell, MRC-5 (Tables 4.2 and 4.3). The IC_{50} values ranged from 64 µg/ml to more than 100 µg/ml.

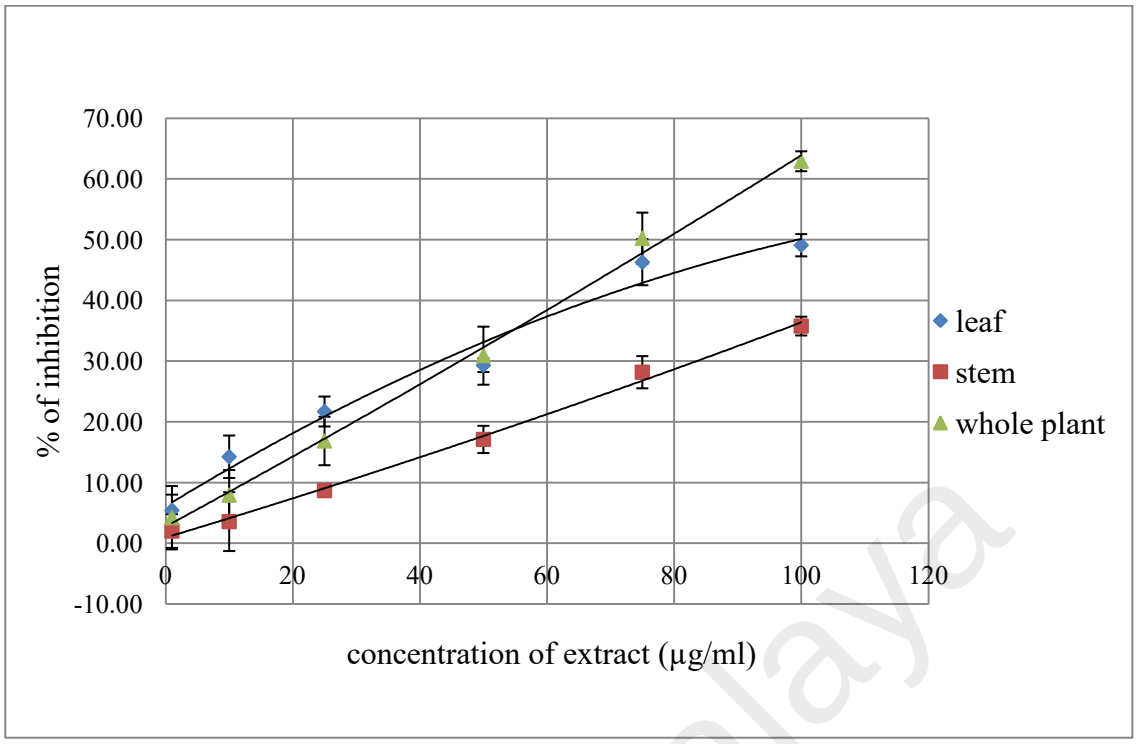


Figure 4.4 (A): The *in vitro* growth inhibition of CaSki cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

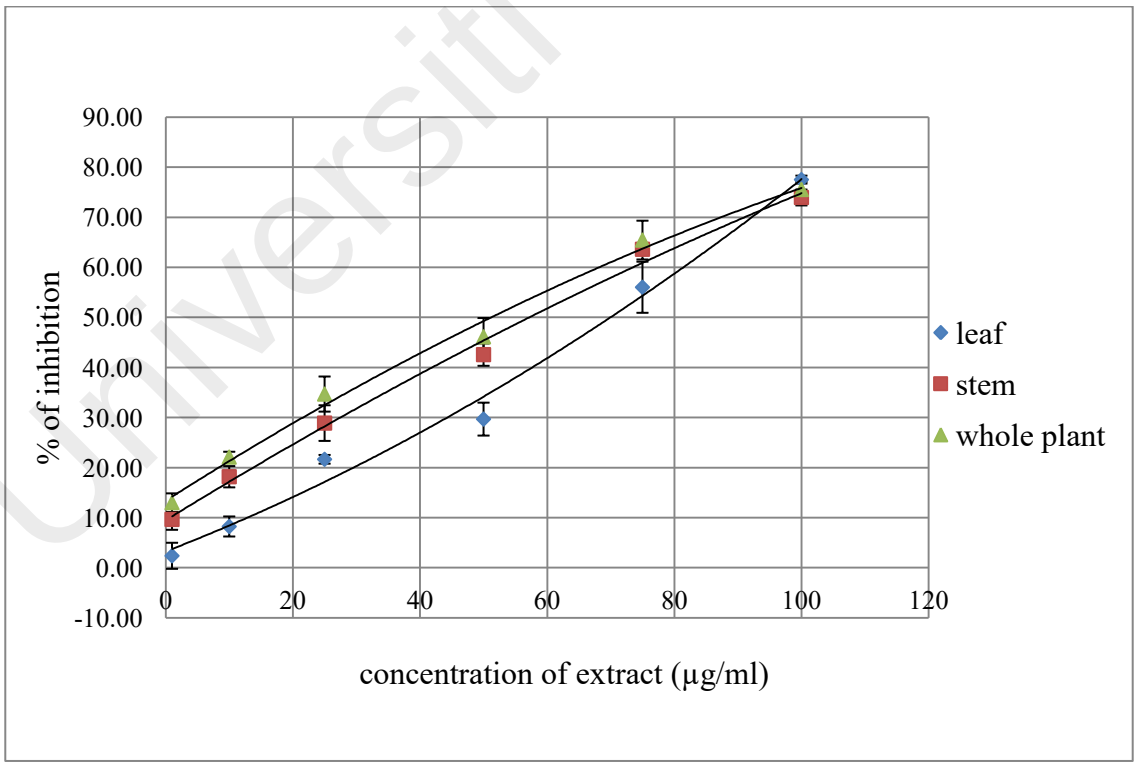


Figure 4.4 (B): The *in vitro* growth inhibition of CaSki cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

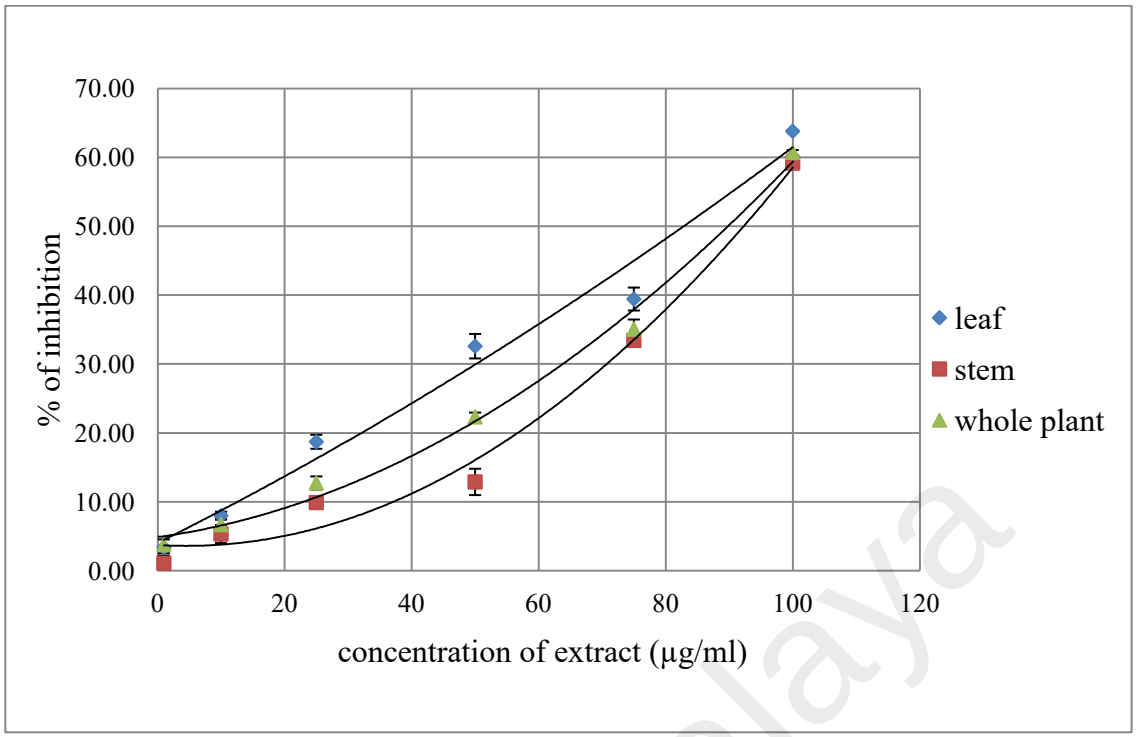


Figure 4.4 (C): The *in vitro* growth inhibition of SKOV-3 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

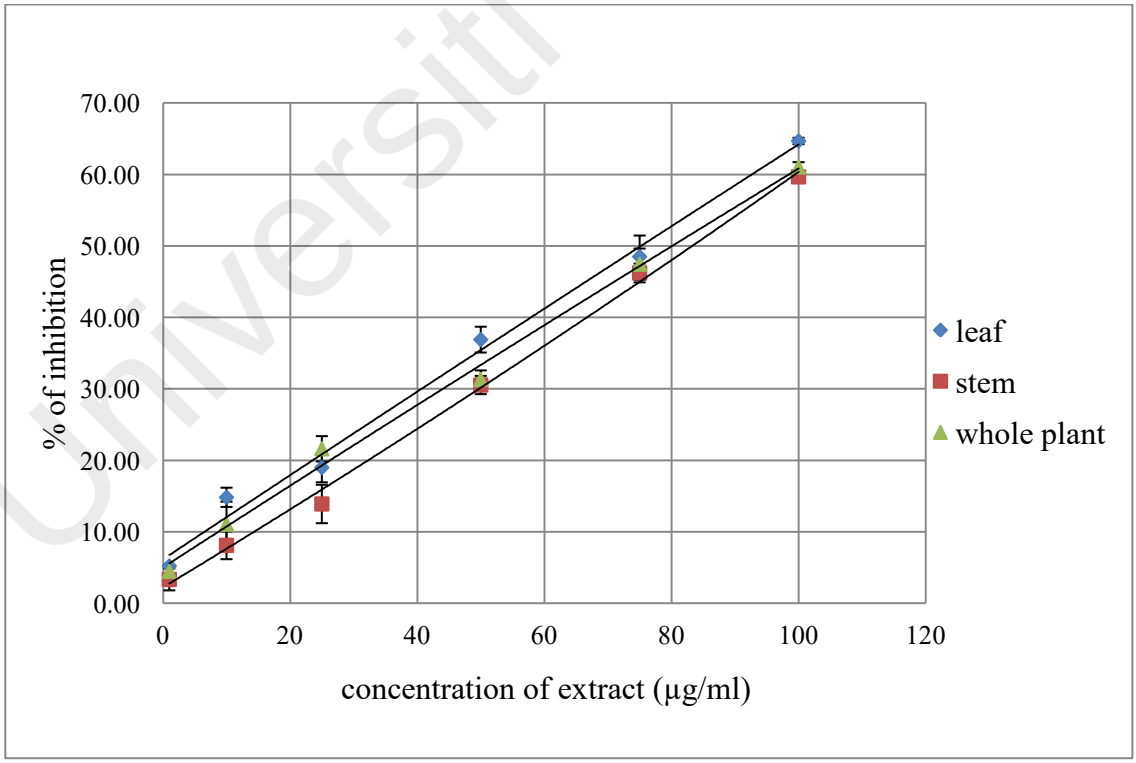


Figure 4.4 (D): The *in vitro* growth inhibition of SKOV-3 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

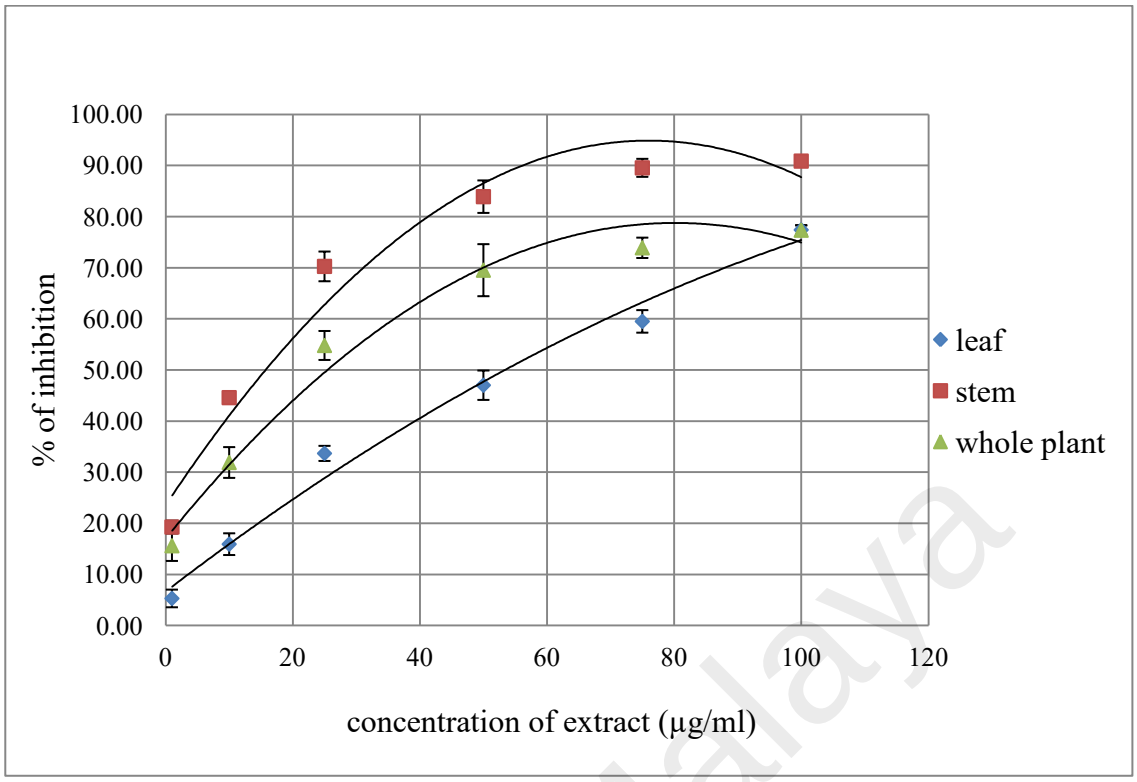


Figure 4.4 (E): The *in vitro* growth inhibition of HT-29 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

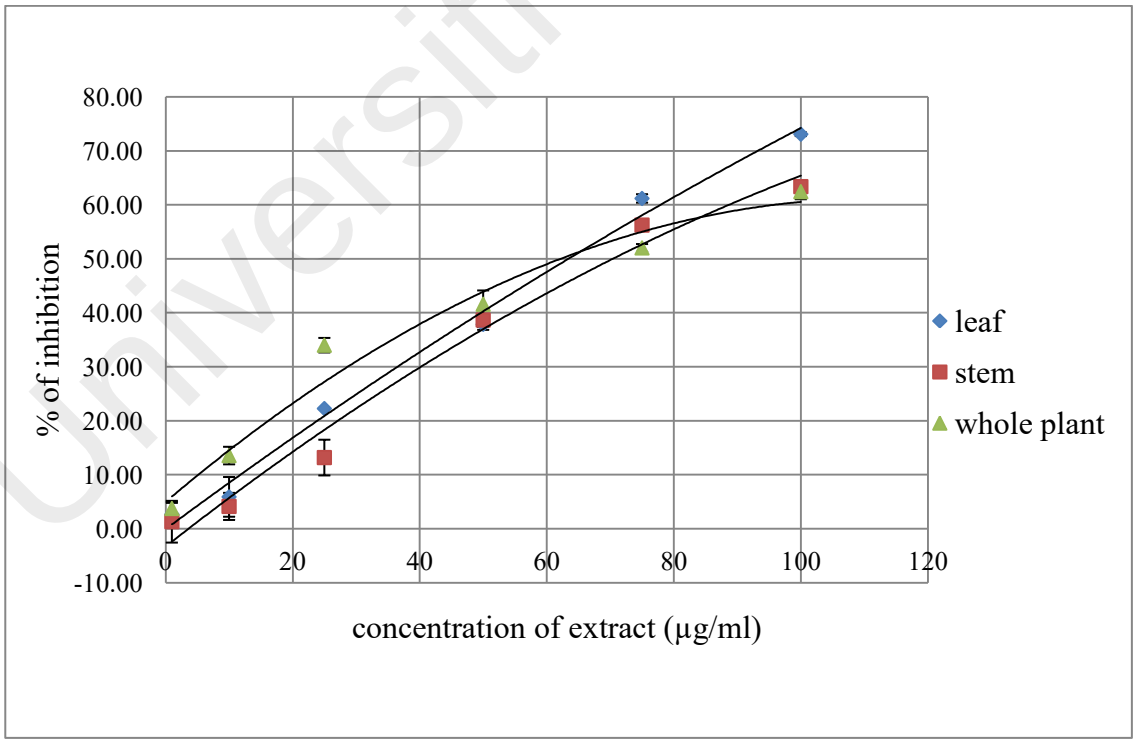


Figure 4.4 (F): The *in vitro* growth inhibition of HT-29 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

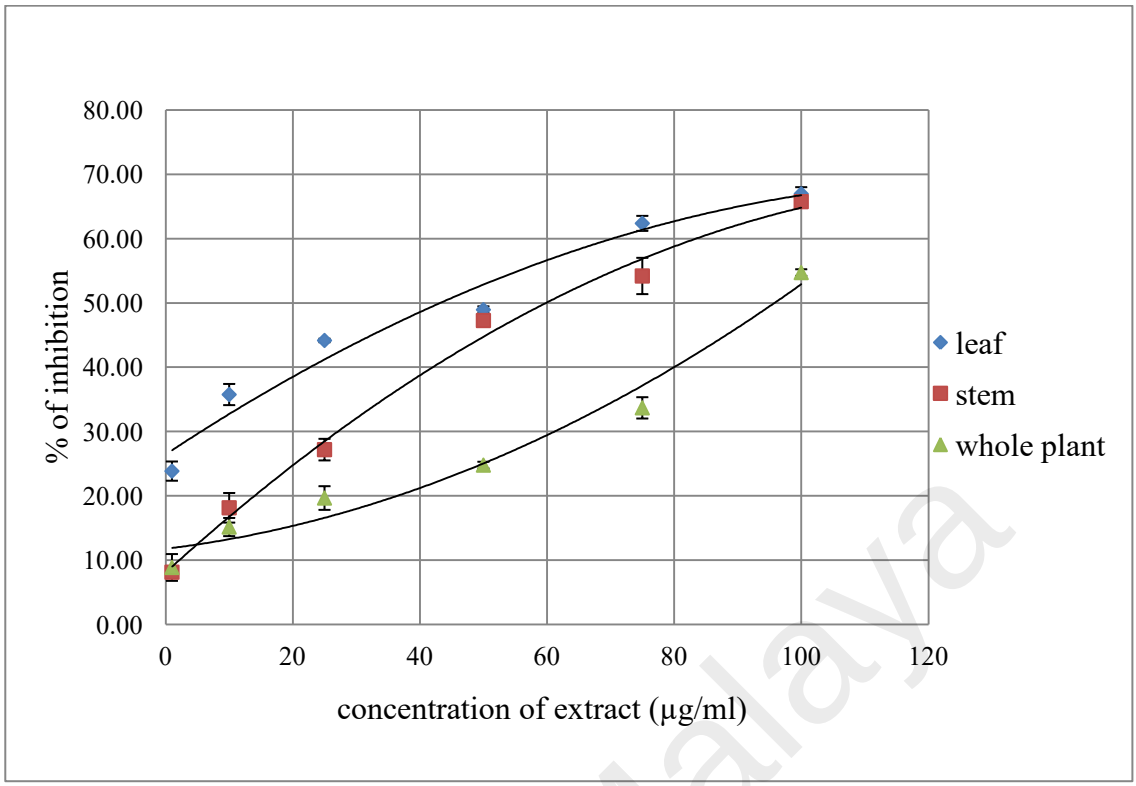


Figure 4.4 (G): The *in vitro* growth inhibition of A549 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

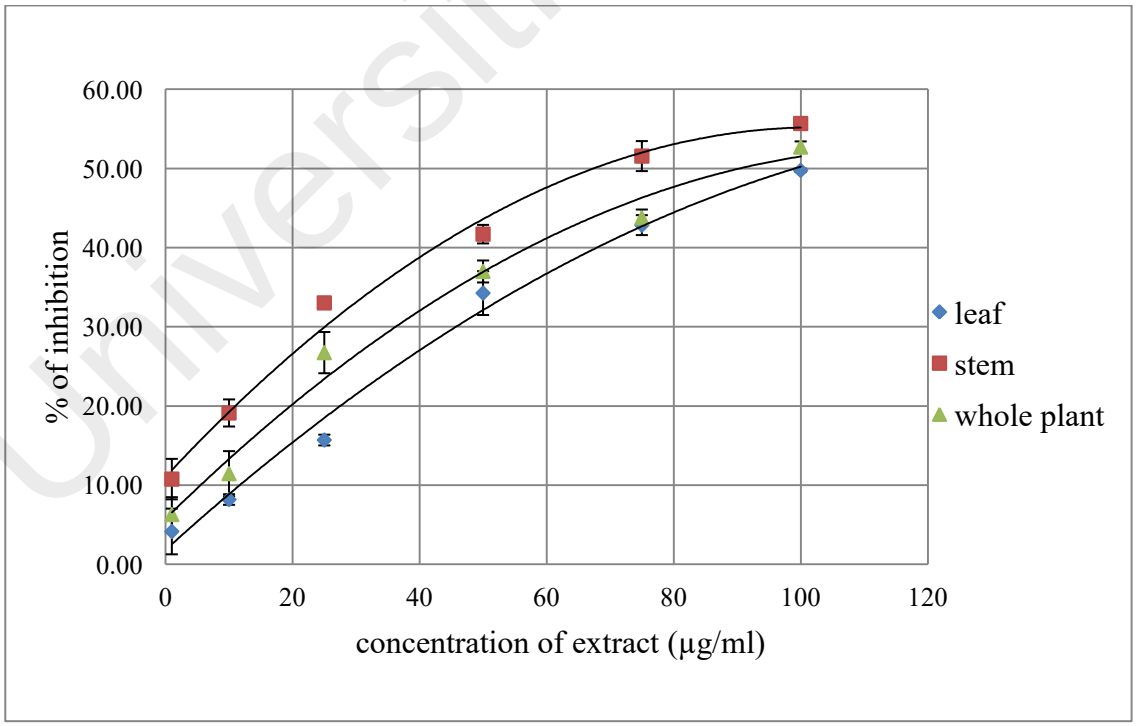


Figure 4.4 (H): The *in vitro* growth inhibition of A549 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

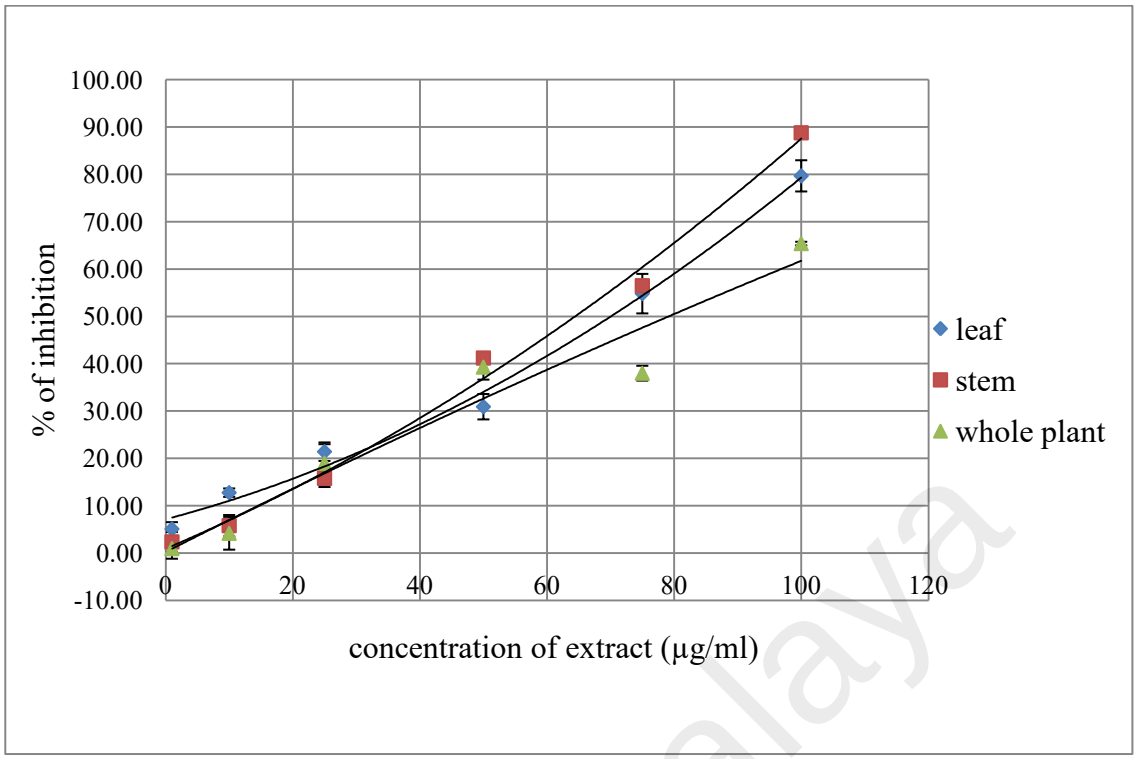


Figure 4.4 (I): The *in vitro* growth inhibition of MCF7 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

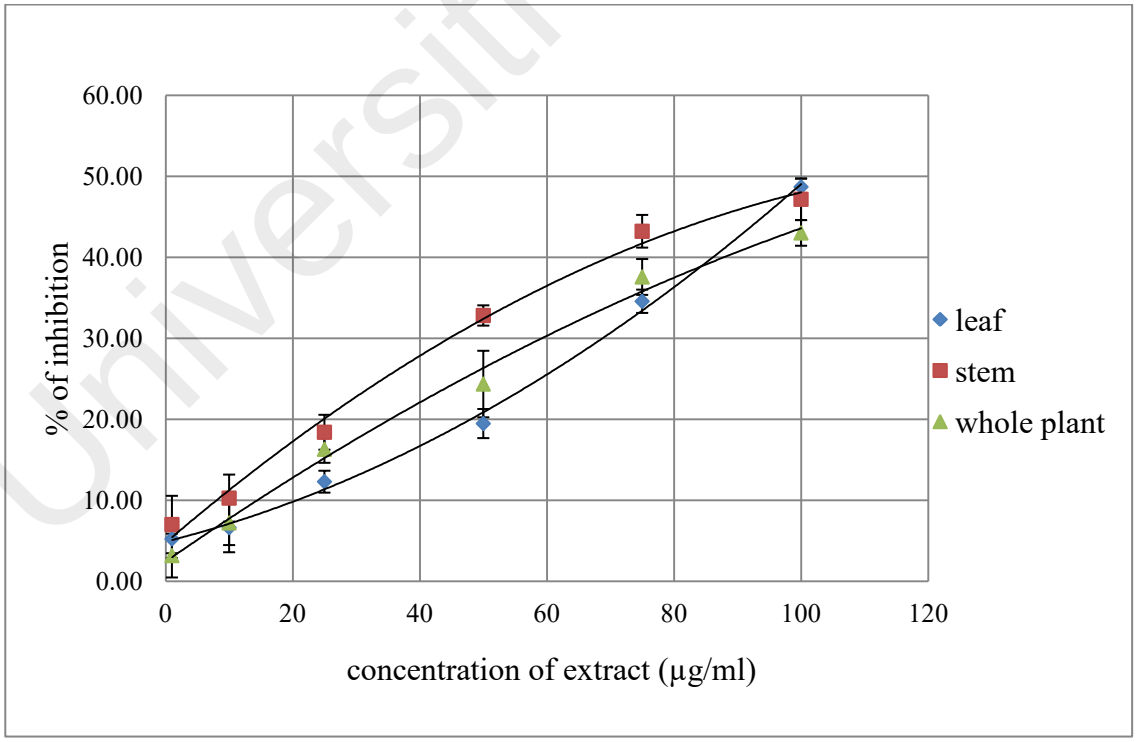


Figure 4.4 (J): The *in vitro* growth inhibition of MCF7 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

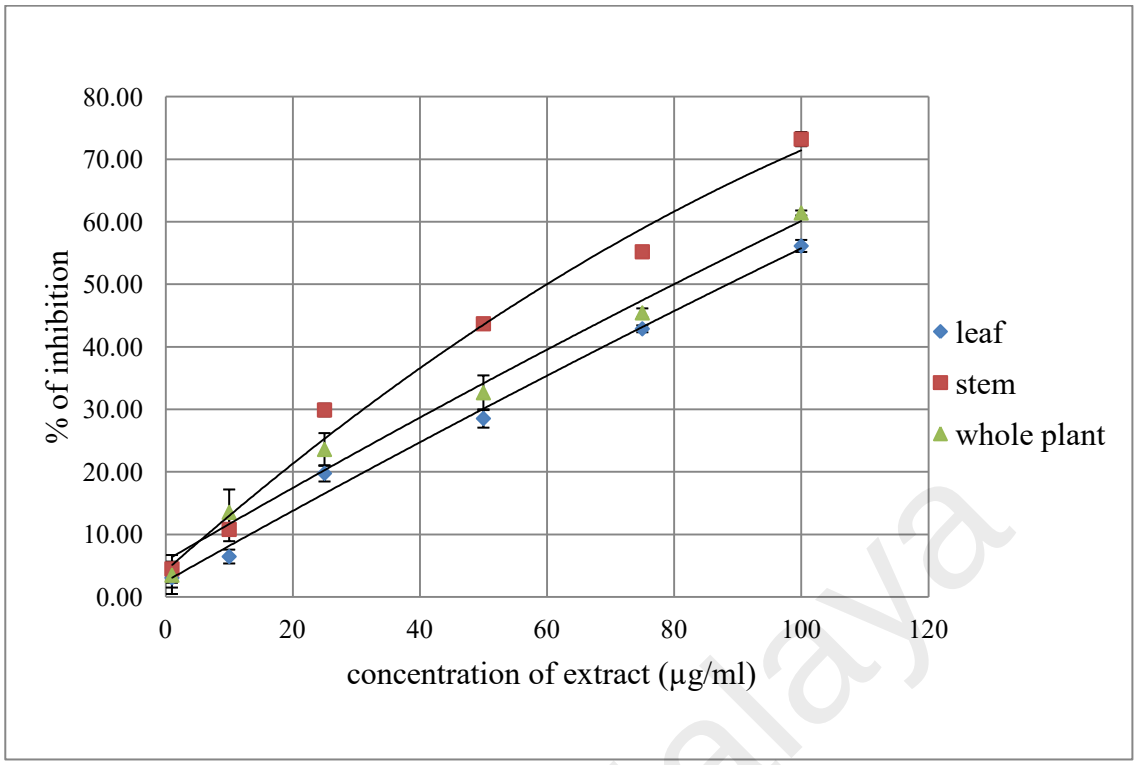


Figure 4.4 (K): The *in vitro* growth inhibition of MRC-5 cells by crude DCM extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

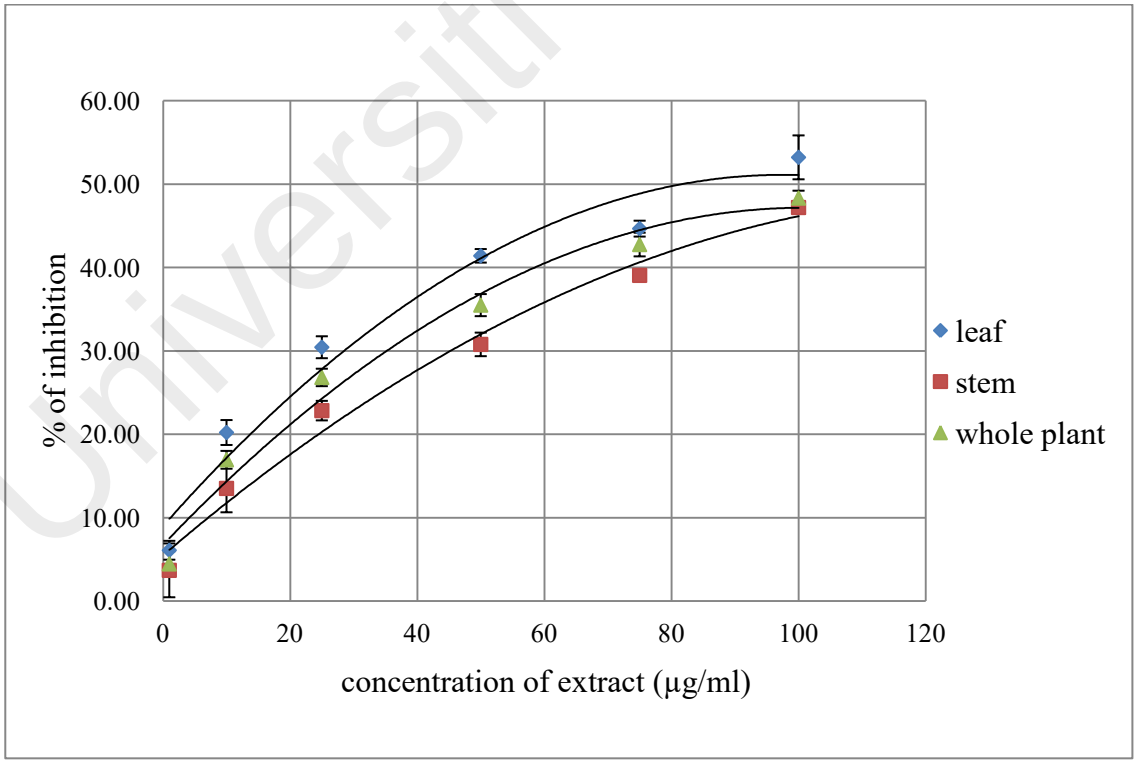


Figure 4.4 (L): The *in vitro* growth inhibition of MRC-5 cells by crude DCM extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

4.4.2.(c) Cytotoxic activity of the water extracts of *P. chinensis* var. *chinensis*

Figures 4.6 (A–L) illustrates the growth inhibition percentages of all the cancer cell lines evaluated against water extracts of *P. chinensis* var. *chinensis*. Different cytotoxic profiles were observed when treated with water crude extracts derived from different plant parts of the lowland and highland varieties. The IC_{50} values for the water crude extract was determined and summarized in Tables 4.2 and 4.3.

i) CaSki, SKOV-3, HT-29, A549, MCF7 and MRC-5

Overall, the IC_{50} values of all the leaf, stem and whole plant crude water extracts derived from both the populations were high with the value of more than 60 µg/ml. The water extracts of *P. chinensis* var. *chinensis* are therefore considered as not actively cytotoxic against all of the cancer cell lines. All crude water extracts derived from both populations showed a low inhibition percentage of less than 40% and IC_{50} values of more than 100 µg/ml when tested on HT-29 and A549 cells at a high concentration of 100 µg/ml.

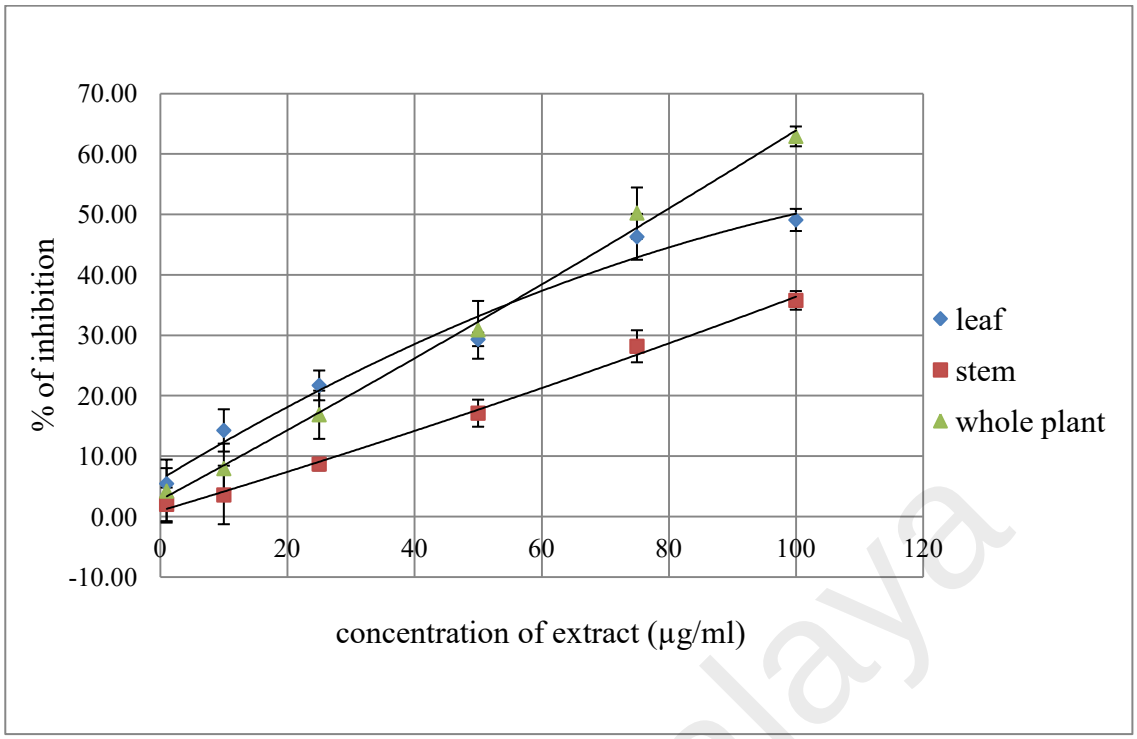


Figure 4.5 (A): The *in vitro* growth inhibition of CaSki cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

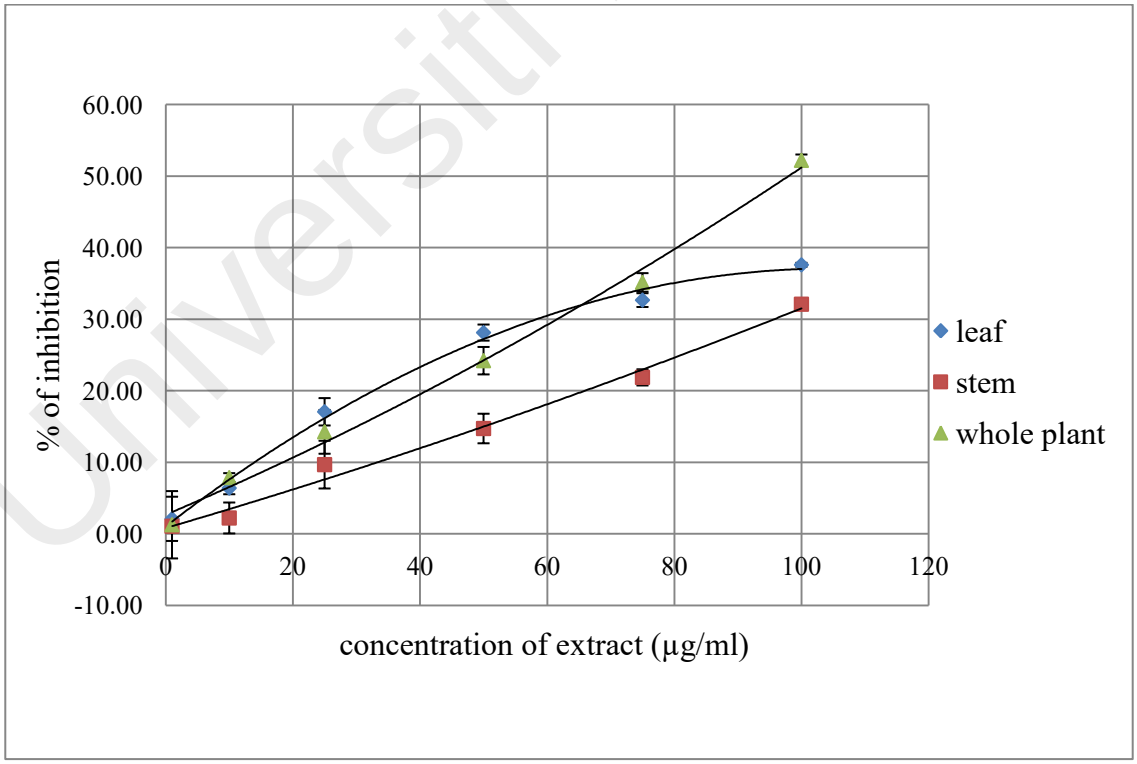


Figure 4.5 (B): The *in vitro* growth inhibition of CaSki cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

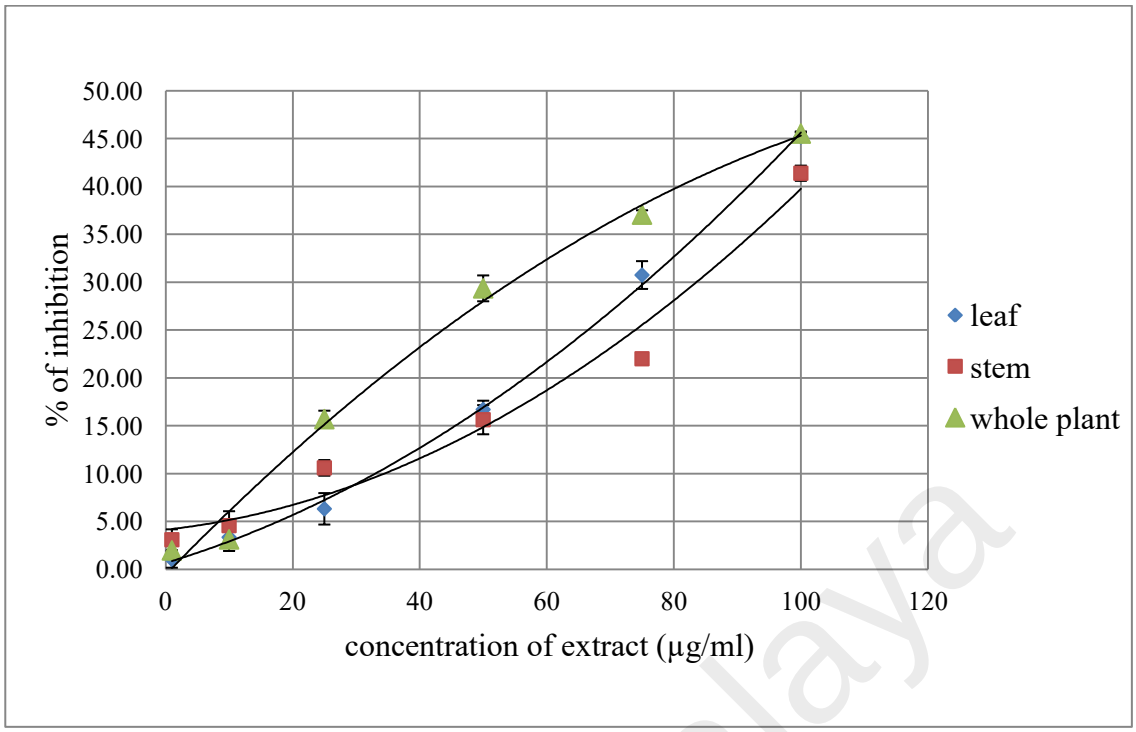


Figure 4.5 (C): The *in vitro* growth inhibition of SKOV-3 cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

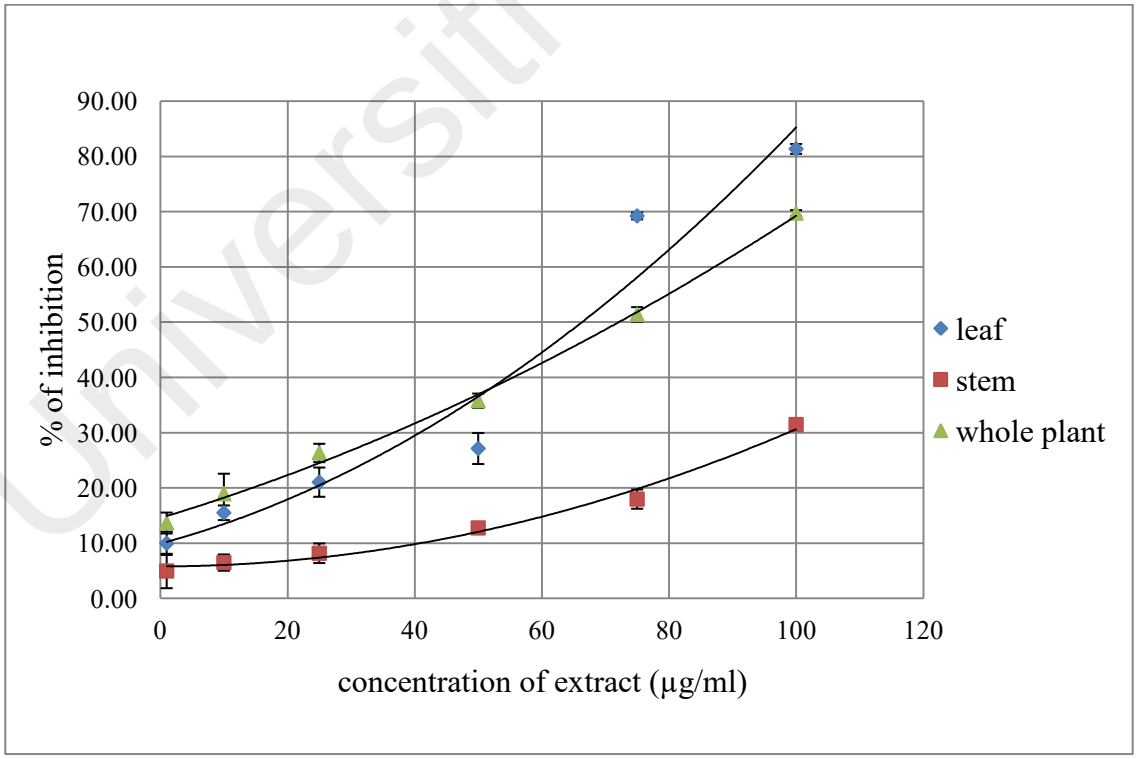


Figure 4.5 (D): The *in vitro* growth inhibition of SKOV-3 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

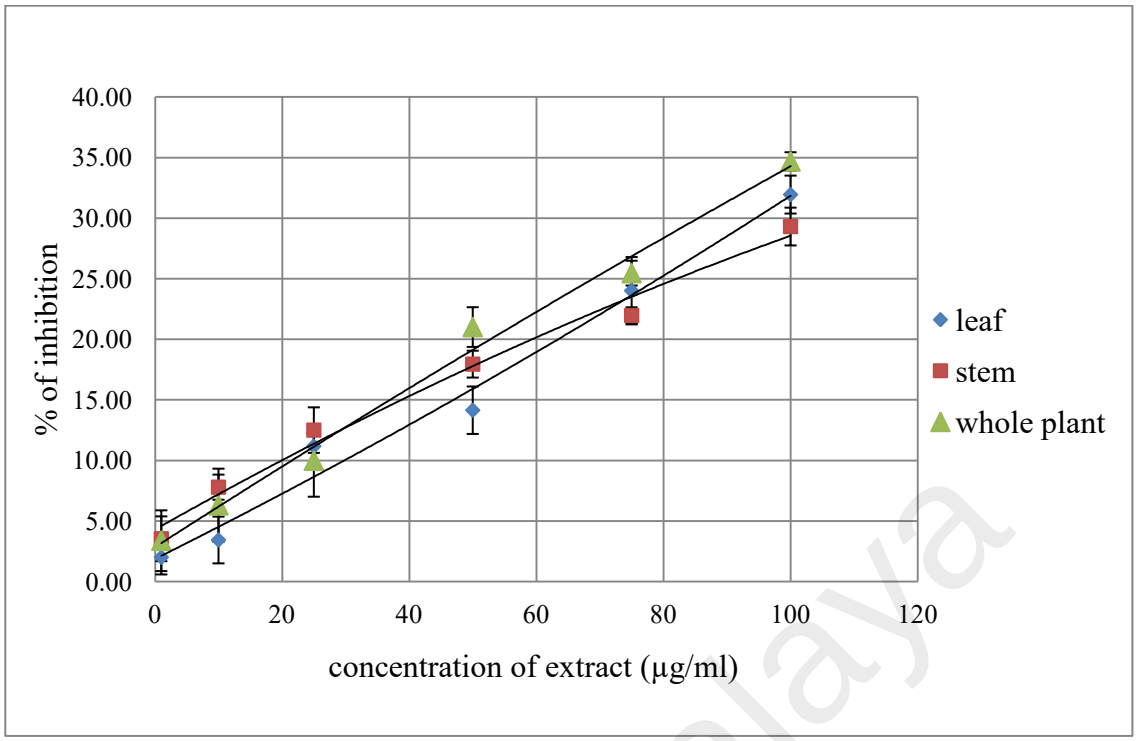


Figure 4.5 (E): The *in vitro* growth inhibition of HT-29 cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

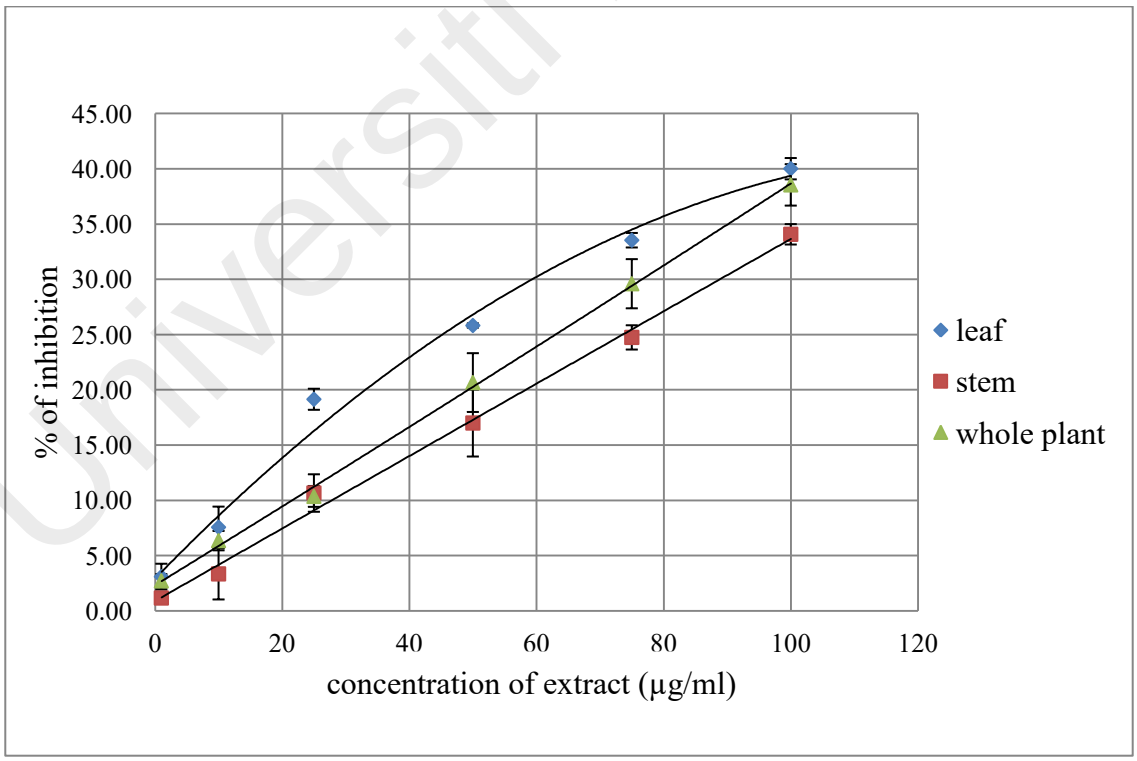


Figure 4.5 (F): The *in vitro* growth inhibition of HT-29 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

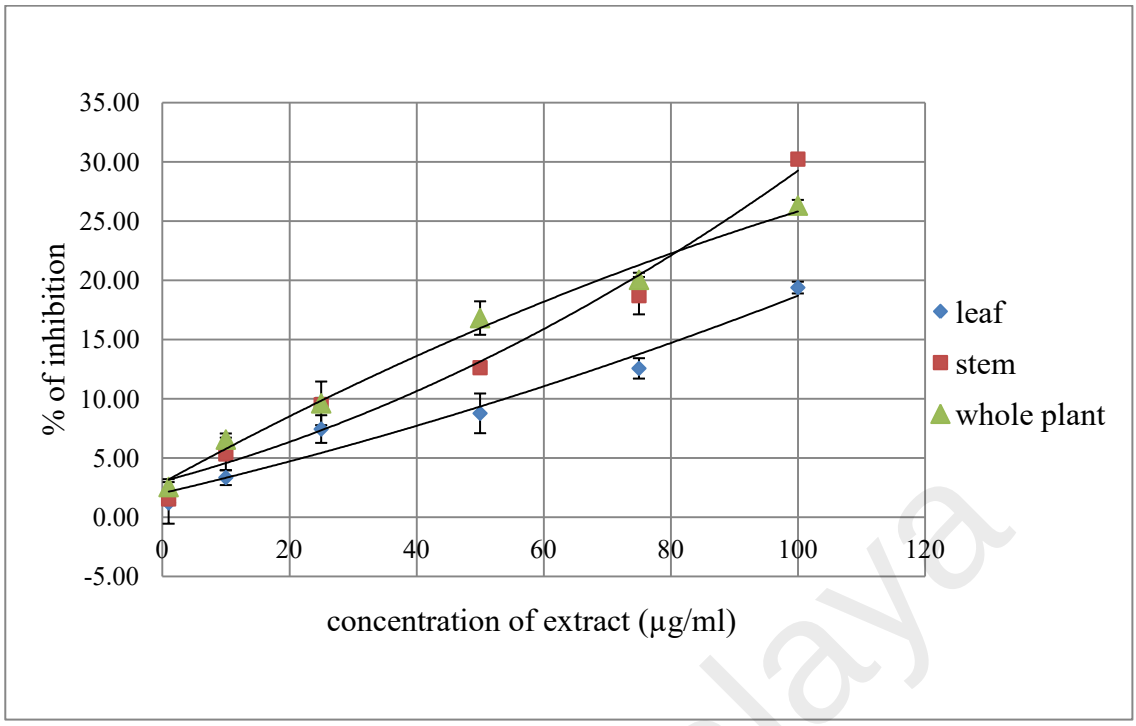


Figure 4.5 (G): The in vitro growth inhibition of A549 cells by crude water extracts of the highland variety of P. *chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

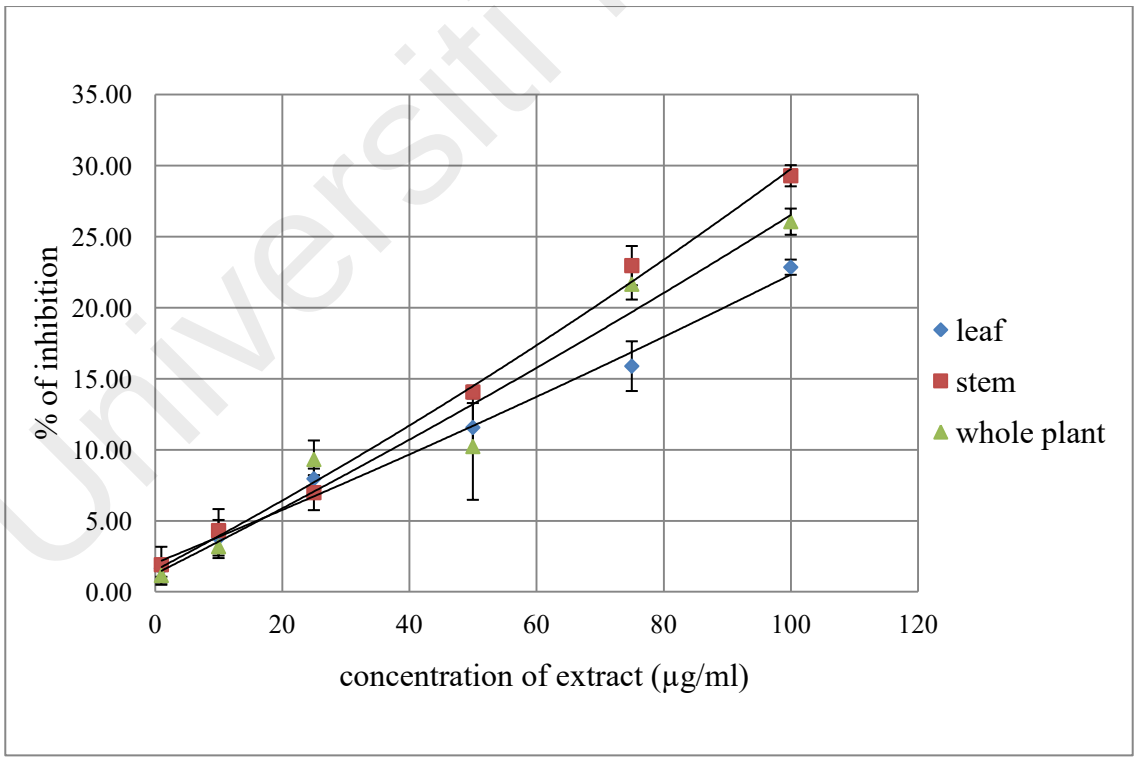


Figure 4.5 (H): The *in vitro* growth inhibition of A549 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

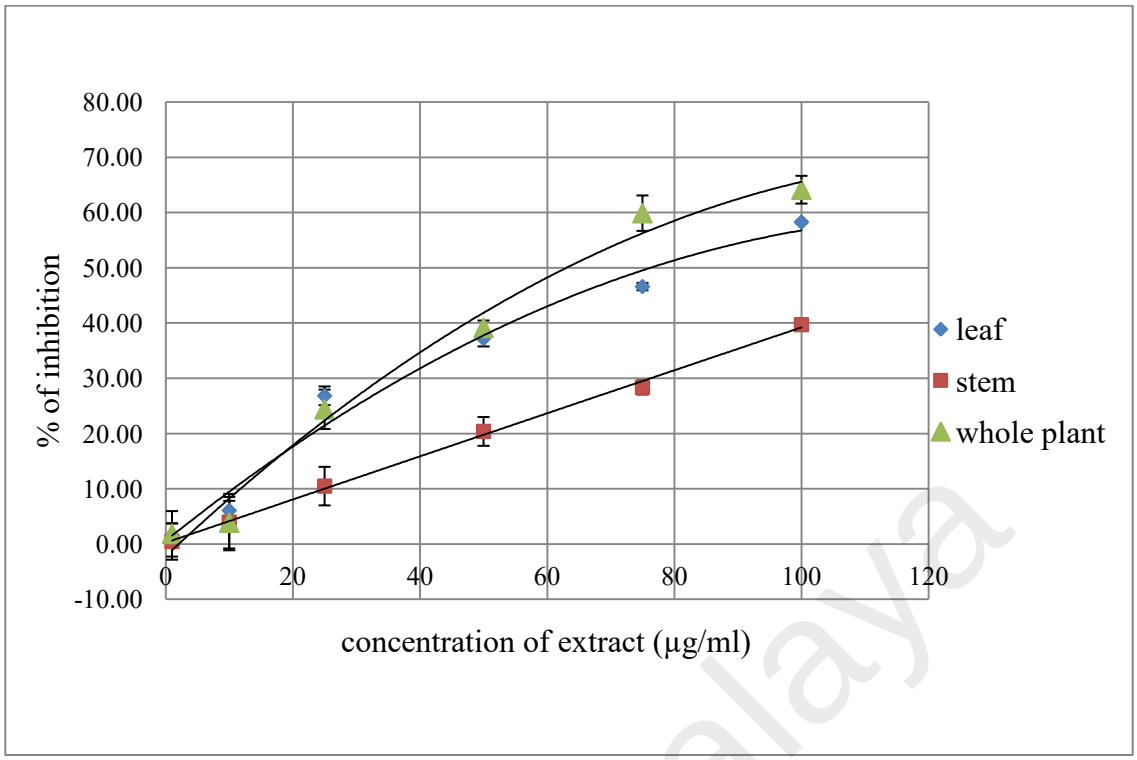


Figure 4.5 (I): The *in vitro* growth inhibition of MCF7 cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

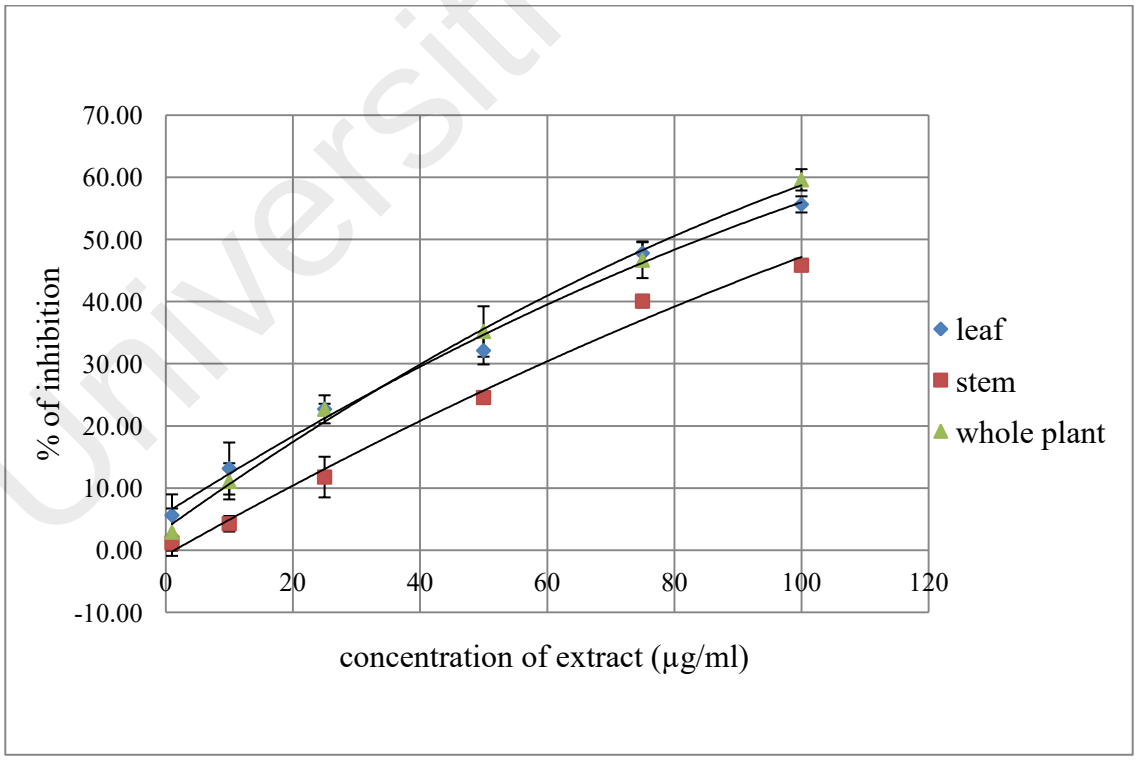


Figure 4.5 (J): The *in vitro* growth inhibition of MCF7 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

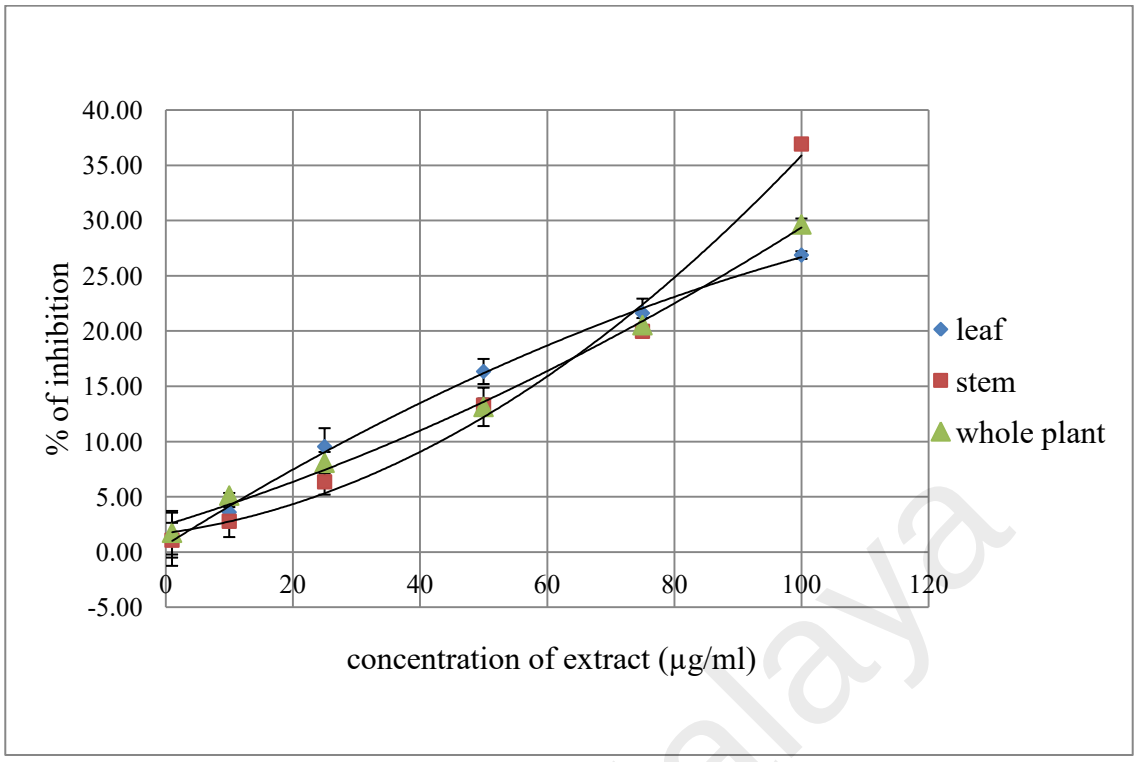


Figure 4.5 (K): The *in vitro* growth inhibition of MRC-5 cells by crude water extracts of the highland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

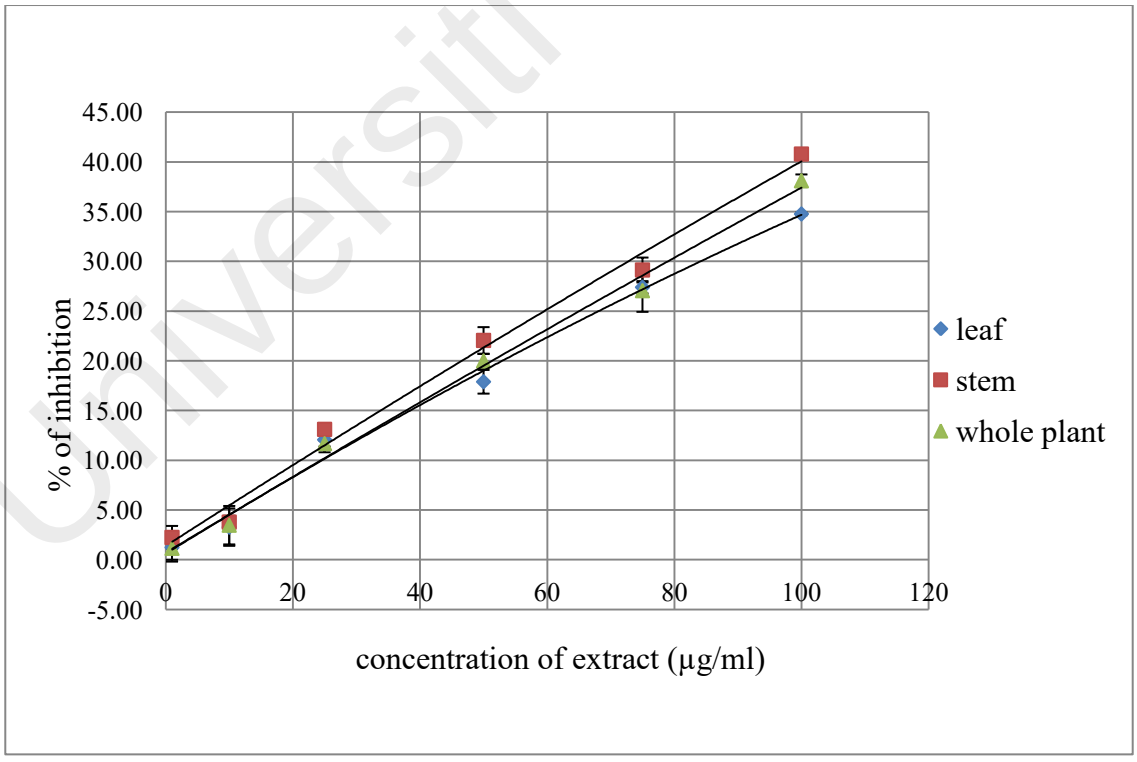


Figure 4.5 (L): The *in vitro* growth inhibition of MRC-5 cells by crude water extracts of the lowland variety of *P. chinensis* var. *chinensis* in the *in vitro* Neutral Red Cytotoxicity Assay.

Table 4.2: The IC_{50} values of crude methanol, dichloromethane and water extracts of the lowland cultivated variety of *P. chinensis* var. *chinensis* against different cell lines in the *in vitro* Neutral Red Cytotoxicity Assay.

Crude extracts of lowland cultivated variety of *P. chinensis* var. *chinensis*		IC_{50} value (µg/ml) against different cells					
		SKOV-3	CaSki	HT-29	A549	MCF7	MRC-5
Methanol	Leaf	39.34	**11.37**	52.95	47.78	**29.27**	>100
	Stem	69.20	85.37	81.54	75.49	69.29	>100
	Whole plant	46.41	54.40	62.73	60.15	60.48	>100
Dichloromethane	Leaf	77.29	69.29	63.04	>100	>100	90.61
	Stem	82.00	58.84	66.15	70.99	>100	>100
	Whole plant	79.67	54.99	70.23	92.37	>100	>100
Water	Leaf	63.57	>100	>100	>100	82.02	>100
	Stem	>100	>100	>100	>100	>100	>100
	Whole plant	72.80	96.78	>100	>100	81.44	>100

Table 4.3: The IC_{50} values of crude methanol, dichloromethane and water extracts of the highland wild variety of *P. chinensis* var. *chinensis* against different cell lines in the *in vitro* Neutral Red Cytotoxicity Assay.

Crude extracts of the highland wild variety of *P. chinensis* var. *chinensis*		IC_{50} value (µg/ml) against different cells					
		SKOV-3	CaSki	HT-29	A549	MCF7	MRC-5
Methanol	Leaf	**19.38**	53.45	59.21	62.22	51.21	>100
	Stem	40.89	76.40	39.68	96.87	49.47	85.36
	Whole plant	**21.71**	56.69	47.01	72.58	59.47	>100
Dichloromethane	Leaf	85.84	>100	55.98	51.97	69.98	88.43
	Stem	91.11	65.04	**13.17**	59.90	64.42	63.71
	Whole plant	89.56	82.90	**21.86**	94.35	85.93	82.15
Water	Leaf	>100	>100	>100	>100	82.28	>100
	Stem	>100	>100	>100	>100	>100	>100
	Whole plant	>100	72.45	>100	>100	63.10	>100

4.5 Discussion

Cytotoxicity assays are commonly used in preliminary *in vitro* antitumour screening tests. It is a rapid, inexpensive, standardized and sensitive method to measure drug-induced alterations in metabolic pathway or structural integrity which may or may not be related directly to cell death (Wilson, 1986). A number of methods have been employed for the detection of cytotoxicity or cell viability, i.e. the methyl tetrazolium assay (MTT), the ATP content of the treated cells, the lactate dehydrogenase leakage assay (LDH), a protein assay, and the neutral red uptake assay (NR). Weyermann *et al*. (2005) reported that, different cytotoxicity assays could give different results depending on the cell death mechanism induced. Fotakis & Timbrell (2006) reported that the MTT assay and the NR assay are the most sensitive cytotoxicity assays that show statistical significance between the treated cells and the controls.

In this study, NR assay was chosen instead of MTT assay to determine the cytotoxic effect of *P. chinensis* var. *chinensis* crude extracts against selected cancer cell lines. This selection was made due to several reasons. The MTT assay gives satisfactory responses using cell membrane damaging agents like triton X-100 and determines the activity of the mitochondria effectively, but this assay could be misleading if the toxic agent only influences intracellular activities, e.g. sodium azide which inhibits the respiratory chain. The MTT assay, which is dependent on enzymatic reactions, might also be influenced by enzyme inhibitors, for example chloroquine. NR assay on the other hand, is an inexpensive assay and is sufficient to measure the cell death (Weyermann *et al*., 2005). Moreover, NR assay requires fewer cells for analysis as a result of the optical density absorbance with the NR assay which is about twice than that obtained in the MTT assay, although both assay yield comparable ranking of cytotoxicity data (Borenfreund *et al*., 1988). In addition, the neutral red assay is also a useful tool to detect lysosomal damage when used in conjunction with other tests in

order to distinguish between cytotoxicity and organelle damage (Fotakis & Timbrell, 2006). However, in some cases, the NR assay is less sensitive (excitoxic model) and is not recommended for use in ion channel studies (Weyermann *et al*., 2005).

Previous studies have reported that, *P. chinensis* var. *chinensis* has produced certain chemical constituents which exhibited anti-inflammatory, anti-allergic properties (Tsai *et al*., 1998) as well as anti-fungal activity (Joshi *et al*., 1997).

Among the 18 tested extracts of *P. chinensis* var. *chinensis*, six extracts showed significant cytotoxic activity against CaSki, SKOV-3, MCF-7 and HT-29, i.e. the leaf methanol crude extract of the lowland and highland varieties, whole plant methanol crude extract of the highland variety, whole plant dichloromethane crude extract of the highland variety and also the stem dichloromethane crude extract of the highland variety. In contrast, none of the extracts assayed exhibited active cytotoxicity against A549 cell line. The presence of the apigenin, isorhamnetin, quercetin and luteolin in *P. chinensis* var. *chinensis* could explain the observed cytotoxic activity (Xie *et al*., 2007).

Apigenin is a flavonoid widely found in many fruits and vegetables. It has been shown to inhibit proteasome activity in breast cancer cells and breast cancer xenografts, induce apoptosis in leukemia cells and human breast cancer, exhibit anticancer activities (Chen *et al*., 2007) and inhibit UV-induced mouse skin tumourigenesis (Lepley *et al*., 1996). Apigenin can reduce the invasion of tumour cells, suppress tumour angiogenesis and therefore inhibit the growth of tumour cells and metastasis (Sun *et al*., 2004).

Luteolin is an important member of the flavonoid family. It has been reported that luteolin can inhibit the proliferation of serial tumour cells including solid tumours, human myeloid leukemia (Zhang *et al*., 2006) and may lead to growth inhibition of tumours and a reduced likelihood of cancer metastasis (Huang *et al*., 1999). Quercetin, a flavonoid, has been reported to have inhibitory effects on tumour cell properties and

proliferation (Huang *et al*., 1999; Wang *et al*., 2003), and also significantly inhibited the growth of the highly aggressive PC-3 prostate cancer cell line (Nair *et al*., 2004).

Isorhamnetin is a member of flavonoid components which has been used in the treatment of heart disease. The *in vitro* anticancer effect of isorhamnetin was observed on human esophageal squamous carcinoma cell line Eca-109 (Ma *et al*., 2007), It also exhibited noticeable anticancer effects on lung carcinoma cells (Yang *et al*., 2004; Zhu *et al*., 2005).

The other members in family Polygonaceae are well-known for their anticancer activities. For instance, *Fallopia japonica* (syn *Polygonum cuspidatum* and *Reynoutria japonica*) has well-documented anticancer activities *in vitro* and in bioassays based on animal models (Kintzios, 2006). The root extracts of *P. cuspidatum* contain the polyphenol resveratrol, (trans-3,4'5-trihydroxystilbene) and this compound has been shown to reduce, significantly, volume and weight of tumours as well as metastasis in highly metastatic Lewis lung carcinoma tumour-bearing mice (Kimura & Okuda, 2001). Emodin, another compound isolated from *P. cuspidatum*, has been reported to have anticancer properties against lymphocytic leukemia in mice (Hsu, 1989; Ogwuru & Adamczeski, 2000) and also acts as a strong inhibitor of a protein tyrosine kinase (Jayasuriya *et al*., 1992).

Recently, *Polygonum bistorta* was screened for its cytotoxicity against selected cancer cell lines and it has been reported to have moderate to very good activity, with the isolated compound, cycloartane-type triterpenoids, showing cytotoxic activities against P338 (Murine lymphocytic leukaemia), HepG2 (Hepatocellular carcinoma), J82 (Bladder transitional carcinoma), HL60 (Human leukaemia), MCF7 (Human breast cancer) and LL2 (Lewis lung carcinoma) cancer cell lines (Manoharan, 2006; Manoharan *et al*., 2007) .

The folk medicinal plant, *Polygonum hydropiper*, contains intense pungent substances in leaf and seed and is used against cancer (Hartwell, 1970). This plant has been reported to contain a potent cytotoxic warburganal, a drimane-type sesquiterpene dialdehyde (Fukuyama *et al*., 1980).

A common vegetable, *Polygonum odoratum*, is also reported to contain some flavonoids such as quercetin, rutin, catechin, isorhamnetin and kaempferol; the flavonoid, rutin, in combination with other flavonoids possess a strong anti-proliferative effect on breast cancer cells (Nanasombat & Teckchuen, 2009). *Polygonum hypoleucum*, a chinese medicinal plant, was found to have emodin and it has been shown to have successfully suppressed activities in various tumour cell proliferations (Kuo *et al*., 1997, 2001).

Techniques for cultivating medicinal plants always follow the same basic pattern but may differ in temperature requirements, pH preferences or the substrate on which these plants are grown. In this case, the highland and lowland varieties of *P. chinensis* var. *chinensis* are definitely grown in different environmental conditions, habitats, temperatures, rainfall, relative humidity, soil pH, soil type, the density of microbial life and so forth. As such in this study, the cytotoxic activities of the lowland and highland varieties of *P. chinensis* var. *chinensis* against different cell lines varied and these variations could be critically influenced by their physicochemical structure which may vary with habitats, cultivated conditions, isolation methods and other factors.

The lowland and the highland varieties of *P. chinensis* var. *chinensis* may produce different pure compounds resulting in different chemopreventive abilities to treat existing cancers. Therefore, these results strongly support further investigations of *P. chinensis* var. *chinensis* which involves looking into its possible antitumour and

anticancer properties; isolation of pure compounds, toxicity studies in order to determine its safety and selectivity index, *in vitro* pharmacological assays and so on.

CHAPTER 5

SUMMARY AND FUTURE STUDIES

5.1 Reproductive biology of the highland and lowland varieties of *P. chinensis* var. *chinensis*

The highland variety of *Persicaria chinensis* var. *chinensis* is a native species found in the tropics growing at lower montane forest at the altitude 1200 m and above. This scrambling herb is monoecious, non-seasonal and bears bisexual flowers. *P. chinensis* var. *chinensis* is heterostylous with short-styled (thrum) and long-styled (pin) flowers. The flowers are borne in compound corymb, usually terminal. The lowland variety of *P. chinensis* var. *chinensis* is an erect herb commonly grown in household compound and easily cultivated by cuttings. *P. chinensis* var. *chinensis* is a highly varied species, i.e. two main differences: the growth habit and leaf shape between the highland and lowland varieties. The lowland variety has yet to set fruit during three years observation in more than three different populations. Usually, the lowland variety is reproduced vegetatively by cuttings as this is frequently used as a traditional medicinal herb in Chinese medicine.

The time taken from the appearance of very young flower buds to the first anthesised flower was 12–14 days for the highland variety and 9–12 days for the lowland variety. The fertilized open flowers developed into mature fruit in about 14–18 days for the highland variety. The average fruit set of the highland variety in Gunung Ulu Kali at the altitude range from 1300 m–1400 m (namely population H2, H3 and H5) was 48% and only 2% in population H4 located at 1600 m. In the lowland variety, there was a high percentage of flower abortion and very high percentage of fruit degeneration; viable fruits had not been collected.

The anthesis time of the wild highland population was at 0600–0730 hours whereas that of the lowland variety was at 0745–0930 hours. The anther dehiscence and stigma receptivity are synchronous.

P. chinensis var. *chinensis* has eight stamens with five epipetalous stamens and three distinct and free stamens. The gynoecium of *P. chinensis* var. *chinensis* is superior, tricarpellary, syncarpous, and each carpel is uniovulate with a single orthotropous, bitegmic and crassinucellate ovule with basal placentation. The anther development and female gametophyte formation are similar in the highland and lowland varieties.

The anther is tetrasporangiate and the wall development conforms to the Monocotyledonous type. The endothecium has fibrous thickenings and this layer together with the epidermis remains until the time of dehiscence. The single middle layer is ephemeral and the cells of the glandular tapetum are initially uninucleate but become multinucleate with 3- to 4- nucleate cells which begin to degenerate after the formation of the microspore tetrads. Cytokinesis in the microspore mother cells is simultaneous forming both tetrahedral and decussate tetrads in the highland variety and mostly tetrahedral tetrads in the lowland variety. The mature pollen grains are shed at the three-cell stage.

The development of the embryo sac conforms to the monosporic *Polygonum* type. The embryo sac elongates more than three times of its original size (from 150 μm to 500 μm) before fertilization. An occurrence of twin eggs within an ovule was observed in the lowland variety. The ovule together with the embryo sac and ovary degenerate in the lowland variety and further development of the endosperm has not been observed. Nucellar beak is well-developed by the time the embryo sac is formed.

In the highland variety, the development of the endosperm follows the *ab initio* Nuclear type and free endosperm nuclei gather around the micropylar, chalazal and also at the periphery of the embryo sac. The endosperm is completely cellular only at the late

heart-shaped embryo stage. The zygote undergoes a short resting period. It initially develops into a dyad, proembryo, globular and later a heart-shaped and finally a slightly curved dicotyledonous embryo. The seed coat is formed from both the outer and inner integuments.

Heterostyly has been observed in the highland variety. The pin flowers have taller style, shorter filaments, less thick and less wide stigma than the thrum flower. The number of pollen grains in a pin flower was higher than that of in a thrum flower. The pollen grains of the thrum flower were bigger than those of the pin flower but they have similar pollen aperture, pollen tectal surface and pollen ornamentation. The pin and thrum pollen grains are 3-colpate, medium-sized, suboblate to oblate spheroidal (oblate according to Hesse *et al*. classification) and have reticulate exine with thick, narrow muri and free clavae in the deep lumina.

Comparing the morphology of the thrum flowers and their pollen in the highland and lowland varieties, the lowland thrum flowers have shorter style, longer filaments, thicker and wider stigma than that of the highland thrum flowers. The size of pollen grain of the lowland variety is bigger than that of the highland thrum pollen; they have similar pollen aperture and tectal surface. However, the exine ornamentation of the lowland thrum pollen is not similar in terms of the lumina and the muri; the Their lumina were less wide and the muri were thicker, less narrow and shorter than those of the highland thrum pollen with free clavae.

The pollen grains of the lowland variety do not germinate in the ‘complex medium’ and the ‘modified medium’. In the highland variety, the optimum sucrose and PEG concentration for germination was 15% and 30% respectively. No germination was found in the medium without PEG and bursting of pollen grains occurred in the medium without PEG. Pollen grains reached maximum germination (50%) in the modified medium with an

average pollen tube length of 140 µm. In the complex medium, the pollen tube produced double tubes and branching, reaching a maximum length of 525 µm while the average length of the pollen tube was 350 µm showing 21% germination.

The highland variety of *P. chinensis* var. *chinensis* was self-compatible. Open pollination produced three times more fruits than self pollination and shows a xenogamous breeding system indicating cross-compatibility and outbreeding. The pollen-ovule ratio suggested the breeding system as facultative xenogamy for the highland and lowland varieties.

The possible pollinating agents of the highland variety of *P. chinensis* are diverse. The most common insect visitors are from the Order of Hymenoptera, followed by Diptera along with a lesser number of Lepidoptera and Coleoptera.

Seed germination is epigeal showing 77% viability. The average percentage of germination for the fresh seeds was 47% under the natural highland environment and 34% under the lowland laboratory environment. The first seedling appears approximately one week after sowing in the highland environment and about 12 days in the lowland laboratory environment.

To improve our understanding of the reproduction of this variety, careful observation on the pollinator behavior and pollen load, seed predation and dispersal need to be further monitored.

5.2 Cytotoxic activity of the highland and lowland varieties of *P. chinensis* var. *chinensis*

The *in vitro* neutral red cytotoxicity assay was successfully applied to screen the cytotoxic activity against 18 crude methanol, dichloromethane and water extracts from the highland and lowland varieties of *P. chinensis* var. *chinensis* derived from the leaf, stem

and whole plant (leaf and stem). Although the results from the *in vitro* assay are not sufficient to predict the final effectiveness of potential natural products in cancer chemoprevention, this practical screening tool is able to identify the active and non-active extracts from the test samples.

Overall results showed that the methanol crude extracts from the leaf samples of the highland and lowland varieties, whole plant methanol crude extract of the highland variety, whole plant dichloromethane crude extract of the highland variety and also the stem dichloromethane crude extract of the highland variety possessed greater cytotoxicity effect against CaSki, SKOV-3, MCF-7 and HT-29 cancer cell lines. Two active extracts obtained from the methanol leaf extracts of the highland and lowland varieties were more effective in suppressing the expression of HPV-16 in cervical cancer-derived, CaSki cells and ovary cancer-derived, SKOV-3 cells. Results indicated that the methanol leaf and whole plant extracts of these highland and lowland varieties maybe a more promising agent for the therapy of HPV-16 associated cervical cancer and ovary cancer as compared to their dichloromethane and water counterparts. In addition, the dichloromethane extract from the stem and whole plant sample of the highland variety showed significant cytotoxic activities against colon cancer-derived, HT-29 cells.

Since the *P. chinensis* var. *chinensis* of the highland and lowland populations showed signs of the selectivity for CaSki, SKOV-3, MCF-7 and HT-29 cells, it is therefore necessary to also evaluate the cytotoxic effect of these extracts on other types of human cancer cells. Further studies should be initiated to isolate and identify the specific active compound(s) from the active extracts which demonstrated significant cytotoxic activities. *In vitro* pharmacological studies on the active metabolites isolated should be carried out to elucidate and understand the toxicity level and mechanism of action of the extracts.

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APPENDIXES

Appendix A: Critical Point Drying Procedures – preparation of specimens for scanning electron microscopy

1. Specimens were fixed in Craft III solution.
2. Suction was applied at 25–30 atmospheric pressure for 10–15 minutes and the specimens were left for at least 48 hours to one week.
3. After 48 hours, specimens were then fixed in alcohol 50% and prepared to use.
4. Specimens were washed in distilled water, 15 minutes with two changed.
5. After washing, specimens were soaked in glutaraldehyde + Sorensen's phosphate buffer (ratio 1:1) for one hour.
6. The solution was then discarded and changed to Sorensen's phosphate buffer + distilled water (1:1)
7. Discarded the solution and changed to osmium + distilled water (1:3) for 14 hours in a fume hood.
8. The specimens were dehydrated through a graded ethanol series from 10% to 90% (15 minutes in each of 10% steps).
9. The specimens were replaced by 95% ethanol and soaked for 15 minutes.
10. The specimens were changed with absolute ethanol for twice, 15 minutes each time.
11. Then specimens were soaked in 100% ethanol + 100% acetone solutions for 20 minutes, each at the ratio of 3:1, 1:1 and 1:3 and finally into pure acetone (100%) for 20 minutes.
12. The specimens were then left for one hour with three changes.
13. The specimens were then critical point dried.
14. The specimens were transferred on to a stub very gentle on a small amount of Electrodag 915 or Silverdag.
15. The specimens were coated with gold using a diode sputter coater.

16. The specimens were ready and can be scanned using a JEOL JSM-6400 scanning electron microscope.

Appendix B: Preparing the solutions for anatomical and embryological studies

1. Craf III Solution, fixing the specimen

1% Chromic acid	30 ml
10% Acetic acid	20 ml
40% Formaldehyde	10 ml
Distilled water	40 ml

2. Safranin "O"

   4 grams of the dye was dissolved in 200 ml of 2-methoxyethanol. Then 100 ml of 95% alcohol and 100 ml of distilled water were added.
   4 grams of sodium acetate in 8 ml formalin was added.

3. Fast green "FCF"

Fast green	1 g
Clove oil	30 ml
2-methoxyethanol	30 ml
Absolute alcohol	30 ml

4. Carbol-xylol

Xylene	300 ml
Phenol crystals	100 g

5. Xylol-alcohol

Xylene	100 ml
95% alcohol	100 ml

6. Egg albumen
   (a) Egg white was cutted with scissors.
   (b) An equal volume of distilled water and glycerine was added, followed by 0.5% sodium benzoate (1% of total volume).
   (c) The mixture was stirred and filtered.
   (d) The solution was then stored in a refrigerator.

7. Alcohol solutions

   50% alcohol = 50 ml 95% alcohol + 45 ml distilled water

   70% alcohol = 70 ml 95% alcohol + 25 ml distilled water

   80% alcohol = 80 ml 95% alcohol + 15 ml distilled water

8. Aerated wax

   (a) 49ºC wax was poured into a clean paper box

   (b) The wax was stirred until it became hard to introduce air into the wax.

Preparation of plant materials for microtome sectioning

1. Fixing: The specimen was cut to the required size and then fixed in Craf III solution for at least 48 hours
2. Pumping: Suction was applied at 25–30 atmospheric pressure for 10–15 minutes and the specimens were left for at least 48 hours to one week.
3. The specimens were washed in 50% alcohol for a few minutes.
4. Dehydration: The specimens were dehydrated through a series of Tertiary Butyl alcohol as follows: -

Step	95% ethyl alcohol (ml)	100% ethyl alcohol (ml)	Tertiary butyl alcohol (TBA) (ml)	Distilled water (ml)
1	25			25
2	25		5	20
3	25		10	15
4	25		17.5	7.5
5	25		25	
6		12.5	37.5	
7			50	
8			50	
9			50	

   Step 9 was consisted of new unused TBA. Step 7 and 8 was utilized the TBA that has been used once or twice.

   Materials may be stored for several days at steps 2 or 3. At any other step, it is best not to keep material more than one day.

5. Infiltration:

   (a) After leaving the specimens in clean and fresh TBA for 12 hours (In step 9), a few chips of aerated wax (49ºC) were added to the vial and kept at room

temperature (28ºC) overnight. If the wax has completely dissolved by the next morning, a few more chips of wax were added and left for a few more hours at room temperature.

(b) When no more wax could dissolve at room temperature, the vials were placed in the oven at approximately 58ºC.

(c) After about two hours, approximately 1/4 of alcohol-wax mixture was poured off and replaced with 1/4 49º C wax mixed with TBA.

(d) After about another two hours, approximately 1/2 of alcohol-wax mixture was poured off and replaced with 1/2 49ºC wax mixed with TBA.

(e) After another two hours, all liquid was poured off and replaced with 49ºC wax.

(f) Four hours later, all 49ºC wax was poured off and replaced with fresh and clean 49ºC wax.

(g) After 12 hours, fresh and clean Paraplast Plus Tissue Embedding Medium was changed (Melting point 56ºC).

(h) After at least 12 hours, suction at 25–30 atmospheric pressure was applied for 20 minutes at 70ºC and this process was repeated 3 times at 4 hourly intervals.

(i) The specimens were ready to be embedded.

6. Embedding

(a) The paraplast plus wax was poured out together with the specimens into a paper boat.

(b) The specimens were arranged properly and the wax block was left to cool in ice water.

7. Sectioning

(a) When the wax block has cooled down, the wax specimens were cut into suitable sizes with proper TS or LS position.

(b) The buds or flowers were sectioned at 6–8µm thickness while fruits at 8–10µm using a rotary microtome in an air-conditioned room.

8. Mounting

(a) A little drop of egg albumin (Appendix 1) was smeared on a clean slide.

(b) A little distilled water was put onto the slide.

(c) A wax ribbon with the specimen was placed on top of the distilled water (the smooth side of the wax ribbon facing downwards).

(d) The slide was warmed on a drying bench to allow the tissue to spread out.

(e) When the tissue was fully stretched, the slide was removed to drain away the excess water.

(f) The slide was dried in the oven at 40ºC for at least 2 days before staining.

9. Staining in Safranin-fast green

(a) The slides were placed in a trough of xylene for 20 minutes to remove the wax.

(b) Then, they were placed in a mixture of xylol-alcohol (1:1 xylene : 95% ethanol) to further remove all the wax traces.

(c) The slides were then transferred to 95%, 80%, 70% and 50% ethanol for 5 minutes in each solution.

(d) They were then stained in 1% safranin "O" in 50% ethanol (Appendix 1) for 12 – 24 hours.

(e) The excess safranin "O" was washed away in a basin of tap water.

(f) The slides were then passed through 50%, 70% and 95% ethanol and for 5 minutes in each step.

(g) The slides were stained for 3–4 seconds in fast-green "FCF" (Appendix 1).

(h) The slides were then differentiated in two changes of xylol-alcohol (5 minutes each) followed by carbol-xylol (3:1 phenol crystals: xylene) and for 15 minutes.

(i) The slides were cleared in two changes of xylene (30 minutes followed by 1 hour).

(j) The specimens were mounted in Canada balsam (1:1 canada balsam : xylene) and dried in the oven at 40ºC for four days.

(k) The slides were ready to be observed under a compound microscope.

Appendix C: Procedure for pollen acetolysis (Erdtman, 1960)

1. The acetolysing mixture was prepared by adding 9 parts of acetic anhydride and 1 part of concentrated sulphuric acid (9:1).
2. Approximately 5 ml of the acetolysing mixture was poured into a clean tube with pollen grains and the tube was put into a water bath (70ºC).
3. The temperature of the water bath was raised slowly from 70ºC to boiling point.
4. The tubes were left in the water bath for about 5 to 10 minutes and the content was stirred occasionally with the glass rod.
5. The tubes were taken out of the water bath when the colour of the solution had turned dark brown.
6. The solution was filtered with a fine wire-mesh and centrifuged at 500-1000 rpm for 15 minutes.
7. The acetolysing mixture was poured off quickly but gently.
8. 5 ml of washing mixture consisting of 3 parts distilled water and 1 part 95% ethanol was added. The tube was shaken and then centrifuged.
9. This washing was repeated for 3 times to get rid of the acid from the specimens.
   Note: Pollen grains which had turned very dark after acetolysis were bleached by adding two or three drops of sodium chlorate, followed by two to three drops of concentrated hydrochloric acid. Chlorine gas evolved would instantly bleach the dark pollen grains. These pollen grains were rinsed three to four times with the washing mixture to get rid of the acid.
10. The washing fluid was poured off and the tubes were inverted on a blotting paper and placed in the oven at 40ºC for approximately 10 to 20 minutes to dry.
11. A small piece of glycerine jelly stained with safranin was cut and dipped into the tube containing the pollen grains.
12. The small piece of glycerine jelly was transferred on the centre of a clean slide.
13. A coverslip was put on the centre of the clean slide and a small piece of wax was put around the periphery of the coverslip.
14. The slide was warmed gently above a spirit lamp to allow the wax and glycerine jelly to melt under the coverslip.
15. When the jelly was spread out, the pollen grains were sealed in by the wax.

Appendix D: Preparation of media and solutions for cytotoxic activity

Basic RPMI 1640 medium, DMEM and MEM media

Medium was prepared by dissolving one bottle of medium powder, 10.4 g, (Sigma) in one litre sterile distilled water. Two grammes of sodium bicarbonate (Merck) and 0.5026 g of N-2-hydroxylethyl-piperazine-N'-2-ethane-sulfonic acid (HEPES, Sigma) were added. The medium was stirred and the pH was adjusted to pH 7.4. The medium was filter sterilized using 0.22 µm filter membrane (Schleicher & Schuell) and stored at 4ºC up to a shelf life of 2 months.

10% supplemented RPMI 1640 medium, DMEM and MEM media

100 ml of 10% supplemented medium was prepared by mixing 90 ml of basic medium, 10 ml of heat inactivated foetal bovine serum (FBS was heated at 56ºC for 30 min, PAA lab), 2 ml of penicillin/streptomycin (PAA lab) and 1 ml of fungizone (PAA lab). The medium was filter sterilized using 0.22 µm filter membrane and stored at 4ºC up to 2 weeks.

20% supplemented RPMI 1640 medium, DMEM and MEM media

50 ml of 20% supplemented medium was prepared using 45 ml of 10% supplemented medium and 5 ml of heat inactivated FBS in a beaker. The medium was filter sterilized using 0.22 filter membrane and stored at 4ºC up to 2 weeks. This 20% supplemented medium was used to revive cells.

Freezing RPMI 1640 medium, DMEM and MEM media

The freezing medium consisted of 50% inactivated FBS, 40% of medium and 10% of DMSO (Sigma). The solution was mixed well using a 20 ml disposable syringe (Terumo) and filter sterilized using 0.22 filter membrane and stored at 4ºC. Freshly prepared freezing medium was using for cryopreservation of cells.

Phosphate buffer saline (PBS) pH 7.2

The PBS was prepared using 1.52 g of disodium hydrogen orthophosphate anhydrous (Na_2HPO_4, BDH), 0.58 g of potassium dihydrogen orthophosphate (KH_2PO_4, BDH) and 8.5 g of sodium chloride (NaCl, BDH), which were dissolved in 1 litre of sterile distilled water. The solution was stirred and the pH was adjusted to pH 7.2. The solution was then filter into a sterilized bottle using filter paper (Whatman 541). The solution was autoclaved and kept at room temperature.

0.4% Tryphan blue

0.4% Tryphan blue was prepared by dissolving 0.2 g of powdered tryphan blue (sigma) in 50 ml sterile distilled water. The solution was kept at room temperature.

Neutral red cytotoxic activity assay

Preparation of solutions

Neutral red stock solution

0.04 g of neutral red (ICN, Biomedicals Inc.) was dissolved in 10 ml of sterile distilled water to provide a final concentration of 4 mg/ml. The stock solution was wrapped with aluminium foil and stored at 4ºC.

Neutral red medium

The neutral red stock solution was further diluted (1:80) in treatment culture medium to give a final concentration of 50 µg/ml in a centrifuge tube. The solution was wrapped with aluminium foil and centrifuge twice at 1500 rpm for 10 minutes to remove any precipitate of dye crystals.

Neutral red washing solution

The washing solution consisted of 1 ml of formaldehyde (Sigma) and 1 g of calcium chloride ($CaCl_2$, Sigma), which were dissolved in 100 ml distilled water and stored at 4ºC.

Neutral red resorb solution

The resorb solution was prepared by adding 1 ml of glacial acetic acid (Merck) into a mixture of 50 ml absolute ethanol (Hamburg) and 49 ml distilled water. The solution was stored at 4ºC.

Appendix E: Pollen morphology in heterostylous variety of *P. chinensis* var. *chinensis*

Pin flower, highland

	Length of style (mm)	thickness of stigma (mm)	width of stigma (mm)	length of filaments, mm (distinct stamen)	length of filaments, mm (epipetalous stamen)
1	2.41	0.125	0.242	1.68	0.99
2	2.38	0.146	0.21	1.62	1.04
3	2.23	0.119	0.256	1.56	1.03
4	2.39	0.141	0.245	1.61	1.01
5	2.36	0.144	0.234	1.59	1.00
6	2.45	0.118	0.223	1.58	0.98
7	2.17	0.129	0.223	1.64	1.04
8	2.29	0.142	0.219	1.60	1.02
9	2.36	0.133	0.212	1.57	1.02
10	2.58	0.147	0.246	1.57	1.03
11	2.67	0.152	0.267	1.64	1.01
12	2.57	0.145	0.225	1.58	1.05
13	2.28	0.154	0.269	1.57	1.02
14	2.29	0.138	0.263	1.56	0.97
15	2.48	0.142	0.223	1.62	0.99
16	2.49	0.141	0.244	1.61	1.00
17	2.4	0.15	0.225	1.61	1.05
18	2.63	0.135	0.23	1.63	1.10
19	2.33	0.133	0.232	1.59	1.12
20	2.67	0.156	0.226	1.63	1.09
21	2.43				
22	2.37				
23	2.34				
24	2.3				
25	2.36				
26	2.41				
27	2.43				
28	2.41				
29	2.32				
30	2.42				
STDEV	0.122	0.011	0.018	0.032	0.040
Mean	2.41	0.140	0.236	1.603	1.028

Thrum flower, highland

	Length of style (mm)	thickness of stigma (mm)	width of stigma (mm)	length of filaments, mm (distinct stamen)	length of filaments, mm (epipetalous stamen)
1	1.17	0.171	0.255	2.16	1.52
2	1.16	0.172	0.266	2.10	1.72
3	1.13	0.197	0.265	2.06	1.62
4	1.06	0.172	0.261	2.19	1.50
5	1.12	0.156	0.268	2.21	1.48
6	1.18	0.204	0.282	2.09	1.63
7	1.06	0.137	0.255	2.02	1.52
8	1.17	0.159	0.268	2.28	1.58
9	1.14	0.161	0.225	2.26	1.63
10	0.99	0.175	0.277	2.13	1.52
11	1.04	0.17	0.258	2.16	1.50
12	1.03	0.17	0.285	2.14	1.64
13	1.00	0.164	0.272	2.05	1.69
14	1.00	0.174	0.25	2.21	1.70
15	1.09	0.179	0.23	2.08	1.54
16	1.15	0.19	0.252	1.99	1.62
17	1.13	0.173	0.257	2.06	1.52
18	1.08	0.183	0.268	2.15	1.57
19	1.14	0.174	0.273	2.01	1.64
20	1.11	0.162	0.275	2.15	1.62
21	1.03				
22	1.08				
23	1.08				
24	1.04				
25	1.04				
26	1.04				
27	1.03				
28	1.06				
29	1.09				
30	1.02				
STDEV	0.056	0.015	0.015	0.081	0.073
Mean	1.08	0.172	0.262	2.125	1.588

Thrum flower, lowland

	Length of style (mm)	thickness of stigma (mm)	width of stigma (mm)	length of filaments, mm (distinct stamen)	length of filaments, mm (epipetalous stamen)
1	0.859	0.184	0.292	2.42	2.07
2	0.871	0.192	0.265	2.41	1.99
3	0.828	0.180	0.285	2.77	2.15
4	0.847	0.199	0.267	2.6	2.01
5	0.849	0.196	0.315	2.73	2.02
6	0.891	0.200	0.305	2.73	2.12
7	0.908	0.152	0.289	2.62	1.91
8	0.91	0.175	0.290	2.62	2.2
9	0.899	0.171	0.258	2.5	2.22
10	0.886	0.177	0.277	2.61	2.12
11	0.871	0.181	0.286	2.74	2.03
12	0.882	0.172	0.262	2.68	1.98
13	0.891	0.167	0.285	2.73	2.21
14	0.926	0.162	0.282	2.62	2.2
15	0.872	0.180	0.273	2.57	2.18
16	0.913	0.175	0.268	2.71	2.09
17	0.865	0.165	0.290	2.67	2.1
18	0.908	0.200	0.307	2.69	2.08
19	0.882	0.184	0.278	2.63	2.13
20	0.902	0.178	0.294	2.62	2.16
21	0.91				
22	0.873				
23	0.877				
24	0.854				
25	0.881				
26	0.862				
27	0.909				
28	0.902				
29	0.904				
30	0.892				
STDEV	0.023	0.013	0.015	0.100	0.087
Mean	0.88	0.180	0.283	2.634	2.099

	Pin flower, highland		Thrum flower, highland		Thrum flower, lowland	
	polar length	equatorial diameter	polar length	equatorial diameter	polar length	equatorial diameter
1	36.083	42.764	40.874	47.499	45.220	49.091
2	35.420	42.201	40.231	46.891	45.555	47.993
3	36.374	41.272	37.752	46.546	41.002	47.117
4	37.063	41.949	42.473	46.009	43.017	48.970
5	36.987	40.683	41.232	46.104	44.956	48.791
6	36.077	41.783	42.517	46.131	44.753	49.473
7	35.400	42.390	41.407	48.978	42.884	47.161
8	37.818	39.329	39.927	45.933	46.231	46.369
9	35.664	42.143	41.461	47.798	43.655	48.070
10	35.826	41.060	41.030	45.176	44.767	49.002
11	36.520	40.674	41.740	47.598	46.442	47.564
12	37.400	41.407	41.867	45.312	42.181	47.110
13	37.833	41.798	41.575	47.730	44.953	49.980
14	37.374	41.534	41.760	45.828	45.527	43.654
15	36.148	40.794	41.029	47.599	46.001	50.646
16	35.717	41.274	42.862	46.116	47.013	45.540
17	37.278	41.035	42.122	46.602	45.940	49.065
18	37.932	43.997	40.110	45.234	44.736	50.004
19	36.935	41.477	41.065	45.449	44.942	50.228
20	37.672	40.869	36.035	45.063	43.521	43.643
21	34.268	40.123	39.006	45.834	45.701	51.752
22	35.754	40.044	41.808	46.927	45.698	47.554
23	35.056	41.050	41.820	47.200	44.653	43.691
24	34.876	41.779	41.897	45.383	42.420	48.009
25	34.232	40.505	41.334	45.882	47.636	48.771
26	37.297	41.344	40.639	47.099	47.851	49.656
27	34.292	40.563	39.790	45.975	47.691	49.799
28	36.907	42.127	41.767	45.160	49.200	47.802
29	36.557	42.644	40.403	47.368	43.471	49.164
30	36.160	41.944	39.217	46.435	45.223	48.140
31	37.148	40.591	40.414	45.549	45.908	51.689
32	36.068	41.960	42.836	46.299	47.985	51.597
33	35.440	42.126	39.709	46.431	41.140	46.312
34	36.999	41.869	42.401	46.147	42.422	47.270
35	35.090	42.102	42.499	46.846	44.691	45.604
36	35.732	41.081	40.162	45.688	42.918	44.404
37	35.421	40.559	39.160	44.729	42.560	48.617
38	36.023	41.604	39.964	45.484	41.341	49.510
39	37.212	41.416	40.526	47.826	44.575	50.197
40	35.297	41.892	40.328	46.782	44.119	48.265
STDEV	1.022359	0.854936189	1.39842712	0.952472	1.964752	2.098153
Mean	36.234	41.444	40.869	46.366	44.762	48.182

Welch Two Sample t-test (Table 3.8 & 3.9)

length of style (highland pin vs highland thrum)
t = 54.1446, df = 40.867, **p-value < $2.2e^{-16}$ (P<0.0001)**
confidence interval = 1.275895 1.374772
mean of pin = 2.407333, mean of thrum = 1.082000

thickness of stigma (highland pin vs highland thrum)
t = -8, df = 34.921, **p-value = $2.089e^{-9}$ (P<0.0001)**
confidence interval = -0.04093608 -0.02436392
mean of pin = 0.13950, mean of thrum = 0.17215

width of stigma (highland pin vs highland thrum)
t = -5.0566, df = 37.269, **p-value = $1.164e^{-5}$ (P<0.0001)**
confidence interval = -0.3697599 -0.01582401
mean of pin = 0.2357, mean of thrum = 0.2621

filament - distinct (highland pin vs highland thrum)
t = -26.7669, df = 24.695, **p-value < $2.2e^{-16}$ (P<0.0001)**
confidence interval = -0.5621897 -0.4818103
mean of pin = 1.603, mean of thrum = 2.125

filament - epipetalous (highland pin vs highland thrum)
t = -30.2256, df = 29.315, **p-value < $2.2e^{-16}$ (P<0.0001)**
confidence interval = -0.597875 -0.522125
mean of pin = 1.028, mean of thrum = 1.588

number of pollen grains in one anther (highland pin vs highland thrum)
t = 17.1585, df = 29.308, **p-value < $2.2e^{-16}$ (P<0.0001)**
confidence interval = 46.3146 58.8433
mean of pin = 151.947, mean of thrum = 99.368

polar length of pollen grain (highland pin vs highland thrum)
t = -16.9222, df = 71.426, **p-value < $2.2e^{-16}$ (P<0.0001)**
confidence interval = -5.181057 -4.088893
mean of pin = 36.23375, mean of thrum = 40.86872

equatorial diameter of pollen grain (highland pin vs highland thrum)
t = -24.3225, df = 77.107, **p-value < $2.2e^{-16}$ (P<0.0001)**
confidence interval = -5.325058 -4.519142
mean of pin = 41.4439, mean of thrum = 46.3660

length of style (highland thrum vs lowland thrum)
t = 17.7738, df = 38.769, **p-value < $2.2e^{-16}$ (P<0.0001)**
confidence interval = 0.1753448 0.2203886
mean of highland thrum = 1.082, mean of lowland thrum = 0.8841

thickness of stigma (highland thrum vs lowland thrum)
t = -1.6652, df = 37.555, **p-value = $1.042e^{-4}$ (P<0.0001)**
confidence interval = -0.016289066 0.001589066
mean of of highland thrum = 0.17215, mean of lowland thrum = 0.17950

width of stigma (highland thrum vs lowland thrum)
t = -4.3962, df = 38, **p-value = $8.574e^{-5}$ (P<0.0001)**
confidence interval = -0.03110845 -0.01149155
mean of of highland thrum = 0.2621, mean of lowland thrum = 0.2834

filament – distinct (highland thrum vs lowland thrum)
t = -17.646, df = 36.455, **p-value < $2.2e^{-16}$ (P<0.0001)**
confidence interval = -0.5669178 -0.4500822
mean of of highland thrum = 2.1250, mean of lowland thrum = 2.6335

filament – epipetalous (highland thrum vs lowland thrum)
t = -20.1143, df = 36.845, **p-value < $2.2e^{-16}$ (P<0.0001)**
confidence interval = -0.5619321 -0.4590679
mean of of highland thrum = 1.5880, mean of lowland thrum = 2.0985

polar length of pollen grain (highland thrum vs lowland thrum)
t = -10.2115, df = 70.445, **p-value = $1.547e^{-15}$ (P<0.0001)**
confidence interval = -4.654162 -3.133338
mean of of highland thrum = 40.86872, mean of lowland thrum = 44.76248

equatorial diameter of pollen grain (highland thrum vs lowland thrum)
t = -4.9841, df = 54.419, **p-value = $6.685e^{-6}$ (P<0.0001)**
confidence interval = -2.546158 -1.085542
mean of of highland thrum = 46.36600, mean of lowland thrum = 48.18185

Appendix F: Pollination experiment

Open pollination, population H2

No.	Flower	fruit
1	91	52
2	58	41
3	24	14
4	110	34
5	104	31
6	71	12
7	91	19
8	110	69
9	138	66
10	75	39
11	92	26
12	78	36
13	79	61
14	24	12
15	34	23
16	41	24
17	136	68
18	38	21
19	93	50
20	82	45
21	46	11
22	15	10
23	13	4
24	102	18
total	1745	786

Population H5

No.	Flower	fruit
1	28	14
2	98	55
3	71	52
4	133	83
5	39	5
6	83	42
7	47	3
8	50	31
9	69	30
10	131	65
11	109	56
12	44	29
13	66	31
14	143	86
Total	1111	582

population H3

No.	Flower	fruit
1	106	52
2	70	5
3	46	34
4	53	11
5	74	27
6	117	53
7	112	74
8	39	8
9	140	78
10	79	35
11	44	24
12	130	75
13	73	35
14	62	44
15	113	38
16	89	37
Total	1347	630

Bagging experiment, population H2

No.	Flowers	fruits
1	58	2
2	51	2
3	41	7
4	43	9
5	63	17
6	49	9
total	305	46

Population H3

No.	Flowers	fruits
1	31	6
2	30	5
3	64	12
4	59	11
5	48	8
6	37	3
total	269	45

Population H5

No.	Flowers	fruits
1	92	20
2	49	9
3	40	6
4	36	4
5	81	16
6	79	13
7	40	5
8	30	4
9	102	19
10	49	10
11	53	11
12	42	9
13	62	16
total	755	142

Appendix G: Cytotoxic activity of the highland and lowland varieties of *P. chinensis* var. *chinensis*

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the highland variety *Persicaria chinensis* var. *chinensis* against CaSki cell line		
	leaves	stem	whole plants
100	67.06±1.86	76.52±0.63	75.00±3.17
75	58.06±2.13	48.43±0.90	64.48±2.57
50	48.71±0.65	31.68±1.45	44.71±1.17
25	28.25±2.29	19.44±0.80	23.89±0.67
10	15.16±2.34	9.21±0.51	11.78±0.58
1	6.09±2.48	3.15±0.14	3.92±0.43

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against CaSki cell line		
	leaves	stem	whole plants
100	85.91±0.91	68.44±0.93	77.42±1.12
75	74.52±0.88	36.94±0.22	67.08±1.04
50	70.75±1.08	21.55±1.73	46.35±0.49
25	60.16±2.02	12.15±0.11	30.04±2.45
10	48.72±2.14	8.22±0.92	16.84±2.04
1	34.96±1.14	1.71±1.79	6.18±1.26

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the highland variety *Persicaria chinensis* var. *chinensis* against CaSki cell line		
	leaves	stem	whole plants
100	45.62±0.93	90.52±1.42	65.02±5.12
75	29.55±5.34	59.72±3.54	43.06±1.52
50	21.19±3.80	35.33±4.31	35.35±5.08
25	12.01±4.40	18.00±2.51	25.61±3.10
10	7.64±3.99	12.46±4.24	10.78±3.56
1	1.02±1.21	7.94±2.17	6.40±3.48

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against CaSki cell line		
	leaves	stem	whole plants
100	77.51±2.60	73.94±2.07	75.53±1.86
75	56.01±2.00	63.60±2.12	65.43±1.07
50	29.68±0.87	42.56±3.56	46.12±3.50
25	21.65±3.29	28.89±2.23	34.68±3.76
10	8.24±5.10	18.18±1.08	22.10±3.87
1	2.38±0.79	9.65±1.58	13.00±1.51

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the highland variety *Persicaria chinensis* var. *chinensis* against CaSki cell line		
	leaves	stem	whole plants
100	49.08±2.57	35.77±2.78	62.91±5.22
75	46.29±3.50	28.17±4.85	50.23±4.13
50	29.31±2.47	17.10±1.04	30.90±3.99
25	21.70±1.12	8.68±2.24	16.84±4.78
10	14.25 ±3.80	3.57±2.65	7.93±4.23
1	5.44 ±1.83	2.02±1.54	4.21±1.64

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against CaSki cell line		
	leaves	stem	whole plants
100	37.59±3.09	32.09±0.39	52.20±4.70
75	32.66±0.86	21.86±2.16	35.14±0.62
50	28.12±1.91	14.71±3.33	24.20±3.06
25	17.05±1.12	9.66±2.07	14.25±1.92
10	6.38±0.96	2.21±1.13	7.86±1.30
1	2.09±0.28	1.05±0.80	1.26±0.82

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the highland variety *Persicaria chinensis* var. *chinensis* against SKOV-3 cell line		
	leaves	stem	whole plants
100	91.43±0.50	86.81±1.17	89.85±0.46
75	90.55±0.65	77.47±1.73	79.53±0.89
50	82.89±1.10	61.48±4.51	62.12±0.57
25	59.82±1.42	29.97±1.90	54.16±1.35
10	33.57±1.78	27.39±1.60	35.21±1.63
1	13.14±0.18	14.94±0.96	16.60±0.61

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against SKOV-3 cell line		
	leaves	stem	whole plants
100	80.38±1.72	80.11±0.43	76.02±0.82
75	78.46±0.25	57.21±0.32	67.86±2.53
50	66.20±1.20	26.12±1.31	55.81±1.47
25	28.22±2.97	17.80±0.91	15.35±1.36
10	18.76±1.69	13.87±1.34	4.62±1.48
1	15.26±0.90	2.52±0.26	2.82±0.64

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the highland variety *Persicaria chinensis* var. *chinensis* against SKOV-3 cell line		
	leaves	stem	whole plants
100	63.79±1.17	59.15±0.71	60.72±1.19
75	39.44±0.59	33.43±1.32	35.06±0.87
50	32.59±1.02	12.89±0.93	22.32±0.95
25	18.71±1.78	9.89±1.91	12.73±0.63
10	7.99±1.67	5.32±0.62	6.67±1.41
1	3.37±0.07	1.07±0.40	3.76±0.35

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against SKOV-3 cell line		
	leaves	stem	whole plants
100	64.71±0.10	59.69±1.53	61.05±0.69
75	48.52±1.34	46.23±1.94	47.46±3.16
50	36.91±2.06	30.56±2.69	31.33±1.77
25	19.00±1.81	13.91±1.28	21.63±1.27
10	14.84±2.96	8.12±1.31	11.08±2.20
1	5.23±0.45	3.35±0.28	4.47±0.69

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the highland variety *Persicaria chinensis* var. *chinensis* against SKOV-3 cell line		
	leaves	stem	whole plants
100	45.17±0.87	41.38±1.09	45.53±0.66
75	30.74±0.74	22.00±1.51	37.06±1.21
50	16.69±1.64	15.68±0.82	29.35±0.88
25	6.32±0.92	10.60±1.52	15.69±1.32
10	3.35±1.45	4.56±0.58	3.13±0.45
1	1.04±0.46	3.09±0.80	1.97±0.21

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against SKOV-3 cell line		
	leaves	stem	whole plants
100	81.34±2.11	31.46±3.11	69.72±1.90
75	69.21±1.33	17.97±1.48	51.37±3.62
50	27.13±2.65	12.75±1.77	35.77±1.66
25	21.03±2.81	8.17±1.00	26.31±1.30
10	15.49±0.62	6.48±1.74	18.94±1.33
1	9.96±0.89	4.95±0.98	13.64±0.54

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the highland variety *Persicaria chinensis* var. *chinensis* against HT-29 cell line		
	leaves	stem	whole plants
100	75.83±2.09	87.75±0.71	76.56±2.08
75	65.59±2.56	77.43±2.44	67.44±0.98
50	40.91±2.65	61.20±2.27	51.61±2.00
25	26.80±3.57	34.07±1.64	38.16±2.58
10	15.31±1.10	22.73±2.52	18.32±3.46
1	5.65±1.89	9.05±1.00	9.96±0.96

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against HT-29 cell line		
	leaves	stem	whole plants
100	71.73±1.75	66.58±3.43	68.83±4.81
75	65.42±4.42	44.12±2.16	58.13±4.08
50	47.94±1.93	32.11±1.73	41.52±1.52
25	34.42±0.35	23.60±1.56	23.73±3.18
10	16.06±3.38	13.42±2.87	14.25±2.05
1	6.52±1.68	3.40±1.05	5.49±0.46

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the highland variety *Persicaria chinensis* var. *chinensis* against HT-29 cell line		
	leaves	stem	whole plants
100	77.40±1.73	90.86±1.26	77.34±2.97
75	59.50±2.12	89.54±1.15	73.90±3.01
50	47.01±1.48	83.90±2.91	69.52±2.83
25	33.67±2.88	70.26±3.18	54.79±5.09
10	15.91±2.20	44.58±1.76	31.89±1.99
1	5.29±0.87	19.26±0.50	15.60±0.99

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against HT-29 cell line		
	leaves	stem	whole plants
100	73.06±2.32	63.35±3.87	62.42±1.04
75	61.16±3.68	56.22±2.51	51.99±1.63
50	37.83±0.09	38.64±3.31	41.56±1.35
25	22.23±0.48	13.15±1.82	33.97±2.55
10	5.87±0.78	4.11±0.83	13.53±0.72
1	2.66±0.35	1.27±0.72	3.72±1.41

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the highland variety *Persicaria chinensis* var. *chinensis* against HT-29 cell line		
	leaves	stem	whole plants
100	31.94±1.42	29.31±1.85	34.68±2.51
75	24.01±1.93	21.96±1.04	25.46±3.02
50	14.15±1.74	17.95±1.88	21.01±2.97
25	11.18±1.96	12.50±1.11	9.98±1.64
10	3.43±2.78	7.79±0.68	6.30±1.02
1	2.01±1.57	3.54±1.56	3.37±0.76

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against HT-29 cell line		
	leaves	stem	whole plants
100	40.00±1.17	34.06±0.51	38.53±0.62
75	33.53±1.87	24.73±2.31	29.59±0.88
50	25.81±0.95	16.98±1.70	20.64±0.95
25	19.13±0.19	10.65±3.03	10.34±2.66
10	7.55±0.66	3.33±1.10	6.34±2.22
1	3.08±0.97	1.15±0.92	2.70±1.87

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the highland variety *Persicaria chinensis* var. *chinensis* against A549 cell line		
	leaves	stem	whole plants
100	66.06±3.77	52.55±2.30	60.76±0.92
75	55.64±2.59	31.88±1.95	51.20±4.92
50	44.61±2.77	20.35±1.54	38.80±1.17
25	21.40±1.31	14.40±1.75	17.51±3.09
10	15.79±0.50	6.13±1.68	5.26±1.30
1	12.90±0.91	3.03±0.96	2.43±0.85

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against A549 cell line		
	leaves	stem	whole plants
100	76.27±3.39	65.57±2.89	75.27±2.36
75	68.92±3.28	49.69±1.69	64.59±2.27
50	52.85±0.54	21.38±0.44	40.03±2.17
25	20.69±1.09	15.23±0.60	24.26±1.56
10	13.84±2.24	12.01±1.48	13.30±1.88
1	5.63±0.47	5.71±0.47	8.06±0.56

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the highland variety *Persicaria chinensis* var. *chinensis* against A549 cell line		
	leaves	stem	whole plants
100	66.95±1.50	65.74±0.57	54.71±2.08
75	62.36±1.64	54.18±2.30	33.69±1.41
50	48.94±0.27	47.26±1.68	24.79±1.84
25	44.16±0.53	27.19±0.49	19.67±0.52
10	35.76±1.17	18.15±2.82	15.16±1.66
1	23.85±1.04	8.14±0.27	8.88±0.52

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against A549 cell line		
	leaves	stem	whole plants
100	49.78±0.10	55.70±1.53	52.73±0.69
75	42.86±1.34	51.58±1.94	43.79±3.16
50	34.27±2.06	41.72±2.69	37.01±1.77
25	15.71±1.81	33.04±1.28	26.76±1.27
10	8.20±2.96	19.13±1.31	11.47±2.20
1	4.16±0.45	10.78±0.28	6.31±0.69

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the highland variety *Persicaria chinensis* var. *chinensis* against A549 cell line		
	leaves	stem	whole plants
100	19.38±1.76	30.22±0.44	26.30±0.66
75	12.56±0.63	18.70±1.38	20.04±0.51
50	8.76±1.17	12.62±0.58	16.81±1.84
25	7.43±1.68	9.49±0.28	9.61±1.41
10	3.34±0.86	5.33±1.58	6.55±0.60
1	1.20±0.50	1.54±0.36	2.54±0.48

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against A549 cell line		
	leaves	stem	whole plants
100	22.84±0.60	29.28±1.26	26.04±0.65
75	15.87±1.25	22.95±1.51	21.64±0.80
50	11.56±0.72	14.05±1.23	10.22±1.34
25	7.95±1.72	6.97±0.45	9.31±3.75
10	3.80±1.75	4.30±1.38	3.17±1.08
1	1.65±0.53	1.90±0.75	1.14±0.92

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the highland variety *Persicaria chinensis* var. *chinensis* against MCF7 cell line		
	leaves	stem	whole plants
100	71.70±3.20	90.22±0.25	73.97±3.73
75	65.76±3.44	84.88±2.16	61.78±0.71
50	49.20±3.05	50.32±1.63	42.85±2.32
25	34.24±1.18	36.26±2.92	36.06±2.99
10	18.88±1.22	23.88±1.14	22.41±3.07
1	11.09±3.65	12.63±1.92	13.19±2.02

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against MCF7 cell line		
	leaves	stem	whole plants
100	76.96±1.63	74.30±4.43	81.59±1.49
75	72.76±1.68	55.38±2.29	71.11±2.86
50	69.35±2.95	31.82±2.89	34.77±4.05
25	46.01±3.15	23.67±1.86	26.53±4.09
10	29.11±1.41	13.23±1.35	20.38±4.68
1	10.12±3.34	7.95±2.44	10.01±3.26

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the highland variety *Persicaria chinensis* var. *chinensis* against MCF7 cell line		
	leaves	stem	whole plants
100	79.68±1.44	88.78±2.08	65.38±2.14
75	54.80±0.89	56.46±2.25	37.99±3.46
50	30.91±1.95	41.19±1.84	39.37±4.03
25	21.41±2.68	15.78±1.26	18.95±2.64
10	12.75±4.17	5.78±0.83	4.17±1.57
1	5.07±3.30	2.34±0.27	0.93±0.37

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against MCF7 cell line		
	leaves	stem	whole plants
100	48.70±2.36	47.17±3.54	43.01±2.70
75	34.57±3.04	43.22±2.90	37.58±2.74
50	19.47±1.35	32.82±2.15	24.35±1.67
25	12.29±1.80	18.40±1.25	16.30±4.11
10	6.62±1.44	10.27±2.02	7.20±2.21
1	5.23±1.03	7.00±2.56	3.17±1.58

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the highland variety *Persicaria chinensis* var. *chinensis* against MCF7 cell line		
	leaves	stem	whole plants
100	58.28±2.13	39.70±3.29	64.13±4.11
75	46.60±1.74	28.34±5.11	59.88±4.66
50	37.18±1.69	20.39±3.49	39.12±3.57
25	26.83±1.41	10.48±2.61	24.39±1.33
10	6.08±0.67	3.98±1.27	3.87±3.21
1	1.60±0.24	0.45±0.26	1.85±2.51

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against MCF7 cell line		
	leaves	stem	whole plants
100	55.64±3.37	45.86±1.24	59.60±3.81
75	47.80±4.18	40.08±1.25	46.67±2.91
50	32.13±0.86	24.57±3.27	35.20±2.26
25	22.71±2.23	11.78±0.75	22.67±4.05
10	13.15±1.89	4.26±0.99	11.09±2.88
1	5.62±1.29	1.18±0.57	2.91±1.72

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the highland variety *Persicaria chinensis* var. *chinensis* against MRC-5 cell line		
	leaves	stem	whole plants
100	47.78±1.81	55.58±3.45	48.51±2.17
75	40.25±3.95	46.11±0.10	33.10±2.43
50	31.50±2.65	39.87±3.98	23.83±0.58
25	22.07±1.29	26.96±1.60	8.87±0.31
10	10.73±1.01	14.25±0.63	4.24±0.99
1	0.78±0.17	5.63±3.84	2.54±0.95

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude methanol extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against MRC-5 cell line		
	leaves	stem	whole plants
100	35.90±2.26	41.71±1.00	38.12±3.12
75	26.39±1.51	30.58±0.68	26.29±1.31
50	20.24±1.36	22.42±0.73	19.20±1.96
25	13.85±3.66	16.50±0.32	13.41±1.14
10	5.07±2.67	11.20±0.69	5.52±0.21
1	2.04±1.56	7.08±1.20	1.79±0.37

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the highland variety *Persicaria chinensis* var. *chinensis* against MRC-5 cell line		
	leaves	stem	whole plants
100	56.13±2.59	73.22±2.22	61.41±1.98
75	42.88±1.11	55.20±1.89	45.43±3.66
50	28.54±1.30	43.69±1.01	32.66±2.62
25	19.76±1.46	29.90±0.49	23.59±2.77
10	6.46±0.55	10.81±0.51	13.53±0.72
1	3.06±0.96	4.49±1.14	3.48±0.40

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude dichloromethane extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against MRC-5 cell line		
	leaves	stem	whole plants
100	53.21±1.13	47.19±3.23	48.32±1.51
75	44.66±1.50	39.05±2.86	42.75±1.07
50	41.40±1.32	30.77±1.17	35.48±1.05
25	30.43±0.81	22.83±1.41	26.81±1.32
10	20.20±0.95	13.50±0.60	16.93±1.42
1	6.08±2.63	3.67±0.45	4.44±0.89

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the highland variety *Persicaria chinensis* var. *chinensis* against MRC-5 cell line		
	leaves	stem	whole plants
100	58.28±2.13	39.70±3.29	64.13±4.11
75	46.60±1.74	28.34±5.11	59.88±4.66
50	37.18±1.69	20.39±3.49	39.12±3.57
25	26.83±1.41	10.48±2.61	24.39±1.33
10	6.08±0.67	3.98±1.27	3.87±3.21
1	1.60±0.24	0.45±0.26	1.85±2.51

concentration (µg/ml)	Average percentage (%) and standard deviation of 3 tests of growth inhibition by crude water extracts of the lowland variety *Persicaria chinensis* var. *chinensis* against MRC-5 cell line		
	leaves	stem	whole plants
100	34.76±1.24	40.78±1.16	38.09±1.34
75	27.39±1.88	29.12±0.64	27.04±1.93
50	17.88±1.26	22.04±0.54	19.98±0.29
25	12.06±1.19	13.10±1.33	11.67±0.70
10	3.27±0.62	3.77±1.25	3.46±2.13
1	1.23±0.14	2.23±0.53	1.14±0.64