Secondary pollen presentation and style morphology in the invasive weed *Mikania micrantha* in South China

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ABSTRACT. *Mikania micrantha* H.B.K. is a successful invasive weed in many parts of the world. Its reproductive biology, specifically, floral functional morphology, growth and behavior of the style during anthesis, and style morphology, was studied in an open *M. micrantha* population in South China during the flowering seasons of 2004 and 2005. Floral biology was studied in detail by examining florets at different developmental stages under a dissecting microscope and a scanning electron microscope. Stigma receptivity and pollen viability was determined by MTT [3-(4,5-dimethylthiazol-yl)-2,5-diphenyl-2H-tetrazolium bromide] staining technique. The results show that *M. micrantha* is protandrous and has a secondary pollen presentation system which characterizes the family Asteraceae. Typically, the flowering period is 6 days and can be divided into six floral stages (A, B, C, D, E and F) based on style morphology and behaviour. At the beginning of anthesis, the style bends to break and enter the tube formed by five fused anthers. Later, the style protrudes the anther tube and moves the pollen out from the anther. During anthesis, two partially overlapping phases, functionally male phase and functionally female phase, can be distinguished by MTT tests: the former is from stage B to D, and the latter is stage E. The style has two style branches in its terminal part. The sweeping hairs, which act as pollen presenter, are located on the tips and on the upper parts of the style branches forming developed stylar appendages. The stigmatic papillae are separated into two ventro-marginal bands along each style branch in symmetric arrangement. The bending behavior of the style and the sweeping hairs play an important role in presenting pollen to pollinator.

Keywords: Invasive plant; *Mikania micrantha*; Secondary pollen presentation; Sweeping hairs.

INTRODUCTION

Biological invasion is a pervasive and costly environmental problem (Vitousek et al., 1996; Kennedy et al., 2002; Perrings et al., 2005; Pimentel et al., 2005). Invasion is defined as the establishment of a species after human-mediated movement beyond its natural range or natural zone of potential dispersal and it is distinct from colonization that is often viewed as natural range expansion (Lee, 2002). Biological invasions are like natural experiments, but their processes are far more rapid than those in colonization (Sakai et al., 2001). For an introduced plant species to become invasive, it must be able to reproduce, even in initially small populations (van Kleunen and Johnson, 2005), and its rate of spread is influenced by the mode of reproduction, the reproductive system and potential for recombination, particularly if continuous adaptation is a prerequisite for the invasion process (Sakai et al., 2001).

Mikania micrantha H.B.K. (Asteraceae) is a fast-growing perennial creeping vine native to Central and South America (Wirjahar, 1976; Holm et al., 1977). It has many-branched stems and reproduces easily through both sexual and vegetative reproduction (Swarmy and Ramakrishnan, 1987; Zhang et al., 2004). It entered South China after 1910, and since the 1980s it has spread and invaded widely (Zhang et al., 2004). In recent years, this notorious weed has caused severe damage to many ecosystems and local economies in Guangdong Province, China and elsewhere in the world (Deng et al., 2004; Yang et al, 2005; Lian et al., 2006; Song et al., 2007). Therefore, it has been listed as one of the 100 worst invasive alien species on earth (Lowe et al., 2001) and as one of the top 10 worst weeds in the world (Holm et al., 1977).

Secondary pollen presentation is the developmental relocation of pollen from the anthers onto another floral organ which then functions as the pollen presenting organ for pollination (Howell et al., 1993). It is a reproductive strategy promoting outbreeding and it occurs in five monocotyledon and 20 dicotyledon families and many publications discuss it in the angiosperms in general (Howell et al., 1993; Ladd and Donaldson, 1993; Yeo, 1993; Ladd, 1994). Secondary pollen presentation is a widespread phenomenon that characterizes the family Asteraceae nearly

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throughout, and it is usually the terminal style that acts as the pollen presenter with active pollen placement (Howell et al., 1993; Ladd, 1994). In Asteraceae, the pollen presenter is sweeping hairs that cover the stigmas, and it can just be the style branches for the species that lack sweeping hairs (Ladd, 1994). Pollen is presented on the terminal section of the modified style where it is actively loaded onto the distal portion of the style as it elongates through a connate ring of anthers (Howell et al., 1993). Within the family Asteraceae, based on the arrangement of the sweeping hairs, different types of secondary pollen presentation have been found: pump mechanism, brushing mechanism, transitions between both that were found in the Asteroideae to which subfamily the Eupartorieae belong, and a special one in the basal subfamily Barnadesioideae (Leins and Erbar, 1990; Yeo, 1993; Erbar and Leins, 1995, 2000; Leins and Erbar, 2006).

Mikania micrantha has very small, compact florets in its inflorescences, and its flower morphology and pollen presentation are nearly unknown. To provide information on the basic reproductive biology of *M. micrantha*, we initiated this study using laboratory and field approaches in an open population at the field station of South China Botanical Garden during the flowering seasons of 2004 and 2005 to investigate: (1) floral functional morphology; (2) growth and behaviour of style during anthesis; and (3) style morphology.

MATERIALS AND METHODS

Study site

The study was conducted at the field station of South China Botanical Garden during the flowering seasons of 2004 and 2005. It is in the suburb of Dongguan City, Guangdong Province, China. Dongguan (22°39’-23°09’ N, 113°31’-114°15’E) is in a subtropical area and is located south of the Tropic of Cancer. It has a lower south subtropical marine monsoon climate. *Mikania micrantha* mostly occurs in open habitats in South China, so at the station we selected a typical population for our studies in an open field where an artificial *Magnolia denudata* forest was cut 10 years ago, and there were almost no other plants except *M. micrantha*.

Flowering phenology

Flowering phenology was observed for 12 *M. micrantha* individuals per season from September to February in 2004 and 2005 flowering seasons. On each plant, 10 capitula buds of similar size were marked and observed at 0800, 1200, 1500 and 1800 h every day until the flowers senesced to collect data on the timing and duration of flowering and fructifying.

Floral biology

We collected at least 80 samples from flower buds to flowers at anthesis from different capitula in 10 *M. micrantha* plants in each season and observed them under a dissecting microscope (SE-CTV, Olympus, Japan). The flowers of *M. micrantha* are co-sexual and we examined the spatial and temporal arrangement of male and female sexual parts within the flowers. We used the information obtained to define six floral morphological stages (A, B, C, D, E and F) based on style morphology and behaviour (Figure 1A-F) and use these stages to classify the flowers we observed during the flowering seasons.

Pollen viability and stigma receptivity. In each flowering season, at least 50 fresh flowers with dehiscing anthers in different floral stages (Figure 1A-F) were randomly collected from 10 individual *M. micrantha* plants. The pollen samples were removed from the anthers and then immersed in a drop of 20 g L^{-1} 3-(4,5-dimethylthiazol-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma M-2128) solution and examined under a microscope (Rodriguez-Riaño and Dafni, 2000). Dark blue staining indicates the pollen is viable. Pollen viability was expressed as mean percentage of stained pollen grains of more than six samples. In each sample, we calculated the percentage number of dark pollens to total number of pollen grains in each of seven randomly selected visual fields under the microscope. The number of dark pollens in each sample was recorded as the average number in the seven visual fields. Due to the secondary pollen presentation in *M. micrantha* (Figure 1A-F), only pollens in anthers were examined to avoid including exotic pollens. The same test was used to determine stigma receptivity (Rodriguez-Riaño and Dafni, 2000).

Fine-scale style and pollen morphology. A scanning electron microscopy (SEM) was used to examine the style morphology. For each floral stage, 20 pistils of fresh flowers were dissected and fixed in 4% glutaraldehyde in 0.1 mol L^{-1} phosphate buffer at pH 7.4. The fixed samples were rinsed three times in the same buffer and fixed again in it with 1% osmium tetroxide added. They were then dehydrated in a graded alcohol series (30% - 50% - 70% - 80% - 90% - 100%) and immediately freeze-dried using a freeze-drying device JFD-310 apparatus (JEOL Ltd., Tokyo, Japan). Then, they were mounted directly on metal stubs using double-sided adhesive tape and sputter-coated with platinum using JFC-1600 auto fine coater (JEOL Ltd., Tokyo, Japan). Morphology of the stigmas was observed with a JSM-6360LV SEM (JEOL Ltd., Tokyo, Japan) at 15 KV. For each floral stage, pollen grains were also randomly selected and treated as above, and then 20 were observed under a SEM to study the pollen morphology (shape, size, and exine).

RESULTS

Flowering phenology

Flowering and fructifying of *M. micrantha* occurred from September to February in both study seasons. The peak bloom occurred between mid November and mid December. Fruiting started as early as the end of October, and almost all fruits had dropped in early February.

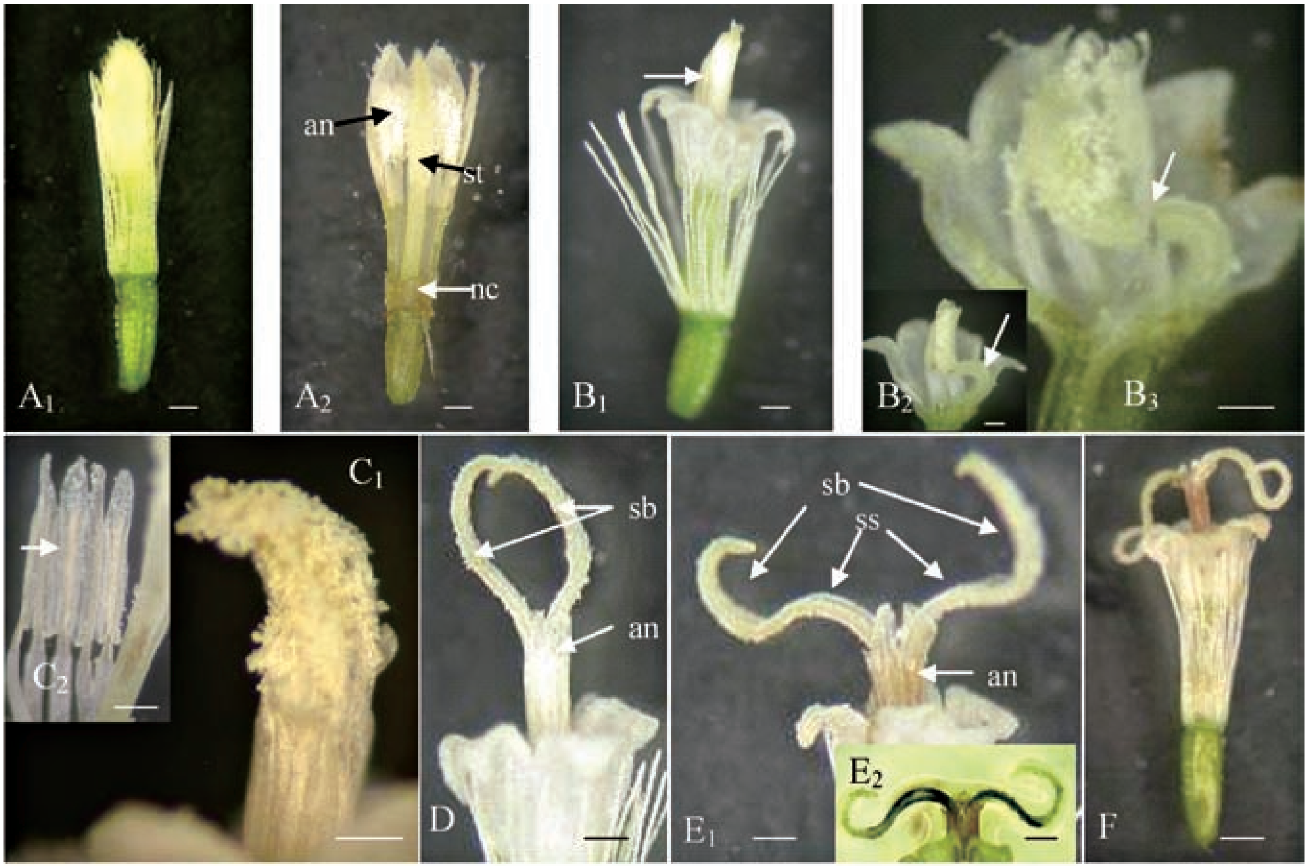

Figure 1. A-F, Floral stages of *Mikania micrantha* showing secondary pollen presentation. A_1, Early floral stage showing flower is beginning to open; A_2, Dissected flower in A_1 showing nectary (nc), anther (an) and style (st); B_1, Flower just opened (the arrow indicates the anther tube); B_2, Dissected flower in B_1 showing the bending part of the style (arrow); B_3, Dissected anthers in B_1 showing the bending part of the style is pushing through the anther tube (arrow); C_1, The style is out of the anther tube; C_2, Dissected flower in C_1 (the arrow shows the empty anthers with few pollen grains); D, Style branches (sb) separate, curved and slightly inflexed, while the anthers (an) are nearly empty; E_1, The style branches (sb) expose the stigmatic surfaces (ss) and the anthers (an) are dry, brown and withering; E_2, Test result of stigma receptivity showing the stigmatic surface appears dark blue when immersed in a drop of MTT solution under a dissecting microscope; (F) Senescence stage showing the two style branches bending towards the centre of the floret. Functionally male phase is from stage B to D, and functionally female phase is stage E. All bars=0.5 mm except E_2=200 μm.

Floral biology

Floral morphology. *Mikania micrantha* is hermaphroditic and its corymbose inflorescence consists of a 4-flowered tight capitula. Its individual florets are 5.87±0.46 mm in length and are actinomorphic. The capitulum is 6.6±0.50 mm in length and 1.65±0.63 mm in diameter. Five anthers attach each other and form a tube, and the stigma and the style grow through the centre of the tube. The flowers produce nectar and pollen. There is a green and ringed ovary-roof nectary which encircles the base of the style (Figure $1A_2$).

Floral stages. The flowers open at any time of day, but the majority do so in the morning. Typically, the flowering period is 6 days and can be divided into six floral stages (A, B, C, D, E and F) based on style morphology and behaviour (Figure 1). In general, within each capitulum, the central florets flower before the surrounding ones. During the 24 h before anthesis (stage A), the growing filament raises the anther tube up to the same level as the style (Figure 1A). During this time of growth, the anthers dehisce inwardly and discharge pollen grains inside the anther tube onto the sweeping hairs of the style. The style branches are aligned and the stigma is not yet receptive.

During anthesis, two partially overlapping phases can be distinguished: (1) Male phase: Between about 6.00-8.00 h of day 1, the flower opens and the tip part (see Figure 1C) of the style breaks through the anther tube formed by five fused anthers, and its lower part (see Figure 1C) bends inside the corolla tube (stage B, Figure 1B). The growing style then unthreads through the tube and loosens the pollen grains which then adhere to the sweeping hairs of the style branches. The green nectary begins to secrete and accumulate nectar within corolla tube at stage B and continues. About 1.5 h later, the lower part of the style straightens, the style begins to protrude out of the anther tube (Figure 1C) and the pollen grains are removed from the anthers by the sweeping hairs (stage C). At this stage, insect visitors were observed. In the afternoon of day 1, the style grows longer until the tip has completely emerged from the anther tube and presented more pollen attached on the sweeping hairs of the style. At the end of stage C, the florets are completely open, and the anthers have released almost all the pollen. The style and style branches present the yellow, clumped pollen grains to the inside of the flower while the style branches are still joined. Insect visitors appear more frequently than before and remove pollen. By day 2, the style branches separate with a slight inward inflexation (Figure 1D) but their lower parts with stigmatic area located on the inner surface and covered with receptive papilla cells are still joined (stage D). At the late male phase, early on the third day after flower opened, tests of stigma receptivity gave negative results. The floral scent is present during daylight hours, and it becomes stronger from stage B to D.

(2) Female phase: From day 3 to 5, the style grows to its full length, its two branches completely open, two layers of receptive papilla cells are exposed, and anthers become dry and brown and begin to wither (stage E, Figure 1E). Nectar secretion and scent emission continue and frequent insect visits occur. By day 6, flowers enter the senescence stage (stage F, Figure 1F) when the two style branches bend towards the center of the floret. From day 7 on, fertilized flowers continue to grow and the green ovary develops into a plump and black seed, while unfertilized ovules shrivel and turn yellow.

Pollen viability. Pollen viability decreases with the progress of floral stages (Figure 2). At stage A, pollen has the highest viability at 95.0%, which then decreases to 86.3% at stage B. At stage C, pollen viability further decreases to about 78.3%. At the late male phase (stage D), early on the third day after flower opened, pollen viability is about 53.8%, while at the early female phase (stage E), late on the third day, viability of the few remaining pollen in the anthers is about 34.4%. In the senescence stage (stage F) the pollen viability decreases to only 9.5%.

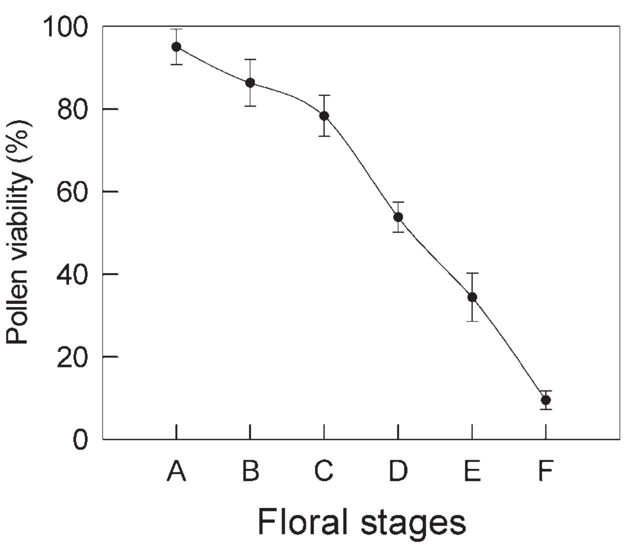

Figure 2. Pollen viabilities in different floral stages (data are presented as means±1SE). See Figure 1 for the descriptions of the floral stages from A to F.

Pollen characteristics. The pollen grains appeared globular or sub-globular under SEM (Figure 3A) and they are 3-colporate. The size of pollen grain is 19.95±1.96 µm in the polar axis and 14.67±0.86 µm in the longest equatorial axis. The ornamentation of exine is echinate, and the length is 1.85±0.33 µm.

Style morphology

The style has two style branches in its terminal part (Figure 3B), and the morphology of one style branch is shown in Figure 3C. Sweeping hairs were concentrated on the tips (Figure 3D) and on the upper parts (Figure 3E) of the style branches forming developed stylar appendages that are sterile structures extended above stigmatic areas. Generally, the sweeping hairs gradually increase in length basipetally, and they act as pollen presenter which is responsible for removing pollen out of the anther tube while the terminal section of the style branches grows through and presenting them. The stigmatic surface is covered with columnar papilla cells that are very close to each other. The stigmatic papillae are separated into two ventro-marginal bands along each style branch in symmetric arrangement (Figure 3F). MTT test showed that the stigmatic area was receptive (Figure $1E_2$).

DISCUSSION

Secondary pollen presentation system is widespread in angiosperms (Howell et al., 1993). It characterizes the family Asteraceae (Howell et al., 1993), and we found that *M. micrantha* is no exception. Our observations show that *M. micrantha* is protandrous, and pollen is shed from the anthers onto the sweeping hairs of the style in the bud stage (Figure 1A) when the style branches are joined and the stigmatic surfaces are not receptive despite that the pollen has the highest viability. This is consistent with the observations on some other species of Asteraceae (Porras and Álvarez, 1999; Roitman, 1999; Cerana, 2004; Grombone-Guaratini et al., 2004). Our results show that the sweeping hairs are both located around the tip of the style branch and reaching below the branching of the style. Thus, *M. micrantha* presumably has attained a combination of a pump pollen presentation mechanism and a brushing mechanism, based on the arrangement of the sweeping hairs for different types of secondary pollen presentation (Yeo, 1993; Erbar and Leins, 1995; Leins and Erbar, 1990; 2006).

We observed during early stages (stages B-C) of anthesis of *M. micrantha* that part of the style is bended. This bending makes it possible for the style tip to break and enter the anther tube sidewise. With the growing of the style, the bending part straightens. This unbending process may generate a pushing force which would not only help the style to protrude out of the anther tube but also facilitate the friction between the sweeping hairs of

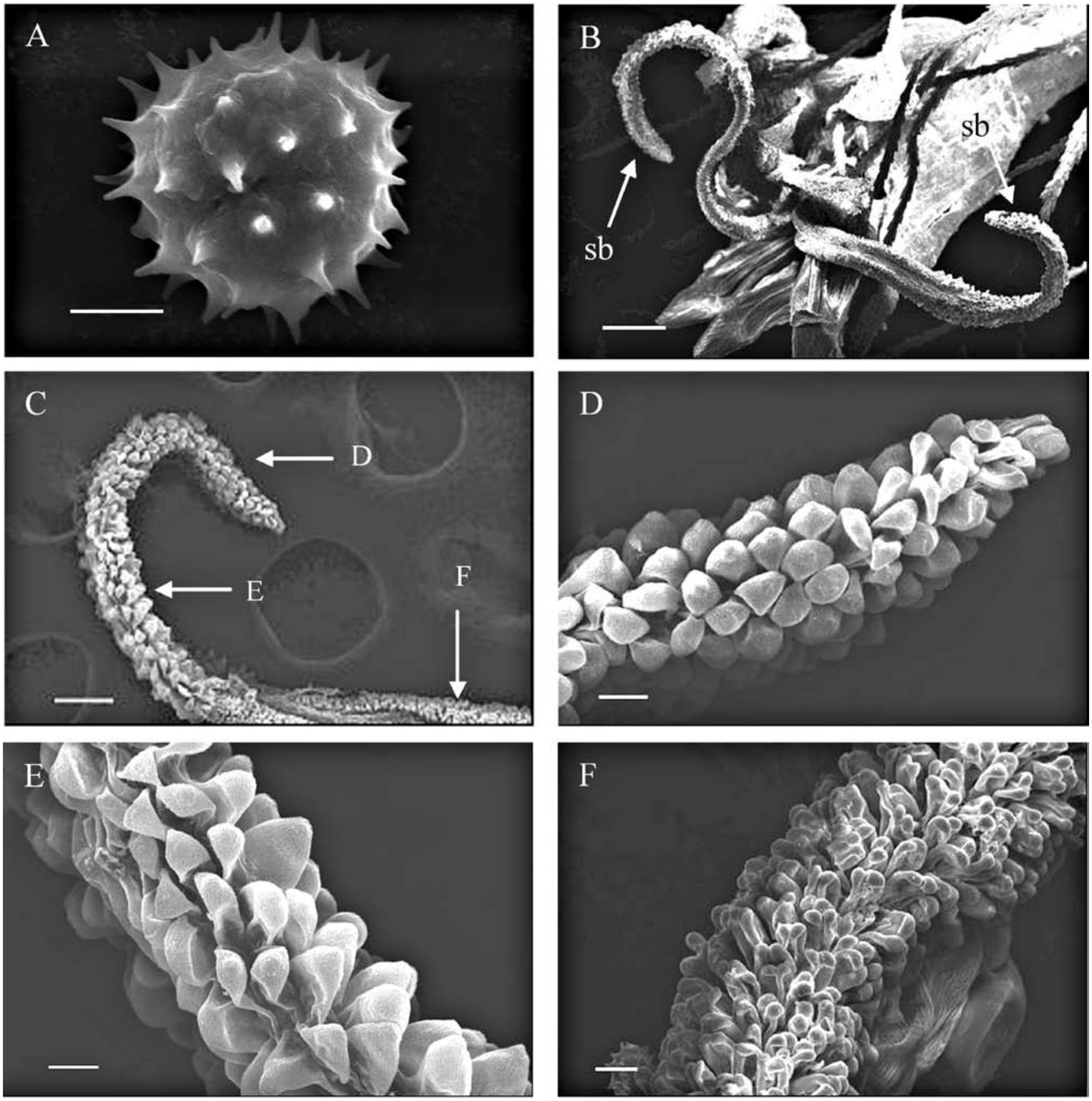

Figure 3. The morphology of pollen and style in *Mikania micrantha* under SEM. A, Pollen (bar=5 μm); B, A style gives off two style branches (sb) (bar=200 μm); C, Detail of a style branch showing the sterile appendage covered by sweeping hairs (D, E) and the stigmatic surface (F) (bar=100 μm); Arrows with figure numbers indicate the approximate position of anatomical sections illustrated in corresponding figures; D, Sweeping hairs at the tip part of the style branch (bar=20 μm); E, Sweeping hairs located on the upper part of the style branch (bar=20 μm); F, Receptive stigmatic surface consisting of columnar papilla cells (bar=10 μm).

the style and the anthers. The pollen grains lodged in the anthers were loosened and adhered to the sweeping hairs of the style. Then, they were brought out of the anther tube with the growing of style. Thus, the bending behaviour of *M. micrantha* style and the sweeping hairs of the style branches may play an important role in presenting pollen to pollinator.

Within each of the style branches, the sweeping hairs gradually increase in length basipetally. Moreover, due to the echinate ornamentation of the exine, the pollens can adhere onto the sweeping hairs. Thus, the sweeping hairs can easily carry the pollens with them out of the anther tube. Therefore, these morphological and structural characteristics of the stylar sweeping hairs and pollen are co-adapted to bring the pollens out effectively. The sweeping hairs of *M. micrantha* are much different from those of Polygalaceae and Fabaceae (Westerkamp, 1999), Cyphiaceae (Leins and Erbar, 2003), because different species display variation in this mechanism, perhaps reflecting differences in mating systems (Anderson et al., 2000; Etcheverry et al., 2003; Leins and Erbar, 2003).

As the style grows out of the anther tube, the outside of the style branches presents pollen for pollination. The receptive papillate stigmatic surface is hidden between two appressed style branches, preventing auto-pollination during the functionally male phase of the floret (Figure 1B-D). Our previous study has suggested that the pollen grains should be transported from the sweeping hairs of one plant to the stigmatic surface of another plant, which has been proven by our pollination treatments indicating that *M. micrantha* needs insect pollination (Hong et al., 2007). During the functionally female phase (Figure 1E), the style branches separate completely, and two layers of receptive papillae on the adaxial surfaces become exposed and are receptive. Thus, the behavior of style determines the timing of the functionally female phase of the floret. In some Asteraceae species, maturation of the flower is marked by the gradual separation of the style branches until they eventually reflex back onto the presenting surface and auto-pollinate though many species are sporophytically self-incompatible (Howell et al., 1993).

Our pollen viability results show that pollen viability began to gradually decrease after flower opened which results in a temporal separation between pollen presentation and pollen viability and diminishes the chance of self-pollination, but some are still viable after the flower have entered female phase. This indicates that there is an overlap between the time when pollen is viable and when the stigma of the same floret is receptive, and thus self-pollination cannot be totally excluded. This shows that the receptive stigma is in general temporally separated from the floret's own viable pollen, but cannot completely avoid the probability of autogamy or geitonogamy. However, our pollination experiments for *M. micrantha* show that seed/ovule ratio was 0.56 for open pollination and 0.0034 and 0.0038 for wind pollination and selfing, respectively (Hong et al., 2007). These indicate that the stigmas almost completely accept outcross pollens. Thus, although flower morphology does not fully prevent self-pollination (and geitonogamy can easily take place), the level of autogamy is very low. Therefore, some self-incompatibility mechanism seems operative in this species (Hong et al., 2007).

Protandry is prevalent throughout the Asteraceae. In self-incompatible species protandry can avoid pollen-stigma interference (Webb, 1985), but this is not fully achieved in *M. micrantha*, since some overlap exists between pollen delivery and receipt, which is a fact in most Asteraceae species.

In conclusion, the results indicate that *M. micrantha* has a system of secondary pollen presentation. During anthesis, the bending behaviour of the *M. micrantha* style and the sweeping hairs of its two branches help the growing style to move the pollens out of the anthers efficiently. This style-developmental morphology and its potential functions would be of great importance to better understand the evolutionary history of floral structure and function in angiosperms. The secondary pollen presentation system and the fine-scale morphology and structure of style indicate that there is some adaptive advantage to be derived from their formation.

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華南入侵雜草薇甘菊的次級花粉展現和花柱形態

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薇甘菊（*Mikania micrantha* H.B.K.），又名小花蔓澤蘭，已在世界上很多地方成為一種成功的入侵雜草。我們從花的功能形態學，花柱在花期的生長和行為特徵，以及花柱的形態等方面，對薇甘菊的繁殖生物學進行了系統研究。本研究於 2004 和 2005 年的兩個薇甘菊花期選擇華南地區的一個開闊地中的薇甘菊種群進行。其中，薇甘菊的花生物學特性通過應用解剖鏡和掃描電子顯微鏡技術對不同發育階段的花進行觀測；柱頭可受性和花粉活力使用 MTT [(3-(4,5-dimethylthiazol-yl)-2,5- diphenyl-2H-tetrazolium bromide)] 染色技術確定。結果表明，薇甘菊的花為雄蕊先熟並具有菊科植物典型的次級花粉展現系統。薇甘菊的單花期一般為 6 天，我們根據花柱的形態和行為將單花的開放過程分為 6 個階段（A，B，C，D，E 和 F）。在開花的初始階段，花柱彎曲在花藥筒內，這種彎曲在花柱伸出過程中所產生的彈力可以幫助花柱突破由 5 個花藥形成的花藥筒，隨後花柱穿過花藥筒並將花粉從花粉囊帶出。MTT 檢測實驗表明，薇甘菊花的雄性功能時期和雌性功能時期並沒有完全分開，仍有少量重疊：其中 B-D 階段為雄性功能時期，E 為雌性功能時期。薇甘菊的花柱頂端具二叉分枝。掃粉毛位於花柱分枝的頂端和上部，起著展現花粉的作用。柱頭狀乳突細胞沿著每個花柱分枝在其腹側邊緣成二條帶狀對稱分佈。在開花初期，花柱的彎曲行為以及柱頭的掃粉毛在薇甘菊的傳粉過程中起著重要的作用。

關鍵詞：入侵植物；薇甘菊；次級花粉展現；掃粉毛。