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FLORAL NECTAR COMPOSITION OF AN OUTCROSSING BEAN SPECIES *MUCUNA SEMPERVIRENS* HEMSL (FABACEAE)

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Abstract

Mucuna sempervirens is a perennial woody climber belonging to family Fabaceae. Hand pollination experiments have proved it to be an out crossing species. In the present study, the chemical composition of *M. sempervirens*'s floral nectar was analysed. Concentrations of sugars, proteins, phenolics, and hydrogen peroxide were determined through spectrophotometry. Free amino acids were identified and quantified, among which aspartic acid was the most abundant. GC-MS analysis showed that aromatic compounds were responsible for the nectar scent which lacked sulphur compounds. ICP-AES analysis determined calcium to be the nectar's major inorganic ion. The presence of high levels of hydrogen peroxide in the nectar might serve as defence against invading microorganisms. Phenolics may act as to repel nectar thieves repeller or serve a defensive function. Our findings show that *M. sempervirens*'s pollination system is different from other neotropical *Mucuna* species. Analysis of these differences may help to better understand how Asian *Mucuna* species adapted and coevolved with pollinators.

Introduction

Mucuna is a genus of about 150 species belonging to the family Fabaceae, which is distributed throughout tropical regions of the Old and the New World (Allen & Allen, 1981). They are common to sandy beaches, coves, bushwood areas, forest borders, thickets, and moist areas. *Mucuna* species in neotropical regions have been well studied, and shown to be bat pollinated (Agostini *et al.*, 2006; Fleming *et al.*, 2009). However, very little is known about the *Mucuna* species in subtropical and temperate regions of Asia, and, to date, no studies on the pollination systems of *Mucuna* species outside the tropical regions have been published. The aim of this study was to see how *Mucuna* species outside tropics, such as the Asiatic *Mucuna sempervirens* Hemsl, differ from their well-documented tropical counterparts, particularly in regard to pollination and nectar composition.

Floral nectar composition is central to understanding a species' pollination system and relationship with potential pollination vectors (Heil, 2011). Different groups of animals differ in their physiology and nutritive requirements; therefore the composition of sugars and amino acids in nectar is usually highly correlated with the specific nutritive requirements of a flower's pollinator (Gonzalez-Teuber & Heil, 2009). It is interesting that insects do not give preference to any specific morph, as they are interested to get nectar instead of any pollen (Abid, 2010). In addition, secondary metabolites and volatile compounds in flower nectar have been associated with both antimicrobial defensive functions and pollinator attraction (Gonzalez-Teuber & Heil, 2009). In some cases, nectar constituents can play an important role in determining the plant-pollinator interaction, and may also help defend the flower against invaders; both the functions are critical and to allow the flower to achieve its ultimate goal of improving the rate of out crossing (Thornburg *et al.*, 2003; Raguso, 2004; Gonzalez-Teuber & Heil, 2009; Gonzalez-Teuber *et al.*, 2009, 2010; Abid & Sarwar, 2012). Species are evolved according to their native habitat (Sajjad & Saeed, 2010).

There are 18 *Mucuna* species found in China, nine of which are endemic and used in Chinese traditional medicine (Sa & Melanie Wilmot-Dear, 2010). *Mucuna sempervirens* is a densely foliaged woody climber species, and is widely distributed in subtropical regions of China, Bhutan, NE India (W Bengal, Manipur, Sikkim), Japan and Myanmar. This species possesses some features common to bat-pollinated flowers: large and strong inflorescence, large quantities of nectar, an unpleasant smell, flower's positioned on branches or tree trunks, and large quantities of pollen (Howe & Westley, 1988). The bees (*Apis cerana cerana* Fabricius and *Bombus montivolans* Richards) are its main pollinators in the Kunming Botanical Garden (Yunnan, China) and in nearby natural habitats. To better understand the relationship between *Mucuna* species and pollinators outside the tropical regions, this study was undertaken to determine the precise composition *M. sempervirens* nectar including sugars, proteins, free amino acids, inorganic ions, phenolics, and volatile compounds and analyze the potential functional aspects of the nectar's components.

Materials and Methods

Plant material and breeding system: The compatibility system of *M. sempervirens* Hemsl was determined by performing controlled pollination experiments at the Kunming Botanical Garden using six *M. sempervirens* plants from the garden's living collection. The experiments were performed in May 2010. Inflorescences were bagged during the bud stage to exclude all potential pollinators. After anthesis, ten flowers on each of six inflorescences were hand-pollinated with pollen from the same plant (self-compatibility test), and another ten flowers on each of six different inflorescences were hand-

pollinated with pollen from different plants (self-incompatibility test). One month after hand pollination fruit set frequencies for self- and cross-pollinated inflorescences were compared.

Chemical analysis: Unless otherwise noted, all chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich Inc (St. Louis, MO, USA). Fresh nectar was collected in May 2010 from *M. sempervirens* plants in the Kunming Botanical Garden, China, using a pipette and autoclaved tips. Each flower produced 20~80µl of nectar. All nectar samples were kept on ice and stored at -20°C prior to use. Wide and narrow range pH test strips (Sigma) were used to test the pH of fresh nectar from 30 randomly selected flowers from 6 different plants. The level of hydrogen peroxide in the nectar from 30 randomly selected flowers was analyzed through a commercially available kit (Beyotime, Jiangshu, China), and measurements were performed in accordance with the manufacturer's directions. Except for pH and hydrogen peroxide tests, all other analyses in this study used pooled nectar samples from six *M. sempervirens* plants. The total sugar content was determined by colorimetric analysis using the phenol–sulphuric acid method (Dubois *et al.*, 1956). The individual concentrations of the main sugars in the nectar (glucose, fructose, and sucrose) were determined through the colorimetric–enzymatic method using a commercial kit (Biosentec, Toulouse, France). The protein content was determined according to the method of Bradford (1976), with bovine serum albumin (BSA) used as a standard. The Folin–Ciocalteu method (Chang *et al.*, 2002) was used to measure total phenolic content. Gallic acid was taken as a standard, and the content was expressed in µg of Gallic acid equivalents (GAE) mL^{-1} of fresh nectar. Following digestion with nitric acid, the concentrations of calcium, phosphate, and other elements in *M. sempervirens* nectar were determined using inductively coupled plasma atomic emission spectrometry (Shimadzu, ICP-AES; ICP 1000-II series, Japan). After most of the proteins were removed from the nectar by ultra-centrifugal filtering with a Microcon YM-3 centrifugal filter (cut-off 3000 Da; Millipore, Bedford, MA, USA), the concentrations of free amino acids in nectar were measured using an Amino Acid Analyzer (Hitachi, L-8800, Japan).

GC-MS analysis: Ten ml of fresh nectar were extracted with *n*-hexane and shaken at room temperature for one hour. The mixture was then centrifuged at 12000g for 30 min at 4°C. The supernatant oils were dried (anhydr. Na_2SO_4), concentrated under a stream of N_2, and subjected directly to either GC [Agilent HP5890, column: 0.32 mm × 30 m (30QC2/AC5)] with temperature programming from 80 to 280°C at 3°C/min, or to GC–MS [Agilent HP6890/5973, column: 0.25 mm × 30 m (HP-5MS)] analysis under the following conditions: electron-impact (EI) mode (70 eV), constant current 1.0 ml/min, and temperature programming from 80 to 240°C at 3°C/min. Identification of volatile compounds was performed using MSD ChemStation software for Windows. Peak identification was made by comparing retention times and the mass spectra of eluting compounds to those in the NIST/Wiley database.

Result and Discussion

Mating system: Neotropical *Mucuna* species are reported to be bat-pollinated in the way that they possess some physical features that suggest self-incompatibility (Agostini *et al.*, 2006). In this study, no fruit set was observed on self-pollinated *M. sempervirens* inflorescences, while the mean fruit set frequency on cross-pollinated inflorescences was 30%. The species was thus shown to be an outcrossing species. It also possesses some features of self-incompatibility, such as large and deep corolla, a strong floral scent, syrupy nectar, etc. Bees (*Apis cerana cerana* Fabricius and *Bombus montivolans* Richards) were observed as its main pollinators.

Sugar, protein, and free amino acids: Sugars, proteins, and free amino acids are the three major components of floral nectar among which sugars are the most dominant (Nicolson & Thornburg, 2007). In general, insect-pollinated flowers produce relatively concentrated nectar, whereas bat pollinated flowers produce dilute and hexose-rich nectar (Baker & Baker, 1983). In this study *M. sempervirens* nectar was found to be both highly concentrated and sucrose dominant (Table 1). The mean total sugar concentration was 29 %, much higher than the mean range of most neotropical bat-pollinated flowers (15-18%) (von Helversen, 1993) and similar to many bee-pollinated flowers (Baker & Baker, 1983). Moreover, the ratio by weight of sucrose to glucose and fructose (S/(G+F)) was 5.38, a figure comparable to, or even higher than, the ratio found in the nectar of flowers pollinated by bees and hummingbirds (Baker & Baker, 1983; Perret *et al.*, 2001). These high levels of sugar concentration indicate that *M. sempervirens* is bee-pollinated, which is consistent with our observations in a local botanical garden and in nearby natural habitats.

The mean value of total protein in *M. sempervirens* nectar was 620µg mL^{-1}. This value is almost the highest total protein concentration in floral nectar reported to date, and far exceeds the mean value of total protein in most other floral nectars (ca. 100 µg mL^{-1}) (Nicolson & Thornburg, 2007). Considering that *M. sempervirens* is a bean species, which are generally nitrogen-fixing, the high protein concentration in its nectar is not surprising, and may be used as an extra reward for potential pollinators.

The concentration of total free amino acids in the nectar was 460µg mL^{-1}, and 16 proteinaceous amino acids were identified and quantified (Table 1). Aspartic acid was most abundant and comprised 61% of the total free amino acids in the nectar. The amino acid balance is thought to be central to determining the nectar's taste (Gardner & Gillman, 2002). It has been suggested that some amino acids are more beneficial to certain pollinators than others and that aspartic acid can increase insect growth (Dadd, 1973). High concentrations of aspartic acid therefore suggest a connection between *M. sempervirens* and insect pollination. Although proline and phenylalanine have been reported to be important components in floral nectar (Petanidou *et al.*, 2006; Nicolson & Thornburg, 2007), these were relatively scarce in *M. sempervirens* nectar. Further study is needed to better understand the relationship between aspartic acid dominant nectars and potential pollinators.

Table 1. The chemical constituents of *M. sempervirens* nectar.

Test[a]	pH	Sugars	Total protein	Free amino acids	Inorganic ions	Volatile compounds		Others
		Details (mg mL^{-1})	(μg mL^{-1})	Details (μg mL^{-1})	Details (μg mL^{-1})	Details (% from the total volatiles)		Details
	4.4±0.2 (n=30)	Total sugar 293.8±5.0 (n=6)	620.3±40.2 (n=6)	Aspartic acid 205.6 ± 3.0	Ca 329.4±38.9	Fatty acid derivatives Tetradecane	5.5	Total phenolics (μg GAE mL^{-1}) 120.4±10.7 (n=8)
				Isoleucine 40.9 ± 1.8	Mg 113.8±16.5			
		Sucrose 137.8±18 .0 (n=10)		Cysteine 14.6 ± 2.8	S 58.5±9.4	Hexadecane	3.5	Hydrogen peroxide (μM) 68.4±60.3 (n=30)
				Valine 10.9 ± 2.4	Cu 4.2±1.4	Erucylamide	3.0	
		Glucose 11.7±1.7 (n=10)		Proline 8.8 ± 2.0	Fe 1.2±0.4	Dodecane	1.9	
				Tryptophan 7.4 ± 1.4	Zn 0.5±0.1	Decane	1.8	
		Fructose		Histidine	P nd[b]	Octadecane	1.4	

Table 1. (Cont'd.).

Test[a]	pH	Sugars	Total protein	Free amino acids	Inorganic ions	Volatile compounds		Others
		Details (mg mL^{-1})	(μg mL^{-1})	Details (μg mL^{-1})	Details (μg mL^{-1})	Details (% from the total volatiles)		Details
				Lysine 2.6 ± 0.8 (n=3)		2,5-Cyclohexadiene-1,4-dione,2,6-bis(1,1-dimethylethyl)	1.0	
						2,6-dt-t-butyl-4-methylene-2,5-cyclohexadiene-1-one	1.0	
						Alpha pinene	0.8	
						Anti-1-(1-hexynyl)-4-metoxy-4-methyl-2,5-cyclohexadien-1-ol	0.8	
						1-Limonene	0.5	
						Nonanal	0.4	
						Gamma-Terpinene	0.3	
						2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	0.3	
						Benzenoids		
						Dibutyl phthalate	6.9	
						1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	4.2	
						Phenol,2,6-bis(1,1-dimethylethyl)-4-methyl	4.0	
						Anthracene	1.6	

Inorganic ions: As shown in Table 1, six inorganic ions were detected and quantified in the nectar. Among these calcium and magnesium were the most abundant, with mean concentrations of 329.4µg mL^{-1} and 113.8µg mL^{-1}, respectively. It has been reported that plants pollinated by bats have higher levels of calcium in their nectar than plants pollinated by other animals (Barclay, 2002). This may be due to the fact that calcium is a limiting resource for bats. Plants that depend on bats for pollination and seed dispersal may have evolved to provide increased calcium as a reward and means of competing for bat visits (Barclay, 2002). Since published data on concentration of ions and their role in floral nectar are uncommon, we can only suggest that high concentrations of calcium and magnesium in *M. sempervirens* nectar might act as attractants for potential pollinators.

pH, hydrogen peroxide, and total phenolics: Most nectars are slightly acidic (Baker & Baker, 1983). The Nectar of *M. sempervirens* has also been found to be acidic, with a pH of 4.4±0.2 (Table 1). Spectrophotometeric analysis showed the presence of hydrogen peroxide and phenolics in the nectar; mean concentrations were 68.4µM, 120.4µg GAE mL^{-1}, respectively (Table 1). The high levels of hydrogen peroxide that accumulate in the nectar of ornamental tobacco are believed to serve as a defensive function, protecting the metabolite-rich nectar from invading microorganisms (Carter & Thornburg, 2004). The hydrogen peroxide detected in nectar might play a similar role. Phenolic substances are quite widespread in nectars (Baker & Baker, 1983; Liu *et al*., 2007). Their accumulation may render the nectar as toxic and repel some visitors (Hagler & Buchmann, 1993). It is possible that nectar of *M. sempervirens* might also be involved in the interaction between the flower and its visitors.

Volatile compounds: *M. sempervirens* nectar has a strong, unpleasant scent, and flowers without nectar were observed to be almost scentless. In this study, 32 volatile compounds were identified in the nectar using GC/MS by comparing retention times and the mass spectra of eluting compounds to those in the NIST/Wiley database (Fig. 1). These 32 identified compounds accounted for 54.2% of the total volatiles (Table 1). They were grouped into three major classes (fatty acid derivatives, terpenoids, and benzenoids) based on their biosynthetic origins. None of these three classes were dominant in *M. sempervirens*. Terpenoids have been identified in the nectars of numerous plant species, and are generally thought to be insect attractants (Tholl *et al*., 2004). In *M. sempervirens* nectar, 9.1% of the total volatiles were terpenoids and might serve as insect pollinator attractants. The floral scent compositions in the few previously studied bat-pollinated species are characterized mainly by sulphur-containing compounds (von Helversen *et al*., 2000). However, no sulphur-containing volatile compounds were detected in *M. sempervirens* nectar, indicating that this species might not be bat-pollinated.

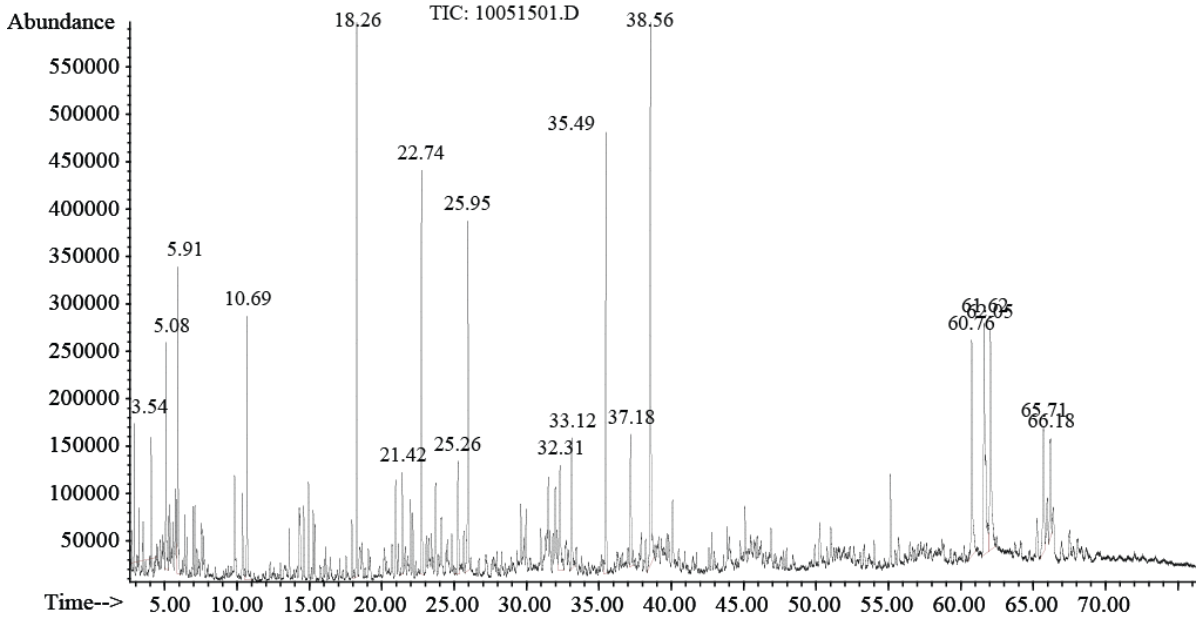

Fig. 1. GC chromatogram of floral nectar samples of *M. sempervirens* Hemsl.

Conclusion

Our results have clearly demonstrate that *M. sempervirens* is an outcrossing species. It is different from other *Macuna* species studied in neotropical regions due to its highly concentrated sucrose dominant floral nectar. These differences suggest that unlike its bat pollinated relatives, *M. sempervirens* is most likely insect pollinated. In addition, nearly the highest total protein content published to date was measured in the nectar of *M. sempervirens*. We suspect that the high level of protein is related to the nitrogen fixing function *M. sempervirens* which may be acting as an extra reward for pollinators. Sixteen free amino acids, among which aspartic acid was

the most abundant, and phenolics were detected in the nectar of *M. sempervirens*. These components might function to attract pollinators. Six inorganic ions and 32 volatile compounds were identified and quantified in the nectar. The particular abundance of calcium indicates that *M. sempervirens* nectar maintains some similarities with tropical bat pollinated *Mucuna* species. However, lack of sulphur-containing compounds in the nectar of this species is an obvious dissimilarity from the nectar composition of most bat pollinated flowers. Hydrogen peroxide present in the nectar, probably plays a role in defending against microbial attack.

In general, this study has added to the further understanding of the *Mucuna* genus through first analyzing the pollination system and nectar composition of a previously unstudied Asiatic *Mucuna* species and then comparing its features with those of well-studied tropical *Mucuna* species. The pollination syndrome in this species is a hybrid feature, typical to both bee and bat pollinated species. Moreover, analysis of the nectar composition has revealed interesting results, particularly exceptionally high protein content in the nectar and the presence of potentially antimicrobial components. The specific functions of the nectar components identified in this study warrant further examination in future research.

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