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BIOLOGÍA REPRODUCTIVA DE *HELICTERES GUAZUMIFOLIA* Y *HELICTERES BARUENSIS* (STERCULIACEAE) COMO ESPECIES SIMPÁTRICAS DEL BOSQUE TROPICAL SECO DE COSTA RICA

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Biología para optar al grado de *Magister Scientiae* en Biología

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ÍNDICE

LISTA DE TABLAS

ARTÍCULO I

ARTÍCULO II

LISTA DE ILUSTRACIONES Y FIGURAS

ARTÍCULO I

ARTÍCULO II

RESUMEN

Los estudios sobre biología de la polinización han llevado al supuesto de que las interacciones entre plantas y los polinizadores animales se han especializado implícitamente por una serie de caracteres asociados que delimitan los síndromes de polinización (Molina-Freaner & Eguiarte 2003, Etcheverry & Trucco Alemán 2005). El conocimiento de los caracteres de estos síndromes constituye una herramienta muy poderosa para predecir el espectro de polinizadores de una especie particular de planta (Vogel 1990, Von Helversen 1993, Murcia 2002). Generalmente, las plantas polinizadas por animales han evolucionado adaptaciones que las hacen atractivas para sus polinizadores específicos y por lo tanto aumentan la probabilidad de transferir polen de una planta individual a otra (Von Helversen 1993).

Los modelos fenotípicos predicen la especialización a un polinizador particular cuando la ganancia marginal de idoneidad (fitness) excede la pérdida marginal de idoneidad (fitness) resultante al ser menos adaptado a otros polinizadores. Los sistemas de polinización especializados evolucionarán cuando los polinizadores efectivos están predeciblemente disponibles tanto en el tiempo como en el espacio (Molina-Freaner & Eguiarte 2003). Sin embargo, estudios recientes han cuestionado la fuerza de la correlación entre los síndromes florales y los ensamblajes de polinizadores, sugiriendo que tanto los polinizadores como las plantas son más generalizados de lo que previamente se había pensado (Muchhala & Jarrín 2002, Etcheverry & Trucco Alemán 2005).

La especialización en la interacción planta-polinizador tiene tres aspectos a favor. En primer lugar, se optimiza la inversión de la planta en néctar o en polen, dado que éstos son consumidos únicamente por los polinizadores más eficientes. Segundo, se garantiza que los estigmas sólo reciben polen de otros individuos de la misma especie. Por último, se maximiza el retorno energético para el polinizador, pues éste mejora su eficiencia para extraer el recurso, asimilar los nutrientes y manejar cualquier sustancia tóxica que esté presente en el néctar o en el polen (Murcia 2002).

La morfología floral es uno de los aspectos más importantes en la interacción planta-polinizador. Determina la accesibilidad del polinizador al néctar, la eficiencia de la deposición de polen en el cuerpo del polinizador, y la eficacia en la adquisición de polen por parte del estigma de los vectores de polen. Las corolas también funcionan como protección de los recursos florales

contra visitantes florales no polinizadores. Aunque las correlaciones entre morfología floral y sistemas de polinización se han discutido repetidamente, la morfología floral ha sido usualmente descrita de forma cualitativa, como tazón, platón o tubular (Sakai *et al.* 1999).

La cuantificación de la morfología floral usualmente está restringida a la profundidad de la flor, la cual se estima está relacionada en algunos casos con la longitud de la lengua del polinizador. Sin embargo, se ha demostrado la importancia de otras características morfológicas aparte de la profundidad de la flor. Más de una característica morfológica causa la diferenciación en los nichos de forrajeo entre los visitantes florales. Una evaluación precisa y una comparación de la morfología floral entre especies emparentadas básicamente con la misma construcción representan un enfoque fructífero (Sakai *et al.* 1999).

Otros caracteres florales también afectan las interacciones entre plantas y sus polinizadores. Caracteres tales como forma, color y fenología, junto con producción de néctar (cantidad y concentración) son caracteres importantes asociados con varias interacciones planta-polinizador. Se ha considerado que han evolucionado y diversificado en respuesta a las preferencias de los animales visitadores de las flores (Bawa 1990, Knudsen & Tollsten 1995). Además el grado de eficiencia del polinizador contribuye con las posibles fuerzas selectivas que pueden estar actuando sobre estas características (Murcia 2002).

Los nectarios son estructuras secretoras de las plantas de diversa morfología, anatomía y función (Koptur 1994). Secretan néctar, como una solución acuosa externa rica en asimilados, siendo sus principales componentes varios azúcares, aminoácidos, y otros constituyentes menos abundantes (Endress 1994, Langenberger & Davis 2002). Su función primaria en un contexto evolutivo es del tipo fisiológico, la eliminación del asimilado superfluo. Sólo de forma secundaria fue que los nectarios adquirieron varias funciones ecológicas (Endress 1994).

Los nectarios median dos interacciones mutualistas importantes entre las plantas y los animales, la polinización y la protección. El néctar producido por los nectarios florales es la recompensa más importante para los visitantes florales, y representa el costo fisiológico primario pagado por la planta en sistemas de polinización basados en el néctar (Koptur 1994, Perret *et al.* 2001, McDade & Weeks 2004). Sin embargo, comparado con otros recursos tales como polen y aceites, en casos donde éstos representan los principales atrayentes de las flores, la producción de néctar es relativamente una baja inversión de las plantas para atraer a los visitantes florales (Langenberger & Davis 2002). Los nectarios no están confinados a órganos florales particulares,

pueden localizarse en tallos y hojas (Endress 1994, Proctor *et al.* 1996). La secreción de néctar está a menudo asociada con la base de los pétalos y estambres (Proctor *et al.* 1996).

Los tres azúcares principales que se encuentran en el néctar son sacarosa, fructosa y glucosa. La sacarosa es un disacárido que puede ser hidrolizado en partes iguales de dos monosacáridos, glucosa y fructosa, por la acción de la enzima invertasa. El néctar puede contener sólo sacarosa, o una mezcla en varias proporciones de los tres o de dos azúcares. Se puede encontrar un rango completo entre néctar dominado por sacarosa y néctares en los que las hexosas constituyen un 90% o más de los azúcares presentes; la glucosa y la fructosa no se presentan necesariamente en cantidades similares (Proctor *et al.* 1996). Las proporciones de sacarosa sobre fructosa y glucosa se han relacionado con diversas clases de polinizadores (Perret *et al.* 2001).

Los aminoácidos son el segundo grupo de componentes más importante que se encuentra en el néctar. Estos componentes pueden ser nutricionalmente significativos para muchos visitantes florales, especialmente para aquellos que no se alimentan de polen u otros materiales ricos en proteínas. Existen correlaciones entre el contenido de aminoácidos del néctar y el tipo de visitante (Proctor *et al.* 1996).

Debido a su papel significativo en la atracción de polinizadores potenciales y en servir como precursor de la miel, el néctar floral ha recibido amplia atención en cuanto a investigación (Langenberger & Davis 2002). Su accesibilidad en relación con la morfología floral, pero también sus propiedades inherentes tales como concentración, volumen, viscosidad y composición química, determinan las relaciones planta-polinizador (Perret *et al.* 2001). Desde los estudios pioneros de Baker y Baker en los años setenta, el néctar de especies de regiones templadas y tropicales ha sido estudiado con cierto detalle (Gottsberger *et al.* 1984, Baker & Baker 1990).

Más información detallada acerca de la biología del néctar (variación intraespecífica en cantidad y cualidad, tasa y costo de producción, y respuesta a la remoción por visitantes florales) está disponible únicamente para muy pocas especies (McDade & Weeks 2004). La mayoría de los estudios de la química del néctar han sido diseñados para revelar la convergencia entre comunidades de plantas ecológicas o geográficas, sin embargo unos cuantos se ocupan de las comparaciones de néctar entre especies que pertenecen a un mismo género o tribu (Perret *et al.* 2001).

La mayoría de las especies de plantas son polinizadas por varias especies de vectores, que a menudo pertenecen a órdenes distintos o incluso a diferentes clases (Bertin 1982). El conocimiento de los polinizadores de los cuales dependen las plantas es esencial para la conservación (Muchhala & Jarrín 2002). Es claro que los vertebrados, tales como murciélagos y colibríes desempeñan una función significativa en la polinización (Endress 1994, Proctor *et al.* 1996).

Los murciélagos son importantes polinizadores de un número considerable de especies de plantas en los trópicos de el Viejo y el Nuevo Mundo. Se ha reportado o se ha asumido que cerca de 750 especies distribuidas en 64 familias de angiospermas son polinizadas por murciélagos dados sus caracteres florales, y de éstas más o menos 590 especies ocurren en el Nuevo Mundo (Von Helversen 1993, Endress 1994, Knudsen & Tollsten 1995, Proctor *et al.* 1996, Horner *et al.* 1998, Muchhala & Jarrín 2002). La inmensa mayoría de investigación en quiropterofilia ha detallado la biología reproductiva de especies de plantas quiropterofílicas a nivel individual. Las plantas quiropterofílicas son a menudo tan dependientes de los murciélagos polinizadores que la planta podría verse seriamente amenazada en caso que desaparecieran sus murciélagos visitantes (Muchhala & Jarrín 2002). Los murciélagos visitadores de las flores se encuentran principalmente donde hay una sucesión de flores aptas para ellos durante todo el año (Proctor *et al.* 1996).

Como en la mayoría de otros sistemas de polinización, las plantas polinizadas por murciélagos son visitadas y polinizadas por diferentes especies de murciélagos. Los murciélagos visitadores de plantas neotropicales viven de una dieta mixta a base de néctar y polen, así como de frutos e insectos (Knudsen & Tollsten 1995). Los murciélagos nectarívoros glosofáginos son un grupo de murciélagos neotropicales altamente especializados para alimentarse de las flores durante la noche (Winter 1998). Ellos tienen una excepcionalmente alta rotación de energía, probablemente cerca del límite máximo para los mamíferos (Von Helversen 1993). La mayoría varían entre 8 y 30 g de peso, y son polinizadores costosos energéticamente desde la perspectiva de las plantas. Ellos requieren como mínimo entre 18-53 KJ de energía diaria (Horner *et al.* 1998). Al igual que los colibríes, los murciélagos glosofáginos usualmente planean mientras visitan las flores, y han desarrollado una cinemática del vuelo especializada para planear eficientemente de una forma única entre los murciélagos (Winter 1998).

Los colibríes son el grupo de aves más especializado en alimentarse de néctar en el Nuevo Mundo (Wolf *et al.* 1976) y son polinizadores fiables de muchas plantas polinizadas por aves en el Neotrópico (Colwell 1973, Linhart *et al.* 1987). La polinización por aves puede ser ventajosa para las plantas ya que los colibríes son polinizadores más fiables que los insectos en un amplio rango de cambios estacionales y climáticos altitudinales. Ellos también pueden transportar una mayor cantidad de cargas de polen en distancias mayores y aumentar la probabilidad de entrecruzamiento (Heinrich & Raven 1972). Como resultado, muchas especies de plantas se han adaptado a la polinización por colibríes (Linhart *et al.* 1987).

Los colibríes se encuentran entre los homeotermos más pequeños, y en consecuencia tienen altos costos metabólicos por gramo de peso corporal (Wolf & Hainsworth 1971, 1972; Colwell 1973, Wolf & Hainsworth 1975). La mayoría pesa menos de 3 g, y unos cuantos pesan más de 10 g (Proctor *et al.* 1996). Ellos obtienen la mayor parte de la energía a partir del néctar floral, que se encuentra presente en flores individuales en cantidades lentamente renovables (Wolf & Hainsworth 1975, Cole *et al.* 1982). La energía obtenida del néctar depende tanto de su producción como de su concentración, los cuales difieren dentro y entre especies de plantas (Wolf & Hainsworth 1971, Colwell 1973). La habilidad para planear está bien desarrollada entre los colibríes (Faegri & Van der Pijl 1979) el cual es el mecanismo más demandante de energía ente los métodos de forrajeo, pero que hace posible lograr las mayores tasas de visitación floral (Proctor *et al.* 1996). En consecuencia, la alta demanda energética tiene un efecto sobre el comportamiento de forrajeo durante los períodos de actividad (Wolf & Hainsworth 1971, Baker 1975, Wolf *et al.* 1976).

Como se sabe que las angiospermas ocupan virtualmente cada punto a lo largo del continuo de los sistemas de polinización, es importante comprender cuales han sido las fuerzas ecológicas que han favorecido la generalización o la especialización en linajes y regiones particulares y documentar la fluidez temporal y espacial de las interacciones planta-polinizador (Molina-Freaner & Eguiarte 2003). Las flores representan un escenario prominente de la radiación adaptativa entre las angiospermas (Vogel 1990).

La radiación adaptativa puede ocurrir en todos los niveles posibles, tales como a nivel de subgénero, género, subtribu o tribu de una manera iterativa y polifilética. Entre la familia Sterculiaceae y dando énfasis a nivel genérico, *Helicteres* representa un ejemplo sobresaliente de diversificación floral (Vogel 1990). Los miembros del género han desarrollado flores

especializadas para diferentes tipos de polinizadores. Los síndromes florales incluyen psicofilia, ornitofilia y quiropterofilia (Vogel 1990, Cristóbal 2001a).

Helicteres brevispira representa un ejemplo de una especie psicofílica (Cristóbal 2001a). No obstante, las especies de colibríes *Amazilia lactea* y *Cholorostilbon aureoventris* han sido reportadas como polinizadores exclusivos de las flores en Brasil (Franceschinelli & Kesseli 1999, Franceschinelli & Bawa 2000). Las especies ornitofílicas polinizadas por colibríes incluyen a *Helicteres guazumifolia*, *Helicteres sacarolha* y *Helicteres heptandra* (Vogel 1990, Cristóbal 2001a). Atluri *et al.* (2000) determinaron que *Helicteres isora* es polinizada principalmente por aves y abejas.

Dentro de las especies quiropterofílicas se incluyen *Helicteres rekoi* y *Helicteres jamaicensis* (Cristóbal 2001a). Sazima & Sazima (1988) estudiaron las interacciones entre las flores de *Helicteres ovata* y el murciélago *Glossophaga soricina* en el sureste de Brasil. Con base en la morfología floral, estos autores consideran que *Helicteres lhotzkyana* es otra especie polinizada por murciélagos, sin embargo se han observado colibríes visitando sus flores. En la zona noroeste de Costa Rica, Von Helversen & Voigt (2002) sugirieron que *Helicteres baruensis* también se encuentra adaptada a la polinización por murciélagos.

A pesar de que el género *Helicteres* posee un diverso número de especies y que ha radiado hacia múltiples tipos florales, se ha realizado poca investigación en miembros del género orientados hacia la biología de la polinización a pesar de los trabajos hechos por Sazima & Sazima (1988) y Von Helversen & Voigt (2002) en dos especies polinizadas por murciélagos, y Atluri *et al.* (2000) en *Helicteres isora*. Los trabajos realizados por Franceschinelli & Kesseli (1999) y Franceschinelli & Bawa (2000) mostraron un enfoque distinto; sus estudios en *Helicteres brevispira* estuvieron orientados hacia la estructura de las poblaciones, flujo génico y sistemas de apareamiento.

Con el fin de comprender el papel de las interacciones planta-polinizador en la especiación, es importante llevar a cabo estudios de tales interacciones con géneros que cuenten con un gran número de especies simpátricas (Bawa 1990). Por ende, el objetivo primordial de esta tesis fue comparar la biología reproductiva de dos especies simpátricas del género *Helicteres*; dos arbustos comunes en el bosque tropical seco de la zona noroeste de Costa Rica: *Helicteres guazumifolia* Kunth y *Helicteres baruensis* Jacq.

Helicteres guazumifolia está ampliamente difundida y cubre el área más grande de América. Se extiende desde el sur de México hasta América Central, desde el noroeste de Cuba hasta Rondonia y el oeste de Mato Grosso y zona vecinas de Bolivia (Cristóbal 2001a). Se trata de un arbusto o árbol pequeño de 0.50 - 5 m de altura, ramificado desde la base o erguido con ramas delgadas (Robyns & Cuatrecasas 1964, Cristóbal 2001a, b). Las flores son axilares, erectas, actinomorfas y tienen una corola tubular con un nectario basal. Los pétalos son rojos y espatulados, y el pedúnculo está alineado con el androginóforo (Robyns & Cuatrecasas 1964, Gentry 1993, Cristóbal 2001a, b). Se encuentra en bosques caducifolios, abiertos y secundarios, bosques de galería, pastizales y zonas de incendios y raleos periódicos, al igual que en matorrales húmedos y laderas arbustivas (Robyns & Cuatrecasas 1964, Cristóbal 2001a, b).

Helicteres baruensis también tiene una amplia distribución en América. Se extiende desde la costa Pacífica de México, el sur de Sonora hasta Oaxaca, en la Península de Yucatán, el Caribe y Suramérica hasta Colombia, Venezuela, Suriname, Guyana y Brasil (Cristóbal 2001a). Se trata de un arbusto de 2-6 m de altura con follaje denso. Las flores son geniculadas y crecen en inflorescencias axilares, usualmente se encuentran de tres a cinco flores y tienen una posición horizontal. Son zigomorfas y tienen una corola tubular con un nectario basal, también poseen dos o más nectarios extra-florales localizados en la base de los pedicelos entre las flores. Los pétalos son de color verde pálido y acintados, y el androginóforo es curvo (Robyns & Cuatrecasas 1964, Cristóbal 2001a, b). Esta especie es característica de bosques secos caducifolios, bosques de encinos y bosques de galería (Cristóbal 2001a, b).

La presente tesis se compone de dos artículos científicos cuyo formato es el establecido por la revista Biotrópica. El primer artículo científico se titula "Patterns of nectar production and composition in *Helicteres guazumifolia* and *Helicteres baruensis* (Sterculiaceae): two sympatric species of the tropical dry forest of Costa Rica" (Patrones de producción y composición del néctar en *Helicteres guazumifolia* y *Helicteres baruensis* (Sterculiaceae): dos especies simpátricas del bosque tropical seco de Costa Rica). El objetivo principal del mismo fue caracterizar los patrones de producción de néctar de las dos especies según la hora del día o de la noche cuando hubo secreción de néctar, con el fin de: (1) determinar la cantidad en términos de volumen (µl) de néctar producido; (2) analizar la composición del néctar por medio de la identificación y cuantificación de los azúcares y aminoácidos; y (3) comparar el tipo de néctar producido en estas dos especies a la luz de sus contrastantes síndromes de polinización.

El segundo artículo científico se titula "Reproductive phenology, morphology of flowers and other structures of *Helicteres guazumifolia* and *Helicteres baruensis* (Sterculiaceae): two sympatric species of the tropical dry forest of Costa Rica" (Fenología reproductiva, morfología de flores y otras estructuras de *Helicteres guazumifolia* y *Helicteres baruensis* (Sterculiaceae): dos especies simpátricas del bosque tropical seco de Costa Rica). Con el fin de obtener un mayor conocimiento acerca de la biología de la polinización y la ecología reproductiva de ambas especies los objetivos de dicho artículo fueron: (1) describir la fenología reproductiva de las poblaciones; (2) analizar el desarrollo y la morfología floral; (3) analizar y comparar la morfología de los granos de polen y otras estructuras como los nectarios florales y extra-florales, anteras, estigmas y pétalos por medio de microscopia electrónica; y (4) estimar el número de granos de polen contenidos en una antera de cada especie.

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ARTICLE I: Patterns of nectar production and composition in *Helicteres guazumifolia* and *Helicteres baruensis* (Sterculiaceae): two sympatric species of the tropical dry forest of Costa Rica

ABSTRACT

Helicteres guazumifolia Kunth and *Helicteres baruensis* Jacq. (Sterculiaceae) represent two sympatric species of shrubs common along the North Western tropical dry forest of Costa Rica. I documented the nectar production patterns of both species according to the time of day or night of nectar secretion. Nectar secretion of flowers of *H. guazumifolia* was restricted to the first day of flower life span, shortly after anthesis at 0600 h and lasted until 1800 h. Flowers secreted on average 15.63 ± 8.45 µl of nectar. Nectar is composed of three main sugars: sucrose, fructose and glucose, and it is a sucrose-rich nectar. A total of 17 different free amino acids were identified in the floral nectar. Proline, arginine, threonine, and tyrosine were the most abundant amino acids, with a concentration above 70 Ng/µl. In contrast, on flowers of its related species *H. baruensis* nectar secretion was confined to the second day of flower life span after anthesis, starting at 1600 h and ceasing at 0600 h of the following day. Flowers secreted on average 77.03 ± 64.99 µl of nectar. Nectar is also composed of three main sugars; however, it showed a tendency to be hexose-rich, having more fructose and glucose over sucrose. Nectar also contained a total of 17 different free amino acids. The most concentrated ones were proline, alanine, tyrosine, arginine, and threonine. Patterns of nectar production are clearly distinguished between the two species related to timing, amount, and composition of nectar secretion.

RESUMEN

Helicteres guazumifolia Kunth y *Helicteres baruensis* Jacq. (Sterculiaceae) son dos especies simpátricas de arbustos comunes en el bosque tropical seco de la zona noroeste de Costa Rica. Registré los patrones de producción de néctar de las dos especies según la hora del día o de la noche cuando hubo secreción de néctar. En *H. guazumifolia* se limitó al primer día del período de vida floral, desde el inicio de la antesis a las 0600 h hasta las 1800 h. Las flores secretaron en promedio 15.63 ± 8.45 µl de néctar. El néctar está compuesto por tres azúcares principales:

sacarosa, fructuosa y glucosa, y es rico en sacarosa. Se identificó un total de 17 aminoácidos diferentes en el néctar floral. Prolina, arginina, treonina y tirosina fueron los aminoácidos más abundantes con una concentración mayor a 70 Ng/µl. En constraste, las flores de su pariente *H. baruensis*, secretaron néctar en el segundo día de vida de la flor, después de la antesis; se inició a las 1600 h y cesó a las 0600 h del día siguiente. Las flores secretaron en promedio 77.03 ± 64.99 µl de néctar. El néctar también está compuesto por tres azúcares principales; no obstante, tiende a ser rico en hexosas, con más fructuosa y glucosa que sacarosa. También contiene 17 aminoácidos libres, siendo los más concentrados prolina, alanina, tirosina, arginina y treonina. Se observan claramente patrones diferentes de producción de néctar entre las dos especies según la hora, la cantidad y la composición del néctar.

Key words: Amino acids; bats; hummingbirds; nectar; pollination; reproductive biology; sugars.

Nectar is one of the major primary attractants and rewards of angiosperm flowers to their pollinators (Baker & Baker 1973b, Gottsberger *et al.* 1984, Freeman *et al.* 1985, Stiles & Freeman 1993, Endress 1994, Galetto 1997). It plays a central role in plant reproduction by mediating plant-pollinator interactions due to its inherent features such as sugar concentration, volume, viscosity and chemical composition (Cruden 1976, Cruden *et al.* 1983, Galetto & Bernardello 1995, Perret *et al.* 2001, McDade & Weeks 2004). Since the pioneer works of Baker and Baker in the seventies, the nectar of hundreds of species from temperate and tropical regions has been studied in some detail (Gottsberger *et al.* 1984, Baker & Baker 1990). Information is available for a few hundred species for the most basic nectar traits such as sugar concentration and volume per flower (McDade & Weeks 2004). In addition, considerable attention has been given to ecological, chemical and phylogenetic investigations of nectar (Gottsberger *et al.* 1984, Baker & Baker 1990, Gottsberger *et al.* 1990).

Sugars are present in all floral nectars in greater amount than any other constituent, except for the water they are dissolved (Baker & Baker 1973b, 1976; Baker 1977). The three most common sugars found in nectar are sucrose, fructose and glucose. They dominate the total solutes and may be present in varying proportions (Freeman *et al.* 1985, Martínez del Río 1990, Stiles &

Freeman 1993, Endress 1994, Proctor *et al.* 1996, Galetto 1997, Baker *et al.* 1998). Nectars may contain sucrose only or different combinations of these three sugars (Cruden *et al.* 1983, Proctor *et al.* 1996) and may vary between species (Murcia 2002).

Amino acids are the second most important group of components found in nectar. Essential amino acids are present in all species (Baker & Baker 1973a, b, 1976; Murcia 2002); however, their importance in the nutrition of pollinators is far from being clear. Also, it is not clear if there are regular patterns of amino acids content in nectar (Gottsberger *et al.* 1989, 1990). Other substances present in minor proportions are lipids, antioxidants, alkaloids, phenolic substances and glycosides (Baker 1977, Endress 1994).

Most studies on nectar chemistry have been designed to reveal convergence among ecological or geographical plant communities, but few concern nectar comparisons between species belonging to the same genus or tribe (Perret *et al.* 2001). Vogel (1990) discussed the parallel radiation of Neotropical plants into different pollination modes by describing the floral syndromes of the family Sterculiaceae and the corresponding pollinators. Members of the genus *Helicteres* provide an outstanding example of floral diversification and pollinator use. Floral syndromes include psychophily, sphingophily, ornitophily, and chiropterophily (Vogel 1990, Von Helversen & Voigt 2002).

Two sympatric species within the genus *Helicteres*, with contrasting pollination syndromes, occur along the North Western tropical dry forest of Costa Rica. *Helicteres guazumifolia* Kunth which is hummingbird pollinated and *Helicteres baruensis* Jacq. known to be bat pollinated. The general objective of this study was to characterize nectar production patterns of both species according to the time of the day or the night in which nectar is secreted, in order to: (1) determine the quantity in terms of volume (µl) of nectar produced; (2) analyse the nectar composition by the identification and quantification of sugars and amino acids; (3) compare the type of nectar produced in these two species in the light of their contrasting pollination syndromes.

METHODS

Study site. This study was conducted in Santa Rosa National Park, Guanacaste Conservation Area (ACG), North Western Costa Rica (10°45' to 11°00' N and 85°30' to 85°45' W). Two life zones are present in the area and the study was conducted in a tropical dry forest, with a moist transition (Holdridge 1967, Hartshorn 1991). The park includes a mosaic of forests of different ages and abandoned pastures (Janzen 1986, Hartshorn 1991, Gerhardt 1993).

In the past, the zone was covered by extensions of tropical dry forest, the most threatened ecosystem of Mesoamerica, it originally covered about 550.000 km^2 from Mazatlan in Mexico until Panama Canal. Actually, only 2% is maintained and 25% of the surface is protected, represented principally in the Guanacaste Conservation Area (Janzen 1986, Fernández Morillo 1998).

The climate is highly seasonal, with a well defined dry season that goes from late November to mid May. Annual rainfall ranges between 800 and 2600 mm, with an annual mean of 1423.4 mm. Annual mean temperature is 25.7°C and annual mean relative humidity is 81% (Rojas Jiménez 2001).

Study species. *Helicteres* is a pantropical genus that contains approximately 60 species, native to the tropics of both hemispheres (Robyns & Cuatrecasas 1964, Gentry 1993, Cristóbal 2001a, Bayer & Kubitzki 2003). It is most abundant in America in which 38 species are distributed from Mexico, Central America, the Caribbean and South America through North Western Argentina and slightly South of the tropics line in Eastern Paraguay and Brazil (Sazima & Sazima 1988, Cristóbal 2001a, b). The members of the genus are shrubs or small trees of dry lowland areas (Sazima & Sazima 1988). They are characterized by having distinctive fruits, which are spiral capsules, many seeded and with a long androgynophore, free or fused (Robyns & Cuatrecasas 1964, Gentry 1993, Bayer & Kubitzki 2003). Two species of *Helicteres* are found in Santa Rosa National Park: *Helicteres guazumifolia* and *Helicteres baruensis*.

Helicteres guazumifolia is widely spread and covers greatest area in America. It extends from Southern Mexico to Central America, North Western Cuba until Rondonia and West of Mato Grosso and neighboring zones of Bolivia (Cristóbal 2001a). It is a shrub or small tree 0.50 - 5 m high, ramificated from the base or erect with slender branches (Robyns & Cuatrecasas

1964, Cristóbal 2001a, b). Flowers are axillary, erect, actinomorphic and have a tubular corolla with a basal nectary. They have short red and spatulated petals, and the peduncle is aligned with the androgynophore (Robyns & Cuatrecasas 1964, Gentry 1993, Cristóbal 2001a, b). It is found on open, secondary and semideciduous forests, gallery forests, pastures and zones of periodic fires and clearings, also on dry or moist thickets, grassy or bushy slopes (Robyns & Cuatrecasas 1964, Cristóbal 2001a, b).

Helicteres baruensis is also widely distributed in the Americas. It extends from the Pacific coast of Mexico, South of Sonora until Oaxaca, in Yucatan Peninsula, the Caribbean and South America until Colombia, Venezuela, Suriname, Guyana and Brazil (Cristóbal 2001a). It is a shrub or slender tree 2-6 m high with dense foliage. Flowers are geniculated and are borne in axillary or oppositifolious inflorescences, usually three to five flowered and have a horizontal position. They are zygomorphic and have a tubular corolla with a basal nectary, and also have two or more nectaries at the base of the pedicels between the flowers. The petals are pale greenish and acintated, and the androgynophore is bent (Robyns & Cuatrecasas 1964, Cristóbal 2001a, b). This species is characteristic of dry caducifolious forests, holms-oak forests and gallery forests (Cristóbal 2001a, b).

Nectar production. Flower production of *H. guazumifolia* and *H. baruensis* was monitored from March 2003 to March 2004. *H. guazumifolia* produced flowers during March to late June 2003, and *H. baruensis* since July to late December 2003. Nectar volume was measured when each species produced flowers.

A total of five mature flower buds per plant were selected randomly and were bagged one day prior to anthesis using cheesecloth bags for each observation period. A total of 15 different plants were selected randomly from each population for every day of observation. The accumulated nectar was sampled each hour, on newly opened flowers over the course of each day. Measurements were conducted for *H. guazumifolia* within the period from 0600 h to 1800 h and for *H. baruensis* from 1600 h to 0600 h of the following day.

To determine nectar production, the volume (µl) of nectar secreted was measured using calibrated micropipettes (Drummond Scientific Company Wiretrol®, for *H. guazumifolia* of 10-20 µl, and for *H. baruensis* of 25-50 µl and 50+100 µl). Nectar production was determined as the

volume of nectar secreted over the course of the day after anthesis. Floral nectar was measured only once on each flower; and they were manipulated with care preventing floral damage, contamination by pollen grains or tissue secretions. Nectar samples were stored in 1.5 ml vials containing 1 ml of 70% ethanol for preservation. They were kept frozen at -20°C until analysis.

Nectar composition. The study of the chemical composition of nectar was conducted in the Biology Laboratories, Ulm University, Germany. These analyses were conducted using High Performance Liquid Cromatography (HPLC) (Linskens & Jackson 1987). Sugar content was determined examining the concentration (micrograms/microliters) of three sugars: sucrose, glucose and fructose. In addition, amino acid concentration (nanograms/microliters) was also determined from nectar samples from the two species of *Helicteres*. Sugar analyses with high performance liquid chomatography allow the determination of nectar composition with greater precision and accuracy (Freeman & Wilken 1987). The analytical methodology has been outlined previously by Elisens and Freeman (1988).

Statistical analyses. Statistical analyses were conducted using Statistica^{TM} (release 6.0). One way analysis of variance was used to compare mean nectar volume produced through the course of the day or night for each species. Multivariate analyses of variance were used to compare mean sugar concentration and mean amino acid concentration for each species.

RESULTS

Nectar production

Helicteres guazumifolia. Nectar production in flowers of *H. guazumifolia* begins shortly after their anthesis, approximately at 0600 h and ceases at 1800 h. It is produced only during the first day of flower life span. Daily nectar production ranged between 8.29 and 22.08 µl. Analysis of variance revealed significant variation in mean nectar production (µl) during the course of the day ($F= 12.30$, $df= 12$ and 396, $p < 0.0001$). On average, a total of 15.63 ± 8.45 µl (N= 409) of nectar were secreted through the day.

Nectar secretion remained fairly constant until 1100 h, it increased slightly at 1200 h, reaching a maximum peak of 22.08 µl at 1300 h. During the following afternoon hours high

quantities of nectar secreted were maintained, ranging from 18.03 µl to 22.08 µl. Secretion ceased drastically by 1800 h in most flowers sampled (Fig. 1).

Helicteres baruensis. Flowers of *H. baruensis* initiate nectar secretion during the second night after anthesis. Flowers secreted nectar only during one single night. Secretion started around 1600 h and ceased around 0600 h of the following day. Mean nectar volume (µl) produced varied significantly during the course of late afternoon and night times ($F= 13.64$, gl= 14 and 148, $p < 0.0001$). Flowers secreted an average amount of *77.03 ± 64.99 µl (N= 163)* of nectar.

Nectar volume secreted between 1600 h and 1700 h was the lowest ranging between 27.88 µl and 25.21 µl. An increase in nectar secretion occurred since 1900 h. The maximum production was recorded around 0200 h of the following day. On average a total of 199.82 µl of nectar were produced at this time. A reduction in secretion occurred after 0400 h. At 0500 h, mean nectar production was only 50 µl. At 0600 h in the early morning most flowers have ceased nectar production (Fig. 2).

Sugar Concentration

Helicteres guazumifolia. Floral nectar of *H. guazumifolia* contained three main sugars: sucrose, fructose and glucose. Mean sugar composition varied significantly ($F=2.37$, df= 33 and 106, $p= 0.0005$) during the course of the day. Nectar differs in concentration and predominant sugar type. Sucrose predominates as the main sugar and it is significantly more concentrated than glucose and fructose ($F= 4.08$, df= 11 and 38; $p= 0.0005$). The mean concentration of sucrose was 292.69 ± 114.99 µg/µl (N=50).

Sucrose concentration in nectar was higher during the first morning hours, reaching a value of 650.76 µg/µl at 0600 h (Fig. 3). It declines steadily until 0900 h, reaching 219.93 µg/µl. A slight peak was recorded at 1300 h and at 1600 h: on average, 334.36 µg/µl and 335.94 µg/µl were produced respectively. Sucrose concentration remained fairly constant during the rest of the day.

Mean concentration of fructose and glucose was low during the course of the day (1.68 ± 2.87 µg/µl and 1.02 ± 2.54 µg/µl, $F= 1.35$, df= 11 and 38; $p= 0.24$ and $F= 1.98$, df= 11 and 38;

p= 0.06, respectively). Sucrose was present in all nectar samples regardless of the time of the day. In contrast, fructose was not found in samples taken at 0800 h and 1600 h. No glucose was found in samples from 0800 h, 0900 h, 1100 h, 1200 h, 1400 h and 1500 h.

Helicteres baruensis. Floral nectar of *H. baruensis* also contained three main sugars: sucrose, fructose and glucose. There were no significant differences in mean concentration between the three types of sugars (F=1.12, df= 36 and 204, p= 0.30). Nectar concentration, as well as the predominant sugar type did not vary significantly through sampling time. Overall mean sugar concentration for sucrose, fructose, and glucose were 41.52 ± 39.31 µg/µl, 41.94 ± 30.32 µg/µl, and 41.11 ± 30.15 µg/µl, respectively. However, a tendency for nectar to have more fructose and glucose over sucrose was observed at the following hours: 1700 h, 1800 h, 2200 h, 2300 h, 2400 h, 200 h, 0400 h and 0500 h.

Amino acid Concentration

Helicteres guazumifolia. The nectar of *H. guazumifolia* contained a total of 17 different free amino acids. There were no significant differences between amino acid concentrations and the different sampling times. On average, proline was the most abundant amino acid, with a mean concentration of 554.22 ± 391.64 Ng/µl (F= 3.69, df= 3 and 20, p= 0.03). As shown in Table 1, arginine, threonine and tyrosine were significantly more abundant (more than 70 Ng/µl) than the rest of the amino acids found in the nectar.

The following amino acids were found in low concentrations (less than 50 Ng/µl) on every sampling period: methionine, lysine, serine, valine, histidine, aspartic acid, leucine and glycine. Cysteine was the only amino acid absent from every sample period. Meanwhile, glutamic acid, alanine, isoleucine and phenylalanine were found only in some samples.

Helicteres baruensis. Similar to its related species *H. guazumifolia*, floral nectar of *H. baruensis* contained a total of 17 different free amino acids (Table 2). There were no significant differences in amino acid concentration between sampling times. Proline, alanine, tyrosine, arginine, and threonine were more concentrated than the other amino acids (more than 20 Ng/µl). Proline showed the highest concentration (146.56 ± 100.82 Ng/µl) among these amino acids.

Even though they were found in low concentrations (less than 10 Ng/µl), valine and lysine were found in every sampling period. Glycine, methionine, and leucine were absent in every sample. Aspartic acid, serine, glutamic acid, histidine, cysteine, isoleucine and phenylalanine were absent in two sampling periods (1700-1800 h and 2100-2200 h).

Both *Helicteres* species share the same types of amino acids that were found to be the most concentrated, with the exception of alanine, which was present on *H. baruensis* and absent from *H. guazumifolia* on sampling time 1200-1400 h.

DISCUSSION

Nectar production

Nectar production is a dynamic process that involves continuous processes in the lifespan of every flower such as secretion, reabsorption and evaporation (Gottsberger *et al.* 1989, 1990). An enormous amount of descriptive work has been published on patterns of floral nectar production. Nectar production may be affected by time of day or season, flower age, size or stage, flower location on the plant, defoliation, soil moisture and weather conditions (Gottsberger *et al.* 1984, Zimmerman 1988, Witt *et al.* 1999). Other selective pressures that can influence nectar production besides pollinator class are flower density, habitat, nectar thieves and breeding system (Cruden *et al.* 1983). As a result, rates of nectar production among plants in populations have been found to differ in variability (Zimmerman 1988). The two *Helicteres* species showed differences in the amount and composition of the nectar produced, as it varies between different species (Witt *et al.* 1999, Murcia 2002).

Helicteres guazumifolia. It has generally been stated that flowers pollinated by high energy requiring animals such as hummingbirds and bats tend to produce high amounts of nectar (Cruden *et al.* 1983, Stiles & Freeman 1993, Proctor *et al.* 1996), such as the ones registered for each *Helicteres* species. Patterns of nectar production are clearly distinguished between the two species. Nectar is secreted in different amounts and at different times of the day and night; in addition, both species provide a great energetic reward.

Flowers pollinated by diurnally active animals produce nectar during the day (Cruden *et al.* 1983) and this pattern was observed for *H. guazumifolia* flowers which started secretion at

0600 h. Regardless of quantification of hummingbird visits to the flowers, several individuals of the hummingbird *Amazalia rutila* were observed hovering at flowers early in the morning after 0700 h and late in the afternoon after 1500 h. After noon and during the rest of the day, flowers contained higher amounts of nectar that could be offered to them. In most species nectar is secreted at a constant rate until some critical amount has accumulated, then ceases at approximately the same time that hummingbirds cease daily activity, approximately at 1830 h (Cruden *et al.* 1983). This may explain why secretion was stopped by 1800 h after having nectar available during most of the day.

In unbagged flowers exposed to pollinators, it is possible that nectar secretion ceases if pollinators are inactive or that is being reabsorbed in old or pollinated flowers (Cruden *et al.* 1983). Reabsorption could occur at the end of the day, when hummingbirds are not active anymore. Further studies under these conditions could help to understand if *H. guazumifolia* flowers show this type of adaptive pattern on the production of nectar. Galetto and Bernardello (1995) in their study of nectar secretion of two ***Lycium*** species, showed that nectar was reabsorbed at the end of flower lifetime. A nectar production pattern with no reabsorption may have an impact on reproductive biology (Zimmerman 1988, Galetto & Bernardello 1995). The seed numbers of a plant may decrease due to the costs of producing nectar (Witt *et al.* 1999). So, plants reabsorb nectar from aging flowers and utilize its carbon in developing seeds with a consequent reproductive advantage (Zimmerman 1988, Galetto & Bernardello 1995).

Helicteres baruensis. *H. baruensis* belongs to the group of plants specifically adapted to the pollination by bats of the subfamily Glossophaginae, *Glossophaga soricina* (Von Helversen & Voigt 2002). According to Cruden *et al.* (1983), flowers that are pollinated by nocturnally active animals produce nectar at night and this was the nectar secretion pattern registered for the species. Secretion of nectar is much greater in bat flowers than in all other pollination syndromes (Von Helversen 1993, Endress 1994, Murcia 2002, Tschapka & Dressler 2002). *H. baruensis* flowers secreted nectar only during a single night, and produced on average 77.03 µl $\pm$ 64.69 (N= 163), which is considered a high amount. Nectar glands of this species are voluminous in comparison to those of related species that are not bat pollinated (Von Helversen 1993). Large flowers with a deeper corolla tube produce a higher volume of more diluted nectar than smaller

flowers (Cruden & Herman 1983, Galetto & Bernardello 1995), and that is why *H. baruensis* secreted more nectar than its related species *H. guazumifolia*. The relatively large flowers and large amounts of nectar are among the traits associated with the syndrome of chiropterophily (Tschapka *et al.* 1999).

The timing of nectar production is correlated with the activity cycle of the pollinator (Cruden 1976, Cruden *et al.* 1983). Nectar secretion started at 1600 h and incremented hourly since 1900 h, initiation of secretion may occur over a period of an hour or more (Cruden & Herman 1983). Initiation of secretion is so timed that sufficient amounts of nectar are present when pollinators become active (Cruden *et al.* 1983). *H. baruensis* flowers initiated nectar secretion around two to four hours prior to bat activity, which was registered to occur from 1800 h to 2300 h by Von Helversen & Voigt (2002). As shown in Figure 2, maximum amounts of nectar started to be produced from 2100 h, five hours later since secretion started. From this time on flowers contained maximum amounts of nectar available for its main pollinator, reaching a peak of 199.82 µl.

After flowers had accumulated critical amounts of nectar available for its pollinator, reduction in secretion started to occur, again while pollinators were inactive. Von Helversen & Voigt (2002) proposed that sugar was probably reabsorbed actively by *H. baruensis* flowers in the morning hours as they had little or no nectar during the day, even in flowers that had been bagged during the whole preceding night. By 0500 h, flowers contained only 50 µl, and none of the flowers sampled at 0600 h contained nectar.

Some *H. guazumifolia* plants were located in an open area close to few individuals of *H. baruensis*. Between 1600 h and 1700 h when the timing of nectar production for the former species started, individuals of *Amazilia rutila* were seen visiting their flowers, probably consuming the smaller amounts of nectar available at those hours. Hummingbirds are known to visit several species of chiropterophilous flowers during late afternoon, but usually they act as nectar thieves (Sazima *et al.* 1994, Muchhala 2003).

Sugar concentration

Helicteres guazumifolia. The chemical constituents of floral nectar are known to vary according to the type of pollinator attracted to the flowers (Gottsberger *et al.* 1989, Baker & Baker 1990, Stiles & Freeman 1993, Baker *et al.* 1998, Witt *et al.* 1999), and in turn may affect the visiting behaviour of potential pollinators (Baker & Baker 1976). The three sugars sucrose, glucose, and fructose, are by far the most common and abundant sugars in nectars (Baker *et al.* 1998) and were contained in the nectar of both *Helicteres* species. However, nectars differed in the concentration of their sugars (Wells *et al.* 1992).

Sucrose is the predominant sugar in the nectars of New World hummingbird pollinated species (Stiles & Freeman 1993, Baker *et al.* 1998) and it is the main sugar in nectar of *H. guazumifolia*. Sucrose was present more than 200 times as much as glucose and fructose at every sampling time. The sugar concentration of the nectar of some hummingbird pollinated species increased during the morning (Cruden *et al.* 1983), and the highest sucrose concentration values were recorded during the first hours of the day. Nectar contained a low content of fructose and glucose; however, the hexoses were found to be fructose-glucose balanced, also typical for hummingbird pollinated flowers (Freeman *et al.* 1985, Stiles & Freeman 1993).

Baker *et al.* (1998) stated that the low sucrose content in nectar represents the ancestral condition for taxa pollinated by volant vertebrate animals. Most likely, the ancestral condition of nectars with low sucrose content was breached by species in the New World where they were in contact with hummingbirds. The nectar chemistry may have shifted from low sucrose content to high more than once, with the repeated evolution of hummingbird-pollinated species. Adaptive convergence in sugar composition presumably reflects taste preferences of hummingbirds. Nestlings will be imprinted with sucrose when they are fed such a predominantly sucrose diet. Due to the establishment of a long lasting preference for sucrose, hummingbirds may actively seek sucrose-rich food sources (Stiles & Freeman 1993, Baker *et al.* 1998), and this type of source was highly available at the flowers during the whole period of nectar secretion.

It is expected that secretion of sucrose rich nectar would be more economical than hexose rich nectar, because most sugar is translocated within the plant as sucrose, which is the major sugar in the phloem sap (the source of sugar in nectar) (Stiles & Freeman 1993).

Helicteres baruensis. Flowers pollinated by neotropical bats are seem to be dominated by the hexose sugars, fructose and glucose (Baker & Baker 1990, Von Helversen 1993). Although *H. baruensis* nectar had a similar overall sugar concentration between sucrose, fructose and glucose, it did showed a tendency for it being composed more of fructose and glucose in the majority of time hours of nectar secretion. *H. baruensis* nectar is one of the principal components in the diet of its main pollinator *Glossophaga soricina*; as it is the only species that provides nectar during most of the rainy season at Santa Rosa (Fernández Morillo1998).

Glossophagine bats with weight ranges between 7-35g are animals with an unusually high energy turn-over. The minimum requirement for a small *Glossophaga* is in the range of 1 mg of sugar (or about 5 µl of a 20 % nectar) average reward for one flower visit (Von Helversen 1993). The glossophagines' ability to hover leads to the exploitation of the flowers that they visit. Hovering visits of bats generally last less than a second and are of a shorter duration than in hummingbirds. Although hovering is an expensive mode of flight, it allows bats, like hummingbirds, to visit a larger number of flowers per time unit and therefore improves total foraging efficiency (Von Helversen 1993, Tschapka & Dressler 2002). Sugars encountered in *H. baruensis* nectar may tend to compensate the high energetic demands imposed by their flight mode. Also, bats use nectar as an additional water source when water is in short supply under seasonally arid environments like the dry forest (Tschapka & Dressler 2002).

Even though, pollinator visits were not quantified in this study, *Glossophaga soricina* was observed visiting the flowers since 2200 h until approximately 0300 h. *G. soricina* visits flowers of the same bush or group of bushes consecutively every 15 to 40 minutes during the first half of the night carrying pollen of the plant in its fur (Fernández Morillo 1998, Von Helversen & Voigt 2002).

Amino Acid Concentration

The amounts of amino acids present in the nectar of most flowering plants, although small, are sufficient to provide pollinators with a useful nitrogen supply (Baker & Baker 1973a).

The overall concentration of amino acids differs among species and appears to be related to their principal pollinator (Gottsberger *et al.* 1989, 1990; Dress *et al.* 1997). In general, it has been argued that amino acid concentration is lower if the principal pollinator has alternate sources of amino acids in its diet (Baker 1977, Gottsberger *et al.* 1984, Dress *et al.* 1997). Pollinators such as birds and bats, which normally eat pollen or insects, do not need to rely entirely on nectar to obtain all amino acids needed for their nutrition (Gottsberger *et al.* 1984, 1990). Nectars are typically expected to be richer in amino acids if flowers are pollinated by settling moths, butterflies and many wasps which, as adults, do not have alternative sources of protein building materials (Baker 1977, Gottsberger *et al.* 1990). This study reveals that there are significant amounts of amino acids in the nectar of these two species, suggesting that their presence may affect the flower-visiting behaviour of potential pollinators (Baker & Baker 1973a, 1976).

Helicteres guazumifolia. It has been shown that hummingbird pollinated flowers often show little amino acid in the nectar (Baker & Baker 1973a, b; Baker 1977, Endress 1994). These results are understandable because hummingbirds can use an alternative source of protein building materials in the insects that they catch (Baker & Baker 1973a, b; Baker 1977). Even though, the nectar of this species contained a total of 17 different amino acids, only four were found to have a concentration above 70 Ng/µl. In contrast, eight amino acids showed concentrations of less than 50 Ng/µl. These findings indicate that *H. guazumifolia,* as a hummingbird pollinated plant, has nectar with low amino acid content.

Other studies have shown that arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are essential nutrients for many insects. Proline and glycine are also essential for some insect species. Other amino acids, while not essential, do increase insect growth like alanine, aspartic acid, glutamic acid, glycine and serine (Dress *et al.* 1997). Several of these essential amino acids for insects, were found in lower concentrations in nectar, *e.g.*, hisitidine, leucine, lysine, methionine, valine and glycine. Isoleucine and phenylalanine were absent in the nectar of *H. guazumifolia*. In addition, two non essential amino acids for insects, aspartic acid and serine, were present in low concentration in nectar. Lastly, alanine and glutamic acid were absent in the nectar. As a consequence, hummingbird pollinators

could actually obtain these amino acids from insects and this could explain why *H. guazumifolia*'s floral nectar is low in amino acid composition.

Helicteres baruensis. The nectars of bat pollinated plants are low in amino acids content (Baker 1977). Bats need substantial quantities of protein building materials, but they can use pollen, fruits, and insects as alternate sources of nitrogen (Baker & Baker 1973b, Baker 1977, Von Helversen 1993). Similar to *H. guazumifolia*, *H. baruensis* floral nectar contained 17 different amino acids, but only five were found with a concentration over 20 Ng/µl. Only two amino acids, valine and lysine had concentrations lower than 10 Ng/µl. Ten out of the 17 total amino acids were not present in all samples: glycine, methionine, leucine, histidine, isoleucine, phenylalanine, aspartic acid, glutamic acid, serine and cysteine. These findings indicate that, with the exception of cysteine, the amino acids present in the nectar of this species match the group of essential and non essential amino acids for insects.

Fernández Morillo (1998) determined the feeding behavior of *Glossophaga soricina* during the dry and the rainy seasons at Santa Rosa. During the dry season bats feed predominantly on pollen and nectar, since these resources are in major abundance from late December and May. Von Helversen (1993) stated that within the evolution of Glossophaginae pollen has become the main protein source, suggesting that pollen of chiropterophilous flowers can satisfy the amino acids needed by bats (Fernández Morillo 1998). Future studies of *H. baruensis* are needed to determine amino acid composition of pollen, and compare it with that of its nectar. In this sense it may be expected that some amino acids in pollen grains have higher concentrations, meanwhile in the nectar have lower concentrations or are absent.

During the rainy season bats consumed insects (lepidoptera, hawkmoths, flies and beetles), fruits (*Muntingia calabura*), pollen and nectar of *H. baruensis* in similar proportions. The amino acids present in insects can satisfy the requirements of flight, pregnancy and lactancy. Lepidoptera are rich in lipids, which provide more energy than sugars in nectar (Fernández Morillo 1998). *H. baruensis* has nectar with low amino acid composition, and the amino acids in nectar that were found in low concentration or absent may be obtained directly from insect consumption.

Nectar amino acid complements of closely related species show a high degree of constancy, and they tend to show similar but not identical complements (Baker 1977, Baker & Baker 1979, Cruden & Hermann 1983). Floral nectar of both *Helicteres* species share four types of amino acids which were the ones found in high concentration: proline, arginine, threonine and tyrosine. They also have two amino acids in common that had lower concentration: lysine and valine. Finally, they also shared three amino acids that were absent from some samples: glutamic acid, isoleucine and phenylalanine. Amino acids can be valuable in taxonomic and phylogenetic studies (Baker 1977, Baker *et al.* 1998). The results obtained in this study agree with those suggested by Gottsberger *et al.* (1989), in the sense that within each species certain amino acids predominate while others are absent or appear only in low concentrations.

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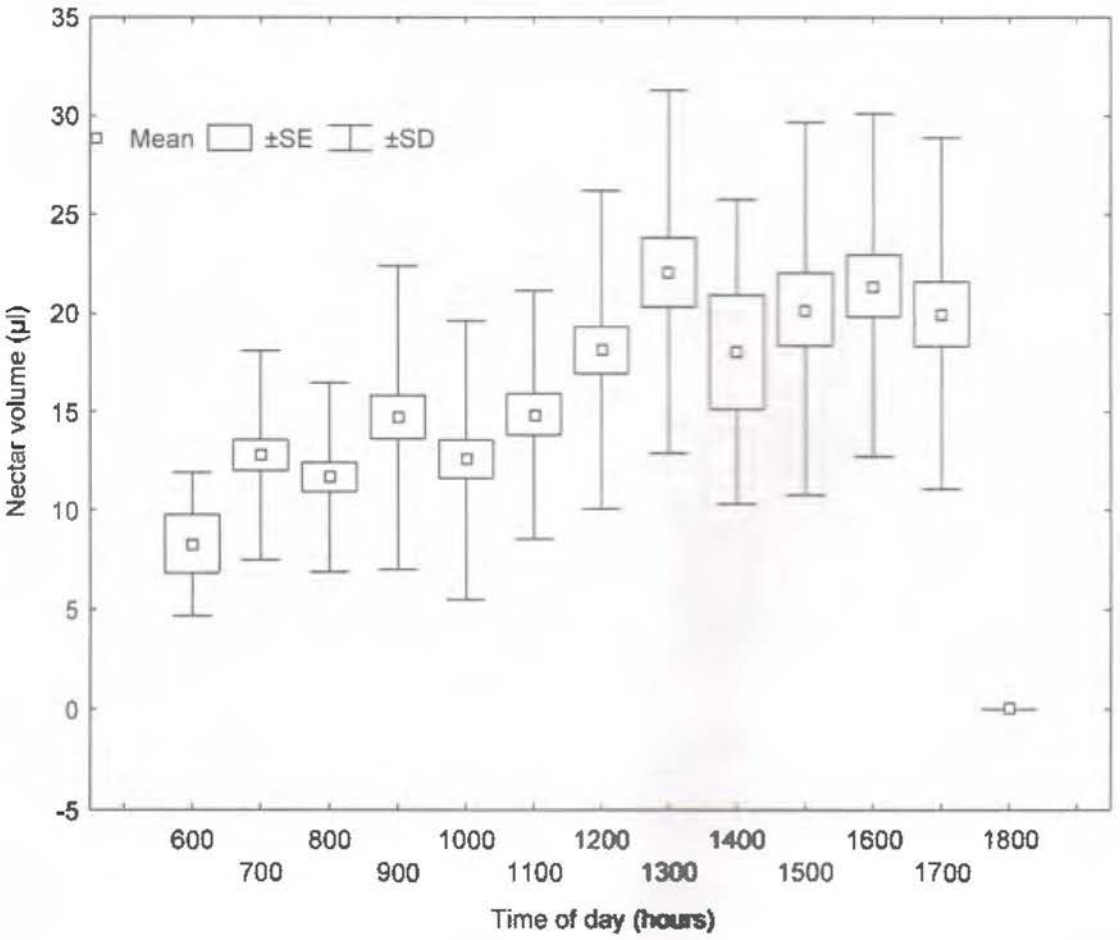

Figure 1. Mean nectar production (µl) by flowers of *H. guazumifolia* during the day. Standard error and standard deviation are shown. Observations were made from 0600 h to 1800 h on the same day.

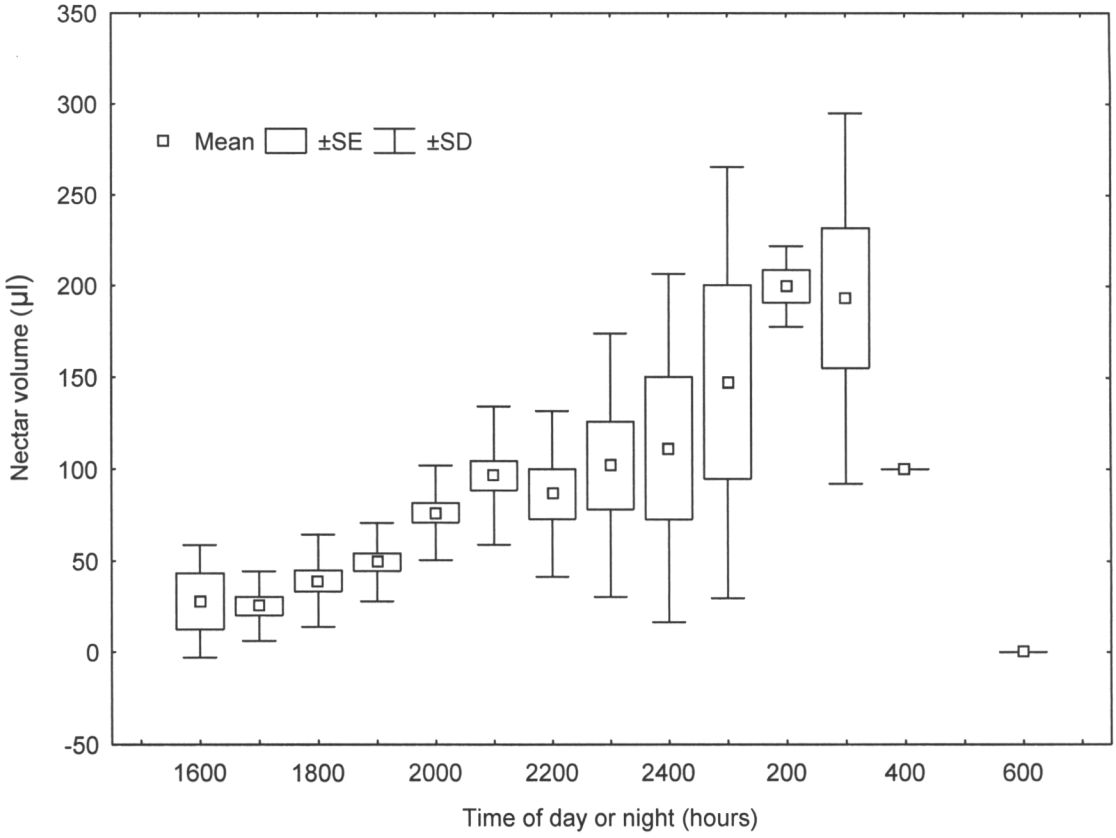

Figure 2. Mean nectar production (µl) by flowers of *H. baruensis* during the day and night.
Standard error and standard deviation are shown.
Observations were made from 1600 h to 0600 h of the following day.

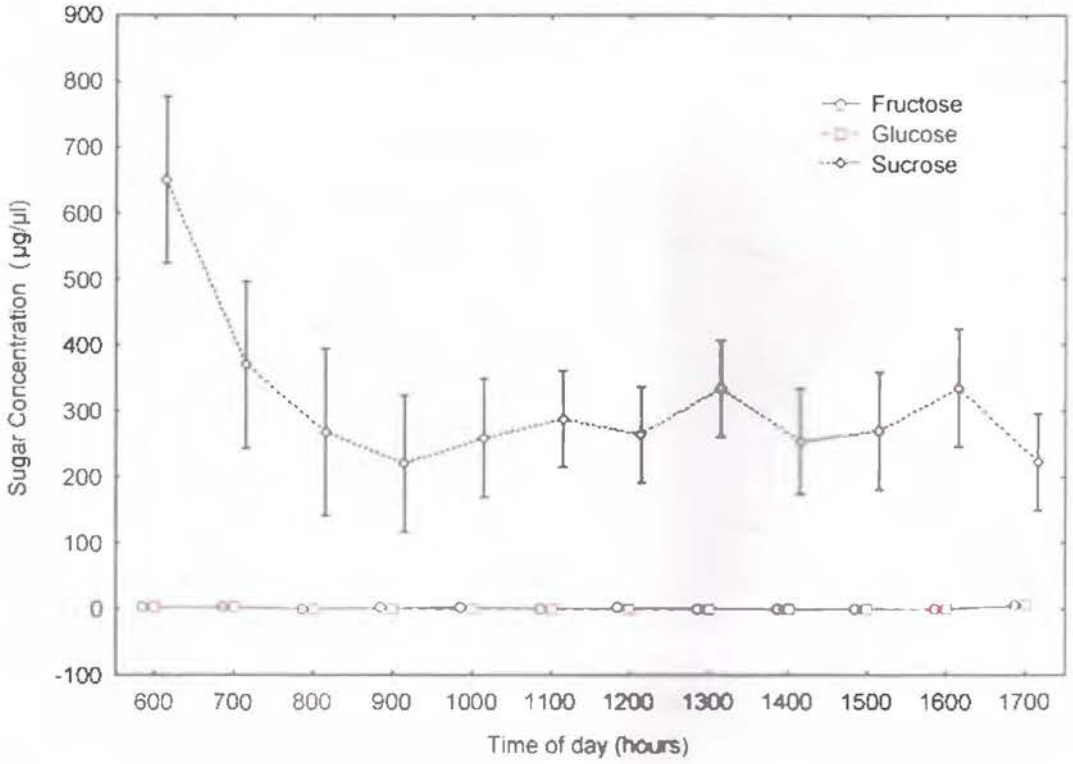

Figure 3. Mean sugar concentration (µg/µl) of nectar of *H. guazumifolia* according to the time of the day. Vertical bars denote 0.95 confidence intervals. Observations were made from 0600 h to 1700 h on the same day.

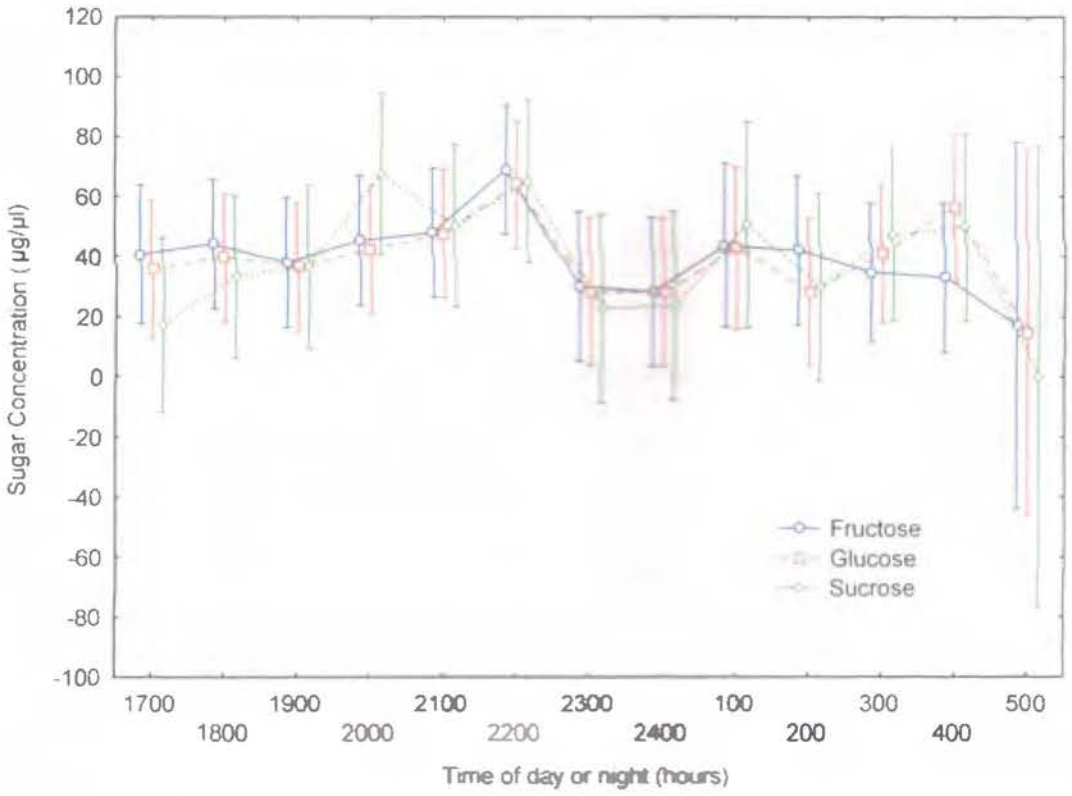

Figure 4. Mean sugar concentration (µg/µl) of nectar of *H. baruensis* according to the time of the day or night. Vertical bars denote 0.95 confidence intervals. Observations were made from 1700 h to 0500 h of the following day.

Table 1. Floral nectar amino acid composition and concentration in Ng/µl of *H. guazumifolia* according to the time of the day.

	0600-0800	0900-1100	1200-1400	1500-1700	Overall means
ASP	2,24 ± 4,19	20,05 ± 40,10	30,18 ± 35,91	15,72 ± 26,34	13,76 ± 25,66
SER	4,94 ± 7,23	104,18 ± 208,36	63,82 ± 96,07	7,72 ± 14,07	29,88 ± 89,82
GLU	-	-	-	32,61 ± 70,77	12,23 ± 44,75
GLY	0,66 ± 1,08	2,04 ± 4,09	4,41 ± 7,65	0,92 ± 1,83	1,46 ± 3,21
HIS	2,33 ± 4,68	44,48 ± 88,96	29,05 ± 50,32	8,54 ± 19,80	15,02 ± 40,55
ARG	204,40 ± 169,65	118,93 ± 91 19	311,59 ± 173,78	187,12 ± 80,57	197,07 ± 132,50
THR	165,42 ± 132,07	115,80 ± 93,90	136,59 ± 106,66	165,30 ± 73,54	153,50 ± 98,58
ALA	0,21 ± 0,59	1,36 ± 2,73	-	3,41 ± 6,52	1,58 ± 4,26
PRO	504,79 ± 375,34	396,32 ± 285,69	1151,85 ± 616,80	469,12 ± 187,06	554,22 ± 391,64
CYS	-	-	-	-	-
TYR	76,04 ± 98,08	121,43 ± 152,88	193,66 ± 284,74	10,22 ± 23,94	73,63 ± 130,68
VAL	3,51 ± 5,31	70,90 ± 104,14	36,49 ± 63,20	12,99 ± 19,41	22,42 ± 49,95
MET	47,38 ± 58,97	32,75 ± 50,59	66,54 ± 47,44	55,20 ± 56,28	50,27 ± 52,82
LYS	38,44 ± 41,76	64,42 ± 90,92	72,90 ± 91,84	28,28 ± 15,18	43,27 ± 52,07
ILE	-	38,83 ± 77,66	23,03 ± 39,89	5,08 ± 10,11	11,26 ± 34,20
LEU	2,19 ± 6,20	8,44 ± 16,89	6,49 ± 11,25	1,15 ± 3,35	3,38 ± 8,49
PHE	-	6,80 ± 13,60	7,75 ± 13,42	0,73 ± 1,94	2,38 ± 7,16

Abbreviations:

ASP aspartic acid
SER serine
GLU glutamic acid
GLY glycine
HIS histidine
ARG arginine
THR threonine
ALA alanine
PRO proline
CYS cysteine
TYR tyrosine
VAL valine
MET methionine
LYS lysine
ILE isoleucine
LEU leucine
PHE phenylalanine

Table 2. Floral nectar amino acid composition and concentration in Ng/µl of *H. baruensis* according to the time of the day or the night.

	1700-1800	1900-2000	2100-2200	Overall means
ASP	-	2,80 ± 8,85	7,44 ± 13,07	3,79 ± 9,78
SER	-	3,46 ± 10,93	1,62 ± 5,12	1,88 ± 7,24
GLU	-	1,94 ± 6,13	2,27 ± 7,19	1,56 ± 5,64
GLY	-	-	-	-
HIS	-	1,58 ± 3,45	-	0,59 ± 2,17
ARG	14,75 ± 20,29	31,69 ± 14,20	21,61 ± 19,40	23,56 ± 18,52
THR	6,49 ± 10,67	25,73 ± 12,94	21,84 ± 24,67	19,30 ± 18,90
ALA	14,55 ± 16,63	28,79 ± 24,65	40,48 ± 43,58	29,43 ± 32,22
PRO	89,72 ± 66,65	193,80 ± 88,25	139,12 ± 116,45	146,56 ± 100,82
CYS	-	0,78 ± 2,47	-	0,29 ± 1,50
TYR	21,01 ± 21,31	32,06 ± 34,14	19,49 ± 26,35	24,54 ± 27,99
VAL	2,55 ± 6,10	10,65 ± 15,72	5,61 ± 10,51	6,68 ± 11,98
MET	-	-	-	-
LYS	0,67 ± 1,78	0,28 ± 0,89	0,25 ± 0,79	0,37 ± 1,12
ILE	-	0,41 ± 1,30	-	0,15 ± 0,79
LEU	-	-	-	-
PHE	-	1,38 ± 4,36	-	0,51 ± 2,65

Abbreviations:

ASP aspartic acid
SER serine
GLU glutamic acid
GLY glycine
HIS histidine
ARG arginine
THR threonine
ALA alanine
PRO proline
CYS cysteine
TYR tyrosine
VAL valine
MET methionine
LYS lysine
ILE isoleucine
LEU leucine
PHE phenylalanine

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Article II: Reproductive phenology, morphology of flowers and other structures of *Helicteres guazumifolia* and *Helicteres baruensis* (Sterculiaceae): two sympatric species of the tropical dry forest of Costa Rica

ABSTRACT

I studied the reproductive phenology of *Helicteres guazumifolia* and *Helicteres baruensis* between March 2003 and March 2004. My results indicated that both species have a steady state flowering pattern, and that there is no overlap in the blooming times among them. *H. guazumifolia* produced flowers from March to late June 2003, with peak flowering time in April. Flower production ceased the rest of the year and it started again in February 2004. Flower production of *H. baruensis* started in late May and proceeded until late December 2003. Most plants of *H. baruensis* bloomed during August, September and October and a peak in number of flowers was observed in September. Flowering time acts as an isolating mechanism that maintains both species at Santa Rosa. Five stages of individual flower phenology were determined for each species.

I also describe the morphology of the pollen grains, floral and extrafloral nectaries, petals, anthers and stigmas using photomicrographs taken with scanning electron microscope. Pollen grains of *H. guazumifolia* are classified as type IX, while the ones of *H. baruensis* are type VII. The number of pollen grains contained in an anther of a flower of *H. guazumifolia* was on average 9631, while an anther of *H. baruensis* contained on average 15884 pollen grains.

RESUMEN

Estudié la fenología reproductiva de *Helicteres guazumifolia* y *Helicteres baruensis* durante el período de marzo 2003 a marzo 2004. Mis resultados indicaron que ambas especies tienen un patrón de floración constante, y que no existe traslape entre los períodos de floración. *H. guazumifolia* produjo flores desde marzo hasta finales de junio 2003, con un pico de floración en abril. La producción de flores cesó por el resto del año, y la siguiente estación de floración ocurrió a partir de febrero 2004. La producción de flores de *H. baruensis* ocurrió a partir de mayo hasta diciembre 2003. La mayoría de las plantas de *H. baruensis* tuvieron flores en agosto,

septiembre y octubre, con un pico de floración en septiembre. El tiempo de floración actúa como un mecanismo de aislamiento de ambas especies en Santa Rosa. Para cada especie se determinaron cinco estadios de desarrollo floral.

También describí la morfología de los granos de polen, los nectarios florales y extra-florales, pétalos, anteras y estigmas usando fotomicrografías tomadas con microscopio electrónico. Los granos de polen de *H. guazumifolia* se clasifican como tipo IX, mientras que los de *H. baruensis* como tipo VII. El número de granos de polen contenidos en una antera de *H. guazumifolia* es en promedio 9631, mientras que una antera de *H. baruensis* contiene en promedio 15884 granos de polen.

Key words: Floral morphology; flower ecology; flower development; flowering time; nectary; pollen.

Plant phenology is concerned with the seasonal timing of recurring events that may be critical to survival and reproduction (Rathcke & Lacey 1985, Newstrom *et al.* 1994, Williams-Linera & Meave 2002). Reproductive phenology is measured by the timing, duration, and synchrony of events such as flowering, fruiting, and germination within and between seasons (Barrett & Eckert 1990). The timing, duration, and the frequency of flowering largely describe the flowering pattern of a population and its constituents. A number of studies reveal that plants display a wide variety of flowering patterns (Bawa 1983, Williams-Linera & Meave 2002). Phenological patterns can be analyzed at several levels including the flower, individual, population, species, guilds and communities (Barrett & Eckert 1990, Williams-Linera & Meave 2002).

Individuals of a population may flower for periods as brief as one day or as long as one year; several times a year, once a year, or once every few years (Bawa 1983). Attempts to explain differences in phenology among species have led to a variety of hypothesis concerned with proximate factors and evolutionary forces shaping species interactions and controlling the timing of reproductive events (Barrett & Eckert 1990, Lobo *et al.* 2003).

Proximate causes principally include short-term environmental events that may trigger phenological patterns, while ultimate causes include evolutionary forces that are responsible for these patterns (Rathcke & Lacey 1985, Lobo *et al.* 2003). Environmental cues such as changes in water level stored by plants, seasonal variations in rainfall, changes in temperature, photoperiod, irradiance, and sporadic climatic events have been mentioned as proximate causes triggering phenological events (Rathcke & Lacey 1985, Lobo *et al.* 2003). In contrast, biotic factors, such as competition for pollinators or pollinator attraction, competition for seed dispersers, and avoidance of herbivory have been interpreted as ultimate causes responsible for phenological patterns in tropical species (Lobo *et al.* 2003).

Flower morphology may play an important role in the study of plant-pollinator interactions. It determines pollinator accessibility to nectar, efficiency of pollen deposition on the pollinator body, and efficiency of pollen acquisition by the stigma from the pollen vectors. The importance of morphological characteristics has been demonstrated in diverse studies. An accurate evaluation and comparison of floral morphology among related species represents a fruitful approach (Sakai *et al.* 1999).

Two sympatric species within the genus *Helicteres*, with contrasting pollination syndromes, occur along the North Western tropical dry forest of Costa Rica. *Helicteres guazumifolia* Kunth and *Helicteres baruensis* Jacq. In order to have a better understanding of the pollination biology and reproductive ecology of these two species the goals of the present study were to: (1) describe the reproductive phenology of populations; (2) analyze individual flower development and morphology; (3) analyze and compare the morphology of pollen grains and other structures such as floral and extrafloral nectaries, anthers, stigmas and petals by aids of scanning electron microscopy; and (4) estimate the number of pollen grains contained in the anthers of both species.

METHODS

Study site. This study was conducted in Santa Rosa National Park, Guanacaste Conservation Area (ACG), North Western Costa Rica (10°45' to 11°00' N and 85°30' to 85°45' W). Two life zones are present in the area and the study was conducted in a tropical dry forest,

with a moist transition (Holdridge 1967, Hartshorn 1991). The park includes a mosaic of forests of different ages and abandoned pastures (Janzen 1986, Hartshorn 1991, Gerhardt 1993).

In the past, the zone was covered by extensions of tropical dry forest, the most threatened ecosystem of Mesoamerica, it originally covered about 550.000 km^2 from Mazatlan in Mexico until Panama Canal. Actually, only 2% is maintained and 25% of the surface is protected, represented principally in the Guanacaste Conservation Area (Janzen 1986, Fernández Morillo 1998).

The climate is highly seasonal, with a well defined dry season that goes from late November to mid May. Annual rainfall ranges between 800 and 2600 mm, with an annual mean of 1423.4 mm. Annual mean temperature is 25.7°C and annual mean relative humidity is 81% (Rojas Jiménez 2001).

Study species. *Helicteres* is a pantropical genus that contains approximately 60 species, native to the tropics of both hemispheres (Robyns & Cuatrecasas 1964, Gentry 1993, Cristóbal 2001a, Bayer & Kubitzki 2003). It is most abundant in America in which 38 species are distributed from Mexico, Central America, the Caribbean and South America through North Western Argentina and slightly south of the tropics line in Eastern Paraguay and Brazil (Sazima & Sazima 1988, Cristóbal 2001a, b). The members of the genus are shrubs or small trees of dry lowland areas (Sazima & Sazima 1988). They are characterized by having distinctive fruits, which are spiral capsules, many seeded and with a long androgynophore, free or fused (Robyns & Cuatrecasas 1964, Gentry 1993, Bayer & Kubitzki 2003). Two species of *Helicteres* are found in Santa Rosa National Park: *Helicteres guazumifolia* and *Helicteres baruensis*.

Helicteres guazumifolia is widely spread and covers greatest area in America. It extends from Southern Mexico to Central America, North Western Cuba until Rondonia and West of Mato Grosso and neighboring zones of Bolivia (Cristóbal 2001a). It is a shrub or small tree 0.50 - 5 m high, ramificated from the base or erect with slender branches (Robyns & Cuatrecasas 1964, Cristóbal 2001a, b). Flowers are axillary, erect, actinomorphic and have a tubular corolla with a basal nectary. They have short red and spatulated petals, and the peduncle is aligned with the androgynophore (Robyns & Cuatrecasas 1964, Gentry 1993, Cristóbal 2001a, b). It is found on open, secondary and semideciduous forests, gallery forests, pastures and zones of periodic

fires and clearings, also on dry or moist thickets, grassy or bushy slopes (Robyns & Cuatrecasas 1964, Cristóbal 2001a, b).

Helicteres baruensis is also widely distributed in the Americas. It extends from the Pacific coast of Mexico, South of Sonora until Oaxaca, in Yucatan Peninsula, the Caribbean and South America until Colombia, Venezuela, Suriname, Guyana and Brazil (Cristóbal 2001a). It is a shrub or slender tree 2-6 m high with dense foliage. Flowers are geniculated and are borne in axillary or oppositifolious inflorescences, usually three to five flowered and have a horizontal position. They are zygomorphic and have a tubular corolla with a basal nectary, and also have two or more nectaries at the base of the pedicels between the flowers. The petals are pale greenish and acintated, and the androgynophore is bent (Robyns & Cuatrecasas 1964, Cristóbal 2001a, b). This species is characteristic of dry caducifolious forests, holms-oak forests and gallery forests (Cristóbal 2001a, b)

Plant phenology. The phenology of *H. guazumifolia* and *H. baruensis* was recorded every two weeks for 67 plants of *H. guazumifolia* and 73 plants of *H. baruensis* from March 2003 to March 2004. The number of flowers, flower buds, developing fruits and mature fruits produced per plant were counted with a manual counter, obtaining a final total for each month of the period evaluated.

Stages of flower phenology. Flower development for *H. guazumifolia* was monitored in April 2004 and for *H. baruensis* in Decemeber 2003 on ten individuals of each species. On each plant ten flower buds ranging in size 2 cm for *H. guazumifolia* and 4 cm for *H. baruensis* were marked, and their sequence of development was observed every 3 hours until the flowers senesced.

Flower morphology. To describe the floral morphology, five floral morphological characteristics were selected and measured with an electronic digital caliper Fowler & Nsk Max-Cal Mas Series EDC; the data were digitalized by using the Optoface program. Sixty flowers in twenty-five different plants for *H. guazumifolia* and a hundred flowers in fifty different plants for *H. baruensis* were removed and measured. Measurements included length of the corolla, length of the bracts (only for *H. guazumifolia*), corolla width (calculated as the greatest width of the

corolla), distance from the base of the corolla to the stigma (corolla-stigma), and distance from the base of the corolla to the anthers (corolla-anthers).

Morphology of pollen grains and of other structures with Scanning Electron Microscopy. Pollen grains morphology. The study of the pollen grains of *H. guazumifolia* and *H. baruensis* was conducted in the Biology Laboratories, Ulm University, Germany.

Acetolysis. Pollen for SEM (scanning electron microscope) was acetolysed following the protocol of the technique of Erdtman (1960). Flower buds on the point of opening were collected and preserved in vials containing 70% ethanol for preservation. They were dissected under light microscope using forceps and a dissecting needle. The pollen from two closed anthers was removed an placed in a vial containing 1 ml 70 % ethanol for preservation. The tube was spun for 10 minutes at 2400 revolutions per minute. Ethanol was removed and 100% acetic acid was added. The step was repeated once and the sample was left still for 3 hr minimum. The tube was spun and decanted. Acetolysis mixture (mixture consisted of 9 vols. of chemically pure acetic anhydride and 1 vol. concentrated sulphuric acid) was added to the vial. It was placed in a water bath and warmed up to 90°C for 15 minutes. The vial was spun and decanted. Acetic acid 100 % was added. The step was repeated once and the sample was left still for 3 hr minimum. The vial was spun and decanted and FDA was added to the sample. The vial was spun and decanted, and the sample was ready for SEM preparation.

Critical Drying Point Technique. The vial containing pollen grains was filled with 2-propanol until 2/3rds of its volume, spun and decanted. The sample of pollen grains and other structures for analysis such as floral nectaries, extrafloral nectaries, anthers, stigmas and petals was stored in small baskets and placed in a holder filled out with 100% 2-propanol medium for enhancing the critical point drying process with the critical point drying apparatus model E3000 (for use with liquid carbon dioxide). Critical drying point is a method of drying tissue without collapsing or deforming the structure. Its major application concerns to the tissue preparation for the scanning electron microscope. Tissues become damaged by normal drying because surface tension forces are created in cavities of small dimensions when there is a liquid-gas interface. As tissue dries the liquid-gas interface travels through the surface of the material collapsing the cavities between projecting structures. The critical point method of drying avoids these effects by

never allowing a liquid-gas interface to develop, in this way tissue is not exposed to surface tension forces.

Vacuum Coating. Dried pollen grains and structures were mounted on microscope stubs and were vacuum coated sputtered with gold. The sputtering device used was model Balzers Union FL-9496. Micrographs were taken using a JSM-SI Scanning Microscope.

Pollen counts. The number of pollen grains contained in an anther was determined using a Casy particle counter (Scharfe System Gmbh). Pollen numbers were estimated from 18 mature flower buds of 15 plants of *H. guazumifolia* and from 37 mature flower buds of 32 plants of *H. baruensis* selected randomly. Two undehisced anthers were removed from each bud and placed on a petri dish containing droplets of Casyton solution and teased with a dissecting needle until all pollen grains were released. The anther tissue was separated from the pollen grains and they were placed in a vial containing 10 ml of Casyton solution. Total pollen counts/ml were obtained from a Casy particle counter using the Casy Excell program.

RESULTS

Plant phenology

Helicteres guazumifolia. *H. guazumifolia* flowered from March to late June 2003, with peak flowering time in April. During August and September, some plants flowered again. Flowering time started later on until February 2004 (Fig.1). Flower buds were detected since March to September 2003, with a major peak during April and a slight peak in September. Plants started to produce buds the next year starting February 2004. Developing fruits were monitored starting early July 2003 until February 2004, with a first major peak between the months of August and September, and a second peak in December. Mature fruits were abundant in the plants during the whole year, however, most of them were observed in March 2003.

Helicteres baruensis. *H. baruensis* flowered from late May to late December 2003. Most plants flowered in August, September and October (Fig. 2), and flower production was higher in September. A reduction in the number of flowers observed per month was due until late December. Flower buds were detected from May to December 2003, with a peak in September. Developing fruits were detected starting September 2003, and every month more developing

fruits were registered. A major peak occurred in January 2004 until no more fruits developed in February. Mature fruits were very common in plants during the entire year, with a slight increase around January 2004.

Stages of flower phenology. The flowering stages of development at the level of individual flowers were determined for each *Helicteres* species.

Helicteres guazumifolia. Five different flowering stages were determined and are described as follows (Figs. 3, 4, 5):

Stage 1: Buds closed. Closed flower buds of one equal size ranging between 2 cm were found in pairs along the ends of branches on every individual plant. Flower buds engrossed at the apex would open on that day (Fig. 3 A-C).

Stage 2: Buds opening. Buds slowly started to open from the tip end, between 1700 h and 1800 h. A diminutive portion of the stigma could be seen, surrounded by the closed petals (Fig. 3 D-F).

Stage 3: Stigma starts to protrude. At 2200 h the stigma starts to protrude out from the corolla, the petals are still unfolded and covered by the outer bracts. There is a clear distinction between the whorl of bracts surrounding the whorl of petals. The position of both whorls at this time of the night is at the same level along the floral tube (Fig. 4 A).

Stage 4: Stamens start to protrude. Between 0100 h and 0200 h the whorl of stamens start to protrude out from the corolla until the complete whorl of stamens and the stigma are not covered by the petals. All the anthers are closed. The stigma projects beyond the anthers and lies in an erect position. Petals unfold and start to grow and elongate (Fig. 4 B-F).

Stage 5: Androgynophore elongation. Between 0400 h and 0500 h, the androgynophore starts to elongate reaching its maximum extension at 0500 h. The stamens form an elongated column adnate to the gynophore. The ten anthers are grouped at the tip of the column and dehisce synchronously and longitudinally. The pollen is exposed to potential pollinators by 0600 h. Flowers are completely developed and are ready to start nectar production beginning at 0600 h (Fig. 5 A-D).

The flowers of this species last for 3 days. Flower buds and flowers at anthesis are bright red. Nectar is secreted only on the first day after flowers opened. The next day in the morning flowers had changed color to dark red. Petals start to wilt and corrugate. Anthers have released all pollen grains. The stigma turns dark. By the next day, the flowers are even more dark red.

Helicteres baruensis. Five different flowering stages were determined and are described as follows (Figs. 6, 7, 8):

Stage 1: Buds closed. Closed flower buds of three different sizes were located at the end of most branches on every plant. They varied in size between 0-1 cm, 1-2 cm and 2-4 cm. Buds were grown in pairs of different sizes, *i.e.* one bud of 4 cm and another one 2 cm long (Fig. 6 A-B). Flower buds of 4 cm long and engrossed at the apex would open on that day (Fig. 6 C).

Stage 2: Buds opening. Buds slowly start to open from the tip end, at night between 2000 h and 2100 h. A portion of the bottom of some anthers is exposed. The curved androgynophore is hidden in the closed bud. Buds remain in this stage until the following day (Fig. 6 E-F).

Stage 3: Androgynophore curved. During the morning hours of the following day, at 0800 h the medial part of the androgynophore protruded from the calyx, with the whorl of stamens and the stigma hidden and covered by the calyx. The ten stamens form an elongated column adnated to the gynophore. Flowers remained in this stage until 1000 h or 1100 h, approximately for two to three hours (Fig. 7 A-B).

Stage 4: Androgynophore starts elongation. The androgynophore grew out of the corolla and unfolds, between 1000 h and 1100 h (Fig. 7 C). It starts to elongate slowly and stretches upwards (Fig. 7 D-F). The ten anthers are grouped at the tip of the column and dehisce synchronously and longitudinally. Pollen is exposed to pollinators and other flower visitors. Pollen grains have a bright yellowish color appearance. The stigma projects beyond the anthers.

Stage 5: Androgynophore completes elongation. The androgynophore reaches maximum extension and stretches upwards completely by 1200 h and 0200 h, reaching a complete curved position and the flower is exposed in a pendant position with respect to the horizontal. Flowers are completely developed and are ready to start nectar production beginning at 1600 h (starting on the second day after anthesis). Even though, pollen has being exposed for

about four hours, the anthers still contain pollen grains, some anthers are still fully covered with pollen while others have less or no pollen grains (Fig. 8 A).

The flowers of this species also last for 3 days. During the morning of day 3, aging flowers gradually change in coloration but don't change flower position. The anthers became brownish and dried, they lack of pollen grains and the stigma is wet. The borders of the calyx start to turn brownish (Fig. 8 B-D).

Fruit development and fruit maturation. Within a few days, the flowers that were successfully pollinated started fructification until the helicoidal capsule was formed (Fig. 9). Developing and mature fruits of *H. baruensis* have the same whitish color appearance, while *H. guazumifolia*'s developing fruits are green and mature capsules black.

Flower morphology. Measurements on distinct characters of flowers demonstrated that length and width of corollas of *H. baruensis* is twice the length and width of the corollas of *H. guazumifolia* (Table 1). Similarily, the distances between corolla and stigma and between corolla and anthers in flowers of *H. baruensis* are more than twice the distances in flowers of *H. guazumifolia*, resulting in a longer androgynophore for *H. baruensis*.

Pollen grains morphology. Figures 11 and 12 show photomicrographs of pollen grains of *H. guazumifolia* and *H. baruensis*. Pollen grains were described based on the sculpture of the exine, as suggested by Pire & Cristóbal (2001). The terminology used is the one proposed by Erdtman (1960) and Sáenz de Rivas (1976). Nine pollen types are recognized in the genus *Helicteres*, and they are ordered according to the presence of different supratectal elements and complexity of perforations. Simple pollen types have a uniform sculpture in the whole grain, while the more complex or advanced ones have a differentiation between the polar and equatorial zones (Pire & Cristóbal 2001). Both *H. guazumifolia* and *H. baruensis* have complex pollen types; pollen grains are classified as type IX and type VII respectively.

Helicteres guazuamifolia. Pollen grains of *H. guazumifolia* are classified as type IX, a complex pollen type. Pollen is tectated-perforated, fossulate, and microechinate along the surface. Along the equatorial zone, the tectum is almost complete, and has small perforations that form a distinctive zone around the area. Grains have a triangular isopolar form and have three circular pores with no ring. Grains have microspines as supratectal elements distributed along the entire

surface and in the borders. A total of eight pollen grains were used to measure the diameter of ten microspines selected randomly along the equatorial zone. On average, the diameter of each microspine was 655.00 ± 129.75 Nm (N= 80) (Fig. 11 A-C; Fig. 12 A-B).

Helicteres baruensis. Pollen grains of *H. baruensis* are classified as type VII, also a complex pollen type. Pollen is tectated-perforated and have supratectal sculptural elements known as verrucae. Grains have a triangular isopolar form and have three circular pores with a psilate ring. The verrucae are well differentiated and are distributed on the whole grain surface, except the rings. A total of seven pollen grains were used to measure the diameter of ten verrucae selected randomly along the equatorial zone. On average, the diameter of each verrucae was 1.23 ± 0.16 Nm (N= 70) (Fig. 11 D-F; Fig. 12 C-D).

Floral nectary morphology. The floral nectary of *H. guazumifolia* is located at the base of the calyx, and has an average length of 1.54 ± 0.34 mm (N= 5) with respect to the total length of the corolla (Fig. 13 A-B). As in *H. guazumifolia*, the floral nectary of *H. baruensis* is also located at the base of the calyx, and has an average length of 2.83 ± 0.70 mm (N= 11) with respect to the total length of the corolla (Fig. 13 C-D). In both species, the floral nectary is formed by a distinct group of trichomes that form a particular carpet (Fig. 14).

Extrafloral nectary morphology. *H. baruensis* has extrafloral nectaries located at the base of the pedicels between the flowers (Fig. 6 D; Fig. 15). Extrafloral nectaries have a bright green color and secreted nectar at night starting at 2100 h. They are like tubercles and have a smooth appearance. *H. guazumifolia* lacks extrafloral nectaries of any type.

Petals. Figure 16 shows the appearance of the petals of *H. guazumifolia* and of *H. baruensis*; both have a smooth surface but differ in the types of hairs above their surface. In *H. guazumifolia*, the petals have trichomatous hairs such as the ones present in the floral nectaries; while *H. baruensis* has stellate hairs.

Anthers and stigmas. Figures 17, 18, 19 and 20 show the appearance of the anthers and stigmas for both species. Flowers have ten anthers grouped in a column adnated to the androgynophore. The stigma is short, formed by five lobes, and projects beyond the group of anthers.

Pollen counts. The number of pollen grains contained in an anther of *H. guazumifolia* was on average 9631 ± 2913 (N= 36 anthers); while an anther of *H. baruensis* contained on average 15884 ± 6730 pollen grains (N= 69).

DISCUSSION

Plant phenology

The flowering pattern of a plant is defined by the duration of the flowering period, as well as the number and temporal distribution of flowers. Among tropical species, the flowering phenology of individual plants varies continuously between two extreme patterns. At one extreme are species with "mass flowering" individuals producing large numbers of new flowers each day over a week or less. At the opposite extreme are species with "steady state" individuals producing small numbers of new flowers almost daily for many weeks (Auspurger 1983, Bawa 1983, Rathcke & Lacey 1985). Both *Helicteres* species have a steady state flowering pattern, with no overlap in blooming times. *H. guazumifolia* produces most of its flowers for four months, while *H. baruensis* produces flowers for six months.

A particular problem for successful pollination lies in avoiding cross pollination between closely related species. Isolating mechanisms can be provided by different flowering times or by attracting different species of pollinators (Newstrom *et al.* 1994, Tschapka *et al.* 1999). Flowering time acts as an isolating mechanism that maintains both *Helicteres* species at Santa Rosa restricting *H. guazumifolia* blooming time to the first months of the year, and *H. baruensis* after July.

The timing of plant reproductive cycles affects not only plants but also animals that depend on plant resources (Newstrom *et al.* 1994). The longer flowering season of *H. baruensis* may be understood due to the fact that as a bat-pollinated plant, it produced flowers over extended periods longer than one or several months. This permits bats to learn the location of a reliable food resource and save energy and time by using this knowledge on consecutive nights (Von Helversen 1993, Tschapka *et al.* 1999).

Both species produce fruits that are wind-dispersed twisted capsules mostly during the dry season. Lieberman (1982) indicates that species with dry fruits tend to fruit in the dry season, specially wind-dispersed species do so by this time when strong winds prevail.

Floral biology. As a hummingbird pollinated species, flowers of *H. guazumifolia* show several features of the typical ornitophylic syndrome.

Attraction: Floral scent and color. Flowers are red colored, predominance of red in hummingbird flowers has been interpreted as exploitation of an ecological niche with regard to the class of bee-pollinated flowers. Red is at the long-wave end of the visible spectrum of electromagnetic radiation. Bees and some other insects are not able to perceive red. Birds have their greatest spectral sensitivity and finest hue discrimination towards the long-wavelength end of the spectrum. At the same time, it may be a cryptic color to avoid visits by insects that could act as pollen robbers or nectar robbers (Endress 1994, Proctor *et al.* 1996). *H. guazumifolia* flowers were scentless at any time of the day.

Accesibility: Floral position. *Helicteres guazumifolia* flowers have an erect position. In *Helicteres* the flower position in respect to the vertical varies between erect, oblique, horizontal or pendant, and these variants are related to the pollination agents (Cristóbal 2001a).

Structure. Robustness may serve to prevent nectar thieving by both insects and birds. However, many flowers of *H. guazumifolia* from different plants were found in the field to have either one or two circular perforations in the calyx, at the base of the flower. Also, *Trigona* bees were observed piercing the flowers. It should be important on future studies to evaluate the effect of nectar robbing on the flowering phase of the species, and its consequences on the reproductive biology. Cristóbal (2001a) indicates that it is common to find over the limit of the calcinial nectary perforations perfectly circular, being destroyed the petals nails. Nectar robbing was also registered in other species such as *H. sacarolha* by wasps *Polybia occidentalis, Vespidae, Polistinae, Polybiini*; in *H. mucosa* and *H. heptandra* by bees of *Trigona, Apidae, Meliponine* (Cristóbal 2001a).

Flowers depending on bat-pollination for reproduction, however, frequently show distinct adaptations (Tschapka & Dressler 2002) and *H. baruensis* flowers show characters specific of the chiropterophilous syndrome.

Attraction: Floral scent and color. Floral scents are important long distance attractants (Von Helversen 1993, Tschapka & Dressler 2002). *H. baruensis* flowers emitted a strong and distinctive scent around 2200 h during the second night of flower life span. Further studies could be oriented toward the analysis of the floral scent components. The greenish color of the calyx and the whitish lobes of the corolla also act as attractants, and as a camouflage hiding the flowers from visually oriented foragers, sphingid moths (Von Helversen 1993, Tschapka & Dressler 2002).

Accesibility: Floral position. Flowers tend to grow very exposed protruding from the foliage (Von Helversen 1993, Tschapka & Dressler 2002). *H. baruensis* flowers grow in axillary inflorescences and after anthesis they acquire a horizontal and pendulous position along the branches.

Structure. Corollas in *H. baruensis* are very robust, a feature necessary in order to withstand the visits of their pollinators (Von Helversen 1993, Tschapka & Dressler 2002). Shapes have been developed in order to optimize pollen transfer by bats (Tschapka & Dressler 2002). Corollas are wider at the upper part of the flower, they have on average 1.24 cm of wide (Table 1), when bats hover at the flowers they approach to the calyx and the anthers and pistil slid ventrally along the bat's belly. The wider calyx opening may facilitate the bats to push their snout into the flower and reach the nectar easily.

Pollen grains. As a genus, *Helicteres* is palynologically rather uniform with respect to the shape and size of the grains as well as the type and number of the apertures. However, the different patterns of exine sculpture have taxonomic and phylogenetic value (Pire & Cristóbal 2001), and this is clearly demonstrated in the two related species under consideration.

Floral nectaries morphology. Members of the family Sterculiaceae possess trichomatous floral nectaries, consisting of multicellular clavate hairs, which release nectar from the top and are usually aggregated in cushions or carpets (Endress 1994, Vogel 2000). Tricome nectaries seem to be relatively rare (Endress 1994); but it is one of the features characterizing core Malvales (Bombacaceae, Malvaceae, Sterculiaceae and Tiliaceae). In Helicteroids floral nectaries maintain a radial arrangement and carpets are mostly calyx-borne (Vogel 2000).

As shown in Figures 13 and 14 each floral nectariferous trichome can be distinguished separately and clearly because they do form a compact carpet, on both *Helicteres* species. They are always multicellular, uni- to masonry-like pluriseriate, with a basal cell rooting in the epidermis, one neck cell, and a filiform, clavate or fusiform glandular body. All nectar carpets are supplied with special innervation and with additional glandular tissue in the subjacent mesophyll, lending the complex an integrated organ like character (Vogel 2000). The glandular tissue is also shown in Figure 14 as a thick layer underneath the layer formed by the trichomatous nectaries.

The dimension of these carpets varies enormously depending on floral and pollinator type (Vogel 2000). As flowers of *H. baruensis* are larger and wider than those of *H. guazuamifolia*, consequently there is more area occupied by the floral nectary of *H. baruensis* which almost doubles the size compared to the one of *H. guazumifolia*. Accordingly, this leads to the production of more quantities of nectar by *H. baruensis* flowers. Nectar glands in bat flowers are often voluminous in comparison to those of related species that are not bat-pollinated, and that is why secretion of nectar is much greater in bat flowers than in all other pollination syndromes (Von Helversen 1993).

Hairs of this anatomical design are by no means confined to floral nectaries but also occur elsewhere on the plant and may have other functions. They may produce extrafloral nectar, water, or even allelopathic substances (Vogel 2000). Trichomatous hairs are present in petals, corolla, stigmas and anthers.

Extra floral nectaries morphology. Extrafloral nectaries are located on vegetative structures and are involved in nonpollination functions. They may appear as tubercles on the pedicels of the flowers (Elias 1983). as exemplified by extrafloral nectaries of *H. baruensis*. According to Zimmerman's classification system, *H. baruensis* extrafloral nectaries could be classified as formless nectaries. These are amorphous nectaries that lack obvious structural specialization at the tissue or organ level but are capable of secreting rich nectar. They are recognized on the plant by the presence of nectar and often a distinct coloring of the site of secretion (Elias 1983). By the time extrafloral nectaries were found active they secreted abundant nectar and their color was bright green on the plants.

Extrafloral nectaries are important in maintaining the mutually beneficial relationship between many plants and certain insects, especially ants, which are attracted to the nectaries and in turn offer the plant varying degrees of antiherbivore protection. Also, they may deter other organisms that may interfere with the pollination process or nectar robbers (Elias 1983). Ants were common visitors to the extrafloral nectaries of *H. baruensis*. Several unidentified ant species were seen foraging on nectar secreted by the extrafloral nectaries. At night, between 2100 h to 2300 h, two species were observed most of the time (Fig.10 C-D). During the day other two species were seen between 0800 h and 1000 h (Fig.10 E-F).

Among other visitors, *Trigona* bees were seen collecting pollen from the anthers, avoiding contact with the stigma (Fig. 10 A-B). Most chiropterophilous flowers attract quite a number of additional visitors, often bees (Apidae and Meliponinae) that can be observed collecting nectar and pollen left in the flowers in the morning hours (Von Helversen 1993).

Pollen counts. Estimates of the number of pollen grains contained per anther of each species revealed that pollen amount is another adaptative character associated to the distinct pollination syndromes of the two *Helicteres* species studied. Von Helversen (1993) indicates that the pollen supply of glossophagine flowers is larger than that of related species with flowers not pollinated by bats for several reasons. Within the evolution of the Glossophaginae, pollen has become the main protein source. Pollen ingestion tends to be high, since bats interrupt their foraging flights for cleaning their fur that catches pollen constantly.

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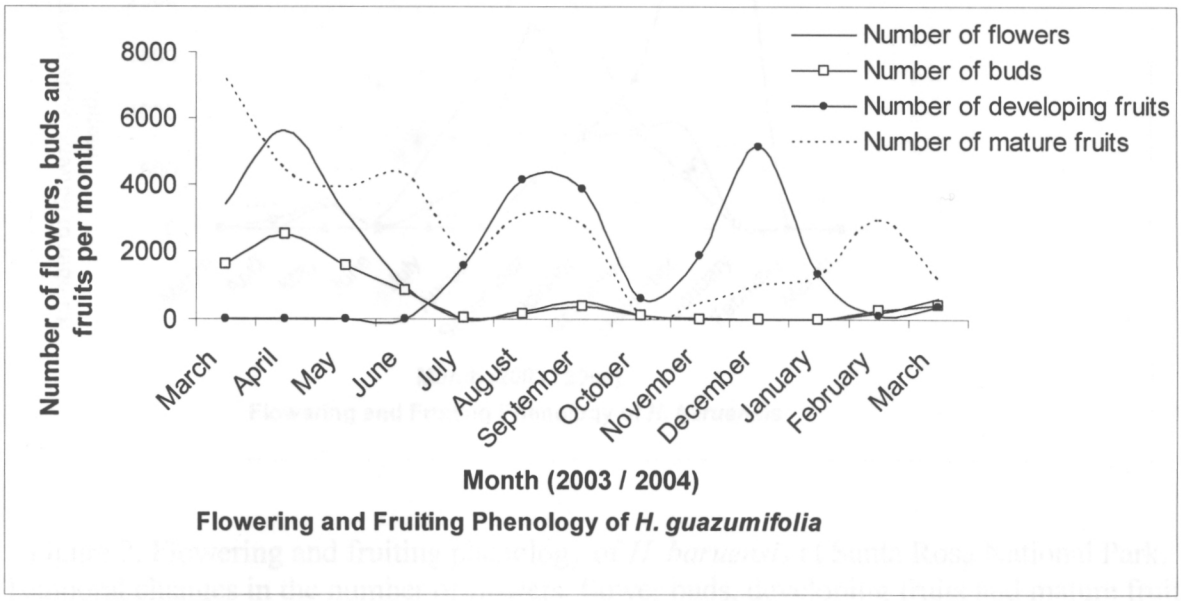

Figure 1. Flowering and fruiting phenology of *H. guazumifolia* at Santa Rosa National Park. Temporal changes in the number of flowers, flower buds, developing fruits and mature fruits observed per month (N= 67 plants).

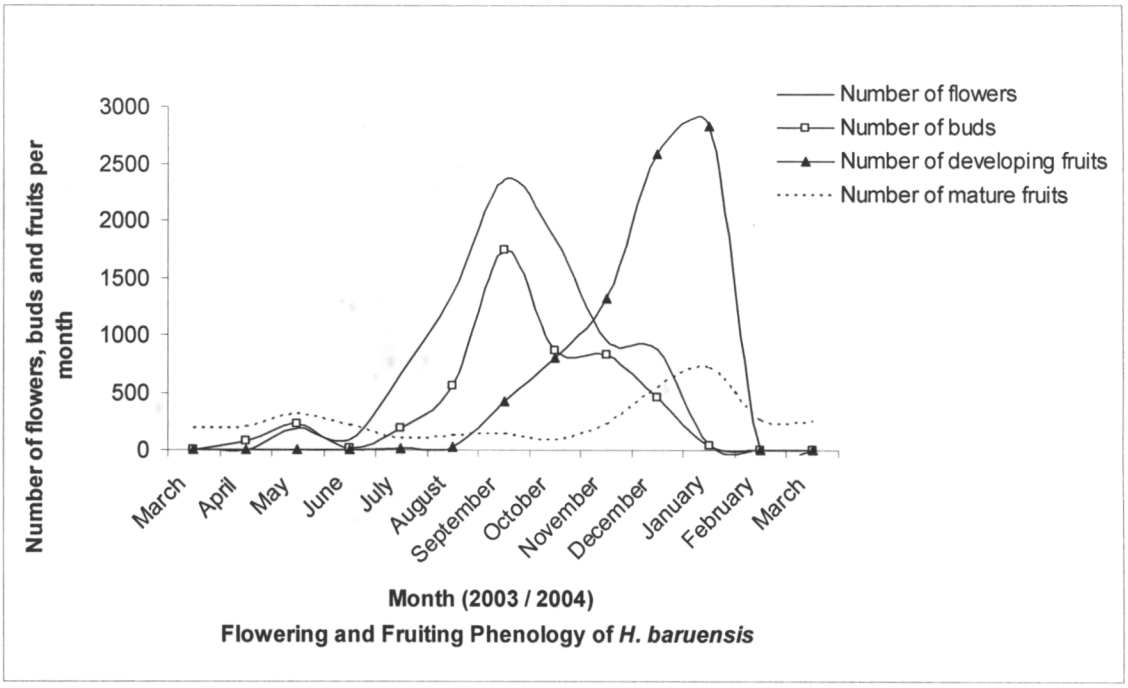

Figure 2. Flowering and fruiting phenology of *H. baruensis* at Santa Rosa National Park. Temporal changes in the number of flowers, flower buds, developing fruits and mature fruits observed per month (N= 73 plants).

Table 1. Floral morphological characters of *H. guazumifolia* and *H. baruensis*. Values represent mean (SD) in cm.

Species	n	Corolla length	Bracts length	Corolla width	Corolla-stigma distance	Corolla-anthers distance
H. guazumifolia	60	2.46 (0.14)	2.15 (0.18)	0.63 (0.06)	4.10 (0.28)	3.81 (0.25)
H. baruensis	100	4.03 (0.34)	-	1.24 (0.17)	10.48 (0.76)	10.12 (0.69)

Figure 3. Floral development of *H. guazumifolia*. Stage 1: Buds closed A. B. C. Stage 2: Buds opening D,E,F.

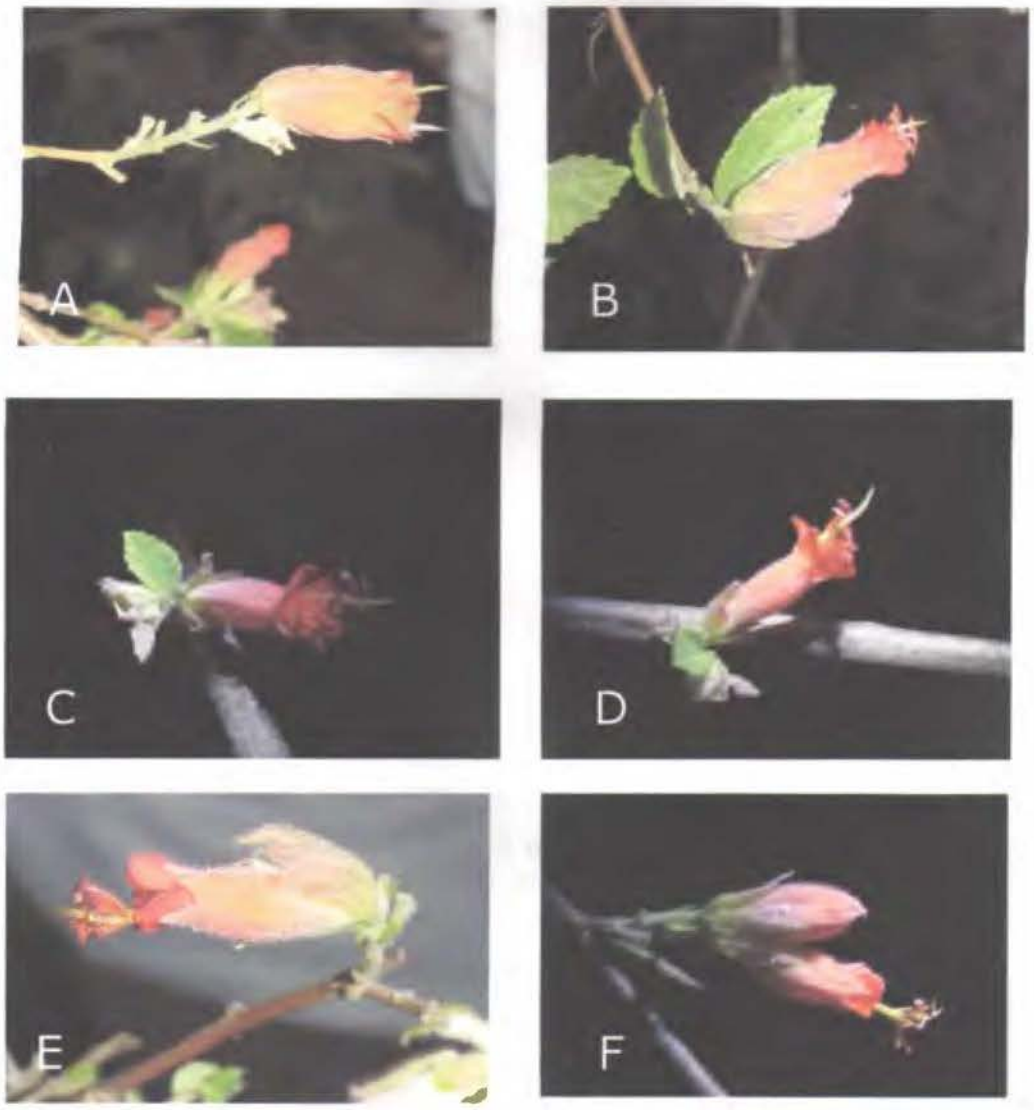

Figure 4. Floral development of *H. guazumifolia*. Stage 3: Stigma starts to protrude A. Stage 4: Stamens start to protrude B, C, D, E, F.

Figure 5. Floral development of *H. guazumifolia*. Stage 5: Androgynophore elongation and complete flower development A, B, C, D.

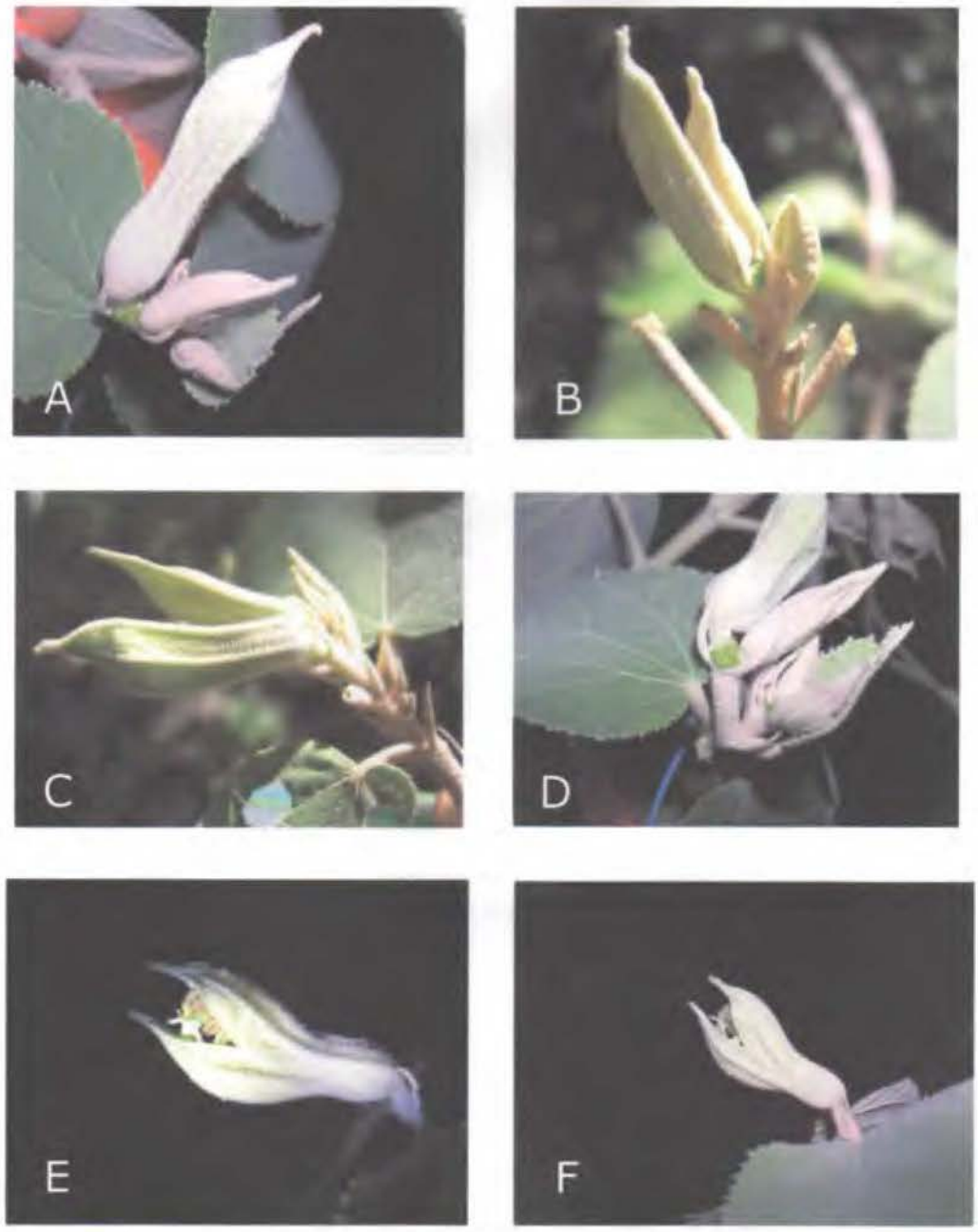

Figure 6. Floral development of *H. baruensis*. Stage 1: Buds closed A,B,C. Stage 2: Buds opening E,F. Extrafloral nectaries D.

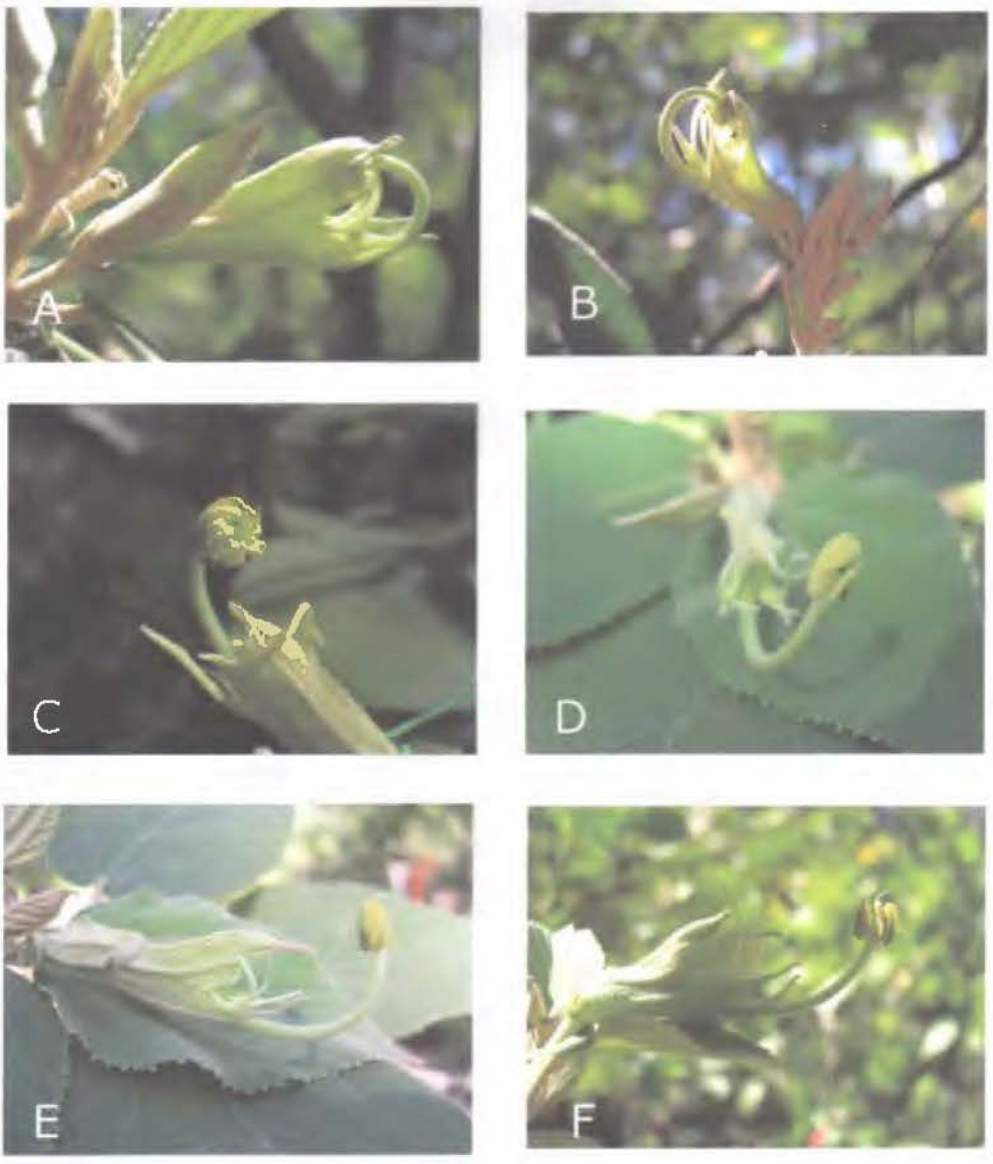

Figure 7. Floral development of *H. baruensis*. Stage 3: Androgynophore curved A,B Stage 4: Androgynophore starts elongation C, D, E, F.

Figure 8. Floral development of *H. baruensis*. Stage 5: Androgynophore completes elongation and complete flower development A. Flowering aging B, C, D.

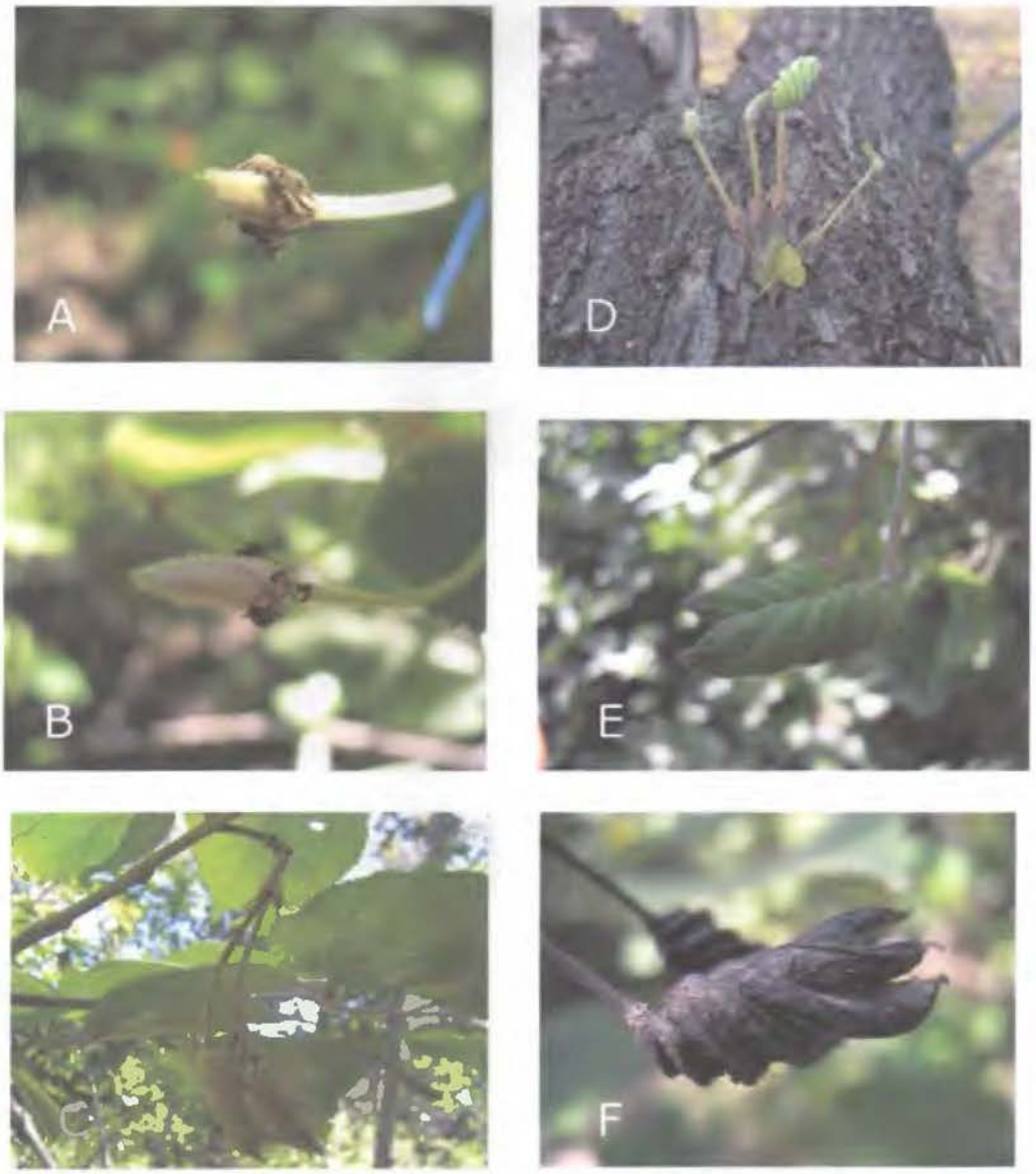

Figure 9. Fruit development and fruit maturation of *H. baruensis* A, B, C and *H. guazumifolia* D, E, F.

Figure 10. *H. baruensis* with flowers and floral visitors. The stingless bee *Trigona* collecting pollen A,B. Several ant species collecting extrafloral nectar C,D,E,F.

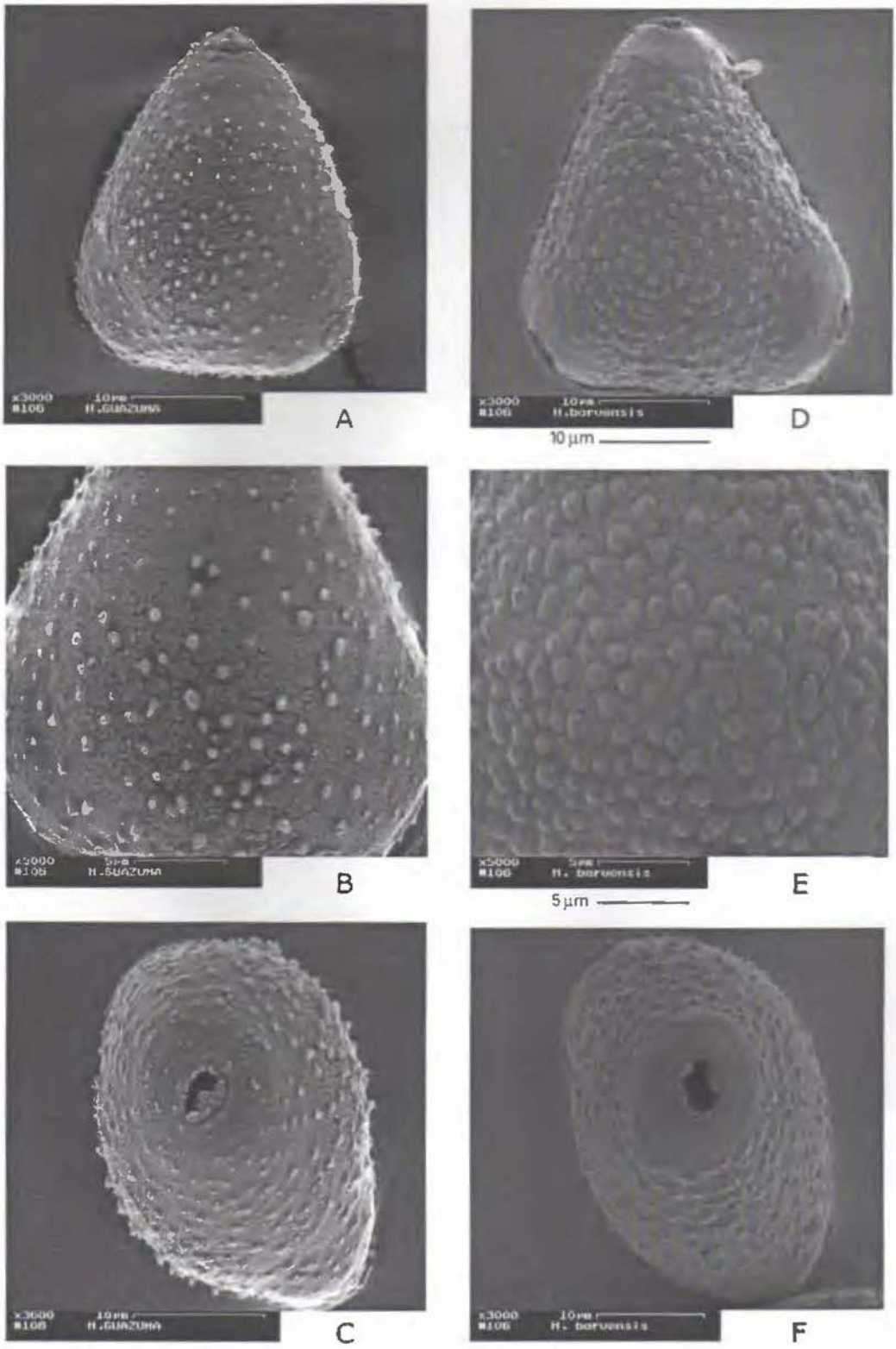

Figure 11. Pollen grains of *H. guazumifolia* A,B,C and *H. baruensis* D,E,F. Bars = 10 µm (A,C,D,F) and 5 µm (B,E).

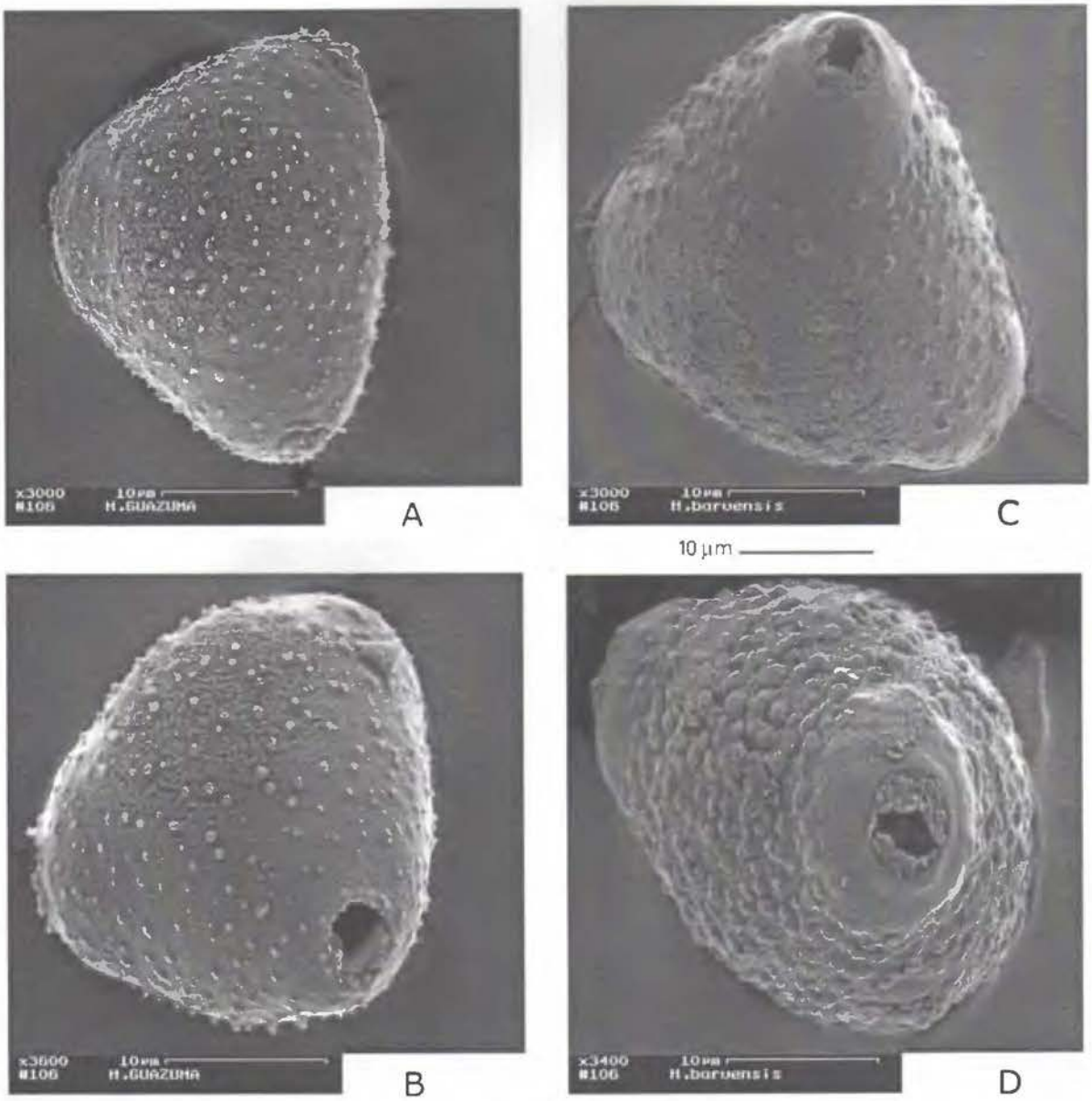

Figure 12. Pollen grains of *H. guazumifolia* A,B and *H. baruensis* C,D. Bars = 10 µm.

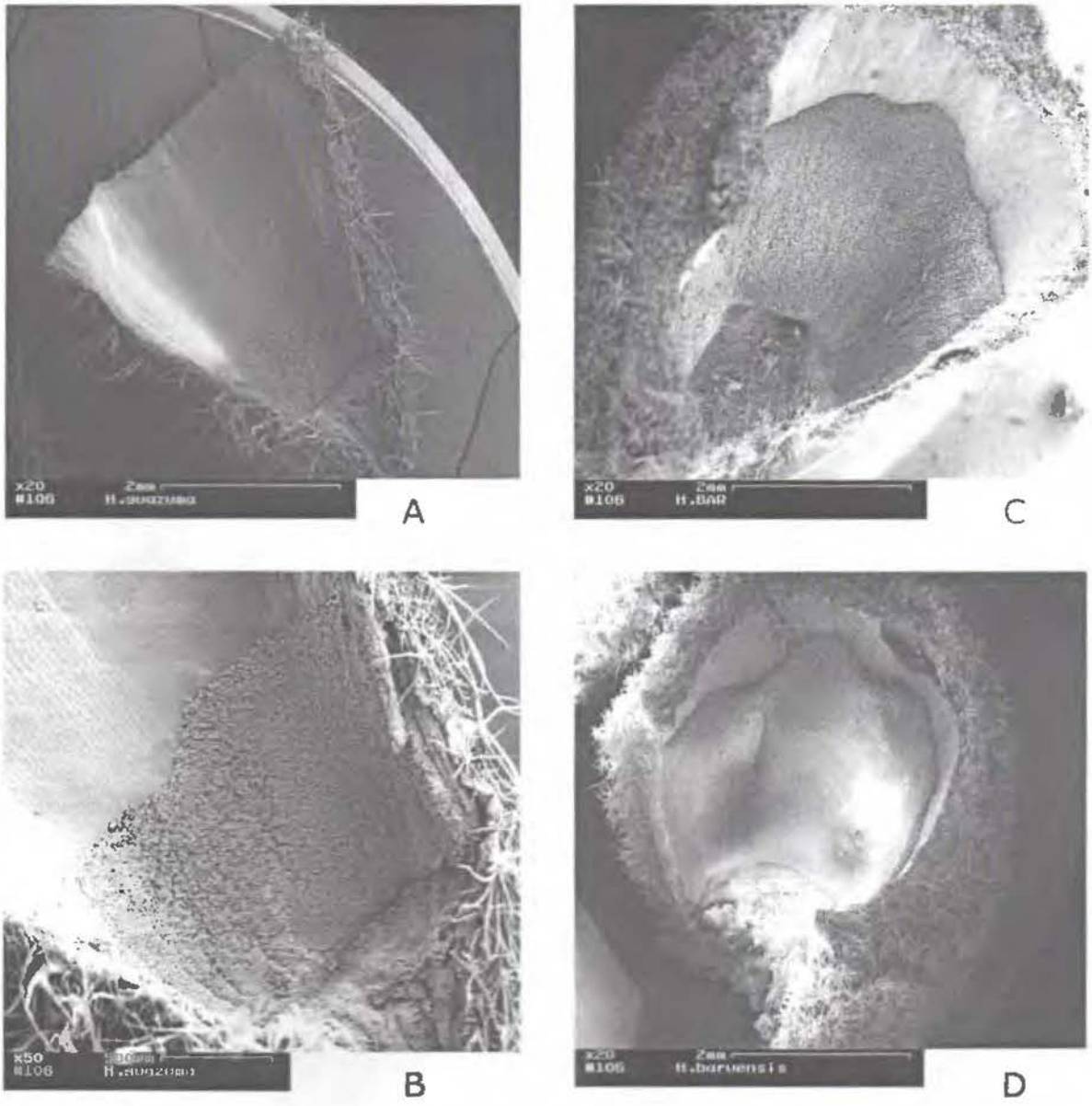

Figure 13. Floral nectary of *H. guazumifolia* A,B and *H. baruensis* C,D. Bars = 2 mm (A,C,D) and 500 µm (B).

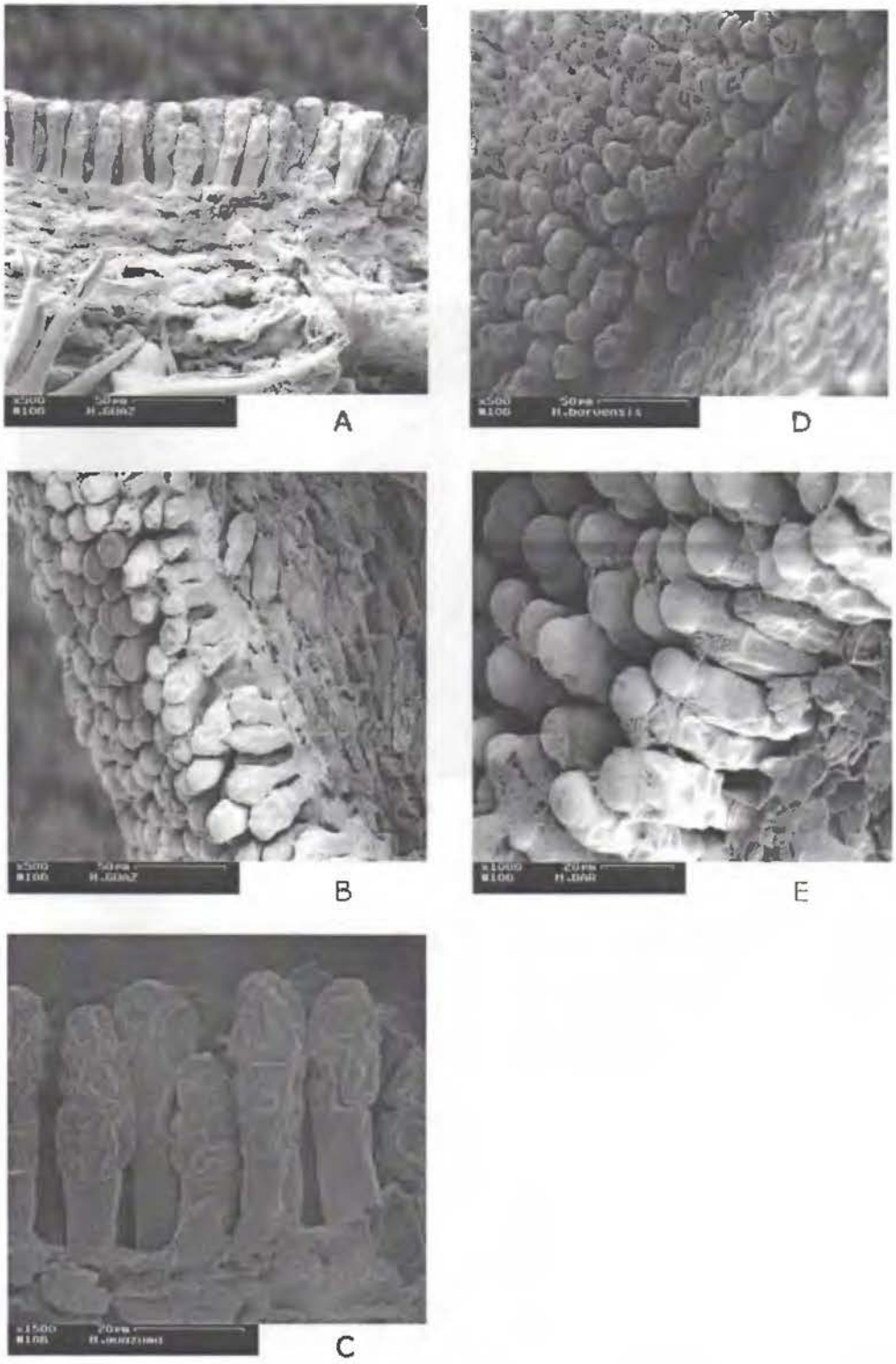

Figure 14. Trichomatous floral nectaries of *H. guazumifolia* A, B, C and of *H. baruensis* D, E. Bars = 50 μm (A,B,D) and 20 μm (C,E).

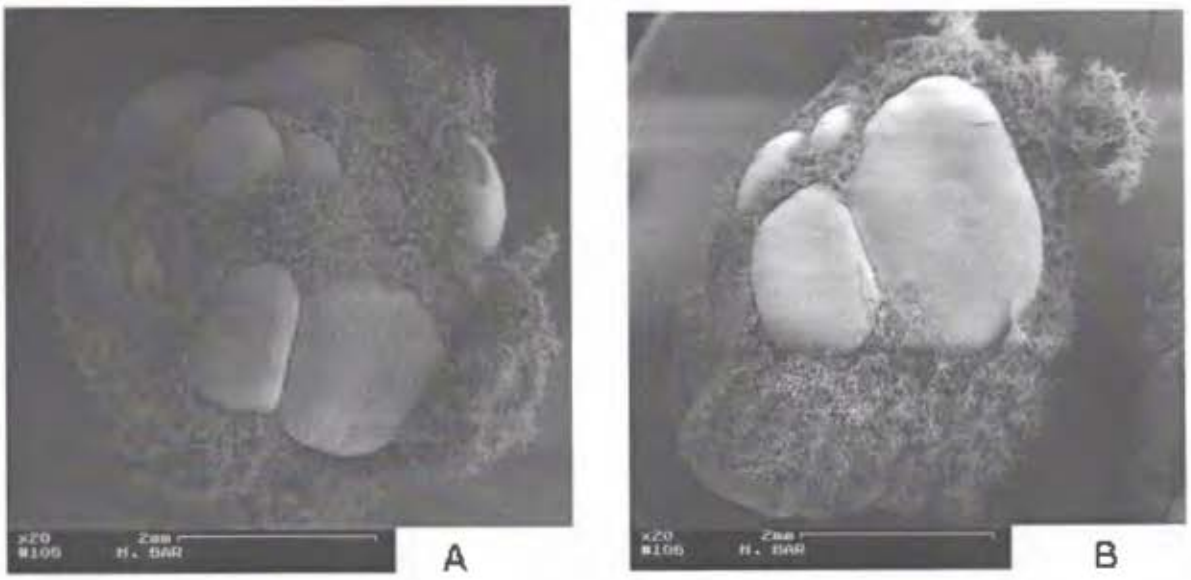

Figure 15. Extra floral nectary of *H. baruensis* A, B. Bars = 2 mm

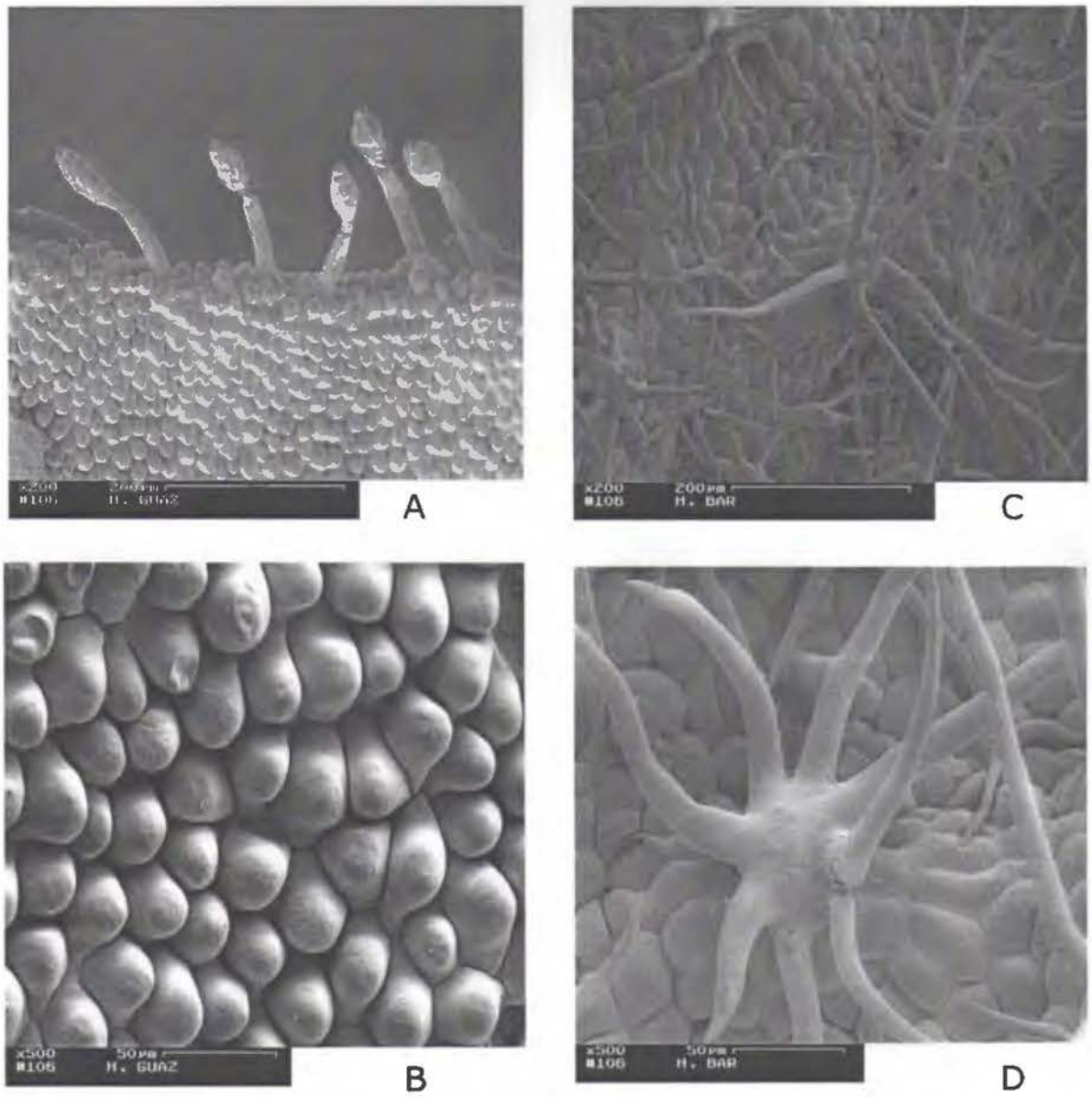

Figure 16. Petal surface of *H. guazumifolia* A, B and corolla surface of *H. baruensis* C, D. Bars = 200 μm (A, C) and 50 μm (B, D).

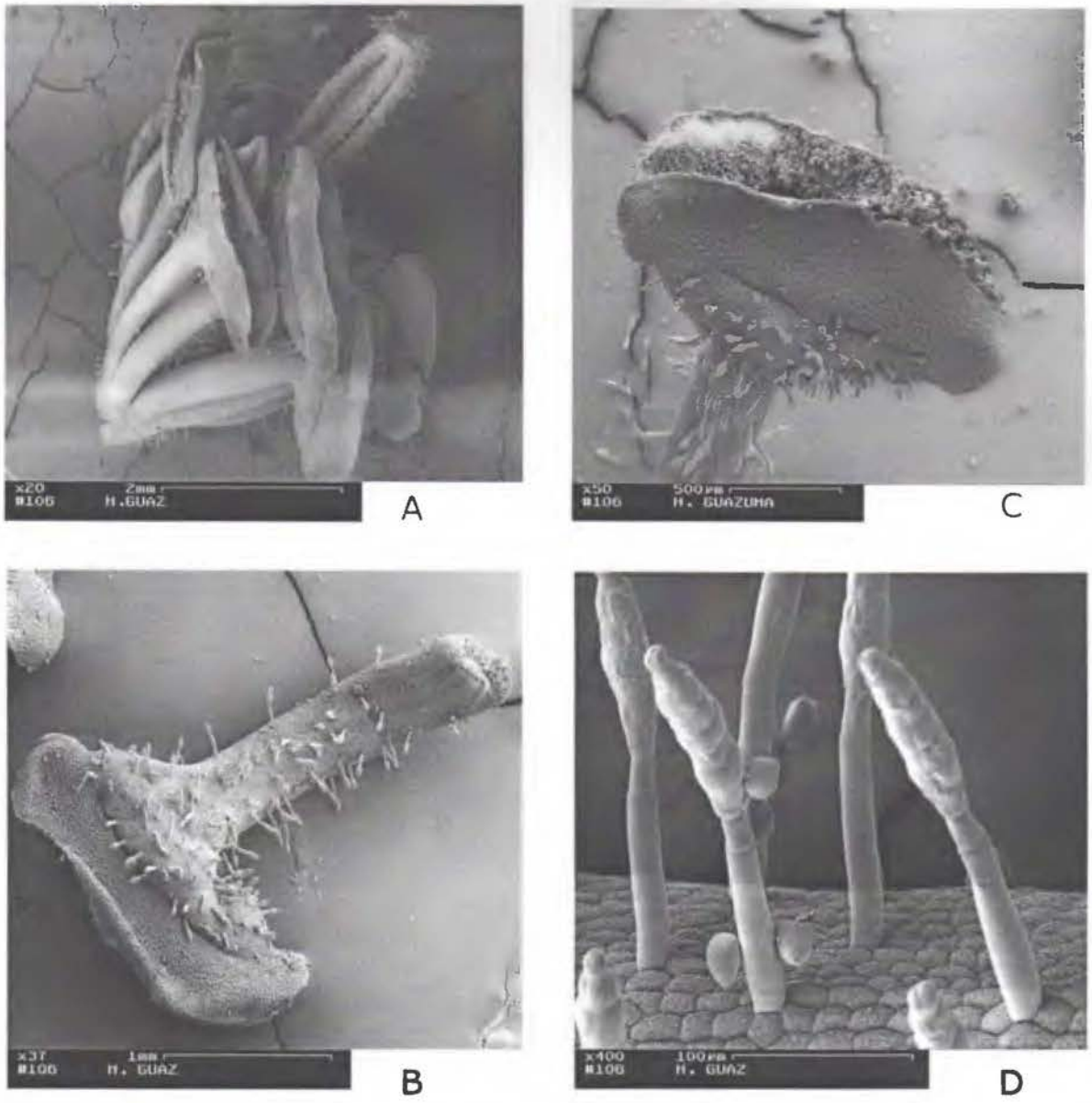

Figure 17. Anthers and stigma of *H. guazumifolia* A. Anther B, C. Trichomatous hairs on anther D. Bars = 2 mm (A), 1 mm (B), 500 µm (C) and 100 µm (D).

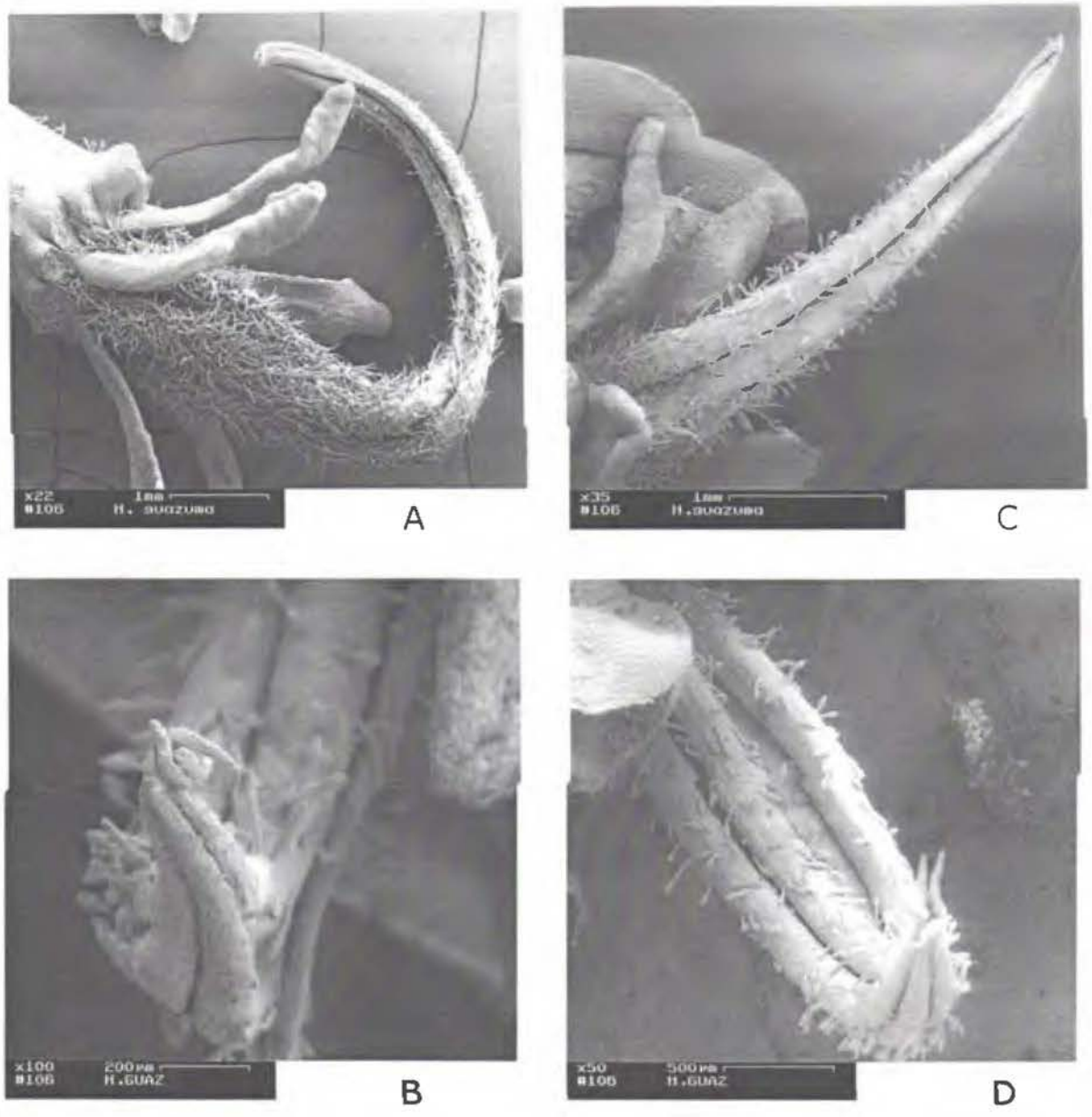

Figure 18. Stigma of *H. guazumifolia* A, B, C, D.
Bars = 1 mm (A, C), 200 μm (B), 500 μm (D).

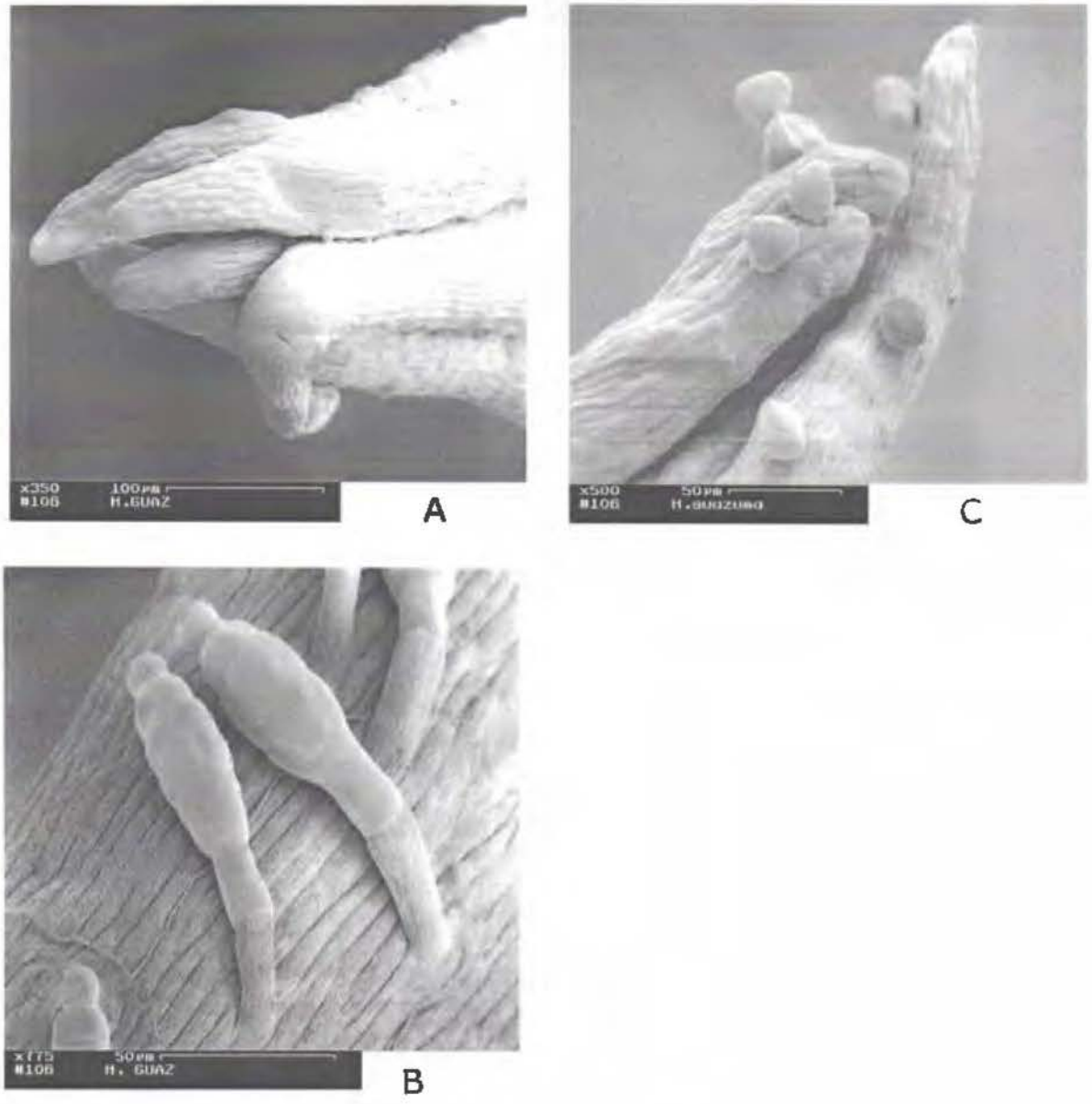

Figure 19. Stigma of *H. guazumifolia* A. Stigma with pollen grains B. Trichomatous hairs on stigma C. Bars = 100 µm (A), 50 µm (B), 50 µm (C).

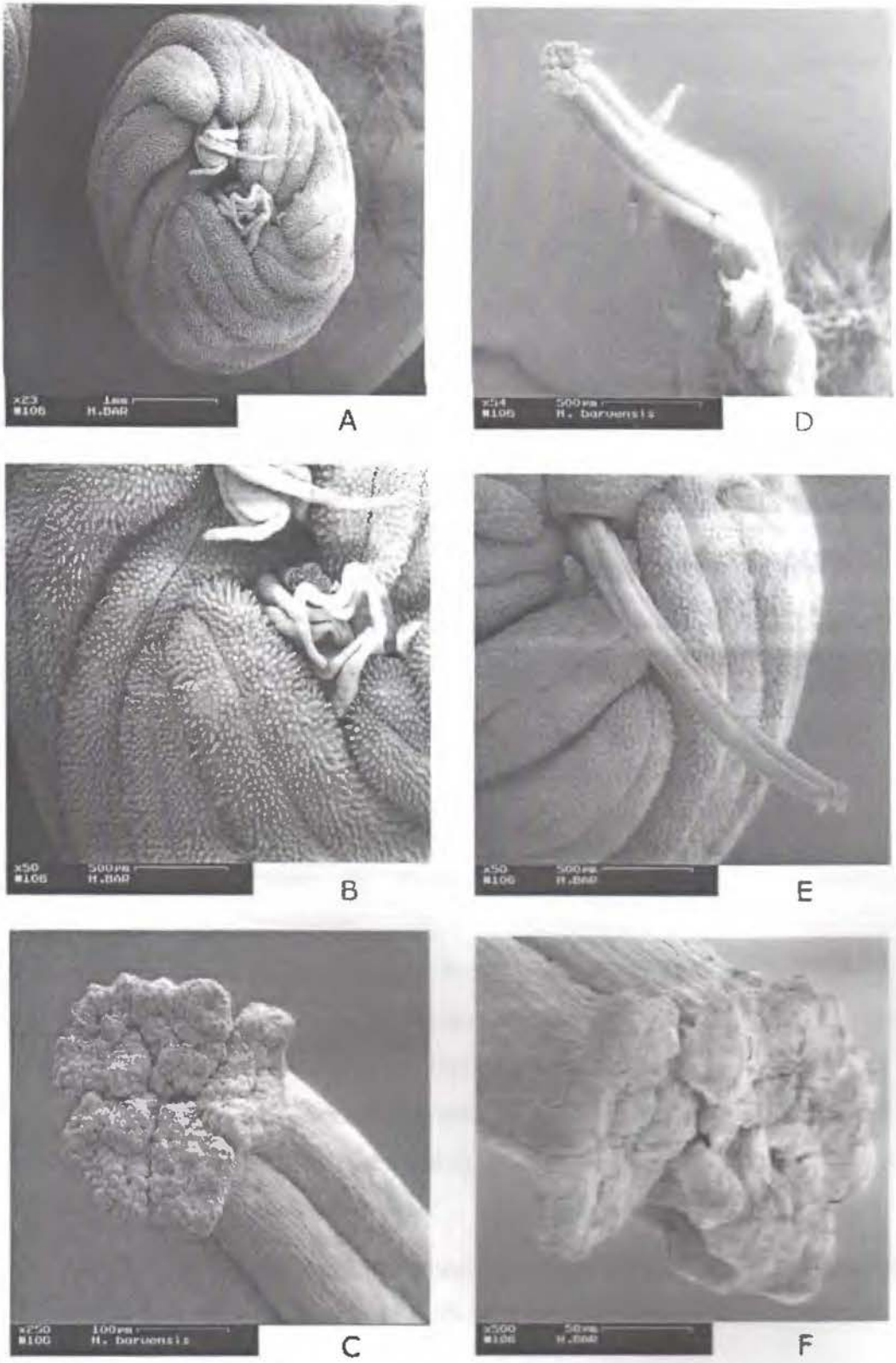

Figure 20. Anthers of *H. baruensis* A,B. Stigma of *H. baruensis* C,D,E,F. Bars = 1 mm *(A), 500 µm (B,D,E), 100 µm (C), 50 µm (F).*

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