New species, pollinator interactions and pharmaceutical potential of Himalayan orchids

Subedi, A.

Chapter 4

Pollination and protection against herbivory of Nepalese Coelogyninae (Orchidaceae)

Abishkar Subedi, Ram P. Chaudhary, Cees van Achterberg, Theodoor Heijerman, Frederic Lens, Tom J.M. Van Doore and Barbara Gravendeel

Although many species of the orchid genus *Coelogyne* are horticulturally popular, hardly anything is known about their pollination. Pollinators of three species were observed in the field in Nepal. This information is urgently needed since many orchid species in Nepal are endangered. Whether the exudates produced by extrafloral nectaries played a role in protection against herbivory was also investigated. Pollinators of *Coelogyne flaccida*, *C. nitida* and *Otochilus albus* were filmed, caught, and identified. Ant surveys and exclusion experiments were carried out. To investigate whether pollinators are needed for fruit set, plants were wrapped in mesh wire bags. Inflorescence stems were examined with microscopy. Fehlings reagent was used to detect sugars in extrafloral exudates. *Coelogyne flaccida* and *C. nitida* need pollinators to set fruit and are pollinated by wild bees identified as *Apis cerana*. *Otochilus albus* was found to be pollinated by *Bombus kashmirensis*. Extrafloral nectar was found to be exudated by nectary-modified stomata and contained high amounts of sugars. Different species of ants were observed collecting these exudates. A significant difference was found between *C. nitida* plants living in trees with ant nests vs. ant-free trees in damage inflicted by flower and leaf-eating beetles.
Floral syndromes include scented and colored trap flowers without reward to their pollinators. All orchids investigated exudate nectar by nectary-modified stomata. This nectar was found to flow from the phloem to the stomata through intercellular spaces in the outer parenchymatous layer of the inflorescence.

Key words: Ants, *Apis cerana, Bombus kashmirensis, Coelogyne,* herbivory, nectary-modified stomata, Nepal, *Otochilus, Pholidota,* pollination.

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Introduction

Orchid species belonging to subtribe Coelogyninae (Epidendroideae) are fairly common epiphytes throughout southeast Asia (Gravendeel et al., 2005). Their flowers are small to medium-sized and often sweetly scented. Although many species are horticulturally popular and commonly cultivated (Clayton, 2002), hardly any published pollinator records exist. Carr (1928) reported that *Coelogyne mayeriana* Rchb.f. is pollinated by wasps in Peninsular Malaysia. Female wasps of the genus *Vespula* (Vespidae) were found removing pollinia from flowers of *Coelogyne fimbriata* Lindl. in China (Cheng et al., 2009). To rectify some omissions about the ecology of these otherwise fairly well-known orchids, we present new pollination records for two species of *Coelogyne* and one species of *Otochilus* from Nepal. This is important information since Nepalese orchids face serious threats due to habitat fragmentation and illegal trade (Chaudhary et al., 2002; Subedi, 2005). Locally endangered species can only be conserved if their natural pollinators and their pollination biology are also known. An illustrative example is the previously endemic orchid species *Cymbidium whiteae* King and Pantl. that vanished from the type locality in India due to local extinction of its pollinator (Lucksom, 2007).

Fig. 4.1. Exudation of extrafloral nectar from the base of the pedicel of *Coelogyne flaccida* (photograph: A. Subedi).

Next to pollinators, plants also interact with other insects, most notably herbivores (Howe and Jander, 2008; Rodriguez-Saona and Frost, 2010). Plants use several strategies to defend themselves against damage caused by herbivores. One of these strategies involves attraction of natural enemies of herbivores for protection against herbivory (Oliveira et al., 1999; Rudgers, 2004; Palmer and Alison, 2007). Many Coelogyninae exudate extrafloral nectar both in their native habitat and under cultivation (Darwin, 1885; Jeffrey et al., 1970). Exudating occurs from the base of the pedicel (Fig. 4.1) and sepals. To investigate whether these exudates attract and sustain ants as a defence strategy against herbivory as recorded for orchid species belonging to *Coryanthes, Oncidium* and *Prosthechea* (Soysa, 1940; Jeffrey et al., 1970) but experimentally verified only for *Schomburgkia* (Rico-Gray and Thien, 1989), we (1) searched for specialized structures producing these exudates in various Nepalese Coelogyninae using light, scanning and transmission electronic microscopy, (2) tested for the presence of sugars in these

exudates using Fehlings' reagent, (3) conducted surveys of *Coelogyne nitida* in which the percentage of floral and leaf damage was compared between orchids growing with vs. without ants, and (4) carried out an ant-exclusion experiment.

Materials and Methods

Pollination

Observations of pollinators of *Coelogyne flaccida*, *C. nitida* and *Otochilus albus* were made at different localities in the vicinity of Pokhara, Central Nepal in May 2008, March-July 2009 and December 2010. Plants with freshly opened flowers were observed at each site from 0500 until 0600 AM and from 0800 AM until 0400 PM for several successive days. Visiting insects were only considered actual pollinators when we observed them removing, carrying and depositing pollinia. Insects were photographed and filmed during observation, caught with a net, killed in a jar containing ether and preserved on either 70% ethanol or silica gel. Specimens were identified by various specialists at the Natural History Museum of Tribhuvan University in Nepal, NCB Naturalis and the Animal Taxonomy section of the Biosystematics Department of Wageningen University in The Netherlands.

To investigate whether pollinators are needed for fruit set, a total of five plants of *C. flaccida* and three plants of *C. nitida* with 4-7 unopened floral buds were wrapped in fine-mesh wire bags in an experimental garden near Pokhara, Nepal, in April and June 2009. For comparison, the bagged plants were placed next to a similar amount of unbagged plants with a similar number of unopened flowers. All flowers were checked several times until the perianth withered and it was recorded whether the ovary was swelling.

Extrafloral nectaries

Inflorescences, pedicels and sepals of various Nepalese Coelogyninae were fixed in FAA (18:1:1 of ethanol (50%), acetic acid formalin, and water). Samples were dehydrated through an ethanol series and critical-point dried. Dried samples were mounted, sputter-coated with platinum, and observed with a JEOL JSM-5300 scanning electron microscope. Structures exudating nectar were digitally photographed.

Small pieces of fresh inflorescence stems sampled from a longitudinal gradient around the base of the pedicel were cut and preserved in FAA. After a few days, the stems were dehydrated in graded ethanol-xylol series and gradually infiltrated with LR White resin (hard grade) (London Resin) or paraffin. For the LR White samples, solutions of 1/3, 1/2, 1/1, 1/2, 1/3 resin/ethanol were used for at least 8 h each. The samples were subsequently stored overnight in pure resin. The next day, samples were placed in closed capsules filled with fresh resin and polymerized at 60 °C for 48 h. Stem sections of 5 µm were cut with a rotary microtome (Reichert Jung 2040 Autocut), heat fixed to glass slides, stained with toluidine blue, and embedded in DEPEX (BDH) before viewing under an Olympus microscope. For the paraffine samples, the stems were dehydrated in graded ethanol - xylol series after a few days of fixation and gradually infiltrated with paraplast (melting point 56-57 °C) using solutions of 1/2, 1/1, 1/2 paraplast/xylol in an incubator at 60 °C for at least 8 h each. The samples were subsequently stored in two steps of fresh paraplast, each during 24 h. The next day, samples were placed in peel away moulds filled

with paraplast and hardened at room temperature. Stem sections of 7 µm were cut with a rotary microtome (Leitz Minot 1212), fixed to glass slides with Haupt's adhesive, dried for 1 h at 35 °C, stained for 4 h with Etzold solution, rinsed in tap water and mineralized water for 1 min each, dried for 1 h at 35 °C, deparaffinized in 3 steps of 5 min each in xylol, and treated similarly as described for the LR White samples.

For transmission electronic microscope (TEM) analyses, small pieces of fresh inflorescence stems sampled from a longitudinal gradient around the base of the pedicel were cut and fixed for 3 h in a modified Karnovsky fixative (2.5% glutaraldehyde, 2% formaldehyde) in a 0.1M sodium cacodylate buffer (pH 7.2). After washing in 0.1 M sodium cacodylate buffer the material was post-fixed for 2 h in 1% osmium tetroxide and then washed in distilled water. After dehydration in a series of ethanol and propylene oxide, the pieces were infiltrated gradually with Epon by emerging them in five subsequent mixtures of propylene oxide and Epon with an increasing amount of Epon. Each step lasted 1 h. After overnight evaporation of the remaining propylene oxide, the material was embedded in fresh Epon and polymerised at 60 ºC for 48 h. Ultrathin sections were cut with an LKB ultratome, mounted on film-coated copper slot grids and post-stained with uranyl acetate and lead citrate (Reynolds, 1963). The sections were examined with a Jeol 1010 TEM.

Extrafloral nectar

In a previous study on the extrafloral exudates of *Coelogyne cristata* Lindl., fructose, glucose and sucrose were discovered (Jeffrey et al., 1970). To detect sugars in the extrafloral exudates of the Coelogyninae studied here, Fehlings' reagent (Fehling, 1849) was used. Amounts of 5 µl of exudate were collected in the field with micropipettes, stored in vials, and transported to the laboratory. After an equal amount of reagent was added, the vials were heated to 90 °C for 5 min and the resulting color changes were recorded to reveal sugar content.

Fig. 4.2. Ant species of the genus *Campanotus* foraging on *Coelogyne nitida* (photograph: A. Subedi).

Ant survey and exclusion experiments

Fieldwork was undertaken from May until August 2009 in the forests around the Panchase mountain, ca. 40 km west of Pokhara, central Nepal. One species of Coelogyninae in Nepal, *C. nitida*, was selected. This species is a common epiphytic orchid in Nepal growing on various host trees. The most common trees and shrubs are *Daphniphyllum himalaense* (Benth.) Muell., *Castanopsis indica* (Roxb.) A. DC., *Quercus semicarpifolia* Sm., *Q. lamellosa* Sm. and *Rhododendron arboretum* Sm. *Coelogyne nitida* occurs between 1200 and 2500 m elevation in temperate forest and secondary vegetation and flowers from March until July. Two ant species are common foragers on this orchid: *Crematogaster* sp. (4-5 mm in size) and *Camponotus* sp. (9-10 mm in size; Fig. 4.2). Both ant species usually occupy the same orchid individual, but foraging activities are separated in time: the first species is mainly active during the day and the second only during the night. Ants forage throughout the reproductive season

of *C. nitida* for the exudates produced at the base of the pedicels. The most dominant herbivores found on *C. nitida* are beetles present in relatively large numbers during floral development, boring holes in the floral buds (Fig. 4.3A); by doing this, the beetles decreased the number of flowers that successfully develop into fruits. The fruits themselves were not attacked by the beetles. The beetles were identified as belonging to *Adalia* sp. (Coccinellidae), *Aderorhinus nepalensis* (Curculionidae), *Athimus* sp. (Cantharidae), *Cerogria basalis* (Lagriidae), *Coccinella septempunctatum* (Coccinellidae), *Epilachna* sp. (Coccinellidae), *Holotrichia* sp. (Scarabaeidae), *Nassophasis cardoni* (Curculionidae) and *Popillia* sp. (Scarabaeidae; Table 4.1; Figs. 4.3B-D).

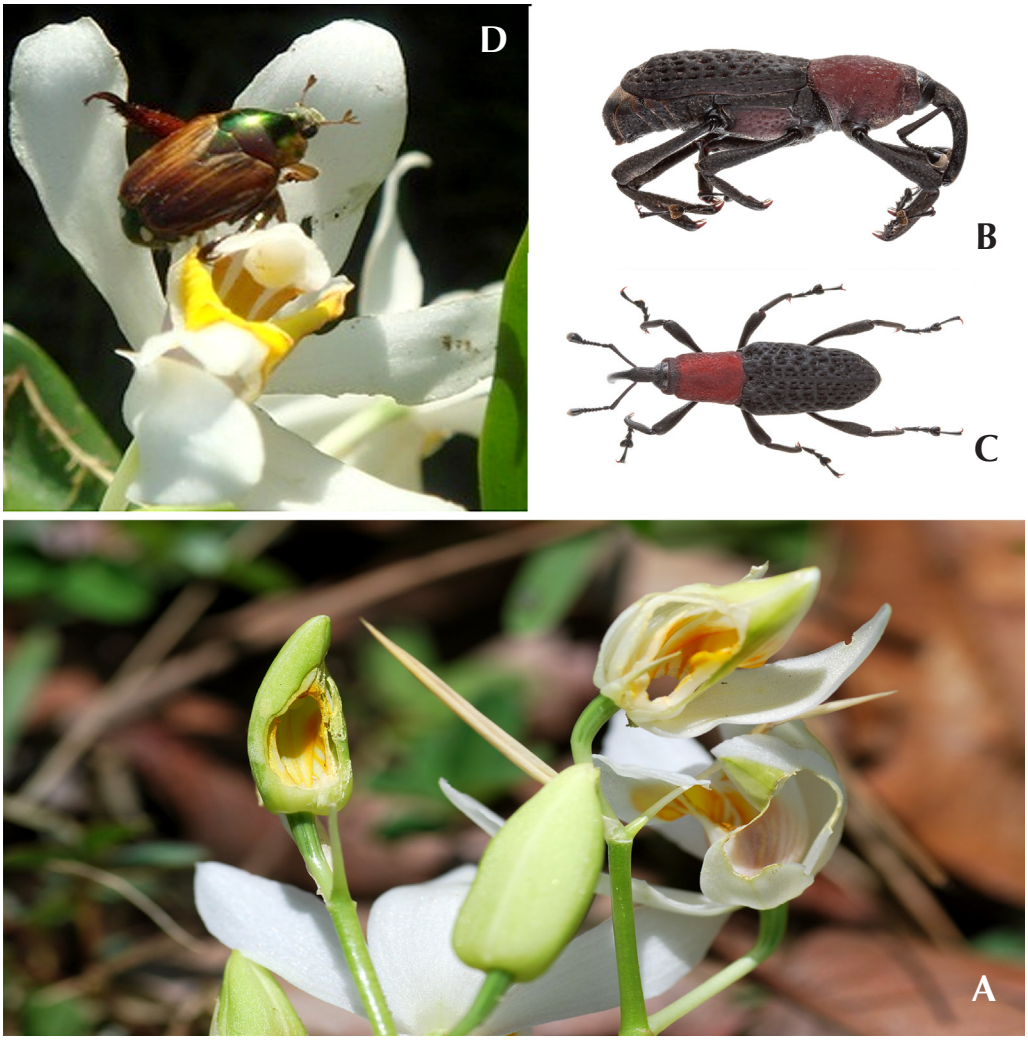


Fig. 4.3. Floral damage of *Coelogyne nitida* (A) inflicted by *Nassophasis cardoni* (B-C), and *Popillia* sp. (D) (photographs: R. Poot, T. Heijerman and A. Subedi).

Two different experiments were designed to determine the effect of ant presence on herbivory of *C. nitida*. The first consisted of field surveys in the Panchase mountains in temperate forest around Deurali (1824-2075 m elevation) and secondary vegetation around Kandé (1836-1888 m elevation) in central Nepal. All the observations were made between 0800 AM and 1530 PM. During this survey, the relative damage to flowers and leaves was recorded for a total of 60 plants, of which 30 were growing in trees with ant nests and 30 in trees without ant nests. The seasonal cycle at the survey sites is characterized by a spring season (February-May), summer or monsoon season (June-August), autumn season (September-October), and winter season (November-January). The average annual rainfall is over 3985 mm and temperature ranges between 24 and 35 ^{0}C (DHM/MoE, 2010).

Table 4.1. Overview of the different insect species observed visiting flowering Coelogyninae in Nepal. Vouchers of the orchids are deposited at TUCH under the collection numbers indicated between brackers. Vouchers of the ants are deposited at the Museum of Comparative Zoology of Harvard University and vouchers of all other insects at NCB Naturalis under the barcodes specified.

Orchid	Visitor	Barcode
Coelogyne cristata	*Hippofion celerio* L. (Noctuidae, Sphingidae)	L0285340
(Subedi 224)	*Romigio fragalis* Fabr. (Noctuidae)	L0285341
Coelogyne flaccida	*Camponotus* sp. (Formicidae)	L0285342
(Subedi 301)	*Kallima inachus* Boisduval (Lepidoptera, Nymphalidae)	L0285343
	Oecophylla smaragdina Fabricius (Formicidae)	L0285344
	Parapolybia nodosa van der Vecht (Hymenoptera, Vespidae)	L0285345
	Romigio fragalis Fabr. (Noctuidae)	L0285346
	Spindasis lohita Horsfield (Lepidoptera, Lycaenidae)	L0285347
Coelogyne nitida	*Adalia* sp. (Coleoptera, Coccinellidae)	L0285348
(Subedi 226)	*Aderorhinus nepalensis* Legalov (Coleoptera, Attelabidae, Rhynchitinae)	L0285349
	Athimus sp. (Coleoptera, Cantharidae)	L0285350
	Bombus kashmirensis Friese (Hymenoptera, Apoidea)	L0285351
	Camponotus sp. (Formicidae)	L0285352
	Cerogria basalis Hope (Coleoptera, Lagriidae)	L0285353
	Coccinella septempunctatum L. (Coleoptera, Coccinellidae)	L0285354
	Crematogaster sp. (Formicidae)	L0285355
	Epilachna sp. (Coleoptera, Coccinellidae)	L0285356
	Holotrichia sp. (Coleoptera, Scarabaeidae)	L0285357
	Nassophasis cardoni Desbrochers (Coleoptera, Curculionidae)	L0285358
	Pheidole sp. (Formicidae)	L0285359
	Popillia sp. (Coleoptera, Scarabaeidae)	L0285360
	Teetramorium sp. (Formicidae)	L0285361
Otochilus albus	*Monomorium* sp. (Formicidae)	L0285362
(Subedi 370)		

In the second experiment, a total of 10 sets of two plants of *C. nitida*, each with one inflorescence with 3-4 intact floral buds, were placed in trees with nests of ants actively collecting extrafloral nectar. Ant access to extrafloral nectar was blocked on one plant by applying duct tape to all extrafloral nectaries; the other plant was used as a control (nectaries not taped; Fig. 4.4A). Relative floral damage was recorded after 2, 4, and 6 weeks by scoring the number of damaged and undamaged flowers per inflorescence.

A control experiment was carried out to assess whether the duct tape itself warded off ants or pollinators. For this experiment, a total of 20 sets of plant pairs of *Otochilus albus*, each pair with inflorescences with the same number (7-14) of intact floral buds, were selected growing in trees with nests of ants actively collecting extrafloral nectar. Duct tape was applied on one plant on the inflorescence far below the extrafloral nectaries one h. before the start of the observations; the other plant was used as a control (inflorescence not taped) (Fig. 4.4B). The number of ants and pollinators visiting the flowering spikes was counted during a period of one h. The experiment was carried out on ten plant pairs per day, in two successive days. Pollinators were only observed on the second day of observations and we therefore only tested for that day whether the presence of duct tape had an effect on their visits.

Fig. 4.4. A. Taped vs untaped plants of *Coelogyne nitida* used in ant-exclusion experiment. B. Taped vs untaped plants of *Otochilus albus* used in control experiment (photographs: A. Subedi).

Proportions of damaged leaves and flowers, relative fruit set and differences in ant and pollinator visits were analyzed using generalized linear models for binomially distributed data with R version 2.9.1 (Crawley, 2007; Ihaka and Gentleman, 1996). Overdispersion in binomial proportions was checked by fitting quasibinomial distributions to the most

complex models. If these indicated substantial overdispersion (dispersion parameter > 2), we fitted quasibinomial models and used F tests for assessing the significance of effects. Otherwise, likelihood ratio tests were used on likelihoods obtained from fitting binomial distributions. We carried out a backward model selection procedure, where non-significant effects were sequentially removed from the models until only significant effects remained. Reported tail probabilities are from such minimum adequate models.

Results

Pollination

The results of our observations on pollinators of *C. flaccida*, *C. nitida* and *O. albus* in Nepal are summarized in Table 4.2.

Table 4.2. Overview of the different insect species observed pollinating flowering Coelogyninae in Nepal. Vouchers are deposited at NCB Naturalis under the barcodes specified.

Orchid	Pollinator	Barcode
Coelogyne flaccida	*Apis cerana* F. (Hymenoptera, Apoidea)	L0285337
Coelogyne nitida	*Apis cerana* F. (Hymenoptera, Apoidea)	L0285338
Otochilus albus	*Bombus kashmirensis* F. (Hymenopter, Apoidea)	L0285335

Wild bees identified as *Apis cerana* (Hymenoptera, Apoidea) were observed approaching open flowers of *C. flaccida* from the early morning to early afternoon (0800 – 1400) in March 2009 (Fig. 4.5).

Fig. 4.5. Pollination of *Coelogyne flaccida* by *Apis cerana* (photograph: A. Subedi).

Fig. 4.6. Pollination of *Coelogyne nitida* by *Apis cerana* (photograph: A. Subedi).

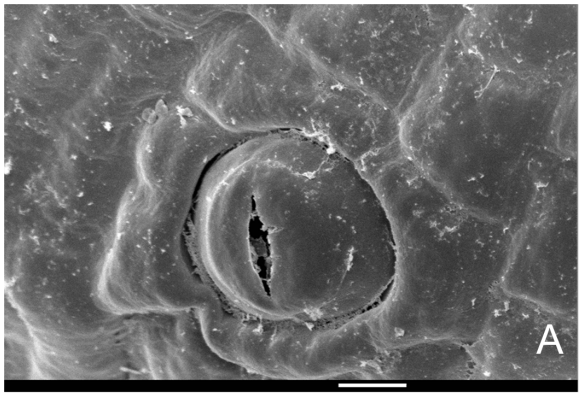
They landed directly on the apex of the lip and then crawled in the flower towards the base of the lip. Given the narrow passage formed by upright lateral lobes of the lip and overhanging column, bees could retreat only backwards from the flowers, during which their thorax touched the anther. Bees carrying no pollinia, always left flowers after the viscidium made contact with their thorax such that pollinia were removed. Bees already carrying pollinia struggled for 40-60 s to free themselves until the pollinia broke off after attachment of the viscidium to the receptive stigma. Wild bees identified as *Apis cerana* were also observed pollinating *C. nitida* (Fig. 4.6) in a similar way as described above in the early morning (0800 – 0900 AM) in June 2008 and June 2009.

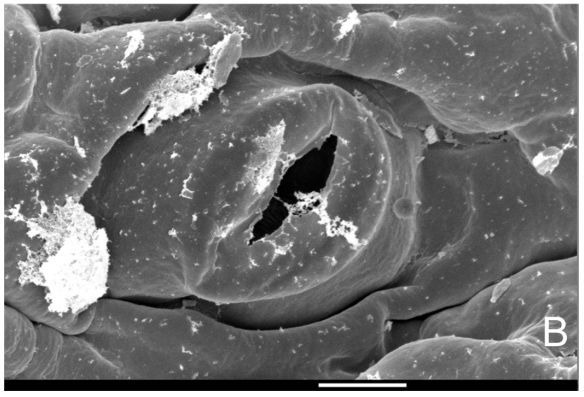
Wild bumblebees identified as *Bombus kashmirensis* were observed pollinating *O. albus* (Fig. 4.7A) in the early morning (1000 – 1100 AM) in December 2010. These bumblebees landed on the open flowers at the apex of the hanging inflorescences and then quickly moved upwards on the flowering spike within 5-7 seconds. Open flowers were aggressively probed for nectar. The pollinia of *O. albus* were observed sticking to the forehead of the bumblebees (Fig. 4.7B).

Fig. 4.7. A. Pollination of *Otochilus albus* by *Bombus kashmirensis*. B. Pollinia of *O. albus* sticking to head of *B. kashmirensis*. (photographs: A. Subedi).

Extrafloral nectar exudating structures

Nectary-modified stomata (Fahn, 1979; 1990; Nepi, 2007) were found on the inflorescences of *C. fimbriata*, *C. flaccida*, *C. nitida*, *C. prolifera*, *Chelonistele sulphurea*, *Otochilus albus* (Fig. 4.8A)*, Pholidota articulata, P. griffithii* and *P. pallida* (Fig. 4.7B) and sepal base of *C. flaccida* (Fig. 4.8B). Although we analysed samples from a longitudinal gradient around the position where the peduncle is attached to the inflorescence, we found only very few nectary-modified stomata just below the peduncle (Fig. 4.9A.) where we also observed exudation of nectar droplets in the field and greenhouse (Fig. 4.1). The nectary-modified





stomata were not found in the complete circumference of the inflorescence but on a limited spot only (Fig. 4.9B). The stomatal aperture of the nectary-modified stomata was observed to be enlarged and raised slightly above the epidermis (Fig. 4.8) in comparison with the leaf stomata (not shown). Well developed intercellular spaces were observed in the subepidermal tissue below the nectary-modified stomata (Fig. 4.9B). Basal cells of absorbing trichomes were also occasionally found in the subepidermal tissue of the inflorescences analysed (Fig. 4.9C-D).

Fig. 4.8. Scanning electron microscope images of (A) stomata on the inflorescence of *O. albus* and (B) sepal base of *C. flaccida*. Scale bar = 10 µm (photographs: B. Gravendeel).

Extrafloral nectar

Results of the Fehlings' reagent analyses of all extrafloral exudates collected are summarized in Table 4.3. In almost all analyses, the color of the reagent changed from blue to green to yellow to orange (to red) after heating indicating a high content of glucoses, fructoses and sucroses. Only the exudate produced by the trichomes on the flowering inflorescence of *C. nitida* remained blue, indicating low sugar content.

Table 4.3. Results of analyses with Fehlings' reagent on extrafloral exudates collected after heating to 90 °C for 5 min.

Orchid species	Exudate source	Final color stage
Coelogyne flaccida	peduncle base	red
Coelogyne nitida	flowering spike	blue
	peduncle base	orange
Otochilus albus	peduncle base	orange

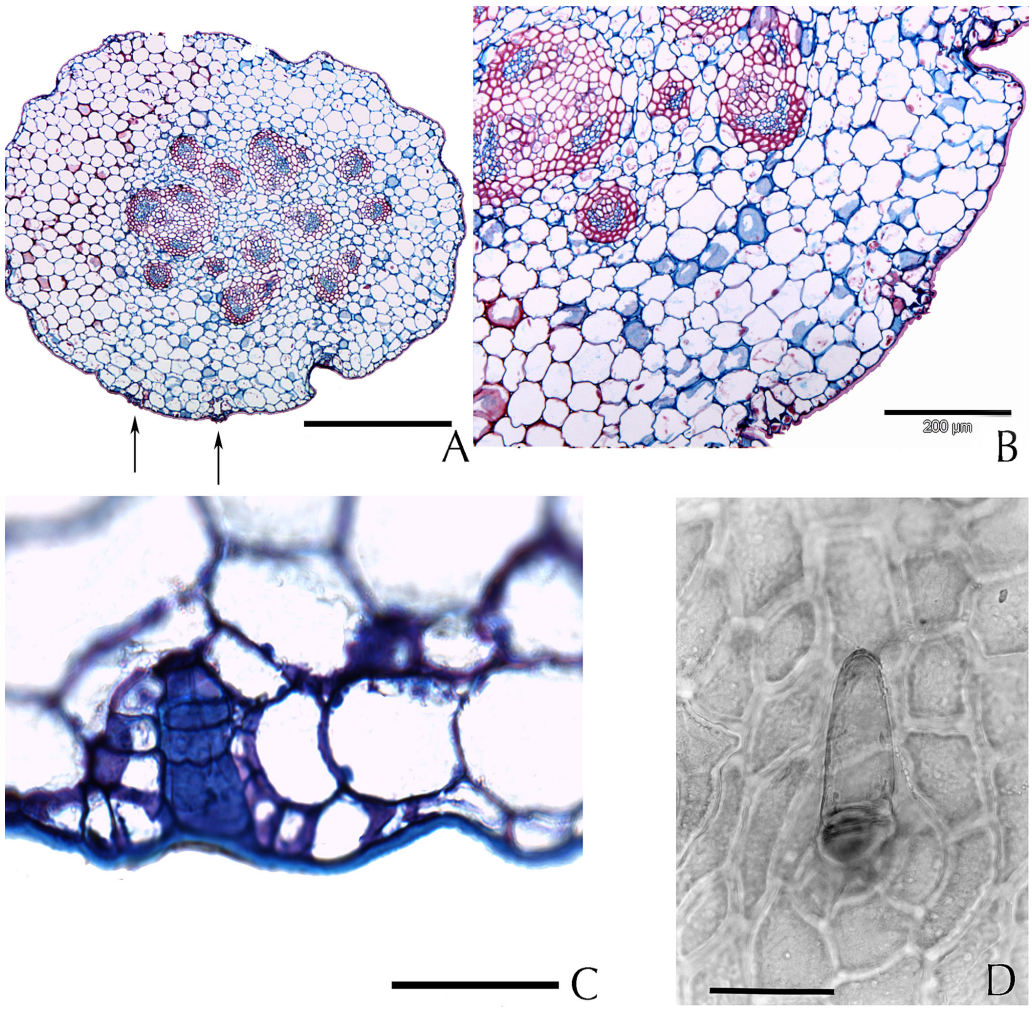


Fig. 4.9. Light microscope images of transverse sections of inflorescences. (A) Overview of the entire transverse section of the inflorescence of *Coelogyne fimbriata*. Arrows indicate nectary-modified stomata. The phloem and xylem in the vascular bundles in the central part of the inflorescence stem are colored blue and red, respectively. (B) Detail of nectary-modified stomata and intercellular spaces in outer parenchymatous layer. (C). Detail of basal cells of trichome on inflorescence of *Chelonistele sulphurea* (photographs: F. Lens). (D). Trichome on inflorescence of *Coelogyne carinata* (photograph: C.G. Koops). Scale bars: = 500 µm (A); 200 µm (B); 50 µm (C-D)

Ant surveys

Results of the ant surveys are summarized in Table 4.4. There was overdispersion in the data for the proportion of damaged flowers, but not for the proportion of damaged leaves. The percentage of damaged flowers and leaves differed significantly between plants growing in trees with vs. without ants. The presence of ants has significant positive effects on the proportions of undamaged flowers ($p = 0.0002$) and leaves ($p = 0.04$).

Table 4.4. Differences in relative floral and leaf damage (%) between *C. nitida* plants occurring on trees with and without ant nests in Deurali and Kandé surveys. Values are means ± s.d.

	Plants with ants (n=30)	Plants without ants (n=30)	Significance of difference
Relative floral damage (%)			
Deurali	19 ± 18	37 ± 20	
			p = 0.0002 *
Kandé	16 ± 17	62 ± 25	
Relative leaf damage (%)			
Deurali	28 ± 20	46 ± 27	
			p = 0.04
Kandé	26 ± 22	42 ± 21	

* F test

Ant-exclusion experiment

Results of the ant-exclusion experiment are summarized in Table 4.5. There was no indication of overdispersion in the data. A significant difference between the proportion of damaged flowers in taped and untaped plants (P = 0.0002) was found. Untaped plants had a smaller proportion of damaged flowers.

Table 4.5. Differences in relative floral damage (%) between control plants of *C. nitida* (nectaries not taped) and treated plants (nectaries taped). Values are means ± s.d.

	Control plants (nectaries not taped) (n=10)	Treatment plants (nectaries taped) (n=10)	Significance of difference
Relative floral damage (%)	41 ± 18	81 ± 20	p = 0.0002

Control experiment

We counted a significantly different number of ants (F = 18.870, p = 0.0001) and pollinators (chi-square = 0.184, df = 1, p = 0.668) visiting the inflorescences on different days. There was no significant difference in the number of ants and pollinators visiting plants with and without tape (Table 4.7). From this, and our observations that both ants and pollinators walked freely over the tape, we conclude that taping itself does not affect insect visits.

Pollinator exclusions

Relative fruit set differed significantly between treatment and control plants (p = 0.004) (Table 4.6). There was no indication of overdispersion in the data.

Table 4.6. Differences in relative fruit set between control plants of *C. nitida* (nectaries not taped) and treated plants (nectaries taped). Values are means ± s.d.

	Control plants (nectaries not taped) (n=10)	Treatment plants (nectaries taped) (n=10)	Significance of difference
Relative fruit set (%)	44.17 ± 20.81	13.33 ± 18.51	p = 0.004

Table 4.7. Differences in ant and pollinator visits in one hr between control plants of *Otochilus albus* (inflorescences not taped) and treated plants (inflorescences taped). Values are means ± s.d.

	Control plants (not taped) (n=20)	Treatment plants (taped) (n=20)	Significance of difference
Ant visits day 1	16.4 ± 9.5	14.3 ± 10.2	
			p = 0.539*
Ant visits day 2	6.7 ± 3.2	6.2 ± 2.9	
Pollinator visits day 1	-	-	
			p = 0.668
Pollinator visits day 2	2.3 ± 1.3	2.6 ± 1.1	

* F test

Discussion

Pollination

The existing hypothesis predicts that flowers of most *Coelogyne* species are pollinated by bees because of their zygomorphic shape, yellow/white colors, prominent landing platform, sweet scent, and presence of nectar guides (van der Pijl and Dodson, 1966). The few published records of pollinators of Coelogyninae and our own observations generally support the hypothesis that insects attracted to light shining through the base of the lip crawl into the flower, become trapped at the base, and then retreat backwards, when they either remove or deposit pollinia. The distance between the lip and gynostemium apex holding the anther and stigma is considerable in flowers of *Otochilus*. Only large sized pollinators such as *Bombus kashmirensis* seem capable of transferring pollinia successfully.

Flowers of *C. flaccida, C. nitida* and *O. albus* lack rewards for pollinators. It seems that only their extrafloral nectaries are rewarding and these were not visited by the pollinating species of bees observed. Floral odors seem to be the primary attractant for the pollinators observed; *C. flaccida*, *C. nitida* and *O. albus* have heavily scented flowers. Additional visual cues might be provided by the bright yellow color patches on the white lip of these species. Bioassay and pollinator choice experiments (e.g. Brodmann et al., 2009; Cheng et al., 2009) need to be carried out to test these hypotheses further.

Because none of the bagged flowers of *C. flaccida* and *C. nitida* set fruit in our

selfing experiment, we conclude that these species need pollinators for successful fruit set. To test whether these species are obligate outcrossers, additional controlled crosses need to be carried out involving both self vs. non-self pollen.

Extrafloral nectar exudation

Exudation of extrafloral nectar in the Coelogyninae studied here was observed to occur through nectary-modified stomata. Nectar exudation through stomata is a common manner of nectar release (Fahn, 1979; Nepi, 2007). Nectar-modified stomata were previously recorded in other epiphytic orchid species belonging to *Catasetum* and *Epidendrum* (Zimmermann, 1932). Zimmermann (1932) already postulated that the nectar flows from the vascular bundles through intercellular spaces to the nectary-modified stomata where it is exudated. We found further proof for this postulation by carrying out LM and TEM analyses. In the LM coupes, we observed many intercellular spaces in the outer parenchymatous layer below the nectary-modified stomata. In the TEM coupes (not shown), we did not observe any cells with relatively large nuclei, increased vacuole volume, or large numbers of ribosomes and/or mitochondria as usually observed in more specialised nectar secreting tissue (Nepi, 2007). The only cells with large nuclei observed in the parenchymatous layer were identified as basal cells of glandular trichomes (Fig. 4.9D), which absorp water in epiphytic orchids (Solereder and Meyer, 1930; Pridgeon, 1981; Rosinksi, 1992).

Exudation of extrafloral nectar of various Nepalese Coelogyninae in the field was highly variable according to our own observations. Often, no nectar seemed to be produced at all. Occasionally, though, especially during steep temperature drops right before heavy rainfall, large (2-3 mm diameter) nectar droplets could be observed emerging from the pedicel and/or sepal base (Fig. 4.1). Several factors could explain this variable exudation pattern. First of all, the volumes of extrafloral nectar exudated seem to be affected by relative humidity and other edaphic factors (Bentley, 1977). Second, extrafloral nectar exudation is inducible by herbivory attacks; in the absence of herbivory, costs of nectar exudation may be avoided, and full costs are incurred only during periods of herbivory (Wackers et al., 2001). Additional experiments should be carried out to find out whether temporal absence of extrafloral nectar exudation in Coelogyninae could be explained by these factors.

Protection against herbivory

Our results show that attraction of ants is a successful defence strategy against herbivory. Ant-orchid interactions are characterized by two benefits to orchids: nutrition and protection. In return, ants are provided with shelter and food (Peakall, 1994). The results of our ant survey and ant-exclusion experiment clearly show that in the presence of ants, *C. nitida* experiences significantly lower herbivory damage. A number of earlier experimental studies on plants with extrafloral nectaries also showed these species are protected by nectar-foraging ants against flower and leaf herbivores and seed predators (Schemske and Pautler, 1984; Buckley, 1982; Koptur, 1984; Beattie, 1985; Rico-Gray and Thien, 1989; Koptur, 1992; Davidson and McKey, 1993; Oliveira, 1997; Goheen and Palmer, 2010). Ant protection is not universal, however, and several other experimental studies demonstrated that ants visiting extrafloral nectaries may not always provide protection of their hosts in return for the food reward received (Rashbrook et al., 1992). Benefits to the plants also depend on the protective abilities of the ants and the fact that not all herbivores are equally

vulnerable to ant deterrence (Koptur, 1992; Freitas and Oliveira, 1996). The reason that we found significantly reduced herbivore damage on *C. nitida* in the presence of ants might be explained by a sufficiently efficient deterrence of *Nassophasis cardoni* (Fig. 4.3B), the most dominant flower- and leaf-eating beetle in the study area, by these ants. This beetle genus is a common orchid pest in Korea (Hong and Hong, 2000) and Taiwan (Morimoto, 1994), and our results show that ants could be used to develop a biological control system of these beetles in orchid nurseries in Asia. Ants are already successfully used in Neotropical nurseries to protect commercially interesting orchids from herbivory (Peakall, 1994).

The distribution of ant nests in the field is subject to high spatial heterogeneity due to variable dispersal patterns of founding queens, prey species, and the proximity of other ant colonies (Bentley, 1977; Rico-Gray and Thien, 1989; Oliveira, 1997). This might probably explain why we found orchids protected by ants in close proximity to orchids without any protection. Arboreal ants are also extremely sensitive to low air temperatures (Bentley, 1977), which might explain the relatively higher protection against herbivory found in the secondary vegetation of the Kandé survey where light intensities were higher due to lower canopy coverage as compared to the temperate forest of the Deurali survey. We also noticed *Coelogyne prolifera* growing in large ant nests on tree trunks in this area. Ants benefit from this orchid species here not only by receiving food but also by the structural support for the nest provided by the orchids' roots.

We found more than one ant species on a given orchid plant, which is different from observations in other studies (e.g. Rico-Gray and Thien, 1989). Both *Camponotus* sp. and *Oecophylla smaragdina* were found together on single plants of *C. flaccida*. *Oecophylla smaragdina* was repeatedly observed displaying aggressive behaviour such as lifting its abdomen and pointing it at intruders and also fierce use of its mandibles towards cockroaches, beetles and other ant species, indicating territoriality. Different species of *Camponotus, Cremagogaster, Pheidole,* and *Teetramorium* were found together on single plants of *C. nitida*. Species of the first genus were found to be predominantly active during the night whereas all other species were observed to be predominantly active during the day. Distinct periods of activity for different ant species have been reported before (Bentley, 1977). All ant species were seen actively collecting extrafloral nectar for adult nutrition and proteins to feed to their larvae (Nishida, 1958; Putman, 1963). In contrast to floral nectaries, extrafloral nectaries usually produce nectar both during the day and night (Bentley, 1977). This might explain why *Camponotus* sp. is able to shift its activity pattern to the night and thereby avoid encounters with other ant species during the day.

Conclusions

We conclude that *C. flaccida* and *C. nitida* are pollinated by bees in Nepal and *O. albus* by bumblebees. Floral syndromes employed include heavily scented, brightly colored trap flowers that offer no reward to their pollinators. *Coelogyne flaccida* and *C. nitida* need pollinators to set fruit. Light, scanning and transmission electronic microscopic analyses showed that extrafloral nectar flows from the phloem to nectary-modified stomata located close to floral buds, the regions of greatest vulnerability to flower-eating herbivores. Experimental evidence was gathered showing that *C. nitida* is protected from herbivore damage by ants collecting extrafloral nectar.