

**POLLINATION AND PROTECTION AGAINST HERBIVORY OF
NEPALESE COELOGYNINAE (ORCHIDACEAE)¹**

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- *Premise of the Study:* Although many species in 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 because many orchid species in Nepal are endangered. Whether the exudates produced by extrafloral nectaries played a role in protection against herbivory was also investigated.
- *Methods:* Pollinators of *C. flaccida*, *C. nitida*, and *Otochilus albus* were filmed, captured, 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. Fehling's reagent was used to detect sugars in extrafloral exudates.
- *Key Results:* *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 exuded by nectary-modified stomata and contained high amounts of sugars. Different species of ants were observed collecting these exudates. A significant difference was found in damage inflicted by flower and leaf-eating beetles between *C. nitida* plants living in trees with ant nests and those in ant-free trees.
- *Conclusions:* Floral syndromes include scented and colored trap flowers without reward to their pollinators. All orchids investigated exude extrafloral 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.

Orchid species in subtribe Coelogyninae (Epidendroideae) are fairly common epiphytes throughout Southeast Asia (Gravendeel et al., 2005). Their flowers are small (<1 cm in diameter) to medium-sized (≤5 cm in diameter) 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 spe-

cies of *Coelogyne* and one species of *Otochilus* from Nepal. This is important information because Nepalese orchids are seriously threatened by habitat fragmentation and illegal trade (Chaudhary et al., 2002; Subedi, 2005). Locally endangered species can be conserved only if their natural pollinators and their pollination biology are known. An illustrative example is the previously endemic orchid species *Cymbidium whiteae* King and Pantl., which vanished from the type locality in India because of local extinction of its pollinator (Lucksom, 2007).

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 exude extrafloral nectar both in their native habitat and under cultivation (Darwin, 1885; Jeffrey et al., 1970). Exuding occurs from the base of the pedicel (Fig. 1) and sepals. Investigations regarding the attraction of ants as a defense strategy against herbivory have been made for *Coryanthes*, *Oncidium*, and *Prosthechea* (Soysa, 1940; Jeffrey et al., 1970), but this mechanism has only been verified for *Schombugkia* (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

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Fig. 1. Exudate of extrafloral nectar from the base of the pedicel of *Coelogyne flaccida* (photograph: Abishkar Subedi).

for the presence of sugars in these exudates using Fehling's reagent; (3) conducted surveys of *Coelogyne nitida* in which the percentage of floral and leaf damage was compared between orchids growing with ants and those growing without them; and (4) carried out an ant-exclusion experiment.

MATERIALS AND METHODS

Pollination—Observations of pollinators of *C. 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 to 0600 hours and from 0800 to 0400 hours for several successive days. Visiting insects were considered actual pollinators only if we observed them removing, carrying, and depositing pollinia. Insects were photographed and filmed during observation, captured 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 four to seven 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 number of unbagged plants with a similar number of unopened flowers. All flowers were checked several times to see whether the perianth of the fully developed flowers started to wither. If so, it was then recorded whether the ovary started to swell and develop into a fruit capsule.

Extrafloral nectaries—Inflorescences, pedicels, and sepals of various Nepalese Coelogyninae (different species of *Chelonistele*, *Coelogyne*, *Otochilus*, and *Pholidota*) were fixed in FAA (18:1:1 of ethanol [50%], acetic acid, and formalin). 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 exuding 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, 2/1, 3/1 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 paraffin 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 molds 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 three 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 0.1-M 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 polymerized at 60°C for 48 h. Ultrathin sections were cut with an LKB ultratome, mounted on film-coated copper slot grids and poststained 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 *C. cristata* Lindl., fructose, glucose, and sucrose were discovered (Jeffrey et al., 1970). To detect sugars in the extrafloral exudates of the Coelogyninae studied here, Fehling's 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.

Ant survey and exclusion experiments—Field work was undertaken from May until August 2009 in the forests around Panchase Mountain, ~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 himalayense* Müll. Arg., *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). 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. 2A–C); by doing this, the beetles decreased the number of flowers that successfully developed 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 1; Figs. 2B–C).

Two different experiments were designed to determine the effect of ant presence on herbivory of *Coelogyne nitida*. The first consisted of field surveys in Panchase Mountain 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 and 1530. 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 >3985 mm and temperature ranges between 24°C and 35°C (DHMMoE, 2010).

In the second experiment, a total of 10 sets of two plants of *C. nitida*, each with one inflorescence with three to four 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. 3). Relative floral damage was recorded after 2, 4, and 6 wk by scoring the number of damaged and undamaged flowers per inflorescence.

A control experiment was conducted to assess whether the duct tape itself warded off ants or pollinators. For this experiment, a total of 20 sets of plant pairs of *O. 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 to one plant on the inflorescence far below the extrafloral nectaries 1 h before the start of the observations; the other plant was used as a control (inflorescence not taped). The numbers of

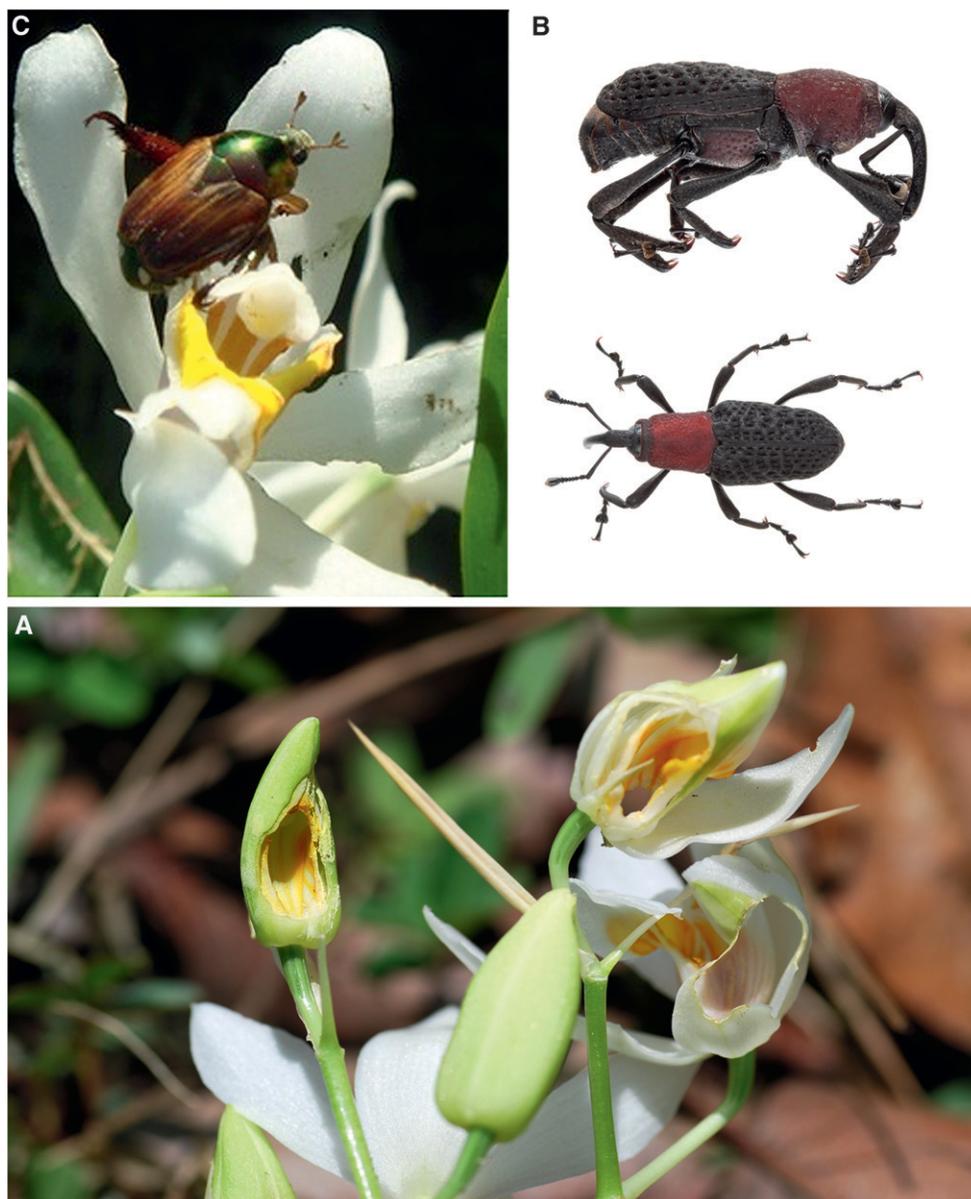


Fig. 2. Floral damage of *Coelogyne nitida* (A) inflicted by *Nassophasis cardoni* (B) and *Popillia* sp. (C) (photographs: R. Poot, T. Heijerman and A. Subedi).

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

ants and pollinators visiting the flowering inflorescences were counted during a period of 1 h. The experiment was carried out on 10 plant pairs per day, in two successive days. Pollinators were observed only on the second day of observations and, therefore, we only tested for that day whether the presence of duct tape had an effect on their visits.

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 (Ihaka and Gentleman, 1996; Crawley, 2007). 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, in which nonsignificant 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 2. 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 hours) in March 2009. They landed directly on the apex of the lip and then crawled in the flower toward the base of the lip. Given the narrow passage formed by upright lateral lobes of the lip and overhanging column, bees could retreat only backward 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 away from their attachment point at the viscidium and were deposited into the stigma. Wild bees identified as *A. cerana* were also observed pollinating *C. nitida* (Fig. 4) in a similar way as described

above in the early morning (0800–0900 hours) in June 2008 and June 2009.

Wild bumblebees identified as *Bombus kashmirensis* were observed pollinating *O. albus* (Fig. 5A) in the early morning (1000–1100 hours) in December 2010. These bumblebees landed on the open flowers at the apex of the hanging inflorescences and then quickly moved upward on the flowering spike within 5–7 s. Open flowers were aggressively probed for nectar. The pollinia of *O. albus* were observed sticking to the forehead of the bumblebees (Fig. 5B). The exact mechanism of pollinia deposition was not observed.

Extrafloral nectar-exuding 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*, *O. albus* (Fig. 6A), *Pholidota articulata*, *P. griffithii*, and *P. pallida* and sepal base of *C. flaccida* (Fig. 6B). Although we analyzed samples from a longitudinal gradient around the position where the peduncle is attached to the inflorescence, we found only very few nectary-modified stomata on the lower portion of the peduncle (Fig. 7A) where we also observed exudation of nectar droplets in the field and greenhouse (Fig. 1). The nectary-modified stomata were not found in the complete circumference of the inflorescence but just below the pedicel only (Fig. 7B). The stomatal aperture of the nectary-modified stomata was observed to be enlarged and raised slightly above the epidermis in comparison with the leaf stomata (not shown). Well-developed intercellular spaces were observed in the subepidermal tissue below the nectary-modified stomata (Fig. 7B). Basal cells of absorbing trichomes were also occasionally found in the subepidermal tissue of the inflorescences analyzed (Fig. 7C–D).

Extrafloral nectar—Results of the Fehling's reagent analyses of all extrafloral exudates collected are summarized in Table 3.



Fig. 3. Taped and untaped plants of *Coelogyne nitida* used in ant-exclusion experiment (photograph: A. Subedi).

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 that this exudate consisted of water only.

Ant surveys—Results of the ant surveys are summarized in Table 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 ants and those in trees 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 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
<i>Coelogyne flaccida</i>	<i>Apis cerana</i> F. (Hymenoptera, Apoidea)	L0285337
<i>Coelogyne nitida</i>	<i>Apis cerana</i> F. (Hymenoptera, Apoidea)	L0285338
<i>Otochilus albus</i>	<i>Bombus kashmirensis</i> F. (Hymenopter, Apoidea)	L0285335

Ant-exclusion experiment—Results of the ant-exclusion experiment are summarized in Table 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.

Pollinator exclusions—Relative fruit set differed significantly between treatment and control plants ($P = 0.004$; Table 5). Fruit set was significantly lower in bagged than in unbagged plants. There was no indication of overdispersion in the data.

Control experiment—We counted significantly different numbers of ants ($F = 18.870$, $P = 0.0001$) and pollinators ($\chi^2 = 0.184$, $df = 1$, $P = 0.668$) visiting the inflorescences on different days. There was no significant difference in the numbers of ants and pollinators visiting plants with and without tape (Table 6). 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.

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 (Carr, 1928; Cheng et al., 2009) 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 backward, when they either remove or deposit pollinia. The distance between the lip and gynostemium apex holding the anther and stigma is considerable (>1 cm) in flowers of *Otochilus*. Only large 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 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 and nonself pollen.

Extrafloral nectar exudate—Exuding of extrafloral nectar in the Coelogyninae studied here was observed to occur through nectary-modified stomata. Nectar exuding through stomata is a common manner of nectar release (Fahn, 1979; Nepi, 2007). Nectar-modified stomata were recorded in other epiphytic orchid species in *Catasetum* and *Epidendrum* by Zimmermann (1932), who postulated that the nectar flows from the vascular bundles through intercellular spaces to the nectary-modified



Fig. 4. Pollination of *Coelogyne nitida* by *Apis cerana* (photograph: A. Subedi).

stomata where it is exuded. We found further proof for this postulation by carrying out LM and TEM analyses. In the LM photographs, we observed many intercellular spaces in the outer

parenchymatous layer below the nectary-modified stomata. In the TEM photographs (not shown), we did not observe any cells with relatively large nuclei, increased vacuole volume, or large numbers of ribosomes, or mitochondria, as usually observed in more specialized 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. 7D), which absorb water in epiphytic orchids (Solereder and Meyer, 1930; Pridgeon, 1981; Rosinski, 1992).

According to our own observations, exudation of extrafloral nectar of various Nepalese Coelogyinae in the field was highly variable. 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 peduncle below the pedicel and/or sepal base (Fig. 1). Several factors could explain this variable exudation pattern. First of all, the volumes of extrafloral nectar exuded 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). We have no found no proof for either of these hypotheses. Additional experiments should be carried out to find out whether temporal absence of extrafloral nectar exudation in Coelogyinae could be explained by these factors.

Protection against herbivory—Our results show that attraction of ants is a successful defense 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. Earlier experimental studies on plants with extrafloral nectaries also showed that these species are protected by nectar-foraging ants against flower and leaf herbivores and seed predators (Buckley, 1982; Koptur, 1984; Schemske and Pautler, 1984; Beattie, 1985; Rico-Gray and Thien, 1989; Koptur, 1992;

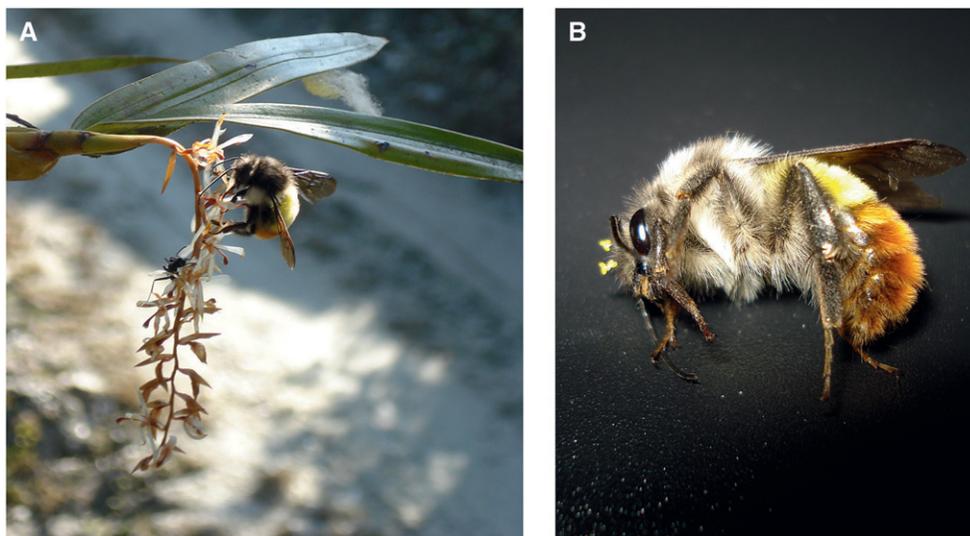


Fig. 5. (A) Pollination of *Otochilus albus* by *Bombus kashmirensis*. (B) Pollinia of *O. albus* sticking to head of *B. kashmirensis* (photographs: A. Subedi).

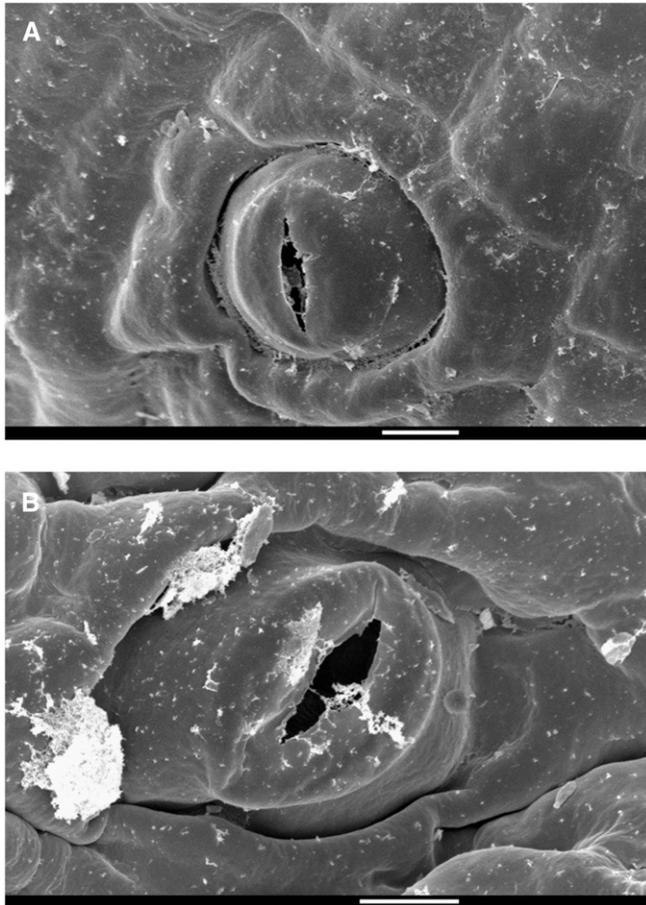


Fig. 6. Scanning electron microscope images of (A) stomata on the inflorescence of *Otochilus albus* and (B) sepal base of *Coelogyne flaccida*. Scale bar = 10 μ m (photographs: B. Gravendeel).

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 (Whalen and Mackay, 1988; 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). Our finding of significantly reduced herbivore damage on *C. nitida* in the presence of ants might be explained by a sufficiently efficient deterrence of *Nassophasis cardoni* (Fig. 2B), 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

TABLE 3. Results of analyses with Fehling’s reagent on extrafloral exudates collected after heating to 90°C for 5 min.

Orchid species	Exudate source	Final color stage
<i>Coelogyne flaccida</i>	Peduncle base	Red
<i>Coelogyne nitida</i>	Flowering raceme	Blue
	Peduncle base	Orange
<i>Otochilus albus</i>	Peduncle base	Orange

TABLE 4. Differences in relative floral and leaf damage (%) between *Coelogyne nitida* plants occurring on trees with and without ant nests in Deurali and Kandé surveys. Values are means \pm SD.

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

* *F*-test.

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). Several factors might explain why we found orchids protected by ants in close proximity to orchids without any protection. First of all, the distribution of ant nests in the field is subject to high spatial heterogeneity because of variable dispersal patterns of founding queens, prey species, and the proximity of other ant colonies (Bentley, 1977; Rico-Gray and Thien, 1989; Oliveira, 1997). 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, in which light intensities and corresponding air temperatures were higher because of lower canopy coverage than in the temperate forest of the Deurali survey. Third, we noticed *C. prolifera* growing around large ant nests on tree trunks in this area. Ants benefit from this orchid species 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 behavior such as lifting its abdomen and pointing it at intruders and also fierce use of its mandibles toward cockroaches, beetles, and other ant species, indicating territoriality. Different species of *Camponotus*, *Crematogaster*, *Pheidole*, and *Teetramorium* were found together on single plants of *Coelogyne 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. It is generally assumed that this extrafloral nectar is used for adult ant nutrition and for proteins to feed to ant larvae (Nishida, 1958; Putman, 1963). In contrast

TABLE 5. Differences in relative floral damage and fruit set (%) between control plants of *Coelogyne nitida* (nectaries not taped) and treated plants (nectaries taped). Values are means \pm SD.

	Control plants (nectaries not taped) (n = 10)	Treatment plants (nectaries taped) (n = 10)	Significance of difference
Relative floral damage (%)	41 \pm 18	81 \pm 20	<i>P</i> = 0.0002
Relative fruit set (%)	44.17 \pm 20.81	13.33 \pm 18.51	<i>P</i> = 0.004

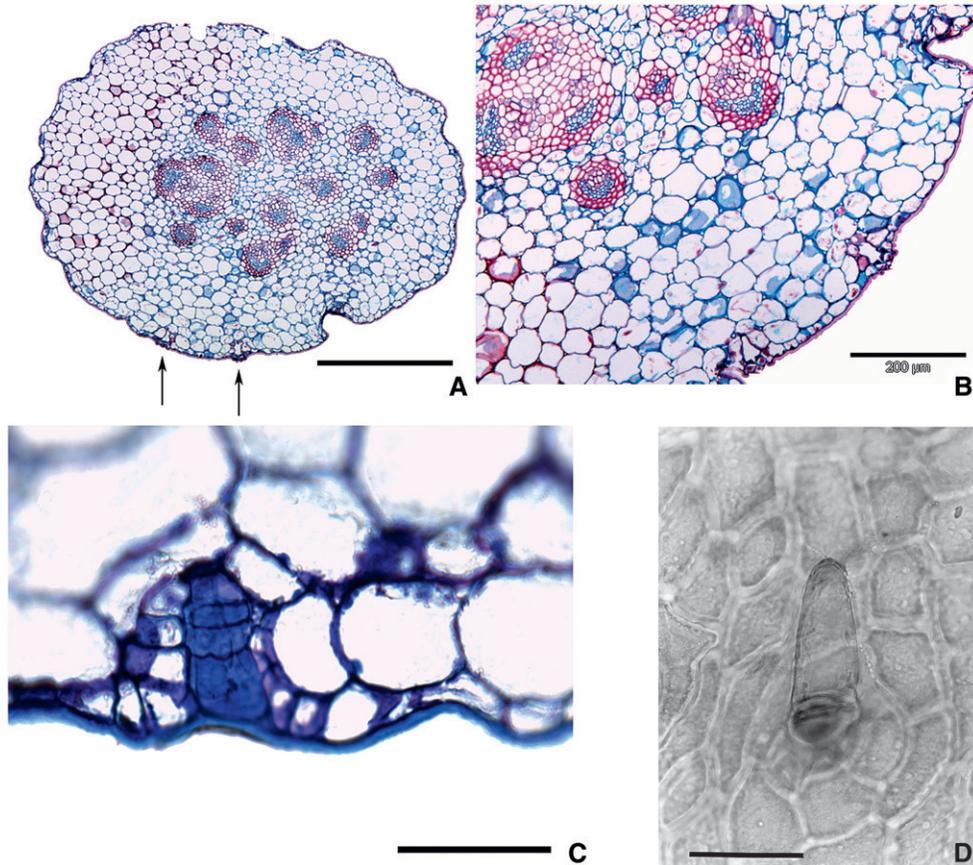


Fig. 7. Light microscope images of transverse sections of inflorescences. (A) Overview of the entire transverse section of the inflorescence of *Coelogyne fimbriata*. Arrows indicate nectary-modified stomata. The phloem and xylem in the vascular bundles in the central part of the inflorescence stem are 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).

to floral nectaries, extrafloral nectaries usually produce nectar during both 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 *Otochilus 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.

LITERATURE CITED

- BEATTIE, A. J. 1985. The evolutionary ecology of ant-plant mutualisms. Cambridge University Press, Cambridge, UK.
- BENTLEY, B. L. 1977. Extrafloral nectaries and protection by pugnacious bodyguards. *Annual Review of Ecology and Systematics* 8: 407–427.
- BRODMANN, J., R. TWELE, W. FRANCKE, Y. B. LUO, X. Q. SONG, AND M. AYASSE. 2009. Orchid mimics honey bee alarm pheromone in order to attract hornets for pollination. *Current Biology* 19: 1368–1372.
- BUCKLEY, R. C. 1982. Ant-plant interactions: a world review. In R. C. Buckley [ed.], *Ant-plant interactions in Australia*, 111–141. Junk Press, The Hague, The Netherlands.
- CARR, C. E. 1928. *Coelogyne mayeriana*. Rchb.f. *Journal of the Malaysian Branch of the Royal Asiatic Society* 6: 61.
- CHAUDHARY, R. P., A. SUBEDI, L. R. SHAKYA, D. KARKI, O. VETASS, AND V. N. P. GUPTA. 2002. Orchid diversity in Arun river and Marsyangdi river basins of Nepal: distribution and conservation priorities. In R. P. Chaudhary, B. Subedi, and O. Vetaas [eds.], *Vegetation and society: their interaction in the Himalayas*, 108–117. Tribhuvan University, Nepal and University of Bergen, Norway.

TABLE 6. 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 \pm SD.

	Control plants (not taped) (n = 20)	Treatment plants (taped) (n = 20)	Significance of difference
Ant visits day 1	16.4 \pm 9.5	14.3 \pm 10.2	$P = 0.539^*$
Ant visits day 2	6.7 \pm 3.2	6.2 \pm 2.9	
Pollinator visits day 1	—	—	$P = 0.668$
Pollinator visits day 2	2.3 \pm 1.3	2.6 \pm 1.1	

* F -test.

- CHENG, J., J. SHI, F. Z. SHANGGUAN, A. DAFNI, Z. H. DENG, AND Y. B. LUO. 2009. The pollination of a self-incompatible, food-mimic orchid, *Coelogyne fimbriata* (Orchidaceae) by female *Vespa* wasps. *Annals of Botany* 104: 565–571.
- CLAYTON, D. A. 2002. The genus *Coelogyne*: a synopsis. Natural History Publications (Borneo) in association with The Royal Botanic Gardens, Kew, UK.
- CRAWLEY, M. J. 2007. *The R book*. John Wiley, New York, New York, USA.
- DARWIN, C. 1885. On the various contrivances by which orchids are fertilised by insects. J. Murray, London, UK.
- DAVIDSON, D. W., AND D. MCKEY. 1993. The evolutionary ecology of symbiotic ant–plant relationships. *Journal of Hymenoptera Research* 2: 13–83.
- DHM/MoE. 2010. The Department of Hydrology and Meteorology (DHM), Ministry of Environment (MoE), Government of Nepal, Nepal.
- FAHN, A. 1979. *Secretory tissues in plants*. Academic Press, London, UK.
- FAHN, A. 1990. *Plant anatomy*, 4th ed. Pergamon Press, Oxford, UK.
- FEHLING, H. 1849. Die quantitative Bestimmung von Zucker und Stärkmehl mittelst Kupfervitriol. *Annalen der Chemie und Pharmacie* 72: 106–113.
- FREITAS, A. V. L., AND P. S. OLIVEIRA. 1996. Ants as selective agents on herbivore biology: effects on the behaviour of a non-myrmecophilous butterfly. *Journal of Animal Ecology* 65: 205–210.
- GOHEEN, J. R., AND T. M. PALMER. 2010. Defensive plant-ants stabilize megaherbivore-driven landscape change in an African savanna. *Current Biology* 20: 1768–1772.
- GRAVENDEEL, B., E. F. DE VOGEL, AND A. SCHUITEMAN. 2005. Coelogyinae. In A. Pridgeon, P. J. Cribb, M. W. Chase, and F. N. Rasmussen [eds.], *Genera Orchidacearum*, vol. 4, 29–88. Oxford University Press, Oxford, UK.
- HONG, K., AND K. J. HONG. 2000. Weevils on orchids in Korea (Coleoptera: Curculionoidea). *Korean Journal of Applied Entomology* 39: 131–134.
- HOWE, G. A., AND G. JANDER. 2008. Plant immunity to insect herbivores. *Annual Review of Plant Biology* 59: 41–66.
- IHAKA, R., AND R. GENTLEMAN. 1996. A language for data analysis and graphics. *Journal of Computational and Graphical Statistics* 5: 299–314.
- JEFFREY, D. C., J. ARDITTI, AND H. KOPOWITZ. 1970. Sugar content in floral and extrafloral exudates of orchids: pollination, myrmecology and chemotaxonomy implication. *New Phytologist* 69: 187–195.
- KOPTUR, S. 1984. Outcrossing and pollinator limitation of fruit set: breeding systems of neotropical *Inga* trees (Fabaceae: Mimosoideae). *Evolution* 38: 1130–1143.
- KOPTUR, S. 1992. Extrafloral nectary-mediated interactions between insects and plants. In E. Bernays [ed.], *Insect–plant interactions*, vol. IV, 81–129. CRC Press, Boca Raton, Florida, USA.
- LUCKSOM, S. Z. 2007. *The orchids of Sikkim and northeast Himalaya*. S.Z. Luckom, Sikkim, India.
- MORIMOTO, K. 1994. Notes on orchid weevils in Japan, with description of a new species (Coleoptera: Curculionoidea). *Transactions of the Shikoku Entomological Society* 20: 233–241.
- NEPI, M. 2007. Nectary structure and ultrastructure. In S. W. Nicolson, M. Nepi, and E. Pacini [eds.], *Nectaries and Nectar*, 129–166. Springer, Dordrecht, The Netherlands.
- NISHIDA, T. 1958. Extrafloral glandular secretion as a food source for certain insects. *Proceedings of the Hawaii Entomological Society* 16: 379–386.
- OLIVEIRA, P. S. 1997. The ecological function of extrafloral nectaries: herbivore deterrence by visiting ants and reproductive output in *Caryocarpus brasiliense* (Caryocaraceae). *Functional Ecology* 11: 323–330.
- OLIVEIRA, P. S., V. RICO-GRAY, C. DÍAZ-CASTELAZO, AND C. CASTILLO-GUEVARA. 1999. Interaction between ants, extrafloral nectaries, and insect herbivores in Neotropical coastal sand dunes: herbivore deterrence by visiting ants increases fruit set in *Opuntia stricta* (Cactaceae). *Functional Ecology* 13: 623–631.
- PALMER, T. M., AND K. B. ALISON. 2007. Mutualism as reciprocal exploitation: African plant-ants defend foliar but not reproductive structures. *Ecology* 88: 3004–3011.
- PEAKALL, R. 1994. Interactions between orchids and ants. In J. Arditti [ed.], *Orchid biology: reviews and perspectives VI*, 103–134. Kluwer, Dordrecht, The Netherlands.
- PRIDGEON, A. M. 1981. Absorbing trichomes in the Pleurothallidinae (Orchidaceae). *American Journal of Botany* 68: 64–71.
- PUTMAN, W. L. 1963. Nectar of peach leaf glands as insect food. *Canadian Entomologist* 95: 108–109.
- RASHBROOK, V. K., S. G. COMPTON, AND J. H. LAWTON. 1992. Ant–herbivore interactions—reasons for the absence of benefits to a fern with foliar nectaries. *Ecology* 73: 2167–2174.
- REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* 17: 208–212.
- RICO-GRAY, V., AND L. B. THIEN. 1989. Effect of different ant species on reproductive fitness of *Schomburgkia tibicinis* (Orchidaceae). *Oecologia* 81: 487–489.
- RODRIGUEZ-SAONA, C. R., AND C. J. FROST. 2010. New evidence for a multi-functional role of herbivore-induced plant volatiles in defense against herbivores. *Plant Signaling & Behavior* 5: 58–60.
- ROSINSKI, M. 1992. Untersuchungen zur funktionellen Anatomie der Laubblattstrukturen epiphytischer Coelogyinae und Eriinae (Orchidaceae). Ph.D. thesis, University of Saarland, Saarbrücken, Germany.
- RUDGERS, J. A. 2004. Enemies of herbivores can shape plant traits: selection in a facultative ant–plant mutualism. *Ecology* 85: 192–205.
- SCHEMSKE, D. W., AND L. P. PAUTLER. 1984. The effect of pollen composition on fitness components in a Neotropical herb. *Oecologia* 62: 31–36.
- SOLEREDER, H., AND F. J. MEYER. 1930. Orchidaceae. In *Systematische Anatomie der Monokotyledonen VI. Scitamineae—Microspermae*, 92–242. Borntraeger Verlag, Berlin, Germany.
- SOYSA, S. W. 1940. Orchids and ants. *Orchidologia zeylanica* 7: 88.
- SUBEDI, 2005. Orchids and sustainable livelihoods: initiatives in Nepal to manage globally threatened biodiversity. In A. Raynal-Roques, A. Roguenant, and D. Prat [eds.], *Proceedings of the 18th World Orchid Conference*, 470–474. Naturalia, Turriers, France.
- VAN DER PIJL, L., AND C. H. DODSON. 1966. *Orchid flowers: their pollination and evolution*. University of Miami Press, Coral Gables, Florida, USA.
- WACKERS, F. L., D. ZUBER, R. WUNDERLIN, AND F. KELLER. 2001. The effect of herbivory on temporal and spatial dynamics of foliar nectar production in cotton and castor. *Annals of Botany* 87: 365–370.
- WHALEN, M. A., AND D. A. MACKAY. 1988. Patterns of ant and herbivore activity on five understory euphorbiaceous saplings in sub-montane Papua New Guinea. *Biotropica* 20: 294–300.
- ZIMMERMANN, M. 1932. Über die extrafloralen Nektarien der Angiospermen. *Beihfte zum Botanischen Centralblatt* 49: 99–196.