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Mini-review

Ergoline alkaloids in convolvulaceous host plants originate from epibiotic clavicipitaceous fungi of the genus *Periglandula*

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ABSTRACT

Ergoline (i.e., ergot) alkaloids are a group of physiologically active natural products occurring in the taxonomically unrelated fungal and plant taxa, Clavicipitaceae and Convolvulaceae, respectively. The disjointed occurrence of ergoline alkaloids seems to contradict the frequent observation that identical or at least structurally related natural products occur in organisms with a common evolutionary history. This problem has now been solved by the finding that not only graminaceous but also some dicotyledonous plants belonging to the family Convolvulaceae, such as *Ipomoea asarifolia* and *Turbina corymbosa*, form close associations with ergoline alkaloid producing fungi, *Periglandula ipomoeae* and *Periglandula turbinae*. These species belong to the newly established genus *Periglandula* within the Clavicipitaceae. The fungus–plant associations are likely to be mutualistic symbioses.

Introduction

Chemotaxonomy is a field at the interface between natural product chemistry and taxonomy. The basis of chemotaxonomy is the frequent observation that identical or at least structurally related natural products reflect the common evolutionary history of different but related organisms (Hegnauer 1962–1992; Eich 2008). The validity of the systematic classification of organisms on the basis of natural products has sometimes been challenged because in some cases natural products occur in evolutionarily unrelated organisms. Among these are maytansinoids, which are found in completely unrelated organisms such as bacteria and higher plants of the family Celastraceae (Pullen *et al.* 2003; Cassady *et al.* 2004). The ergoline alkaloids represent another case. They are products of fungi belonging to the family Clavicipitaceae, but are also present in higher plants such as *Ipomoea asarifolia* and *Turbina corymbosa* (Schultes & Hofmann 1992; Groeger & Floss 1998; Leistner & Steiner 2009), members of the family Convolvulaceae. For the disjointed occurrence of ergoline alkaloids, a horizontal gene transfer or a repeated origin of the ergoline alkaloid biosynthetic pathway was suggested but remained unproven (Mothes *et al.* 1985, Groeger & Floss 1998).

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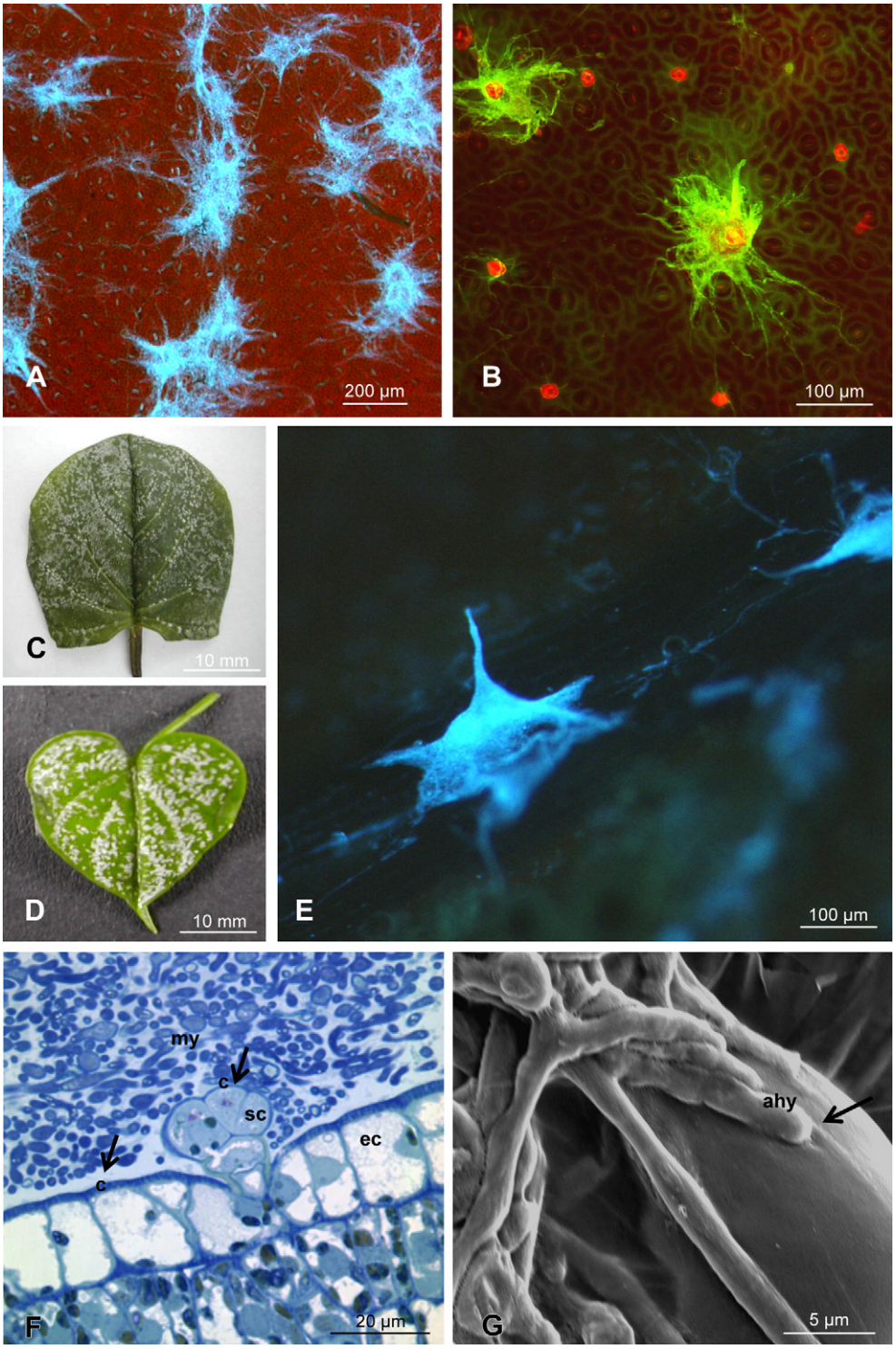

Fig 1 – Epibiotic clavicipitaceous fungi on the adaxial leaf surface of plants belonging to the family Convolvulaceae: (A) Colonies of fungal mycelia (blue) on the adaxial leaf surface of *I. asarifolia*; (B) Fungal colonies (green) that are closely associated with oil containing peltate glandular trichomes (red); (C, D) Manually opened leaf buds of *I. asarifolia* (C) and *T. corymbosa* (D) showing dense white mycelium; (E) Synnema-like structure (blue) formed by a well developed colony of *P. ipomoeae* on *I. asarifolia*; (F) Epibiotic development of mycelium (my) concentrated over a peltate glandular trichome with secretory cells (sc) covered by the cuticle (c) which is thinner (arrow) than over epidermal cells (ec) (arrow); (G) Appressoria-like hyphal structures (ahy) (arrow) adhere to the cuticle of a glandular trichome. The oil cavity of glandular trichomes stained red with Nile red (Fig 1B). Mycelium stained either blue with diethanol (Fig 1A, E), or toluidine blue (Fig 1F) or green with Oregon green labelled wheat germ agglutinine (Fig 1B).

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We show in the present article that the occurrence of ergoline alkaloids in Convolvulaceae is neither due to a horizontal transfer of genes from fungi to higher plants nor to a repeated origin of a rather complicated biosynthetic pathway, but rather that convolvulaceous plants may be colonized by clavicipitaceous epibiotic fungi responsible for the presence of ergoline alkaloids in these plants. These are the first ergoline alkaloid producing Clavicipitaceae described that are apparently mutualistic symbionts of dicotyledonous plants (Leistner & Steiner 2009; Steiner *et al.*, in press). The colonisation of Convolvulaceae by clavicipitaceous fungi indicates the ecological role of ergoline alkaloids in a fungus-plant symbiosis. Several studies have shown that ergoline alkaloids can confer environmental tolerance, fitness, drought resistance, insecticidal activity and feeding deterrence to their graminaceous hosts and very likely also to their dicotyledonous host plants. Books (Schultes & Hofmann 1992; White *et al.* 2003; Schulz *et al.* 2006; Varma *et al.* 2008) and reviews (Groeger & Floss 1998; Tudzynski & Scheffer 2004; Bacon & Lyons 2005; Bischoff & White 2005; Schardl & Leuchtmann 2005; Schardl *et al.* 2006) testify to the roles of ergoline alkaloids in ecological, agronomical, genetic, biochemical, pharmacological and anthropological research.

Host plants and microscopic appearance of fungal colonies

The identification of plant associated fungi responsible for the presence of ergoline alkaloids in some species of the family Convolvulaceae was based on several lines of evidence (Leistner & Steiner 2009). One of these involves the colonies of an epibiotic fungal mycelium that was detectable by visual inspection of leaves of *T. corymbosa*. When opened manually the surfaces of leaf buds even show a white dense fungal layer (Fig 1D). While a fungus on expanded leaves of *I. asarifolia* is not readily observed by the naked eye, the opened leaf buds of this plant (Fig 1C) also show a dense white mycelium similar to that on *T. corymbosa* (Ahimsa-Müller *et al.* 2007). Microscopic investigation of the leaf surface of *I. asarifolia* and *T. corymbosa* revealed the presence of fungal colonies that are closely associated with secretory oil glands on the adaxial leaf surface (Kucht *et al.* 2004; Steiner *et al.* 2006; Leistner & Steiner 2009). While oil glands were stained with Nile red, the associated fungal structures were highlighted using diethanol and Oregon green labelled wheat germ agglutinine that reacts with chitin as a component of the fungal cell wall (Fig 1B; Kucht *et al.* 2004). Fungal hyphae usually extend from a mycelium to oil glands located in their vicinity (Fig 1B). In some cases, hyphae curl around the basal and stalk cells of secretory glands (Kucht *et al.* 2004). Appressoria-like hyphal structures can be found on oil glands of both plant species (Fig 1G). Moreover, synnema-like structures without spores (Fig 1E) are formed by the fungus on *I. asarifolia* as well as on *T. corymbosa*. Mycelial colonies of *T. corymbosa* very characteristically are lined up along leaf veins (Leistner & Steiner 2009), whereas colonies on *I. asarifolia* are located more randomly on the leaf surface. The mycelial colonies occur only on the adaxial, but not on the abaxial leaf surface and are absent from cotyledons (Leistner & Steiner 2009).

We proposed the name *Periglandula* for the new fungal genus with reference to its association with oil glands (Fig 1B, F, Fig 2) (Steiner *et al.*, in press). The genus comprises the fungal species *Periglandula ipomoeae* and *Periglandula turbinae*. *P. ipomoeae* is present on leaves of both the white- and the red blooming *I. asarifolia* plants. The fungus on the former plant was previously annotated IasaF13 whereas the one on the latter plant was annotated IasaredF01 (Steiner *et al.* 2006; Ahimsa-Müller *et al.* 2007). The fungi on both hosts are indistinguishable by morphological and molecular biological criteria (Steiner *et al.*, in press). The systematic name *P. turbinae* (previously annotated TcorF01, Ahimsa-Müller *et al.* 2007) is proposed for the fungus present on *T. corymbosa* (Steiner *et al.*, in press).

The genus *Ipomoea* comprises the largest number of species within the Convolvulaceae with at least 500 species. Many of these species are concentrated in the Americas. *I. asarifolia* occurs in Bolivia, Brazil, Colombia, Ecuador, Jamaica, Panama, Peru and Venezuela (Austin & Huáman 1996). Eich (2008) lists 41 *Ipomoea* species devoid of ergoline alkaloids, a series of *Ipomoea* plants in which the occurrence of ergoline alkaloids is doubtful, and 23 *Ipomoea* species which are unambigously ergoline-positive. It is to be expected that this latter group of plants is also colonized by clavicipitaceous fungi. The genera *Argyreia*, *Strictocardia* and *Turbina* contain also ergoline alkaloids. The pantropical genus *Turbina* comprises 15 species but only two plant species, *T. corymbosa* and *Turbina abutiloides* (Eich 2008) were analyzed and turned out to be ergoline alkaloid positive.

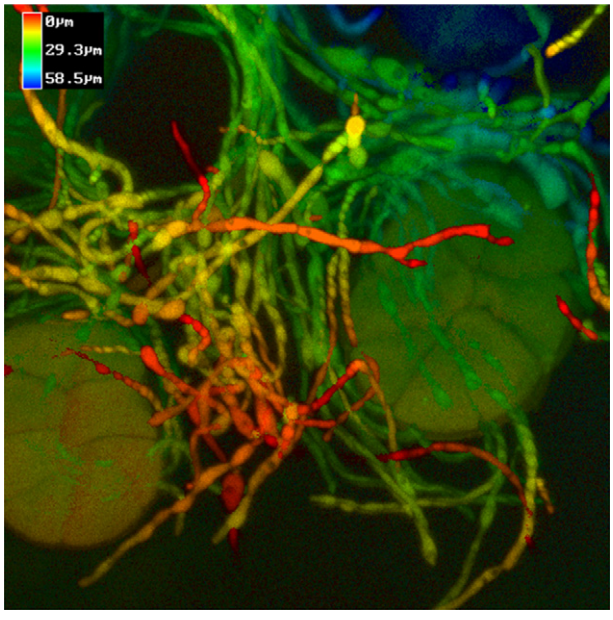

Fig 2 – Mycelium of the ergoline alkaloid producing epibiotic clavicipitaceous fungus *Periglandula ipomoeae* in close association with peltate glandular trichomes on the adaxial leaf surface of the plant *Ipomoea asarifolia* (Convolvulaceae) (Topographical image obtained by CLMS, three-dimensional reconstruction of 40 optical sections after staining with acid-fuchsin, different depths of the object between 0 and 58.5 µm are rendered as different colours).

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Molecular characterization of epibiotic strains

Experiments using the single strand conformation polymorphism (SSCP) technique revealed that, of the fungi associated with *I. asarifolia*, (Steiner *et al.* 2006) and *T. corymbosa* (Ahimsa-Müller 2007), the epibiotic fungi were the most abundant ones. These fungi are also present in the host seeds. Most importantly, only *P. ipomoeae* and *P. turbinae* grew from surface sterilized seeds of *I. asarifolia* and *T. corymbosa* plants, respectively, that had germinated under axenic conditions (Steiner *et al.* 2006; Hellwig 2007). These plants contained ergoline alkaloids. Three months after germination, the alkaloid fraction in the *T. corymbosa* plant (5.7 $\mu g\,g^{-1}$ fresh weight) consisted of chanoclavine, elymoclavine, ergonovine and lysergic acid amide, and isolysergic acid amide. The *I. asarifolia* plant contained 1.4 μg ergoline alkaloids g^{-1} fresh weight, consisting of chanoclavine, ergonovine, isolysergic acid amide and additional unidentified alkaloids staining positive with Van Urks reagent (Ahimsa-Müller *et al.* 2007; Hellwig 2007). These results made it seem plausible that the epibiotic fungi *P. ipomoeae* and *P. turbinae* are responsible for ergoline alkaloid biosynthesis on both plant species.

To further test this hypothesis, four groups of plants were treated with a regime of four different systemic fungicides (Benomyl®, Switch®, Pronto Plus®, Folicur®) for 18 weeks. The four fungicides eliminate fungi by different reaction mechanisms. Two of these fungicides from the class of azoles (Pronto Plus®, Folicur®) were most effective in the removal of the fungi associated with secretory glands on the leaf surfaces of *I. asarifolia* and *T. corymbosa* (Kucht *et al.* 2004; Hellwig 2007). The fungicide-treated plants showed no hyphae or mycelium mats around the glands, whereas on control plants fungal structures were associated with 93 % of all secretory glands. The absence of the fungus was also confirmed by SSCP (Steiner *et al.* 2006). Plants were analyzed before and after fungicide treatment for the presence of alkaloids using capillary electrophoresis and thin layer chromatography. Confirming our hypothesis, we found that fungicide-treated plants as opposed to the control group had lost all ergoline alkaloids (Kucht *et al.* 2004). We conclude that

(i) the epibiotic fungus and ergoline alkaloids either co-occur or are both absent from the intact plant (Ahimsa-Müller *et al.* 2007), suggesting that the fungus is the alkaloid producing organism (Kucht *et al.* 2004; Steiner *et al.* 2006).
(ii) during fungicide treatment, biosynthesis of ergoline alkaloids ceases because the fungi are eliminated. Since natural products may be subject to a turnover (Amrhein & Diederich 1980, Barz & Koester 1981) alkaloids accumulated before fungicide treatment are likely to be degraded. We hypothesize that in untreated plants ergoline alkaloid biosynthesis and degradation are in balance as long as a viable fungus is present on the plant.

In addition to the alkaloids the volatile oils of the secretory glands of *I. asarifolia* were also analyzed in these experiments and was mainly found to consist of five different sesquiterpenes: α-copaene, (E)-β-caryophyllene, α-humulene, germacrene, and δ-cadinene (Kucht *et al.* 2004). As opposed to ergoline alkaloids, the sesquiterpenes are biosynthetic products of the plants (Markert *et al.* 2008). These five sesquiterpenes were present in the *I. asarifolia* plant before and after fungicide treatment. We therefore conclude that fungicide treatment does not affect secondary metabolism in general, but only and specifically fungal metabolism including the biosynthesis of ergoline alkaloids (Kucht *et al.* 2004).

Sequencing of the 18S rDNA revealed that of the 12 endophytic and one epibiotic fungus isolated from *I. asarifolia* only the epibiotic colony forming *P. ipomoeae* was placed within the order Hypocreales, an order that includes the family Clavicipitaceae. Sequencing of the internal transcribed spacer (ITS) indicated that the epibiotic fungi *P. ipomoeae* and *P. turbinae* belong to the family Clavicipitaceae (Steiner *et al.* 2006; Ahimsa-Müller *et al.* 2007). This was again a strong indication that these particular fungi are indeed responsible for the presence of ergoline alkaloids in convolvulaceous plants. The taxonomic placement of the epibiotic mycelia within the family Clavicipitaceae was confirmed by construction of phylogenetic trees from sequences of five nuclear and one mitochondrial gene (Leibner 2009; Steiner *et al.*, in press). Finding genes responsible for ergoline alkaloid biosynthesis in both fungal species is in agreement with these conclusions (Markert *et al.* 2008).

The *dmaW* (also annotated *cpd1*) gene is responsible for the determinant step in ergoline alkaloid biosynthesis. As with the genes from other ergoline alkaloid producing fungi, this gene consists of three exons and two introns with exon number two comprising 122 base pairs in all genes that initiate ergoline alkaloid biosynthesis. The gene from *P. ipomoeae* was overexpressed in yeast, the enzyme characterized, its kinetic data and the substrate specificity determined. The gene is responsible for the introduction of a dimethylallyl residue into the 4-position of tryptophan. Therefore, the enzyme is called 4-(γ,γ-dimethylallyl)tryptophan synthase. A reverse genetic experiment localized the transcription process of this gene to the fungus *P. ipomoeae*. The gene is part of a cluster encoding the enzymes catalyzing ergoline alkaloid biosynthesis (Markert *et al.* 2008).

Although the fungi are clearly the site of ergoline alkaloid biosynthesis (Markert *et al.* 2008) the alkaloids are hardly detectable in extracts of fungal mycelium collected from the leaf surface of *I. asarifolia* or *T. corymbosa*. Ergoline alkaloids were found, however, when a very sensitive analytical method was employed. Introduction of fungal mycelium directly into the injection port of a combined gas chromatograph-mass spectrometer made it possible to detect agroclavine in the fungal hyphae. Moreover, the developmental situation of the leaf bud seems to be crucial for the detection of the alkaloids within the associated fungus (K. Ploss & W. Boland, personal communication). Another experiment gave a hint as to the whereabouts of the bulk of alkaloids. Both fungi are epibiotic and a penetration of the plant epidermis was never observed. It is for this reason that mycelium and plants can be separated by ultrasonic treatment. Subsequent analysis of both mycelium and plant detected almost all of the ergoline alkaloids within the plant material (Markert *et al.* 2008). It is therefore evident that a transport system must exist that translocates the alkaloids from their site of synthesis within the hyphae into the plant where they are sequestered.

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Fungus-host plant specificity

It was also desirable to investigate the host plant specificity of the fungi *P. ipomoeae* and *P. turbinae*. Experiments employing different hosts would be desirable but are not yet possible since the fungi *P. ipomoeae* and *P. turbinae* are currently not culturable and even fail to grow after reinoculation of fungicide-treated host plants (Steiner *et al.* 2006, 2008).

An inoculation experiment with *Penicillium roquefortii* seemed to be promising, however, because this fungus is an *in vitro* producer of isofumigaclavine A, an ergoline alkaloid. Moreover, the fungus had been isolated from an *I. asarifolia* plant using a method employed to detect endophytes and thus lives asymptomatically within this plant. The *P. roquefortii* fungus indeed colonized the plant which, however, remained devoid of any alkaloids (Steiner *et al.* 2008).

When removed from the leaf by a fungicide and reapplied to the leaf surface *P. ipomoeae* and *P. turbinae* do not resume growth. We found by chance, however, an *in vitro* method to establish a plant-fungus association. Plant callus and cell suspension cultures of *I. asarifolia* were easily established in different media. It was soon realized that these cultures also contained fungal cells, which seemed to have escaped the sterilisation process during establishment of the cell culture. The *P. ipomoeae* was identified by microscopic techniques, by SSCP and by sequencing of the ITS region. This showed beyond doubt that the fungus lived within the plant cell cultures. The fungus was not detectable by the naked eye and the callus culture appeared normal and grew asymptomatically. Despite the fact that the clavicipitaceous fungus was present, neither the callus nor the cell suspension cultures contained any detectable ergoline alkaloids (Hussein 2004). Apparently, the morphological differentiation of the host plant is a prerequisite for the production of ergoline alkaloids. After changing the hormonal regime of the callus culture, a differentiated shoot developed, and after again changing the medium a root system appeared. Mycelium of *P. ipomoeae* covered the young plantlet and it contained ergoline alkaloids. This experiment (Steiner *et al.* 2006, 2008) indicates that: (i) the plant integrates the *P. ipomoeae*-fungus into its own developmental program; and (ii) a morphologically differentiated plant is essential for alkaloid synthesis. Since the fungus is usually associated with secretory glands, these structures may possibly play a crucial role in the interaction and the metabolic dialogue between both associated organisms.

How do propagules spread within the host plant?

The seeds of our ergoline alkaloid containing convolvulaceous plants carry a clavicipitaceous fungus destined for seed transmission. When seeds germinate the fungus appears on the adaxial leaf surface and alkaloids occur within these leaves. It is not known, however, how the fungus spreads from the seed to shoots and leaves. The fungi are epibionts that never seem to penetrate the plant epidermis. With a grafting experiment (Hellwig 2007) we hoped to shed light on this unresolved question. *I. asarifolia* and *T. corymbosa* plants were raised from cuttings within three months. One set of plants was generated from fungicide-treated plants whereas another set was from untreated plants, and consequently carried clavicipitaceous fungi and ergoline alkaloids. A shoot with fungus and alkaloids was grafted into the shoot system of a plant devoid of fungus and alkaloids. In the corresponding experiment a shoot from a plant devoid of fungus and alkaloids was grafted into a shoot system carrying both fungi and alkaloids.

After the grafting process the plants were kept in the greenhouse for 18 weeks. Chemical and microscopic analyses of the shoot systems of both sets of plants revealed that those shoots containing alkaloids and covered with fungus before the grafting experiment also had these at the end of the grafting experiments. Shoots devoid of fungus and alkaloids before the experiment were also devoid of fungus and alkaloids after the experiment. Apparently, alkaloids were not translocated within the *I. asarifolia* and the *T. corymbosa* shoot systems during the 18 week period; no migration of fungi within the plant or on the leaf surface was observed under these experimental conditions.

Conclusions

Phylogenetic analysis as well as morphology, molecular biological evidence, and host and chemotype data described herein supported the description of two new clavicipitaceous epibiotic fungi belonging to the genus *Periglandula* within the family Clavicipitaceae (Steiner *et al.*, in press). The fungi are seed transmitted and are responsible for the occurrence of ergoline alkaloids in the convolvulaceous host plants. This is in agreement with the ecological role of ergoline alkaloids. Our conclusions give an explanation for the disjointed occurrence of ergoline alkaloids in clavicipitaceous fungi and higher dicotyledonous plants. They dispute the hypothesis that ergoline alkaloids have multiple origins in different taxa during evolution or that a horizontal gene transfer occurred shifting a set of genes responsible for ergoline alkaloid biosynthesis from fungi to a progenitor species of today's convolvulaceous higher plants (Leistner & Steiner 2009).

It is not yet known how the fungi spread in and on their respective host plants, nor how a molecular and metabolic dialogue is established between a host plant and an epibiotic fungus that never seems to penetrate the plant epidermis. Microscopic observations, however, may be helpful in establishing a hypothesis. As pointed out above, fungal hyphae are in close contact with the oil secretory glands and occur above and underneath the cuticle covering the oil containing cavity of the secretory glands (Leistner & Steiner 2009). These structures may play a major role in the metabolic interaction between the fungus and its host. Darwin (1875) suggested, and other authors agreed (Barthlott *et al.* 2004), that carnivorous plants use the trichomes of their leaves for the uptake of nutrients derived from digested insects. Apparently, glands may function as uptake systems. This model may be suitable to direct further research in the laboratory in order to identify the entrance gate into the convolvulaceous host plant and the molecular dialogue between both organisms.

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