

## Commentary

# The latest news from biological interactions in orchids: in love, head to toe

In the *The Blue Angel* (1930), Marlene Dietrich became famous by singing ‘*Ich bin von Kopf bis Fuß auf Liebe eingestellt*’ (‘in love, head to toe’). Orchids can also be viewed to be in love from head to toe, since they have evolved tight biological interactions from head (pollination by insects) to toe (mycorrhizal symbiosis with fungi). Love, or mutualism, can be questioned in the many cases where the orchid does not reward its partner, but even these potentially cheating orchids participate in biological interactions, head to toe. Orchids are thus excellent models for investigating the ecology and evolution of biological interactions. For example, in the past 20 yr, *New Phytologist* has published no less than 46 papers on biological interactions in orchids. They were also the focus of the 31<sup>st</sup> New Phytologist Symposium ‘Orchid symbioses: models for evolutionary ecology’ which was held in Calabria, Italy on 14–16 May 2013 (Fig. 1). As reported by Bronstein *et al.* in this issue of *New Phytologist* (pp. 373–375), this symposium highlighted active research along the mutualism–parasitism continuum.

This commentary introduces the reports and discussions from this meeting, which are published in this and recent issues of *New Phytologist*, within the framework of the latest advances in this field.

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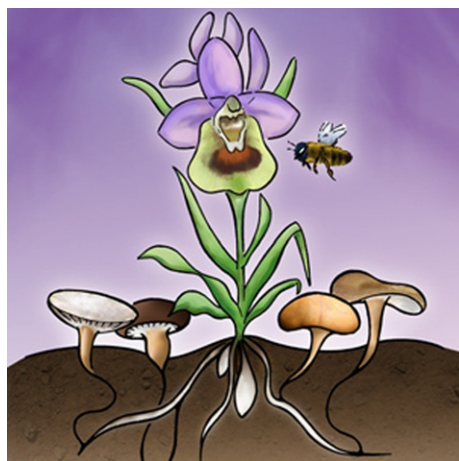
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Most land plants form mycorrhizas and depend on soil fungi for their mineral nutrition. Orchids nevertheless present two peculiarities: first, most of their mycorrhizal fungi belong to a polyphyletic assemblage of saprotrophic taxa called ‘rhizoctonias’ which is specific to this family (Dearnaley *et al.*, 2013). Second, seeds are small and reserveless so germination depends on colonization by a mycorrhizal fungus, resulting in an achlorophyllous, heterotrophic seedling called a protocorm (Eriksson & Kainulainen, 2011). Since the fungus provides nutrient and carbon (C) at germination, the reward, and thus the mutualism, have been questioned. On the one hand, the reversion of C flow at adulthood (Cameron *et al.*, 2008), when green leaves are formed, allows a net C benefit to the fungus, at least in some associations. On the other hand, nonphotosynthetic or even some photosynthetic orchids still obtain C from mycorrhizal fungi at adulthood (e.g. Yagame *et al.*, 2012; Stöckel *et al.*, in this

issue of *New Phytologist* pp. 606–615). Full heterotrophy has evolved in more than 30 independent orchid lineages (Merckx, 2013), and partial heterotrophy is considered to be an evolutionary step, and a predisposition to the emergence of full heterotrophy (Roy *et al.*, 2013). These partially or fully heterotrophic orchids do not associate with saprotrophic rhizoctonias, but with other fungal taxa that are saprotrophic or mycorrhizal on other nearby autotrophic plants (see Dearnaley *et al.*, 2013, for a review). Heterotrophic orchids cast doubts on whether mutualism is the rule in the orchid mycorrhizal symbiosis. However, discussions on mutualism are often based on C flow, and ignore other possible benefits for the fungus, such as the delivery of vitamins, or the protection of hyphae within roots, which are indeed less easy to address. Thus, a rigorous evaluation of the effect of the orchids on fungal fitness is still pending.

Pollination by insects is common among land plants, but the ancestors of orchids evolved a particular feature: pollen is produced within two modified stamens, the pollinia, and highly-derived floral morphologies allow the dispersion of whole pollinia (or large pollinia pieces) by visiting insects. Some inflorescences have even evolved morphological adaptations to pollinators’ behaviour, in order to enhance pollination success, but also to limit self-pollination due to pollinators visiting too many flowers from the same inflorescence (geitonogamous selfing; Iwata *et al.*, 2012). Although many orchids reward insect pollinators with nectar, cheating strategies evolved repeatedly, and at least 30% of orchid species present food deception (e.g. by mimicking rewarding flowers; Johnson *et al.*, 2004) or sexual deception (where olfactory and/or visual cues concur to attract naïve males; Vignolini *et al.*, 2012). Deceptive flowers usually select avoidance on the insect side, either by learning at the individual level or by natural selection over generations, which may limit the number of pollinator visits. However, in orchids only a few pollinator visits are required for dispersion or reception of the pollinia, a feature that probably predisposed orchids to the evolution of floral deception. The avoidance of deceptive flowers by pollinators also reduces the likelihood of repeated visits to flowers from the same inflorescence, and thus increases allogamy. For this reason, floral cheating was thought to be evolutionarily stable, since a more rewarding plant would undergo a greater number of repeated visits on its inflorescence, and therefore more inbreeding depression (Johnson *et al.*, 2004). However, recent phylogenetic analyses of the genus *Disa* recently demonstrated the repeated evolution of rewarding flowers from deceptive ancestors (Johnson *et al.*, 2013). Thus, biological interactions clearly oscillated between mutualism and exploitation on the flower side too, although the underlying selective pressures beyond transitions remain unclear.

Other plant families display very small seeds with symbiotic germination (Eriksson & Kainulainen, 2011; Hashimoto *et al.*, 2012) or pollen dispersion via pollinia (e.g. some Asclepiadaceae;



**Fig. 1** *Von Kopf bis Fuß auf Liebe eingestellt*: orchids can be viewed to be 'in love, head to toe', because of their strong biological interactions from head (pollination by insects) to toe (mycorrhizal symbiosis with fungi). These interactions were the focus of the 31<sup>st</sup> New Phytologist Symposium, held in Calabria, Italy, on 14–16 May 2013, which was devoted to Orchid symbioses: models for evolutionary ecology.

Wyatt *et al.*, 2000). Yet, orchids are unique in combining the two features, which are indeed complementary. The germination success of reserveless seeds is low, since it requires a fungus (see McCormick & Jacquemyn, in this issue of *New Phytologist*, pp. 392–400), but the production of numerous seeds (up to  $10^6$  per fruit; Arditti & Ghani, 2000) compensates for this. Such seed production would be challenged by pollen limitation, if pollinia did not allow for the massive, simultaneous delivery of pollen grains. One can even speculate that the joint evolution of these traits allowed orchids, one of the largest plant families with 27 000 species, to undergo greater diversification than families displaying symbiotic germination or pollinia dispersal alone. Interestingly, pyrolids, a tribe of Ericaceae with small seeds, symbiotic germination (Hashimoto *et al.*, 2012) and large seed production (Johansson *et al.*, 2013), also tend to transfer pollen clusters by way of insects, that is, in tetrads or even larger aggregates (Takahashi, 1986). This striking convergence suggests that symbiotic germination and transfer of pollen aggregates sometimes reinforce each other in evolution.

Despite this evolutionary link between the above- and below-ground associations, remarkably few works simultaneously addressed the head (pollination) and the toe (mycorrhizas) of orchid interactions hitherto. At an evolutionary level, investigations on the South African orchid subtribe Coryciinae suggested that speciation is associated with pollinator shifts, while fungal shifts do not occur during speciation, but rather when species coexist sympatrically (Waterman *et al.*, 2011). In a study published in this issue of *New Phytologist*, Jacquemyn *et al.* (pp. 616–627) identified divergent mycorrhizal partners from seven sympatric species on a 625 m<sup>2</sup> Mediterranean plot; similarly, Těšitelová *et al.* (2013) recently showed that sympatric diploid and tetraploid cytotypes of Central European *Gymnadenia conopsea* had different rhizoctonia partners. In the latter case, triploid *G. conopsea* hybrids mixed parental partners, supporting a genetic determinism, and sympatry exacerbated segregation of partners between diploids and

tetraploids. Such a pattern can be viewed as a niche partitioning process allowing for the stable coexistence of orchid species. A picture thus emerges where pollinator shifts drive speciation, likely by interrupting gene flow between sister species, while the mycorrhizal interaction adapts to sympatric coexistence when required. This may explain why some partners' fidelity exists at a lower phylogenetic level (where related orchid species tend to have identical or related fungal partners, for example, within tribes and subtribes; Waterman *et al.*, 2011; Martos *et al.*, 2012), but not at a higher phylogenetic level, where fidelity to ancestral symbionts is progressively lost by selection for other symbionts whenever sympatry occurs. However, in the specific case of heterotrophic orchids at least, shifts of mycorrhizal fungi may also drive speciation (Dearnaley *et al.*, 2013). This view of the respective evolutions of mycorrhizal and pollination associations should now be challenged by more studies, in diverse phylogenetic frameworks, that consider simultaneously the two interactions. The development of network analysis of interactions of numerous orchids with their diverse fungal partners over a given geographic area, in a phylogenetically-formed framework (Martos *et al.*, 2012), is a promising tool to obtain wide ecological and evolutionary views of these interactions, and it should now be applied to tripartite fungus–orchid–pollinator networks.

Coming back to mycorrhizal interactions, two new tools have recently been introduced: secondary ion mass spectrometry (SIMS) and proteomic and transcriptomic methods. SIMS allows isotopic analysis of cell compartments at a micrometric scale, and the tracing of nutrient exchanges in protocorms after isotopic labelling of the fungus. SIMS now sheds new light on a long-debated question: hyphal pelotons live first within the host cells, separated by the plasma membrane from the cytosol, and then undergo a final senescence often described as a 'lysis', but when do nutrients move to the orchid? From living pelotons? During, and thanks to, the peloton lysis? Or at both stages? Ironically, two recent studies reach divergent conclusions. In this issue of *New Phytologist*, Kuga *et al.* (pp. 594–605) investigated heterotrophic protocorms of *Spiranthes sinensis*, a rhizoctonia-associated species that turns green at adulthood. Carbon (C) and nitrogen (N) transfer to the host plant occurred from both living and senescent pelotons, within 24 h (Kuga *et al.*). Bougoure *et al.* (2014) investigated a different species, which is heterotrophic at adulthood. *Rhizanthella gardneri* obtains C and other nutrients from a fungus mycorrhizal on nearby green plants, as is the case with many heterotrophic orchids. Using a tripartite microcosm culture, Bougoure *et al.* (2014) undertook a clever labelling experiment, where they provided <sup>13</sup>CO<sub>2</sub> to the green plant that was feeding the shared fungus, and they monitored <sup>13</sup>C transfer to linked *R. gardneri* protocorms. Carbon-13 (<sup>13</sup>C) was transferred to the protocorm but unexpectedly remained in living fungal pelotons for up to 216 h after labelling, without transfer to the host cells or to senescing pelotons. This supports first, a totally different kinetics, and second, that lysis is required, since intact pelotons did not transfer C. The discrepancy between Kuga *et al.* and Bougoure *et al.* (2014) can be explained by the different physiologies of their respective models: the way heterotrophic orchids acquire nutrients from their host may differ from the way green, rhizoctonia-associated orchids exploit the fungus at the protocorms stage. Heterotrophic orchids are sometimes

considered to be neotenic species, expressing a protocorm-like heterotrophy at adulthood, but their exploitation of fungal resources may well be a full evolutionary innovation, as suggested by their shift to taxonomically and ecologically different fungal partners (as already stated). Indeed, Stöckel *et al.* emphasize in this issue another difference: in heterotrophic orchids, protocorms and adults collected *in natura* are naturally enriched in  $^{13}\text{C}$  and  $^{15}\text{N}$  as compared with autotrophic plants, which reflects the enrichment of their specific mycorrhizal fungi. Conversely, protocorms of green, rhizoctonia-associated orchids are less enriched, or not enriched at all. We still cannot know whether this results from the  $^{13}\text{C}$  enrichment of rhizoctonias (whose isotopic content remains hitherto unknown), or from a different isotopic fractionation during the transfer to the host.

Transcriptome analyses, corroborated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) evaluation of the expression of selected genes, have recently allowed a comparison between non-symbiotic and rhizoctonia-associated protocorm tissues. Although the use of different methods complicates the comparison, colonized tissues of the green orchids *Dendrobium officinale* (Zhao *et al.*, 2013) and *Serapias vomeracea* (Perotto *et al.*, 2014) have revealed strikingly few fungal transcripts, and an up-regulation of diverse orchid genes involved in metabolism, gene expression or transport. Strikingly few plant genes involved in defence against pathogens were found, suggesting a ‘friendly plant–fungus relationship’ (*dixit* Perotto *et al.*, 2014), at least on the plant side. Together with the possibility of nutrient transfer through living pelotons (Kuga *et al.*), this questions some concepts pointing to an aggressive symbiosis that dates back to the pioneering work of Noël Bernard in the 1900s. Bernard was trained with Pasteurian microbiologists who were used to seeing microbial relationships as pathogenic (see Selosse *et al.*, 2011). He introduced words such as ‘phagocytosis’ or ‘lysis’ for the final peloton degradation, and he first used the name ‘rhizoctonia’, a fungal genus once described as pathogenic. The connotations of such words should not be misleading. Peloton degradation may simply be a domestic recycling of old structures, while on the fungal side, rhizoctonia species tend to be phylogenetically distinct from related parasitic taxa (Dearnaley *et al.*, 2013; Veldre *et al.*, 2013). Thus, the mycorrhizal association between green orchids and rhizoctonias may be closer to true ‘love’ than to parasitic interactions.

From the crucial role of fungi in germination, it is often assumed first, that generalist orchids, with a large fungal spectrum are more widespread than specific ones, and second, that fungal distribution limits that of orchids. In this issue, McCormick & Jacquemyn review evidence falsifying these assertions. First, some fungi are so common in soils that they do not constrain the range of their specific hosts. Second, some experimental seed-sowing at sites potentially suitable but devoid of adults, or even ecologically unsuitable, revealed the presence of fungi triggering germination (Těšitelová *et al.*, 2012). Thus, seed dispersal and/or some other biotic and abiotic constraints may rather be limiting. Seeds successfully germinating between ecologically unsuitable sites (Těšitelová *et al.*, 2012) also imply that the limitation acts at later developmental stages. Indeed, the transformation of heterotrophic protocorms into green adults is a drastic nutritional and developmental transition. Its

impact on orchid demography and physiology is hitherto overlooked, although recent proteome analysis of greening *Oncidium sphacelatum* protocorms emphasizes drastic metabolic changes and the accumulation of stress proteins (Valadares *et al.*, 2014). This transition may reveal new requirements, or different susceptibilities to environmental factors, and thus may limit the demography and distribution of orchids, but this, again, awaits further studies.

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Many other biological interactions in orchids remain poorly investigated, as was clear from the 31<sup>st</sup> New Phytologist Symposium (Bronstein *et al.*). For example, one symbiosis extending from toes to head in orchids remains overlooked: endophytic fungi, which grow within living tissues, without apparent infection, and range from mutualists to commensals or weak pathogens. They are often obtained during attempts to isolate mycorrhizal fungi *in vitro*, or during barcoding of root fungi. In the 454 pyrosequencing data handled by Jacquemyn *et al.*, 39% of all fungal sequence reads are related to nonmycorrhizal taxa, and this extends to up to 80% in some individuals. Beyond their diversity, the roles of endophytes in roots, as well as in shoots, also deserves further study: a rare report on the addition of fungicide revealed the potential for both positive and negative effects of fungi on orchid growth and survival (Bayman *et al.*, 2002).

More and more ecological or evolutionary views of orchid interactions are being published, and at the same time, new analytical tools are becoming available, such as SIMS, or proteomic and transcriptomic approaches, which point toward genes involved in orchid–fungal interactions. We may soon learn more of the way that orchids are *von Kopf bis Fuß auf Liebe eingestellt*—deeply in love, head to toe.

## Acknowledgements

The author wishes to thank Salvatore Cozzolino (University of Naples, Italy), Giuseppe Pellegrino (University of Calabria at Cosenza, Italy) and Lee Taylor (University of Alaska at Fairbanks, USA) and the *New Phytologist* Central Office for the organization of the 31<sup>st</sup> New Phytologist meeting. The author also thanks Sarah Lennon, Florent Martos and Silvia Perotto for helpful comments on earlier versions of this commentary.

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**Key words:** biological interactions, mycorrhizas, orchids, pollination, symbioses, 31<sup>st</sup> New Phytologist Symposium.

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