Commentary

Selenium and the elemental defense hypothesis

There are innumerable studies that report on how plants defend themselves against insect herbivores. Most focus on chemical traits (Harborne, 1993), morphological adaptations (Schoonhoven *et al.*, 2005), compensatory strategies (Trumble *et al.*, 1993), or genetic variations that allow escape in time or space (Denno & McClure, 1983). In 1992, a new strategy was proposed and named the elemental defense hypothesis (Boyd & Martens, 1992). This novel strategy suggested that some plants (termed hyperaccumulators) sequester exceptionally high concentrations of metals as a defense against herbivores. This hypothesis was extended by Coleman *et al.*, in 2005, to include plants that accumulated more modest amounts of trace elements. While there have been an excellent series of studies conducted in the laboratory that generally support this hypothesis (Boyd, 2007 and references therein), and many plant species have been discovered that can accumulate these elements (Reeves & Baker, 2000), field-based studies investigating herbivore responses to metal sequestration have been lacking. The limited field research to date has primarily focused on systems with a single plant and insect (but see Boyd, 2007 for studies with nickel). The paper by Galeas *et al.*, in this issue of *New Phytologist* (pp. 715–724), is the first to use a field survey approach to test the elemental defense hypothesis across a range of insect species feeding on plants containing elevated concentrations of selenium (Se).

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The importance of selenium in the environment

Although the periodic table contains 87 elements that are considered to be metals or metalloids (elements that act like metals, but lack luster), fewer than 15 of these are commonly found at elevated concentrations in plants. These key elements, which include arsenic (As), aluminium (Al), lead (Pb), cobalt (Co), chromium (Cr), copper (Cu), cadmium (Cd), mercury (Hg), manganese (Mn), nickel (Ni), Se and zinc (Zn), have all been demonstrated to be toxic to arthropods (Heliovaara & Vaisanen, 1993 and references therein). Of these, Se is a particularly important problem. Although Se is an essential trace nutrient important to most animals as an antioxidant (Mayland, 1994), this widespread metalloid displays a narrow margin between concentrations that are beneficial and those that are toxic. No continent on earth is free of soils with significant concentrations of Se (McNeal & Balistrieri, 1989), but some specific regions do have Se-poor soils. The many Se-contaminated sites in North America have been reviewed by Mayland *et al.* (1989). In California, the San Joaquin Valley alone has over 213 000 ha of soil with elevated amounts of Se, resulting primarily from agricultural activities (http://tin.er.usgs.gov/geochem/doc/averages/se/usa.html). In fact, virtually all countries that burn coal as an energy source, or that mine metals such as copper, have significant point source contamination with Se (McNeal & Balistrieri, 1989). Thus, while the primary evolution of plants and insects with adaptations to elevated concentrations of Se has almost certainly occured at geologically stable sites with rainfall leaching selenate from naturally seleniferous soils, there are now many anthropogenic sources of Se contamination where new relationships may evolve.

Selenium and ecosystem function

Terrestrial herbivores, particularly arthropods, are critical to the effective functioning of ecosystems. Because these organisms are active at the base of the food web, changes in population densities of arthropods can have profound effects on higher-level organisms that depend on them as primary food sources. Many arthropods are beneficial, serving to keep pest populations under control, thereby preventing damaging outbreaks. Other arthropods pollinate plants, disseminate seeds and produce structures used by countless other animals. Disruption of any of these activities can have substantial (and usually deleterious) effects on an ecosystem. Thus, arthropods are often used as bio-indicators when ecosystems become polluted. While many studies have examined insect diversity at sites with anthropogenic metal contamination, it is somewhat surprising that relatively little information is available from sites with seleniferous soils where elevated concentrations of Se occur naturally. Nonetheless, there is unambiguous information that Se has significant fitness effects on arthropods.

Terrestrial insect herbivores have to acquire their nutrients, minerals and trace elements from food, and toxic ions can readily cross an arthropod's midgut epithelium and enter the hemolymph. The early ecotoxicology literature suggested that

intake of metals and metalloids by arthropods was dependent primarily on the concentrations in the food (Dallinger & Rainbow, 1993). A subsequent review by Jensen & Trumble (2003) cites numerous cases across several insect orders where a dose-dependent concentration effect has been documented for a variety of elements. However, the soil concentrations of Se are not always indicative of the concentrations of Se in plants because of the rapid transport and uptake (Mayland *et al.*, 1989). Typically, sodium selenate is leached from rocks or soil by rainwater or agricultural irrigation water, and then transformed within plants to other forms of Se. Plants can acquire Se as either sodium selenate or sodium selenite, or in organic forms such as selenomethionine or selenocysteine, but the most common soluble form of Se in water is sodium selenate. When acquired as sodium selenate, plants generally convert it to sodium selenite. The Se in sodium selenite is then substituted for sulfur in certain amino acids, commonly producing selenomethionine, selenocysteine or selenocystine. Selenomethionine is the most common form of Se found in plants (Daniels, 1996). This form, which was found to be highly toxic to insects in laboratory feeding studies (Jensen *et al.*, 2006), is not detected by some insects, allowing rapid ingestion of toxic doses (Trumble *et al.*, 1998). The various forms of Se are known to be transferred between plants and herbivores, with a tendency for biomagnification (Vickerman & Trumble, 2003).

Comparatively few studies have examined the relative toxicological responses of higher trophic levels to metals accumulated in prey. Boyd & Wall (2001) fed four different predator species (two insects and two arachnid species) with prey containing high concentrations of nickel. They found that while three of the species were not affected, one of the spider genera had a significant decrease in survival. Vickerman & Trumble (2003) found that hemipteran predators exposed to Se in their prey (a caterpillar) had significantly higher mortality and weighed less than control predators fed prey with low Se concentrations. Detrimental effects were observed for predators despite the prey containing more Se than the predators. Thus, a trace element in the food chain can have detrimental effects, even in the absence of biomagnification. In a contrasting study, Merrington *et al.* (2001) tracked Cd and Zn from fertilizer applications through wheat plants to aphids, and then to their lacewing predators. They found that the aphids accumulated concentrations of Cd and Zn some 24 and 140 times greater, respectively, than the concentrations in the fertilized soil on which the wheat plants were grown. However, the predatory lacewings fed high-Cd/Zn prey did not accumulate Cd or Zn any differently from the controls. The authors speculated that this was because of the piercing and sucking method of feeding by the predator and the location of contaminants in the body of the prey. Therefore, higher trophic level predators or parasites may be differentially affected by trace elements based on variable feeding strategies or detoxification mechanisms. This variability provides an opportunity for the evolution of plants, herbivores and higher tropic level organisms in areas with elevated metal or metalloid contents in soil.

Evolution of plant accumulation of metals and metalloids

As early as 1957, a possible evolutionary role for plants accumulating metalloids was suggested when Tadros (1957) reported that plants adapted to high Se environments were attacked more readily by pathogens if they were grown in soils containing low concentrations of Se. Subsequently, Boyd & Martens (1992) listed five hypotheses that have attempted to explain the evolution of plants that accumulate high concentrations of metals. These hypotheses predict that the ability to accumulate metals and metalloids evolved to provide (1) tolerance to, or disposal of, the elements from the plant, (2) a drought-resistance strategy, (3) a means of avoiding competition from plants less tolerant to the elements, (4) inadvertent uptake of elements and (5) defense against herbivores or pathogens. All of these hypotheses have been tested to varying degrees (Boyd, 2004), and there is some indication that each has merit. Of these, the best studied is the elemental defense hypothesis, for which considerable support has been found. However, not surprisingly, the available literature shows that this hypothesis is not universally applicable, as some herbivores, pathogens and parasitic plants can successfully attack hyperaccumulator species (Boyd, 2007). Interestingly, the literature treats all five proposed hypotheses as independent, and even mutually exclusive, evolutionary processes. We believe that these hypotheses are compatible and can act jointly to reinforce each other in driving the evolution of plant accumulation of elements.

The importance of the study of Galeas *et al.* is that their research provides a comprehensive field survey of a naturally seleniferous site, including a combination of herbivore and predator occurrence data, in conjunction with Se concentrations in the plants, insects and predators. These data permit a detailed analysis of associations that has not been previously possible. This is a necessary step in unraveling why some plants have evolved the ability to accummulate substantial amounts of Se, and how some insects appear to be compensating. In addition, the assessment they provide of the prevalence of insect adaptation or tolerance to Se across feeding guilds is particularly intriguing. The plant–insect associations presented in the paper by Galeas *et al.* may even find application in the developing fields of phytoremediation and phytomining (Reeves & Baker, 2000; Vickerman *et al.*, 2004).

Testing for elemental defense against herbivores

Using the reasoning employed by Berdegue *et al.* (1996) to examine the theory of enemy-free space, we suggest that three falsifiable null hypotheses can be constructed to test the theory of elemental defense for Se-containing plants. The first

hypothesis is that the fitness of a plant without Se and without herbivores is equal to the fitness of a plant without Se and in the presence of herbivores. Disproving the first null hypothesis demonstrates that herbivores have fitness effects for this plant. While it can be argued that herbivores are known to harm plants, and therefore experimental testing is unnecessary, herbivory may not be limiting plant fitness in a particular system at a particular time (see Trumble *et al.*, 1993).

The second hypothesis states that the fitness of a plant with Se and in the presence of herbivores is equal to the fitness of a plant without Se and in the presence of herbivores. Disproving the second null hypothesis demonstrates that the presence of Se has fitness consequences for herbivores. The second hypothesis has been tested in the laboratory and to a lesser extent in the field (Freeman *et al.*, 2007; see also Galeas *et al.*), providing evidence that insect herbivory is in fact reduced by the presence of Se. The limitation of these studies is that they do not directly measure plant fitness, but rather focus on the amount of leaf eaten, or the survival or development of the insect herbivore.

The third hypothesis predicts that the fitness of a plant with Se but without herbivores is equal to the fitness of a plant without Se and without herbivores. This final hypothesis looks for a cost or a benefit of Se accumulation in the absence of herbivores. If Se is costly, there is support for the elemental defense hypothesis. However, if Se is beneficial in the absence of herbivores, then defense against herbivores is probably not the primary advantage of Se accumulation. Note that failing to disprove this last hypothesis does not disprove the elemental defense hypothesis, but merely suggests that alternative hypotheses explaining Se hyperaccumulation should also be considered.

Looking forward

The opportunities for compelling research on these fascinating metal-accumulating systems are nearly endless, particularly as they relate to current physiological and ecological theories. Although some recent research has suggested physiological mechanisms by which Se may be detoxified in insects (see Jensen, 2006), substantially more information is needed before we can begin to understand fully the physiological ecology of the independent and joint effects of metals and metalloids. Minimal information is available on the effects of Se on fitness and population dynamics of parasitoids in either natural or agricultural systems. No studies have addressed the relative importance of top-down (natural enemies) vs bottom-up (plant nutrition) processes at contaminated sites. In addition, the possible role that Se may play in mediating competitive interactions among herbivores, or among natural enemies, is completely unknown. No reports are available on the effects of Se on pollination ecology or on the possible interactions of Se accumulation with the increasing temperatures predicted as a result of global warming. How these various ecological processes will be altered by the presence of metals, such as Se, should provide exciting research opportunities for the foreseeable future.

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Fungal fidelity in the myco-heterotroph-to-autotroph life cycle of Lycopodiaceae: a case of parental nurture?

The nature and identities of the mycorrhizal fungal partners in lower vascular plants have long been a matter of intrigue, acquiring major evolutionary significance following the suggestion of Pirozynski & Malloch (1975) that the co-evolution of plants and arbuscular mycorrhizal (AM) fungi was critical for the colonization of land by plants. This view has subsequently gained considerable support from palaeobotanical (Remy *et al.*, 1994) and molecular-clock (Simon *et al.*, 1993) evidence. However, the identities of fungal partners in extant lower vascular plants, which may be the closest living relatives of the early land plants, have only recently started to be determined (Kovács *et al.*, 2007; Winther & Friedman, 2007a). In this issue of *New Phytologist*, Winther & Friedman (2007b, pp. 790–801) provide the first molecular identification of the fungal symbionts in seven species spanning two major genera in the Lycopodiaceae, an ancient family descended from some of the earliest known land plants (Wikström & Kenrick, 2001). The new study addressed three important questions.

(1) What is the identity of AM fungi in sporophytes?
(2) Do gametophytes and sporophytes share the same AM fungi?
(3) How are the AM fungi in Lycopodiaceae phylogenetically related to AM fungi found in other initially myco-heterotrophic lower plants and fully myco-heterotrophic higher plants?

'Are the fungal symbionts of gametophytes sometimes so restricted in their distributions or host ranges that parental nurture is more important than epiparasitism of unrelated plant species?'

In this study, Winther & Friedman (2007b) confirmed that Glomeralean AM fungi are symbionts in the Lycopodiaceae. In resolving this long-standing uncertainty, they demonstrated that gametophytes and sporophytes of *Huperzia* share the same AM fungi and established that *Lycopodium clavatum* and *Huperzia* spp. show considerable fungal specificity, associating with a single clade of fungi in *Glomus* group A. In seven species of Lycopodiaceae sampled from six locations, they found (based on ribosomal DNA sequences) only four AM phylotypes, with one phylotype present in six of the plant species and at five of the locations. The clade of *Glomus* A fungi associated with the seven Lycopodiaceae species is sister to the clades found in the myco-heterotrophic gametophyte and photosynthetic sporophytes of the fern *Botrychium* (Winther & Friedman, 2007a) and to an achlorophyllous myco-heterotrophic Lilialean plant *Arachnitis uniflora* (Bidartondo *et al.*, 2002).

These findings contribute to the increasing evidence that myco-heterotrophs dependent on AM specialize on restricted fungal clades within the *Glomus* A group. These advances also raise important new hypotheses relating to plant–fungus co-evolution, mycorrhizal fungal specificity and lifetime fidelity, intergeneration carbon subsidies and myco-heterotrophic plant–fungal interactions. The study provides further compelling support for the view that AM fungi can play a critical role in supporting the establishment of some plants through myco-heterotrophy by enabling interplant carbon transfer, and may enable intergeneration carbon subsidies from autotrophic adults to progeny that depend on myco-heterotrophy for establishment.

Alternation of mycorrhizal functioning across alternating generations?

A defining feature of the lower vascular plants is their alternation of generations (Kenrick, 1994), in which their fungal

partners often appear to play critical, but distinct, roles (Read *et al.*, 2000). The majority of lower plants are photosynthetic, but in approx. 1000 species of lower tracheophytes, the gametophyte generation develops in soil or litter and is achlorophyllous, and is entirely dependent on mycorrhizal fungi to supply organic nutrients by myco-heterotrophy (Winther & Friedman 2007a). Key genera with heterotrophic gametophytes include *Tmesipteris*, *Psilotum*, *Ophioglossum*, *Botrychium*, *Huperzia* and many species of *Lycopodium* (Leake 1994; Read *et al.*, 2000; Duckett & Ligrone, 2005). In contrast, the sporophytes of these genera normally produce chlorophyll, and, after an initial phase in which they are temporarily parasitic upon the gametophyte, are presumed to have conventional mycorrhizal functioning whereby they supply carbon to fungi in return for mineral nutrients. This raises important questions concerning the taxonomic and functional relationships of symbionts between the two life phases of these plants (Read *et al.*, 2000), to which Winther & Friedman (2007b) have now started to provide some answers.

Early evidence and late proof of AM symbionts in Lycopodiaceae

The cryptic nature of myco-heterotrophic gametophytes in nature, and their very localized occurrences, have presented significant barriers to their study. In the first description of *L. clavatum* gametophytes, Lang (1899) remarked that 'In no group [of lower vascular plants] has information regarding the life history been accumulated more slowly than in the case of the Lycopodiaceae'. In over a century of detailed microscopic observations of myco-heterotrophic and chlorophyllous gametophytes of lower vascular plants, including those in the Lycopodiaceae, the consistent presence of aseptate fungi with some of the characteristics of AM fungi has been demonstrated (e.g. Treub, 1884; Lang, 1899; Bruchmann, 1908; Peterson *et al.*, 1981; Schmid & Oberwinkler, 1993; Duckett & Ligrone, 2005). However, the absence of arbuscules, and the formation of unusually small vesicle structures in myco-heterotrophic gametophytes and in some sporophytes of lower tracheophytes, have cast some doubts on their fungal affinities. In their study of *L. clavatum*, Schmid & Oberwinkler (1993) stated: 'It is very questionable whether the endophytic fungus, although producing vesicles, belongs to the Glomales', a view echoed for *Psilotum nudum* by Schüßler (2000). The study of Winther & Friedman (2007b) is thus an extremely important landmark, laying to rest over a century of speculations and uncertainty, by confirming, with molecular identification, that the fungal associates of *L. clavatum* and *Huperzia* species are species of *Glomus*. This establishes that the distinctive fungal structures found in many lower plants can indeed be formed by AM fungi, thereby providing further support for the view that lower tracheophytes are almost universally associated with glomeralean fungi (Read *et al.*, 2000).

Mycorrhizal infection of alternating generations: are the fungi the same or different?

In lower plants with myco-heterotrophic gametophytes buried in soil or litter (including many epiphytic species), the sporophyte generations are initially parasitic upon the gametophyte until their tissues emerge above their substrate into light. For example, in *L. clavatum* the first scale leaves on the subterranean developing sporophyte are colourless, but higher up the stem, out of the soil, they develop into ordinary green leaves (Lang, 1899). It is perhaps surprising that, as a constant feature, there is no direct intergeneration transfer of mycorrhizal infection through gametophyte–sporophyte interfaces. Sporophytes, although initially dependent on carbon supplied by the gametophyte, are themselves unable to participate directly in myco-heterotrophy, at least during their early establishment phases (e.g. *L. clavatum*; Lang, 1899). Even in species with photosynthetic surface-dwelling gametophytes, including *L. cernuum* (Duckett & Ligrone, 1992), and ferns in the Gleicheniaceae (Schmid & Oberwinkler, 1995), the gametophyte–sporophyte interface is fungus free. The universal exclusion of fungi from this interface is probably an adaptation to prevent exploitation of the carbohydrate and nutrient fluxes from gametophyte to sporophyte.

An important consequence of the lack of direct intergeneration transfer of mycorrhizal fungus is that symbionts in sporophytes may differ from those in gametophytes. In *L. clavatum* (Lang, 1899) and *Botrychium* (Winther & Friedman, 2007a), mycorrhizal infection of the sporophyte occurs *de novo* into primary roots, a feature also now confirmed for *Huperzia hypogeae* by Winther & Friedman (2007b). Furthermore, because the functioning of mycorrhizal symbiosis in the gametophyte and sporophyte generations are probably physiologically very different and may not be optimally met by a single fungus, we might expect differences in the fungal partners in the two life stages. In autotrophic life phases, mycorrhizas are presumed to be primarily involved in mineral nutrition. Arbuscules, the defining structure of AM and the main site of fungus-to-plant phosphate transfer, are normally present in the sporophytes of *Psilotum* and *Botrychium* sp. (Read *et al.*, 2000) and in the green gametophytes of *L. cernuum* (Duckett & Ligrone, 1992) and of ferns in Gleichinaceae (Schmid & Oberwinkler, 1995). By contrast, arbuscules are absent from the myco-heterotrophic gametophytes of *L. clavatum*, *Psilotum* and *Botrychium* spp. (Read *et al.*, 2000). However, these observations have not resolved whether the different fungal–plant interfaces in the alternating generations are controlled by either functional or taxonomic differences, or by both.

In their recent studies of *Botrychium* and now of *Huperzia*, Winther & Friedman (2007a,b) established that the alternating heterotrophic and autotrophic generations of the species they examined share the same specific symbionts with very high fidelity. The close proximity of sporophyte roots to gametophyte tissues may allow intergeneration fungal

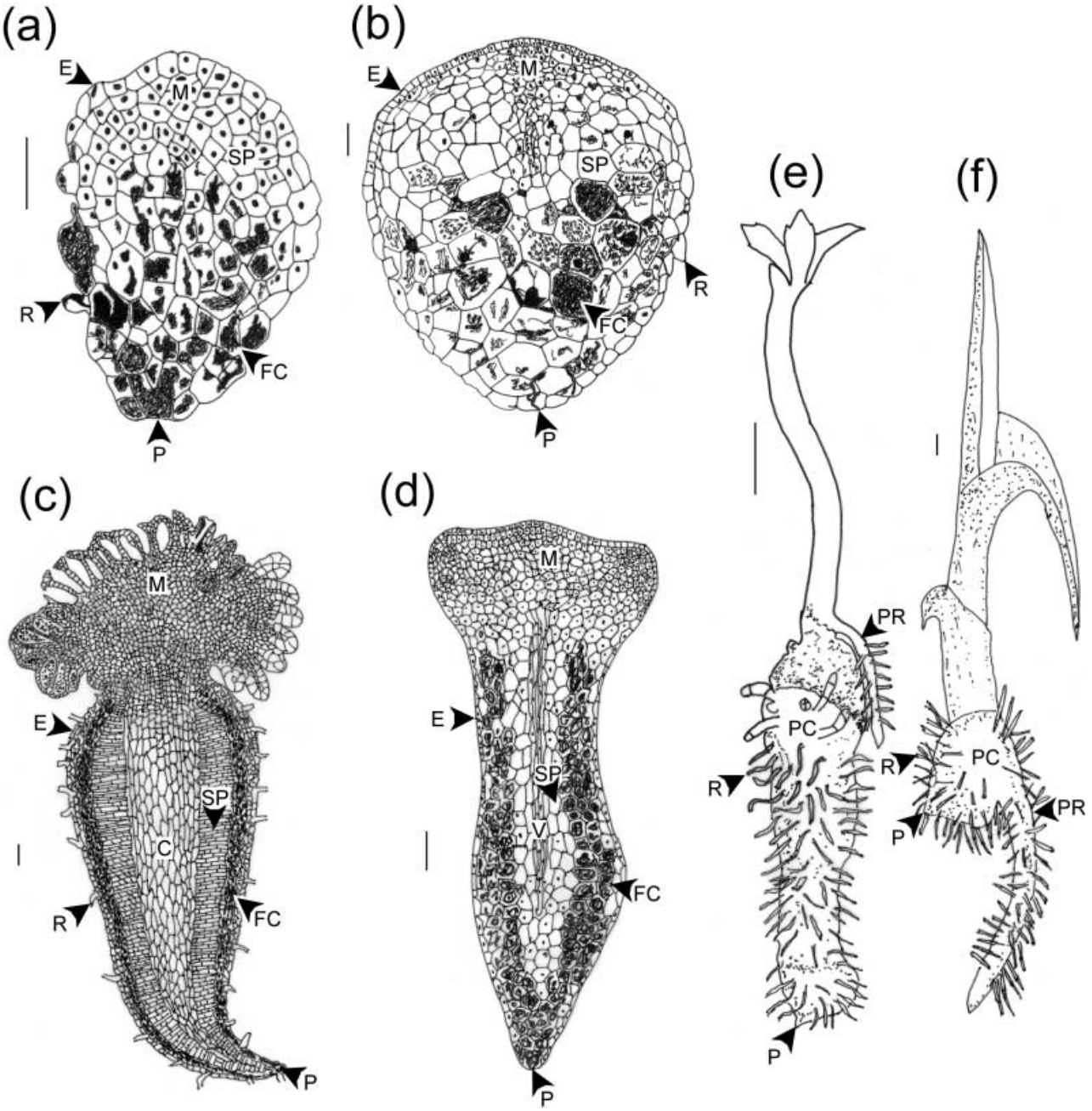

Fig. 1 Convergent evolution of myco-heterotrophic protocorm structures in lower tracheophyte gametophytes and orchid seedlings. (a) *Ophioglossum pendulum* gametophyte (after Lang, 1902), (b) *Dactylorhiza majalis* protocorm (interpreted from Rasmussen 1990), (c) *Diphasiastrum complanatum* gametophyte (after Bruchmann, 1908), (d) *Neottia nidis-avis* protocorm (after Bernard, 1902), (e) *Lycopodium obscurum* gametophyte with a sporophyte at the three-leaf stage, growing from the protocorm (after Spessard, 1917) and (f) *Calanthe veitchii* orchid seedling at the three-leaf stage attached to its original protocorm (after Arditti, 1992). Scale bars = 100 mm except for 500 mm in (e). Penetration point of initial fungal infection (P), fungal coils (FC), rhizoid (R), storage parenchyma, normally containing starch (SP), meristematic region (M), which is fungus-free, and epidermal cells (E) with occasional fungal infections. Vascular tissue (V) is fungus-free, as is the central cortex (C); primary roots (PR) are intially uninfected but grow in close proximity to the rhizoids of protocorms (PC) formed by the gametophytes and seedlings, from which they may become infected with the same fungi.

infection (Fig. 1e), especially as hyphae sometimes grow out of gametophyte rhizoids (Lang, 1899). In *Huperzia*, the same three AM phylotypes were found in gametophytes and sporophytes (Winther & Friedman, 2007b). However, in *Botrychium crenulatum*, the diversity of fungal partners increased through the transition from myco-heterotroph to autotroph, commencing with two AM phylotypes in the subterranean gametophyte, increasing to four phylotypes in subterranean

sporophytes and peaking with nine phylotypes in the photosynthetic sporophytes (Winther & Friedman, 2007a). This accords with the findings of Kovács *et al.* (2007), who found between five and seven phylotypes of AM fungi in sporophytes of *B. virginianum*, all but two of which were in *Glomus* group A. Whilst the gametophytes appear to have the very high fungal specificity characteristic of myco-heterotrophic partnerships (Bidartondo *et al.*, 2002), some sporophytes presumably benefit from a wider range of AM fungi for mineral nutrition. However, despite the loss of fungal specificity in *Botrychium* sporophytes, the two gametophyte-associated AM phylotypes in *B. crenulatum* were consistently retained in all developmental stages of the plant and were found in the adjacent angiosperm, *Caltha palustris* (Winther & Friedman, 2007a).

Is mycorrhiza providing an intergeneration power line supporting undercover sex?

By freeing the gametophyte generation from the requirements to photosynthesize, myco-heterotrophy enables sexual reproduction to occur undercover in moist soil and litter environments that provide substantial protection from desiccation. The fidelity of fungal partners demonstrated by Winther & Friedman (2007a,b) suggests that AM mycelial networks can connect between the generations and allow autotrophic sporophytes to supply the small, but critical, amounts of carbohydrates required to support heterotrophic gametophyte generations – a form of parental nurture. Other species hosting the specific mycorrhizal fungi required by the gametophytes (e.g. *C. palustris* with *B. crenulatum*) may provide a similar service via epiparisitism. Records of gametophytes of *Lycopodium*, *Huperzia* and other species with myco-heterotrophic haploid generations have mostly come from sites with established sporophytes, although this will reflect sampling bias. Experimental evidence is now required to establish the relative importance of parental vs epiparasitic nurture. Are the benefits of inter-generation nurture so great for some species that they offset any costs to the sporophyte in excluding other mycorrhizal fungi that may provide greater access to mineral nutrients? Are the fungal symbionts of gametophytes sometimes so restricted in their distributions or host ranges that parental nurture is more important than epiparasitism of unrelated plant species? Which other plant species support the crucial fungi?

Fungal benefits from fidelity in a 'give now-but get more later' carbon economy?

Why do the fungi remain with the plants? Are there significant pay backs? The fidelity of specific fungal partners between the myco-heterotrophic and autotrophic life phases of lower plants, shown by Winther & Friedman (2007a,b), may enable fungal partners to gain long-term benefit from a 'give now, but get-more later' carbon economy like that recently demonstrated in the life cycle of an orchid (Cameron *et al.*, 2006). The carbon invested in myco-heterotrophic orchid seedlings by their fungal partners can later be returned 'with interest' once seedlings become autotrophic, providing that fungal fidelity is maintained across the two life stages (Cameron, *et al.*, 2006). Further studies are required to confirm the extent to which mycorrhizal fungi participate in relationships based on initial carbon investment for long-term return and whether this has provided an easy route to the cheating behaviour of fully myco-heterotrophic species (Leake, 1994). In long-lived perennial and evergreen species, such as many members of the Lycopodiaceae, there is considerable scope for long-term fungal reward with plant carbohydrate from sporophytes.

Convergent evolution between myco-heterotrophic gametophytes of lower trachaeophytes and myco-heterotrophic higher plants

Myco-heterotrophy in lower plant gametophytes allows their sporophytes to invest less reserve carbohydrates in individual spores, so that prodigious numbers of tiny propagules can be produced – acting as emissaries for finding their fungal partners and colonizing new habitats. The equivalent strategy of maximizing seed production and wind dispersal, by minimizing maternal investment in individual seeds and relying on myco-heterotrophy to make good a deficiency in reserve carbohydrates for seedling establishment, is a characteristic feature of orchids – one of the most advanced groups of higher plants (Cameron *et al.*, 2006). Indeed, there is striking convergent evolution of plant–fungus structure–function and myco-heterotroph-to-autotroph life cycle relationships between the achlorophyllous gametophytes of lower tracheophytes and achlorophyllous seedlings of orchids (Fig. 1). The subterranean orchid seedlings and lower plant prothalli are both tuberous structures that share the same unique term – protocorm. These have parallel adaptations for securing and sequestering fungal-derived carbon and have requirements for economy of carbon use. This convergence is especially remarkable in view of the wide plant and fungal divergence in the evolutionary origins of these partnerships, the lower plants being associated with AM fungi and the orchids mainly with basidiomycetes.

Because myco-heterotrophy results in plant structural simplification (Leake, 1994), morphological analyses alone are unable to establish whether myco-heterotrophic gametophytes are an ancient or a relatively recent trait (Duckett & Ligrone, 1992), but there is little doubt that it has been an extremely successful one. The fact that extant members of some of the earliest ancestral groups of land plants depend upon myco-heterotrophy for establishment from spores indicates either that this is an ancestral trait or that it has been more recently evolved to overcome the structural and functional limitations to competitive success imposed by the small stature and

relatively slow growth rates that are characteristic of basal plant groups.

By establishing that AM fungi are the essential symbionts involved in nurturing the gametophyte generations of some members of the Lycopodiaceae, Winther & Friedman (2007b) give impetus to the requirement for functional studies of these associations, to understand their evolutionary origins and roles in early land plant terrestrialization and radiation.

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Key words: arbuscular mycorrhiza, convergent evolution, fungal specificity, gametophyte, inter-plant carbon transfer, orchid.

Predicting evolutionary consequences of pollinator declines: the long and short of floral evolution

Recent research indicates that pollinator populations across the globe are declining (Allen-Wardell *et al.*, 1998; Biesmeijer *et al.*, 2006; National Research Council Committee on the Status of Pollinators in North America, 2007). This finding has been a prominent feature in recent news stories and headlines in the popular press, for the expectation is that the large fraction of plants that rely on animal pollinators to reproduce may therefore suffer reduced reproduction, and under some circumstances, population reductions (Knight *et al.*, 2005, Biesmeijer *et al.*, 2006; National Research Council Committee on the Status of Pollinators in North America,

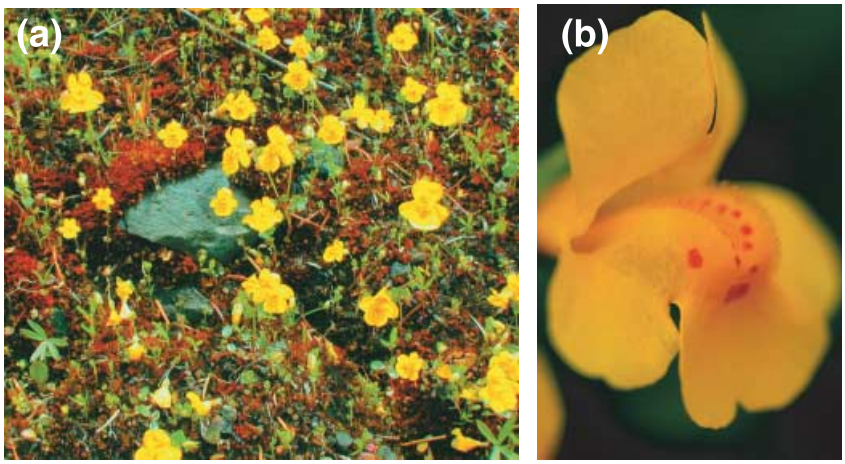

Fig. 1 *Mimulus guttatus* (yellow monkeyflower). (a) A small portion of the Iron Mountain (Oregon) population of *M. guttatus* (Phrymaceae). Photo courtesy of Megan Hall, Duke University. (b) Close-up of a single flower of *M. guttatus*. Fishman & Willis found that when pollinators are present, selection favors larger *M. guttatus* flowers, but when pollinators are absent, selection favors smaller flowers with anthers and stigma close together. The anthers and stigma are inside the corolla and are not visible in this view. Photo courtesy of Megan Hall, Duke University and NYU.

2007; Pauw, 2007). Closer to the hearts, and the stomachs, of most newspaper readers are the potential consequences of pollinator declines for human food supplies (National Research Council Committee on the Status of Pollinators in North America, 2007; Klein *et al.*, 2007). Both of these ecological effects are certainly of immediate concern, but little attention has been given to the potential evolutionary consequences of reduced pollinator populations. In this issue of *New Phytologist*, Fishman & Willis (pp. 802–810) show that the direction and magnitude of selection on floral traits can change dramatically when pollinators are not available. Selection when pollinators are scarce favors smaller flowers that are better able to self-fertilize when unvisited. This raises the possibility that as pollinators decline, flowers may evolve to become smaller and less noticeable, which may accentuate the problem. Evolutionary effects will take longer to arise than will ecological effects, but may be more insidious, fundamentally altering the phenotypic and genetic makeup of plant populations, and the composition of native plant communities.

'These results imply an antagonism between selection for selfing when pollinators are rare, and selection for a different suite of floral traits when pollinators are more abundant.'

Fishman & Willis worked with *Mimulus guttatus*, an annual plant that reproduces mostly through outcrossing when pollinators are present. However, when unvisited, the flowers are also able to self-pollinate automatically. This can happen because the female receptive surface (stigma) in the flower is positioned very near the pollen-producing structures (anthers). This separation between anther and stigma is known as herkogamy and varies substantially among plants. The capacity for autonomous seed production provides reproductive assurance when mates or pollinators are scarce (Kalisz *et al.*, 2004), but has the drawback that it often results in fewer, inbred seeds. Inbreeding depression is substantial in this species, reducing offspring performance by 69% (Willis, 1993), so this is not a minor concern. Still, self-pollination is a common reproductive strategy in plants, and many close relatives of *M. guttatus* (such as *Mimulus micranthus*) reproduce exclusively through selfing. Those selfing species generally have smaller flowers and very little herkogamy (Fig. 1).

To understand in greater detail the consequences of pollinator scarcity, Fishman & Willis applied a factorial design of pollen supplementation and pollinator exclusion to over 400 plants in an Oregon population of *M. guttatus.* They found that pollen supplementation increased seed production and pollinator exclusion strongly reduced it, indicating that seed production was pollen-limited. A particularly novel part of this study was that the authors also quantified selection on floral characters for these plants, allowing more powerful analysis and inference (Ashman & Morgan, 2004; Ashman *et al.*, 2004). When pollen was abundant, selection via seed production favored plants with relatively long and wide flowers. When pollinators were not available, reproduction was much reduced, but selection favored narrow-flowered plants with little herkogamy, a morphology that facilitates autonomous self-pollination.

These results suggest that *M. guttatus* plants experiencing different pollinator abundances will be subject to strongly divergent types of selection. Importantly, the pattern of selection when pollinators are absent is in the direction of the patterns found in selfing relatives. If pollinator abundance varies strongly in time or space, as seems to be common in wild populations of many plants (e.g. Moeller, 2005), the selective surface (averaged over years and sites) for *M. guttatus* might be visualized as having two fitness peaks (Fig. 2). One of the peaks applies when pollinators are available but plants

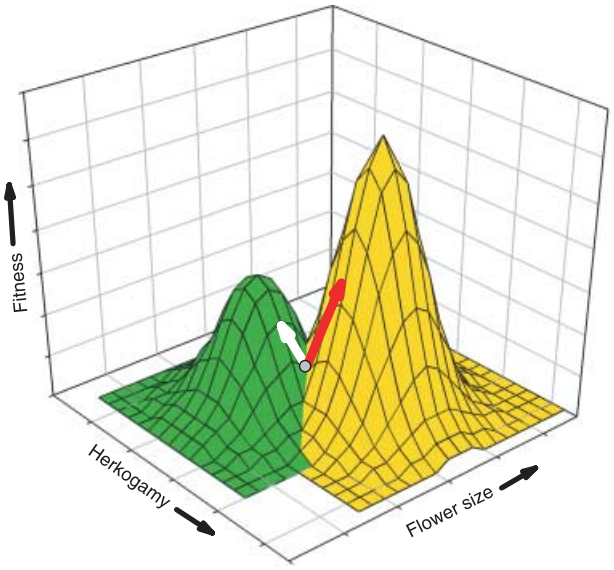

Fig. 2 Hypothetical fitness surface for plants that experience variation in selection as a result of variation in the pollinator environment. The green area (peak on the left) applies when pollinators are rare and most flowers will only produce seeds through autonomous selfing. The yellow area (peak on the right) applies when pollinators are sufficiently abundant that most flowers produce some outcrossed seeds, but not so abundant that all flowers are fully pollinated. The gray dot indicates the location of a population on the fitness surface, and the two arrows show expected trajectory under two conditions: no pollinators (white arrow on the left); or pollinators present (red arrow on the right). The direction of evolutionary change depends on the pollinator environment at that time.

must compete for visitation. In this situation, selection favors large flowers with substantial herkogamy. The other peak occurs when pollinators are rare. There, selection favors small flowers with little herkogamy. The results of Fishman & Willis suggest that over time (and assuming that fitness through seed production mirrors total fitness) the morphology of *M. guttatus* populations will wander between these two fitness peaks, with the direction of travel related to the history of pollinator abundance and the genetic correlations among these traits. The position and relative height of the peaks might change if seed fertility does not correlate well with overall fitness. This depends on the extent to which male function (pollen donation) and survival to reproductive age covary with these morphological characters. Other complicating factors to consider include the quality of pollen arriving on stigmas (Aizen & Harder, 2007) and the level of inbreeding depression. Future research that more fully documented selection via lifetime total fitness would be valuable, as would work that assessed a range of pollinator abundances, and thus would allow exploration of and characterization of the entire selective surface.

What is especially intriguing is that these results also imply an antagonism between selection for selfing when pollinators are rare, and selection for a different suite of floral traits when pollinators are more abundant. The balance between these deserves both empirical and theoretical investigation, which could provide answers to important questions about, for example, the conditions and amount of time required to generate a selfing species such as *M. micranthus* from a primarily outcrossed species like *M. guttatus*, or the evolutionary conflicts faced by plants with mixed-mating systems (for example, is a jack-of-all-trades phenotype possible, and, if so, what features would it have?).

The factorial manipulation of pollen addition and pollinator exclusion, as used in this study, allows another surprising insight. In the open-pollinated treatment, selection favored larger flowers. Most studies of selection on floral traits include only such unmanipulated plants. That part of the analysis for this population of *M. guttatus* indicates that the cause of selection is pollinator response to flower size. The role of pollinators could, in principle, be confirmed or denied with pollinator observations. However, large-flowered plants were also favored when pollen was added to plants that were never visited by pollinators, a treatment that equalizes pollen receipt of large-flowered and small-flowered plants, and eliminates the opportunity for pollinator preference to operate. Fishman & Willis suggest that this association between large flowers and high seed production, regardless of pollinator activity, may be mediated by correlations of floral traits with other factors such as individual environment or inbreeding history. More generally, this result joins a growing literature suggesting that floral traits often are developmentally correlated with other traits that have important effects on fitness (Herrera, 1995; Frey, 2004), and raises important questions about the extent to which floral phenotypes serve only to affect pollination (Galen, 1999).

One might be tempted to conclude that the results of Fishman & Willis suggest that in a world of declining pollinator populations the flowers themselves may begin to evolve to be less attractive and less reliant on pollinators, which might then reinforce pollinator declines. But in truth, their results highlight how little we know mechanistically about the link among pollinator abundance, natural selection and likely evolutionary trajectories.

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Key words: floral evolution, flower size, herkogamy, inbreeding depression, *Mimulus guttatus*, plant natural selection on floral traits, plant reproduction, pollinator decline.

Letters

The observer effect in plant science

What is known of observer effects

Plants have been widely documented to respond to mechanical stimuli such as wind and touch. Well-known and long-studied examples of these are carnivorous plants (e.g. Darwin, 1893), but nonspecialized plants are also sensitive and responsive to mechanical perturbation. Studies on this phenomenon, called 'thigmomorphogenesis' (Jaffe, 1973), have been conducted for several decades, revealing complex signaling and response pathways (Braam, 2005). Common thigmomorphogenetic responses include altered shoot elongation vs radial expansion ratios, delayed flowering, changes in chlorophyll content, etc. (see Biddington, 1986 and Cahill *et al.*, 2002 for a review and a concise overview, respectively). In nature, such changes usually occur in response to wind and as a result of contact with neighbouring plants. Humans can unwillingly mimic these effects when studying plants, as several studies have shown that the mere act of touching plants by hand can have significant effects (Braam & Davis, 1990; Cahill *et al.*, 2001). Moreover, in a considerable number of plant studies, measurements are not limited to touching plant tissue but include destructive sampling of leaves, roots, etc. It is apparent that if such (repeated) plant measurements, whether destructive or nondestructive, affect plant functioning, this could have far-reaching implications. Nevertheless, the attention given to such 'observer effects' in plant science has been limited.

Implications

If studying plants indeed implies involuntarily altering their morphology and/or physiology, then two main problems could arise. First, in studies on the state of nature (e.g. ozone damage in European forests, Ferretti *et al.*, 2007), the presence of an observer effect could cause such assessments to deviate from reality, leading to erroneous conclusions. Second, in studies with an experimental treatment, a further problem arises if handling plants results in different effects in the different treatments (as already suggested by Cahill *et al.*, 2001). Such a treatment × handling interaction would again distort the study's results and conclusions, as it implies inflation or understatement of the treatment effects. As treatment studies are often future oriented (e.g. investigating the effects

of elevated CO_2 concentrations or increased temperatures), this could subsequently lead to an incorrect impact assessment of several global changes.

Experimental example: POP-EUROFACE

As an example from an actual experiment, we processed data from POP-EUROFACE in Central Italy (42°22′N, 11°48′E), a large-scale experiment for studying the long-term effects of elevated CO_2 concentrations on carbon sequestration and bio-energy production in a short rotation coppice. An overview of the set-up can be found in Scarascia-Mugnozza *et al.* (2006). During six consecutive years (1999–2004), poplars were exposed to elevated CO_2 concentrations (550 ppm) in three free air CO_2 enrichment (FACE) areas, and three areas with ambient CO_2 concentrations served as a control. Each area was divided into six sectors that were planted with three different poplar species (*Populus alba* L., *P. nigra* L. and *P.* × *euramericana*). Throughout these 6 yr, over 10 different research teams carried out measurements in this plantation, from the leaf level up to the canopy scale. Most of the wide array of common ecological measurements took place in 'permanent growth plots' (PGPs), and consisted of both destructive (e.g. leaf chlorophyll, nitrogen, rubisco, leaf area, soil coring) and nondestructive (e.g. tree diameter, height, canopy light transmission) measurements, with a similar intensity in each year. The PGPs consisted of a group of six adjacent trees within each sector, surrounded by at least one row of trees of the same genotype and treatment (Supplementary material Fig. S1). To assess the occurrence of observer effects, trees with very limited exposure to handling were randomly selected from the remaining poplars inside each sector (i.e. 18 trees during the first rotation (until 2001), and nine trees during the second rotation (until 2004)). In this study, we compared poplar biomass production (scaled up from stem diameter via allometric relations, cf. Calfapietra *et al.*, 2003; Liberloo *et al.*, 2005, 2006) inside and outside the permanent growth plots, to assess the following: whether an observer effect was detectable; whether there was an interaction of this effect with the CO_2 treatment; whether the three poplar species responded differently to handling; and whether any of the observer effects changed over time. To this end, data sets from 2000, 2001, 2003 and 2004 were used. Stem diameter was the only measurement consistently made inside and outside PGPs during the course of the experiment, but is considered a suitable parameter for a general assessment of observer effects because of its nondestructive nature and its use as a proxy for tree vigour and health.

Data were examined in SAS (SAS 9.1; SAS Institute, Cary, NC, USA), first using an analysis of variance (ANOVA) with repeated measures in time on the biomass data (log transformed for normalisation) to test the significance ($P < 0.05$) of observer effects. The design was a randomized complete block, with CO_2 treatment, species, year and plot identity (PGP or non-PGP) as fixed factors, block (i.e. the combination of one control and one treatment area) as a random factor, and plot as the unit of replication. Upon confirmation of the significance of observer effects, an identical-repeated measures ANOVA was then performed on the observer effect itself (i.e. the percental difference in above-ground biomass between PGPs and non-PGPs (these data had a normal distribution after removal of one outlier)), to test specifically for effects of treatment, species or year (fixed factors). An *a posteriori* comparison of means was performed with the Bonferroni correction for multiple comparisons.

The analysis showed that trees inside and outside PGPs differed significantly ($P < 0.001$), with biomass reduced by up to 50% because of handling (Fig. 1). The observer effect differed between species ($P < 0.01$), with significantly lower adverse effects of handling in *P. alba* compared with the other two species ($P < 0.05$ after correction). Observer effects were furthermore strongly affected by the measurement year ($P < 0.001$) as they only reached significance in the last 2 yr of the study (2003–2004). Finally, *a posteriori* comparison revealed a trend towards differences in the size of observer effects in both treatments for *P.* × *euramericana* ($P = 0.10$ after correction), which was also visible in Fig. 1. Other factors and interactions were not significant.

Discussion of experimental results

The experiment that we scanned for observer effects yielded several interesting results. In general, handling was found to decrease productivity and did so by proportionally the same extent under treatment and control conditions, even though there were indications that the observer effect differed somewhat between treatments for one species. Our data thus provide further evidence to disprove the assumption of researchers as 'benign observers', as indeed the act of conducting an experiment can alter the experimental results (Cahill *et al.*, 2001). The general absence of observer effects in 2000 and 2001, and the markedly steep decline in biomass inside vs outside PGPs in the last 2 yr of the study, furthermore suggest that adverse handling effects can build up (even across coppicing events), as the measurement intensity was similar in all years. Such an effect would be of particular importance in long-term experiments in which the same plots and plants are sampled continuously (similar to POP-EUROFACE). The recent trend towards such long-term studies (e.g. Wullschleger & Hanson, 2006; Mohan *et al.*, 2007), invoked by their great scientific value, especially in determining impacts of global changes beyond single growing seasons, could therefore lead to a growing relevance of observer effects. It must be noted that, because there was no general treatment × handling interaction, conclusions of the POP-EUROFACE studies regarding effects of elevated CO_2 concentrations on poplar growth were probably correct.

Apart from direct effects of measuring, observers can also cause indirect effects that affect plant functioning. Among these are altered incidence of herbivory or plant diseases (Latimer & Oetting, 1999; Niesenbaum *et al.*, 2006), soil compaction

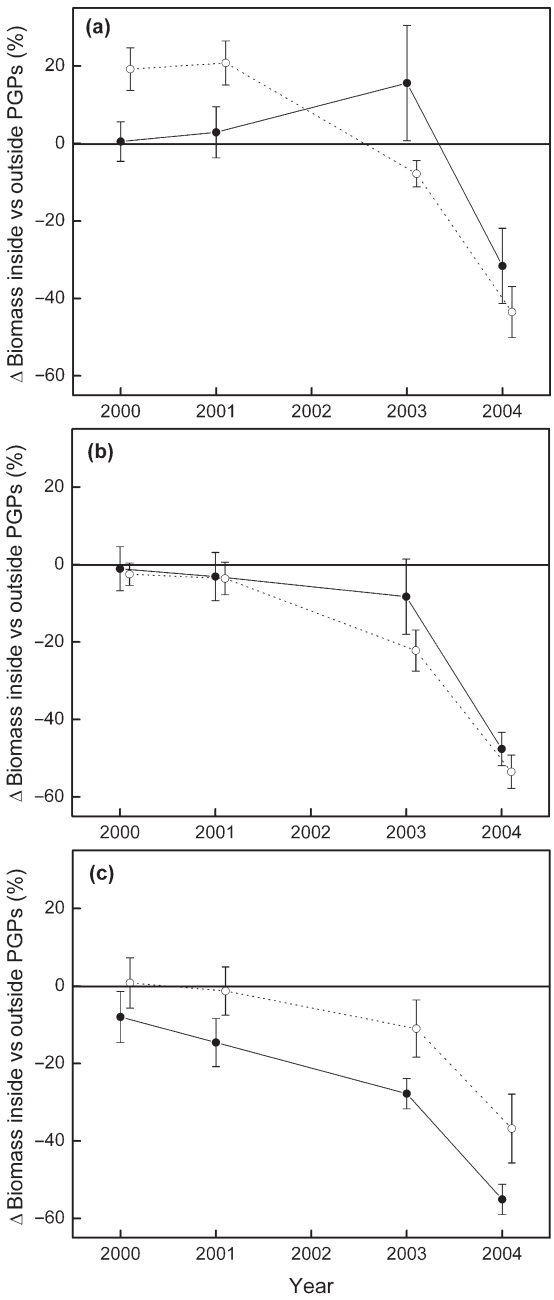

Fig. 1 The percental difference in above-ground biomass (calculated from stem diameter via allometric relationships) between trees inside and outside permanent growth plots (PGPs), growing under ambient (open symbols, dashed line) or elevated (closed symbols, solid line) CO_2 concentrations. Three *Populus* species are depicted: (a) *P. alba*; (b) *P. nigra*; and (c) *P.* × *euramericana*. Only averages and standard errors are shown. Symbols are slightly shifted, with respect to the *x*-axis, for clarity.

(Hik *et al.*, 2003; Andres-Abellan *et al.*, 2005) and changes in light conditions (Cahill *et al.*, 2001). An indirect effect that probably caused a proportion of the observer effect in the POP-EUROFACE experiment was the combination of a windy site and the presence of scaffolding towers, causing mechanical damage to (predominantly) the PGP trees. We rule out that observer effects were solely attributable to the towers, as these were located at one side of the PGPs (Supplementary material Fig. S1) and therefore did not impact all PGP trees (affirmed by visual inspection of the damaged tree tops). As significant biomass reduction was found throughout the PGPs, negative direct impacts of measurements and sampling must have contributed to the observed growth reduction. The multitude of possible observer effects, both direct and indirect, renders it extremely difficult to predict their combined outcome. Moreover, sampling has been documented to affect different species in different ways (Hik *et al.*, 2003), which was confirmed by the POP-EUROFACE data. This further increases the difficulties of quantifying observer effects, and hence makes it paramount to avoid or minimize such effects in the first place.

Dealing with observer effects

In animal studies, and especially those concerned with behaviour, observer effects have long been known and acknowledged (Wade *et al.*, 2005). In that field of research, avoidance is also deemed the best strategy for coping with observer effects rather than taking these into account somehow (e.g. Baker & McGuffin, 2007). In plant science, noninvasive techniques exist as an alternative to certain destructive measurements (such as leaf area determination). However, accuracy problems often make these alternatives less reliable (e.g. Broadhead *et al.*, 2003). In many cases it is unavoidable that researchers do exert an influence, as, for example, cuvette measurements (which can damage leaves) provide data that are often essential but currently impossible to collect in less intrusive ways. Given the constraints imposed by the measurement technology currently available, the most appropriate solution to minimize observer effects seems to be to lower the measurement intensity. This can be achieved either by taking fewer samples per unit of time, or by spreading out the measurements over a larger number of study objects (plants, communities, etc.). Two main problems are associated with this. In the first case, reducing the number of samples would lower the statistical power, whereas the second proposed solution goes against the often-adhered researcher's philosophy to make full use of the money granted by maximizing the amount of data collected per unit of currency funded. Nevertheless, to avoid the risk of the experimental results becoming flawed, either of our two proposed solutions should be considered. Of course, only in hindsight can it be confidently stated whether the applied intensity affected plant functioning. It would nevertheless be prudent to design all sampling protocols for minimal disturbance while maintaining a statistically adequate number of data samples. This is especially relevant for treatment-type studies, in which the same limited number of experimental objects are sampled continuously and which therefore seem much more prone to

oversampling than state-of-nature studies that usually have a lower sampling intensity.

Expanding knowledge

Field experiments regarding observer effects have almost uniquely been conducted to test the 'herbivory uncertainty principle', which states that researcher visitation and plant measurements may alter herbivore and pathogen damage (Cahill *et al.*, 2001; Schnitzer *et al.*, 2002). The manipulations in these type of experiments can be considered as mild, as they consist mainly of visual observations and height measurements. Even under these conditions were observer effects, although not consistently (e.g. Bradley *et al.*, 2003; Cahill *et al.*, 2004). We have demonstrated that in a long-term experiment with frequent and invasive measurements, observer effects are potentially larger (although the largest effects were observed only in the later stages). To elucidate the uncertainties associated with observer effects, research is needed: to unveil the generality of observer effects (i.e. whether they are more outspoken in certain ecosystems (e.g. tundra) and species or functional groups than in others); to clarify the relationship between measurement intensity and effect (i.e. is there a dose–response relationship (linear or otherwise) or are there thresholds?); to assess which types of measurements have the largest impact; and to uncover which plant process is the most sensitive to handling. To help resolve these important questions, we advise leaving part of any experiment unsampled, allowing for an *a posteriori* assessment of observer effects (such as for POP-EUROFACE).

Conclusions

From contacts with international colleagues, we understand that a majority of scientists dealing with long-term experiments are aware of the existence of observer effects. However, by quantifying these effects, we have shown that the often underlying assumption that they are negligible is not necessarily true. Observer effects should therefore always be considered in setting up new experiments and drawing up sampling strategies, by focusing on minimizing disturbance. Such considerations are, in our eyes, vital to further plant research. Indeed, the issue of observer effects is a genuine concern, which will not be resolved by ignoring its existence.

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Key words: FACE, handling, manipulation, observer effects, plants, thigmomorphogenesis.

Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Layout of the POP-EUROFACE plantation with two poplar fields, divided by a country road, and six experimental areas (black = free air CO_2 enrichment (FACE)).

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2007.02329.x
(This link will take you to the article abstract).

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Two allopolyploid ascomycete fungal plant pathogens were not rescued by vertical transmission

In a recent letter to *New Phytologist*, Selosse & Schardl (2007) convincingly argued that, after the allopolyploid hybrid grass endophytic *Neotyphodium* spp. were first formed, they were 'saved' by conversion to vertical transmission, with the primary sexual horizontal transmission mechanisms of the 'parent' species being lost. They further argued that the hybrids probably arose through parasexual crosses after the dual colonization of a plant species which was an 'unusual' host for both the 'parental' pathogenic *Epichloë* species. Rightly, in our opinion, they stated that their model 'emphasizes the role and consequences of hybridization in fungal evolution', adding that '[it] may apply to a much wider range of fungi'. Whilst we agree with these authors on the important role that interspecific hybridization giving rise to allopolyploid hybrids can play in fungal evolution and accept that their model may apply to other fungi, particularly other endophytes, we wish to point out that it does not apply to the two known cases of allopolyploid fungal plant pathogens.

Verticillium wilts

Vascular wilt diseases caused by *Verticillium* species occur in a wide range of dicotyledonous hosts. The pathogens are soil-borne hemibiotrophs, infecting via roots and colonizing the plant before long-lived resting structures (microsclerotia or dark resting mycelium for the two most important species) are produced during a necrotrophic stage. Resting structures are returned to the soil in plant debris and may persist for several years in the absence of hosts (Pegg & Brady, 2002). Long-spored *Verticillium dahliae* isolates have been shown to be allopolyploid (or amphihaploid, a term we have previously used to describe the specific case of two almost entire haploid genomes, each derived from different species, occurring in a single nucleus; Barbara & Clewes, 2003; Collins *et al.*, 2003) hybrids between haploid, short-spored *V. dahliae* and a second species, initially thought to be *V. albo-atrum* (Karapapa *et al.*, 1997) but which is molecularly distinct from known isolates of this species and may represent a currently undescribed species (Barbara & Clewes, 2003; Collins *et al.*, 2003; E. Clewes *et al.*, unpublished). Molecular studies have divided the allopolyploid isolates into three types, which in part parallel host specificity in the field (Collins *et al.*, 2003; E. Clewes *et al.*, unpublished). With very few possible exceptions the hybrids occur only in crucifers and with two main exceptions only long-spored isolates have been obtained from crucifers.

Both long- and short-spored isolates occur in horseradish (*Armoracia rusticana*) in the USA (Babadoost *et al.*, 2004) and in horticultural crucifer crops (notably Chinese cabbage (*Brassica rapa*) and Japanese radish (*Raphanus sativus*)) in Japan (Horiuchi *et al.*, 1990). Many other species can be infected with allopolyploid isolates experimentally but at present there is no evidence that natural infection of species outside the Brassicaceae is important or that they actually act as reservoirs for such isolates in the field, as has been suggested might occur (Johansson *et al.*, 2006). Whilst both allopolyploid and haploid isolates of *V. dahliae* can be seed-borne (Pegg & Brady, 2002), occasionally at high rates, for example, in lettuce (*Lactuca sativa*) (Vallad *et al.*, 2005), the levels of allopolyploid isolates in seed from naturally infected plants, where studied, appear to be low (e.g. Heppner & Heitefuss, 1995) and the primary route of transmission of allopolyploids in crucifers is horizontal via resting structures (microsclerotia) returned to the soil in plant debris.

At present, when or where these three groups of hybrids arose is not known, or even if they represent three separate hybridizations or divergent lines from a single event, but unless they have subsequently reverted to horizontal transmission (which seems unlikely) the hybrids have not been 'saved' by switching to vertical transmission in the continuing presence of horizontally transmitted 'parents' as proposed for endophytes by Selosse & Schardl (2007). However, the novel host part of the endophyte model may apply to the *Verticillium* hybrids. Apart from the exceptions already mentioned, nonhybrid isolates of either *V. dahliae* or *V. albo-atrum* are not isolated from crucifers in the field. As the 'non*dahliae*' parent has not been unequivocally identified, be it either *V. albo-atrum* (as originally suggested by Karapapa *et al.*, 1997) or another species (Collins *et al.*, 2003, 2005), we cannot rule out the possibility that it infects crucifers (perhaps only occasionally or in a little-studied species or geographic region) but as candidate isolates with the appropriate molecular attributes have been sought in crucifers but not found (Barbara & Clewes, 2003; Collins *et al.,* 2003, 2005) we assume that it normally occurs in a noncrucifer host. Therefore it is probable, but not certain, that the hybrid has an altered host range relative to its 'parents'.

As these fungi have no sexual stage, we assume that hybridization occurred through hyphal fusion followed by nuclear fusion. In accordance with the model of Selosse & Schardl (2007), the most obvious site for this would be in a dually colonized crucifer which is a poor host for both the parental haploid species; spread of the hybrids to other crucifers and host selection would then have led to the development of a widespread host-specific pathogen. The identity of the host in which hybridization occurred is not known. Horseradish or the horticultural crucifer crops of Japan seem unlikely, as haploid *V. dahliae* infect them commonly and they would not have provided the necessary selective advantage. A crucifer slightly more resistant to haploid isolates than these but still susceptible to low-level infection seems more likely. An alternative scenario is that fusion between the 'parent' haploid species occurred in a doubly-infected noncrucifer plant which is a good host for both species. However, in such a host, there would be no immediate selective advantage for the hybrid and the fused nuclei, presumably few in number relative to those of the 'parental' species, would have to have been incorporated into microsclerotia, returned to the soil and infected a new (crucifer?) host resistant to the 'parental' species before they had any selective advantage. A second alternative is that hyphal fusion occurred before root penetration following germination of resting structures of the two 'parent' haploid species physically close in the soil. In this scenario, it is possible that the first host of the hybrid was actually a complete nonhost for the haploid species (as long as it was capable of stimulating germination of the resting structures) and would therefore apply much stronger selection pressure for the novel pathogenicity of the hybrid. Whether fusion between the hypha from germinating resting structures is possible has not been studied.

Botrytis species causing onion neck rots

Neither part of the model proposed for grass endophytes by Selosse & Schardl (2007) appears to hold for allopolyploid hybrids of *Botrytis* which occur exclusively on onion (*Allium cepa*) and related species and can cause neck rot, most often in storage. These hybrids and their 'parents' are soil-borne pathogens overwintering as sclerotia on debris or free in the soil. Although they can be seed-borne, transmission is primarily horizontal. *Botrytis allii* has been divided into two groups, one with small conidia and 16 chromosomes and the other with large spores and 32 chromosomes (Shirane *et al.*, 1989). Molecular studies using a number of markers have convincingly shown that isolates of the 32-chromosome type are not autodiploids derived from the 16-chromosome type as suggested by Shirane *et al.* (1989) but arose as an allopolyploid hybrid between *Botrytis aclada* and *Botrytis byssoidea* (Nielsen *et al.*, 2001; Nielsen & Yohalem, 2001; Yohalem *et al.*, 2003; Staats *et al.*, 2005). It has now been proposed that the name *B. allii* be applied specifically to the hybrid species (Yohalem *et al.*, 2003). None of the three species has a sexual stage and it has been suggested that sclerotia may have acted as a recipient for spermatia (microconidia) produced by *B. aclada* (Staats *et al.*, 2005). As all three species infect *Allium*, the hybridization presumably took place either on a common host or in the soil near it. There seems no reason to invoke a species that is a poor host for the 'parents' but good for the hybrid as no new host specificity has arisen.

As with *Verticillium*, transmission of both the hybrids and haploid 'parents' remains primarily horizontal and a switch to vertical transmission does not account for the success of the hybrids. This may need re-investigation, however. Transmission of *B. allii* from seed to seedling has been reported, but, both in the original study (Tichelaar, 1967) and in more recent studies on seed transmission (du Toit *et al.,* 2004; Chilvers

et al., 2007), *B. allii* and *B. aclada* were not differentiated and it may be that only one of the species is transmitted in this way, possibly giving it an advantage. In contrast to *Verticillium*, it is also difficult to make a case for a new host specificity 'saving' the hybrid as all three species cause neck rots of onion with little, if any, difference between them (D. Yohalem, pers. com.). There may be some so far unnoticed ecological difference between the species that led to the hybrid persisting in the continued presence of the parental species, but to date we have seen no suggestions as to what advantage the hybrid *B. allii* might have.

Summary

As noted at the beginning of this letter, Selosse & Schardl (2007) have proposed a convincing model to explain the initial success of allopolyploid hybrid grass endophytes, based on a switch to vertical transmission and the development of new host specificities. Two examples of allopolyploid plant pathogens are known. New host specificity may be sufficient to explain the existence of the hybrid *Verticillium* isolates in cruciferous hosts, but neither hybrid has switched to primarily vertical transmission and to date we have seen no explanation for the success of *B. allii* on onions. In conclusion, the evidence suggests that their model cannot be applied directly to the currently known plant pathogenic allopolyploid hybrids.

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Key words: allopolyploid, *Botrytis*, endophytes, *Epichloë*, interspecific fungal hybrid, *Verticillium*.

New Phytologist

Meetings

Fungal networks made of humans: UNITE, FESIN, and frontiers in fungal ecology

UNITE/FESIN meeting, Dragør, Denmark, September 2007

The recent bloom of fungal community ecology can be credited in large part to the development of molecular tools for identification of fungi (Koljalg *et al.*, 2005). These tools have been largely self-assembled, as individual researchers have borrowed techniques from other fields such as molecular systematics and medical diagnostics and applied them to particular studies. As a result, the development of the field has had some resemblance to an unplanned boomtown; growth has been breathtakingly rapid but weak on development of coordinated infrastructure. To address these challenges, researchers have started to assemble into larger networks. Two such groups, the Fungal Environmental Sampling and Informatics Network (FESIN; http://www.bio.utk.edu/fesin/title.htm) and the User-friendly Nordic ITS Ectomycorrhizal database group (UNITE; http://unite.ut.ee/) held a joint meeting in September in Dragør, Denmark to struggle with the problems of building and maintaining infrastructure for the burgeoning field of fungal ecology.

'The relationship between biodiversity and ecosystem function remains a "holy grail" issue in ecosystem science ...'

UNITE and FESIN

UNITE led the way 5 yr ago by focusing on the problem of unreliable names and undersampled ectomycorrhizal taxa in GenBank and creating a sequence database for the identification of ectomycorrhizal fungi. FESIN is a newly formed group whose broad goals are to make fungi accessible to ecologists by (1) coordinating the development of rapid identification and information retrieval tools, (2) fostering interactions between mycologists and ecologists, and (3) supporting educational initiatives in fungal ecology. The UNITE/FESIN meeting was coordinated between the two groups because of the strong overlap in goals and interests. Both organizations are funded as research coordination networks, which means that they have funds to organize meetings, but not to do any actual research. It was realized by both groups that, to transcend this constraint, the best outcome of the Dragør meeting would be to spearhead community research proposals. To that end, the first part of the meeting had a strong focus on methods, so that current tools, and their limitations, were in the forefront of participants' minds.

Microarrays

Microarrays provided the axis for much discussion regarding the development of new methods in fungal ecology. Three different types of array were discussed. Gary Andersen (Lawrence Berkeley National Laboratory, CA, USA) presented a talk on development of oligonucleotide arrays for fungal identification. His group had previously developed such tools for prokaryotic taxa using Affymetrix chips (Wilson *et al.*, 2002; DeSantis *et al.*, 2005), and they are now directing their efforts to a fungal chip based on nuclear internal transcribed spacer (ITS) region and large subunit rRNA (LSU) sequences. The advantages of an Affymetrix array approach are manifold. Firstly, chips have a capacity of 500 000 probes in a 1.28-cm^2 array, which allows multiple probes for each operational taxomic unit (OTU) as well as mismatch probes to test for specific hybridization. Secondly, a single hybridization experiment can be used for each biological replicate and will simultaneously identify tens of thousands of taxa from mixed assemblages. Thirdly, results from array experiments yield presence/absence determinations for organisms differing in abundance by roughly five orders of magnitude; this method is currently much more sensitive at detecting low-abundance targets than sequencing of clone pools (although see pyrosquencing below). In addition, semi-quantitative changes in abundance can be measured between hybridizations (i.e. samples and replicates). The cost per array is estimated to be $200 USD, but this is after substantial development cost, and assumes one has access to the equipment needed to read the chips.

Overall, the UNITE/FESIN group saw the Affymetrix chips as an incredibly useful but untapped tool for analyzing fungal communities at large scales, and they discussed ways that the community might be involved in developing a consortium to lower the price and to test the chip across multiple habitats.

The main disadvantage with the identification array is that one cannot identify taxa that are not already known and sequenced. For this reason, it became obvious to the group that one of the best community-wide actions would be to ensure that Andersen's group (Lawrence Berkeley National Laboratory, CA, USA) has access to all available data, so that the chip would be widely useful across diverse study areas.

Functional gene arrays

Chris Schadt (Oakridge National Laboratory, TN, USA) presented an overview of 'functional gene arrays' (FGA) which target protein-coding genes important for ecosystem function. The relationship between biodiversity and ecosystem function remains a 'holy grail' issue in ecosystem science, and FGAs offer a rapid way to compare functional capacity across systems and conditions. In their current form, these arrays are printed 'in-house' on glass slides, and probes are relatively long and therefore tolerate more mismatches than the short oligonucleotide probes used in Affymetrix arrays. Environmentally extracted RNA is used to probe the arrays. This approach provides a view of functions that are expressed in the microbial community, and when coupled with random amplification methods it is sensitive enough to detect activities in even minute samples (Gao *et al.*, 2007). As with current identification arrays, the functional genes are primarily drawn from prokaryotic organisms (Wu *et al.*, 2001; He *et al.*, 2007), with the exception of genes for cellulose and lignin degradation which are heavily drawn from the fungi. However, the potential for extending the coverage of fungal functions is increasing as more fungal genome sequences become available.

Oligonucleotide fingerprinting

James Borneman (University of California Riverside, USA) discussed his oligonucleotide fingerprinting approach (Valinsky *et al.*, 2002). This is essentially a reverse array in which environmental clones are spotted and probed with a battery of oligonucleotide probes. He uses the approach to compare conditions, such as suppressive and nonsuppressive soils, and to pick out key organisms that are enriched by particular environmental conditions. Unlike the other arrays, this method is used for finding 'a needle in a haystack' rather than to enumerate all members of the haystack. It also allows one to identify previously unknown taxa because the probes are only used to screen clones, which are later sequenced if they prove interesting in their patterns of occurrence. The main limiting factor is that it is work-intensive, and the number of clones one can screen is still fairly modest (approx. 9600) – at least in relation to hyperdiverse systems such as soil. To work around this problem, Borneman is exploring the use of 'polonies', which are tiny colonies amplified from single molecules on acrylamide-coated slides (Mitra & Church, 1999). This would increase the screening capacity to over a million colonies per slide.

Sequencing and the future

Advances in sequencing technology are poised to increase the amount of fungal sequence by orders of magnitude in the next few years. It is clear that much of this increase will be from environmental samples – that is to say, sequences that are retrieved directly from complex substrates containing multiple unidentified fungi. Two examples were reported at the meeting. Lee Taylor (University of Alaska–Fairbanks, USA) discussed his work in boreal forest at Bonanza Creek Long-term Ecological Research (LTER) site. His group teamed up with the genome sequencing facility of the Broad Institute at Massachusetts Institute of Technology to produce over 70 000 sequences that included both the ITS and LSU regions. Ari Jumpponen (University of Kansas, USA) made it clear in his talk on pyrosequencing that even 70 000 sequences will be a small number in the near future. Although still costly, pyrosequencing avoids most of the PCR biases, eliminates cloning entirely, and yields megabases of sequence in a single run (Ronaghi *et al.*, 1996; Ronaghi *et al.*, 1998). Length of sequence reads has been a limitation, but improvements in chemistry and protocols are predicted to substantially increase the read length in the near future (Mashayekhi & Ronaghi, 2007). With sequence capacity increasing at such a rapid rate it seems possible that array approaches might be quickly replaced by direct sequencing, although the trade-off between resolution and replication will likely persist until the costs of sequencing drop substantially. Furthermore, it is clear that sequencing capacity has temporarily outpaced bioinformatics approaches; in both Taylor's and Jumpponen's work, one of the main bottlenecks has been analyzing the huge volume of sequences.

Fungal genomics projects represent another huge source of new fungal sequences that will revolutionize fungal ecology. Francis Martin (French National Institute for Agricultural Research (INRA)-Nancy, France) presented an update on these projects focused on taxa that might be of special interest to ecologists (e.g. *Laccaria*, *Tuber* and *Melampsora*). These data will have at least three applications in ecology: (1) elucidating gene expression and signaling between symbiotic partners; (2) inferring the functional capacity of sequenced taxa based on gene content; and (3) deriving population and phylogenetic markers from sequenced taxa and expanding their use to related taxa.

Although the methodological talks painted a bright future for fungal ecology, they also underlined the huge need on the bioinformatics side of the field. Simple tools that allow sequences to be used for identification such as BLASTN, galaxieblast (Nilsson *et al.*, 2004), and emerencia (Nilsson *et al.*, 2005) all require significant human interpretation to avoid inaccurate classification. This is largely because GenBank is filled with inaccurately identified sequences (Bridge *et al.*, 2004; Nilsson *et al.*, 2006) and because BLAST hits are strongly affected by sequence length. The option of expanding third-party databases such as the UNITE database is certainly available, but the permanence of these is not guaranteed and the vast majority

of data will always be in GenBank. Furthermore, it is almost certain that the bulk of future sequences will be environmental samples and thus will not be tied directly to an identified specimen. For these reasons, meeting participants were united in their call for third-party annotation of GenBank sequences (something that is not currently allowed), and for using community-based annotation in a concerted effort to clean up the problems in GenBank. This is a critical need for the field in the near term; existing data need to be corrected and reliable automated classification systems need to be developed before the onslaught of new data makes these goals much more difficult.

Global collaborations

With these methodological advances in mind, further discussion centered on large-scale community ecology projects that would apply such tools at continental or global scales and would involve collaborations among laboratories across Europe and North America. The basic idea was that by scaling up we could achieve a view of fungal systems that is unobtainable by individual research groups. This is likely to be an ongoing discussion over the next several years and there is no reason that it should be limited to development of only one such project, as there are many pressing questions that would benefit from community-wide efforts. The only danger in higher level coordination is that it can stifle innovation of individual researchers: as more resources are funneled into large community-wide projects, less may be available for independent studies. This danger may be avoided by integrating fungal ecology more fully into microbial and ecosystems studies so that it becomes a necessary part of many fields.

A list of participants, individual presentations, and more details on group discussions are available at the FESIN website (http://www.bio.utk.edu/fesin/title.htm). All interested are encouraged to attend the first public meeting of FESIN at the annual meeting of the Ecological Society of America in Milwaukee, Wisconsin in August 2008.

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