MINIREVIEW

Fungal cannons: explosive spore discharge in the *Ascomycota*

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Abstract

The ascomycetous fungi produce prodigious amounts of spores through both asexual and sexual reproduction. Their sexual spores (ascospores) develop within tubular sacs called asci that act as small water cannons and expel the spores into the air. Dispersal of spores by forcible discharge is important for dissemination of many fungal plant diseases and for the dispersal of many saprophytic fungi. The mechanism has long been thought to be driven by turgor pressure within the extending ascus; however, relatively little genetic and physiological work has been carried out on the mechanism. Recent studies have measured the pressures within the ascus and quantified the components of the ascus epiplasmic fluid that contribute to the osmotic potential. Few species have been examined in detail, but the results indicate diversity in ascus function that reflects ascus size, fruiting body type, and the niche of the particular species.

Introduction

Spores – the most recognizable of fungal cells – are produced in prolific quantities and sent forth to colonize fresh substrates. Members of the Kingdom Fungi disperse their spores through air and water using a variety of techniques (see Deacon, 2006). The members of the phylum *Ascomycota*, which constitute nearly 75% of all described fungi, produce their sexual spores (ascospores) in tubular sacs called asci; some asci act like small water cannons and expel the spores into the air (Fig. 1). Shooting asci have evolved to stretch upward and thrust the spores through a pore in the top of the sac (Figs 2 and 3). The mechanism has long been thought to be driven by turgor pressure within the extending ascus (DeBary, 1887). Dispersal of spores by forcible discharge is critical for the dissemination of nonmotile propagules of many fungal plant disease organisms as well as many saprophytic fungi. Despite the prevalence of this dispersal mechanism among economically important fungi, the mechanism has not been well studied in any fungus. Thus, in this review, the structure and function of the shooting ascus will be explored.

The phylum *Ascomycota* comprises three monophyletic subphyla: the Taphrinamycotina and the Saccharomycotina, which lack a complex fruiting structure, and the Pezizomycotina, which produce the multi-tissue fruiting bodies (Figs 2 and 3). Each subphylum contains members that forcibly discharge their spores. The Taphrinomycotina is the earliest diverging clade (James *et al.*, 2006). The genus *Taphrina*, which is the causal agent of peach leaf curl and produces naked asci that fire individually, and the woodland saprotroph *Neolecta*, which produces small fruiting bodies with asci that discharge their spores through a slit in the ascus apex (Landvik *et al.*, 2003), are examples from this group. *Metschnikowia bicuspidate*, a member of the subphylum Saccaromycotina, exhibits perhaps the most unusual use of the discharging ascospore. This brine shrimp pathogen fires its spear-shaped spores from the intestinal lumen of its host to colonize the body cavity (Lachance *et al.*, 1976). Thus, it uses the discharge capabilities as an infection method instead of as a dispersal method.

In the subphylum Pezizomycotina, the *Eurotiomycetes* is the only class devoid of discharging members. Members of this class form fruiting bodies with prototunicate (spherical) asci that release their spores passively as the walls of the asci degrade. The remaining six classes, the *Sordariomycetes*, *Leotiomycetes*, *Lecanoromycetes*, *Lichinomycetes*, *Dothideomycetes*, and *Pezizomycetes*, contain both species that do and do not discharge their spores. The nonshooting members may have fragile asci, which dehisce and subsequently extrude spores through the neck; whole asci, which are extruded intact; or asci that simply do not forcibly fire spores.

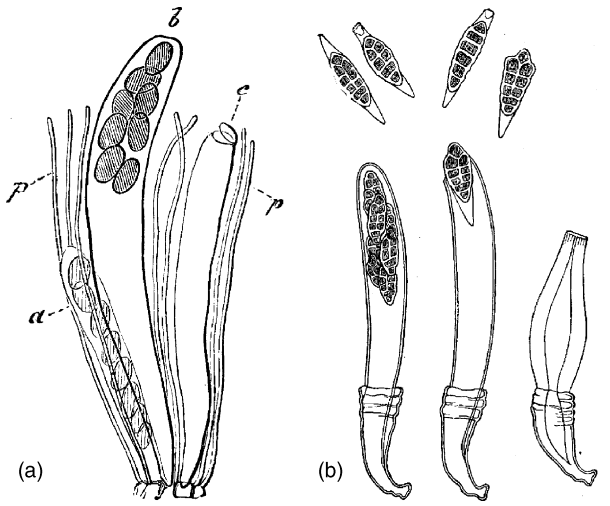

Fig. 1. Ascus structure and function. (a) Unitunicate asci with mature spores (*a*, *b*) and accompanying paraphyses (*p*) from *Ascobolus* sp. Ascus extending up to fire spores (*b*). Discharged ascus showing operculum (*c*). (b) Stages of ascospore discharge in a bitunicate ascus of *Pleospora scirpi*. Outer wall has dehisced and inner wall is extended (left). Spores are released from inner wall (center). Ascus following discharge (right). From (DeBary, 1887).

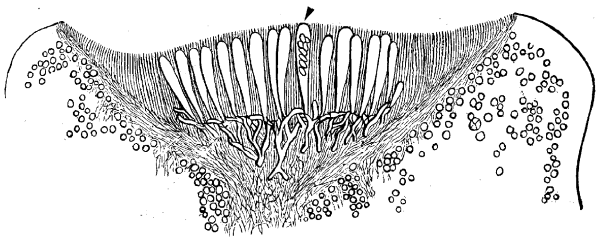

Fig. 2. Apothecium of *Lecanora subfusca* showing mature ascus (arrowhead) developing in fertile layer. Asci fire in coordinated bursts of hundreds of asci called 'puffing'.

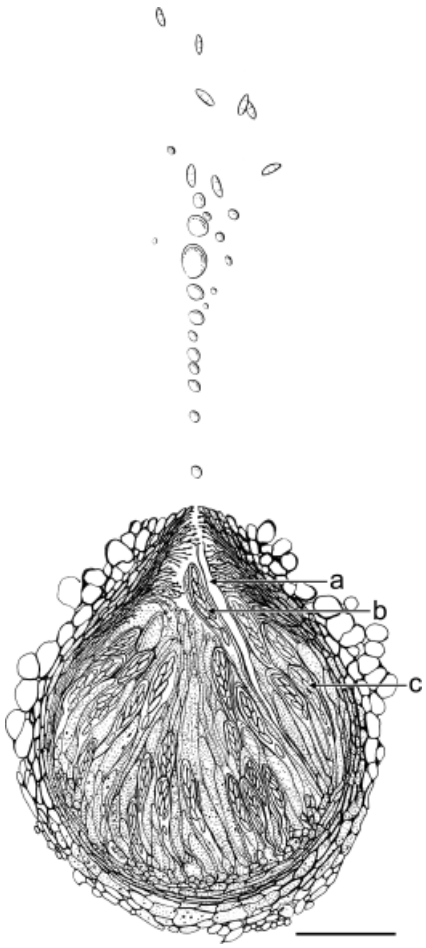

Fig. 3. Perithecium of *Gibberella zeae* showing ascus (a) discharging its contents of spores and epiplasmic fluid (droplets), a mature ascus extending upward ready to fire (b), and a mature unextended ascus (c). Scale bar = 50 μm. Reproduced from Trail *et al*. (2005), by permission.

That almost all taxonomic categories within the phylum *Ascomycota* contain ascospore-discharging species suggests that forcible discharge is a primitive condition. Berbee & Taylor (1992) used the 18S rRNA gene to compare the phylogenetic ranking with phenotypic characteristics of asci and fruiting bodies in a set of perithecium-producing fungi. They concluded that the ability to forcibly discharge spores has been lost at least three times during the course of evolution of the species studied. If forcible discharge of ascospores is common to the two groups that diverged earliest, Taphrinamycotina and the Saccharomycotina, and if future investigations indicate that the mechanism of discharge is conserved between these groups, then it is probably ancestral. However, until that information is available, one cannot rule out the independent acquisition of discharge by diverse taxa. However, the work compiled here shows an amazing diversity among the *Ascomycota* in the use of structure and physiology to propel their sexual spores.

An early history of research on ascospore discharge

The phenomenon of ascospore discharge has captured the interest of biologists since the late 1800s. In *Comparative Morphology and Biology of the Fungi Mycetozoa and Bacteria*, Anton DeBary (1887) described his extensive work on these fungi and also referenced earlier work. It is impressive how much was known about development and function at that time; our knowledge has progressed strikingly little since. DeBary clearly described the basis of ascospore discharge as a developmentally regulated increase in turgor pressure in the ascus tip. He suggested the importance of lateral

pressure from paraphyses (sterile hyphae among the asci) as contributing to the ejection of spores (more on this below).

In his *Researches on Fungi*, A.H. Reginald Buller (1909) explored forcible ascospore discharge in the large-spored, apothecium-producing species *Ascobolus immersus. Ascobolus immersus* is a dung fungus; it produces its spores on dung, launches them out of the bovine 'zone of repugnance' back onto the forage plants, where they may be consumed. Propagules are then excreted – placing the spores back into the prized substrate. Buller explored many of the biomechanical facets of ascospore discharge, such as the breakup of the ascus jet in flight and the physical principles driving spore dispersal.

Ascus structure

Three kinds of asci exist that forcibly discharge spores. *Inoperculate unitunicate asci* have a single wall, and a pore at the tip of the ascus that opens to release the spores. 'Single wall' refers to the wall, which acts as a single unit, no matter how many layers one can distinguish (reviewed in Beckett, 1981). Although the pore has no obvious cap, it may have a plug that is pushed out when the ascus dehisces; this phenomenon has been observed, for example, in *Sordaria humana* (Beckett, 1981). Inoperculate asci have a huge array of pore designs, from simple to complex (Bellemere, 1977). *Operculate unitunicate asci* have a single wall and a hinged lid that opens to release the spores (Fig. 1a). The operculum develops early in ascus formation by an in-pinching of a region of the ascus tip (Hung, 1977; Minter & Cannon, 1984). *Bitunicate asci* have two walls that act as two distinct units. The outer wall is thin and stiff and breaks open as the ascus expands (Fig. 1b), whereas the inner wall stretches through the outer wall and fires the spores. *Bitunicate asci* usually have a pore at the tip of the inner wall through which the spores are released. Ascus structure and development has been reviewed by Read & Beckett (1996).

A tether may attach spores to each other and to the ascus pore. For example, in *Sordaria macrospora* an actin filament runs through the middle of the ascus, attaching to each spore, and adjusts the spore's position in the ascus. One end is attached to the pore, the other end of the strand is free (Thompsoncoffe & Zickler, 1993). It has been suggested that this would allow multiple spores to remain as one projectile initially, thereby allowing their momentum coming out of the ascus to be maximized. Spores may be bound together in other ways as well. Buller (1909) reported finding *A. immersus* ascospores bound together in a gelatinous sheath, and Czymmek & Klomparens (1992) found microtubules attached to the ends of ejected spores of *Thelebolus crustaceus*, which remained clustered. In some situations, aggregation of spores may be advantageous because it increases relative propagule size and will increase shooting distance.

The ascus wall is a structure unique to the fungi. It stretches directionally and rapidly and bursts at a precise time and place. Reynolds (1971) provides the most extensive analysis of (bitunicate) ascus wall function as well as clues to the apparent stretchability of the ascus. He observed microfibrils in the inner wall in the upper half of the ascus, which gave the wall an undulated appearance. Reynolds hypothesized that these striae disappear when the ascus expands before discharge: thus, they provide the extra wall and membrane needed for expansion. Parguey-Leduc & Janex-Favre (1981) reported that the inner wall of the ascus of several *Leptosphaeria* species, as well as *Massaria foedans*, and *Pleospora herbarum* were similarly structured. Microfibrils in the outer wall of the bitunicate ascus run parallel to the length of the ascus, whereas those that are in the inner, expanding wall run obliquely to the length of the ascus (Reynolds, 1971). This structure may account for the stretchability of the inner wall and the stiffness of the outer wall. Funk & Shoemaker (1967) also observed fibers in the inner wall of the bitunicate ascus; the fibers appeared to be horizontal but became helical in orientation as the ascus expanded.

A helical orientation of wall fibers characterizes many other pressurized designs found in nature, from pressurized herbaceous plant stems to shark skin. Interestingly, this orientation of wall fibers enables the body design to dictate the mode of expansion: to elongate upon increased internal pressure, the helical fibers must be oriented such that the angle of the fiber with respect to the long axis of the cylinder is $> 55^\circ$ (Vogel, 1988). Angles $< 55^\circ$ result in the expansion of width. The wall fibers of asci are tricky to observe and these angles have not yet been measured.

The ascus cannot expand limitlessly. The wall itself can provide stiffness (tension) to counterbalance the water pressure, but pressure from the outside of the ascus can also serve to minimize expansion of the ascus circumference. The asci of some fungi are packed up against expanding cells. In closed fruiting bodies in the perithecium- and locule-forming species, many fungi have sterile hyphae called paraphyses or pseudoparaphyses (depending on their location of origin within the central cavity) dispersed between the asci. For example, the pseudoparaphyses in *Gibberella zeae,* which are multicellular and senesce by the time the asci are mature (Trail & Common, 2000), retain functional membranes after senescence, and the cells can be seen expanding in squash mounts of asci in water. The largest cells occur at the base of the asci and may serve to apply pressure to the ascus, which would assist in ascus extension and discharge. However, not all paraphyses appear to have evolved for this purpose. For example, the apothecium-forming ascomycetes frequently have paraphyses that are not senescent and that can be quite slender compared to the asci; these do not appear to function to aid in extension. Interestingly, the majority of

species with bitunicate asci appear to lack paraphyses or pseudoparaphyses, with notable exceptions in the order *Pleosporales*. Because the outer wall of bitunicate asci appears to be quite stiff, it may be serving to counterbalance water pressure and minimize expansion in circumference.

Spore design

To be dispersed, ascospores need to (1) be propelled far enough to fly beyond the boundary layer of air surrounding the fruiting body, (2) remain suspended in the air currents to be distributed appropriately, and (3) precipitate out at the right time and place. For very small propagules, takeoff is strongly impacted by drag, hence high launch speeds must be achieved to reach air currents. In species that rely on forcible discharge, evolution of spore shape likely has been impacted by drag constraints. A study of a wide range of forcibly discharged ascospores provided evidence that minimization of drag might be a primary driving force for spore shape (Roper *et al.*, 2007). This study suggested that drag limits spore size to between 1.8 and 30 μm (using a radius of a sphere of equal volume). However, forcibly ejected spores come in a wide variety of shapes and sizes, including those of *Podospora,* which exceed this ideal size, and, furthermore, have large appendages that increase drag. In these latter spores, selection for maximization of a criterion other than size (e.g. maximizing landing) is likely to have occurred.

Biomechanics of ascospore discharge

Ascospore discharge is no small feat of evolution. A precisely timed increase in osmolytes within the ascus draws in water to increase turgor pressure. Turgor pressure then provides the force for stretching the ascus and ultimately for firing the spores. What is in the ascus that acts as an osmolyte? Ingold measured osmotic pressures as 1–1.3 MPa in asci of *Sordaria fimicola* using incipient plasmolysis and later speculated that a combination of glucose and ions served this function (Ingold, 1966, 1971). Similarly, he estimated those of *Ascobolus stercorarius* to be 1.3 MPa for the mature isolated asci (Ingold, 1939). The first reported quantitative analysis of the osmotic contents of the epiplasmic fluid of the ascus was performed for *G. zeae* (Trail *et al.*, 2002, 2005). Potassium ion channel inhibitors were found to inhibit discharge when applied to mature perithecia. Of the sugar derivatives, mannitol had the highest concentration, but quantification of the relative contributions of Cl^-, K^+, and mannitol to the epiplasmic fluid revealed that mannitol exists in very low levels in comparison with the ions and thus makes an insignificant contribution to turgor generation (Trail *et al.*, 2005). The pressure necessary to propel the eight ascospores from inside the ascus was estimated from measurements of the distance traveled by the spores (an average of 0.46 cm) and spore size; the ion concentration would nearly generate the 1.54 MPa necessary to drive discharge (Trail *et al.*, 2005).

Fischer *et al.* (2004) studied the osmolytes in the epiplasmic fluid of the apothecium-producing fungus *Ascobolus immersus* and found that glycerol and proline were the major components. (The presence of ions was not examined.) They measured pressure within the asci using a pressure microprobe, which presses nondestructively on the ascus surface. This type of measurement was possible because this genus has large asci, which are exposed in the apothecial fruiting body. The mean turgor was 0.31 MPa, and a significant portion of that pressure could be explained by the quantities of glycerol and proline in the epiplasmic fluid. In this study, Fischer et al. also made a few direct measurements using an invasive microprobe and reported turgor to be ~0.45 MPa. These are the only direct measurements of ascus fluid reported in the literature.

The pressures reported for *A. immersus* and *G. zeae* range from 0.31 to 1.54 MPa. (The measurements of Ingold (1939, 1966) on *S. fimicola* have been neglected due to their imprecise nature and the fact that they were carried out using mature asci, not discharging asci.) Is there a biological explanation for this wide range? The most obvious answer is that pressure scales inversely with ascus size. The asci of *A. immersus* are quite large [500–600 μm length by 45–50 μm radius; (Seaver, 1928) compared with those of *G. zeae* (76 μm × 2–4 μm; Trail *et al.*, 2005)]. This can be illustrated using a stretched spring (the expanded ascus) that stores the energy in its stretch and releases it as it returns to normal size (after firing). The turgor pressure drives the stretching, which may act to stretch wall fibers. The energy stored in the spring (stretched wall) is proportional to the pressure squared and radius cubed. For example, using the data from *G. zeae* and *A. immersus* and assuming that the asci of these two species are stretched the same distance (*A. immersus* is actually stretched farther, but this assumption will simplify the calculations and provide a minimum value), the ratio of the pressures (*A. immersus* to *G. zeae*) is 0.21. The ratio of the radii (*A. immersus* to *G. zeae*) is 12.5. Therefore, the energy stored in the *A. immersus* ascus will be minimally 8.8-fold higher than that of *G. zeae* despite the difference in pressure. Thus, for a lower pressure, there is the potential to store much higher energy in the asci of *A. immersus*.

Interestingly, from a materials viewpoint, the lower pressure in the asci of *A. immersus* is probably essential to its function. All ascus walls are made of essentially the same materials with the same limitations. The most important limitation is the tension in the ascus walls that prevents an uncontrolled blowout. Wall tension is measured as the difference in pressure (between outside and inside the ascus) times the radius. Thus, for the same wall tension, a much

larger radius should scale with a much smaller pressure to prevent such a blowout. Conversely, a very small radius can stand to have a higher pressure inside (Vogel, 2003).

In fact, asci have evolved to rupture only at the tip. They burst at this opening before the spring is stretched beyond its capacity to spring back. The ascus cylinder has two kinds of tensions in its wall: that on the circumference (hoop stress) and that on the length (longitudinal stress). Hoop stress is twice the longitudinal stress, so as the tension increases, the chance of a lengthwise blowout increases. However, the tip appears to be the weakest point. Enzymatic digestion of the wall may occur during maturation to allow for the blowout at this weakest point, although the mechanism of weakening is as yet undescribed.

What happens to the pressure within the ascus as the ascus stretches and ultimately discharges its contents? Intuitively, one would think that the pressure would decrease as the volume increases and as the ascus fires its contents. LaPlace's law describes the relationship among pressure, tension, and radius for curved vessels, which include asci. The pressure buildup in the ascus must be evenly distributed to prevent a blowout at a weak spot. For even distribution to occur, the wall needs to become stiffer as it is stretched. In mammalian arteries, this is accomplished by the presence of collagen, which stretches very little and is folded up in the nonpressurized artery and straightens out as pressure builds. Similarly, as the ascus fills with fluid, tension increases on the outer wall and the microfibrils stretch horizontally, thereby limiting further ascus expansion in that direction and directing the pressure upward. Once the ascus begins to expand toward discharging, it expands rapidly. LaPlace's law says that tension (force per unit length) of the wall will increase on the surface as the ascus expands, but pressure (force per unit area) will not change considerably (Vogel, 2003). As the ascospores leave the ascus, the pressure inside will remain relatively stable until the ascus contents are shot upward. An observation that seems to support this scenario is that in many species, no spores remain in the asci after discharge (Hung, 1977; Trail *et al.*, 2005).

Gibberella zeae holds the record for the organism, in any kingdom, with the highest reported acceleration: An ascospore of this species can accelerate from 0 to 8 500 000 m s^{-2} within the ascus (Trail *et al.*, 2005). Although this appears to be an astounding number, the relationship between size and acceleration makes this feat possible without amazing machinery (see Vogel, 2005). Basically, Newton's law (F = ma) shows that mass is inversely proportional to acceleration for the same force. Thus, for very minute bodies, astonishing accelerations can be reached. The build-up of speed is important for very small bodies because drag becomes a prominent factor as soon as the spore leaves the ascus. Larger spored species need less acceleration, as there is less drag on them when they hit the air. As an example, sporangia of the zygomycete dung fungus *Pilobolus*, which are ~200 μm in diameter, accelerate to only 500 000 m s^{-2}.

For propagules as small as spores, drag increases with surface to volume ratio. Thus, larger propagules travel longer distances. One strategy to reduce drag among the ascospores of the apothecium-forming fungi is to shoot simultaneously, behaving as a large mass, reducing both surface area and drag. This allows these fungi to travel a farther distance than they would alone. Many of the largest apothecia (13 cm in diameter) are formed by the forest-floor inhabitants (*Peziza* and relatives). These large fruiting bodies may serve to maximize the mass of spores shot at one time in an environment in which the boundary layer can be 30 cm or more on a quiet day. In contrast, for *G. zeae* the initial shooting distance is not so important because these very small spores move on air currents and the boundary layer of air in a typical field is quite thin. In addition, because propagule distribution is so important, these spores do not remain attached to each other but disperse once they have left the ascus, thereby maximizing the number of independent propagules available for distribution.

As an aside, puffing has implications on the calculations of shooting distance. *Ascobolus* puffs its spores, which creates a draft situation and permits them to go farther with a lower initial velocity. Measuring projectile range for these spores is error prone for another reason as well. One would think that convection currents present in shooting chambers would simply increase scatter, but in a variety of chambers it was found that these currents greatly increased spore launch distance (8.5 cm with currents vs. 0.45 cm without currents; Trail *et al.*, 2005; F. Trail, unpublished data). Therefore, initial velocity calculations, on which calculations of the force necessary to project the spores are based, can be greatly affected. Aylor & Anagnostakis (1991) have described the chamber sizes that limit convection currents.

Ascospore discharge: a model

Figure 4 illustrates a model of ascospore discharge based on studies of *G. zeae* (Trail *et al.*, 2002, 2005; F.Trail et al., unpublished data). In this species, the discharge process appears to be developmentally regulated and perithecia are ephemeral – once development has been initiated, the process is continuous until discharge has occurred (Hallen *et al.*, 2007). The model in Fig. 4 suggests a two-stage increase in pressure. The first stage is a preliminary increase in turgor, which pushes the ascospores to the upper end of the ascus and primes the ascus for discharge. For bitunicate asci, this would be the stage at which the outer wall is engorged but has not burst yet. In *G. zeae,* the initial engorging of the asci (Fig. 4b) may be driven by accumulation of mannitol, which is not sufficient to propel the spores, but is the sugar derivative found in the highest amounts in

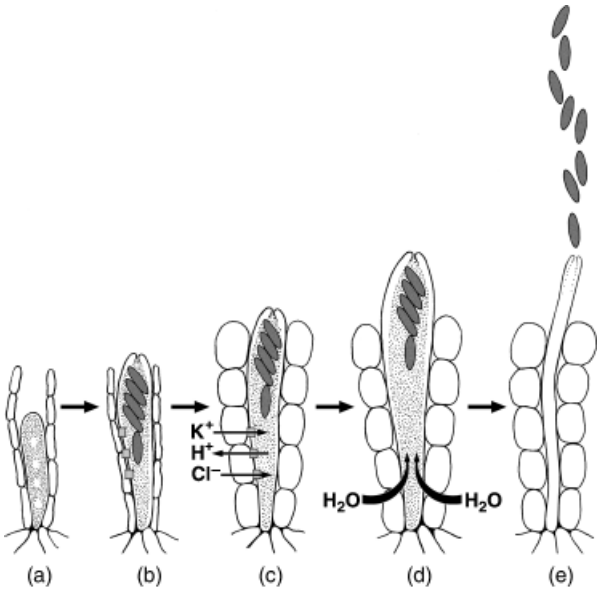

Fig. 4. Model for forcible discharge showing the development of an ascus based on *Gibberella zeae*. (a) Four-nuclei stage following meiosis. Mature paraphyses flank the young ascus. In this species, paraphyses are senesced, but retain functional membranes. (b) The ascus wall is fully developed and initial increases in turgor may be driven by accumulation of mannitol. Spores (grey) are mature. Once the walls of the spores are formed, the nuclei are no longer in contact with the epiplasm and are unlikely to direct development. Therefore, all gene expression related to ascus formation probably occurs before the formation of spore walls. Ion channels are in place (small rectangles on left side of the ascus). Pore is present at tip. (c) The rapid ascus expansion has been initiated by the efflux of H^+ and the influx of K^+ and Cl^-. Paraphyses are fully expanded. (d) Water flows through the ascus membrane to drive the extension of the ascus wall. (e) Spores are fired along with the epiplasm and the ascus collapses.

the ascus (Trail *et al.*, 2002). This preliminary osmolyte accumulation would stimulate the second stage of discharge, in which the ascus rapidly stretches and fires (Fig. 4c–e). The accumulation of mannitol initiates stretching of the ascus wall as water flows into the ascus and stimulates an as yet unidentified proton-pumping ATPase to begin pumping protons out of the ascus. A hypothesized proton motive force drives the influx of K^+ ions, and the influx of Cl^- ions balances the charges (Fig. 4c). It is this buildup of ions that drives the influx of water, resulting in the hydrostatic pressure that causes the ascus to stretch rapidly and fire. The control of ion influx may be regulated by Ca^{2+}. Calcium ion channel inhibitors were shown to suppress ascospore discharge (Trail *et al.*, 2002) and the specific disruption of the calcium ion channel Cch1p has been shown to arrest ascospore discharging in *G. zeae* (Hallen & Trail, unpublished data). Figure 4 also shows the presence of paraphyses, which are not common to all discharging ascomycetes, but are shown here to be engorged, providing the necessary counter-pressure within the perithecium to drive the ascus upward. Paraphyses of *G. zeae* have been observed to engorge in freshwater mounts (Trail & Common, 2000). The ascus wall stretches, storing potential energy that is released when the ascus bursts, allowing the spring to resume its natural size (Fig. 4e).

Concluding remarks

Forcible discharge of ascospores is an important part of basic fungal biology, but the mechanism is poorly understood. Ascospores initiate the disease cycle for a great many plant pathogens. Thus, in practical terms, understanding ascospore dispersal will contribute to understanding of disease. Interestingly, despite the fact that several known human pathogens fall into the Pezizomycotina, there are no known human pathogens that rely on ascospore discharge as an epidemiological component. There is no obvious reason for this disparity. It may be artificial due to the very small number of known fungal human pathogens (about 150 in total) when compared with fungal plant pathogens [more than 8000 species; (Agrios, 1988)]. In addition, a trend toward suppression of the sexual cycle may exist in human pathogens, which may suppress dependence on sexual propagules (Heitman, 2006). However, sexual cycles may be present and not yet appreciated epidemiologically. For instance, the recent discovery in pigeon droppings of a sexual stage of *Cryptococcus neoformans*, although not an ascomycete, has strong implications for the epidemiology of that disease (Nielsen *et al.*, 2007).

The most effective controls for any disease are those that interrupt the life cycle. Elimination of ascospore distribution would be a very effective control for ascomycetous plant pathogens, but most effective on those that use ascospores as their primary inoculum (e.g. *Venturia inaequalis*, the apple scab pathogen; *Sclerotinia sclerotiorum*, white mold of beans and other vegetables; *G. zeae*, causal agent of head blight of wheat). More knowledge of the basic mechanism and the variations throughout the Kingdom Fungi should facilitate the design of controls targeting this life cycle stage.

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