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Review

The mechanism of ascus firing – Merging biophysical and mycological viewpoints

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ABSTRACT

The actively discharging ascus is the unique spore-bearing cell that is responsible to dispatch spores into the atmosphere. From a physical perspective, this type of ascus is a sophisticated pressure gun that reliably discharges the spores at an extremely high velocity, without breaking apart. We identify four essential steps in discharge of spores whose order and timing may vary across species. First, asci that fire are mature, so a cue must be present that prevents discharge of immature spores and signals maturity. Second, pressure within the ascus serves to propel the spores forward; therefore a mechanism should be present to pressurize the ascus. Third, in ostiolate fruiting bodies (e.g. perithecia), the ascus extends through an opening to fire spores into the air. The extension process is a relatively unique aspect of the ascus and must be structurally facilitated. Fourth, the ascus must open at its tip for spore release in a controlled rupture. Here we discuss each of these aspects in the context of understanding the process of ascus and fruiting body function. While there is great diversity among fungi, we focus on discharge in a few model species, and then discuss how this framework may vary in other fungi. Our goal is to tie the physiological and molecular studies of ascus function with concepts in engineering that dictate structure.

1. The problem of dispersal

As predominantly nonmotile organisms, fungi have evolved diverse ways to enhance their distribution. The more fascinating dispersal mechanisms involve forcible launching of spores into the air. Two groups of higher fungi, the Ascomycota and the Basidiomycota, have each taken a different approach to forcible spore discharge. Species of the Ascomycota form spores endogenously within cellular sacs called “asci”, which in many species function as cellular cannons, using turgor pressure to launch spores into the air. The Basidiomycota form their spores exogenously, on the tips of pointed cellular appendages. The basidiospores are then propelled from their appendages due to a sudden change in their center of mass resulting from the rapid motion of a fluid droplet, called ‘Buller’s drop, over the spore surface (Webster *et al.*, 1984; Pringle *et al.*, 2005; reviewed by Money, 1998). Despite the importance of spore release to fungal ecology and plant disease, the

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mechanism of spore release remains largely undescribed. In this review, we will focus on the active mechanism of release of ascospores in the Ascomycota, orchestrating molecular processes with the physics of ascus deformation and we will discuss the accumulated literature in light of these concepts.

A fundamental mechanism of ascospore discharge appears common to all of the ascus-forming fungi that have forcibly discharging asci; however there is much variation, some of which affects how and when the spores are dispersed. New advances in high speed video microscopy have enabled close observation of the process of spore firing (Yafetto *et al.*, 2008; Roper *et al.*, 2008). However, asci are usually located within fruiting bodies (Fig 1) and hardly function in isolation; thus the microscopic dynamics of ascus expansion and discharge remain not readily experimentally accessible. In particular, how the structure of the ascus, including wall, cell membrane and cytoskeleton features permit unidirectional expansion and controlled release, are virtually unknown.

Asci are derived from a single cell, within which meiosis occurs. In many species, nuclei undergo an additional division following meiosis, and a double membrane bounding the ascospore wall forms around each nucleus, yielding eight spores in a tubular ascus (Fig 1; see Thompson-Coffe and Zickler, 1993; reviewed by Read and Beckett, 1996). The majority of asci have an opening (via a pore, slit or operculum) at their tip through which the spores are released along with the "epiplasmic fluid" in which they are suspended. Multiple (often hundreds) of asci are packaged together in a fruiting body. Ascomycete fruiting bodies are diverse in shape and structure (Fig 1), reflecting niche requirements. Fruiting body structure dictates whether asci within a single fruiting body fire singly or simultaneously. In flask-shaped fruiting bodies (locules and perithecia), asci fire one at a time in succession. In cup-shaped fruiting bodies (apothecia), asci commonly fire together, in a process called "puffing". Ascus structure reflects the difference in fruiting bodies in which they function.

Before uncontrolled rupture, the ascus opens up at its apex and forcibly releases its content. A variety of mechanisms and morphologies allow opening: asci can unseal through an apical pore or canal; or be released through an outer wall (Fig 1; see Schoch *et al.*, 2006). The ascus opening must be finely controlled, as a mismatch in timing may cause delays in firing, resulting in deterioration of the ascus, uncontrolled rupture of the ascus, or the dispatch of spores that are not mature.

2. Generalized model of ascus function

The ascus is an unusual cell that undergoes extreme morphological deformations perfectly orchestrated to reliably fire the spores. From a physical perspective, the ascus is a pressure gun that realizes accelerations of more than 10^5 g to discharge mature spores at an extremely high speed, without uncontrolled bursting. For the process to succeed, we identify four steps whose timing as well as molecular implementation may vary across species. We explore these four steps based on recent studies of ascus function:

A. *A signal for maturity and a signal to fire.* The process of maturation of spores and asci is diverse and can be very slow. For example, maturation of fruiting bodies of some plant pathogenic fungi occurs across seasons, while others can be initiated and mature within a few days. A signal must be produced upon maturation to prevent discharge of immature spores. In mature asci, the nuclei are embedded in the spores, which suggests that asci have limited ability to repair and regenerate. Thus, for some species it may be expedient to fire spores shortly after maturation is reached to prevent retention of mature spores until the ascus membrane degrades and firing is no longer feasible.

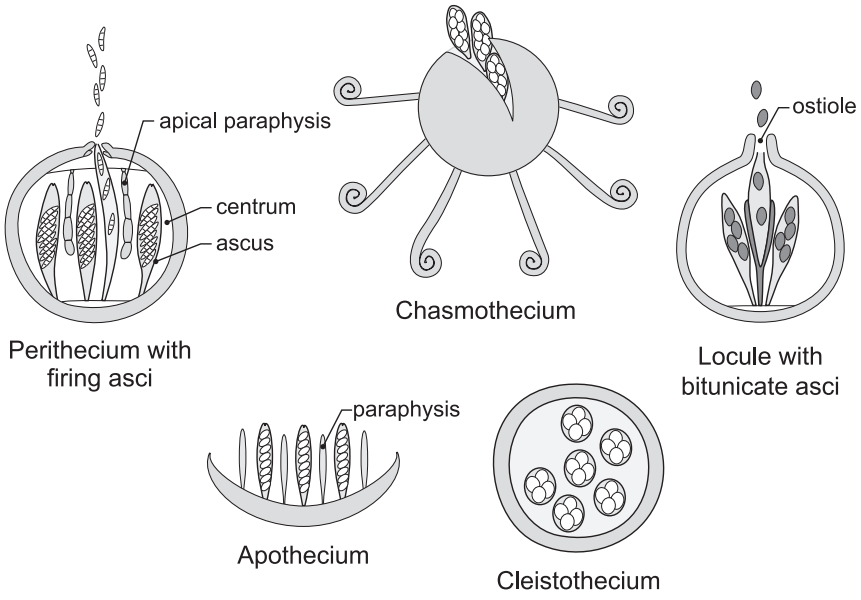

Fig 1 – Fruiting body types and structure among the Ascomycota. The fruiting bodies are associated with the following taxa: perithecia (Sordariomycetes), chasmothecia (Erysiphales); locules (Dothidiomycetes); cleistothecia (Eurotiomycetes); apothecia (Pezizomycetes and Leotiomycetes).

B. *Turgor pressure buildup*. Turgor pressure has generally been thought to be due to an increase in osmolyte concentration inside the ascus, due to accumulation of sugars or ions. Water tends to flow into the ascus and the ascus wall opposes mechanical resistance to swelling, thus yielding turgor pressure buildup.
C. *Ascus elongation before firing*. In closed fruiting bodies, such as the perithecia and locules (Fig 1), asci extend and fire one at a time. Where does the signal come from for each successive ascus to fire, or is it the outcome of competition among expanding asci within the fruiting body?
D. *Opening of ascus and release of spores*. Before uncontrolled rupture, the ascus opens up at its apex and forcibly releases its content.

These steps may not occur in the same order in all fungi or be physiologically similar, but they must be functionally present.

A signal for maturity and a signal to fire

The timing of ascus discharge should coordinate with ascus maturity and this information needs to be transferred to the machinery that initiates the spore discharge. Whether the origins of the cues for discharge are molecular or physical is an open question. However, the decision to fire would require an acknowledgment of ascus maturity. Signals for maturity likely vary among species and classes. In *Venturia inaequalis*, red light is required for asci to fire (Brook, 1969). Another possible signal would be a slight increase in turgor pressure of the membrane, which could set up a cascade of events leading to discharge. In *Fusarium graminearum*, turgor pressure associated with ascus firing is generated by ions (see next paragraph). At maturity, the ascus contains mannitol (Trail, 2007) and glycerol (Min *et al.*, 2010) which are the predominant sugars found in the epiplasmic fluid. The amounts of these sugars are too small to play a role in the turgor pressure needed for ascus discharge in this fungus (Trail, 2007). However, their accumulation may promote a slight increase in turgor that could be used as a signal of maturity, perhaps stimulating ion channel opening. In *Ascobolus immersus*, which produces very large asci, discharge is driven by glycerol accumulation (Fischer *et al.*, 2004), not by ions, indicating that this mechanism may not be shared by all asci-producing fungi.

Turgor pressure buildup

The buildup and release of turgor pressure drives ascospore discharge as was first hypothesized by Pfeffer (1881). Later DeBary (1887) emphasized the idea that asci swell as they mature, with a buildup of turgor pressure within asci, and suggested that osmolytes within asci drive an influx of water to generate the pressure needed for discharge. Studies by Ingold investigated the pressures obtained within asci for discharge. The osmotic pressure in mature asci of *Ascobolus stercorarius* (Ingold, 1939) and *Sordaria fimicola* (Ingold, 1966) were estimated to be 1.0–1.3 MPa and 1.0–3.0 MPa, respectively, as assessed by incipient plasmolysis analysis and freezing point depression measurements of diluted ascus epiplasmic fluid. Ingold (1939, 1966, 1971) suggested that breakdown products of glycogen, sugars, and ions serve as osmolytes in asci, although he never published supporting data.

Recent technical advances have now facilitated the analysis of ion concentration and the process of pressure buildup. Measurement of the turgor pressure in *A. immersus* asci by a pressure microprobe found an average pressure of 0.31 MPa due to glycerol accumulation in the ascus epiplasmic fluid (Fischer *et al.*, 2004). A biomechanical study of *F. graminearum* ascospore discharge estimated that 1.54 MPa of pressure was needed to achieve the estimated launch acceleration of 970,000 g and the trajectory characteristics observed. Analysis of epiplasmic fluid from asci showed that cellular levels of K^+ and Cl^- generated turgor pressure close to the estimate needed for discharge (Trail *et al.*, 2005). Physiological measures in *F. graminearum* showed that K^+ is the main ion responsible for the increase in osmotic pressure (1.1 MPa out of an estimated 1.5 MPa; Trail *et al.*, 2005). Cl^- ions have been shown to have a lower concentration, that is not sufficient to balance the cellular charge due to K^+ influx. Pharmacological drugs were screened for their inhibition of *F. graminearum* ascospore discharge. Several K^+ channel blockers inhibited ascospore discharge by up to nearly 50% (Trail *et al.*, 2002), supporting a primary role for K^+ in the discharge of asci in this species. It has been suggested that ascus size may dictate the osmolyte used for discharge (Trail, 2007) but research on more species is essential to determine if this is true. Asci are very variable in size (e.g. the asci of *A. immersus* are 7–8 times the length of asci of *F. graminearum*).

Firing asci do not function in isolation except in very few species, such as some of the Taphrinomycotina, which produce naked asci on the surface of plants. Maturity may thus be identified collectively at the fruiting body level, rather than at the single ascus level. Asci are usually embedded in a fruiting body among tens, if not hundreds of other asci, and the proximity implies that the activity of these asci will affect neighboring structures. Interspersed among the asci are paraphyses, sterile hyphae that have long been presumed to assist in fruiting body function. In largely closed fruiting bodies, such as perithecia and locules (Fig 1), the wall of the fruiting body could assist in pressurization of the contents within (i.e. asci surrounded by closely appressed paraphyses and mucilage). In *F. graminearum*, paraphyses have been shown to undergo programmed cell death as the asci develop, which leaves their cells devoid of contents but with functional membranes, allowing them to engorge in the presence of moisture and contributing to pressure within the perithecium (Trail and Common 2000; Sikhakolli *et al.*, 2012). Thus the entire centrum contents (asci and paraphyses) contribute the counterpressure on the mature ascus, ready to fire.

In cup shaped fruiting bodies (Fig 1), paraphyses play an important role in discharge. Paraphyses of the Leotiomycetes and Pezizomycetes, which form apothecia, are linear or branched, and slender, becoming turgid at maturity (Wang *et al.*, 2006). Studies on the paraphyses of the woodland cup fungi *Urnula geaster* and *U. craterium* revealed that the paraphyses are strings of cells that become turgid and assist in expansion of the cup hymenium to direct the asci to expel their spores away from the cup interior (Seaver, 1937; Wolf,

1958). In *Sclerotinia sclerotiorum*, a small group of asci were shown by high-speed video to trigger a wave of ascospore discharge as they fire (Roper *et al.*, 2010). The firing of the first few asci releases pressure in the rest of the fruiting body to stimulate discharge. Paraphyses, which are interspersed between the asci and remain turgid in the mature fruiting body, are hypothesized to be involved in generating this wave by readjusting their position as the asci among them fire, thus propagating the wave (Roper *et al.*, 2010). A similar process is likely to occur in flask-shaped fruiting bodies (e.g. perithecia) with turgid paraphyses.

In some fungi, paraphyses may not be essential for discharge of spores. In the Dothidiomycetes, which produce locules (Fig 1), the asci are bitunicate. The stiff outer wall of these asci may provide the counter-pressure needed by the asci, and indeed, one major order within this class, the Dothideales, does not produce lingering paraphyses (Schoch, *et al.*, 2006), but is still able to discharge spores. The chasmothecium-forming Erysiphales, also do not produce interascal tissue (Kirk *et al.*, 2001), and have just a few asci, which eject spores after the fruiting body splits (Fig 1). How or if the fruiting body contributes to the firing mechanism in these fungi is not clear.

The biology and biomechanics of ascus elongation

Ascus swelling is a peculiar example of morphological deformation. After maturation, no gene expression affects ascus structure and function, as the spores seal and the nuclei no longer contribute to cellular processes. The ability of the ascus to stretch unidirectionally in response to environmental and developmental cues is thus not mediated by gene expression. Few cells in nature perform in this unusual way, and the ascus wall has evolved remarkable properties that permit its unique function. The major components of fungal cell walls are chitin (polymer of N-acetyl-glucosamine), β(1-3)- and β(1-6)-glucans, and mannans (polymers of mannose). The basal layer of the wall is composed of chitin fibrils, which are usually appressed to the membrane. The middle layer is composed of glucans, which are long, branched chains to which oligosaccharides may be attached. Proteins and mannans are also present. These elements can be cross-linked, which will add rigidity (Riquelme *et al.*, 2011). As with all fungal cells, the wall plays a role in determining ascus shape, and in the laboratory, enzymatic digestion of the cell wall results in a spherical ascus (Trail, unpublished). Naturally spherical asci of several species fail at forcible discharge, suggesting that the tubular shape is at the same time an important aspect of ascus shape and a requirement for forcible discharge.

Ascus wall

The thickness of the ascus wall and the degree of cross-linking of its components may impact the unique ability of the wall to stretch unidirectionally as the ascus extends to discharge (Fig 2). The ascus wall appears very thin in many species, which is likely due to its need for flexibility, but histological examination of asci in some species indicate a multilayered structure, a quality not restricted to bitunicate asci (Beckett, 1981; Parguey-Leduc and Janex-Favre, 1982; Minter and Cannon, 1984; Read and Beckett, 1996). The multiple layered wall

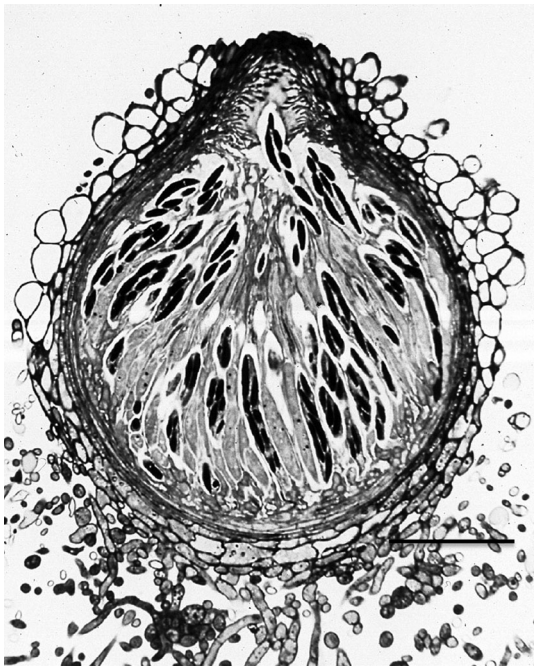

Fig 2 – Cross section of a perithecium of *F. graminearum* showing emerging ascus, ready to fire. Collapsed paraphyses are apparent among asci. Bar = 50 μm. Reprinted with permission from Trail and Common (2000).

structure present in some unitunicate asci is difficult to reconcile with the need for flexibility and stretching. Among the cell wall polymers, a case can be made for chitin to be essential for accommodating stretch. Chitin is synthesized from membrane-bound chitin synthases, and most filamentous fungi have seven to eight chitin synthase genes (Kong *et al.*, 2012, Horiuchi, 2009; Riquelme *et al.*, 2011). Studies of chitin synthase knockouts have revealed that different chitin synthase genes encode proteins with different functions. In the yeast *Candida albicans*, two forms of chitin have been identified, chitin made up of long fibrils, and chitin made up of short fibrils. The two distinct forms of chitin are synthesized by different chitin synthases (Lenardon *et al.*, 2007). In filamentous fungi, the short form (called rodlets) predominates in yeast-like cells and the longer fibrils are more common in mycelial fungi (Gow and Gooday, 1983). Although only vegetative cells have been examined to date, the long chitin fibrils would be a natural fit for function in the stretchable ascus walls.

Chitin synthases are analogous to cellulose synthases that are responsible for cellulosic walls of plants. In plants, cellulose synthases migrate along cytoskeleton microtubules, leaving long (as long as 7 um have been documented) cellulose microfibrils in their wake (Paredez *et al.*, 2006), and resulting in coalignment of microfibrils and microtubules. The stiff microfibrils run perpendicular to the axis of cell expansion resulting in anisotropic growth (Baskin, 2005), and depolymerization of microtubules changes the orientation of the microfibrils. In plant cells, it has been demonstrated that mechanical stress within the cell dictates the orientation of microtubules in the cytoskeleton. Anisotropy of mechanical

stress in stems results in transverse orientation of the microtubules (Hamant *et al.*, 2008). Does chitin act in a similar way during ascus development? In fungal cells, interactions between the cytoskeleton and the cell wall are well characterized in vegetative cells (reviewed in Lichus *et al.*, 2011). More research is needed to better understand the parallels of chitin and cellulose organization and function in determining cell wall structure in the ascus.

Unidirectional elongation

Swollen asci are approximately cylindrical, and their exact shape is determined by constraints such as pore shape and size. In addition, taxonomic classes of fungi have asci that differ in structure and shape (Fig 1). To understand how the ascus accomplishes unidirectional elongation, we compare the ascus to a balloon. The walls of an inflated cylindrical balloon are under uneven tension: in the longitudinal direction, tension is half the tension of the cross sectional direction. In other words, if the walls are linearly elastic, it is easier to bulge the balloon than to elongate it. This is a fundamental constraint that is known to shape the mechanical response of several biological systems with internally pressurized structures. For example, arteries avoid bulging (aneurysms) by activating a non-linear response when stress is too intense (Vogel, 2003). Alternatively bulging can be avoided with the use of helical fibers. The angle of the fibers determines their ability to resist longitudinal versus orthogonal stresses. If the fibers run more circumferentially, they are able to resist a larger circumferential stress, thus avoiding bulging. If the fibers run more lengthwise, they tend to resist elongation while allowing bulging. Fibers at a 55° angle with the length will impart twice the resistance in the circumferential direction than the longitudinal direction (Wainwright *et al.*, 1982). At this angle, the cylindrical balloon inflates without changing shape. Fiber angles more than 55° will increase in length with increased pressure (Vogel, 2003). Vogel (2003) noted that, despite a wide variety in function, internally pressurized structures across the tree of life hinge on the presence of helical fibers. These are by far the most common wrappings sustaining internal pressure, and are found in the stems of young herbaceous plants, flatworms, sea anemones, the outer mantle of squids, and shark skin.

Chitin fibrils are nearly inextensible, with an elastic modulus of about 15 GPa, well above the pressures that would be exerted by a swollen ascus. Thus, if the asci are no exception in the tree of life, and function with helical fibers, the most prominent candidate to form helical wrappings in the ascus wall is chitin. There is some evidence for a helical arrangement of wall fibers in asci. Several studies of ascus wall sections examined by transmission electron microscopy revealed fibrils that could correspond to undulating or helical arrangements (Reynolds, 1981; Bezerra and Kimbrough, 1982; Parguey-Leduc and Janex-Favre, 1982; reviewed in Read and Beckett, 1996, Trail 2007).

Ascus membrane

To enable volume growth as the ascus expands, additional membrane must be added. If no membrane was newly synthesized nor stored in wall invaginations, ascus volume would shrink, as it is geometrically impossible for an axisymmetric object to elongate while increasing in volume and preserving the same surface area. In *F. graminearum* the base of the ascus remains attached to the centrum and the ascus must then swell to the full height of the perithecium (Fig 2). Asci expand from an initial length of approximately 76 microns and a volume of 3700 μm^3 to the full length of the fruiting body, about twice their initial length (Trail *et al.*, 2005). To double in length and yet preserve their volume, these asci must shrink their cross section of a factor 2, thus their radius by a factor of $\sqrt{2}$. This amounts to an increase in their surface area by $2/\sqrt{2}$. Typical double lipid membranes only stretch about 5%: some unknown mechanism must be in place to allow for such a large extension. The exact amount of surface increase depends on the details of ascus shape, which is not perfectly cylindrical: we observe that mature asci are smaller at the base and wider at the top (Fig 2), due to the pressure of paraphyses and other asci in the centrum. For this species, discharge of spores through the ostiole of the perithecium can be directly observed under a microscope as successive asci extend upward and fire their spores. Under optimal conditions of moisture, one ascus fires every 45 s (Cavinder *et al.*, 2011). Thus, assuming these asci fire as soon as they are fully extended, the tip moves at a speed of about 2 microns/second, about 5 times faster than the fastest reported growth for pollen tubes in vitro, 25 microns/min (Hill *et al.*, 2012). Thus, extra membrane will be required for extension of the ascus, either through invaginations of the wall and membrane in unextended asci, or by rapid expansion of the membrane and wall during ascus extension. In several species, there is evidence of membrane and wall invagination (Reynolds, 1971; Parguey-Leduc and Janex-Favre 1981; Minter and Cannon, 1984). Minter and Cannon (1984) suggest that "latitudinal striations" observed on the discharged asci of some species indicate wall stretching has occurred. However, examination of other species, including *Sordaria humana* (Read and Beckett, 1996), show no indication of additional wall and membrane material in pre-extension asci. Further investigations are needed to account for the exact shape of these asci and to determine how they implement the geometrical constraints imposed by the limited stretchability of the membrane.

How does the ascus shoot before breaking?

The ascus pore has the important role of regulating the release of the ascus contents. As stated above, the asci may resist circumferential stresses through helical cell wall reinforcement. In addition, in closed fruiting bodies, the presence of swollen paraphyses among the asci and mucous, which will swell in the presence of water, cause the buildup of counterpressure against the asci. In fruiting bodies that are open, or do not have paraphyses, stiffer ascus walls may perform the same function. Thus, the ascus apex would be the only area not receiving counterpressure (Minter and Cannon, 1984).

The ascus pore is a source of great diversity among the ascomycetes, and has been used in the past as a taxonomic character (Beckett 1981). Pores can be extremely complex or simple. Recent studies have demonstrated the importance of the pore during the firing process. Fritz *et al.* (2013) found that the morphologies of 52 poricidal species (having an apical pore) across the Ascomycota are nearly optimal fungal cannons. In these

species, the dissipation of energy during spore discharge is minimal according to the detailed elastohydrodynamics of this process. The data are consistent with the hypothesis that pressure inside the ascus reaches a maximum level proportional to the elasticity of the apical pore. These findings suggest that pore opening occurs at a prescribed pressure corresponding to a safety level to avoid uncontrolled rupture of the ascus. To enforce such a safety criterion, the body of the ascus needs to communicate with the apex to time the opening of the tip upon reaching the correct internal pressure.

The molecular mechanism that controls the timing of pore opening is unidentified. However, in *F. graminearum* calcium has been shown to be a major regulator of ascus firing. Stretched-gated and voltage-gated Ca^{2+} channels synergistically activate discharge (Hallen and Trail, 2008; Cavinder *et al.*, 2011). The knockouts of Ca^{2+} channels result in impaired firing, but the wild type phenotype is rescued by an increased concentration of extracellular Ca^{2+}. This demonstrates that Ca^{2+} plays a key role in this process. It is possible that calcium flows in and interacts with the actin network, either directly or through molecular motors (myosin). The gated calcium channels may sense stretch, and are thus natural candidates to activate the opening before the ascus breaks. The actin network may direct reshaping of the ascus and/or potentially open the apical pore.

3. Conclusions

In this review, we have revisited the microscopic mechanism of ascus firing, enumerating the important checkpoints that need to be in place to ensure successful discharge. We have then suggested possible connections between the physical constraints on ascus firing and the molecular processes responsible for their implementation. Information on these processes is surprisingly sparse, given the fundamental importance of ascus firing in the spreading of fungi across the globe. Further research is needed to quantitatively pursue these leads and advance our understanding of ascus firing.

Following discharge, the ascospores are launched into the air. Fungi have evolved adaptations to overcome some of the issues of air dispersal, a topic that has been recently reviewed elsewhere (Pringle *et al.*, submitted for publication). Individually launched spores are dramatically decelerated after discharge due to air drag and drag is more severe as size is reduced (Trail *et al.*, 2005). The Pezizomycetes produce cup-shaped apothecia that use their fruiting bodies to uniquely affect air drag. Apothecia discharge their asci simultaneously, a phenomenon known as "puffing" resulting in a self produced wind that carries the spores 20 times farther than the range of individually fired spores (Buller, 1958; Roper *et al.*, 2010). Spore shape is another important determinant of spore range. A theoretical analysis and comparison with real data of over 70 ascomycete species, shows that individually launched spores are constrained to remain within 1% of the minimum possible drag for their size (Roper *et al.*, 2008). Recently, a novel mutation resulted in a change in ascospore shape in *F graminearum* from crescent shaped to round. Accompanying the shape change was an increase in the mean distance the spore traveled in still air. Higher turgor pressure was also demonstrated in the mutant. Analysis showed that both the shape and the higher pressure on discharge contributed to the increased shooting distance (Min *et al.*, 2010).

A holistic view of spore dispersal entails an understanding of the connection between microscopic and planetary scales. Spores sediment at a distance that can range from few mm to hundreds or even thousands km from the fruiting body. This impressive variability is determined by a number of factors, including the shape and size of the spores, the parameters of discharge, the fluid dynamics of air in close proximity of the fruit body, the canopy where the spores are released and the large-scale atmospheric circulation. While fungi control discharge at the slimmest precision, the species fate at the global scale is subdued to the extremely fluctuating atmospheric circulation. Future research will have to bridge this gap and explore how the stochastic fate of spore dispersal shapes the evolution of the species.

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