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Review

Go with the flow: mechanisms driving water transport during vegetative growth and fruiting☆

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ABSTRACT

Fungi need water for all stages of life. Notably, mushrooms consist of ~90% water. Fungi degrade organic matter by secreting enzymes. These enzymes need water to be able to break down the substrate. For instance, when the substrate is too dry, fungi transport water from moist areas to arid areas by hydraulic redistribution. Once nutrients are freed from the substrate, they are taken up by transporters lining the cell membrane. Thereby an intracellular osmotic potential is created which is greater than that of the substrate, and water follows by osmosis. Aquaporins may facilitate water uptake depending on the conditions. Since fungi possess a cell wall, the cell volume will not increase much by water uptake, but the cell membrane will exert higher pressure on the cell wall, thereby building up turgor. Fungi have tightly coordinated osmotic regulatory controls via the HOG pathway. When water is getting scarce, this pathway makes sure that enough osmolytes are synthesized to allow sufficient water uptake for maintaining turgor homeostasis. The fungal network is interconnected and allows water flow when small pressure differences exist. These pressure differences can be the result of growth, differential osmolyte uptake/synthesis or external osmotic conditions. Overall, the water potential of the substrate and of fungal tissues determine whether water will flow, since water flows from an area of high- to a low water potential area, when unobstructed. In this review we aim to give a comprehensive view on how fungi obtain and translocate water needed for their development. We have taken *Agaricus bisporus* growing on compost and casing soil as a case study, to discuss water relations during fruiting in detail. Using the current state-of-the-art we found that there is a discrepancy between the models describing water transport to mushrooms and the story that water potentials tell us.

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1. Introduction

From the moment a fungal spore germinates, the fungus depends on water for its growth (Heaton *et al.*, 2010; Lew, 2011), substrate degradation (Guhr *et al.*, 2015), nutrient uptake, reproduction and spore dispersal (Dressaire *et al.*, 2016). Mushrooms even consist of ≥90% water (Kalberer, 1985). Water and nutrients are transported throughout the fungal network by tip-directed mass flow that is the result of internal pressure differences (Eamus and Jennings, 1984). It is not clear in which part(s) of the mycelium water is taken up, while this has huge implications for the extent and direction of transport. For example, some hyphae can be coated in hydrophobins (Wösten, 2001), which would prevent water uptake (Winandy *et al.*, 2019). Moreover, hyphae of *Agaricus bisporus* can be covered with calcium oxalate crystals (Masaphy *et al.*, 1987). Does this prevent water uptake? Some imply that water must be taken up in the colony centre or along the hyphae in the colony (reviewed by Lew, 2011; Fricker *et al.*, 2017) to enable long-distance tip-directed mass flow, while it is also suggested that water is taken up locally at the hyphal tips to sustain hyphal extension (by Lew, 2011; Tegelaar, 2017, 2020). If all the water, required for hyphal extension, is taken up at the hyphal tip, the mass flow observed towards the colony margin (Lew, 2005), would not be possible, since it could counteract the sub-apical pressure differences needed for tip-directed mass flow. Likewise, on the other hand, if the centre of the colony is the sole zone of water uptake, this would counteract the observed oscillations and bidirectionality in transport directions (Tlalka *et al.*, 2007; Muralidhar *et al.*, 2016 Schmieder *et al.*, 2019). This suggests that these proposed models might be incomplete and can co-exist or that water uptake is regulated in space and time and can be heterogeneous between single hyphae. This is discussed in more detail below. Although extensive efforts have been made to understand the water relations in *A. bisporus* cultures, it is not clear how water would be transported from compost to the mushrooms, since mushrooms have higher water potentials than the compost (Kalberer, 1983, 1985, 1987, 2006) and water should flow from areas with high to low water potentials.

In this review, that focusses mainly on saprotrophic mushroom forming fungi, we explore what factors influence water uptake and transport in the vegetative mycelium and transport to the mushrooms, and how water availability in the substrate may limit mushroom formation in *Agaricus bisporus* as an example.

2. Water uptake and transport

2.1. *Aquaporins*

Water needs to be transferred across the fungal cell membrane in order to enter the cell. Although the cell membrane is permeable to water, aquaporins increase membrane permeability and allow control by gating. Four classes of fungal aquaporins have been proposed based on phylogenetic analysis: orthodox aquaporins, aquaglyceroporins, small basic intrinsic proteins (SIP) like aquaporins and X intrinsic proteins (XIP; Verma *et al.*, 2014). However, basidiomycetes only possess orthodox aquaporins or aquaglyceroporins (Xu *et al.*, 2013; Verma *et al.*, 2014).

Orthodox aquaporins only allow permeation of water molecules. The crystal structure of the Aqy1 in *Pichia pastoris* indicates that water molecules can be taken up in single file, but that water is not allowed to exit the cell. Gating is regulated by changes in membrane tension and phosphorylation (Soveral *et al.*, 2008; Fischer *et al.*, 2009). Aquaglyceroporins transport water, glycerol (Nehls and Dietz, 2014), urea, and arsenite (Heller *et al.*, 1980). They show the highest number of functional subgroups in fungi compared to all other organisms that have been investigated (Verma *et al.*, 2014), indicating that they have specialized roles. For example, yeast aquaglyceroporin Fps1 closes in response to hyperosmotic conditions (Tamás *et al.*, 1999; Lee *et al.*, 2013). Remarkably, some aquaglyceroporins found in the basidiomycete *Laccaria bicolor* are better water transporters than the orthodox aquaporins (Dietz *et al.*, 2011; Xu *et al.*, 2013). Genomic analysis of *A. bisporus* has revealed only four aquaporin genes. They all belong to the aquaglyceroporin class. Homologs of these genes influence membrane water permeability in *L. bicolor* (Dietz *et al.*, 2011). The function of these missing orthodox aquaporins might thus be taken over by the aquaglyceroporins in *A. bisporus* (Nehls and Dietz, 2014). Although, the plasma membrane is usually already sufficiently permeable to water, aquaporins may facilitate water uptake at lower temperatures, when water permeability of the plasma membrane becomes limiting, or in situations in which bulk volumes of water are required.

Expression analysis has shown that aquaporins are upregulated during fruiting body formation in *L. bicolor* and *Flammulina filliformis*. High transcript levels are found during the primordial stage. In later stages either the stipe or cap shows high expression of aquaporins (Nehls and Dietz, 2014; Xu *et al.*, 2016; Liu *et al.*, 2020). These studies provide an interesting starting point on testing the functioning of aquaporins in water permeation during fructification. Their role could be imperative given that mushrooms expand in size exponentially (Straatsma *et al.*, 2013), which requires bulk water transport in a relatively short time span. In analogy with this, human renal tissue expresses aquaporins to facilitate reabsorption of water, reaching volumes of 150 L per day (Noda *et al.*, 2010)! Studying which aquaporins are expressed where and when, and their gating, and whether they impact water uptake during specific conditions, such as fruiting, will improve our understanding of water transport in fungi.

2.2. *Osmoregulation*

Turgor is regulated by the High-osmolarity glycerol (HOG) pathway (Reiser *et al.*, 2003; Schaber *et al.*, 2010). The HOG pathway does not only respond to osmotic stress, but also to stresses such as cold (Panadero *et al.*, 2006), citric acid (Lawrence *et al.*, 2004) and heat (Winkler *et al.*, 2002). The HOG pathway structure of *Saccharomyces cerevisiae*, as well as a timeline following hyperosmotic shock have been previously reviewed (Hohmann, 2002; O'Rourke *et al.*, 2002; Hohmann *et al.*, 2007; Saito and Posas, 2012). The HOG pathway consists of two branches that sense osmotic changes differently

(Fig. 1). These branches are termed the Sho1 and Sln1 branch and integrate their signal transduction at the MAPKK Pbs2, which in turn activates the MAPK Hog1 (Maeda *et al.*, 1995). The HOG pathway in yeast encompasses an immediate response aimed at maintaining vital cellular processes, followed by a more long term transcriptional response to counteract the hyperosmotic conditions. The fast response involves mediating ion fluxes (Proft and Struhl, 2004; Lew, 2011), whilst the transcriptional response induces the synthesis of compatible solutes, such as glycerol, which lowers the internal osmotic potential (Albertyn *et al.*, 1994).

Considerably less is known about the HOG pathway in filamentous fungi. Comparative genomic analysis between yeast species, filamentous ascomycetes, and the basidiomycete *Ustilago maydis*, highlight a 'consensus' HOG pathway consisting of the Sho1 and the Sln1 branch converging on Pbs2. However, designs of both branches differ notably in the pathway input between these species (Krantz et al., 2006a, 2006b, 2006b). Activation of the HOG pathway via the Sln1 branch in the filamentous ascomycete *Neurospora crassa* has been recently reviewed (Lew, 2019). Unlike in *S. cerevisiae*, the Sho1 branch in filamentous fungi presumably does not function in response to osmotic changes, but in the filamentous growth MAPK pathway (Krantz *et al.*, 2006a). Indeed, the HOG pathway of *Aspergillus nidulans* is solely activated by proteins orthologous to the Sln1 branch of *S. cerevisiae* (Furukawa *et al.*, 2005).

The sensor Sln1 is the only hybrid histidine kinase in *S. cerevisiae*. Fungal histidine kinases generally have sensor functions to respond to a variety of stresses (Bahn et al., 2007). Most Basidiomycetes have multiple histidine kinases, implying a more complex input of extracellular signals. A classification system of these proteins has been proposed by Lavín *et al.* (2010). *A. bisporus* has four hybrid histidine kinases, one of which is a type IA (AGABI2DRAFT_228355; Lavín *et al.*, 2013). The hybrid histidine kinase type IA Tco1/Nik1 regulates the HOG pathway in *Cryptococcus neoformans* (Bahn *et al.*, 2006). The histidine kinase Le. Nik1 is important during fruiting body formation in *Lentinula edodes*. Expression of Le. Nik1 increases as primordia mature into fruiting bodies. High amounts of transcripts are found in the trama cells, that support the gills, and expand rapidly by absorption of water during the later stages of fruiting body development (Szeto *et al.*, 2008). This suggests that the HOG pathway does not only mediate the regulation of osmotic potential during vegetative growth, but also the development of fruiting bodies. Functional analysis of this pathway should be done in *A. bisporus* to provide definitive conclusions. We will discuss how fruiting bodies of *A. bisporus* generate an osmotic potential in paragraph 3.7.

2.3. *Turgor and tip-directed mass flow*

Filamentous fungi maintain turgor for optimal growth, but also differences in turgor pressure exist in a fungal colony. Eamus and Jennings (1984) have proposed pressure-driven mass flow as a mechanism for translocation of water and solutes in the direction of the growing hyphal tips in Basidiomycetes. This flow is achieved by internal small pressure differences along the hyphae that result from differential (i) osmolyte take up, including ions and nutrients (Lew, 2005); (ii) osmolyte synthesis, such as glycerol (Lew, 2011); (iii) extracellular osmotic or matric potentials (Amir *et al.*, 1995; Muralidhar *et al.*, 2016); or (iv) growth (Heaton *et al.*, 2010; Lew, 2011, 2019). Filamentous fungi usually have higher pressures in the centre than in the periphery of the colony (Luard and Griffin, 1981; Granlund *et al.*, 1985; Thompson *et al.*, 1985; Eamus and Jennings, 1984, 1986). These pressure differences can generate tip-directed mass flow, only if water is allowed to exit the translocation pathway, since water is incompressible. During normal conditions the most predominant way for water to exit is likely through growth. When hyphae extend, the new growth volume needs to be filled with water (Heaton, 2010). This water can move from adjacent compartments into the apical compartment, thereby generating long distance mass flow (Fig. 2A). By branching and extending, hyphae can increase the tip-directed mass flow velocity (Fig. 2B; Heaton *et al.*, 2010). However, tip-directed mass flow can never completely reach the apical compartment. On this micrometre scale other means of translocation, such as through tubular vacuoles, cytoplasmic mixing, or diffusion, should take over to deliver nutrients into the apical compartment (Darrah *et al.*, 2006; Bleichrodt *et al.*, 2015a).

The colony margin usually has a lower osmotic potential than the centre of the colony (Luard, 1982a, 1982b, 1982b). The high concentration of solutes in the margin thus also attracts water through osmosis. This water could be all derived from mass flow (Fig. 2A; see Heaton *et al.*, 2010; Fricker *et al.*,

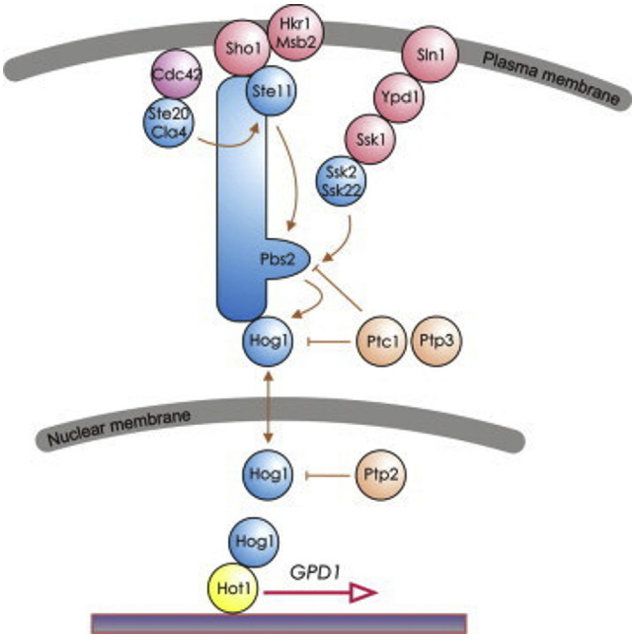

Fig. 1 – Signal transduction in the yeast HOG pathway. Membrane-localised sensors and regulators are shown in red, protein kinases in blue, protein phosphatases in orange and transcription factors in yellow. Two branches converge at the level of Pbs2 to activate Hog1, which accumulates in the nucleus under stress. Ste11, Ssk2 and Ssk22 are MAPKKKs, Pbs2 a MAPKK and Hog1 the MAPK in the system. See text for further details. Used with permission of [John Wiley and Sons], from [Control of high osmolarity signalling in the yeast *Saccharomyces cerevisiae*, Hohmann, 583, 2009]; permission conveyed through Copyright Clearance Center, Inc.

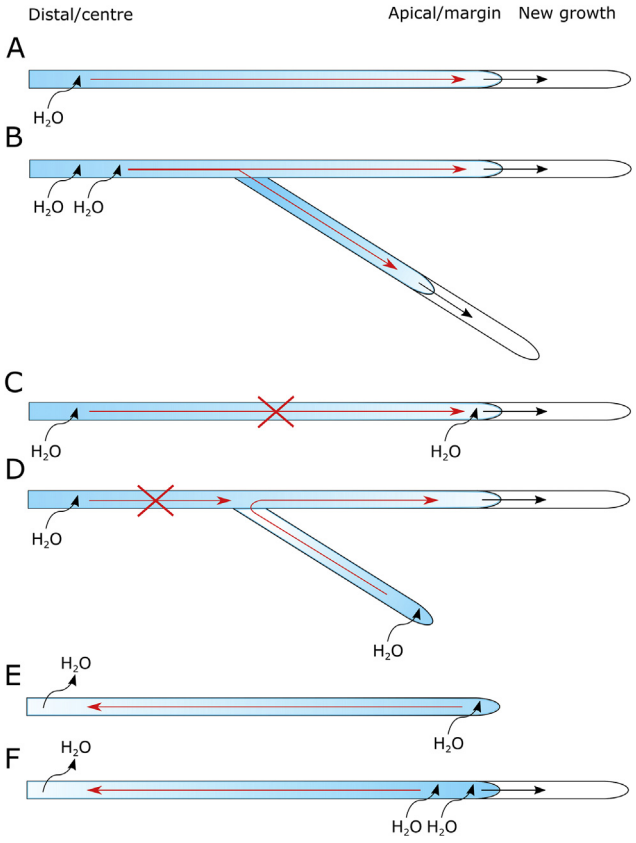

Fig. 2 – Mass flow in hyphae depending on local water uptake, water loss and growth. A) Tip-directed mass flow. If hyphae take up water distally, pressure increases locally and mass flow is initiated towards the colony margin, provided that the apex extends to enable water to exit the translocation pathway in the newly extending tip, since water is incompressible. The amount of water taken up should be equal to the amount of water needed to fill the new growth volume. B) Branching of hyphae doubles the flow rate. If hyphae branch and water is taken up distally, double the amount of water is needed to maintain the original extension rate in each tip. This doubles the flow rate in the compartments located distally from the branch. In theory, simultaneous uptake of water distally and at the apex could happen, but at each of these locations this needs to be half the amount of water required to maintain extension. In this case the original flow rate is maintained in the compartments before the branch, but mass flow within each branch is halved. However, this reduces the benefits of growth-induced mass flow to maximally supply water and nutrients for the extending hyphae that are acquired distally. C) No mass flow. When the hyphae take up all the water needed to fill the extending tip apically, mass flow is not initiated since water from the translocation pathway cannot enter the new growth zone. D) Reversed flow in branching hyphae. If one of the branches stops growing and takes water up at the tip an over pressure is generated that reverses the flow and provides the other branch with mass flow to drive extension. E) Reversed mass flow. If hyphae stop growing and take up water at the apex, this would generate an over pressure at the apex and would generate a mass flow towards the colony centre. This flow can only be brought about if water can exit at the centre via exudation, evaporation or new growth such as forming aerial hyphae or reproductive structures. If water could not exit the translocation pathway the turgor would build up, but no mass flow would be initiated. F) If hyphae take up more water at their apex than the amount needed to fill the extending tip, a reversed flow towards the colony centre could emerge, provided that water can exit the translocation pathway at the centre. It cannot be excluded that alternatively hyphae transiently increase their extension rate to accommodate the excess water uptake and as a result no reversed mass flow would be triggered. Transient increase in extension has been observed in algae after increasing turgor pressure at the tip (Zhu and Boyer, 1992). Curved black arrows indicate water uptake or loss. Red arrows indicate mass flow and its direction. Straight black arrows indicate hyphal tip extension. When red arrows are marked with a red cross, this means that mass flow is not possible. The assumptions are that the cytoplasm is continuous, the cell wall is rigid and that there is no resistance to flow within hyphae.

2017), but it could also be taken up by the apical compartment itself (Lew, 2011). However, if all the water required to fill the new growth volume is taken up by the tip, this would prevent water to exit the translocation pathway, and the observed mass flow would be not possible (Fig. 2C). By applying hyperosmotic conditions to different zones within the fungal colony, mass flow can be temporarily altered. When applied to central or subapical zones, water leaks out locally and tip-directed mass flow stops or even reverses (Thompson *et al.*, 1985; Lew, 2005, 2011; Abadeh and Lew, 2013) and as a result growth stops or reduces its rate (Clarke *et al.*, 1980). When applied at the colony margin, water leaks out of the tips, and the velocity of mass flow increases (Lew, 2005, 2011; Abadeh and Lew, 2013). Again this principle is based upon that water exits the translocation pathway, in this case by exudation. The more water exits, the higher the velocity of mass flow, provided that water is taken up at another location than where water exits (Fig. 2).

Mass flow supports the growing margin with nutrients that are derived subapically or from the centre of the colony (Lew, 2011). This is beneficial during exploration of low nutrient substrates with patchy food sources. However, in the laboratory most experiments have been performed using nutrient rich conditions. When the second compartment of leading hyphae is ruptured by laser ablation, the apical compartments that survive, continue growth at a similar rate, as if the hyphae were still attached to the colony (Tegelaar and Wösten, 2017; Tegelaar *et al.*, 2020). Thus, at least in case of an emergency, apical compartments can take up sufficient water and nutrients to maintain normal extension rates during nutrient rich conditions, and do not rely on resources delivered by long-distance mass flow. Nonetheless, on nutrient rich conditions, Aspergilli still show tip-directed mass flow of sugars to the colony margin, indicating that, although not required, mass flow is still eminent during nutrient rich conditions (Bleichrodt *et al.*, 2015b). Mass flow is thus happening regardless the nutrient conditions, but is only essential to sustain growth in nutrient poor conditions.

Although, mass flow has classically been regarded as tip-directed (Eamus and Jennings, 1984), this long standing view is being challenged by observations of reversed mass flow during normal conditions (thus not regarding hypo- or hyperosmotic conditions). Oscillations of nutrient transport have been observed in *Phanerochaete velutina* (Tlalka et al., 2002, 2003, 2007), while single hyphae switching mass flow direction over time have been reported in *Coprinopsis cinerea* (Schmieder et al., 2019). These observations contrast the model of tip-directed mass flow. Dynamic opening and closing of septa has been proposed as a mechanism to revert mass flow (Schmieder et al., 2019). However, it is at present unclear how the fungus would regulate this concerted dynamic opening and closing of septa, and closed septa do not seem to form a barrier for water and sugar transport, at least in Aspergillus (Bleichrodt et al., 2015b). It is not known whether closed septa of Basidiomycetes block water flow, but they have at least a different architecture than Ascomycete septa (Van Peer et al., 2010). We propose two alternative models for reversed mass flow: (i) when growth stops and hyphae start taking up water at the tip, this would reverse the direction of mass flow so that it is directed towards the colony centre (Fig. 2E); (ii) if hyphal tips take up more water than is needed to fill the new growth volume, this could result in a reversed flow (Fig. 2F), if the hyphal extension rate keeps constant. In both cases, the hyphae should lose water distally by evaporation or exudation. Therefore the sites and volumes of water uptake and water exiting the translocation pathway in a mycelium are of critical importance in understanding how turgor pressure differences mediate mass flow. Identifying the locations and unravelling dynamics of water uptake/loss and subsequent pressure differences, at both the single hypha level and the level of distinct zones within the colony, should provide the starting point to resolve these seemingly contrasting models and observations.

3. Water requirements during fruiting body formation in *A. bisporus*

3.1. *Commercial culturing of* A. bisporus

To provide an overview of the water balance of *A. bisporus* during fruiting, we first describe how the fungus is commercially cultivated. *A. bisporus* is grown on compost that is based on a mixture of horse/chicken manure, wheat straw, gypsum and water. Composting is performed in two phases. In phase I mesophilic bacteria self-heat the compost during 3–6 days and degrade proteins and (hemi)cellulose, and produce ammonia. Gradually thermophilic bacteria are selected due to temperatures as high as 80 °C. Phase II starts at 50 °C, followed by 60 °C for 2 days and finally a 3 day period at 45 °C. In this phase, actinomycetes and the thermophilic fungus *Mycothermus thermophilus* aid in ammonia removal and the high temperatures kill undesirable microorganisms (Gerrits, 1988; Straatsma and Samson, 1993; Jurak, 2015; Jurak et al., 2015). This makes the compost selective for *A. bisporus* (Ross and Harris, 1983; Straatsma et al., 1989, 1994). Spawn, usually rye colonised with mycelium, is mixed through the compost. *A. bisporus* colonises the compost in 16 days, feeding on lignin, (hemi)cellulose and/or bacteria (Jurak, 2015; Jurak et al., 2015; Vos et al., 2017). The colonised compost is transported to the grower, where it is applied as a ~25 cm thick layer and topped with a 5 cm thick casing layer (Fig. 3), composed of peat and lime. After colonisation of the casing layer, primordia formation is induced by lowering the air temperature and lowering CO_2 levels by ventilation (Visscher, 1988). Few of the primordia develop into mature mushrooms, that are harvested in 2–3 flushes at weekly intervals (Vos et al., 2017).

Previously, typical water contents of casing soil and compost were 50–56% and 67–69%, respectively (Gerrits, 1971; Kalberer, 1985). Of note, the casing soil that is nowadays used, has a higher water content of 80% at a water potential of ~0 MPa. This may impact the conclusions that are drawn below, when applied to modern day casing. Previously, casing contained 2 volumes black peat, 2 volumes black soil and 1 volume ground chalk or lime cakes (Kalberer, 1985; or similar, Kalberer, 1990b), while presently casing contains only peat and lime (Vos et al., 2017).

Mushrooms contain more than 90% water (Kalberer, 1987), thus their water supply is paramount for development. In the next paragraphs we will describe from which layers (*i.e.* casing and compost) in the culture the mushrooms take their water, what the mechanism is for driving this water uptake/transport, and discuss which factors may limit mushroom development in relation to water availability.

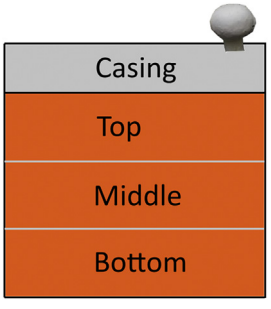

Fig. 3 – *A. bisporus* mushroom culture. Colonised compost is topped with a layer of casing soil to produce mushrooms. In some studies the compost was subdivided in three equal sized layers (Top, Middle and Bottom) to analyse their individual water content or water/osmotic potentials.

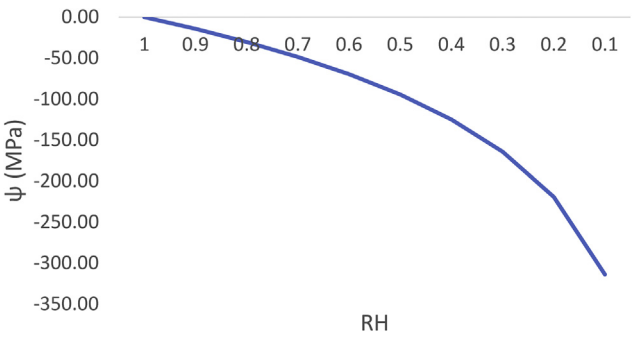

Fig. 4 – The relation between the water potential (ψ) of air and relative humidity (RH). Calculated using eq. 4 in box 1.

3.2. *The casing layer and compost differentially supply mushrooms with water*

A 5–6 cm thick casing layer on ~17.5 cm compost contributes on average 33% of the water in mushrooms of the first flush, whilst a thinner 3–3.5 cm casing layer decreases this contribution to 16% (Kalberer, 1983, 1985). These observations are similar to Gerrits (1968) and Flegg (1974). Moreover, subsequent flushes draw more water from the casing, until in the third flush almost equal parts of the water are taken from the compost and the casing (Kalberer, 1985). The water content after the second flush is 54.1% for casing and on average 71.1% for compost, while at start these are 56.0% and 71.9%, respectively. Together, this shows that although casing has a lower water content, mushrooms still take a considerable amount of water from the casing. This is because the osmotic potential of casing (−0.26 MPa) is higher than that of compost (−1.33 MPa on average; Kalberer, 1987; see also Box 1), since casing has a lower solute concentration. Thus, water can be more easily extracted from the casing than from the compost by the mycelial network to supply the mushrooms.

Interestingly, thin (3–3.5 cm) casing layers produce mushrooms with a higher dry matter than casing layers with 5 cm casing layer. In addition, young mushrooms (stages 1–2, Fig. 5) have a higher dry matter content than old mushrooms (stages 5–7, Fig. 5) on a 5 cm casing layer. This is in agreement with the facts that young and old mushrooms take 37% and 46% of their water from casing, respectively, and that

Box 1: Water potential (ψ)

The water potential is the sum of all forces that work on water. When unobstructed, water flows from an area with high water potential to an area of lower water potential. This can be sometimes tricky to imagine, since the water potential can be between zero and a negative value. Thus water would flow from an area with a water potential of *e.g.* −2 MPa to an area of −4 MPa. When a substrate dries out, as a consequence of water usage by the fungus or evaporation, the water potential will drop. The relationship between gravimetric water content and water potential is not linear and can be depicted in a soil-water characteristic curve (Tuller and Or, 2005).

The water potential is given by the following equation (Eq. 1):

$\Psi = \Psi_\pi + \Psi_p + \Psi_s + \Psi_v + \Psi_m (Pa = kg \cdot m^{-1} \cdot s^{-2} = Nm^{-2})$ where:

The osmotic potential (Eq. 2) $\Psi_\pi = -i \cdot \Phi \cdot C \cdot R \cdot T$, *where* i = *ionization constant or Van't Hoff factor (the number of molecules that the substance disintegrates in when dissolved in water),* Φ = *osmotic coefficient (is the deviation of a solvent from ideal behaviour, which is usually close to 1 (*mounir *et al., 2020)*) C = *molarity* ($mol \cdot l^{-1}$), R = *gas constant* ($8314\ l \cdot Pa \cdot K^{-1} \cdot mol^{-1}$), *and* T = *absolute temperature* (K).

The (hydrostatic) pressure potential ψ_p or turgor is the internal pressure of the cell and is usually positive, in contrast to all other factors contributing to the water potential that are negative. If a cell is at equilibrium with a bath of pure water at atmospheric pressure, then its turgor pressure is equal to its osmotic pressure (Beauzamy *et al.*, 2014). However, this holds only for a cell that is not constrained by a rigid cell wall, which fungi do possess. In walled fungi the turgor becomes increasingly greater with higher osmotic potential differences, since the cell wall gets more rigid at higher internal pressures (Lew, 2011). The turgor pressure can be measured using atomic force microscopy (Arnoldi *et al.*, 2000; Bovio *et al.*, 2019) or a pressure probe (Lew, 2005).

The gravitational potential (Eq. 3) $\Psi_s = \frac{m \cdot g \cdot h}{v}$, *where* m = *mass (kg),* g = *gravitational acceleration* ($9.81\ m \cdot s^{-2}$), h = *height difference (m),* v = *volume* (m^3).

The gravitational potential in a mushroom bed is negligible, since 1 kg of water transported over 0.25 m height difference, results in a pressure of just $(1 \cdot 9.81 \cdot 0.25)/(1\ kg/997\ kg\ m^{-3}) = -0.0025$ MPa.

The humidity potential (Eq. 4) $\Psi_v = \frac{RT}{V_w} \ln(RH)$, *where* R = *gas constant,* T = *absolute temperature* (K), V_w = *partial molar volume of liquid water* ($18E-6\ m^3 \cdot mol^{-1}$), *and* RH *is the relative humidity of the air as a fraction, thus 80% RH* = *0.8.*

The water potential quickly drops with decreasing relative humidity of the air (Fig. 4). In fact, the low water potential of air (−70 MPa at 60% RH and 22 °C) is usually the biggest factor for water loss, due to evaporation. In plant research the concept of the soil plant atmosphere continuum explains why and how water flows from the soil via root uptake through the plant and finally ends up in the air by evaporation from the leaves. Soil, root, stem, leaves, and air have increasingly lower water potentials (Taiz *et al.*, 2014). This ensures a continuous flow of water through the system that can be used for nutrient transport.

The matric potential is defined as the attractive forces between water and the soil, such as capillary pressure exerted by van der Waal's and electrostatic forces, given by the Young–Laplace equation (Eq. 5), $\psi_m = -\frac{2\gamma Cos(\theta)}{r}$ where γ is the surface tension of water ($N \cdot m^{-1}$), θ is the contact angle between the water and soil at the air–soil–water interface (°), and r is the radius (m) of the pore in hydrophilic soils. Usually the matric potential is not calculated, but determined by subtracting the measured osmotic potential from the water potential. In hydrophobic soils, the matric potential (Eq. 6) $\Psi_m = \Psi_p + \Psi_\pi$, where ψ_p is hydrostatic pressure, and ψ_π is osmotic potential, but in this case these cannot be dissected experimentally (Whalley *et al.*, 2013).

Water potential can be measured by several methods. For example, thermocouple psychrometry relies on the relation between the vapour pressure and the water potential of a sample. Water with a higher concentration of solutes has a lower vapour pressure, and thus in a closed chamber the surrounding air has a lower humidity. In a closed chamber, where temperature and pressure are in equilibrium, the humidity can be determined in various ways using a thermocouple (Boyer, 1995). From these humidity readings the water potential is calculated.

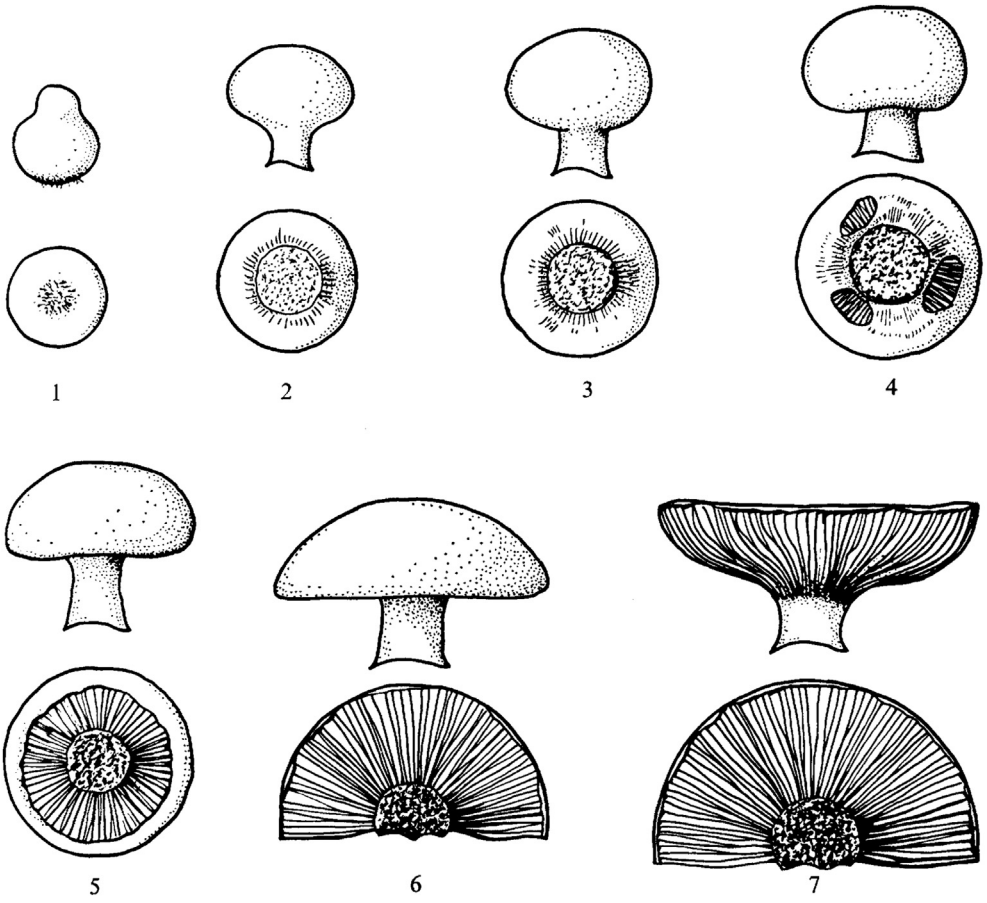

Fig. 5 – Appearance of sporophores at stages 1–7. Used with permission of [Microbiology Society], from [Carbohydrate Metabolism in *Agaricus bisporus* (Lange) Sing.: Changes in Soluble Carbohydrates during Growth of Mycelium and Sporophore, Hammond and Nichols, 93, 1976a]; permission conveyed through Copyright Clearance Center, Inc.

mushrooms take less water from a thinner casing layer. Mycelial biomass is lower in thin casing layers (Kalberer, 1983), and thus has a lower water absorbing capacity, hence explaining the higher dry matter content of mushrooms. Over three flushes combined, yields were 1.16x higher ($p < 0.01$) on thick casing compared to thin casing layers. This can be explained by a higher water content of the mushrooms (Kalberer, 1985). The fact that thicker casing layers result in mushrooms with higher water contents, indicates that casing mainly supplies water, while compost supplies water and dry matter to the mushrooms.

3.3. *The vegetative mycelium supplies mushrooms differentially depending on the compost depth*

For all flushes, most water is lost from the top layer of compost (Fig. 3), followed by the middle layer, while the bottom layer hardly exhibits changes in water content (Kalberer, 1985, 1987). Flegg (1981) has shown that the top layer of the compost also supplies most nutrients to the mushrooms. *A. bisporus* has been shown to transport water from zones of abundant- to zones of scarce water availability by hydraulic redistribution, to aid substrate degradation (Guhr *et al.*, 2015). However, the mycelium of the bottom compost layer does not seem to transport much water to the layers above. In compost the mycelium forms a fine network of mainly individual hyphae, while in casing predominantly bundles of hyphae (cords) are formed, of which some hyphae have higher diameters (Cairney, 1990). The volume flow rate in cords is strongly dependent on the radius (r^4; Herman *et al.*, 2020). Therefore, it is likely that the distance from the lower compost layer is so far located from the mushrooms, that the resistance of the fungal network therein is too high to significantly transport to the mushrooms. Supporting this, is the finding that a network architecture having predominantly cords shows 5-fold more nutrient transport to mushrooms than a network of predominantly fine mycelium (Herman *et al.*, 2020). On the other hand, the bottom compost layer may accumulate water due to percolation of water when the casing is watered. More research is needed to uncover the contribution of water percolation, hydraulic or external redistribution of water and water uptake by the fungus on the total water balance of cultures during cropping.

3.4. *Evaporation by the mushrooms and the casing layer*

By evaporation from the casing and through the mushrooms, and the mushroom harvest itself, the cultures lose water.

Although, relative humidity is usually kept between 95 and 99%, air still has a lower water potential (Eq. 4 in Box 1: −7.0 to −1.4 MPa at 22 °C; Fig. 4) than that of the casing and mushrooms (Kalberer, 1987), thereby stimulating evaporation. Evaporation totals to 9–50% of the water content of the harvested mushrooms, depending on the conditions (San Antonio and Flegg, 1964; Kalberer, 1987). Evaporation from the casing is constant over a large range of gravimetric water contents (Flegg, 1974). Mushrooms evaporate at the same rate as a free water surface. This is independent on age, developmental stage or air temperature (San Antonio and Flegg, 1964). 2 cm diameter mushrooms (stage 1, Fig. 5) evaporate 1 ml/day, while 6 cm diameter mushrooms (stage 4–5, Fig. 5) evaporate 2–4 ml/day, this is up to 3 mg/cm^2/h. When evaporation is increased to 6–8 mg/cm^2/h, growth ceases and the pileus becomes cracked, scaly and discoloured. This indicates that the hyphal network attached to the mushroom can only supply up to 3 mg/cm^2/h plus the water needed for expansion of the mushroom.

Thus, the rate of water transport to mushrooms must be greater than their evaporation rate, otherwise mushrooms could not expand exponentially in volume (Straatsma *et al.*, 2013). This suggests that reducing evaporation may increase expansion rate. However, when mushrooms are grown under water saturating conditions, mushrooms expand at the same rate as mushrooms under control conditions (Mader, 1943; Riber Rasmussen, 1959). This shows that evaporation is not required for mushroom expansion. Nevertheless, evaporation by the mushroom is important for spore dispersal (Dressaire *et al.*, 2016).

3.5. *Effect of watering*

Watering can increase the moisture content of the casing and to a certain degree that of the compost (Kalberer, 1985), since water percolates through the casing layer into the compost (Fig. 3). Colonisation of the casing layer is markedly affected by watering. In relatively dry casing, the mycelium grows dense, while in wetter casing the mycelium forms thick hyphal cords leaving areas of uncolonized casing (Flegg, 1962, 1974). Watering the casing layer shows a positive correlation with yield (Flegg, 1974). This suggests that cord formation correlates with mushroom yield. It is not clear whether this is due to better transport connections between the compost and the mushrooms or better water uptake from the casing.

Mushroom cultures that are given the same amount of water, but with different watering regimes, do not differ in yield by more than 10%. Yet, yield is optimal when watering is applied at regular intervals, during colonisation of the casing and fructification. When watering is delayed, yield is reduced and the casing is covered with more mycelium. When no water is applied, still 2/3 of the yield is obtained compared with regular watering, indicating that the substrate stores a considerable water reserve that supplies mushrooms under development (Flegg, 1975). At any point in development, the amount of water given to the casing needs to be ~50% of the wet weight of the fruiting bodies at that time, to keep the water level in casing constant. Watering of cultures results in higher moisture levels of the casing, top and middle compost layers (see Fig. 3). The yield is significantly higher in watered than in unwatered cultures, but this is only when harvesting after 3 weeks after filling, but not earlier. Fruiting bodies from watered cultures have significantly lower dry matter contents than unwatered cultures (Kalberer, 1990b).

3.6. *Hydrophobins: friend and foe*

Fungi secrete hydrophobins that assemble at the air–water interface. This helps the fungus escape from the substrate into the air to bridge gaps within the substrate or form reproductive structures (Wösten, 2001). During cultivation of *A. bisporus* both the compost, but predominantly the casing soil become more and more hydrophobic, which hampers water uptake after watering, thus reducing water availability for mushroom production. On the other hand, casing soil contains cells or pockets filled with water (Noble *et al.*, 1999). Once uncolonised casing soil is dried out too much, these pockets cannot be regenerated by watering. Thus both hydrophobins and collapse of casing pockets contribute to lower water uptake by casing. In colonised casing, hydrophobins may coat these pockets or coat water-filled gaps in the compost. The hydrophobins would lower the surface tension of water, thus lowering the capillary forces (see Box 1) of walls of air pockets, thereby increasing the matric potential, and thus free up scarce water for the fungus. This said, matric potential is quite low in commercial mushroom compost (Kalberer, 2006), thus this only comes into play at lower water availability. Thus paradoxically, on one hand hydrophobins might help to 'squeeze' out the last bits of water from the substrate, while on the other hand, they prevent water uptake by the casing and compost during watering, since they repel water.

It is unclear whether hydrophobin coating affects water uptake by hyphae. At least, when stones are coated with fungal hydrophobins they become impermeable for water, but keep them permeable for water vapour (Winandy *et al.*, 2019). If the same would hold for fungal hyphae, the location of water uptake would be dictated by hydrophobin secretion. However, if water could still evaporate from hyphae, this could locally give rise to drops in internal pressure, potentially driving mass flow. However, for hyphae that dive in the substrate this would not hold, since hydrophobins only establish on hydrophobic–hydrophilic interfaces (Wösten, 2001). More research is needed to discover the role of hydrophobins in water uptake in mushroom cultures.

3.7. *Mushrooms pull water from the vegetative mycelium by creating an osmotic potential*

Mushrooms synthesise compatible solutes to produce an osmotic potential, that drives water flow from the supporting mycelial network to them. These solutes are usually sugars, like fructose, arabinose and trehalose or sugar alcohols, such as mannitol and arabitol. The abundance and ratio of these is dependent on the species (Hong and Kim, 1988; Heleno et al., 2009, 2012; Reis *et al.*, 2012). *A. bisporus*' main osmolyte is mannitol and comprises 30–60% of the mushroom dry weight (Hammond and Nichols, 1976a; Tan and Moore, 1994; Stoop and Mooibroek, 1998).

Enzymes that are involved in compatible solute synthesis, such as trehalose phosphorylase (TP), mannitol dehydrogenase (MD), glucose-6-phosphate dehydrogenase (G6PD), and glycogen phosphorylase (GP) are developmentally regulated in contrast to hexokinase (HK) that is active during glycolysis (Wells *et al.*, 1987; Wannet *et al.*, 1999). The former three enzymes have higher levels in aerial hyphal aggregates, that precede primordium formation, than in vegetative mycelium. Before fruiting the mycelium accumulates high trehalose, glucose and sucrose levels, that drop during fruiting, but mannitol levels do not increase in the vegetative mycelium (Hammond and Nichols, 1976a; Wannet *et al.*, 1999). Trehalose translocates to the fruiting bodies and each molecule is then rapidly converted to a glucose and a glucose-1-phosphate molecule by TP (Wannet *et al.*, 1998), thereby potentially doubling the osmotic potential. These serve as carbon source for growth and for synthesis of the osmolyte mannitol via fructose through MD (Wannet *et al.*, 1999). Mannitol mainly accumulates in the stipe and pileus of mushrooms (Hammond and Nichols, 1976a). Higher G6PD and MD activities are consistently found in stage 1–2 sporophores (see Fig. 5) harvested between flushes, while their activity is minimal during flushes (stage 2) and as a result mannitol and trehalose levels are highest in stage 1 sporophores harvested between flushes (Hammond, 1981). Hammond and Nichols (1979) present conflicting evidence that mannitol levels are highest during flushes.

Glycogen has also been detected in mushrooms (Hammond and Nichols, 1979), but it is unclear if glycogen is produced in the mycelium and translocates to the mushrooms, and is then converted to glucose to serve mannitol synthesis, or whether glucose is transported and converted to glycogen and stored in the primordia until needed for mushroom expansion. The former explanation is less plausible, since glycogen is stored as 20 nm cytosolic particles (Northcote, 1953; Mundkur, 1960), that might not be able to pass the septa between neighbouring compartments. Glycogen levels of mycelium and fruiting bodies appear to be similar (Hammond, 1979) and can reach up to 22% of the dry weight (Hammond and Nichols, 1979). In another study, levels of young mushrooms were found to be smaller (stage 1–2 Fig. 5; 2–4% of the dry weight) than that of mature opened mushrooms (stage >5 Fig. 5; 5–8%; Hammond and Nichols, 1976b), indicating that glycogen is not degraded to build up an osmotic potential on one hand. On the other hand, small mushrooms that emerge just before a flush show maximum glycogen levels (Hammond and Nichols, 1979). Moreover, the sum of their peak trehalose and glycogen levels correlates with the yield of the first flush (Hammond and Nichols, 1979). This indicates that both trehalose and glycogen are converted to produce osmolytes, to pull water from the mycelium to support mushroom expansion during flushes.

This leads to a model in which the mycelium prepares for fruiting during before/inter flush stages, by accumulating sugars and trehalose. When mushrooms form, these compounds are transported to the mushrooms by (i) growth induced mass flow and are converted into osmolytes such as mannitol which pull water from the supporting mycelium; or (ii) uptake/synthesis of nutrients/osmolytes in the vegetative mycelium that attract water by osmosis from the substrate, which in turn creates an over pressure in the mycelium that pushes water to the mushrooms. These forces combined, work in harmony to supply the exponentially growing mushrooms (Straatsma *et al.*, 2013) with water and nutrients. It is not clear in what ratio cell division and cell expansion contribute to mushroom expansion. The facts that more mature mushrooms have higher water contents (Kalberer, 1983) and that mushrooms before a flush have higher mannitol levels (Hammond, 1981), indicate that expansion is mostly due to cell expansion by water uptake. Interestingly, total free sugar (alcohol) content is correlated with mushroom size (Table S1). Large mushrooms have 3x higher sugar (alcohol) content than smaller mushrooms ($p = 0.009$, t-test). Since larger mushrooms drain more water from their substrate than small mushrooms, they need to accommodate the drop in water potential of the substrate, by creating a more negative osmotic potential. The higher level of free sugar (alcohols) in larger mushrooms would facilitate this.

When NaCl or PEG is added to casing soil, which decrease osmotic- and matric potential (see Box 1), respectively (Ramirez *et al.*, 2004), mushroom yield is reduced (Kalberer, 1990a; Stoop and Mooibroek, 1998). Moreover, cropping is delayed, and mushrooms have higher dry weight percentages (Kalberer, 1990a). Interestingly, total crop dry weight is similar to controls and in experiments when salt is added to the casing soil. Moreover, NADP-dependent mannitol dehydrogenase (MtDH; EC 1.1.1.138) shows increased activity, abundance and mRNA accumulation, indicating elevated mannitol levels. Additionally, *MtDH* transcript level increases during mushroom maturation (Stoop and Mooibroek, 1998). This shows that *A. bisporus* can regulate the mushroom osmotic potential depending on the water potential of the substrate, at least between certain limits. However, when the water potential of the substrate becomes too low, no more mushrooms will develop on the bed, since water can be less easily extracted by the fungus. This explains why subsequent mushroom flushes have lower yields.

The big open question remains: what determines which primordia or pins will develop into fully mature mushrooms? After colonization of casing soil and venting, thousands of mushroom initials develop per m^2 while only a few of these mature. Previously, it has been hypothesized that a certain compound needs to reach a threshold to induce mushroom maturation (Chanter and Thornley, 1978; Chanter, 1979). This compound could be trehalose and/or glycogen. However, it is not clear what determines which pins will receive most. A plausible explanation would be that primordia tap in to different parts of the mycelial network. The differential network architecture could then determine which primordia receive most trehalose by cytoplasmic mass flow. Another explanation may be that outgrowing primordia compete, thereby limiting the number that will develop into mushrooms (Straatsma *et al.*, 2013).

3.8. *Mushrooms should not get water from the compost according to the story the water potentials tell*

To look into why mushrooms can more easily extract water from the casing than from compost, we have to look at the water potentials of the respective layers in the mushroom

culture and of the mushrooms (see Box 1 for an explanation of the water potential). If not obstructed, water would freely move from areas with high water potential to areas with lower water potentials.

The osmotic potential of casing press juice has been found to be −0.07 MPa at filling and −0.26 MPa after the second flush (Kalberer, 1987). Since casing matric potential is very small (0.04 MPa; Noble *et al.*, 1999), the casing osmotic potential is a good approximation of its water potential (see also Box 1). Watering is performed so that the moisture content of the casing is brought to about the same level as before each flush, but the top compost layer decreases in water content over time (Kalberer, 1987). Top, middle and bottom compost layers (Fig. 3) show osmotic potential changes from −1.46 to −2.16 MPa, −1.71 to −2.44, and −1.43 to −1.55, respectively, before flush 1 until the end of flush 4. The water content changes from 63.3% to 50.4%, 60.1%–51.7%, and 63.1%–65.1%, respectively (Kalberer, 2006). Since changes in water content are quite similar for the top and middle layer, but the middle layer shows lowest water and osmotic potentials, this indicates that the middle layer produces more osmolytes. The bottom layer does not lose water, indicating that it is not supplying much water to the mushrooms, as found earlier by Kalberer (1985). However, the higher the thickness of the compost bed, the higher the yield is (Leyh and Blok, 2017). Thus, since the bottom layer is not supplying much water to the mushrooms, it must have another function.

The water potential of mushrooms is on average −0.72 ± 0.10 MPa, while the osmotic potential is −1.13 ± 0.13 MPa. Pressure potentials of mushrooms are on average 0.4 MPa, during the cropping process (Kalberer, 2006). This is calculated by subtracting the osmotic potential from the water potential. However, this ignores the matric potential of the mushroom tissue. Thus, in practice the pressure potential could be even greater (see Box 1). However, in plant tissues matric potentials are close to zero at physiological water contents (Wiebe, 1966). Using the data of Kalberer (1987), we have calculated a correlation of 0.9 between the yield and the respective osmotic potential of the caps of the mushrooms over three flushes. The osmotic potentials of the caps increase (thus become less negative) during each following flush (Kalberer, 1987), suggesting that transport of osmolytes (or precursors thereof) to the mushrooms, decreases due to lowering osmotic potentials of the compost.

The water potential of the casing is always higher (>-0.26 MPa; Kalberer, 1987) than that of the mushrooms (−0.72 MPa), suggesting that mushrooms can readily take up water from the casing. In contrast, the water potentials of the compost (<-1.40 MPa) are always lower than that of the mushrooms (−0.72 MPa; Kalberer, 2006). It is therefore contradictory, that mushrooms take most of their water from the compost (Kalberer, 1983, 1985, 1987), since the water potential differences would oppose this water transport. However, to our knowledge the water/osmotic potential and pressure potential of the mycelium within the compost and casing were never measured during fruiting. Pressure potentials range from 0.86 to 3.32 MPa in mycelium of other fungi and are on average 2.03 MPa (Luard and Griffin, 1981). If *A. bisporus* mycelium would have similar pressure potentials during fruiting, it is likely that the vegetative mycelium (turgor 2.03 MPa) pushes water into the expanding mushrooms (turgor 0.4 MPa) due to their differential internal pressures. Internal pressure is dependent on the osmotic potential and this is usually close to that of the substrate (Luard, 1982b; Lew, 2011). Thus, the vegetative mycelium needs to accommodate its osmotic potential during decreasing water potentials of the compost during cropping to maintain turgor to push water to the mushrooms. The mushrooms keep synthesising sugar (alcohols) to maintain low osmotic potentials to pull water from their supporting mycelial network. Thus, both work in harmony to generate water transport to the mushrooms.

4. Conclusions

We have a limited understanding of the accessibility of water, water uptake and its translocation during the fungal life cycle. Notably, there are seemingly contrasting views and observations regarding water transport in the vegetative mycelium, but also to the mushrooms. For example, central water uptake facilitates mass flow to the colony margin, supplying nutrients and water for growth. If leading hyphae take up all the water required for extension, tip-directed mass flow would stop. Moreover, oscillations and bi-directionality of transport have been observed, which conflict with tip-directed mass flow. To complete the picture, it is key to unravel when, where and how much water is taken up and how this is regulated, but also where water exits the translocation pathway as exudation, water vapor or growth. Single cell measurements are required to understand the phenomenon of bi-directional translocation and oscillations observed in fungal colonies. According to the current account of the water potential data during fruiting, we cannot explain the rapid translocation of water from the compost to the mushrooms. We point to the internal pressure of the mycelium during fructification as the missing link in the story of water potentials. This should be measured using *e.g.* atomic force microscopy during flush and inter-flush periods to understand transport to mushrooms. We also foresee a role for aquaporins in facilitating bulk water uptake that is required for the exponentially expanding mushrooms.

Declaration of competing interest

The authors declare to have no competing interests.

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Appendix A. Supplementary data

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