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A Study of Water Relations of *Epichloë amarillans* White, an Endophyte of the Grass *Agrostis hiemalis* (Walt.) B.S.P.

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Abstract

In this study we measured water losses from various excised parts of plants of *Agrostis hiemalis* and stromata of the endosymbiont fungus *Epichloë amarillans*. Measurements were made by excising culms from plants, then inserting cut ends into microsedimentation tubes containing water and measuring water consumed under defined conditions. It was found that water was lost from the stroma at a rate 10 times faster than that from the surface of the emergent leaf blade. Comparative studies of stroma-bearing culms with those free of stromata showed that overall water losses in stroma-bearing culms were only slightly higher than water losses in culms without stromata. However, it was found that stroma-bearing culms were significantly reduced in mass when compared to culms free of stromata. This mass reduction is suggested to have a compensatory effect on water losses. It is suggested that the enhanced rate and unregulatability of the evaporation of water from stromata may have been a key factor in selecting for endophytes that do not produce stromata.

Keywords: *Epichloë*, endophytes, Clavicipitaceae

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1. Introduction

Epichloë amarillans White (Clavicipitaceae; Ascomycotina) is one of several species of fungi that live endosymbiotically in grasses (Clay, 1989; Schardl et al., 1991; Leuchtmann, 1992; White, 1994). This and other species of *Epichloë* (Fr.) Tul. grow in the intercellular spaces of leaves and tillers of grasses. When an infected grass individual flowers in the spring, the endophytes proliferate in inflorescence primordia tissues and may be incorporated into carpels and seeds, and thus are seed-transmitted to the next generation of the grass. However, another means of reproduction is frequently evident where the endophyte emerges from the grass and reproduces sexually. Here the endophyte proliferates in the inflorescence primordia and embeds that tissue and a surrounding leaf sheath in a mycelium to produce a structure referred to as a stroma (Kirby, 1961; Figs. 1, 3, and 4). On the surface of the stroma, spermatia (gametes) are produced. Spermatia are vectored between compatible mating types of the fungus by flies of the genus *Phorbia* (Bultman et al., 1995). When spermatia are deposited on a stroma of a compatible mating type, the ascoma containing perithecia develops (Fig. 3, arrow). Ascospores are ejected from perithecia onto surrounding vegetation. It is believed that

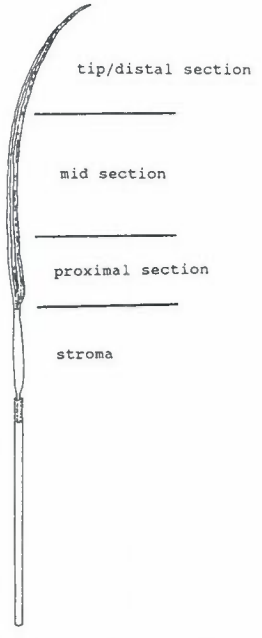

Figure 1. Illustration of stroma in microsedimentation tube showing approximate locations of sections.

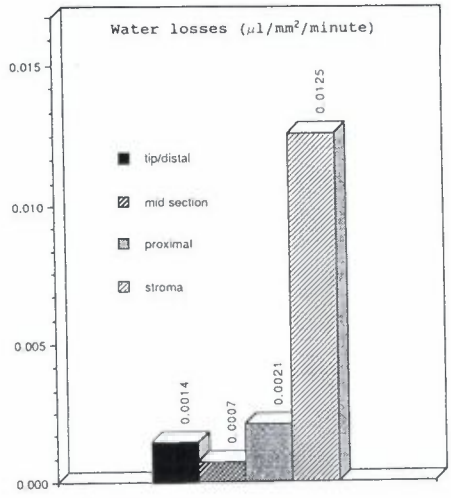

Figure 2. Water losses ($\mu l/mm^2/min$) for each section of stroma and emergent leaf blade.

these ascospores or secondary conidia developing from them possess the capacity to infect uninfected grasses, however, natural infections have not been demonstrated (Bacon and Hinton, 1988).

The endophyte remains entirely intercellular during all stages of its life cycle. It is believed that energy compounds for endophyte growth are obtained through absorption of nutrients that leak into the apoplast of tissues in which mycelium grows (Thrower and Lewis, 1973; Lam et al., 1994). Species of *Epichloë* produce an array of alkaloids that render host species toxic to animals and resistant to attack by insects (Clay, 1989; Lewis et al., 1993). Because of the enhanced resistance to insect pests, the symbiosis between endophytes such as *E. amarillans* and host grasses has been referred to as a defensive mutualism (Clay, 1988).

Because *Epichloë* produces its sexual reproductive structures on the surface of the entrapped inflorescences of the host, among the possible costs to the grass of this association with *E. amarillans* is a reduced production of seed-bearing culms (White and Chambless, 1991). Thus, fecundity of the host may be affected by the endophyte. Studies on water relations have demonstrated that *Epichloë* draws water from host tissues for activities such as ascospore liberation (Ingold, 1948; Raynal, 1991). These and a more recent study on translocation of dyes in stroma-bearing and nonstroma-bearing culms suggest that moisture relations may be an important element in the endophyte-host relationship (White et al., 1993). In this latter study the comparatively rapid accumulation of dyes in leaves emergent from the apex of stromata on excised culms was taken as an indication that the transpiration rate is increased in

culms on which stromata are produced. The study reported here was undertaken in order to quantify and elaborate on the characteristics of stromata and associated plant tissues with respect to moisture relations to the host and environment. The overall hypothesis to be evaluated is that production of stromata by the endophyte increases water losses from tissues of the grass.

2. Materials and Methods

Experimental material

Plants of the grass *Agrostis hiemalis* infected by *Epichloë amarillans* were obtained from several different wild populations in the Montgomery area and maintained in the field. Plants that did not possess necrotic or chlorotic regions and which bore freshly-formed spermatial stromata were selected for water loss experiments.

Measurement of water losses from the stroma and emergent leaf

To estimate water losses, culms with stromata were excised from plants and the cut ends immediately immersed in water to prevent air from entering the xylem. Culms were trimmed so that they had approximately 8–10 mm of stem tissue below the stroma. This stem segment was inserted into one end of a plastic microsedimentation tube (internal volume = 0.7 µl/mm length; J. T. Baker Diagnostics, Bethlehem, Pennsylvania). Silicone grease was used to seal the junction between culm and tubing (See Fig. 1). The tube with attached stroma was then placed in an upright test tube with the stroma and emergent leaf protruding from tube. Each stroma was placed under a gentle flow of air generated using a fan on the low speed setting placed approximately 1 meter from stroma. The ambient temperature was 22–24°C and the relative humidity ranged 50–60%. All experiments were conducted on a lab bench with normal room lighting being supplied by florescent bulbs (34 watt; Philips F40CW/RS/EW-II) at a distance of approximately 2 meters from plants. Water consumption of the intact stroma and emergent leaf blade was measured for a 10 minute period. Following this measurement, a segment of leaf was excised from the tip of the emergent stromal leaf (Fig. 1). The cut end of the truncated stromal leaf blade was sealed using tape so that evaporation from the cut was minimized. Care was taken to cover only 1–2 mm of the blade with tape so as not to reduce evaporation from the remaining stroma and leaf blade. Water loss from the stroma and truncated stromal leaf blade was measured for

a 10 minute period. The mid section was then excised and the cut end sealed and water loss measured as above. Finally the proximal section was excised and the cut end of the leaf blade emerging from the stroma was covered with tape, and water loss again measured for a 10 minute period. The lengths and estimated surface areas of each of the four stromata and stromal leaf segments are given in Table 1. Estimation of surface areas were made by treating the tip segment as triangular and the mid and proximal sections as rectangular, while the stroma was considered cylindrical.

Table 1. Water losses from the stroma and emergent stromal leaf of *Epichloë*

Section[a]	Length (mm)	Surface area (mm^2)	$\mu l\ H_2O$/min	$\mu l\ H_2O/mm^2$ /min
Distal	94.2 ± 18.2	322.2 ± 89.9	0.4364 ± 0.2402	0.0014 ± 0.0008
Mid	44 ± 8.5	338.3 ± 81.6	0.2413 ± 0.1524	0.0007 ± 0.0005
Proximal	21.2 ± 6.2	185.4 ± 64.4	0.3499 ± 0.1716	0.0021 ± 0.0012
Stroma	21.5 ± 3.2	100.6 ± 29.5	1.4077 ± 0.2978	0.0126 ± 0.0021
Total	180.8 ± 25.7	951.7 ± 165.4	2.4045 ± 0.587	0.0168 ± 0.0021

[a]See Figure 1 for an explanation of sections.

After measurements were made for a stroma and its emergent leaf, calculations of water loss in μl/min and $\mu l/mm^2$ of surface area/min were made for each segment (Table 1). To calculate water loss for a distal section, loss from the stroma and leaf after the tip had been excised was subtracted from the loss measured with tip intact. The difference in water loss was taken to approximate that of the intact tip section. Similarly, to calculate loss of water from the mid section, loss with the mid section excised was subtracted from loss with that section intact. The same procedure was used to calculate losses of water from the proximal section and stroma. All measurements are reported as mean ± standard deviation (Table 1) and represent 20 separate measurements. To examine translocation of dye into mycelium of stromata, 10 culms were excised from plants and immersed in a solution of blue food color (McCormick & Co., Inc., Hunt Valley, Maryland) at a concentration of 3 drops/10 ml of distilled water. Stromata were maintained under constant air flow as described above. Stromata were examined over a four hour period for accumulation of dye in the emergent stromal leaf and stroma (Figs. 4 and 5).

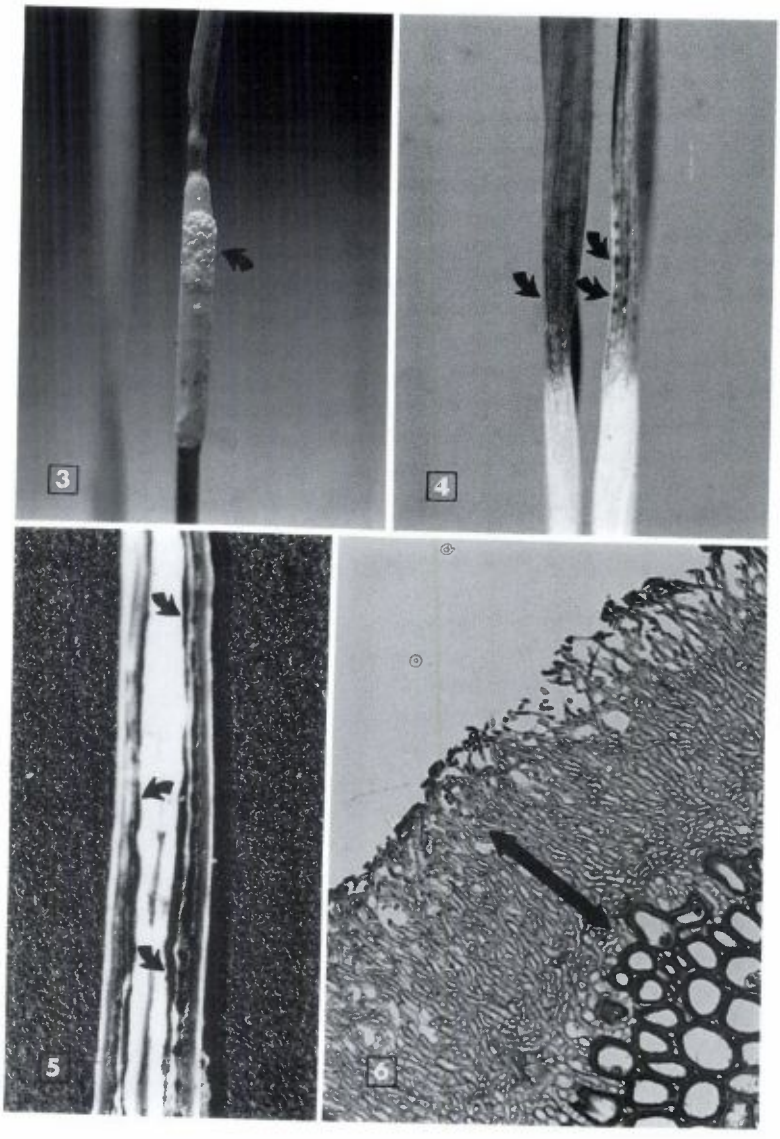

Figure 3. Stroma showing development of perithecia (arrow); number square = 2 mm.
Figure 4. Stromata showing accumulation of blue dye (food coloring) in leaves above stroma (arrows); number square = 1.5 mm.
Figure 5. Stroma split longitudinally to show accumulation of blue dye in tissues of leaves (arrows) and exclusion from mycelial tissues; number square = 1 mm.
Figure 6. Cross-section of stroma showing plant tissues (lower right corner) covered by a fungal cortex layer (thickness indicated by the double headed arrow); arrow = 30 μm.

Measurement of water losses from culms with and without stromata

To assess the impact of stromata on overall water losses from entire grass culms, measurements of losses were made using culms with young inflorescences and those with stromata, both excised at the plant crown. After culms were excised they were immediately immersed in water to prevent air from entering the xylem. Cut ends were then inserted into microsedimentation tubes and water losses measured as previously described (Table 2). Following measurement of water losses, culms were weighed (wet weight), then dried approximately 15 minutes in an oven at 200°C, and weighed again (dry weight). Data are presented as mean ± standard deviation (Table 2).

Table 2. Comparisons of culms of *Agrostis hiemalis* with and without stromata[a]

	Culms without stromata	Culms with stromata
Wet weight (mg)	*488.7 ± 149.3	*272.8 ± 79.7
Dry weight (mg)	*124.7 ± 45.5	*81.7 ± 44.5
Water content (µl)	*343.1 ± 122.3	*217.6 ± 63.3
H_2O loss/min (µl)	2.6379 ± 1.5275	3.2064 ± 1.3773
H_2O loss/gram/min (µl)	*5.4622 ± 2.6913	*11.4826 ± 1.9119

[a]An asterisk before a measurement indicates that means (with and without stromata) are significantly different according to the Student's T-test ($P<0.05$).

3. Results and Discussion

Water losses of the stroma and emergent stromal leaf

Water losses have previously been suggested to be high in plants bearing stromata (White et al., 1993). This conjecture was based on studies involving translocation of dyes in excised culms with and without stromata, where the rate of accumulation of dyes in the leaves associated with stromata was greater than that of leaves not associated with stromata. However, until this study, no measurements of water losses from plant or fungal tissues have been available. The average water loss from the surface of the stroma was approximately 1.41 µl/min (Table 1). This loss is slightly greater than the loss

from the much larger leaf blade that emerges from the top of the stroma, where measurements averaged 1.03 µl/min. However, the surface area of the emergent leaf blade was estimated to be 845 mm^2, while that of the stroma was 100 mm^2 (Table 1). This is more than an eight fold difference. It is thus apparent that the rate of loss of water from the surface of mycelium (0.0126 $\mu l/mm^2/min$) is much higher than the average loss from leaf tissues (0.0014 $\mu l/mm^2/min$). The rate of evaporation from the surface of the stroma is approximately 10 times faster than that from the surface of the emergent leaf blade for a comparable size area. A probable explanation for this large difference in water loss from stroma and leaf tissues is that leaf tissues are covered by a waxy cuticle layer that reduces evaporation from the surface, while the stroma of *Epichloë* lacks any moisture containment layers. It is notable that water loss from the emergent blade differed depending of the section examined (Fig. 2; Table 1). The mid section of the stromal leaf blade showed a lower rate of water loss per mm^2 than the tip and the proximal section. The leaf tip is expected to show a higher rate of water loss since the cuticle is thin over this young tissue. The proximal region of the emergent blade is more mature tissue, however, previous studies on leaf cells proximal to stromata have shown that cells here show structural anomalies (White et al., 1993). By some mechanism, as yet unknown, the endophyte stimulates enlargement of mesophyll parenchyma and epidermal cells in leaf tissues for a centimeter or so above the stromal mycelium. These altered tissues show an absence of air spaces in the mesophyll and absence of cuticle layer on the leaf surface. The leaf tissues proximal to the stroma show an increased loss of water, perhaps due to rapid evaporation from this structurally altered leaf tissue.

The alterations to the cells of the leaf proximal to the stroma may be similar to changes that the fungus induces in plant tissues embedded within the mycelium of the stroma. It is reasonable that the purpose of such alterations to host tissues may be to enhance the transfer of nutrients and moisture to the fungus. Previous anatomical studies of stromata (White et al., 1991) have shown that tissues of xylem and phloem, in addition to the mesophyll parenchyma of leaf and inflorescence tissues embedded in stromata, are permeated intercellularly by endophytic mycelium. The presence of mycelium, particularly in the conducting tissues, seems to suggest that the stroma is adapted for nutrient and moisture transfer to the reproductive cells of the endophyte. Ultrastructural studies will be necessary to document any additional alterations of the host tissues that may facilitate this process.

The mycelial barrier to the environment

Although water freely evaporates from the surface of the stroma, other compounds such as sugars do not appear to leak from the tissues. This is evident since there is never any accumulation of sugar droplets on the surface of the stromata. This is in contrast to the fungus *Claviceps purpurea* (Fr.: Pers.) Tul. where sugars are known to leak from plants to form droplets of sugar solution (Taber, 1985). In addition, in dye translocation experiments, dye will pass through plant tissues of the stroma to the emergent stromal leaf (Fig. 4) and will accumulate in tissues of the grass embedded within the mycelium (Fig. 5), but will not pass across the mycelial layers to the surface of the stroma even after several hours. This may be due to the presence of a special layer of tightly packed mycelium (Fig. 6). This layer, referred to here as the 'cortex layer', lies between the superficial hymenial layer and the epidermis of the sheath of the leaf whose blade emerges from the apex of the stroma. The mycelium of the cortex layer consists of tightly packed parallel hyphae (Fig. 6) that may prevent solutes from passing intercellularly through the apoplast to the exterior of the stroma. It is logical that any substances that pass from the interior of the plant to the exterior of the stroma must be absorbed by mycelium and transported within hyphae of the cortex layer. In this respect the stroma may be viewed as a selectively permeable barrier to the environment through which water may pass freely but other substances are excluded (eg. the dye) or selectively absorbed.

Water losses from entire culms with and without stromata

Although water loss from the surface of stromata is high, the overall rate of water loss from entire culms bearing stromata was only slightly higher than that from culms without stromata and the difference was not statistically significant (Table 2). On the other hand, water loss per gram (dry weight) of tissue (l/gram/min) is much higher in stroma-bearing culms (Table 2). The reason for the large difference here is that the overall mass of stroma-bearing culms is reduced. Culms with stromata had only about 65% of the mass (dry weight) of culms without stromata (Table 2). The reduction in mass of the stroma-bearing culms reduced the water lost from those culms so that the loss from culms with stromata approaches the loss from the much larger and heavier culms without stromata (Table 2). This is likely a function of surface area. Culms without stromata have an inflorescence, more leaves and a much larger surface area over which water is lost. While stroma development on a culm increases water losses due to a low efficiency of the fungal tissue to prevent evaporation, reduction in size of the entire culm compensates for the

higher rate of water loss from the stroma and overall water loss is only slightly increased.

Ecological considerations

In culms free of fungal stromata, the stomatal pores of leaves may close and the leaves roll to conserve moisture within plant tissues. However, loss of water from the fungal stroma is unregulatable. Perhaps, because *A. hiemalis* tends to occur in very moist soils, increased water loss does not compromise survivability of host or fungus individuals. On the other hand, many grasses with endophytes (e.g., tall fescue and perennial ryegrass) are known to grow in soils with little moisture. Such grasses would likely be severely compromised by water losses from stromata, if they were commonly formed on individuals of these species. Thus there is likely a strong selection against stroma-forming ability in places where moisture is lacking. The endophytes of grasses in dry habitats show seed transmission but do not produce stromata. Stromata of *Epichloë* are known to occur most frequently in areas where soil water is abundant (Petch, 1932). Loss of the ability to produce stromata may be viewed as an adjustment in the relationship between endosymbiont and host that allows the symbiosis to persist under conditions where stroma formation would compromise survival of the association.

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