Osmoregulatory Responses of Fungi Inhabiting Standing Litter of the Freshwater Emergent Macrophyte *Juncus effusus*

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Standing litter of emergent macrophytes often forms a major portion of the detrital mass in wetland habitats. Microbial assemblages inhabiting this detritus must adapt physiologically to daily fluctuations in temperature and water availability. We examined the effects of various environmental conditions on the concentrations of osmoregulatory solutes (polyols and trehalose) and the respiratory activities of fungal assemblages inhabiting standing litter of the freshwater emergent macrophyte *Juncus effusus*. Under field conditions, the concentrations of osmolytes (polyols plus trehalose) in fungal decomposers were negatively correlated with plant litter water potentials ($r = -0.75, P < 0.001$) and rates of microbial respiration ($r = -0.66, P < 0.001$). The highest concentration of osmolytes (polyols plus trehalose) occurred in standing litter exposed to desiccating conditions (range from wet to dry, 0.06 to 0.68 μmol·mg of fungal biomass$^{-1}$). Similar fluctuations in polyol and trehalose concentrations were observed in standing litter wetted and dried under laboratory conditions and for four predominant fungal decomposers of *J. effusus* grown individually on sterilized *Juncus* leaves. These studies suggest that fungal inhabitants associated with standing litter of emergent macrophytes can adjust their intracellular solute concentrations in response to daily fluctuations in water availability.

In many emergent macrophytes, leaf abscission is absent and collapse of shoot material to the sediment surface does not occur until long after senescence. Large amounts of standing dead plant matter can accumulate in wetland and salt marsh habitats (9), where it begins to decay in an upright aerial position without detachment from the parent rhizomes (3, 43, 44, 51). Diverse assemblages of microorganisms, principally fungi, are known to colonize standing litter in freshwater wetland and salt marsh habitats (2, 6, 31).

Previous studies have established that water availability is a major factor affecting the activity of microbial decomposers in standing litter of emergent macrophytes (19, 21, 33, 45). Recently, Kuehn and Suberkropp (33) reported that the rate of CO$_2$ evolution by microbial assemblages inhabiting standing litter of *Juncus effusus* L. increases (>100 μg of CO$_2$·C·g of organic matter$^{-1}·$h$^{-1}$) after exposure to wetting conditions and continues to be high until the plant litter becomes dry. In the absence of precipitation, microbial respiratory activity exhibits a distinct diel response, with respiration rates increasing during the evening following dew condensation on plant litter. Exposure of standing litter to increasing temperatures during the day contributes to the desiccation of litter, which leads to decreases in microbial respiration. Thus, the respiratory activity of microbial assemblages inhabiting standing litter changes rapidly in response to daily changes in water availability.

Since microorganisms possess no active cross-membrane transport mechanism for water, they must raise the intracellular water potential relative to the external environment to meet the physiological demands for cellular maintenance and growth (46). Intracellular turgor pressure is an important factor that affects the rate of hyphal extension growth and provides the driving force for invasive fungal growth through organic debris (40, 41). The magnitude of hyphal turgor is controlled by a complex osmoregulatory system that controls the internal cytoplasmic osmotic potential (14, 39). Osmotic adjustment is achieved by the uptake or export of inorganic ions (K$^+$, Na$^+$) across the cell membrane and by the biosynthesis or degradation of organic compounds (5). Acyclic sugar alcohols (i.e., polyols) and trehalose have been shown to be important carbohydrate storage products in fungi (27, 34, 54), as well as the dominant osmolytes produced in response to increased water stress (4, 5, 23, 27, 39). These solutes are often referred to as compatible solutes, because their accumulation in the cytoplasm does not interfere with or inhibit the normal physiological functions of the cell (4, 5).

Most investigations assessing the osmoregulatory responses of fungi to increased water stress have been laboratory-based studies in which pure fungal isolates, primarily yeast isolates, were grown in liquid media containing various concentrations of solutes (e.g., NaCl, KCl, polyethylene glycol) (4, 5, 23). Glycerol is consistently reported to be the main cytoplasmic polyol produced in fungi under conditions of increased solute-associated water stress (4, 5, 23). In addition, trehalose has been documented to be important as a general stress-protective solute in fungi during periods of increased desiccation, high and low temperatures, and toxic chemical (metal) exposure (16, 17, 24, 42, 49, 56). In contrast, few studies have assessed the osmotic responses of fungi to changes in matric potential water stress within naturally decaying substrates. Several researchers have reported that the dominant polyol pool in fungal cultures can vary depending on the age, state of growth, and specific stress solute used (22, 58).

The present study was conducted to examine the effect of increased desiccation on the osmoregulatory response of fungal decomposers inhabiting standing litter of the freshwater emergent macrophyte *J. effusus* under field and laboratory conditions. Changes in concentrations of fungal osmolytes (polyols
and trehalose) were assessed in response to changing water potentials, and the organic mass of the leaf pieces.

**RESULTS**

Increasing temperatures (Fig. 1A) during the day contributed to the desiccation of standing J. effusus litter, which led to decreases in plant litter water potentials (Fig. 1B). Both microbial respiratory activities (Fig. 1C) and the concentrations of fungal compatible solutes (polyols and trehalose) were used to determine retention times and to establish optimal chromatographic separations. Derivatized sugars in samples were identified and quantified based on a comparison with these known compounds.

**Statistical analysis.** Statistical analysis of the data was performed by using SAS software (52). Data are presented below as means ± standard errors. Values were considered significant at $P < 0.05$.

**Fungal biomass.** Fungal biomass was determined by extracting and quantifying ergosterol from plant litter samples (20). Ten leaf pieces (length, 2 cm) from each litter sample were collected, placed in 5 ml of methanol (high-performance liquid chromatography [HPLC] grade) and stored at −20°C in the dark until they were extracted. Additional replicate leaf pieces (10 pieces from each sample) were dried at 60°C and combusted overnight at 550°C to determine the organic mass of the leaf material in preserved samples. The ergosterol in plant litter samples was extracted by refluxing in alcoholic KOH (25 ml of methanol plus 2.5 ml of 6% KOH in 95°C for 30 min (53)). The resulting extract was partitioned into n-pentane and evaporated to dryness under a stream of nitrogen gas at 37°C. Dried ergosterol extracts were redissolved in 2 ml of methanol (HPLC grade), filtered, and stored tightly capped at −20°C in the dark until they were analyzed. 

**Polyols and trehalose.** Polyols and trehalose were extracted from plant litter by using methods modified from those of Karsten et al. (28) and Richardson et al. (50). Twenty leaf pieces (length, 2 cm) from each sample were collected, placed into 10 ml of 70% ethanol, returned to the laboratory, and stored at −20°C until extracted. The leaf pieces were homogenized with a Polytron apparatus (Brinkmann) at speed setting 5 for 15 s, and each sample was transferred to a 100-ml round-bottom flask along with an additional 10 ml of 70% ethanol. The homogenized plant tissue was extracted by refluxing for 2.5 h at 80°C. The resultant extract was cooled to room temperature and filtered (pore size, 0.7 μm; type GF/F, Whatman). An aliquot of the filtered extract was evaporated to dryness under a stream of nitrogen gas at 75°C. The polyols and trehalose in dried extracts were redissolved and converted to their oximes by adding 500 μl of hydrolyzable pyridine in pyridine (25 μg/ml) containing phenyl-β-D-glucoside (200 μg/ml in pyridine) as an internal reference standard (8). The resultant mixture was heated for 30 min at 75°C with periodic vortexing. Sugars were derivatized by adding 500 μl of N-O-bis(trimethylsilyl)trifluoroacetamide–1% trimethylchlorosilane (Pierce Chemical Co.) and were heated for an additional 20 min at 75°C. An aliquot (ca. 150 μl) of hydrolyzable sodium sulfate was added after derivatization reactions to absorb any water. Samples were stored tightly capped at −20°C in the dark until they were analyzed.

Separation and analysis of trimethylsilyl-derivatized polyols and trehalose were performed by injecting 1-μl samples into a type DB-1 fused-silica capillary column (0.25 mm by 15 m; film thickness, 2.5 μm; Alltech, Inc.) connected to a Hewlett-Packard model HP-5890 series II gas chromatograph equipped with a flame ionization detector. The chromatographic results were analyzed by using a model HP-3385 Chemstation (software version 3.5; Hewlett-Packard, Inc.). The detector and detector temperatures were maintained at 225 and 280°C, respectively. The oven temperature was initially kept at 125°C for 4 min and then was programmed to increase at three program levels. In level one, the oven temperature was increased from 125 to 166°C at a rate of 1°C min−1 and kept at 166°C for 1 min. In level two, the temperature was increased from 166 to 172°C at a rate of 0.5°C min−1 and kept at 172°C for 4 min. In level three, the temperature was increased from 172 to 300°C at a rate of 10°C min−1 and kept at 300°C for 5 min and then decreased to 125°C at a rate of 10°C min−1, and used as the carrier gas at a flow rate of 1.9 ml/min. A split flow injection mode was used with a split flow rate of 100 ml/min. The air and hydrogen flow rates to the detector were 250 and 24 ml/min, respectively. Standard trimethylsilyl-derivatized polyols and trehalose (Sigma Chemical Co.) were used to determine retention times and to establish optimal chromatographic separations. Derivatized sugars in samples were identified and quantified based on a comparison with these known compounds.
341 μmol · mg of fungal biomass−1; trehalose range, 0.024 to 0.334 μmol · mg of fungal biomass−1). Mannitol was the predominant polyol identified (79% ± 1% of the total polyol concentration), followed by arabitol (12% ± 2%) and glycerol (7% ± 1%). At night, decreasing temperatures and increasing relative humidities (Fig. 1A) resulted in dew formation on standing litter, which led to increased plant litter water potentials (Fig. 1B) and decreased microbial water stress. The rates of CO2 evolution from plant litter subsequently increased (Fig. 1C), and the total polyol and trehalose contents decreased (Fig. 1D).

The results of field studies conducted in June 1994 illustrate the effect of rain and prolonged daytime water saturation of standing plant litter on the metabolic status of microbial inhabitants (Fig. 2). In the morning hours, before rainfall, the plant litter was exposed to increasing temperatures (Fig. 2A). These environmental conditions contributed to desiccation of the plant litter, which resulted in significant decreases in plant litter water potentials (Fig. 2B) and the respiration rates of the microbial inhabitants (Fig. 2C). However, following rainfall, both plant litter water potentials and rates of CO2 evolution from standing litter increased significantly (P < 0.0001, ANOVA) (Fig. 2B and C). High rates of CO2 evolution continued throughout the day. Significant decreases in polyol concentrations in litter were noted following precipitation (Fig. 2D) (P < 0.05, ANOVA), and the concentrations remained low until the litter was exposed to drying conditions the following morning (polyol concentration range, 0.031 to 0.21 μmol · mg of fungal biomass−1). Mannitol was the predominant polyol identified during these studies (72% ± 8% of the total polyol concentration), followed by glycerol (15% ± 7%) and arabitol (8% ± 1%). The trehalose concentrations in standing litter (Fig. 2D) remained low throughout the day and night until the litter was exposed to drying conditions the following morning (trehalose concentration range, 0.013 to 0.066 μmol · mg of fungal biomass−1). When combined data from both field studies were used, the concentrations of fungal osmotic solutes (polyols and trehalose) in standing litter were negatively correlated with the rates of carbon dioxide evolution and plant litter water potentials (Table 1).

The results of experiments conducted under controlled laboratory conditions were similar to the results obtained during field studies. Microbial assemblages in field-collected samples responded rapidly when dry standing litter was wetted in the laboratory, with significant increases in the rates of CO2 evolution occurring within 5 min (from 4 to 96 μg of CO2-C · g of organic matter−1 · h−1) (P < 0.001, ANOVA) (Fig. 3A). Carbon dioxide evolution continued at high rates for up to 48 h after initial wetting with no significant fluctuations in the rates of CO2 release (P > 0.05, ANOVA, Tukey). When plant litter was exposed to drying conditions, the rates of CO2 evolution...
declined significantly ($P < 0.05$, ANOVA, Tukey). Plant litter water potentials (Fig. 3B) were positively correlated with microbial respiratory activities ($r = 0.65$ and $P < 0.001$, Spearman), rising from $-7.9$ to $-0.7$ MPa in 5 min after the litter was wetted and decreasing from $-0.2$ to $-6.5$ MPa after the litter was exposed to drying conditions. The fluctuations of fungal osmolytes in standing litter also changed in response to wetting conditions (Fig. 3C). A significant decrease ($P < 0.05$, ANOVA) in trehalose content was observed after exposure of plant litter to water-saturating conditions. This decrease was followed by a slight but significant increase in trehalose concentration after exposure to drying conditions ($P < 0.05$, ANOVA, Tukey). The total polyol concentrations in standing plant litter followed a pattern similar to the trehalose pattern, but differences in polyol concentrations were not significant ($P = 0.15$, ANOVA). Mannitol was the predominant polyol identified from plant litter during these studies ($47\% \pm 7\%$ of the total polyol concentration), followed by glycerol ($29\% \pm 5\%$) and arabitol ($19\% \pm 3\%$). The combined accumulation patterns of both polyls and trehalose were negatively correlated with both rates of CO$_2$ evolution and plant litter water potentials ($r = -0.65$ and $-0.72$, respectively; $P < 0.01$, Spearman). The fluctuations in ambient temperatures (17 to 23°C) were smaller than those observed during field studies. Fungal biomass ranged between 47 and 78 mg/g of organic mass (237 to 388 $\mu$g of ergosterol/g of organic mass), and no significant differences among samples were observed ($P > 0.05$, ANOVA, Tukey).

The four fungal isolates examined exhibited significant increases in the total concentration of osmolytes (polyls plus trehalose) when they were exposed to drying conditions in the laboratory at a constant temperature of 22°C ($P < 0.05$, Student’s $t$ test) (Table 2). However, considerable variation in the level of polyls and trehalose was observed among isolates. Only two of the isolates (Drechslera sp. and P. copelandii) exhibited polyol concentrations within the range observed in laboratory and field samples. Phoma sp. and C. ligenica had polyl concentrations that were considerably lower. All of the isolates except P. copelandii had trehalose concentrations that were similar to those obtained for laboratory and field samples; however, the concentration of trehalose in P. copelandii was nearly four times the concentration of trehalose in the laboratory and field samples (Table 2).

**DISCUSSION**

Results obtained in the present study provide evidence that fungi associated with standing J. effusus litter are physiologically adapted to the cyclic desiccation periods experienced in the standing dead phase. During periods of decreased water availability, the fungi adapted by accumulating intracellular organic solutes (polyls and trehalose). Fluctuations in the concentrations of polyls and trehalose, in response to litter drying and wetting conditions, were observed in litter under both field and laboratory conditions, indicating that fungal inhabitants can adjust their internal solute concentrations in response to changes in external water availability. Fungal respiratory activities in standing litter decreased concomitantly, as indicated by the significant negative correlation of total polyl and trehalose contents with rates of microbial respiration and plant litter water potentials (Table 1).

The total polyl and trehalose concentrations reported in the present study are within the range reported previously for other species of filamentous fungi (1, 18, 36–38, 58), even though the type of water stress experienced by microorganisms in the present study differed markedly from the type of water stress in majority of the investigations cited above. During the present study, the increased water stress of microbial assemblages inhabiting J. effusus resulted from a decrease in the plant litter matric water potential. Previous studies have focused primarily on solute-induced water stress, and the water availability in liquid growth media was manipulated by varying the osmotic potential. In addition, in the prior studies the authors

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**TABLE 1. Spearman correlation matrix showing the relationships among rates of CO$_2$ evolution, plant litter water potentials, polyol and trehalose concentrations, and environmental conditions during field studies**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Spearman correlation coefficient</th>
<th>Temp</th>
<th>Relative humidity</th>
<th>Water potential</th>
<th>Trehalose concn</th>
<th>Polyol concn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon dioxide evolution</td>
<td>$-0.52$ (75)$^b$</td>
<td>0.44 (75)$^b$</td>
<td>0.86 (45)$^b$</td>
<td>$-0.40$ (45)</td>
<td>$-0.66$ (45)$^b$</td>
<td></td>
</tr>
<tr>
<td>Temp</td>
<td>$-0.81$ (75)$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative humidity</td>
<td></td>
<td></td>
<td>0.61 (45)$^b$</td>
<td>0.38 (45)</td>
<td>0.42 (45)</td>
<td></td>
</tr>
<tr>
<td>Water potential</td>
<td></td>
<td></td>
<td>0.63 (45)$^b$</td>
<td>$-0.45$ (45)</td>
<td>$-0.43$ (45)</td>
<td></td>
</tr>
<tr>
<td>Trehalose concn</td>
<td></td>
<td></td>
<td>$-0.53$ (45)$^b$</td>
<td>$-0.75$ (45)$^b$</td>
<td>0.62 (45)$^b$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The values in parentheses are the numbers of samples examined.

$^b$ $P < 0.05$. $P$ values were adjusted for multiple comparisons (15) by using the Bonferroni adjustment method.

**FIG. 3. Changes in rates of CO$_2$ evolution (A), plant litter water potentials (B), and total polyl and trehalose concentrations (C) in decomposing standing litter of J. effusus after wetting and drying under controlled conditions in the laboratory. The data are means ± standard errors (n = 3). OM, organic matter.**
described polyol accumulation patterns in growing cultures of single species, which contained high concentrations of labile carbon (e.g., glucose) and other nutrients (N plus P). The values reported in the present study reflect the polyol and trehalose concentrations of a mixed assemblage of fungal inhabiting plant litter that is considerably more recalcitrant (i.e., lignocellulose) and nutrient poor (N, <1.0%; P, <0.05%) than most culture media (32).

In the present study, laboratory experiments revealed an increase in total polyol and trehalose concentrations in plant litter upon exposure to drying conditions. However, the changes in the concentrations of these solutes were not as pronounced as the changes observed under natural field conditions. In addition, there was considerable variation in the concentrations of polyols and trehalose in the isolates examined, suggesting that some fungal species may rely on either polyols, trehalose, or possibly other organic solutes as a means of osmoregulation. Amino acids can also play a role in fungal osmoregulation (15, 47); however, these specific solutes were not measured in the present study. Furthermore, it should be noted that the temperature fluctuations during laboratory studies were smaller (17 to 23°C) than those recorded during field studies (19 to 33°C). It is possible that increasing temperatures along with desiccation stress may have a synergistic effect on the accumulation of trehalose and polyols in fungi inhabiting standing litter, as has been demonstrated previously for several fungal species (24, 55, 56). Hottiger et al. (24) reported that large amounts of trehalose accumulated in cells of Saccharomyces cerevisiae in response to heat shock and that changes in trehalose concentrations were closely correlated with fluctuations in thermotolerance and desiccation tolerance. In the present study, higher concentrations of total polyols and trehalose in plant litter were observed during periods of increased temperatures (Fig. 1 and 2), suggesting that exposure of microbial inhabitants to higher temperatures may result in greater intracellular accumulation of these solutes.

Although there is clear evidence of the importance of glycerol in fungal osmoregulation, other polyols have also been implicated as important osmolytes (22, 30, 38, 58). Hocking (22) reported that glycerol contents declined significantly in five species of filamentous fungi as cultures aged and sporulation increased. Wethered et al. (58) found that growing cultures of Dendryphiella salina (G.K. Sutherland) Pugh & J. Nicot accumulated either glycerol, arabitol, or mannitol in response to increased solute concentrations in the growth media and that the predominance of any specific polyol was dependent on the specific stress solute used. Furthermore, Wethered et al. (58) also confirmed previous findings of Jennings (26), who found that in nongrowing mycelia of D. salina, only mannitol and arabitol were produced in response to increased salinity. The presence and predominance of mannitol in fungal mycelia that are not actively growing are noteworthy, since in the present study mannitol was the predominant polyol identified and was observed only during periods of low microbial respiratory activity. The low respiratory rates exhibited by fungal assemblages during these desiccation periods suggest that there is no active mycelial growth in litter. During these dry periods, fungal inhabitants apparently remain in a state of interrupted growth, in which metabolic activities proceed only at a level that maintains cellular integrity and viability. Since the accumulation of glycerol appears to be associated only with actively growing (metabolizing) mycelia under conditions of increased water stress, this may explain the predominance of mannitol compared with glycerol in this study.

The polyol and trehalose concentrations reported in the present study reflect the amounts of these solutes per unit of fungal dry mass. However, since the total cell water contents of fungi have been reported to decrease in response to solute-induced water stress, these concentrations may be underestimated (18, 48 [but see references 10 and 29]). If fungi inhabiting standing litter experience a similar dehydration pattern, then even a slight change in the amount of polyols or trehalose per unit of fungal biomass could be equivalent to a significant change in concentration on a molar basis.

In addition to increasing the cytoplasmic solute concentration, the presence of polyols, trehalose, and other sugars has been shown to increase the physical stability of cellular structures to the adverse effects of dehydration and thermal denaturation (11, 12). These osmolytes can interact and replace water around the polar groups of membrane phospholipids and proteins, which maintains integrity and increases the stability of membranes during thermal desiccation (11, 12). Additional studies have also documented the role of trehalose and polyols in stabilizing soluble cytoplasmic enzymes from both thermal and desiccation denaturation (7, 12, 13, 25, 35). Therefore, the production and accumulation of these compounds by fungal assemblages inhabiting standing litter may play a role in both osmotic solute adjustment and protection of cellular components during periods of exposure to increased desiccation and high temperature. The ability of fungi to synthesize and accumulate these solutes appears to represent a key adaptive strategy that facilitates the survival of these organisms and their predominance in the environmentally harsh standing-dead habitat.

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