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Authors: Grimmett, Ivan J., Smith, Keegan A., and Bärlocher, Felix

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Tar-spot infection delays fungal colonization and decomposition of maple leaves

Ivan J. Grimmer¹, Keegan A. Smith², AND Felix Bärlocher³

Department of Biology, Mount Allison University, Sackville, New Brunswick, Canada E4L 1G7

Abstract. Tar spot is a common leaf disease of *Acer* caused by *Rhytisma* spp. (Ascomycetes). Initial symptoms are small green-yellow spots. These infected areas turn into black fungal stromata, which resemble blobs of tar. Stromata with apothecia overwinter and release ascospores in the following spring. We measured rates of decomposition of leaf litter of infected and uninfected Norway maple (*Acer platanoides*). In addition, we assessed changes in leaf-litter N and P and rates of sporulation of aquatic hyphomycetes. We incubated leaf disks from areas covered with tar spot, disks touching but not overlapping with tar spot (adjacent disks), and disks from uninfected leaves in a stream. The exponential decay rate of uninfected disks was 50% greater than those for infected and adjacent disks. Initial N and P concentrations were highest in infected disks. N and P concentrations dropped in all treatments during the 1st wk in the stream and subsequently increased. Conidium release by aquatic hyphomycetes in the laboratory was lowest from infected disks and highest from uninfected disks. Cumulative sporulation over 10 wk of stream exposure increased from infected (109 conidia mg⁻¹ d⁻¹) to adjacent (215 conidia mg⁻¹ d⁻¹) to uninfected (521 conidia mg⁻¹ d⁻¹) disks. Similarity of fungal communities was highest between adjacent and uninfected disks (71.5%) and lowest between infected and uninfected leaves (51.9%).

Key words: leaf decomposition, aquatic hyphomycetes, tar spot, *Rhytisma acerinum*, *Acer platanoides*, Norway maple, endophyte.

The analysis of food chains and webs has emphasized distinct, functionally homogeneous trophic levels or species. Aquatic-detritus food chains generally start with plant litter, which is conditioned by microbes and ingested by invertebrates. In streams, these levels are represented by autumn-shed leaves, aquatic hyphomycetes, and leaf-shredding invertebrates (Bärlocher 2005). The potential effect of parasites and pathogens on food webs has almost always been ignored by researchers. Early exceptions were Cummins and Wilzbach (1988), who concluded that >80% of natural mortality of stream invertebrates might be caused by pathogens and not predation, competition for food and space, or environmental stress. More recently, the role of parasites and pathogens in food webs has been recognized as influencing species richness, number of links and food-chain length, and various foodweb statistics (Lafferty et al. 2006, 2008). In freshwater habitats, pathogenic chytrids can cause the collapse of algal

blooms (Wurzbacher et al. 2010). Viruses may play a similar role, especially in the littoral zones of lakes (Middleboe et al. 2008).

Plant litter decomposition is a key ecosystem function. Investigators examining decomposition in streams generally have used unblemished leaves (e.g., Bärlocher et al. 2011, 2012). However, many naturally shed leaves show visible signs of insect herbivore attack. In a study of 12 tree species by Knepp et al. (2005), the average leaf area lost to herbivores was 3.1%, with large variances for individual species. Herbivore damage induces various plant responses, including changes in morphology and phenolics and lignin metabolism (Karban and Myers 1989). Some of these changes affect the decomposability of the leaves. Therefore, excluding partly eaten leaves in studies of decomposition introduces a potential bias.

Leaves also can be attacked by microbes and viruses. The pathogenic fungus *Taphrina deformans* (Berk.) Tul 1866 (Protoascomycetes) causes infected leaves to curl up (Webster and Weber 2007), and such leaves are unlikely to be used in decomposition studies. Even in the absence of pathogens, freshly shed leaves carry a diverse community of saprophytic phylloplane fungi (Bärlocher and Kendrick 1974),

¹ E-mail addresses: ijgrimm@mta.ca

² kasmith@mta.ca

³ To whom correspondence should be addressed. E-mail: fbaerlocher@mta.ca

whose participation in decomposition generally has been ignored. Endophytic fungi are ubiquitous in almost all plant species (Saikkonen et al. 1998). Their presence in grasses tends to slow decomposition in terrestrial environments (Rudgers and Clay 2007). Endophytic fungi are also common in roots (Sridhar and Bärlocher 1992) and needles (Sokolski et al. 2006) of riparian trees, but these infections rarely result in visible symptoms.

An exception is tar spot on several maple species. The infection is caused by the endophyte/borderline pathogen *Rhytisma acerinum*, which forms 1 to 2-cm-wide black lesions (tar spots) on the leaves of sycamore maple (*Acer pseudoplatanus* L.; Webster and Weber 2007) and Norway maple (*Acer platanoides* L.; Hsiang et al. 2008). These lesions initially appear as green-yellow spots in June or July. They arise from ascospores released from fruiting bodies on overwintered leaves and enter the leaf through stomata. Sycamore maple leaf patches infected by *R. acerinum* had higher N and P contents than control patches, and 2 aphid species reached higher adult mass and potential fecundity in 2 autumnal generations (Gange 1996). Cornelissen et al. (2000) reported identical N values from infected (black) and uninfected patches (brown or yellow) but much higher P values from infected patches. These observations suggest that the fungus may inhibit N or P translocation back to the tree before leaf abscission. As a consequence, infected patches potentially provide enriched food to herbivores.

Leaf decomposition in streams is often limited by N or P (Suberkropp and Chauvet 1995, Sridhar and Bärlocher 2000, Bärlocher and Corkum 2003). Therefore, greater N or P content of *Rhytisma*-infected leaf patches may increase decay rates because fungal access to substrate nutrients decreases their dependence on external (stream water) sources (Cheever et al. 2012). However, the opposite was the case with bigleaf maple (*Acer macrophyllum* Pursh) infected with *Rhytisma punctatum*. In a stream experiment, infected patches lost mass more slowly than control patches despite significantly higher P and N levels (LeRoy et al. 2011). Cornelissen et al. (2000) reported the same result from a soil-decomposition experiment with tar-spot-infected sycamore maple leaves.

The objectives of our study were to compare the decomposition of *R. acerinum*-infected Norway maple leaves to decomposition of uninfected leaves in a stream. If the endophyte slows decomposition, we would expect a concurrent significant decline of colonization by aquatic hyphomycetes. We also were interested in following the dynamics of N and P during decomposition. If these nutrients occur primarily in a soluble form, leaching by stream water

might remove them rapidly from the substrate before they can be used by newly arriving fungi.

Methods

Study site

We conducted a field experiment in Boss Brook in Fenwick, Nova Scotia, Canada (lat 45°43.000'N, long 064°09.56'W). This 1st-order stream runs through a mixed forest dominated by white birch (*Betula papyrifera* Marsh.), several maple species (*Acer rubrum* L., *Acer saccharum* Marsh., *Acer spicatum* Lam., and rarely *A. platanoides*), and white spruce (*Picea glauca* (Moench)Voss). At the experimental site, the stream is 2 to 3 m wide and 20 to 50 cm deep, with a stream bed consisting of stones and gravel. A more detailed description of the stream is given by Bärlocher (1987).

Leaf collection and deployment of leaf bags

We collected leaves from 1 tree (*A. pseudoplatanus*) on the campus of Mount Allison University in autumn 2010. We used only leaves that had been on the ground for ≤ 2 d. We air-dried leaves and stored them at room temperature. We soaked leaves overnight in tap water, and then prepared 3 types of leaf disks (15 mm diameter) with a cork borer. We punched tar-spot disks from the black circles formed by fungal stomata and adjacent disks from areas that touched the black circles but did not contain any visible stomata. We punched uninfected disks from leaves without visible fungal stomata. During leaf senescence, an ~ 1 -mm yellow-green ring surrounds the tar spot (Jones 1925). The green ring may be caused by diffusible substances released by the fungus or a hypersensitive response by the plant. We dried the disks to constant mass at room temperature, and weighed 35 disks of each type individually to estimate initial mass.

We placed preweighed sets of 15 (for mass-loss estimates) or 10 (sporulation estimates) disks in nylon mesh bags (20 \times 15 cm; 2-mm mesh). We submerged litter bags in the stream on 5 July 2011 and fastened them to 100-cm-long steel rods driven into the stream bed. We collected 5 litter bags of each leaf disk type on each sampling date (1, 2, 4, 6, 8, and 10 wk) for measurements of leaf mass loss and nutrient concentrations. We collected an additional 4 litter bags for estimation of sporulation by aquatic hyphomycetes. On each sampling date, we measured pH with a portable instrument (Oakton 35614-80; Eutech Instruments, Singapore) and took water samples to the laboratory in acid-washed glass bottles for NO₃⁻ and PO₄³⁻ analyses (Hach Spectrophotometer #DR/2010;

TABLE 1. Physicochemical variables of Boss Brook during field study.

Date	Temperature (°C)	pH	Conductivity ($\mu\text{S}/\text{cm}$)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)
July 5	17.0	5.53	56	0.20	0.04
July 12	16.7	6.24	65	0.23	0.04
July 19	16.6	6.04	51	0.33	0.03
August 3	16.5	5.9	45	0.37	0.03
August 17	17.2	5.9	58	0.63	0.04
August 29	18.3	5.96	42	0.53	0.04
September 12	13.8	6.29	70	0.34	0.03
September 26	12.9	6.76	66	0.23	0.02

Hach, Loveland, Colorado) using manufacturer's instructions. We measured temperature in situ every 30 min throughout the experimental period with a HOB0® Water Temp Pro V2 (Onset, Bourne, Massachusetts).

Mass loss and fungal sporulation

We rinsed the disks from 5 bags (= 5 replicates) with running tap water to remove adherent material, air-dried them to constant mass at 50°C, and weighed them to measure mass remaining.

To estimate spore production, we rinsed sets of 5 disks, each set from a different litter bag, suspended them in 50 mL of distilled, autoclaved water in 200-mL Erlenmeyer flasks, and placed them on a shaker (100 rpm, 15°C, 48 h). We repeated this procedure 4 times for 4 replicates. We filtered the suspension through an 8- μm membrane filter (Millipore Corporation, Bedford, Massachusetts) and stained the filter with cotton blue in lactophenol (50 mg/L). We used a light microscope (400 \times) to identify and count spores on a section of the filter with ≥ 500 spores. We dried the leaf disks at 50°C to constant mass, and expressed results as spores $\text{mg}^{-1} \text{d}^{-1}$.

Chemical analyses

We measured C and N content of freeze-dried leaf disks on a Vario EL II CHNOS Elemental Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). We measured total P after hydrolysis of leaf disks ground in liquid N following the procedures given by Wetzel and Likens (1991).

Statistical analyses

We estimated mass-loss rates by fitting the data from the 3 disk types to an exponential decay equation (nonlinear curve-fitting; Prism 5.0d for Mac; GraphPad Software, La Jolla, California). We compared decay coefficients (k) for the 3 disk types with an extra sum-of-squares test (Motulsky and Christopoulos 2004).

We analyzed sporulation rates on the various sampling dates by 2-way analysis of variance (ANOVA) with time and substrate disk type as fixed factors. We corrected spore release rates on the 6 sampling dates by mass remaining and added the products as a measure of cumulative sporulation over the experimental period. We analyzed them with a 1-way ANOVA, followed by a Tukey–Kramer multiple comparison test.

We compared initial values of C, N, and P percentages of the 3 disk types with a 1-way ANOVA, followed by Tukey–Kramer's multiple comparisons test. We analyzed changes in these variables during decomposition with a repeated measures ANOVA.

Results

During the field experiment, the pH of Boss Brook varied between 5.53 and 6.76, and the temperature decreased from 17.0 to 12.9°C (mean = 15.8; Table 1). Conductivity was consistently low, as were NO_3^- and PO_4^{3-} values.

Initial average mass (± 1 SD) was 24.8 ± 0.8 mg, 8.4 ± 0.7 mg, and 8.3 ± 0.6 mg for single tar-spot, adjacent, and uninfected disks, respectively. Tar-spot disks were significantly heavier than the other 2 disk types ($p < 0.001$). Remaining mass of the 3 types of leaf disk over time is shown in Fig. 1. k was significantly higher for disks from uninfected leaves than from tar-spot or adjacent disks (Table 2).

Sporulation patterns were broadly similar on all 3 disk types, with low values in the first sample(s), followed by an increase and subsequent decline (Fig. 2). Disk type, time, and their interaction all affected sporulation (all $p < 0.0001$). Peak sporulation was highest and occurred earliest in uninfected disks. Cumulative sporulation rates on the 6 sampling dates, adjusted by mass remaining, differed significantly among disk types ($p < 0.0001$), and was significantly higher on uninfected disks than on the other 2 disk types ($p < 0.05$; Table 3).

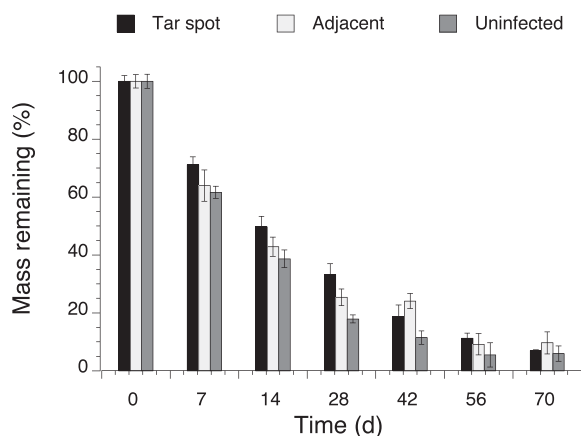


FIG. 1. Mean (± 1 SE) remaining mass of Norway maple leaf disks.

More aquatic hyphomycete species were found on uninfected disks (13) than on tar-spot (10) or adjacent disks (12) (Table 4). Percent similarity was greatest between communities on adjacent and uninfected disks (71.5%), lowest between communities on tar-spot and uninfected disks (51.9%), and intermediate between communities on tar-spot and adjacent disks (56.2%).

Percent C, N, and P differed significantly among disk types at time 0 (before stream exposure) and time 7 (after 1 wk of stream exposure, post-leaching values) (Table 5, Fig. 3A–C). Overall % C, N, and P differed among disk types (repeated measures ANOVA, $p \leq 0.036$). Tukey–Kramer’s test revealed that % C and % N of tar-spot disks differed from % C and % N of adjacent and uninfected disks, whereas % P of tar-spot disks differed only from % P of uninfected disks ($p < 0.05$).

Discussion

Tar-spot-infected maple leaves lost mass less rapidly than uninfected leaves (Fig. 1, Table 2). This result confirms findings by LeRoy et al. (2011), who investigated the decay of bigleaf maple infected with *R. punctatum*. However, k was considerably greater in our study than in the study by LeRoy et al. (2011).

TABLE 2. Daily exponential decay rates (k) of tar-spot infected, adjacent, and uninfected Norway maple leaf disks. Rates with different superscripts are significantly different ($p \leq 0.05$).

Disk type	$k \pm SD$	Intercept (%)	R^2
Tar spot	0.041 ^a \pm 0.002	97.6	0.97
Adjacent	0.044 ^a \pm 0.003	95.0	0.92
Uninfected	0.062 ^b \pm 0.004	98.2	0.96

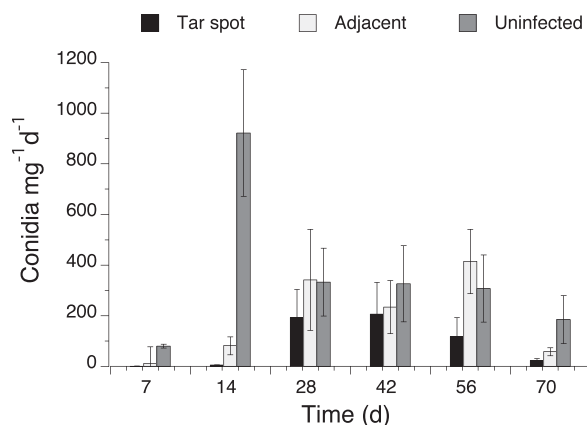


FIG. 2. Mean (± 1 SE, $n = 4$) number of conidia released from tar-spot infected, adjacent, and uninfected Norway maple leaf disks exposed in Boss Brook.

In our study, 7.0% (tar spot) and 5.6% (uninfected) of the original mass remained after 70 d in our study, whereas 50.3% and 31.4%, respectively, remained after 77 d in the study by LeRoy et al. (2011). k -values, estimated over the entire experiment, also were higher in our study than the study by LeRoy et al. (2011) (tar spot: 0.041 vs 0.0088, uninfected: 0.062 vs 0.0135).

Adjacent disks did not lose mass significantly faster than tar-spot disks. This result suggests that *R. acerinum* affected nearby tissues that lacked macroscopic signs of infection. Jones (1925) reported only minimal presence of endophyte mycelia beyond the macroscopically visible black area, despite the presence of an ~ 1 -mm yellow-green ring surrounding the tar spot.

Aquatic hyphomycetes are regarded as the main fungal drivers of leaf decomposition in streams (Krauss et al. 2011), and maximum spore release from recovered leaves usually is correlated with decay rate (Gessner and Chauvet 1994, Maharning and Bärlocher 1996, Bärlocher et al. 2012). We have insufficient data for a comparative analysis, but the trend among the 3 disk types supports the notion that aquatic hyphomycetes are important decomposers. Sporulation

TABLE 3. Sporulation rates, adjusted by remaining mass, summarized over 6 sampling dates from tar-spot infected, adjacent, and uninfected Norway maple leaf disks. Rates with different superscripts are significantly different ($p \leq 0.05$).

Disk type	Rate	SD
Tar spot	109 ^a	64
Adjacent	215 ^a	138
Uninfected	522 ^b	147

TABLE 4. Fungal species identified and % contribution to spore production during field study on tar-spot infected, adjacent, and uninfected Norway maple leaf disks.

Taxon	Tar spot	Adjacent	Uninfected
<i>Anguillospora filiformis</i>	23.5	34.2	47.5
<i>Anguillospora longissima</i> (?)	34.5	–	0.4
<i>Articulospora tetracladia</i>	20.1	36.7	14.9
<i>Clavariopsis aquatica</i>	2.0	0.5	0.1
<i>Clavatospora longibrachiata</i>	9.6	24.0	19.4
<i>Culicidospora aquatica</i>	2.1	–	0.01
<i>Cylindrocarpon</i> sp.	6.0	1.6	1.4
<i>Flagellospora curvula</i>	–	0.4	13.7
<i>Heliscus lugdunensis</i>	0.8	1.7	0.6
<i>Lunulospora curvula</i>	0.01	0.03	0.02
<i>Mycocentrospora</i> sp.	–	0.03	0.01
<i>Sigmoidea</i> sp.	–	0.2	–
<i>Tricladium chaetocladium</i>	1.4	0.12	1.6
<i>Tricladium splendens</i>	–	0.48	0.36
Total number of species	10	12	13

from tar-spot disks occurred much later and was much less than sporulation from uninfected disks (maximum of 207 conidia mg⁻¹d⁻¹ after 43 d vs 922 conidia mg⁻¹d⁻¹ after 14 d, respectively; Fig. 2). This difference is also reflected in cumulative spore production over the experimental period (Table 3). Results from adjacent disks were intermediate between tar-spot and uninfected disks. However, the scarcity of spores released from tar-spot disks must be balanced against the fact that mass per area was roughly 3× greater for tar spot disks than for adjacent or uninfected disks. This result suggests that the fungal endophyte manipulates the plant host to increase local production, which may be channeled directly into fungal tissues. As a rough estimate, we conclude that tar-spot decreases spore production of aquatic hyphomycetes by at least 40% (100 vs 500 spores per unit mass, 3× greater mass of tar-spot disks; Table 3).

Decay rates of plant litter generally are negatively correlated with lignin content and positively correlated with N concentration in the initial substrate (Melillo et al. 1982, Cornelissen et al. 2000). N:lignin and N:C ratios

and the types of decomposers involved can affect which element ultimately limits decomposition (Güsewell and Gessner 2009). An additional complication is that N and P may be provided externally, especially in streams where the water current continuously replenishes nutrients, although higher levels in the water column also can accelerate decomposition and fungal growth (Suberkropp and Chauvet 1995, Suberkropp et al. 2010). These factors result in complex patterns of elemental mineralization and immobilization in response to internal and external nutrient pools and composition of microbial consortia (Cheever et al. 2012).

Initial N and P concentrations were significantly higher in tar-spot disks than in uninfected disks (Table 5), a finding that confirms results by Cornelissen et al. (2000) and LeRoy et al. (2011). In contrast to LeRoy et al. (2011), we found that P also was significantly higher in adjacent disks, another result suggesting that changes in leaf chemistry may extend beyond the region characterized by the visible black stromata. *Rhytisma acerinum* is able to sequester and concentrate nutrients. The average C:N ratio for temperate broadleaf litter has been estimated as 58.4, and the average C:P ratio as 1702 (McGroddy et al. 2004, Cleveland and Liptzin 2007). Tar-spot disks had more favorable ratios (Table 5). Contrary to expectations, but in line with LeRoy et al. (2011), N- and P-enriched tar-spot disks were more resistant to decomposition than unenriched uninfected disks. They also were a poorer substrate for hyphomycete sporulation.

Several mechanisms may contribute to slower decomposition and delayed fungal colonization of tar-spot disks. First, they may be deficient in useable C sources despite their high overall C content (Table 5). According to Jones (1925), the endophyte mycelium initially grows primarily in the upper epidermal cells of the host leaf and later extends into the mesophyll and lower epidermal cells. The vertical walls of the upper epidermal cells eventually rupture, suggesting enzymatic degradation. Therefore, plant polysaccharides and pectins, which are attacked

TABLE 5. Mean (±1 SD) % C, % N, and % P of disk dry mass and molar elemental ratios of tar-spot infected, adjacent, and uninfected Norway maple leaf disks before stream exposure (time 0, 10 replicates) and after 7 d of stream exposure (time 7; 4 replicates). Values with different superscripts are significantly different (*p* ≤ 0.05; comparisons within same time period).

	Time	% C	% N	% P	C:N	C:P	N:P	C:N:P
Tar spot	0	47.4 ^a ± 0.3	1.24 ^a ± 0.05	0.114 ^a ± 0.002	44.6	1073	24.1	1074:24:1
	7	47.8 ^A ± 0.4	0.91 ^A ± 0.04	0.054 ^A ± 0.002	61.3	2286	37.3	2286:37:1
Adjacent	0	44.9 ^b ± 0.2	0.73 ^b ± 0.04	0.098 ^b ± 0.002	71.8	1185	16.5	1184:16:1
	7	44.9 ^B ± 0.2	0.79 ^B ± 0.06	0.043 ^B ± 0.001	66.3	2697	40.7	2697:41:1
Uninfected	0	45.2 ^c ± 0.1	0.73 ^b ± 0.01	0.072 ^c ± 0.001	72.2	1621	22.5	1621:22:1
	7	45.2 ^B ± 0.1	0.76 ^B ± 0.05	0.039 ^B ± 0.005	69.4	2993	43.2	2293:43:1

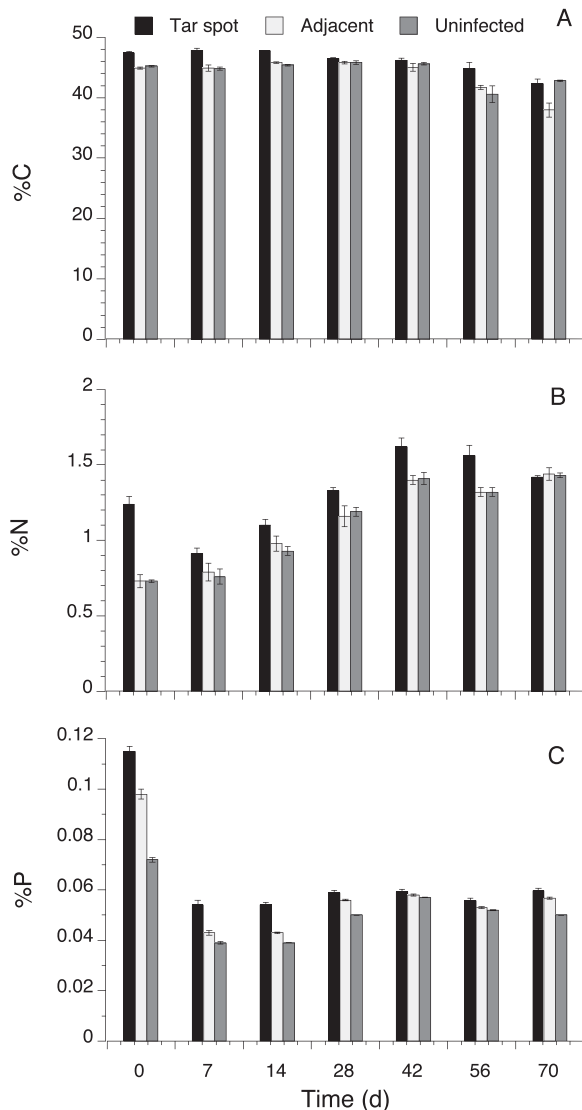


FIG. 3. Mean (± 1 SE) % C (A), % N (B), and % P (C) of tar-spot infected, adjacent, and uninfected Norway maple leaf disks exposed in Boss Brook.

successfully by most aquatic hyphomycetes, will be replaced by fungal polymers, among them chitin, which is less readily available to aquatic hyphomycetes (Chamier 1985). Second, the deposits of black pigments, first in the cells of the upper epidermis, may further limit the effectiveness of fungal exoenzymes (Webster and Weber 2007). Last, N and P may not be in useable form or may be leached too quickly to be accessible to new colonizers. Our results show some evidence supportive of both scenarios. In the 1st wk of stream immersion, when leaching dominates, both N and P decreased substantially (Fig. 3B, C), and C:N and C:P ratios increased (Table 5). This phase was followed by a gradual increase in % N and % P,

presumably caused by fungal immobilization from the stream water. Percent N and, less consistently, % P were highest in tar-spot disks. This result combined with the low values for spore production in tar-spot disks suggests that some of these nutrients are locked up in endophyte/host complexes and are inaccessible to aquatic hyphomycetes.

Aquatic hyphomycetes are important agents of leaf conditioning because they predigest plant polysaccharides and provide fungal lipids and proteins for leaf-shredding invertebrates (Krauss et al. 2011). Therefore, their inhibition by endophytes may impede subsequent trophic levels. A few preliminary observations indicated that infected leaf disks are rejected by the amphipod *Gammarus tigrinus* (FB, unpublished data), but this topic warrants more investigation.

Tar spot is one the most readily visible and identifiable endophytes/diseases (Webster and Weber 2007). Therefore, its frequency of occurrence is relatively easy to estimate. In the US Pacific Northwest, bigleaf maple is a dominant riparian species, and in 2006–2007, >70% of all leaves were infected by *R. punctatum* (LeRoy et al. 2011). We did not attempt a systematic estimate of the extent of the infection in our region, but all of the ~40 Norway maples in the vicinity of Mount Allison campus had tar spots. The leaf area covered by spots varied between ~10 and 40%. Annual variation in the rate of infection is considerable. Unless freshly fallen leaves land and overwinter in relatively moist spots, *R. acerinum* is unlikely to survive (Jones 1925). We followed the procedures published by Weber and Webster (2002) but were unable to revive the fungus in our air-dried material. The metabolic status of *R. acerinum* may affect the success of aquatic hyphomycetes. Generally, leaves that fall directly from the tree into a stream are less readily colonized than leaves that first land on soil and may be partly dried, or leaves that are predried in the laboratory (Bärlocher 1997).

Most endophytes produce no or hidden symptoms in infected plants, but they can profoundly lower the susceptibility of plant parts to pathogens and herbivores. Changes in leaf chemistry induced by infections often survive senescence and death of the plant (Grime et al. 1996, Lemons et al. 2005), so their effects on decomposition processes and nutrient cycling deserve closer attention.

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