Influence of pH on the growth and leaf-maceration ability of fungi involved in the decomposition of floating leaves of *Nymphaea alba* in an acid water

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Ten species of fungi were isolated from floating leaves cut from plants of *Nymphaea alba* in various initial stages of decay, which were collected from an acidic moorland pool. None of the fungal species isolated belonged to the aquatic Hyphomycetes *sensu* Ingold or the aero-aquatic fungi *sensu* Van Beverwijk. Growth experiments were conducted with five of the species on media containing glucose, polysaccharide or isolated cell walls of *N. alba*, each at three pH values. The fungi investigated were capable of growth on a variety of structural polysaccharides, indicating their potential importance in leaf degradation. Low pH inhibited growth on media containing glucose, pectin or cell wall fraction. Utilization of carboxymethylcellulose and crystalline cellulose did not differ much within the pH range studied. The fungi isolated were grown on *N. alba* leaf strips at three pH values to study the influence of pH on leaf maceration. All the fungi investigated could develop at low pH (4.0), but maceration was only observed at pH 5.5 or 7.5. It is likely that inhibition of pectin degradation is an important factor causing suppression of leaf fragmentation at low pH. This may contribute to the inhibition of the decomposition of macrophyte remains in acid aquatic systems.

Introduction

Decomposition of plant remains in aquatic systems is mainly a microbiological process. The contribution of microbes to the mass loss of macrophyte material is about 60–70% (Harrison & Mann, 1975; Brock, 1984). The remainder of the material is lost by leaching of small molecules after cell death, autolysis and consumption by detritivores. The specific role of fungi and bacteria in the decomposition process is not clear. Contradictory reports exist in the literature on the quantitative contribution of these groups to the total mass loss of detritus during decomposition. Some authors stated that fungi are more important than bacteria (Kaushik & Hynes, 1971; Padgett *et al.*, 1985), while other investigators found bacteria to be dominant (Brock, 1984; Benner *et al.*, 1986). These discrepancies may arise from the use of methods which are inadequate to distinguish between bacterial and non-bacterial metabolism (Yetka & Wiebe, 1974), or from differences in the techniques applied (Benner *et al.*, 1986). There is, however, little doubt that fungi dominate the decomposition processes in acid waters (Traaen, 1980; Rheinheimer, 1985; Benner *et al.*, 1986; Leuven & Wolfs, 1988).

It is extensively documented that the decomposition rate is lower in acid systems than in non-acid systems (e.g. Traaen, 1980; McKinley & Vestal, 1982; Hoeniger, 1985). However, there is little information on the mechanisms underlying the inhibition of decomposition at low pH. MacKay & Kersey (1986) suggested that the absence of decay-stimulating detritivores in acid systems is a major cause of the low decomposition rate. This cannot be the only cause, since inhibition of decomposition by acid is also found in laboratory experiments without fauna (Brock *et al.*, 1985; Kok & van de Laar, 1991). Other authors state that the low pH in acid systems inhibits the growth and activity of bacteria (McKinley & Vestal, 1982; Rao & Dutka, 1983). However, this does not explain why the decay rate in acidic media is lower than in media in which antibiotics inhibit the growth and activity of bacteria (Brock, 1984; Brock *et al.*, 1985; Leuven & Wolfs, 1988). So it seems that even acid-tolerant fungi are in some way inhibited by low pH.

Since the soluble compounds of dead plant material are rapidly lost by leaching, the main substrate of saprophytic micro-organisms is the insoluble part of the material, mainly consisting of the cell wall fraction

(Chamier & Dixon, 1982*b*). Under non-acid conditions aquatic fungi are able to produce all the exoenzymes necessary for complete degradation of the cell wall (Chamier, 1985; Benner *et al.*, 1986). Therefore, it is likely that, even though fungi can grow at low pH, there is some kind of inhibition of the exoenzymic degradation of the detritus under acid conditions. Kok *et al.* (1990) reported that the maceration of floating leaf blades of *Nymphaea alba* and *Nuphar lutea* was inhibited in an acid water. These authors suggested that this was due to inhibition of the breakdown of cell wall components. Chamier & Dixon (1982*b*) showed that pectin degradation and leaf maceration by selected aquatic Hyphomycetes was inhibited at pH < 5. Pectate lyase, an important enzyme in tissue maceration (Friend, 1977, and references therein) was not found in cultures grown at pH < 6.5. Inhibition of pectin degradation and the resulting absence of tissue maceration would strongly reduce the decomposition rate of macrophyte material.

To test the hypothesis that the slow decomposition of leaf material under acid conditions is caused by the inhibition of pectin degradation, the influence of pH and carbon source (glucose and structural polysaccharide components) on the growth of fungi isolated from floating leaf blades of *Nymphaea alba* was investigated. The effect of pH on tissue maceration by isolated fungi was also studied.

Methods

Isolation of fungi. Floating leaves of *Nymphaea alba*, which were still connected to the plant, were collected in the Voorste Choorven (The Netherlands; lat. N 51° 34' 54", long. E 5° 12' 18"), an acidic moorland pool (median pH 4.6 in 1988). The leaves were selected according to their appearance. Three categories were distinguished, viz. green leaves with small necrotic spots, leaves with large necrotic areas and totally necrotic leaves. Leaves were collected in October 1988 and October 1990. In 1990, leaf fragments from the sediment detritus layer were also collected. The samples were transported to the laboratory in polyethylene bags.

From the 1988 leaf sample, seven leaves were chosen for each of the three decomposition stages and five leaf segments (about 4 cm²) were cut out from the spotted or necrotic areas of each leaf. These segments were surface-sterilized with a solution of 1% (v/v) hypochlorite in tap water for 3 min and subsequently washed for 30 min in sterile water. Leaf cuttings were placed on 2% and 4% (w/v) malt agar in 9 cm Petri dishes at room temperature. Developing mycelia were transferred to new dishes and identified.

The leaves collected in 1990 were treated specially to detect the presence of aquatic Hyphomycetes *sensu* Ingold (Ingold, 1942, 1975) and aero-aquatic fungi *sensu* Van Beverwijk (Van Beverwijk, 1953). After rinsing with sterile water, leaf segments were cut from five leaves from each decomposition stage as described above. In addition, five samples of necrotic but non-fragmented leaf material from the sediment of the Voorste Choorven were treated in the same way. Four leaf sections per leaf were incubated on 0.1% malt agar containing 50 mg chloramphenicol l⁻¹ at 15 °C under light. Developing colonies were transferred to 2% malt agar, containing 50 mg chloramphenicol l⁻¹, grown at 20 °C and identified. If a colony failed to sporulate, agar

strips were incubated in water in flasks with enforced aeration (Webster & Towfik, 1972; Webster, 1975). In order to detect aero-aquatic fungi, leaf segments were incubated in moist chambers at 15 °C under light. In addition, leaf segments were incubated in water in aerated flasks (Fisher, 1977; Fisher & Webster, 1978). The moist chambers and the flasks were regularly examined during four weeks for the presence of the typical spores indicating the presence of aquatic Hyphomycetes or aero-aquatic fungi.

Experimental design. In order to determine the interactive effect of carbon source and pH on the growth of fungi, flasks with liquid culture media at pH 4.0, 5.5 or 7.5 and different carbon sources were inoculated with mycelium, and the growth (amount of protein produced) after 3 weeks incubation was determined. The pH values were chosen to represent severely acidified, moderately acidified and circumneutral waters, respectively. This experiment was carried out with five species of fungi which were frequently isolated from the decaying floating leaf blades.

To study the influence of pH on leaf maceration, leaf disks (1.2 cm diameter) were cut from green leaves and incubated in basic culture medium. The pH was adjusted to 4.0, 5.5 or 7.5 with 1 M-HCl. The flasks were inoculated with mycelium as described below. After 3 weeks incubation, the degree of leaf maceration (fragmentation) was estimated by filtration of the leaf material over a screen with 0.5 cm mesh size. The leaf material retained after gentle rinsing was blotted dry, weighed and the amount expressed as a percentage of the total amount of material. All growth experiments were carried out in triplicate.

Growth media. The media consisted of a basic culture medium and 1% (w/v) carbon source. The composition of the basic medium was (g l⁻¹): NH₄NO₃, 2.5; KH₂PO₄, 2.0; Na₂HPO₄, 3.0; KCl, 0.5; MgSO₄·7H₂O, 0.5; FeSO₄·7H₂O, 0.01; yeast extract, 0.05; chloramphenicol, 0.05; potassium hydrogen phthalate, 10.0. The carbon sources added were glucose, pectin, carboxymethylcellulose (CMC), cellulose, and cell wall material from floating leaf blades of *N. alba*. Chloramphenicol (50 mg l⁻¹) was added to suppress bacterial growth.

All chemicals used for the preparation of the media were of analytical grade. Pectin (Sigma) was derived from citrus fruits and was 10% methoxylated. The cellulose used was Sigmacell, type 50. The cell wall fraction was prepared from floating leaf blades of *N. alba* by a serial extraction method (modified from Harborne, 1984). Leaf blades were homogenized in 70% (v/v) acetone with an Ystral homogenizer. After filtration, the residue was extracted in 96% (v/v) ethanol (0.5 h), then in boiling 1% (w/v) NaCl (1 h), and suspended overnight in an 1% (w/v) amylase solution (porcine pancreas α -amylase, Sigma). After filtration, the residue was extracted in 1 M-NaOH (1 h, 95 °C). The insoluble fraction was washed twice with distilled water and dried at 50 °C. The remaining cell wall fraction contained the lignocellulose and tightly associated hemicellulose (Harborne, 1984).

The media were sterilized at 121 °C, for 30 min. Glucose and pectin were dissolved in distilled water and autoclaved separately to prevent degradation.

In order to keep the pH at the initial level during the whole experiment, it was necessary to include strong buffers in the basic medium. A combination of KH₂PO₄, Na₂HPO₄ and potassium hydrogen phthalate provided the required buffering capacity. This was checked by measuring the pH of the medium before and after the incubation period. Pilot experiments showed that the fungi used in these experiments were unable to utilize phthalic acid as sole carbon source and that growth on oatmeal agar with and without phthalic acid was not significantly different. Thus an influence of phthalic acid on the growth of these fungi can be ruled out.

The culture flasks (250 ml Erlenmeyer flasks, containing 100 ml growth medium) were inoculated with pieces of mycelium cut with a flamed cork borer (diameter 0.8 cm) from the margin of a colony

growing on glucose agar. The flasks were placed on a laboratory shaker at 15 °C in the dark.

After 3 weeks the contents of the flasks were filtered and the whole residue was homogenized and extracted with 1 M-NaOH (1 h, 95 °C). The samples were centrifuged (13000 g, 10 min) and the protein concentration in the supernatant was estimated by the Bradford dye-binding assay as modified by Read & Northcote (1981). Bovine serum albumin was used to calibrate the method. The protein production was corrected for the protein content of the inoculum, which was estimated by determining the amount of protein in a similar amount of mycelium, cut from the same colony.

Statistical procedures. After testing the assumptions of ANOVA, the significance of the effects of the treatments on the fungal growth was tested with ANOVA and Fisher's Least Significant Difference test. Results were considered significant if $P < 0.05$. Calculations were performed using the SAS computer package (SAS Institute, 1985).

Results

Occurrence of fungi in the field

Ten species of fungi were found on the leaf blades of *Nymphaea alba* from the Voorste Choorven. The species and their occurrence in the three decomposition stages distinguished are listed in Table 1. No fungi belonging to the aquatic Hyphomycetes *sensu* Ingold or aero-aquatic fungi *sensu* Van Beverwijk were detected, either by direct isolation, or by spore production in moist chambers or incubation flasks.

Growth experiments

Five fungal species were selected for the growth experiments: *Colletotrichum nymphaeae*, *Epicoccum nigrum*, *Trichoderma viride*, *Botrytis cinerea* and *Fusarium avenaceum*. All species were able to grow on the substrates tested (Fig. 1). Only *F. avenaceum* showed little protein production on the cell wall fraction. All species grew more slowly at pH 4.0. This effect of pH was clearly seen in the media containing glucose, pectin and the cell wall fraction, and was less pronounced in the CMC and cellulose media.

C. nymphaeae had the highest protein production in the glucose (pH 5.5 and 7.5) and pectin (pH 7.5) media. The growth at pH 4.0 was significantly lower than at higher pH, for all media. The difference in protein production between pH 5.5 and 7.5 was only significant in the incubation series with pectin (Fig. 1a).

B. cinerea had the highest protein production in the glucose (pH 5.5) and pectin (pH 7.5) media (Fig. 1b). The growth on CMC and cellulose was not influenced by pH. In the other media the protein production was significantly lower at pH 4.0.

E. nigrum (Fig. 1c) showed a similar growth pattern to *B. cinerea*. Its protein production on CMC and cellulose

Table 1. Occurrence (number of mycelia isolated) of fungi associated with different stages of degradation of floating leaf blades of *N. alba*

Species	Stage*		
	1	2	3
<i>Colletotrichum nymphaeae</i>	freq	freq	freq
<i>Epicoccum nigrum</i>	freq	freq	freq
<i>Botrytis cinerea</i>	3	–	freq
<i>Trichoderma viride</i>	3	–	–
<i>Ulocladium oudemansii</i>	3	–	–
<i>Arthrinium phaeospermum</i>	–	2	–
<i>Pythium undulatum</i>	–	2	–
<i>Fusarium avenaceum</i>	–	2	–
<i>Coprinus</i> sp.	1	–	–
Sterile mycelium	1	–	–

*Stage 1, leaves with small necrotic spots; stage 2, leaves with large necrotic areas; stage 3, totally necrotic leaves. Freq, isolated more than three times.

Table 2. Maceration of leaf strips by isolated fungi at different pH

The results are expressed as the percentage of material, $\pm$ SD, retained by 0.5 cm mesh after 3 weeks of fungal growth.

Species	pH		
	4.0	5.5	7.5
<i>C. nymphaeae</i>	93 $\pm$ 2	53 $\pm$ 8	32 $\pm$ 6
<i>T. viride</i>	89 $\pm$ 4	44 $\pm$ 6	43 $\pm$ 3
<i>P. undulatum</i>	96 $\pm$ 3	89 $\pm$ 6	65 $\pm$ 4
<i>A. phaeospermum</i>	92 $\pm$ 1	85 $\pm$ 3	43 $\pm$ 7
<i>E. nigrum</i>	90 $\pm$ 3	65 $\pm$ 6	23 $\pm$ 3
<i>U. oudemansii</i>	89 $\pm$ 5	75 $\pm$ 4	45 $\pm$ 2
<i>F. avenaceum</i>	97 $\pm$ 1	91 $\pm$ 3	42 $\pm$ 5
<i>B. cinerea</i>	92 $\pm$ 3	87 $\pm$ 6	35 $\pm$ 7

was independent of pH. The highest production was found in the glucose medium (pH 5.5). Protein production on pectin was highest at pH 7.5. The growth in the media containing glucose, pectin and cell wall fraction was strongly inhibited at pH 4.0.

F. avenaceum was the only fungus used in this experiment that could not grow well on all substrates. Its growth on the cell wall fraction medium was very low and independent of the tested pH (Fig. 1d). *F. avenaceum* showed high protein production on glucose (pH 5.5) and CMC (pH 5.5 and 7.5). Low pH inhibited growth in the glucose, pectin and CMC media.

T. viride showed high protein production in the glucose (pH 5.5) and cell wall fraction (pH 7.5) media. Growth on CMC and cellulose was not influenced by pH. On all other substrates the production was inhibited at pH 4.0 (Fig. 1e).

Fig. 1. Influence of pH and carbon source on growth (protein production) of selected fungi in laboratory experiments after 3 weeks incubation. G, glucose; P, pectin; CMC, carboxymethylcellulose; C, native cellulose; CWF, cell wall fraction. Bars represent means ($n = 3$). The vertical rule represents the least significant difference (LSD), for $P < 0.05$. (a) *Colletotrichum nymphaeae*; (b) *Botrytis cinerea*; (c) *Epicoccum nigrum*; (d) *Fusarium avenaceum*; (e) *Trichoderma viride*.

belong to the Hyphomycetes. However, none of the aquatic Hyphomycetes *sensu* Ingold or the aero-aquatic fungi *sensu* Van Beverwijk was isolated. Hyphomycetes *sensu* Ingold are dominant in the degradation of leaf material in many freshwater ecosystems in moderate climate zones (Suberkropp & Klug, 1976; Chamier & Dixon, 1982a; Chergui & Pattée, 1988), and it is remarkable that no species of this group were encountered on the decaying leaves of *Nymphaea alba* in the Voorste Choorven. This group of fungi is generally characteristic for unpolluted water and these fungi seem sensitive to low pH (Webster & Descals, 1981; Chamier, 1987). The conditions in the Voorste Choorven, characterized by extremely low pH (median pH 4.6 in 1988), high NH_4^+ concentrations and intensive sulphate reduction in the organic matter accumulated on the sediment, are probably inhibitory to aquatic Hyphomycetes. Aero-aquatic fungi are characteristic of stagnant or slowly running waters and can be expected to colonize floating leaves by means of their unsinkable spores. There are indications that these fungi can survive in the water layer under low-oxygen conditions, comparable to the situation in the Voorste Choorven (Fisher & Webster, 1979; Webster & Descals, 1981). The absence of this group of fungi, even from the material collected from the sediment, probably means that these fungi do not tolerate the low pH of the Voorste Choorven. So it seems that the environmental conditions of the Voorste Choorven inhibited fungi usually associated with leaf litter decomposition, thereby giving a competitive advantage to the species we isolated.

Most of the species found in the Voorste Choorven are cosmopolitan, and are usually classified as soil fungi (Domsch *et al.*, 1980). Only *C. nymphaeae* seems to be specific to *Nymphaea alba*; it was also isolated from

Maceration experiments

The results of the maceration experiments are shown in Table 2. All species of fungi investigated showed growth on the leaf strips in all treatments. Very little maceration was found at pH 4.0. At pH 5.5, five of the eight species investigated caused at least some leaf fragmentation. At pH 7.5, all species caused the leaf strips to fragment to a large extent.

Discussion

Although it is unlikely that our list of isolated fungal taxa is complete, it is interesting that most of the taxa found

floating leaf blades of *N. alba* in non-acid systems (Van der Aa, 1978). *P. undulatum* was originally isolated from *Nymphaea alba* and *Nuphar lutea* in Denmark and this species is commonly associated with aquatic macrophytes as a saprophyte or perhaps as a weak pathogen (Van der Plaats-Nifterink, 1981).

All the fungal species investigated here were able to utilize a diversity of substrates. The potential to grow on all the purified polysaccharide media tested and on the cell wall fraction indicates that the species investigated are potentially able to decompose the major part of the insoluble plant compounds.

The five species used in the growth experiments are commonly associated with plants as decomposers or pathogens. There are numerous reports on their carbon nutrition, indicating that all species should be able to use the whole range of plant cell wall polymers (Domsch *et al.*, 1980). The poor growth of *F. avenaceum* on the cell wall fraction and the native cellulose may be explained by the varying capacities of different strains of this species for decomposition of polysaccharides (Domsch *et al.*, 1980).

Our results show that pH is an important factor regulating the growth of the fungi isolated. Even though all the investigated fungal species could develop over the whole pH range tested, the ability to degrade certain types of substrate was strongly reduced at low pH.

Growth on pectin was very strongly inhibited at pH 4.0 and inhibition could be demonstrated even at pH 5.5, for all species. These results support the findings of Chamier & Dixon (1982b), who found that of seven species of aquatic Hyphomycetes investigated, only two grew well on polypectate agar at pH 5, whereas all species did so at pH 7. The fungi used in our investigation were somewhat more acid-tolerant, possibly because of a selection in an acidic environment. One of the reasons for the slow growth on pectin at low pH is the high pH optimum (8.5–9) of the enzyme pectin lyase (Rexová-Benková & Marcovic, 1976).

Growth on CMC and cellulose was relatively insensitive to pH. Only *F. avenaceum* and *C. nymphaeae* were affected by low pH on CMC. This can be explained by the low pH optima of the exoenzymes involved in the degradation of crystalline and amorphous cellulose (Chamier, 1985). The faster degradation of the substrate at low pH probably compensated for possible detrimental effects of low pH on the development of the mycelia.

Low pH inhibited the growth of all species on the cell wall fraction substrate, except for *F. avenaceum*, which showed very little growth on this substrate, irrespective of the pH. It is possible that the inhibition of growth on this substrate is due to the presence of hemicelluloses, since pH did not influence the development of the fungi on cellulose, and the pectins had been removed by the

extraction procedure (Harborne, 1984). Accumulation of hemicelluloses in *Nymphaea alba* detritus under acid conditions is known to occur (Brock *et al.*, 1985). It has been shown that hemicellulase production by *T. viride* is inhibited by low pH (Dean *et al.*, 1989), so it is likely that the decomposition of hemicellulose is inhibited at low pH.

Another possible explanation for the low protein production of the fungi on cell wall substrate at low pH is the occurrence of tannins in the cell wall fraction. *N. alba* is known to contain considerable amounts of hydrolysable tannins (McClure, 1970), and tannins can inhibit the growth of fungi (Knudson, 1913, cited in Domsch *et al.*, 1980; Harrison, 1971). Furthermore, it is known that the inhibitory action of hydrolysable tannins is most intensive under acid conditions (Loomis, 1974).

The findings of the growth experiments are supported by measurements of exoenzymic activity in decomposing *N. alba* leaf blades at different pH values (Kok & Van der Velde, 1991). At low pH, pectinase and pectin lyase activities were not detectable. Hemicellulase activity was also low at low pH. Cellulase activity, however, was not influenced by a pH of 4.0.

The results of the leaf maceration experiments support the observations of the growth experiments. Absence of maceration probably indicates inhibition of pectin degradation. The fact that the fungi tested were able to grow on the leaf disks at pH 5.5 without fragmenting the leaf material much also suggests specific inhibition of pectic enzymes rather than general reduction of growth at this pH. Fragmentation of decaying material strongly stimulates the decomposition rate (Larsen, 1982). Furthermore, it is likely that the intact pectin in the middle lamella and the primary cell wall hinders the degradation of other cell wall polysaccharides. Therefore, inhibition of pectin degradation may be a major factor in the low decomposition rate of macrophyte remains in acid waters.

Our results indicate that the degradation of major groups of structural carbohydrates by fungi is inhibited by low pH. Inhibition of growth of the fungal species investigated here is, however, not caused by the effect of low pH on the exoenzymic degradation of the substrate alone. Growth on glucose was also inhibited by low pH. It is not known which process is responsible for this inhibition, but it is possible that high H^+ concentrations interfere with the uptake of other cations or with the regulation of intracellular pH.

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