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Enzymatic analysis of leaf decomposition in freshwater by selected aquatic hyphomycetes and terrestrial fungi

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Alder (*Alnus glutinosa*) leaf discs were inoculated singly with cultures of nine aquatic hyphomycetes and seven terrestrial fungi isolated from submerged decomposing leaves to compare the maceration capability and enzyme production under submerged conditions. In general, leaf discs inoculated with aquatic hyphomycetes had a higher loss of mass and tensile strength than leaf discs inoculated with terrestrial fungi. Aquatic hyphomycetes had a significantly ($P < 0.05$) higher activity of cellulases C_x and C_1 than terrestrial fungi. Softening was significantly correlated with fungal enzyme activity ($P < 0.05$), especially with xylanase ($P < 0.001$). In general, the results confirm the widespread notion that aquatic hyphomycetes play a key role in the degradation of leaves in freshwater.

Keywords: ecology, aquatic hyphomycetes, physiology, degradation.

Decomposition is an important process in ecosystem functioning allowing the recycling of nutrients. In streams and rivers, the major energy source is detritus originating either in the rivers themselves or in the riparian zone.

In the early stages of decomposition, microbial activity is responsible for the breakdown of the complex polysaccharides in the plant cell walls. As a consequence of microbial attack, leaves become softer and their nitrogen content increases (Kaushick & Hynes, 1968; Suberkropp & Arsuffi, 1984; Graça & al., 1993). There is now a general consensus that fungi rather than bacteria are the dominant agents involved in the degradation of leaf tissue. In the final stages of decomposition, however, bacteria may become more important (Suberkropp & Klug, 1976). Detritivores preferentially feed on microbially colonised leaves (Suberkropp, 1992; Graça, 1993), promoting leaf fragmentation and thereby accelerating decomposition.

Aquatic hyphomycetes dominate the fungal community of submerged leaves (Bärlocher & Kendrick, 1974). Their ability to depolymerize complex polysaccharides has been confirmed by several

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studies (Suberkropp & Klug, 1980; Chamier & Dixon, 1982a, b; Suberkropp & al., 1983; Chandrashekar & Kaveriappa, 1988). In spite of the apparent importance of aquatic hyphomycetes in freshwaters, terrestrial fungi have also been isolated from submerged leaf substrates (Kaushik & Hynes, 1968; Godfrey, 1983; Rossi & al., 1983). Little is known, however, of the role of terrestrial fungi in the degradation of submerged plant detritus. It has been shown that leaves entering streams are already colonized by terrestrial fungi that remain alive under submerged conditions, but are rapidly overgrown by the aquatic hyphomycetes. The advantage of aquatic hyphomycetes over terrestrial fungi in freshwater has been related to their more effective dispersal due to the tetraradiate and sigmoid spores, high sporulation rate stimulated by water turbulence (Webster & Descals, 1981), and growth at lower temperatures (Koske & Duncan, 1974).

Graça & Ferreira (1995) showed that at both low (4 C) and high (20 C) temperatures, aquatic hyphomycetes had a higher capability to degrade leaf tissue than terrestrial fungi isolated from streams. Here we further examine the role of terrestrial fungi in lotic environments by comparing the enzymatic capabilities of selected aquatic and terrestrial fungi isolated from streams.

Materials and methods

Fungi

Nine species of aquatic hyphomycetes were obtained as single spore isolates from leaf litter sampled from streams in central Portugal: *Articulospora tetracladia* Ingold; *Lemonniera terrestris* Tubaki; *Lemonniera aquatica* de Wild; *Heliscus lugdunensis* Sacc. & Therry; *Lunulospora curvula* Ingold; *Tetrachaetum elegans* Ingold; *Mycocentrospora angulata* Petersen; *Tricladium angulatum* Ingold and *Tricladium gracile* Ingold.

Seven species of terrestrial fungi sampled from submerged leaves in the same streams were obtained by vigorously shaking the leaves with stream water in sterile plastic bags. Drops of this water were used as inoculum on malt extract agar plates (MEA, Difco, 36 g/L). The specimens isolated included: *Cladosporium herbarum* (Persoon) Link *ex* Gray; *Eupenicillium shearii* Stolk & Scott; *Aureobasidium pullulans* (de Bary) Arnaud; *Rhizopus oryzae* Went & Princen Geerlings; *Cylindrocladium scoparium* Morgan; *Alternaria alternata* (Fries) Keissler and *Rhizoctonia solani* Kuhn.

Leaf maceration

Entire abscised alder (*Alnus glutinosa*) leaves were collected from a single tree and air-dried at room temperature. Before use,

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they were leached in running tap water for five days and leaf discs of similar weight (8.0 ± 0.3 mg) were obtained (see Graça & al., 1993), oven dried at 55° C and weighed to the nearest 0.1 mg.

Seven leaf discs were added to each 250 ml Erlenmeyer flask containing 50 ml of an inorganic salt solution: KNO_3, 1.0 g/L; NaCl, 0.11 g/L; KH_2PO_4, 0.41 g/L; K_2HPO_4, 0.52 g/L; $MgSO_4.7H_2O$, 0.49 g/L; $CaCl_2.2H_2O$, 0.15 g/L; pH7 (Suberkropp & al., 1983) and autoclaved (121 C, 15 min).

Three replicates for each fungus were inoculated with 1 ml of a hyphal homogenate containing 3 to 5 mg (dry mass) of hyphal material obtained from pure cultures grown on malt extract broth. Uninoculated flasks served as initial and final controls. Cultures were incubated in an orbital shaker (GFL3017, 100 rpm) at 15 C for 40 days.

Leaf mass loss due to fungal activities was determined from differences between initial and final dry mass (2 days at 55 C). The tensile strength required to tear apart the leaf was also measured before and after incubation in the following way: A leaf disc was secured by two pegs. One of the pegs was fixed whereas the other was connected to an aluminium cup (200 ml) via a pulley. Sand was gradually added to the cup until its weight caused the leaf disc break. The sand weight was then determined to the nearest 0.1 g. Values were expressed as a percentage of weight and resistance loss, and comparisons between terrestrial and aquatic fungi were assessed by the Mann-Whitney test (Zar, 1984).

Enzymatic assays

At the end of the growth period, the culture was filtered (Whatman No. 50 filter paper), the pH of the culture fluid was measured and the fluid treated with thimerosal (Sigma, final concentration 0.01%) to inhibit microbial growth, and refrigerated at 4 C until assayed for enzyme activities.

Polygalacturonase, xylanase and endo-b-1,4-D-glucanase (C_x) assay. – Activity of these enzymes was estimated by mixing 1 mL of 0.2 M potassium acetate buffer (pH 5.0) with 1 mL substrate (respectively, 1% polygalacturonic acid, Sigma; 1% xylan, Sigma, purified fraction V by the procedure of Baker & al. (1977) or 1% carboxymethyl cellulose (Sigma) and 1 mL of the culture filtrate (Suberkropp & al., 1983). Reducing sugars were determined with the dinitrosalicylic acid reagent (Miller, 1959) after incubation at 30 C for 3 hours. Activity (mg per milliliter per hour) was determined by subtracting absorbance of control (enzyme added after incubation) and comparing the results with standard curves prepared with

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known concentrations of galacturonic acid, xylose and glucose, respectively.

Pectin lyase. – The activity was determined by reacting 1 mL 0.2 M bicine (with 0.03 M $CaCl_2$, pH 8.0) with 1 mL 1% pectin (Sigma), dialysed and centrifuged (9000 g, 20 min), and 1 mL of the culture filtrate (Suberkropp & al., 1983). After incubation at 30 C for 3 hours, the reaction mixtures were assayed for the unsaturated products by reaction with the thiobarbituric acid reagent (Ayers & al., 1966). Activity (unit per ml per hour, where 1 unit represents 1 absorbance unit at 550 nm) was determined as the difference in absorbance of controls (enzyme added after incubation) and experimental mixtures.

Cellobiohydrolase (C_1). – The activity of C_1 was measured in terms of filter paper activity, by incubating a mixture containing a 50 mg strip (10 × 60 mm) of Whatman No. 1 paper; 2 mL 0.2 M potassium acetate (pH 5.0), and 2 mL of the culture filtrate. The mixtures were incubated at 40 C for 24 h and reducing sugars were determined with the dinitrosalicylic acid reagent, by comparing absorbance with a standard curve of glucose. Control assay values were subtracted from experimental values as described above.

Triacylglycerol lipase. – Fungi were grown in a medium containing 8 g/L of peptone (Sigma), 10 mL/L Tween 80 (Sorbitan polyxyethylene mono-oleic, Sigma), 0.1 g/L $CaCL_2.2H_2O$ and 20 g/L agar (Sierra, 1957). The occurrence of a white precipitation ring of fatty acid crystals around the colony after 10 days incubating at 15 C, was considered as a positive test of lipolytic activity.

Phenol oxidase. – Agar plates containing 15 g/L malt extract (Difco), 20 g/L agar and 0.8 g/L tannic acid (Merck) were used (Abdullah & Taj-Aldeen, 1989). Fungi were grown for 10 days at 15 C and screened for the presence of a dark-brown zone around the colony.

Statistical analysis

The Mann-Whitney test was used to compare the maceration ability and enzymatic activity of terrestrial and aquatic fungi. The role of the enzymes on softening leaf discs was investigated by the application of both Spearman rank correlation and stepwise regression analysis.

Results

Leaf discs incubated with pure fungal cultures for 40 days showed clearly visible mycelial growth, especially in flasks inoculated with *T. angulatum* and *T. gracile*. Flasks inoculated with *R. oryzae*

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showed minimum growth. Mass loss and decrease in tensile strength in leaf discs were highly correlated ($r_s = 0.91$; $P < 0.001$). There were no significant differences in the maceration ability between terrestrial and aquatic fungi ($U < 46.5$; $n = 16$; $P > 0.05$) (Fig. 1). When

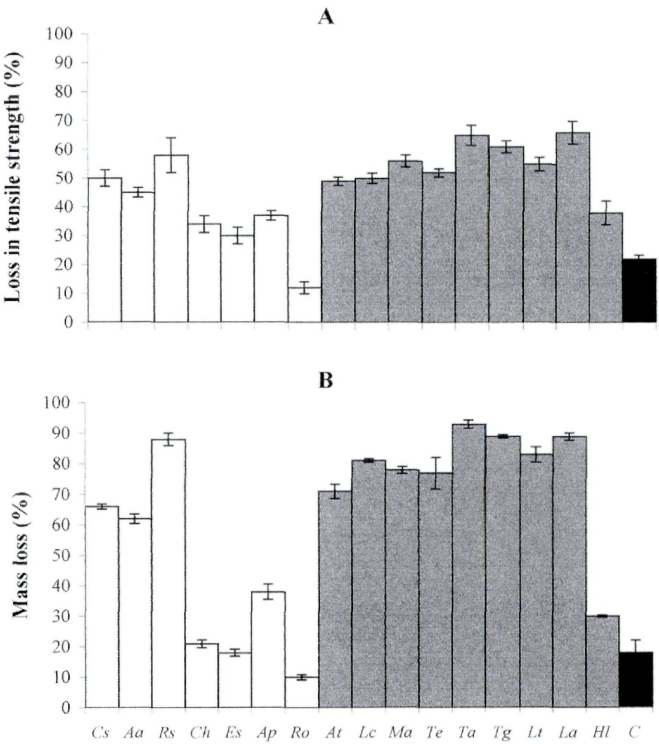

Fig. 1. – Decreases in tensile strength (**A**) and mass (**B**) in alder leaf discs inoculated with pure cultures of terrestrial (white columns) and aquatic (grey columns) fungi (means and standard error). – *Cs = Cylindrocladium scoparium; Aa = Alternaria alternata; Rs = Rhizoctonia solani; Ch = Cladosporium herbarum; Es = Eupenicillium shearii; Ap = Aureobasidium pullulans; Ro = Rhizopus oryzae; At = Articulospora tetracladia; Lc = Lunulospora curvula; Ma = Mycocentrospora angulata; Te = Tetrachaetum elegans; Ta = Tricladium angulatum; Tg = Tricladium gracile; Lt = Lemonniera terrestris; La = Lemonniera aquatica; Hl = Heliscus lugdunensis; C* = control.

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Heliscus lugdunensis, an aquatic species, is eliminated, mass loss and decrease in tensile strength were significantly higher in aquatic hyphomycetes ($U > 43$; $n = 15$; $P < 0.01$). Leaves incubated in uninoculated flasks also decreased in mass and tensile strength. Leaf discs inoculated with *R. oryzae* had a mass and tensile strength loss similar to control discs. The terrestrial *C. herbarum*, *E. shearii*, *A. pullulans* and the aquatic *H. lugdunensis* showed low macerating capability, whereas *T. angulatum*, *T. gracile* and *L. aquatica* (all aquatic) showed high macerating capabilities (Fig. 1).

The species that were less effective in macerating leaves (*R. oryzae*, *C. herbarum*, *E. shearii*, *A. pullulans* and *H. lugdunensis* - Fig. 1) had, in general, lower polygalacturonase/pectin lyase activities (Fig. 2). The exception was polygalacturonase in *A. pullulans*. Aquatic fungi had significantly higher C_x and C_1 enzymatic activities than terrestrial fungi (Fig. 3) ($U = 51.5$; $n = 16$; $P < 0.05$). No differences were observed for other enzymes ($U < 43.5$; $n = 16$; $P > 0.05$). However, among the 16 species screened, the 5 highest activities were observed in aquatic hyphomycetes and the 4 lowest activities were observed in 3 terrestrial and 1 aquatic species.

Leaf softening was correlated with the activity of all enzymes ($r_s = 0.943$, $P < 0.001$ for xylanase; $r_s = 0.607$–0.673, $P < 0.05$ for the other enzymes) (Fig.4). Moreover, a stepwise regression analysis revealed that, among all enzymes, only the activity of xylanase accounted for litter maceration in terms of loss in tensile strength ($r^2 = 0.59$; $b = 0.040$; $t = 4.741$; $P < 0.001$).

All tested species, except *R. oryzae*, were able to produce lipase (Tab. 1). Aquatic hyphomycetes (except *H. lugdunensis*) were much more effective in the degradation of tannic acid (often used as a preliminary indicator of the capacity for lignin degradation) than terrestrial fungi; in this group, only *C. scoparium*, *R. solani* and *C. herbarum* had a positive reaction (Tab. 1).

Discussion

The relevant observations of this work can be summarised as follows: (1) Aquatic hyphomycetes and terrestrial fungi differed in their ability to macerate leaves in the water; (2) leaf degradation was strongly correlated with xylanase activity; (3) terrestrial and aquatic fungi differed in their ability to produce C_x and C_1 degrading enzymes; and (4) there was little agreement with published work on the enzymatic capabilities of some fungi.

Aquatic hyphomycetes and terrestrial fungi differed in their ability to macerate alder leaves in the water. The observation that terrestrial fungi are capable of causing significant mass loss in submerged leaves is consistent with Godfrey's (1983) results. Graça &

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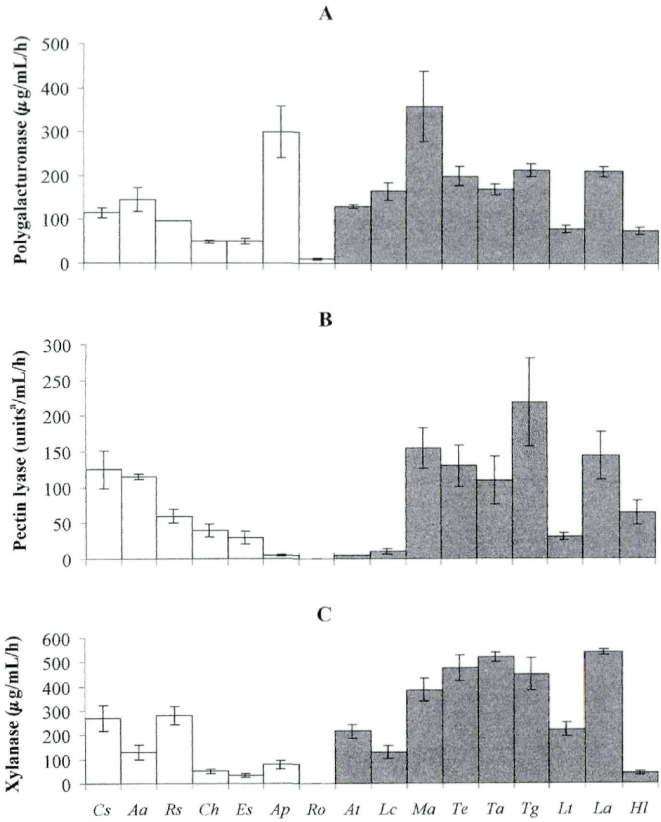

Fig. 2. – Extracellular polygalacturonase (**A**), Pectin lyase (**B.** [a] - One unit of activity = a change of 0.001 absorbance unit at 550 nm) and Xylanase (**C**) activities in alder leaf culture fluid of terrestrial (white columns) and aquatic (grey columns) fungi under laboratory conditions. – *Cs = Cylindrocladium scoparium; Aa = Alternaria alternata; Rs = Rhizoctonia solani; Ch = Cladosporium herbarum; Es = Eupenicillium shearii; Ap = Aureobasidium pullulans; Ro = Rhizopus oryzae; At = Articulospora tetracladia; Lc = Lunulospora curvula; Ma = Mycocentrospora angulata; Te = Tetrachaetum elegans; Ta = Tricladium angulatum; Tg = Tricladium gracile; Lt = Lemonniera terrestris; La = Lemonniera aquatica; Hl = Heliscus lugdunensis; C* = control.

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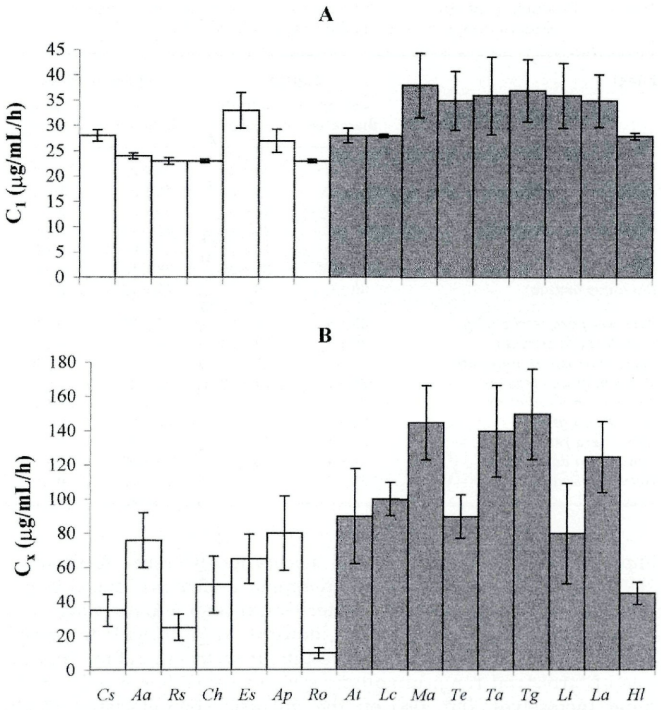

Fig. 3. – C_1 (**A**) and C_x (**B**) activities in alder leaf culture fluid of terrestrial (white columns) and aquatic (grey columns) fungi under laboratory conditions. – *Cs = Cylindrocladium scoparium; Aa = Alternaria alternata; Rs = Rhizoctonia solani; Ch = Cladosporium herbarum; Es = Eupenicillium shearii; Ap = Aureobasidium pullulans; Ro = Rhizopus oryzae; At = Articulospora tetracladia; Lc = Lunulospora curvula; Ma = Mycocentrospora angulata; Te = Tetrachaetum elegans; Ta = Tricladium angulatum; Tg = Tricladium gracile; Lt = Lemonniera terrestris; La = Lemonniera aquatica; Hl = Heliscus lugdunensis; C* = control.

Ferreira (1995), however, reported that terrestrial fungi were not capable of macerating leaves of *Castanea sativa*. The reason for these differences may be related to the type of leaf used, since *Alnus glutinosa* (also used by Godfrey, 1983) is known for its fast decay rate, presumably because of its high nitrogen and low lignin content (Webster & Benfield, 1986). The initial lignin content seems to be an

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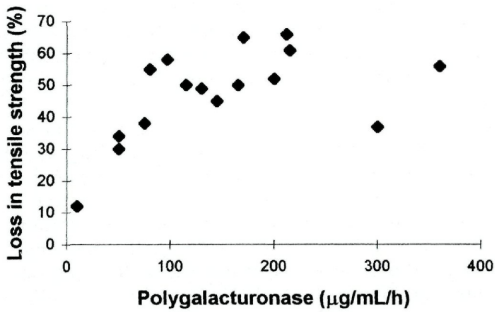
Tab. 1. – Production of lipase and phenol oxidase and growth of terrestrial and aquatic fungi on solid media (mean values ± SD; n = 3).

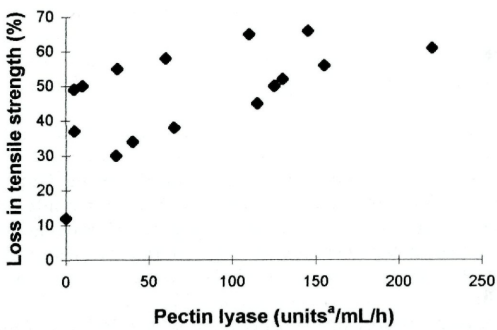
Fungi	Lipase		Phenoloxidase	
	colony diameter	activity zone	colony diameter	activity zone
Cylindrocladium scoparium	50±5	23±4	60±2	3±2
Alternaria alternata	35±2	13±3	48±3	0±0
Rhizoctonia solani	52±6	17±9	59±5	3±1
Cladosporium herbarum	30±6	23±11	45±17	8±2
Eupenicillium shearii	32±2	17±11	57±8	0±0
Aureobasidium pullulans	26±10	37±2	58±13	0±0
Rhizopus oryzae	80±0	0±0	70±10	0±0
Articulospora tetracladia	25±5	16±8	43±12	10±8
Lunulospora curvula	20±3	23±13	36±9	11±8
Mycocentrospora angulata	28±3	20±8	30±2	15±3
Tetrachaetum elegans	28±2	27±12	32±2	13±3
Tricladium angulatum	25±2	13±7	27±3	9±1
Tricladium gracile	23±3	23±8	32±2	8±2
Lemoniera terrestris	25±10	13±5	33±15	8±6
Lemoniera aquatica	24±4	14±5	45±18	10±7
Heliscus lugdunensis	52±7	13±3	52±13	0±0

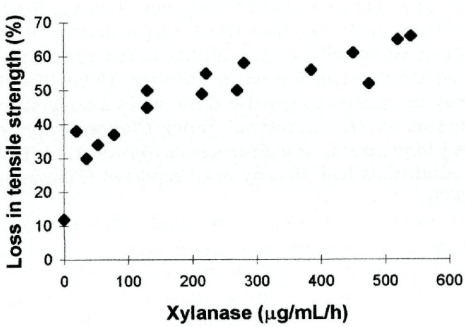
important factor controlling leaf maceration (Gessner & Chauvet, 1994). Phenol oxidase activity, a preliminar indicator of the ability to break down lignin, was lower in terrestrial than aquatic fungi. Due the qualitative nature of the test, however, it is difficult to present any further interpretation of the results. It seems, therefore, that the role of terrestrial fungi in aquatic systems depends not only on the fungi themselves, but also on the intrinsic characteristics of the leaves and probably on the physical environment (Chergui & Pattée, 1988).

Leaf softening was highly correlated with xylanase activity. Previously published results attribute the leading role in leaf softening to pectinases. Pectic polysaccharides are the major component of primary cell walls (about 34% w/w) and are the most immediately available polymers in non-lignified plant tissues. Their degradation exposes the other plant components (the hemicelluloses and celluloses, 24% and 19% respectively) to enzymatic attack (Chamier & Dixon, 1982b). The attack of pectolytic enzymes causes the release of

Fig. 4. – Correlation between xylanase (r_s = 0.943, $P<0.001$), pectin lyase ([a]- One unit of activity = a change of 0.001 absorbance unit at 550 nm; r_s = 0.673, $P<0.05$) and polygalacturonase (r_s = 0.607, $P<0.05$) activities in 16 fungal species and loss of tensile strength in alder leaves (as percentage of initial value).







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epidermal and parenchymal cells into the water column (Suberkropp & Klug, 1980). Jenkins & Suberkropp (1995) showed that pectin lyase was closely associated with the softening and maceration of leaf detritus, confirming that pectin degradation is a key process in the initial stages of leaf breakdown.

Here we tested pectic lyases and polygalacturonases, two enzymes involved in pectin degradation. According to Chamier & Dixon (1982b) pectin lyase and esterase have maximum activity at high pH (> 8.0) while polygalacturonases are most active at lower pH values (5.0–6.0). In our study all species tested differed in their polygalacturonase and pectin lyase activity. *R. oryzae* had no pectin lyase activity. Other studies confirmed the widespread production of this enzyme (*e.g.* Zemek & al., 1985; Suberkropp & al., 1983; Suberkropp & Arsuffi, 1984). Whereas Suberkropp & al. (1983) reported high activity for *Heliscus lugdunensis* and medium activity for *Lemonniera aquatica*, we observed the reverse. As in the cited work, the activity for *Lemonniera terrestris* was low. This same pattern was observed for polygalacturonase (Suberkropp & al., 1983). Very high polygalacturonase activities were observed in *A. pullulans* and *M. angulata* and the same was true for pectin lyase activity in *T. gracile*.

D-xylans are polysaccharides found in the hemicellulosic fraction of higher plant cell walls. Xylanase production in aquatic hyphomycetes was reported by Suberkropp & Klug (1980), Suberkropp & al. (1983) and Zemek & al. (1985). In our study all species were able to degrade xylan except *R. oryzae*. Moreover, the significant relationship between the activity of this enzyme and the decrease in the tensile strength of leaves suggests that this enzyme plays an important role in maceration of leaf tissue.

In contrast to previous publications where *Heliscus lugdunensis* was reported to have a very high xylanase activity (e.g. Suberkropp & al., 1983), this species ranked lowest here (except for *R. oryzae*). The low activity of *H. lugdunensis* towards pectin, cellulose and xylans was consistent with its low ability to macerate alder (present work) and chestnut leaves (Graça & Ferreira, 1995). The differences observed may be related to genetic differences among strains. Xylanase production in the terrestrial fungi *Cladosporium cladosporioides* (Fres.) (one strain) and *Epicoccum nigrum* Link (five strains) in aquatic conditions had already been reported (Flannigan, 1970 in Godfrey, 1983).

It is not clear why in other studies leaf softening was correlated with pectinase activity whereas in the present work a more significant correlation was observed with xylanase activity. pH could be an explanation. Pectin lyases are known to have an optimum at pH 8–9 (Chamier, 1985; Jenkins & Suberkropp, 1995). Polygalacturonases

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and xylanases, also involved in the digestion of the pectic compounds of the cell wall, have an optimum pH of 6 (Chamier, 1985; Jenkins & Suberkropp, 1995). Our tests were run at the intermediate pH value of 7. Litter maceration, on the other hand, is the result of a joint action of several enzymes and no single enzyme can account alone for the process.

Terrestrial and aquatic fungi differed in their ability to produce C_x and C_1 enzymes: This result suggests that aquatic hyphomycetes dominate cellulose degradation under submerged conditions. Colonisation of submerged leaves by terrestrial fungi may take place when leaves are still on the trees or freshly fallen to the ground, before being transported to the water by wind. In this case, only earlier colonisers are present and it is not surprising that they might be unable to use cellulose as an energy source. On the other hand, we recognise that the number of species / strains tested here was too low to make any generalised statements.

Cellulose decomposition by aquatic hyphomycetes has been widely investigated with various methods. Accordingly, the reported results are variable. Nevertheless, there are several lines of evidence suggesting that the ability to degrade cellulose is widespread in aquatic hyphomycetes (Suberkropp & Klug, 1980; Singh, 1982; Suberkropp & al., 1983; Zemek & al., 1985; Chandrashekar & Kaveriappa, 1988; Abdullah & Taj-Aldeen, 1989). All species tested in the present work produced the enzymatic complex C_x-C_1 which is consistent with the previous information. Suberkropp & al. (1983), Suberkropp & Arsuffi (1984) and Abdullah & Taj-Aldeen (1989) found high C_x activity for *Heliscus lugdunensis*. In our study, however, this species ranked last among aquatic hyphomycetes. For the terrestrial species, Godfrey (1983) and Park (1982 in Godfrey, 1983) reported cellulase activity in cultures of the terrestrial *C. cladosporioides* and *E. nigrum* isolated from aquatic habitats.

Fungi are able to degrade lignin and use it as a carbon source (Kirk & al., 1977 in Abdullah & Taj-Aldeen, 1989; Zemek & al., 1985; Gessner & Chauvet, 1994). The oxidation of tannic acid was considered an indicator of polyphenol oxidase activity, an enzyme presumably involved in the degradation of lignin (Davidson & al., 1938 in Abdullah & Taj-Aldeen, 1989). Among terrestrial fungi, only *C. herbarum, C. scoparium* and *R. solani* oxidised tannic acid, while of the aquatic hyphomycetes only *H. lugdunensis* was unable to do so. Zemek & al. (1985) noticed lignin degradation in all aquatic hyphomycetes tested including *H. lugdunensis*. Fisher & al. (1983) found that only 6 out of 16 species of aquatic hyphomycetes were able to produce phenol oxidising enzymes, but they used other substrates. Negative results were observed not only in *H. lugdunensis* but also in *Lemonniera aquatica* and *Articulospora tetracladia*.

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Lipolytic activity was detected in all tested species, but enzyme production was not correlated with mycelial growth, unlike the results obtained by Abdullah & Taj-Aldeen (1989) in *H. lugdunensis*, *L. aquatica* and *L. curvula*.

There was little agreement with published work on the enzymatic capabilities of some fungi. The reasons for differences in enzymatic capabilities in species such as *H. lugdunensis* are beyond the scope of this study. On the other hand, other studies have shown that this species was poorly equipped to macerate leaf tissue (e.g. Butler & Suberkropp, 1986).

It is plausible that strains of a widely distributed species differ genetically in different localities. This subject has been poorly investigated for aquatic hyphomycetes. What seems to be clear from this and related studies is that terrestrial fungi have a secondary role in leaf degradation in aquatic systems.

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Artikel/Article: Enzymatic analysis of leaf decomposition in freshwater by sected aquatic hypomycetes and terrestrial fungi. 160-173