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ORIGINAL PAPER

Control and possible applications of a novel carrot-spoilage basidiomycete, *Fibulorhizoctonia psychrophila*

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Abstract A novel cold-tolerant fungus, *Fibulorhizoctonia psychrophila*, was isolated from a refrigerated carrot storage facility and identified as an anamorph of *Athelia*, often classified in *Rhizoctonia* s.l. Growth of this fungus was observed between 0 and 20°C with an optimum at 9–12°C, while incubation of mycelium grown at 15–32°C resulted in absence of growth even after the fungus was transferred back to 15°C. Growth was inhibited in the presence of the antifungals sorbic acid or natamycin, in particular when the fungus was incubated at 18°C. *F. psychrophila* produces polysaccharide degrading enzymes that, when compared to enzymes from the ascomycete fungus *Aspergillus niger*, retain a larger proportion of their activity at lower temperatures. This indicates that *F. psychrophila* could be used as a source for novel industrial enzymes that are active at 4–15°C.

Keywords *Fibulorhizoctonia psychrophila* · Carrot-spoilage · Cold-active enzymes · Cold-tolerant fungus

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Introduction

Fungi occupy every natural environment on earth as well as man-made indoor environments. In fact, some fungi have ecological requirements and amplitudes that are ideally suited to some artificial (human made) environments. Some of these fungi create large problems with respect to human health (e.g. *Aspergillus*, *Candida*) or to food quality (e.g. *Penicillium*). Among standard methodologies to prevent food spoilage are the use of preservatives and refrigeration of food. The latter method, however, is not effective when cold-tolerant spoilage fungi are present. Cold-tolerant fungi are commonly found in nature and comprise species of different branches of the fungal kingdom, such as *Geomyces*, *Leucosporidium* (Panikov and Sivoza 2007), *Cystofilobasidium* and *Mrakia* (Nagakawa et al. 2004).

Cell walls form the majority of the plant biomass and consist mainly of polysaccharides. As fungi are not able to import polymeric compounds into their cells, they rely on extracellular enzyme systems for the degradation of polysaccharides into monosaccharides and short oligosaccharides. Hydrolytic enzymes acting on polysaccharides are commonly produced by fungi. These enzymes have many applications in the food and feed, paper and pulp, textile, and pharmaceutical industries and have therefore been the subject of many studies (de Vries and Visser 2001; de Vries 2003). Most of these studies and applications involve enzymes of saprophytic ascomycete

fungi (mainly species from *Aspergillus* and *Trichoderma*) that have optimal growth temperatures between 30 and 37°C. Consequently, most of these enzymes have optimum activities between 30 and 50°C and their activity rapidly decrease at lower temperatures (de Vries and Visser 2001). Efficient use of these enzymes in industrial applications is therefore often only possible by incubation above 30°C. This not only increases production costs due to the energy required to reach this temperature, but can also cause spoilage problems, especially with respect to food and feed applications.

One of the most commonly known cold-tolerant spoilage fungi is the basidiomycete *Rhizoctonia carotae* (Adams and Kropp 1996; Jensen 1969; Jones and Aldwinckle 1990; Punja 1987), which is found in storage facilities of several vegetables such as carrot. Although the optimum growth-temperature of this fungus is between 15 and 20°C (Punja 1987), this fungus causes crater rot characterized by sunken lesions and abundant mycelial growth on carrots stored at temperatures between 1 and 4°C. The anamorph genus *Fibulorhizoctonia* (Adams and Kropp 1996) (as *Fibularhizoctonia*) was proposed to accommodate *Rhizoctonia carotae* and *R. centrifuga* Lév (Rader 1948). The teleomorphs of these species belong to *Athelia* Pers., while those of the *Rhizoctonia solani*-complex belong to *Thanatephorus* Donk, currently placed in a different order (Hibbett et al. 2007). The teleomorph of *F. carotae* was identified as *Athelia arachnoidea* (Berk.) Jülich (Adams and Kropp 1996). However, there is some controversion considering the scope of this species, as the name has been used for litter decomposing specimens and lichen parasites. Arvidsson (1976) concluded that the use of the name *A. arachnoidea* should be confined to the lichen parasite, while the material of Adams and Kropp (1996) contained only litter decomposers.

Other basidiomycete species reported from cold storage are: *Corticium centrifugum* (Bielenin 1986; Stalpers and Loerakker 1984; Weresub and Illman 1980), from stored apples and pears, causing fisheye rot. Although the name *C. centrifugum* is connected with *Athelia*, the species concerned differs by having constant clamps at the septa, narrower hyphae and no sclerotia, and the production of a basidiome in culture, which is now known as *Butlerelfia eustacei* (Weresub and Illman 1980). This species is known from Europe and North America.

In this paper we describe a new carrot-spoilage fungus, *Fibulorhizoctonia psychrophila*, which is a predominant species detected in refrigerated storage facilities for carrots in The Netherlands. We have analysed the cold-tolerance of this fungus and its sensitivity to commonly used fungicides. In addition, we have analysed *F. psychrophila* for production of plant polysaccharide degrading enzymes to assess its potential as a source of cold-active enzymes.

Materials and methods

Strains and growth conditions

F. psychrophila CBS 109695 (IMI 395943) was isolated as a mycelial sample by H.A.B. Wösten from a wooden crate containing *Daucus carota* in a refrigerated storage facility (4°C) in Bant, The Netherlands in 2002. *Athelia arachnoidea* (Berkeley) Jülich CBS 418.72 was isolated from fallen leaf-litter of *Populus* sp. in The Netherlands. The type strain of *Fibulorhizoctonia carotae* (Rader) G.C. Adams & Kropp CBS 464.48 and *Aspergillus niger* N402 were described previously (Adams and Kropp 1996; Bos et al. 1988).

F. psychrophila CBS 109695 and *A. niger* N402 were routinely propagated on malt extract agar and minimal medium (de Vries et al. 2004), respectively. For growth of *F. psychrophila* on carrot, potato and onion, these vegetables were ground using a coffee grinder. The vegetable pulp was used at a concentration of 10% in 1.5% agar plates in water. Liquid cultures of *F. psychrophila* and *A. niger* were performed in *Schizophyllum commune* minimal medium (Dons et al. 1979) and *Aspergillus* minimal medium (de Vries et al. 2004), respectively, using 1% of a crude arabinoxylan preparation obtained from wheat after extraction of starch and proteins as the substrate. Plate cultures to determine the influence of several fungicides on growth of *F. psychrophila* were performed on malt extract agar plates. Comparison of the optimal growth temperature for *F. psychrophila*, *Athelia arachnoidea* and *F. carotae* was performed on malt extract agar and cherry decoction agar at temperatures of 0–27°C with intervals of 3°C.

Powdered wood was obtained by grinding wood shavings of a storage crate in a coffee grinder until a fine powder was obtained. Of this powder, 1 g was added to 1.5 g agar and 100 ml water and autoclaved

to prepare solid media. The crude arabinoxylan preparation was a gift from Latenstein (Nijmegen, The Netherlands) and is in fact a waste stream of a protein and starch extraction process from wheat.

Molecular biology methods

Genomic DNA of CBS 109695 was extracted using the FastDNA kit (Bio 101 Systems, Q-Biogene). The 5.8S gene and flanking ITS1 and ITS2 were amplified using the primers ITS1 and ITS4 (White et al. 1990) and the sequence was deposited at genbank (Acc. Nr. EF492880). The sequence was compared with ITS sequences from *F. carotae* (U85789) and *A. arachnoidea* (U85791).

Enzyme assays

F. psychrophila and *Aspergillus niger* were grown at 15 and 30°C, respectively in liquid medium containing a crude arabinoxylan preparation. Culture filtrate was harvested over a nylon filter after 2 days of growth and analysed for enzyme activities at 4, 15 and 30°C. Enzyme activity was determined using *p*-nitrophenyl-*β*-D-galactopyranoside, *p*-nitrophenyl-*β*-D-glucopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, and *p*-nitrophenyl-*β*-D-xylopyranoside (Sigma) as a substrate. A mixture was made consisting of 10 µl culture filtrate, 50 µl 50 mM sodium acetate (pH 5.0) and 30 µl sterile MiliQ water. The reactions were performed in triplicate and were started by the addition of 10 µl of 0.1% stock of the *p*-nitrophenyl-linked substrate and incubated for 2 h at 25°C, unless stated otherwise. The reactions were stopped by the addition of 100 µl 0.25 M Na_2CO_3 and measured at 405 nm in a micro plate reader (model 550, Bio-RAD). The amount of free *p*-nitrophenol was calculated using a calibration curve.

Results and discussion

Taxonomy

Fibulorhizoctonia psychrophila Stalpers & de Vries spec. nov. (Fig. 1).

Mycelium ad 9°C cotoneum, albidum, ad 18°C cremeum, avellaneum vel brunneum, velutinum vel crustosum. Liquor exsudatus brunneus. Sclerotia presentia vel absentia, irregularia, in statu maturitate subfusca a liquore exsiccata. Hyphae hyalinae vel subfuscae, (2.5–)3–6 µm. Septa fibulatae vel afibulatae. Cellae sclerotiorum doliformes, tenui-tunicatae vel crasse-tunicatae, hyalinae vel fuscae. Fungus psychrophilus, ad temperaturas −3° ad 21°C crescens.

Typus: CBS 109695

Colonies at −3 to 9°C are cottony, rather high and reaching the lid of the Petri dish, white. Margin raised, rather dense. At increasing temperatures the mycelium grows less high and becomes nearly velvety at 18°C and nearly crustose at 21°C. At 12° the centre of the colony is cream-coloured, at 15°C becoming Light Cinnamon Drab to Avellaneous (Munsell 7.5YR6/2, 10YR6/2, 10 YR6/3). At these temperatures exudate droplets are not present. At 18°C the colony is low, nearly velvety, Ochraceous Tawny (7.5YR5/8) caused by exudate drops and at 21°C there is hardly any growth; the mycelium is crustose and Cinnamon Brown to Prouts Brown (5YR4/3, 5YR4/4), and finally Mummy Brown. Sclerotia are generally not produced within 6 weeks, but have occurred in a tube; they start as white semiglobose pustules, generally up to 1 mm diameter, aggregated, often fusing, and producing a brown exudate, which dries in, leaving a dark brown sclerotium, up to 5 mm diam, finally Mummy Brown.

Marginal hyphae at −3 to 3°C are irregular, rather long-celled, with swellings and granular contents, thin-walled, (2.5–)3–6 µm wide, swellings up to 12 µm wide. Clamps present, not abundant. Branching at various angles, sometimes a constriction is present at the base of the side branch. At 6°C and above the hyphae are regular, thin- to firm-walled, 2.5–6(–7.5) µm wide, contents hyaline to pale brownish, with abundant clamps, especially at the wider hyphae, but the narrower hyphae (2.5–3.4–4 µm) generally have clampless septa. Cell wall hyaline, but irregular brownish encrustations present at some hyphae, probably dried exudate.

Aerial mycelium regular, thin- to firm-walled, (2.5–)3.2–6.5 µm wide, with hyaline to brownish contents. Most septa with clamps; clamps regular, but sometimes of the medallion type.

Sclerotia rarely produced, uniform, not forming a distinct cortex, consisting of thin- to thick-walled hyphae, usually consisting of slightly elongated swollen cells with granular contents, 4–10 µm wide, not unlike the (thin-walled) advancing hyphae at lower temperatures.

Fig. 1 Colony morphology of *Fibulorhizoctonia carotae* (464.84), *Athelia arachnoidea* (418.72) and *Fibulorhizoctonia psychrophila* (109659) after 2 weeks. (**a**) 0°C; (**b**) 6°C; (**c**) 18°C

Cardinal temperatures: minimum below −3°C, optimum range between 9 and 12°C, maximum 20°C.
Holotype: CBS 109695 (IMI 395943), also preserved dried in herb. CBS.
Mycobank number: MB501325.

The sterile, sclerotium producing mycelia are traditionally classified in the artificial genus *Rhizoctonia*, which is currently restricted to basidiomycetous fungi and comprises anamorphs of various, not closely related genera such as *Tulasnella*, the *Ceratobasidium–Thanatephorus* complex and *Athelia* (Stalpers and Anderson 1996). These groups have been elevated to the genus level as *Epulorhiza*, *Rhizoctonia* s.str. and *Fibulorhizoctonia*. Although teleomorphs have not been observed in *F. carotae* and *F. psychrophila*, morphological, physiological and molecular characters indicate that *F. psychrophila*, *F. carotae* and *Athelia arachnoidea* are closely related and belong to *Athelia*, a corticioid genus, currently classified in the Atheliales (Rader 1948).

F. carotae has been described from cold stored carrots. It differs from *F. psychrophila* in growing between 18 and 27°C (Fig. 2), abundant production of sclerotia, and colour of the mat above 15°C. *A. arachnoidea* has been described from both lichens and leaf litter. It differs from *F. psychrophila* in its growth at higher temperatures (Fig. 2), less abundant aerial mycelium, and pale colour at higher temperatures. ITS sequencing of *F. psychrophila* revealed 95% identity to the ITS sequence of *A. arachnoidea* and *A. carotae* (data not shown), indicating that *F. psychrophila* is very closely related to these two species.

Both morphological and molecular characters indicate without doubt, that *F. psychrophila*, *F. carotae* and *Athelia arachnoidea* are closely related.

Temperature-tolerance and growth of *F. psychrophila*

F. psychrophila was inoculated on malt extract agar plates and incubated between 0 and 27°C. Growth was monitored by measuring the colony diameter. Optimal growth occurred at 9–12°C (Fig. 2) and

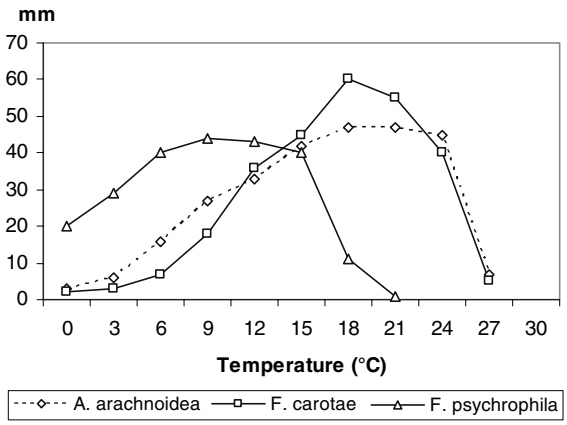

Fig. 2 Growth comparison at different temperatures of *Fibulorhizoctonia psychrophila*, *Fibulorhizoctonia carotae* and *Athelia arachnoidea*. The means of duplicate values are represented

growth was absent above 20°C. When plates incubated for 3 days at 20 and 25°C were placed back at 15°C the fungus re-initiated growth, but incubation for 3 days at 32°C prevented growth of *F. psychrophila* after the plates were transferred to 15°C (data not shown). Optimal growth of *F. carotae* and *A. arachnoidea* was at between 18 and 21°C (Fig. 2). The strong decrease in growth of *F. psychrophila* when the incubation temperature is raised above 15°C, suggests an even stronger adaptation of this fungus to cold biotopes.

Growth of *F. psychrophila* was tested on three vegetable crops that are commonly stored under refrigerated conditions (carrot, potato and onion) in comparison to agar plates without carbon source. Water agar alone already permits growth of *F. psychrophila*, but growth is significantly improved in the presence of carrot (data not shown). The presence of potato only resulted in a small increase in growth compared to water agar, but onion reduced growth of *F. psychrophila*. These data demonstrate a preference of *F. psychrophila* for carrot as a substrate. The reduced growth on onion is likely caused by the high levels of phenolic compounds in onions.

Inhibition of growth of *F. psychrophila* using antifungals

Sorbic acid and natamycin are compounds commonly used for the inhibition of growth of spoilage fungi and the effectiveness of these compounds was tested on *F. psychrophila* on malt- and water agar plates with powdered wood. *F. psychrophila* is believed to survive on the crates used for the storage of carrots and therefore causes repeated spoilage when these crates are re-used. The presence of 250 mM sorbic acid resulted in a significant reduction in growth on malt extract agar, but not on powdered wood at 4°C (Fig. 3a). However, higher levels of sorbic acid (625

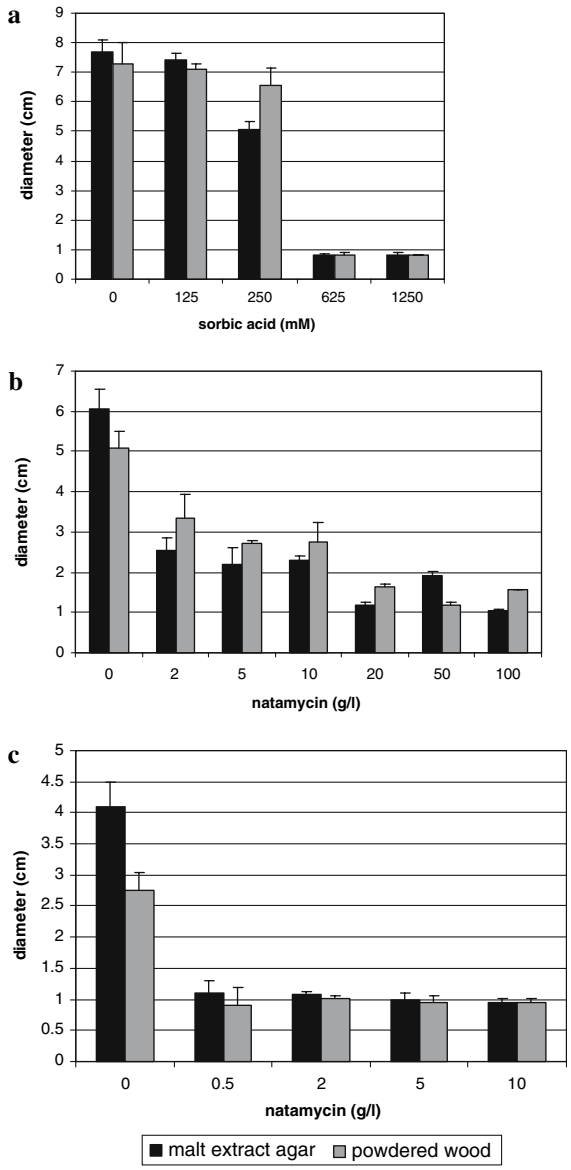

Fig. 3 Influence of antifungals on the growth of *Fibulorhizoctonia psychrophila* grown on malt extract agar (MA) or powdered wood from storages crates (HW). (**a**) sorbic acid, 18°C; (**b**) natamycin, 4°C; (**c**) natamycin, 18°C

and 1,250 mM) prevented growth of *F. psychrophila* on both substrates. In light of the high concentrations of sorbic acid required to inhibit growth, the expected effectiveness of this compound in preventing spoilage by *F. psychrophila* is limited.

Natamycin was more effective in preventing growth of *F. psychrophila*, resulting in a significant decrease in growth at 2 g/l at 4°C. Increasing levels of natamycin reduced growth of the fungus at 4°C similarly on both malt extract agar and powdered wood, although a significant difference was observed at 50 and 100 g/l (Fig. 3b). However, at 100 g/l natamycin, some residual growth could still be observed for powdered wood. A stronger inhibition with natamycin was observed when the incubations occurred at 18°C instead of 4°C, resulting in nearly complete inhibition of growth on both media at 0.5 g/l natamycin (Fig. 3c). The results described here indicate that spraying wooden crates with natamycin and incubating them at 18°C or higher likely reduces spoilage *F. psychrophila*.

F. psychrophila produces cold-active hydrolytic enzymes during growth on polysaccharides

Both carrots and powdered wood consist largely of polysaccharides. As these are the main carbon sources for *F. psychrophila* during growth in the storage facilities, it can be expected that the fungus produces polysaccharide degrading enzymes that are active at low temperatures. To study whether these enzymes have in fact a higher relative activity at low temperatures than those currently used in industrial applications, we compared enzyme activities from *F. psychrophila* to those from *Aspergillus niger*. *A. niger* is one of the most commonly used fungi for the production of industrial enzyme preparations and produces a wide range of polysaccharide degrading enzymes (de Vries and Visser 2001). However, these enzymes are mainly active at higher temperatures.

For *A. niger*, enzyme activities dropped to 30–50% when incubation at 15°C was compared to 30°C (Fig. 4a). At 4°C, only 10–20% of the 30°C-activity was observed. For *F. psychrophila* only a 5–35% drop was detected when comparing 30°C to 15°C and 30–60% of the activity at 30°C was still observed at 4°C (Fig. 4b). The strongest difference was observed

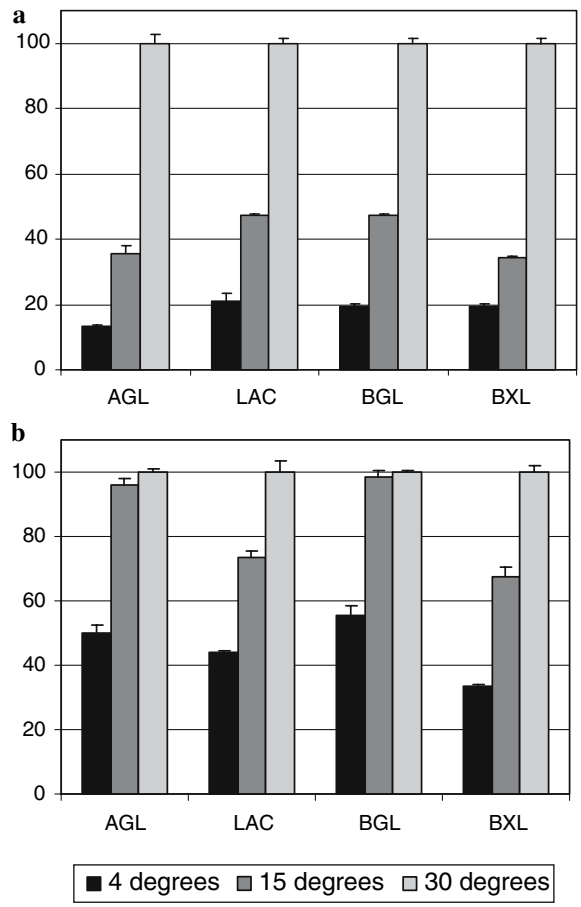

Fig. 4 Relative hydrolase activities in culture filtrate of *Fibulorhizoctonia psychrophila* and *Aspergillus* at different temperatures. (**a**) *A. niger* culture filtrate; (**b**) *F. psychrophila* culture filtrate. AGL = α-galactosidase, LAC = β-galactosidase, BGL = β-glucosidase, BXL = β-xylosidase. Activity at 30° is set at 100%

for α-galactosidase and β-glucosidase, where more than 90% of the activity was still detected at 15°C for *F. psychrophila*, while for *A. niger* only 35–45% of the activity was detected at this temperature.

F. psychrophila and other cold-tolerant fungi are potential sources for enzymes with high activity at low temperatures. This idea is supported by studies with cold-tolerant yeast and fungi, which were shown to produce cold-active pectinases (Nagakawa et al. 2004, 2005a, b), although in these studies a direct comparison with industrially used enzymes was not made. Cold-active enzymes can be important for many applications, for example in detergents or for the removal of lactose from milk for lactose-intolerant people.

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