Antibacterial Activity of Wild Mushroom Extracts on Bacterial Wilt Pathogen *Ralstonia solanacearum*

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Abstract

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In total, 150 protein extracts from 94 different basidiomycete and ascomycete wild mushroom species were tested for antibacterial activity against the quarantine plant-pathogen bacterium *Ralstonia solanacearum*. In in vitro microtiter plate assays, 15 extracts with moderate to high antibacterial activities were identified: 11 completely inhibited bacterial growth and 4 showed partial inhibition. Of these 15 extracts, 5 were further tested and 3 extracts slowed disease progression and reduced disease severity in artificially inoculated tomato and potato plants. However, the in vitro activities of the extracts did not always

correlate with their in vivo activities, which emphasizes the importance of performing early screening tests also in vivo. Testing of selected extracts against 12 *R. solanacearum* strains identified 6 with potential for broader applicability. Further analysis of extracts from *Amanita phalloides* and *Clitocybe geotropa* showed that the active substances are proteins with an approximate size of 180 kDa. To our knowledge, this is the first in vitro and in vivo study that demonstrates that mushroom protein extracts can be promising for treatment of bacterial wilt caused by *R. solanacearum*.

Bacterial diseases continue to pose threats to human, animal, and plant health, a situation that is exacerbated by increased global travel and trade and by climate change. Increased incidence of antibiotic-resistant bacteria, lack of development of new antibiotics (Payne et al. 2007), and limitations imposed on the use of phytopharmaceuticals in agriculture necessitates alternative methods of disease control to complement conventional therapies.

The quarantine plant-pathogen bacterium *Ralstonia solanacearum* (Smith, 1896) Yabuuchi et al. 1996 is a species complex that comprises four phylotype groups (I to IV) (Fegan and Prior 2005b) that can infect a wide range of host plants, including potato, tomato, eggplant, tobacco, banana, pelargonium, and ginger. Overall, *R. solanacearum* causes approximately \$950 million of annual losses worldwide (Elphinstone 2005).

Although there have been extensive studies on *R. solanacearum* that have included its biological control (Anith et al. 2004; Hong et al. 2011; Ji et al. 2005; Maji and Chakraborty 2014; Messiha et al. 2007) and its importance in agronomy, at present there is no efficient chemical or biological agent available for its control. Disease management is based on the use of pathogen-free plant material, planting under pest-free conditions of production, and early detection and removal of diseased plants to prevent the disease from spreading (Fegan and Prior 2005a). In the search for natural agents to control bacterial wilt, promising results have been obtained with biocontrol bacteria and fungi. The oomycete *Pythium oligandrum* (Hase et al. 2008), bacteria from the genera *Acinetobacter* and *Enterobacter*

(Xue et al. 2009), *Bacillus amyloliquefaciens* (Tan et al. 2013; Wei et al. 2011), and other bacteria from the tomato rhizosphere (Aliye et al. 2008) have been shown to reduce bacterial wilt disease incidence of tomato and potato plants, while *B. amyloliquefaciens* also promotes plant growth (Tan et al. 2013; Wei et al. 2011). However, field trials performed across different seasons have shown that the *R. solanacearum* and potential biocontrol agent populations vary between seasons, and that the efficacy of any biocontrol agent is highly dependent upon environmental conditions (Pal et al. 2006; Sundin et al. 2009). Other antibacterial substances, including essential oils, have been tested as soil biofumigants for the control of bacterial wilt; for example, thymol has been shown to reduce disease severity and has the potential for use as a preplanting treatment of the soil (Ji et al. 2005; Pradhanang et al. 2003). Moreover, a cocktail of palmarosa, lemongrass, and eucalyptus essential oils also reduced bacterial wilt of ginger planted in essential-oil-pretreated potting medium (Paret et al. 2010).

In contrast to more thoroughly investigated sources of active substances with antibacterial properties, such as microorganisms, filamentous fungi, and plants, the higher fungi have been relatively underexplored and might represent a valuable source for substances with various activities, including antibacterials (Alves et al. 2012a; Erjavec et al. 2012). Higher fungi (i.e., Basidiomycetes and Ascomycetes) form compact tissue structures with fruiting bodies (also known as mushrooms) that contain compounds that have been shown to have antibacterial, antifungal, antiviral, antiparasitic, antitumor, immunomodulatory, insecticidal, and nematotoxic activities (Lindequist et al. 2005; Ng 2004). Fungal proteins have been investigated due to their often unique characteristics, their thermal and pH stabilities, and their improved efficacies. Several proteins have been isolated from mushrooms that have unique characteristics in terms of their stabilities and function-related properties. Such proteins can be, and have been, used in a variety of applications across medicine, biotechnology, and agriculture (Erjavec et al. 2012).

To date, only a few fungal proteins have been tested in the field of agricultural crop protection. As an example, tamavidin is a biotin-binding protein that can be expressed in transgenic rice and, as such,

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it provides resistance to the important rice-pathogenic fungus *Magnaporthe oryzae* (Takakura et al. 2012). Lectins and proteinase inhibitors of fungal origin have been shown to have insecticidal, bactericidal, and fungicidal properties in genetically modified plants. Of these, the mycocybins, which are cysteine protease inhibitors unique to the basidiomycete mushrooms, have detrimental effects against Colorado potato beetle larvae (Šmid et al. 2013, 2015). Similarly, lectins with specific galactose-binding or lactose-binding activities from *Clitocybe nebularis* have insecticidal and nematocidal activities that can be used in the field of plant protection (Pohleven et al. 2011, 2012). The only example of mushroom proteins that are active against both bacterial and fungal pathogens is from the study of Zheng et al. (2010), who isolated an antibacterial protein from dried fruiting bodies of the mushroom *C. sinopica* that is active in vitro against *Rhizobium rhizogenes*, *R. radiobacter*, *R. vitis*, *Xanthomonas oryzae*, and *X. malvacearum*. However, they did not perform any in vivo tests.

The aim of the present study was to explore mushrooms as a source of antibacterial proteins against *Ralstonia solanacearum*. In total, 150 mushroom protein extracts from 94 different species were tested in vitro. Of these, selected extracts were further evaluated in vivo, with an examination of their influence on disease severity and bacterial concentrations in plants, with the latter determined using real-time polymerase chain reaction (PCR). Moreover, selected extracts were tested for potential broad-spectrum inhibition of 12 *R. solanacearum* strains that belong to different phylotypes, and of *R. mannitolilytica* and *Escherichia coli*.

Materials and Methods

Bacterial cultures and inoculum preparation. The National Collection of Plant-Pathogenic Bacteria strain 4156 *R. solanacearum* (Smith, 1896) Yabuuchi et al. 1996 (phylotype IIB, race 3, biovar 2) (Wullings et al. 1998) was isolated from potato in 2001 in The Netherlands, and was used as the study reference isolate for the primary in vitro screening. The inhibitory specificities of the extracts were tested using further *R. solanacearum* strains that belong to different phylotypes, and also *R. mannitolilytica* (CFBP 6737) (De Baere et al. 2001) and *E. coli* (GSPB 48). The bacteria were grown at 28°C on yeast peptone glucose agar (YPGA) plates (5 g of yeast extract, 5 g of proteose peptone, 10 g of glucose, and 12 g of agar per liter; pH 7.2 to 7.4). Bacterial suspensions were prepared in 0.01 M phosphate-buffered saline (PBS) (1.071 g of Na₂HPO₄, 0.4 g of NaH₂PO₄ · 7H₂O, and 8.0 g of NaCl per liter; pH 7.2). The bacterial concentrations were determined according to absorbance at 595 nm (A₅₉₅), and they were dilution plated on YPGA (CFU/ml).

Protein extract preparation from sporocarps. All of the mushroom materials (as species from the phyla Basidiomycota and Ascomycota) were collected from mycelia growing in forest stands or grasslands in central and western Slovenia. The fungi sporocarps analyzed in this study originated from forested areas that were not under any protection regime, and none of the fungi species analyzed were RED listed (Official Gazette 42/2011). Individual collections originated from areas near the town of Grosuplje (N 45° 57' 18.45", E 14° 40' 22.32"), Rajndol (N 45° 34' 8.26", E 14° 57' 6.44"), and Sežana (N 45° 41' 4.12", E 13° 52' 41.55"). The sporocarp identification followed the morphological species concept, with identification using general field guides (Bon 2011; Courtecuisse and Duhem 1995) or specialized identification keys for particular groups and genera (Breitenbach and Kränzlin 2000). These guides were based on their macromorphological and micromorphological characteristics (Harrington and Rizzo 1999), as assessed under a microscope (Zeiss Axio Imager.Z2 microscope, ×100 to ×1,000 magnification; Carl Zeiss Microscopy GmbH).

The fungi collected were cleaned of soil debris and potential rotten or otherwise infected parts (insects, other fungi), and frozen at –20 or –80°C. Of 150 mushrooms, 141 were identified to the species level and 9 to the genus level. Crude mushroom extracts were prepared by homogenizing the thawed fruiting bodies. The insoluble material was removed by centrifugation at 16,000 × g for 5 min at 4°C. The proteins were extracted from these supernatants using acetone

precipitation, by adding four volumes of cold (–20°C) acetone and leaving the samples on ice for 40 min. Following centrifugation at 10,000 × g for 20 min at 4°C, the pellets were air dried at 4°C, resuspended in PBS buffer, and stored at –20°C until testing (Rosenberg 2005).

The extracts were tested as prepared from the mushrooms (i.e., they were not calibrated to any particular concentration before testing).

Fungal culture and protein extract preparation from mycelia.

A protein extract from mycelia of the *C. geotropa* mushroom was included in the study because of the high proteolytic activity that it showed in our previous study (Sabotič et al. 2007). Here, *C. geotropa* mycelia were isolated from fresh fruiting bodies collected in nature. The mycelia were cultured in liquid sucrose malt yeast extract medium (10 g of sucrose, 10 g of malt extract, and 4 g of yeast extract per liter). Flasks with 200 ml of liquid sucrose malt yeast extract medium were inoculated with the mycelia and incubated for 6 weeks at room temperature in the dark and without shaking. The mycelia were collected and used for protein extraction.

The mycelium protein extracts were prepared by homogenization in liquid nitrogen using a mortar and pestle, and they were then stored at –20°C. Protein pellets were dissolved in 0.05 M Tris-HCl with 0.1 M NaCl (pH 7.4), and centrifuged at 16,000 × g for 5 min at 4°C to remove any remaining insoluble material. The supernatants were filter sterilized through syringe-driven 0.20-μm filters (Millex-LG; Merck Millipore) and frozen at –20°C for short-term storage or –80°C for long-term storage. The extracts were tested as prepared (i.e., they were not calibrated to any particular concentration before testing).

A specimen of the mycelia is stored in the collection of ectomycorrhizal fungi at the Slovenian Forestry Institute Mycotheca and Herbarium (as part of the University of Ljubljana Herbarium) under voucher number EMG 169 (SFI-LJU-CLIGEO 201007).

In vitro screening. For the initial screening for the activities of the mushroom protein extracts against *R. solanacearum* NIB Z 30 (NCPPB 4156), the minimum inhibitory concentration method (European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) 2003) was adapted. Liquid YPG medium (YPGA without agar) and 24-h incubations at 28°C in 96-well microtiter plates allowed good bacterial growth, and the measurement of A₅₉₅ enabled clear distinction between inhibited and noninhibited bacterial growth.

Altogether, 149 mushroom extracts and 1 mycelium extract from 94 different species were tested. The extracts and bacterial suspensions were mixed at a 1:10 ratio for all of the samples for both the in vitro and in vivo experiments. The 200-μl testing wells contained YPG (75 μl), the suspension of *R. solanacearum* (10⁷ cells/ml, 75 μl), 0.01 M PBS (42.5 μl), and the mushroom extract (7.5 μl). The following controls were included for each plate: positive controls (PC; no mushroom extract added), negative controls (no extract and *R. solanacearum* added), and controls of extract sterility (no *R. solanacearum* added). All controls were supplemented to 200 μl with 0.01 M PBS. Each sample (mushroom extract) and control was tested in at least two parallel wells. Two technical replicates were used in the in vitro screening, and triplicates were used in the determination of the specificity of inhibition. Microtiter plates were incubated in a thermo shaker (PST-60HL-4; Biosan) at 400 rpm for 24 h at 28°C. Bacterial growth was monitored spectrophotometrically (A₅₉₅; Tecan Genios, Magellan, v. 6.2 software) with at least four measurements over the 24-h time period. After 24 h, 30 μl of the mixture from each well was plated onto fresh YPGA plates to evaluate whether the effects were bactericidal (i.e., no bacterial growth observed when transferred onto fresh medium) or bacteriostatic (i.e., bacterial growth observed when transferred onto fresh medium).

The *R. solanacearum* inhibition was determined as percentages of the PC growth (A₅₉₅; % PC). Extracts with absorbance (A₅₉₅) within the limits of variation of the PC (3σ rule) were considered not to inhibit the bacteria. Two groups of extracts with growth between 15% PC and 60% PC (strong inhibition) and between 61% PC and 84%

Table 1. Mushroom protein extracts tested against *Ralstonia solanacearum* in the in vitro microtiter plate screening^a

Fungal family and species (Ni/Nt) ^b	Edible ^c	Inhibition of growth ^d
Basidiomycota		
Agaricaceae		
<i>Agaricus campestris</i>	Yes	No
<i>A. moelleri</i>	No	Yes*
<i>A. silvaticus</i>	Yes	No
<i>Coprinus comatus</i> (2)	Yes	No
<i>Lepiota ignivolva</i>	No	No
<i>Leucoagaricus leucothites</i> (2)	Yes	No
<i>Macrolepiota</i> sp.	Yes/no	No
Amanitaceae		
<i>Amanita caesarea</i>	Yes	No
<i>A. lividopallescens</i> (2)	Yes?	No
<i>A. muscaria</i>	No	Yes
<i>A. phalloides</i> (7/10), #T	No	Yes
<i>A. vaginata</i> (2)	Yes?	No
<i>A. virosa</i>	No	Yes
Cortinariaceae		
<i>Cortinarius caeruleus</i>	Yes?	No
<i>C. glaucopus</i>	Yes	No
<i>C. sanguineus</i>	No	No
<i>Cortinarius</i> sp.	Yes/no	No
<i>Hebeloma crustuliniforme</i>	No	No
Entolomataceae		
<i>Entoloma rhodopolium</i>	No	No
Fistulinaceae		
<i>Fistulina hepatica</i>	Yes	No
Hygrophoraceae		
<i>Hygrophorus chrysodon</i>	Yes	No
<i>H. pudorinus</i> (2)	Yes	No
<i>H. eburneus</i>	Yes	No
<i>H. fagi</i>	Yes	No
<i>H. erubescens</i>	No	No
<i>Hygrocybe ovina</i>	No	No
Lycoperdaceae		
<i>Bovista nigrescens</i> , #T	Yes?	Yes*
<i>Lycoperdon perlatum</i>	Yes?	No
<i>L. pyriforme</i>	Yes?	No
Physalacriaceae		
<i>Armillaria mellea</i> (2)	Yes?	No
Pluteaceae		
<i>Volvariella bombycina</i>	Yes	No
<i>Volvariella gloiocephala</i>	No	No
Strophariaceae		
<i>Hypholoma fasciculare</i>	No	No
<i>Hypholoma</i> sp.	Yes/no	Yes
Tricholomataceae		
<i>Clitocybe alexandri</i>	Yes	No
<i>C. geotropa</i> (9/12), #TP	Yes	Yes
<i>C. geotropa</i> mycellum extract (2)	Yes?	Yes
<i>C. gibba</i>	Yes	No
<i>C. nebularis</i> (1/2)	Yes	Yes*
<i>C. odora</i>	Yes	No
<i>Collybia confluens</i>	Yes	No
<i>Laccaria amethystine</i>	Yes	No

(continued in next column)

^a Classification follows the Index Fungorum database of fungal names (<http://www.indexfungorum.org/>); edibility follows identification keys of Rogers mushrooms (<http://www.rogersmushrooms.com/>).

^b Nt = number of extracts tested, Ni/Nt = number of extracts showing inhibition as designated in the table/number of extracts tested, #T = extract tested for inhibition of *R. solanacearum* in tomato pathogenicity test, and #TP = extract tested for inhibition of *R. solanacearum* in tomato and potato pathogenicity tests.

^c Inhibition of *Ralstonia solanacearum* NIB Z30 growth: yes = complete inhibition, yes* = partial inhibition, and no = no inhibition.

^d Edibility: yes = edible and no cooking prior to consumption, yes/no = genus with edible and nonedible or poisonous species, yes? = some species only edible as young fruiting bodies or after cooking or cutting off certain parts of the fruiting body, no = nonedible or poisonous, and na = edibility data not available.

Table 1. (continued from preceding column)

Fungal family and species (Ni/Nt) ^b	Edible ^c	Inhibition of growth ^d
<i>Lepista glaucocana</i> (2)	Yes?	No
<i>L. inversa</i>	Yes	No
<i>L. nuda</i>	Yes?	No
<i>Mycena galeae</i>	No	No
<i>Tricholoma atrosquamosum</i>	Yes	No
<i>T. bufonium</i> (3)	No	No
<i>T. imbricatum</i> (2)	Yes?	No
<i>T. mutabile</i>	Na	No
<i>T. pardinum</i> (2)	No	No
<i>T. saponaceum</i> (8/9), #TP	Yes?	Yes
<i>T. sejunctum</i>	Yes?	No
<i>Tricholoma</i> sp.	Yes/no	No
<i>T. terreum</i>	Yes	No
<i>T. ustale</i>	No	Yes*
<i>T. vaccinum</i> (2)	Yes?	No
<i>Tricholomopsis rutilans</i> (3)	Yes?	No
Boletaceae		
<i>Boletus badius</i> (2)	Yes	No
<i>B. calopus</i>	No	No
<i>B. luridiformis</i>	Yes?	Yes
<i>Leccinum scabrum</i>	Yes	No
<i>Xerocomus chrysenteron</i>	Yes	No
Gomphidiaceae		
<i>Gomphidius glutinosus</i>	Yes	Yes
Suillaceae		
<i>Suillus bovinus</i> (6)	Yes	No
<i>S. granulatus</i> (3)	Yes	No
<i>S. luteus</i> (3)	Yes	No
<i>Suillus</i> sp. (5)	Yes	No
<i>S. variegatus</i> , #TP	Yes	Yes*
Tapinellaceae		
<i>Tapinella atrotomentosa</i>	Yes?	No
Cantharellaceae		
<i>Cantharellus</i> sp.	Yes?	No
<i>Craterellus cornucopioides</i>	Yes	No
Hydnaceae		
<i>Hydnum repandum</i>	Yes	No
Gastraceae		
<i>Gastrum rufescens</i>	No	No
Gomphaceae		
<i>Clavariadelphus</i> sp.	Yes/no	No
<i>Ramaria botrytis</i>	Yes	No
<i>R. flava</i>	Yes	No
<i>R. formosa</i>	No	No
Phallaceae		
<i>Phallus</i> sp.	Yes?	No
Albatrellaceae		
<i>Albatrellus ovinus</i>	Yes	Yes
<i>A. pes-caprae</i>	Yes	No
Russulaceae		
<i>Lactarius blennius</i>	No	No
<i>L. citriolens</i>	No	No
<i>L. deliciosus</i>	Yes	No
<i>L. illyricus</i>	No	No
<i>Lactarius</i> sp.	Yes/no	No
<i>Russula albonigra</i>	No	No
Sparassidaceae		
<i>Sparassis crispa</i>	Yes	No
Thelephoraceae		
<i>Sarcodon imbricatus</i>	Yes?	No
Ascomycota		
Morchellaceae		
<i>Morchella esculenta</i>	Yes?	No
Tuberaceae		
<i>Tuber aestivum</i>	Yes	No
<i>T. excavatum</i>	Yes	No
<i>T. fulgens</i>	Yes	No
<i>T. magnatum</i>	Yes	No
<i>T. mesentericum</i>	Yes	No

PC (weak inhibition) were considered to be partially inhibitory. Absorbance (A_{595}) within the range of negative control variation (3σ rule) was regarded as complete inhibition.

Selected mushroom protein extracts that showed complete, strong, and weak inhibition against *R. solanacearum* NIB Z30 were tested against 11 further strains of *R. solanacearum* that represented its different phylogenotypes and biovars. *R. mannitolilytica* isolated from contaminated autoclave fluids was also included, because it is genetically close to *R. solanacearum* (Coenye et al. 2003), while *E. coli* was chosen as an unrelated gram-negative bacterium and a potential production organism for the mushroom recombinant proteins. In the present study, we had limited amounts of *Bovista nigrescens* and *Suillus variegatus* fruiting bodies and, therefore, we were not able to include *B. nigrescens* and *S. variegatus* extracts in all of the experiments. This is also one of the major limitations of preparing extracts from wild mushroom species.

Tomato and potato pathogenicity test. Tomato plants (*Solanum lycopersicon* ‘MoneyMaker’) were potted in soil substrate and grown in a quarantined greenhouse at a constant temperature of 21 °C and a 16-h photoperiod at 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon irradiance (L36W/77 lamp; Osram). The plants were inoculated at the two- to three-true-leaf stage with mixtures of the *R. solanacearum* suspensions and mushroom extracts (10:1 ratio). The bacteria and extracts were mixed together and immediately inoculated into the plants. The bacterial concentration in the inoculation suspension was 10^5 CFU/ml, which was confirmed in preliminary experiments to be the lowest *R. solanacearum* concentration that reproducibly led to typical symptoms for all of the inoculated plants tested (Supplementary Fig. S1). The *R. solanacearum* suspension and 0.01 M PBS were used for the inoculation of the positive and negative control plants, respectively. Using a sterile needle (Icogamma plus, 0.6 by 25 mm; Novico), each suspension was inoculated between the cotyledons, as follows: the syringe plunger was pushed in until a drop of the sample appeared at the tip of the needle, the plant stem was pierced so that the needle pierced through the drop into the stem and out on the other side, another drop was pushed out at the tip of the needle, and the needle pulled back out (Janse 1988). Approximately 20 μl of each suspension was used per plant.

In total, 40 plants were inoculated with each extract, 42 plants with the PC, and 20 plants with the negative controls. After the inoculations,

the plants were grown at a 28 °C day temperature, with a 16-h photoperiod at 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon irradiance, and at a 20 °C night temperature. The severity of the symptoms was evaluated regularly over 14 days, following the numerical grades of Winstead and Kelman (1952), where 0 = no symptoms, 1 = one leaf wilted, 2 = two to three leaves wilted, 3 = all leaves except the tip of the plant wilted, 4 = all leaves and the tip of the plant wilted, and 5 = plant dead.

For the pathogenicity testing on potato plants, 4-week-old tissue cultures of micropropagated potato plants *S. tuberosum* ‘ Désirée’ were rooted in soil substrate. After 2 weeks of incubation at a 21 °C day temperature, with 16-h photoperiod at 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon irradiance, and a 20 °C night temperature, the plants were injected with the bacterial suspensions with the mushroom extracts (10:1 ratio), 1 cm above the soil substrate. The inoculated plants were incubated at 25 °C day and night temperature, with a 16-h photoperiod at 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon irradiance. For each mixture of bacteria and mushroom extract, 42 plants were inoculated, with 42 PC plants (only bacteria, no mushroom extract), and 20 negative control plants (only 0.01 M PBS). The severity of the symptoms was evaluated following the numerical grades of Winstead and Kelman (1952) (see above).

To compare symptom severity and disease progression in plants inoculated with mixtures of *R. solanacearum* and mushroom extracts with the PC plants, χ^2 tests were used. The area under the disease progress curve (AUDPC) was used as a measure of quantitative disease resistance, as calculated for the pathogenicity tests (Madden et al. 2006) using the R-statistical (R Development Core Team 2008) Agricolae package (De Mendiburu 2013). For easier comparison of the AUDPC between samples, linear regression was used for the *C. geotropa* tomato pathogenicity tests, to obtain values at 14 days postinoculation (dpi) instead of 15 dpi (Supplementary Fig. S3). Overall influence of time and treatment with extracts on the disease severity was estimated with repeated ordinary linear regression using repolr R package (Parsons et al. 2009).

Quantification of *R. solanacearum* in tomato plants using real-time PCR. Correlation of bacterial wilt symptom severity and *R. solanacearum* concentrations was examined in the stem nodes of artificially inoculated tomato plants with and without mushroom extracts using real-time PCR. On average, five plants were analyzed for each symptom level within each treatment group.

Tomato stem tissue was sampled at the first and second nodes above the inoculation point by cutting 5-mm-long stem sections in half and vortexing each with 500 μl of 0.01 M PBS. After 10 min of incubation at room temperature, 400 μl of the suspension was transferred to a fresh microcentrifuge tube and stored at –20 °C until testing. The *R. solanacearum* was quantified by real-time PCR (primers RS-I-F and RS-II-R; probe RS-P) (Weller et al. 2000) using absolute quantification through comparison with a standard curve. The standard curve of *R. solanacearum* (bacterial suspension in 0.01 M PBS), using final concentrations from 1 to 10^8 CFU/ml were included in each run (Supplementary Fig. S2). DNA extraction was used because the DNA is efficiently released from the cells during the DNA polymerase activation step (10-min step at 95 °C), as demonstrated by Dreo et al. (2014); the inhibition effect was excluded by analyzing both undiluted and 1:10 diluted samples. The real-time PCR reactions were performed on an ABI PRISM 7900 HT Sequence Detection system (Applied Biosystems), using the following universal cycling conditions: 2 min at 50 °C, 10 min at 95 °C (polymerase activation step), followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The reaction volumes of 10 μl contained, as final concentrations, 900 nM primers (Eurofins MWG Operon), 200 nM probe (Eurofins MWG Operon), 1 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems), and 2 μl of sample DNA. The real-time PCR results are expressed as quantification cycle values: the real-time PCR cycle at which the fluorescence exceeded the threshold value. Previous experiments have shown that the variation between technical replicates is minimal; therefore, 0 $\times$ in 10 $\times$ dilution were analyzed in one reaction each. The data were collected with the SDS 2.2.3 software, and exported to and analyzed with Microsoft Excel and R statistical (R Development Core Team 2008).

Fig. 1. Different levels of *Ralstonia solanacearum* NIB Z30 growth inhibition in the presence of representative mushroom protein extracts in microtiter plate assays. Growth of *R. solanacearum* was measured spectrophotometrically at 595 nm (A_{595}) over 24 h. Examples show no inhibition (A_{595} within the variation of the positive control), partial inhibition (a delayed exponential growth phase or slower growth of bacteria compared with *R. solanacearum* NIB Z30 positive control), and complete inhibition (A_{595} within the variation of the negative control).

Fig. 2. Influence of selected mushroom extracts on the bacterial wilt disease progression in artificially inoculated potted MoneyMaker tomato plants. Plants were stem inoculated with *Ralstonia solanacearum* (positive control, 10^5 CFU/ml) or a mixture of *R. solanacearum* and mushroom extracts. For each time point, symptoms are expressed as percentage of plants, with symptoms graded according to Winstead and Kelman (1952) represented by different colors, where no symptoms = white; one leaf wilted = light gray, stripes; two to three leaves wilted = light gray; all leaves except tip of the plant wilted = medium gray; all leaves and tip of the plant wilted = medium gray, dots; and dead plant = dark gray. Symptoms were recorded up to 14 days postinoculation. Within groups, the time points at which the distribution of symptoms differs from the corresponding distribution in the positive control group, are marked by asterisks above columns (χ^2 test; * and ** indicate $P < 0.05$ and 0.01 , respectively). Plants in the negative control group did not show any symptoms.

Size-exclusion chromatography and temperature stability of mushroom extracts. The antibacterial fractions were obtained from *Amanita phalloides* and *C. geotropa* extracts (both active in vitro) by size-exclusion chromatography using Sephacryl S-200 equilibrated in 0.02 M Tris-HCl, pH 7.5, with 0.3 M NaCl. The protein concentrations in the individual fractions were determined by measuring absorbance A_{280} . Inhibitory properties of the fractions against *R. solanacearum* were analyzed in vitro as described above, and expressed relative to the PC (% PC).

Selected extracts that inhibited *R. solanacearum* in the in vitro testing were exposed to the high temperatures to confirm that the active

substance is a protein. Extracts and fractions were heated for 10 min at 40, 60, 80, and 95°C, cooled on ice, and tested in vitro against *R. solanacearum*, as described above.

Results

In vitro screening of mushroom protein extracts. Mushroom fruiting bodies were collected in Slovenian forests over several seasons and identified to the species level (Table 1). Altogether, 150 mushroom extracts (including one mycelium extract) from 94 different species that represented 27 families of higher fungi were tested (Table 1). Of these, 15 samples (14 mushroom extracts and 1

Fig. 3. Influence of selected mushroom extracts on the bacterial wilt disease progression in artificially inoculated potted Désirée potato plants. Plants were stem inoculated with mixture of *Ralstonia solanacearum* and mushroom extract or *R. solanacearum* only (positive control, 10^6 CFU/ml). For each time point, symptoms are expressed as percentage of plants, with symptoms graded according to Winstead and Kelman (1952) represented by different colors, where no symptoms = white; one leaf wilted = light gray, stripes; two to three leaves wilted = light gray; all leaves except tip of the plant wilted = medium gray, dots; all leaves and tip of the plant wilted = dark gray; and dead plant = dark gray. Symptoms were recorded up to 14 days postinoculation. Within groups, the time points at which the distribution of symptoms differs from the corresponding distribution in the positive control group, are marked by asterisk above columns (χ^2 test; * and ** indicate $P < 0.05$ and 0.01 , respectively). Plants in the negative control group did not show any symptoms.

C. geotropa mycelium extract) inhibited *R. solanacearum* in vitro (Table 1). These extracts showed different levels of inhibition against *R. solanacearum* NIB Z 30 compared with the PC, as complete inhibition (*Albatrellus ovinus*, *Amanita phalloides*, *A. muscaria*, *A. virosa*, *Boletus luridiformis*, *C. geotropa*, *C. geotropa* mycelium extract, *Gomphidius glutinosus*, *Tricholoma saponaceum*, and *Hypholoma* sp.) and partial inhibition (*Agaricus moelleri*, *Bovista nigrescens*, *Suillus variegatus*, and *Tricholoma ustale*). Levels of inhibition of representative extracts in comparison with a representative extract with no inhibition (*C. nebularis*) are shown in Figure 1.

For 20 mushroom species, the activities of more than one extract of the same species were analyzed. Of note, these extracts from different mushrooms of the same species inhibited *R. solanacearum* to different extents: (i) eight *T. saponaceum* extracts showed complete

inhibition, while one showed partial inhibition; (ii) nine *C. geotropa* extracts showed complete inhibition, while three showed partial inhibition; (iii) seven *Amanita phalloides* extracts showed complete inhibition, while three showed partial inhibition; and (iv) one *C. nebularis* extract showed partial inhibition, while one showed no inhibition (Table 1). This appears to be due to high variation in the composition and content of protein and other substances in these mushrooms, which will depend on the growth substrate, weather conditions (e.g., water availability and temperature), and age of the fruiting bodies (Yildiz et al. 2002).

Pathogenicity tests on tomato and potato plants. Tomato plants were used as a model of *R. solanacearum* bacterial wilt disease and were chosen for the in vivo testing of the activities of the mushroom protein extracts. Potato plants were also included as a model because,

Table 2. Comparison of in vitro and in vivo activities of the mushroom protein extracts

Fungal species extract	In vitro tests ^b		Pathogenicity tests ^a			
	Level of inhibition	Type of activity	Moneymaker tomato		Désirée potato	
			Antibacterial activity	AUDPC (% PC)	Antibacterial activity	AUDPC (% PC)
<i>Amanita phalloides</i>	+++	C	No	114	nt	nt
<i>Bovista nigrescens</i>	+	S	No	94	nt	nt
<i>Tricholoma saponaceum</i>	+++	C	Yes	80	Yes	63
<i>Suillus variegatus</i>	++	S	Yes	68	Yes	75
<i>Clitocybe geotropa</i>	+++	C	Yes	76	Yes	48

^a AUDPC = area under the disease progress curve, quantitative summary of disease intensity over time, calculated for tomato and potato plants infected with mixtures of *Ralstonia solanacearum* NIB Z 30 and protein mushroom extracts. Symptom severity evaluated 14 days postinoculation. AUDPC expressed relative to tomato or potato positive control (% PC); nt = not tested.

^b Symbols: +++ = complete inhibition of bacteria: no growth observed ($\leq 15\%$ positive control, absorbance at 595 nm [A_{595}]), ++ = partial (strong) inhibition of bacteria: growth significantly slower compared with positive control (15 to 60% positive control A_{595}), + = partial (weak) inhibition of bacteria: growth significantly slower compared with positive control (60 to 84% positive control A_{595}), S = bacteristatic effect of the extract = bacterial growth observed when transferred onto fresh medium, C = bactericidal effect = no bacterial growth observed when transferred onto fresh medium, and nt = not tested.

Fig. 4. Correlation of bacterial wilt symptom severity and *Ralstonia solanacearum* concentrations in the stem nodes of artificially inoculated tomato plants with and without mushroom extracts. Plants were stem inoculated with a mixture of *R. solanacearum* and mushroom extract or *R. solanacearum* only (positive control, 10^5 CFU/ml). Numerical grades of the Winstead and Kelman scale were used for symptom severity evaluation (Winstead and Kelman 1952). Bacterial concentrations (logarithmic scale, CFU/ml, represented by diamonds) were determined by direct real-time polymerase chain reaction in the **A**, first and **B**, second node above the inoculation point. Except for the *Clitocybe geotropa* and *R. solanacearum* coinoculated plants, for which no plants with symptom severity grade 5 were analyzed, five plants, on average, were analyzed for each group and symptom severity level.

economically, potato represents the most important host of *R. solanacearum* in the European area.

Five mushroom extracts that were active in the in vitro microplate screening were selected for pathogenicity tests on Moneymaker tomato (Table 1): (i) the *A. phalloides*, *C. geotropa*, and *T. saponaceum* extracts, which completely inhibited *R. solanacearum* in vitro; and (ii) the *B. nigrescens* and *S. variegatus* extracts, which showed partial inhibition in the in vitro microplate screening. Although, in some cases, more than one extract of the same species was tested in the in vitro testing, here we selected the one that completely inhibited *R. solanacearum* or showed the strongest inhibition.

Of the extracts tested, *S. variegatus* and *C. geotropa* had the strongest effects, which were seen as delayed symptom development in the tomato and potato plants for *S. variegatus* and *C. geotropa*, respectively (Figs. 2 and 3). These observations were also supported by the AUDPC analysis (Table 2). The symptom severity for the tomato plants coinoculated with the *S. variegatus* extract (which showed only partial inhibition in in vitro tests), and *R. solanacearum* was significantly lower compared with those observed in PC plants at 4, 5, 7, and 8 dpi ($P < 0.01$). Although the number of plants coinoculated with the *T. saponaceum* extract and *R. solanacearum* that expressed symptoms at 15 dpi (95 and 100%, respectively) were not different from the PC group, the symptom severities were significantly lower at 4 and 7 dpi for *T. saponaceum* ($P < 0.05$). This was also seen as a lower AUDPC of 80%. The *C. geotropa* extract that completely inhibited growth of *R. solanacearum* in vitro had less effect in tomato plants, with an overall AUDPC of 76%. Instead, in potato plants, it significantly delayed symptom development from 5 to 14 dpi ($P < 0.01$; AUDPC of 48%). Coinoculation of the *A. phalloides* and *B. nigrescens* extracts with *R. solanacearum* in vivo did not confirm the inhibitory effects against *R. solanacearum* seen in the in vitro testing. Similar results were obtained also after repeating the experiment for *A. phalloides* and *C. geotropa* extracts on tomato plants, with the overall AUDPC of 98% for *A. phalloides* and 75% for *C. geotropa*. Repeated ordinary linear regression analysis suggests that there is an overall influence of time and individual extracts on the disease severity. Parameter »treatment:dpi« estimates with positive signs are associated with lower probability of increased symptom severity in plants treated with *C. geotropa*, *S. variegatus*, and *T. saponaceum* extracts. Parameter estimates with negative signs are associated with higher probability of increased symptom severity in plants treated with *A. phalloides* and *B. nigrescens* (Supplementary File S1).

The symptoms of the potato plants inoculated with *T. saponaceum* and *R. solanacearum* also appeared at 4 dpi; however, only 3% of the

potato plants showed wilting (Fig. 3). At 5 dpi, there was already a significant difference in a number of wilted plants compared with the PC ($P < 0.05$). Symptoms for the potato plants coinoculated with the *C. geotropa* and *S. variegatus* extracts and *R. solanacearum* appeared at 5 dpi, with 15 and 24% of the plants showing symptoms, respectively; this was significantly lower compared with the 57% wilted PC plants ($P < 0.01$ and $P < 0.05$). At 14 dpi, 92% of the PC potato plants showed symptoms, compared with 63, 67, and 44% for the plants inoculated with the *T. saponaceum*, *S. variegatus*, and *C. geotropa* extracts, respectively, and *R. solanacearum*. These data also corresponded to AUDPC of 63, 75, and 48% of the PC, respectively (Table 2). In general, disease progress was slower in the potato plants than in the tomato plants, which could contribute to the apparently different reactions of the tomato and potato plants to infection in the presence of some of the extracts (e.g., those of *C. geotropa* and *S. variegatus*).

Concentrations of *R. solanacearum* in tomato plants. In natural infections, the *R. solanacearum* concentrations correlate with symptom severity (Elphinstone 2005). Using real-time PCR, the *R. solanacearum* concentrations were determined in plants with different symptom severities in the presence of the mushroom extracts and *R. solanacearum* (Fig. 4). Compared with the PC plants, more variations in the *R. solanacearum* concentrations were observed in the plants inoculated with the mushroom extracts and *R. solanacearum*. *R. solanacearum* was detected in all of the experimental groups at 3 dpi, and very high concentrations ($>10^6$ CFU/ml) were reached before symptoms were observed.

At the time of sampling, the bacterial concentrations were at similar high levels in the PC plants inoculated with *R. solanacearum* only, regardless of the symptom severity and the sampled node. Plants that were coinoculated with the *S. variegatus* extract that significantly delayed symptom expression and *R. solanacearum* had similar concentrations of *R. solanacearum* in both nodes, as did the PC plants. Of the plants that were coinoculated with the extracts that showed few effects in the in vivo testing along with *R. solanacearum*, in comparison with the PC plants, the data indicate that *A. phalloides* had lower *R. solanacearum* concentrations in the second node, *T. saponaceum* had lower *R. solanacearum* concentrations in both nodes, and *S. variegatus* had similar *R. solanacearum* concentrations in both nodes.

Despite having little effect on disease progression, the plants that were coinoculated with the *A. phalloides* extract and *R. solanacearum* had similar concentrations of *R. solanacearum* in the first node above the inoculation point and lower concentrations in the second node, as observed in the PC group.

These data indicate that, in the presence of mushroom extracts, the symptom severity might not always correlate with bacterial concentrations;

Table 3. Specificity of inhibition against different *Ralstonia solanacearum* phylotypes and other bacteria in vitro

Strain (NIB Z)	Host (origin)	Collection ^b	Phylotype	Biovar	Level of inhibition according to mushroom protein extract ^a							
					<i>Tricholoma saponaceum</i>		<i>Amanita phalloides</i>		<i>A. virosa</i>		<i>Boletus luridiformis</i>	
<i>Ralstonia solanacearum</i>												
1625	<i>Lycopersicon esculentum</i> (Zimbabwe)	LMG 2296 (RA 1.64)	III	1	+++	C	+++	C	+++	C	+++	C
1693	<i>Solanum tuberosum</i> (Brazil)	CFBP 4613/NCCPB 3989/CIP 226/SMT 45	II	2T	+++	C	+++	C	+++	C	+++	C
1759	<i>Heliconia caribaea</i> (Martinique, France)	CFBP 6783/ANT 25/RUN 0017	IIB	1	+++	C	+++	C	+++	C	+++	C
1691	<i>Zingiber</i> (Philippines)	CFBP 4616/NCCPB 4005/SMT 49	I	4	+++	C	+++	C	+++	S	+++	S
30	<i>Solanum tuberosum</i> (The Netherlands)	NCCPB 4156	IIB	2	+++	C	+++	C	+++	S	+++	C
1779	<i>Musa</i> sp. plantain (Venezuela)	CFBP 6438/UW181/MLG28/RUN0454	IIA	3	+++	C	+++	C	+++	S	+++	C
1727	<i>Solanum nigrum</i> (Cameroon)	CFBP 7038/CMR 66/RUN 0166	III/23	/	+++	C	+++	S	+++	S	+++	C
1734	<i>Solanum tuberosum</i> (Brazil)	CFBP 6446/NCCPB 3987/CIP217/JT677/RUN 0081	IIB	N2	+++	C	+++	C	+++	S	+++	C
1760	<i>Solanum tuberosum</i> (Martinique, France)	CFBP 3257/CFBP 2972/RUN 0030/JS734/MPT1/	IIA	1	+++	C	+++	C	+++	S	+++	C
1626	<i>Solanum tuberosum</i> (Fiji)	LMG 17144 (RA 1.72)	I	3	+++	C	+++	C	+++	S	+++	S
1732	<i>Musa paradisica</i> L. 'Saba' (Philippines)	CFBP 6925/MOLK 2/RUN 0074	IIB/3	/	+++	C	+++	C	+++	C	+++	C
1629	<i>Lycopersicon esculentum</i> (Peru)	CFBP 3928 (RA 3.20)/NCCPB 3996	I	3	+++	C	+++	C	+++	C	+++	C
<i>R. mannitolilytica</i>												
1631	Contaminated autoclave fluids (United States)	CFBP 6737/LMG 6866 (F 5.35)	...	...	+++	C	+++	C	+++	C	+++	C
<i>Escherichia coli</i>												
703	...	GSPB 48	...	...	+++	C	+++	S	–	S	–	S
(continued on next page)												

(continued on next page)

^a Symbols: +++ = complete inhibition of bacteria: no growth observed ($\leq 15\%$ positive control, absorbance at 595 nm [A_{595}]), ++ = partial (strong) inhibition of bacteria: growth significantly slower compared with positive control (15 to 60% positive control A_{595}), + = partial (weak) inhibition of bacteria: growth significantly slower compared with positive control (60 to 84% positive control A_{595}), - = no inhibition ($\geq 84\%$ positive control A_{595} value), S = bacteriostatic effect of the extract = bacterial growth observed when transferred onto fresh medium, C = bactericidal effect = no bacterial growth observed when transferred onto fresh medium, nt = not tested, and na = not applicable.

^b Bacterial collections: NCCPB = National Collection of Plant-Pathogen Bacteria, The Food and Environment Agency, York, UK; CFBP = Collection Francaise des Bacteries Phytopathogenes, Institut National de la Recherche Agronomique, Beaucauzé Cedex, France; BCCM/LMG = Belgian Co-Ordinated Collections of Micro-Organisms, Laboratorium voor Microbiologie, Universiteit Gent (UGent); IVIA = Instituto Valenciano de Investigaciones Agrarias, Spain; and GSPB = Göttinger Collection of Phytopathogenic Bacteria, Göttingen, Germany.

however, this should be further examined by assays that determine not only the presence but also the viability of the *R. solanacearum* cells.

Specificity of inhibition against different *R. solanacearum* phylogenotypes and other bacteria. To determine the potential for a broader application of the mushroom extracts identified as inhibitory during the screening in the present study (Table 1), these extracts were tested against different strains of *R. solanacearum* and two other bacteria, *R. mannitolilytica* and *E. coli* (Table 3). The *R. solanacearum* strains were selected to represent different phylogenotypes and biovars, and were isolated from different host plants in different geographical regions. Because we had limited amounts of *S. variegatus* fruiting bodies, the *S. variegatus* extract was not included in all of the experiments. This is also one of the major limitations in the preparing of extracts from wild mushroom species.

Of 10 tested extracts, two (*A. phalloides* and *T. saponaceum*) inhibited all of the bacteria tested, including *R. mannitolilytica* and *E. coli*. In addition to these two extracts, those of *A. virosa*, *Boletus luridiformis*, *C. geotropa*, and *G. glutinosus* completely inhibited all of the *Ralstonia* strains, including *R. mannitolilytica*, but did not inhibit *E. coli*. More variability in the patterns of inhibition was seen with the *Agaricus moelleri*, *T. ustale*, and *Albatrellus ovinus* extracts, which only partially inhibited *R. solanacearum* NIB Z 30 during screening. Although the *Agaricus moelleri* extract only partially inhibited most of the *R. solanacearum* strains (with the exception of NIB Z 1625), it completely inhibited *E. coli*.

Of the 10 mushroom extracts that were identified in the screening, inhibitory activity against *R. solanacearum* NIB Z 30 was confirmed for 9 extracts. The activity was not confirmed for the *C. nebularis* extract, which was one of the two extracts identified as partially inhibitory in the screening. This correlated with the kinetics of *R. solanacearum* growth in the presence of the two extracts: *C. nebularis* showed weaker effects that were only noticeable in the final few hours of the measurements, as opposed to the *T. ustale* extract, the effects of which were observed throughout the logarithmic growth phase. Possible reasons for this discrepancy include the resolution of the initial screening, and the stability or concentration of the active substances in the extracts, which might lead to stochastic effects in testing. Interestingly, despite the discrepancy of the result against *R. solanacearum* NIB Z 30, the *C. nebularis* extract showed inhibition against *R. solanacearum* NIB Z 1691 that was isolated from ginger and *R. solanacearum* NIB Z 1625 from tomato.

There were no correlations observed between the levels of inhibitory activity and the classification into phylogenotypes, which indicated

that, for the tested mushroom extracts, the sensitivities of the different strains were not associated with the characteristics that defined the mushroom phylogenotypes.

Size-exclusion chromatography and temperature stability of mushroom extracts. As a further step in the identification of the antibacterial substances observed in mushroom extracts, the *Amanita phalloides* and *C. geotropa* extracts were fractionated by size-exclusion chromatography and their activities against *R. solanacearum* were tested in the microtiter assay. For both extracts, 4 consecutive fractions (approximate size of 180 kDa) of 30 collected fractions inhibited *R. solanacearum* in vitro (Fig. 5). Protein was detected spectrophotometrically (A_{280}) in all of the active fractions; however, as expected, protein was also detected in fractions that did not inhibit *R. solanacearum*.

To further test the nature of the active substances, the thermal stabilities of the extracts and selected fractions were determined. The extracts of *A. phalloides* and *C. geotropa* were stable at up to 60°C but completely lost their antibacterial activity after treatment at 80 and 95°C. Their active fractions were stable at up to 60 and 40°C, respectively.

For the other tested extracts, those of *A. virosa*, *G. glutinosus*, and *T. saponaceum* were no longer active over 60°C, while the extract of *B. erythropus* was not active over 40°C.

Discussion

At present, there are no effective biological or chemical agents available for the control or eradication of *R. solanacearum*. The present study is the first to investigate protein extracts from a large number of wild-grown mushrooms for their inhibitory effects against *R. solanacearum* under in vitro conditions, which were then followed up by in vivo examination of these inhibitory extracts in planta. In addition, the specificities of the inhibition of these mushroom protein extracts were tested against other selected isolates of *R. solanacearum*, to determine the potential for their broader applicability.

The screening of the mushroom protein extracts for their antibacterial activities against *R. solanacearum* in the in vitro microplate assay identified 15 extracts with inhibitory activity out of 94 different species. The active extracts were not limited to specific genera or species; however, several active extracts belonged to the families Amanitaceae and Tricholomataceae, which include species that are known to have low and high molecular weight inhibitory molecules (Antonyuk et al. 2010; Guo et al. 2005; Pohleven et al. 2012; Vetter 1998; Yoshikawa 2002; Zheng et al. 2010).

Table 3. (continued from preceding page)

Level of inhibition according to mushroom protein extract ^a											
<i>Clitocybe geotropa</i>		<i>Gomphidius glutinosus</i>		<i>Agaricus moelleri</i>		<i>T. ustale</i>		<i>Albatrellus ovinus</i>		<i>C. nebularis</i>	
+++	C	+++	C	+++	S	+++	S	+++	S	+	S
+++	C	+++	C	++	S	++	S	+++	S	–	na
+++	C	+++	C	++	S	++	S	+++	S	–	na
+++	C	+++	C	++	S	++	S	++	S	++	S
+++	C	+++	C	+	S	+++	S	+++	S	–	na
+++	C	+++	C	+	S	+	S	+	S	–	na
+++	C	+++	C	+	S	+	S	++	S	–	na
+++	C	+++	C	+	S	+	S	–	na	–	na
+++	C	+++	C	++	nt	–	nt	–	nt	nt	nt
+++	C	+++	S	+	S	–	na	++	S	–	na
+++	C	+++	C	+	S	–	na	++	S	–	na
+++	C	+++	C	++	S	++	S	++	S	–	na
+++	C	+++	C	+	S	+	S	–	na	–	na
–	S	–	S	+++	S	–	na	–	na	nt	nt

The data from this temperature stability testing shows that the mushroom extracts are stable and retain their antibacterial activities over a wide temperature range, confirming observations of the unique properties of mushroom proteins in previous studies (Erjavec et al. 2012). Together with the estimated molecular mass after size-exclusion chromatography, the present data indicate that these antibacterial substances in the mushroom extracts are, indeed, proteins.

To the best of our knowledge, to date, only one study has tested mushroom proteins against plant-pathogen bacteria and fungi in vitro (Zheng et al. 2010). Other studies have mostly involved crude extracts (Alves et al. 2012a), culture filtrates (Chen and Huang 2010; Pandey 2013), or alcohol extracts (Alves et al. 2012b; Schwan et al. 2010). Although crude extracts contain a mixture of all of the molecules that are in the fruiting bodies, culture filtrates or alcohol extracts are abundant in smaller molecules that are mostly secondary metabolites.

In the present study, the prevalence of inhibitory activity against *R. solanacearum* (16% of 150 extracts) is in the range seen for the studies of Chen and Huang (2010; 11% of 27 culture filtrates) and Pandey et al. (2013; 13%, 70 culture filtrates and sporocarp extracts). This is higher than the inhibition prevalence observed in the screening of wild mushrooms against *Acinetobacter baumannii* and *Burkholderia cepacia* (Schwan et al. 2010; 0.9% of 330 crude extracts). There have been other reports on antibacterial screening of mushroom extracts but they have mostly involved smaller numbers of extracts that were tested against one or several pathogens (Alves et al. 2012a,b; Pandey 2013; Wasser 2011). Based on the high percentage of protein extracts that inhibited the gram-negative *R. solanacearum* in the present study, we can conclude that these mushroom extracts do, indeed, appear to be good sources of antibacterial proteins.

Our pathogenicity testing in vivo complemented the in vitro testing by providing further data on the efficacy of the extracts tested in the more natural environment of the xylem tissues of host plants. The in vitro inhibitory effects of the *T. saponaceum*, *S. variegatus*, and *C. geotropa* extracts that completely or partially inhibited *R. solanacearum* were confirmed in vivo. These effects are quite remarkable considering the invasive inoculation method, the presumed dilution of the extracts in the xylem, and the movement of *R. solanacearum*, which can potentially enable them to escape the inhibitory molecules found in the extracts. Also, the inoculation of the mixtures of the extracts and *R. solanacearum* into the plant stems immediately introduces high bacterial concentrations into the plant vascular system, which will give little time for the plant defense responses to act (Walters 2009). Interestingly, the *S. variegatus* extract did not completely inhibit *R. solanacearum* in vitro but significantly slowed disease progression in vivo. This suggests that, with *S. variegatus*, apart from a direct effect on *R. solanacearum*, other mechanisms have a role here. This could include induction of the plant defenses; however, further investigations need to be carried out to confirm this hypothesis, which should include an analysis of the expression levels

of the defense-related genes of the plants. The *T. saponaceum* and *C. geotropa* extracts, however, completely inhibited *R. solanacearum* in vitro and had bactericidal effects that indicate a more direct effect on the bacterial cells. However, an agent with bactericidal activities might not be appropriate for the control of diseases; therefore, further studies should be carried out that take into consideration dose effects and environmental factors which can significantly influence the efficacy of an antibacterial agent (Glare et al. 2012; Lemessa and Zeller 2007).

On the other hand, bactericidal effects in vitro do not always mean that a protein or protein extract will be as efficient in vivo, as seen for the *Amanita phalloides* and *Bovista nigrescens* extracts in tomato plants. This suggests that their mechanisms of action might not be compatible with the experimental set-up of the pathogenicity testing; or, indeed, that their effects are limited to the controlled contact between the extract and bacteria. Despite this, the *A. phalloides* extract or its individual components might still be useful for applications such as surface disinfection.

Discrepancies between in vitro and in vivo efficacies have also been reported before (Lemessa and Zeller 2007). Our data suggest that it is appropriate to perform early screening tests on plants (i.e., in vivo) to observe inhibitory effects. In addition, such an approach might identify growth-promoting effect on plants. We conclude here that all of the extracts that show at least some inhibitory activity in vitro should be taken into consideration as potential plant protection agents.

The quantification of *R. solanacearum* in tomato leaf nodes at different times during disease development indicated that, in the presence of mushroom extracts, the severity of the symptoms might not always correlate with the bacterial concentrations. Although further studies are needed to confirm this, the observation is in line with the hypothesis that the mechanisms of action of the different extracts are not limited solely to direct effects on the bacteria. In particular, this can be seen in the mushroom extract that showed the strongest inhibition in vivo (i.e., the *S. variegatus* extract).

Disease progress was slower in the plants that were coinoculated with the *T. saponaceum*, *S. variegatus*, and *C. geotropa* extracts and *R. solanacearum*, although the concentration of the bacteria detected in the tomato stem was very high, which indicated some degree of pathogen tolerance by these plants. This is also important, because enhancing plant defenses is often more desirable compared with direct effects on a pathogen (Glare et al. 2012). Natural products that use such a system, such as biopesticides (e.g., Regalia) that are active against several soilborne pathogens (i.e., *Rhizoctonia solani*) are already on the market (i.e., Marrone BioInnovations). However, to evaluate the potential of such mushroom extracts or proteins for use as biocontrol agents, field experiments still need to follow. Moreover, the successful industrial production of wild mushrooms, the harvesting of their mycelia, and the extraction of their proteins under standardized conditions is a precondition for the final product.

Fig. 5. Antibacterial activity and protein concentrations of *Amanita phalloides* and *Clitocybe geotropa* fractions obtained by size-exclusion chromatography. Antibacterial activity of the fractions is expressed as percent growth of *Ralstonia solanacearum* in the presence of the fraction relative to the positive control (*R. solanacearum* only), both measured spectrophotometrically (absorbance at 595 nm [A_{595}]) after 24 h incubation. Protein concentrations in the individual fractions were determined spectrophotometrically (A_{280}).

Some of the *Ralstonia solanacearum* strains were susceptible to more mushroom extracts than others; however, no correlations were apparent according to phylotype or biovar classification, host plant, or geographic origin. A broad-spectrum antibacterial agent that covers all of the *R. solanacearum* phylotypes would be of great advantage. However, from the present data for the specificity testing, we can conclude that it would be difficult to predict the efficacy of any identified mushroom extracts against other strains that were not included in the present study. On the other hand, in previous studies, the antibacterial substances from mushrooms were only tested against one strain of *R. solanacearum* (Chen and Huang 2010; Deberdt et al. 2012; Ji et al. 2005; Pandey 2013; Paret et al. 2010; Pradhanang et al. 2003). This, in turn, underlines the importance of including several strains of the same bacterial species in studies of antibacterial efficacies.

As expected, as a phylogenetically close species to *R. solanacearum*, *R. mannitolilytica* was also susceptible to 8 of the 10 mushroom extracts tested here. This is also important from the perspective that *R. mannitolilytica* is an opportunistic pathogen that has caused hospital epidemics, mainly through contamination of medical equipment (Block et al. 2013; De Baere et al. 2001; Gröbner et al. 2007; Jung et al. 2007). Therefore, mushroom proteins that inhibit *R. mannitolilytica* might also find application in medicine or for coating or disinfection of hard surfaces.

In the present study, we successfully cultured *C. geotropa* mycelia on solid and in liquid media, and we have shown that the mycelium extracts completely inhibited all of the *R. solanacearum* strains tested, which indicates that the same or a similar active protein that is present in the extract can be produced in the mycelium culture. This is of particular importance for the production of the active substances in greater quantities and, hence, for industrial applications (Erjavec et al. 2012; Lindequist et al. 2005).

Therefore, this combination of different approaches to determine inhibitory activities of mushroom protein extracts has provided new evidence that mushrooms are, indeed, a good natural source of antibacterial proteins.

Conclusions

We have shown here that mushrooms are a good source of antibacterial proteins that are active against the plant pathogen *R. solanacearum*. In the present study, 16% of these protein extracts from a wide variety of mushroom species were active in the *in vitro* tests. These not only showed potent inhibition of bacterial growth but, more commonly, they also had bactericidal effects, thus killing the bacterial cells (rather than being bacteriostatic). The majority of the extracts tested for their pathogenicity *in vivo* were active, which resulted in slower disease progression and reduced disease severity in artificially inoculated tomato and potato plants. However, the *in vitro* activities did not always correlate with the *in vivo* activities, which emphasize the importance of early screening tests also *in vivo*. The use of this *in vivo* testing also indicates different types of phytoprotective mechanisms of these extracts (i.e., direct effects on the bacteria and indirect effects on the plant defense system, or a combination of these).

When selected mushroom protein extracts were tested against several other strains of *R. solanacearum*, no correlations were seen for their susceptibilities or their phylotypes, biovars, host plants or geographic origins, which stresses the importance of including a large number of strains in such studies. Some extracts also inhibited *R. mannitolilytica*, a bacterium that is phylogenetically related to *R. solanacearum*, thus identifying the potential for the broader application of such extracts.

Taken together, the often unique properties of mushroom proteins should be beneficial in the fields of medicine, biotechnology, waste management or bioremediation, and agriculture.

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