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Evaluation of the effect of chitin-rich residues on the chitinolytic activity of *Trichoderma harzianum*: *In vitro* and greenhouse nursery experiments

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ABSTRACT

The chitin-rich residues obtained from several industries have numerous applications in diverse fields such as biotechnology, pharmacology, medicine and agriculture. In this study, three different chitin-rich residues were evaluated with regard to enhancement of the chitinolytic activity of the mycoparasitic fungus *Trichoderma harzianum*, used for biological control of *Fusarium oxysporum* in greenhouse nurseries. Chitosan (CHIT), shrimp shell powder (SSP) and mushroom wastes (MW) activated the expression of the *T. harzianum* genes encoding the NAGase and chitinase activities (*exc1*, *exc2*, *chit42*, *chit33* and *chit37*), the highest *in vitro* activities of *T. harzianum* being seen with MW. Under greenhouse nursery conditions, the treatments involving amendment with these residues and with *T. harzianum* increased the NAGase activity (between 5- and 20-times higher than peat) and the chitinase activity (between 3- and 6-times higher than peat) of the growing media and maintained the shoot dry weight of plants infected with *F. oxysporum*. Moreover, the incorporation of these residues into the growing media enhanced the growth (first leaf length, stem length and shoot dry weight) of muskmelon seedlings. The use of these residues with *T. harzianum* as amendments of growing media enhanced the growth of muskmelon seedlings and decreased the weight loss due to the pathogen, giving an added-value to these residues and enhancing their management.

1. Introduction

The use of specific microorganisms (biological control agents, BCAs) that interfere with plant pathogens and pests is a nature-friendly, ecological approach to overcome the problems caused by standard chemical methods of plant protection, which are not always effective for some diseases [1]. One of the most-used BCAs in agriculture is the fungus *Trichoderma harzianum*, which has shown good results against *Fusarium oxysporum* f. sp. *melonis* [2].

The beneficial effect of *Trichoderma* is due to a complex of different mechanisms such as: direct mycoparasitism, antibiotic production, nutrient and space competition, enhancement of plant resistance to pathogens and systemic induced or acquired plant resistance [3,4].

The mycoparasitism process allows the penetration of the host mycelium, by secretion of a complex group of extracellular hydrolytic enzymes that include proteases, glucanases and chitinases [1]. The latter have been reported to be a key factor in this process [5].

T. harzianum expresses from five to seven different chitinolytic enzymes [6], classified as endochitinases (CHIT42, CHIT33 and CHIT37) [7,8] and *N*-acetylglucosaminidases (EXC1 and EXC2) [9]. The two types of enzyme differ in their chitin cleavage patterns: endochitinases (EC 3.2.1.14) belong to glycoside hydrolase (GH) families 18 and 19 and catalyse the hydrolysis of β-1,4-linkages in chitin, while *N*-acetylglucosaminidases (EC 3.2.1.52) belong to GH family 20 and hydrolyse the terminal *N*-acetylglucosamine residues [10]. These enzymes are encoded by genes whose expression is activated by the presence of chitin or the products of its degradation [1]. Therefore, the use of chitin-rich compounds can enhance the fungal chitinolytic system.

Chitin is the second-most-abundant polymer in the biosphere after cellulose; it is found in the exoskeletons of crustaceans and insects and in the cell walls of most fungi [10]. There are several industries that produce materials rich in chitin as waste, the seafood industry being the most important [11]. Purified chitin can be obtained by the demineralisation and deproteinisation of crustacean shells and squid bones. In some cases, the chitin is deacetylated to obtain a soluble form of chitin, known as chitosan. Other sources of these compounds rich in chitin are the fungal-based waste materials accumulated in the mushroom production and fermentation industries, where no demineralisation treatment is required due to the low levels of inorganic materials in fungal mycelia [12]. All these residues are non-toxic, biodegradable, biocompatible with plant and animal tissues and exhibit unique properties that allow their use in multiple fields such as cosmetics, food processing, water engineering, biotechnology, pharmacology

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and medicine [13,14]. In agriculture, chitin and its derivates, such as chitosan and oligomers, have been shown to act as potent agents that elicit defence reactions in plants and inhibit the growth of pathogenic fungi and bacteria [15].

There are several studies that have used chitin-rich compounds and *T. harzianum* strains in the control of plant pathogens with good results [16–18].

The objective of this work was to study in depth the effect of three different chitin residues, one purified chitin source (chitosan) and two non-purified chitin sources (shrimp shell powder and mushroom wastes), on the chitinolytic activity of the BCA *T. harzianum*. For this purpose, *in vitro* and greenhouse nursery experimental approaches were used: the *in vitro* assay was carried out by studying the relative expression of *T. harzianum* genes that encode for different chitinolytic activities described in the literature. The response was followed by measuring chitinase and *N*-acetylglucosaminidase activities in a pure culture of *T. harzianum* supplemented with the different chitin sources, in comparison with glucose. The *in vivo* assay was carried out at the greenhouse nursery level, where melon seedlings were grown in growing media amended with *T. harzianum* and the different chitin sources, evaluating chitinolytic activities and the plant response to different chitin sources and their interaction with *T. harzianum* during crop infection with the pathogen *F. oxysporum*.

2. Materials and methods

2.1. Chitin sources

Three different sources of chitin were used: purified chitosan (France Chitine, France), shrimp shell powder (SSP; Microgaia Biotech, Spain) and mushroom wastes (MW; *Agaricus bisporus*, dried and ground). The main chemical characteristics of these compounds are described in Table 1. The percentage of chitin in each source was calculated according to De Boer et al. [19], measuring chitin richness as the glucosamine content. The carbon and nitrogen contents were determined using a LECO TRUSPEC CN carbon/nitrogen analyzer. The rest of the nutrients were determined by digestion with HNO_3 and H_2O_2, using a Milestone Ethos I microwave, followed by analysis by ICP (ICAP 6500 Duo Thermo). The composition of the chitin residues was different according to the nature of the compounds (Table 1). Chitosan showed the highest chitin concentration (measured as glucosamine), which was up to 5- and 10-times higher than in SSP and MW, respectively. In relation to the macronutrient concentrations, the SSP showed the highest values for N, P and Ca, while the highest K concentration was found in the MW.

2.2. Fungal inoculums

The *T. harzianum* isolate T-78 (Th T-78) (CECT 20714, Spanish Type Culture Collection) was grown on potato dextrose agar (PDA) (Scharlau, Spain), previously autoclaved at 121 °C for 20 min and amended with 100 mg L^{-1} sterilised streptomycin, and incubated at 28 °C for 7 days.

Table 1
Composition on dry weight basis of the three chitin-rich residues used in our study.

	Chitosan	Shrimp shell powder	Mushroom wastes
Chitin[a] (mg/g)	503.98	106.37	57.75
Total C (%)	44.50	41.65	40.44
Total N (%)	6.28	8.79	5.84
Total K (%)	0.01	0.28	3.25
Total P (%)	0.05	1.81	0.97
Total Ca (%)	0.13	6.04	0.08
Total Mg (%)	0.07	0.39	0.10
Total Na (%)	0.47	0.38	0.02

[a] Percentage of chitin was measured as glucosamine content.

The pathogen *F. oxysporum* f. sp. *melonis* (FOM) was isolated from melon plants showing disease symptoms, using the method of Nash and Snyder [20]. Conidia of FOM were produced by growing three discs (5 mm diameter) of 7-day-old mycelia grown on PDA (Scharlau, Spain), autoclaved at 121 °C for 20 min and amended with 100 mg L^{-1} sterilised streptomycin, in 250 mL of potato dextrose broth (PDB) (Scharlau, Spain), autoclaved at 121 °C for 20 min. The culture was maintained at 28 °C in a rotary shaker at 150 rpm for 8 days, to achieve a final concentration of 10^8 conidia/mL. The conidia were recovered by centrifugation (6000*g*, 20 min), rinsed twice with sterile, distiled water and filtered through 101 quartz wool (Panreac, Spain). The concentration was determined with a haemocytometer and the conidia diluted to the desired concentration.

2.3. In vitro assay

One millilitre containing 10^6 conidia of *T. harzianum* T-78 (Th T-78) was grown in 150 mL of minimal medium (g/L): KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; $Mg_2SO_4{\cdot}7H_2O$, 0.3, $CaCl_2{\cdot}2H_2O$, 0.3; $FeSO_4{\cdot}7H_2O$, 0.005; $ZnSO_4{\cdot}7H_2O$, 0.002; $MnSO_4{\cdot}H_2O$, 0.002; and 0.05% peptone, with different 0.1% chitin sources as the sole carbon source: 0.1% chitosan (CHIT); shrimp shell powder (SSP); and mushroom wastes (MW). Glucose (GLU) was used as the control to check the normal growth of the isolate. The fungal cultures were maintained in darkness at 28 °C, in a rotatory shaker at 220 rpm, for 72 h. Forty millilitres of each culture were sampled at 24, 48 and 72 h, filtered through Miracloth (Calbiochem, Germany) and washed with sterile water. The mycelia were collected, frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.3.1. Enzyme activities

Chitinase and *N*-acetyl-β-D-glucosaminidase (NAGase) activities were measured as described by López-Mondéjar et al. [21]. The total activities were measured in samples containing mycelium and the extracellular activities in the supernatant of centrifuged samples (13,000*g* at 4 °C, for 10 min). The activities were measured as nkat and normalised with the biomass values (nkat/μg protein). The biomass was measured using 1-mL samples from each flask culture, according to López-Mondéjar et al. [21].

2.3.2. RNA isolation, cDNA synthesis and qRT-PCR

The RNA was isolated from frozen mycelia as described by Chomczynski and Sacchi [22], and its concentration and quality were measured with NanoDrop ND-100 (Thermo Fisher Scientific). One microgram of RNA was treated with DNase (Sigma) following the procedure described by the manufacturer. The cDNA was synthesised with oligodT using the ThermoScript RT-PCR System (Invitrogen), following the manufacturer's protocol. The relative expression of each gene was measured using the LightCycler System (Roche), and each run with a total volume of 10 μL included: 2 μL of a 1:5 diluted sample of cDNA, 5 μL of Quantimix EASY SYB (Biotools, Spain), 0.375 μM of each primer, 0.5 mg/mL of bovine serum albumin (BSA) and DEPC-water. The amplification conditions were: a denaturation step of 30 s at 95 °C, followed by 45 cycles of 5 s at 95 °C, 25 s at 60 °C and 1 s at 82 °C, and a final step to obtain the melting curves of the amplicons: 0 s at 95 °C, 10 s at 65 °C and 0 s at 95 °C. The eukaryotic elongation 1-alpha gene (*tef*) and the glyceraldehyde-3-phosphate dehydrogenase gene (*gadph*) were used as housekeeping gene controls for RT-PCR. The data were analysed with LightCycler Software 4.0 (Roche). Primers sequences for the *chit42*, *chit33*, *exc1*, *exc2* and *tef* genes were obtained from López-Mondéjar et al. [21]. The primers for *chit37* and *gadph* were designed with LightCycler Probe Design 2