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Evaluation of fungal antagonists to control black mold disease under field conditions and to induce the accumulation of antifungal compounds in onion following seed and set treatment

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ABSTRACT

Three isolates, AS3 (non-aflotoxigenic *Aspergillus flavus* Link), TRIC7 and TRIC8 (*Trichoderma harzianum* Rifai), from onion (*Allium cepa* L.) growing soils were recently found to control black mold disease caused by *Aspergillus niger* (An) van Tieghem and to increase the accumulation of antifungal compounds in pot-grown onion sets. Their ability to increase bulb diameter and total soluble solids in marketable bulbs, to control black mold and to induce the production of antifungal compounds were tested in sets and marketable bulbs raised from treated seeds and sets, respectively, in naturally An-infested field soils at two locations. These isolates significantly controlled the disease at both locations, but they did not have any enhancing effect on set or bulb diameter and soluble solids in marketable bulbs. AS3 and TRIC8 in particular led to defense reactions with accumulation of antifungal compounds in sets and marketable bulbs in both locations. Different compounds were also identified in the fractions with highly antifungal effects.

1. Introduction

Onion (*Allium cepa* L.) is an economically important vegetable crop grown in Turkey. It is commercially grown as an annual plant for its leaves and bulbs, providing a profitable rotation alternative to sunflower and cereals. Most common production of bulb onion is done using direct seeding and onion sets. Low yield of onion sets and marketable bulbs are attributed to factors such as its susceptibility to black mold disease caused by *Aspergillus niger* Van Tieghem (An) (Özer and Köycü, 2004). The pathogen is transmitted by contaminated seed or soil. The infection usually begins at germination of onion seeds and may continue throughout storage (Hayden and Maude, 1992; Hayden et al., 1994a, b; Köycü and Özer, 1997; Sirois et al., 1998). Visual symptoms are not observed on sets developing from seeds infected with pathogen because of latent infection (Özer and Köycü, 1997), although visible external and internal symptoms of black mold occur on infected marketable bulbs (Sumner, 1995; Sinclair and Letham, 1996).

Options for the control of An are limited by unsatisfactory chemical control (Hayden et al., 1994b; Sinclair and Letham, 1996; Özer and Köycü, 1998) and lack of highly resistant cultivars (Özer, 1998; Ko et al., 2002). Use of biocontrol agents is a promising approach for managing seed and soil borne diseases; however, little is known about the microorganisms that can protect onion bulbs against An (El Neshawy et al., 1999). The potential of using antagonist fungal species by seed and set treatment to control black mold has not been tested in onion under field conditions.

Biological control can also be accomplished through induced resistance (Cook et al., 1996; Haran et al., 1996; Elad, 2000; El Hassni et al., 2007). This mode of action involves applying a microorganism to seeds, leaves, or roots of plants to elicit a resistant phenotype (Cook et al., 1996). Accumulation of antifungal compounds is one of several biochemical defense responses in plants (Nicholson and Hammerschmidt, 1992; Hunt et al., 1997). Naturally occurring compounds such as water-soluble phenols and flavones in red or yellow pigmented onion bulb scales have antifungal properties. These compounds provide resistance by preventing spore germination and penetration of fungal pathogens (Link and Walker, 1933). Induced accumulation of phenolic compounds in onion has been correlated with the degree of resistance expressed to black mold infection during seed germination (Özer et al., 1999). A previous study conducted by Özer (2011) demonstrated the possible role of seed treatment with isolates of

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non-aflatoxigenic *Aspergillus flavus* (AS3) and *Trichoderma harzianum* (TRIC7 and TRIC8), in the accumulation of antifungal compounds coinciding with increased protection against An in onion sets in pot experiments. These isolates also had no non-target effects on seed, seedling and set bulb development, based on the parameters of seed germination percentage, abnormal seedlings, shoot length and set size. However, they have not been evaluated against An on onion sets and marketable bulbs under field conditions. In addition, to our knowledge, there are no reports of induced resistance in onion by seed or set treatment with any antagonist fungus against black mold caused by An under field conditions.

Considering these points, the present study was conducted to determine the effects of these potential antagonists (AS3, TRIC7 and TRIC8) on black mold disease, studying their potential role in some bulb characteristics, and induction of antifungal compounds in onion sets and marketable bulbs under field conditions.

2. Materials and methods

2.1. Plant material

Onion (*A. cepa* L.) cultivar Kantartopu, which is susceptible to *Aspergillus niger* (Özer, 1998), was used as the host plant. To check for potential infection, onion seeds and sets were surface sterilised by immersing them in a 1% solution of sodium hypochlorite for 5 min, rinsing in sterile distilled water and air drying on sterile filter paper. Sterilized seeds and tissues of 0.5 cm dissected from sterilized set bulbs were placed aseptically on potato dextrose agar (PDA) (Oxoid, Unipath Ltd.) and sterile filter paper (Blotter method) moistened with sterile distilled water in 9 cm Petri dishes and incubated at 23 °C for seven days in the dark (Köycü and Özer, 1997). Eight replicates of 25 seeds and 25 set tissues were used for each medium. No seed- or set-borne onion pathogens (Köycü and Özer, 1997) were recovered throughout the experiments.

2.2. Antagonist fungi

Isolates of potential antagonist fungi used in this study had previously been recovered from onion growing soils fungistatic toward An (Özer et al., 2009), and were found highly effective against *Aspergillus niger* in inhibiting growth in dual culture tests and in pot experiments (Özer, 2011). These include *A. flavus* (AS3) and *Trichoderma harzianum* (TRIC7 and TRIC8). The *A. niger* isolate (An6) obtained from naturally infected onion seeds (Köycü and Özer, 1997) in Turkey was used as the pathogen. This isolate was identified as the most aggressive among the An isolates tested (Özer and Köycü, 1997). All fungi were grown on PDA (Oxoid) for 7 days at 25 °C in the dark.

2.3. Antagonist treatment and field experimental design

Seeds and sets were separately placed in 50 ml and 2000 ml conical flasks, respectively, and treated with spore suspensions (1×10^7 spores ml^{-1}) of each antagonist fungus in aqueous Tween 20 (Merck, Darmstadt, Germany) (0.1% v v^{-1}). The flasks were gently rotated on a reciprocating shaker (Electro-Mag, İstanbul, Turkey) for 1 h at 40 strokes min^{-1}. Non-treated seeds and sets were used as controls.

Two onion locations in Tekirdağ province, which were known to be naturally infested with An (3×10^4 CFU/g soil), were selected for the field experiments. Location I (Kayı village) was located at 41°1′37.05″ N, 27°31'27.83″ E at an altitude of 243 m above sea level; Location II (Köseilyas village) was located at 41°1′06.73″ N, 27°34'55.70″ E at an altitude of 112 m above sea level. In location I, the soil was clay (sand: 23.51%, silt: 25.19%, clay: 51.30%) containing 11.25 ppm P_2O_5, 17.5 ppm K_2O and organic matter of 1% with a pH of 7.6; in location II, the soil was clay (sand: 23.74%, silt: 31.39%, clay: 44.87%) containing 18.32 ppm P_2O_5, 47.6 ppm K_2O and organic matter of 1.58% with a pH of 6.7. No fertilizer application of any kind was carried out during the experiment.

There were two series of field experiments, one for onion seed and the other for set treatments at each location. Seed experiments were carried out in a 1 m^2 plot for each replication of a treatment using 5 g seed m^{-2}. In the set experiments, 50 onion sets were used in a 1.50 m^{-2} plot for each replication. Daily average temperature and relative humidity were 18 °C and 79%, respectively, in field experiments. All trials were arranged as a randomized block design with four replicates.

2.4. Evaluation of potential fungal antagonists for their effects on bulb size and dry matter content

Four months after sowing or planting, the sets and marketable bulbs of 25 plants which were taken randomly from each plot at two locations were evaluated. The diameter of each set and marketable bulb was measured at their equator. In addition, four bulbs were taken randomly from the marketable bulbs in each replication and dry matter content, which is the major factor affecting onion flavor, storage losses and dehydration of processing (Lin et al., 1995; Raines et al., 2009), was determined. The dry outer skin of bulbs was removed manually. Two rectangular scale pieces were cut from the second and third layers (counting from the outer scale) of each bulb and crushed into a hand garlic press (Sinbo, İstanbul, Turkey). The total dry matter content of juice was determined using a hand refractometer and was expressed as the percentage of total soluble solids (Lin et al., 1995).

2.5. Evaluation of potential fungal antagonists for their effects on disease incidence by An in onion set and marketable bulbs

Onion sets and marketable bulbs, which were harvested as described previously, were evaluated for disease incidence by An at both locations. In the evaluation of onion sets for disease incidence, each onion set was surface sterilized as described in Section 2.1, then cut horizontally. Tissues near the base were dissected and cultured on PDA to examine the presence of latent infection by An (Özer and Köycü, 2004). Disease incidence was recorded as the percentage of sets infected with black mold caused by An.

The disease incidence in marketable bulbs was calculated by counting the total number of bulbs with visible black mold infection divided by the total number of harvested bulbs and expressed as a percentage.

2.6. Extraction from onion sets and marketable bulbs of compounds absorbing ultraviolet light and their antifungal activity

The set bulbs raised from treated seeds and marketable bulbs raised from treated sets in fields at each location were separately collected and sets or marketable bulbs of 20 plants from each replicate at each location were homogenized in 2 ml 95% ethanol per gram fresh weight. After 24 h extraction at 25 °C in the dark, ethanolic extracts were sterilized through a membrane filter with a 0.22 μm pore size (Millipore, Millipore Co., Billerica, MA, USA). Similarly, ethanolic extracts were prepared from sets and marketable bulbs raised from untreated seeds and sets, respectively. Separation of extracts by thin layer chromatography (TLC) on silica gel plates (TLC plates 60 F254, Merck, Darmstadt, Germany), spectral analysis of each band and antifungal effects of fractions on An spores were performed following the methods used by Özer (2011). Ethanolic samples of pure 0.1 M catechol (Sigma