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SHORT COMMUNICATION

Ascochyta blight of chickpea reduced 38% by application of *Aureobasidium pullulans* (anamorphic Dothioraceae, Dothideales) to post-harvest debris

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In 2004–2005, application of non-amended suspensions of *Aureobasidium pullulans* conidia to post-harvest chickpea debris resulted in 37.9% fewer Ascochyta blight lesions on chickpea test plants relative to controls. Analogous tests in 2006–2007 resulted in 38.4% fewer lesions. Ascospores released from debris were predominantly *Davidiella* sp. (anamorph, *Cladosporium* sp.), followed by *Didymella rabiei* (anamorph, *Ascochyta rabiei*, agent of Ascochyta blight).

Keywords: *Ascochyta rabiei*; *Cicer arietinum*; *Cladosporium*; *Davidiella*; *Didymella rabiei*

Introduction

Ascochyta blight has long presented challenges for chickpea (*Cicer arietinum* L.) production, especially under reduced tillage practices. The causal agent, *Didymella rabiei* (Kovachevsky) Arx [asexual stage = *Ascochyta rabiei* (Pass.) Lab.] over-winters in post-harvest debris on the soil surface (Kaiser 1992). Ascospores from debris are considered the primary inoculum in many chickpea-growing areas (Milgroom and Peever 2003; Chilvers, Peever, Akamatsu, Chen, and Muehlbauer 2007). Prior research includes epidemiology and population dynamics (e.g., Trapero-Casas and Kaiser 1992a,b; Navas-Cortés, Trapero-Casas, and Jiménez-Díaz 1998; Peever, Salimath, Su, Kaiser, and Muehlbauer 2004), sources of resistance (e.g., Meuhlbauer and Chen 2007), and host specificity and phylogenetic relationships (Peever 2007). Comprehensive reviews of chickpea breeding and disease management are available (e.g., Davidson and Kimber 2007; Yadav, Redden, Chen, and Sharma 2007), but little information is published on prospective biological control of *D. rabiei*.

Aureobasidium pullulans (de Bary) G. Arnauld (AuP) is a cosmopolitan fungus used for experimental biological control of plant diseases of a variety of crops, e.g., apple, cherry, grapes, pear, strawberry and greenhouse-grown vegetables (e.g., Wittig, Johnson, and Pscheidt 1997; Barreiro, Santos, Ramos, Pais, and Silva 2006; Elmer and Reglinski 2006), or for experimental control of ochratoxin-producing *Aspergillus*

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spp. in vineyards (Battilani et al. 2007; Visconti, Perrone, Cozzi, and Solfrizzo 2008). It is an active ingredient in Blossom Protect™, used against *Erwinia amylovora*, the bacterial agent of fire blight disease of apple and pear (Kunz 2004). Criteria for safe utilization of fungi for biocontrol, and the suitability of AuP with regard to these criteria, have been summarized (Dugan, Lupien, Hernandez-Bello, Peever, and Chen 2005), although AuP is recently described as potentially 'under-recognized' as an allergen in certain situations or localities (Taylor et al. 2006).

AuP is present in post-harvest chickpea debris, and complete suppression of both sexual and asexual reproduction of *D. rabiei* on chickpea stems was achieved *in vitro* (Dugan et al. 2005). We conducted small-scale field and greenhouse trials with post-harvest chickpea debris inoculated with non-amended (without added carbon source, stickers, spreaders, etc.) AuP in fall or winter and left to over-winter in the field. Lesion development on susceptible chickpea trap plants served as a surrogate measure of ascospore release. In addition to assessing the capacity of AuP to limit disease, we present further information on fungi producing ascospores from chickpea debris.

2004–2005 Field trial

Design

The treatment consisted of post-harvest chickpea debris subjected to early winter inoculation with AuP. The control consisted of debris with no inoculation (i.e., immersion in tap water only). The treatment and control, each consisting of two replicates, were left to over-winter in the field prior to planting of 24 chickpea trap plants surrounding each replication in the following spring as described below.

Production of AuP inoculum

Single-spore isolate AuP26 from chickpea debris (Dugan et al. 2005) was grown on solid half strength V8 (½V8) (Stevens 1981). Conidia were harvested into sterile water, transferred to ½V8 broth maintained under cool white + near ultraviolet lights at ambient lab temperature and 130 rpm, then centrifuged (20 min at 16,900 $\times g$, 4°C), washed, re-suspended, and diluted to 10^6 conidia/ml.

Collection and inoculation of debris

Chickpea debris remaining after the September 2004 harvest was collected into mesh bags and stored indoors until immersion in AuP inoculum suspension (until runoff, $<$1 min) on 10 December 2004, then transported to the field. Debris applied to control plots was immersed in tap water only. Debris was known to be infested with both *D. rabiei* mating types (Peever et al. 2004).

Production of chickpea trap plants for transplanting to the field

Chickpea plants (cultivar 'Burpee') were grown in a greenhouse with natural and supplemental light, and irrigated with nutrient solution. Plants 7–13 cm tall were

randomly assigned to each replication in the above design, and acclimated for 5 days under a tarp prior to transplanting to the field on 19 April 2005.

Plot establishment

Treatment and control plots were separated by 500 m on sites of similar soil type and exposure. Each plot and its single replicate were 4 m apart, with debris scattered 4–5 cm deep in a rectangle of 1.2×1.8 m and covered with mesh to minimize debris dispersal. Twenty-four trap plants, ca. 10 cm tall, were planted in a rectangle surrounding the debris of each plot at a distance of 1 m from the edge of the mesh (Figure 1A). Two groups of 24 plants each were established at distance of 1 km from the treatment plots to monitor background infection from long distance drift of ascospores. Plants were watered by hand as needed.

Rating of infection

On 26 and 27 May 2005, lesions diagnostic for Ascochyta blight (Kaiser and Hannan 1988) were counted on each plant in replications 2 and 1, respectively, of each treatment + control.

2006–2007 Field/greenhouse trials

Design

The treatment consisted of post-harvest chickpea debris inoculated in early winter with AuP. The control consisted of debris with no inoculation (i.e., immersion in tap water only). At each of two sites (F2A06 and Whit06), the treatment and the control, each consisting of four replications in a stratified block, were left to over-winter in the field prior to the debris being removed to the greenhouse the following spring for exposure of chickpea trap plants as described below. (Nearby commercial plantings of chickpea in 2006 left post-harvest debris with potential inoculum, so trap plants were exposed in the greenhouse rather than the field). Twenty-five chickpea plants, not adjacent to any debris, were used to monitor possible background infection in the greenhouse.

AuP inoculum production

Single-spore isolate AuP12 from chickpea debris (Dugan et al. 2005) was amplified for application to debris as described above for AuP26, to yield suspensions of 10^6 conidia/ml.

Collection and inoculation of debris

Field debris was collected September 2006 following harvest of chickpeas (cultivar Burpee), stored indoors until 14 October and homogenized. The homogenized debris was randomly divided into 16 portions [(1 treatment + 1 control) $\times$ 2 sites $\times$ 4 replications/site = 16] of equal volume. Debris was immersed for two min in AuP suspensions or (for the controls) in tap water, and transported to the field on

15 October 2006. Individual replicated plots were 1 m^2 with debris 4–5 cm deep, and separated by a distance of one meter. Debris was placed onto the soil surface and covered with mesh as above.

Production of chickpea trap plants

Chickpea seeds (cultivar Burpee) were sown in potting mix and plants were grown for 13 days in the greenhouse with natural and supplemental light. Six pots of trap plants with three plants (ca. 20–25 cm tall) per pot were placed in each misting tent

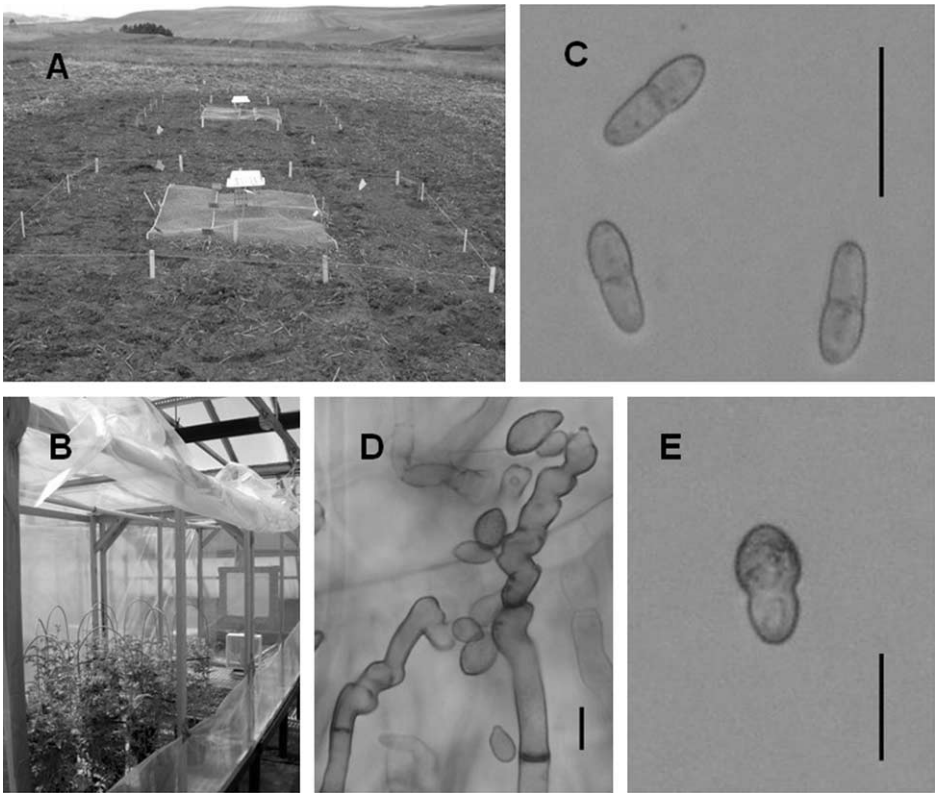

Figure 1. (A–E) Field and greenhouse trials. (A). Replications 1 & 2 of the treatment plot in the field study of 2004–2005. Wire mesh confines the debris, stakes and twine indicate placement of chickpea seedlings, and inverted white trays protect petroleum-jelly covered glass trap slides. (B). One chamber in the 2006–2007 trials, in which chickpea plants were exposed to over-wintered debris transported to the greenhouse. A chamber wall has been rolled up to expose the interior. In each chamber (replication), a fan (rectangular box in the background) blew air from debris (on floor of chamber between fan and plants) onto chickpea plants on which lesions were later quantified. (C–E) Mycoflora of chickpea debris. Figure 1C. Ascospores of *Davidiella* discharged from chickpea debris, trapped on water agar, and photographed in air at 100 ×. Bar = 30 μm. (D) Conidia and conidiophores of *Cladosporium* isolate #7-09, from transfer of a single *Davidiella* ascospore. Conidiophores are typical for *C. herbarum*. Bar = 5 μm. (E) An ascospore of *Didymella rabiei* from chickpea debris, trapped on water agar and photographed in air at 100×. Bar = 20 μm.

as described below and exposed to debris sampled from the field plot of a single replication.

Colonization rates in over-wintered debris 2006–2007

On 14 March 2007, debris samples were collected from all treatment and control plots at each field location (F2A06 and Whit06) for a total of 16 samples. Samples were dried in paper bags prior to removal of ten 2.5-cm long stem segments from each bag. Segments were washed in running tap water for 10 min, transferred to malt extract agar amended with antibiotics for suppression of bacteria, and incubated 3–5 days. The number of segments with AuP colonies was recorded by treatment and location. Identification of AuP was confirmed by methods of de Hoog and Yurlova (1995). Procedures were repeated with 16 analogous samples taken 7–8 May.

Rating of infection

Over-wintered debris was removed from the F2A06 field site on 7 May and from the Whit06 field site on 8 May 2007. A sample of debris (250 gm) was placed into each of 16 plastic tents in the greenhouse in a randomized block design corresponding to that established in the field. In each tent, a fan blew air over debris, directing ascospores toward test plants (Figure 1B). Plants and debris were misted with water at programmed intervals. Numbers of lesions per pot of trap plants were recorded for each replication of treatments and controls from both field sites on 25 May 2007 by six persons, with each person counting one pot per replication over all replications.

Ascospores released from debris over-wintered in 2006–2007

Chickpea debris was collected from field plots on 7 and 8 May 2007. Stem segments were soaked in sterile water for 2 hr, and mounted over 3% water agar for discharge of ascospores (Dugan et al. 2005). Ascospores were examined at 100 × on agar surfaces, photographed and measured. Representative spores were transferred with a needle to fresh agar. Two resulting isolates (#2-07, #9-07), representative of a putative *Davidiella* sp., were grown for DNA extraction, amplification, and sequencing of partial *ACT* (actin) and *EF* (elongation factor 1-alpha) genes following procedures of Schubert et al. (2007). ACT and EF sequences of #2-07 were deposited with Genbank as EU730605 and EU730603, respectively, and corresponding sequences for #9-07 were deposited as EU730606 and EU730604, respectively. Eight representative isolates resembling *A. rabiei* were tested for pathogenicity against green-house grown 'Burpee' chickpea plants along with water controls.

Statistical analysis

Results were analyzed by analysis of variance (estimate model, LSD) in SYSTAT 9.0, (SPSS, Inc., Chicago, IL).

Results and conclusions

2004–2005 Field trial

Application of AuP to debris reduced ($P < 0.001$) the mean number of lesions/plant from 89.4 in controls to 55.5 in the treatment. Plants used for monitoring background ascospores ('background plants') were non-symptomatic.

2006–2007 Field/greenhouse trials

Application of AuP to debris subsequently placed in the field for over-wintering and then transported to the greenhouse reduced ($P < 0.001$) the mean number of lesions on plants in the greenhouse from 19 (6.3 per plant) in the controls to 11.7 (3.9 per plant) in the treatment. Site F2A06 had a mean of 8.5 lesions (2.8 lesions per plant), differing ($P < 0.001$) from a mean of 22.2 (7.4 lesions per plant) at Whit06. No lesions were seen on the plants used to monitor background infection in the greenhouse.

In the March 2007 sample, inoculation with AuP had increased ($P < 0.001$) the mean rate of colonization by AuP (mean number of colonized stem segments per sample of 10 segments) from 1.4 in the controls to 7.3 in the treatments. In May, the corresponding increase was from 4.4 in the controls to 9.9 in the treatments ($P < 0.001$). Differences between location and replication were insignificant for each sampling period.

Application of AuP to post-harvest debris reduced numbers of lesions in the field trial of 2004–2005 ($P < 0.001$) and the field/greenhouse trial of 2006–2007 ($P < 0.001$). It is probably coincidental that both sets of trials produced results in which disease incidence in treatments was reduced by ca. 38% relative to controls, but P values indicate strong statistical significance. Ascospores of *D. rabiei* are well documented in literature (Milgroom and Peever 2003; Chilvers et al. 2007) as constituting the primary inoculum. In the greenhouse, there was no opportunity for water splash to disseminate conidia, which are produced in a mucilaginous matrix and require rain-splash for dispersal (Pearse et al. 2001). We therefore infer that ascospores constituted the majority of the disease inoculum in our experiments. We conclude that it is possible to suppress disease development by bolstering populations of AuP in post-harvest debris, and the mechanism of that suppression is most likely suppression of ascospore formation or release.

In both sets of trials, treatment consisted of simple (i.e., non-amended – no stickers, spreaders, or nutrient sources) suspensions of conidia in water. In 2004–2005, freezing temperatures followed soon after treatment; and in 2006–2007 heavy rain occurred the night after placement of debris in the field. Our monitoring of persistence of AuP in debris in 2007 indicated that final levels of AuP in treated debris were on the order of twice that in untreated debris. Although it is unlikely that 38% suppression would constitute cost effective control, we strongly suspect that application of AuP, amended with a suitable sticker and/or other adjuvants and applied under more favorable meteorological conditions, would result not only in greater persistence of AuP, but in effectiveness superior to the 38% disease suppression twice attained here.

Ascospores released from debris over-wintered in 2006–2007

Nearly all ascospores were of two kinds, each of which was measured and photographed. The most common (Figure 1C), observed in all instances in which ascospores were detected, matched proportions and dimensions in Arx (1949), Corlett (1988) and Schubert et al. (2007) for *D. tassiana* (De Not.) Crous & U. Braun, the teleomorph of *C. herbarum* (Pers.: Fr.) Link, or the closely related *D. macrocarpa* Crous, K. Schub. & U. Braun, the teleomorph of *C. macrocarpum* Preuss. Ascospores individually transferred to agar media produced colonies provisionally identified as *C. herbarum*, and two representative isolates (#2-07, #9-07) keyed to that species in Schubert et al. (2007). Compared to sequences from Schubert et al. (2007), *ACT* sequences from #2-07 most closely matched *C. herbarum* (1 nucleotide difference), with *C. macrocarpum* as the next closest match (differing by three nucleotides), whereas #9-07 was identical to *C. macrocarpum* by *ACT*; *EF* sequences from both isolates most closely matched *C. macrocarpum*, with *C. herbarum* as the next closest match. But, conidiophore morphology (Figure 1D) was congruent with *C. herbarum* rather than with *C. macrocarpum* by criteria in Schubert et al. (2007). Many similar *Davidiella*-like ascospores (but only a few *D. rabiei*-like ascospores) were trapped on petroleum jelly-covered glass slides in 2005 (data not shown). We tentatively assign our *Davidiella* isolates to *D. tassiana*. Debates over conspecificity of *C. herbarum* with *C. macrocarpum* are summarized by Schubert et al. (2007). *C. herbarum* and *C. macrocarpum* are extremely common, cosmopolitan fungi reported from an immense variety of plants (Farr, Rossman, Palm, and McCray n.d.).

The second kind of ascospore (Figure 1E), observed on ca. 25% of the dishes on which ascospores were detected, conformed to descriptions and/or illustrations of *D. rabiei* in Kovachevsky (1936), Müller and von Arx (1962), Trapero-Casas and Kaiser (1992b) and Wilson and Kaiser (1995). Transfer of individual ascospores to water agar resulted in colonies typical of *A. rabiei*, and eight representative isolates produced symptoms typical of *A. rabiei* when inoculated to chickpea plants. No disease was obtained with water controls.

It would be desirable to use an organism consistently present in chickpea debris for control of *D. rabiei* in that substratum. *Cladosporium* spp., *Alternaria* spp. and most other fungi predominant in chickpea debris are quite allergenic or, like several *Fusarium* spp., consistently act as plant pathogens (Day and Ellis 2001; Samson, Houbraken, Summerbell, Flannigan, and Miller 2001; Dugan et al. 2005). Given the lesser allergenicity of AuP, its record as an experimental and commercial biocontrol agent, and considering the results presented here, we believe that AuP represents a fungal candidate for biological control of *D. rabiei* in post-harvest debris.

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