Measuring Root Disease Suppression in Response to a Compost Water Extract

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ABSTRACT

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Commercial application of compost to prevent plant disease is hindered by variable performance. Here, we describe the use of a growth pouch assay to measure impact of a compost water extract (CWE) on root infection under controlled conditions. Most pea roots ($\geq 95\%$) inoculated with *Fusarium solani* or *Phoma pinodella* spores rapidly develop a single local lesion in the region of elongation. In the presence of CWE, infection of pea roots grown in pouches was reduced by 93 to 100%. CWE used as a drench on pea seedlings grown in sand also resulted in 100% protection

but, in a heavy clay soil, infection was reduced by $<50\%$. CWE filtered to remove microorganisms did not inhibit frequency of *F. solani* infection, and resulted in increased local lesion development on individual roots. CWE inhibited mycelial growth of both pea- and cucumber-infecting isolates of *F. solani* in culture but exerted $<40\%$ protection against cucumber root infection. CWE treatment of pea but not cucumber was associated with retention of a sheath of border cells interspersed with bacteria covering the region of elongation. Growth pouch assays may provide a system to monitor effects of specific compost mixtures on root-rhizosphere interactions, and to identify variables influencing disease control.

In recent decades, global interest in sustainable agriculture increasingly has focused on reduced reliance on commercial fertilizers and pesticides (25,26,33). Urgency in developing biological control methods for soilborne diseases has increased as efforts to cease application of methyl bromide have progressed (14,16). Compost in various forms has been used for centuries but concerns about food safety, reproducibility, and phytotoxicity have hampered systematic application of such approaches in agriculture (4,13,29,38). The United States Department of Agriculture has outlined a critical need to define, measure, and determine mechanisms of compost function in plant health and development in diverse climate and soil environments (USDA.gov). Although chemically defined pesticides and fertilizers can predictably influence crop production, composts are heterogeneous mixtures whose intrinsic variability complicates efforts to achieve reproducible results. Defining biological control mechanisms even with specific microbial populations under controlled conditions remains a long-standing challenge (24). Defining mode of action for any given solid compost preparation sufficiently to predict efficacy for a given crop may not be a realistic goal. Water extracts of compost, by contrast, yield a liquid product that is amenable to chemical and biological analysis, and can be applied as a drench to plant surfaces (9,20,34,43). Promising effects of compost treatments on important soilborne pathogens, including species of *Pythium* (5,8,35), *Phytophthora* (31), *Fusarium* (12,23,32), *Rhizoctonia* (35), and *Meloidogyne* (43), reveal the benefits to be gained by a

better understanding of how disease is reduced with some crop species in some conditions.

Little is known about physiological effects of compost during early root development. Root tips (≈ 1 to 2 cm from the apex) of higher plants contain primary sites for the establishment of beneficial and pathogenic root-microbe associations and can influence the architecture of the entire plant (1,10,36). Therefore, understanding how compost influences root tip and rhizosphere structure and function may be critical to designing reliable approaches to crop improvement. Unfortunately, the fact that roots grow underground, out of sight, is an obstacle to evaluating the dynamic cellular and biochemical bases for root tip responses to additives (11). A rapid, cost-effective growth pouch assay has been used for decades to describe mechanisms of root infection and colonization by *Rhizobium* spp. and other bacteria, including species isolated from compost mixtures (2,19). Clapp and co-workers (7) introduced the growth pouch assay as a tool to quantify plant responses to soluble nutritional factors from humic substances commonly used in compost preparations. We have used the pouch assay to document mechanisms by which root border cell populations released from root caps function in defense (17,21,22). In the current study, we report effects of a specific compost water extract (CWE) on susceptibility of roots to fungal infection, using the soilborne pathogen *Fusarium solani* f. sp. *pisi* (*Nectria haematococca* mating population VI) and its host, *Pisum sativum* L., as the primary model system.

MATERIALS AND METHODS

Plant material. Pea (*P. sativum* L.) seed of 'Little Marvel' (Meyer Quality Seeds, Baltimore, MD) and 'Alaska' (Chesmore Seed Company, St. Joseph MO) were treated as described previously (17,18). After immersion for 10 min in 95% ethanol fol-

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains two supplemental tables.

lowed by 60 min in 5.0% sodium hypochlorite, seeds were rinsed six times in distilled water and then imbibed in sterilized water for 6 h. Seeds that floated to the surface were discarded. Imbibed seeds were placed on 1% water agar overlaid with germination paper, then incubated for 48 h at 24°C in the dark. Maize (*Zea mays* L., 'Golden Cross Bantam') (Ferry-Morse Seed Company, Fulton KY) seeds were treated for 5 min in 95% ethanol followed by 10 min in 2.5% sodium hypochlorite, then rinsed six times in distilled water. Cucumber (*Cucumis sativa* L.) (Ferry-Morse Seeds) seeds were treated for 5 min in 95% ethanol followed by 5 min in 2.5% sodium hypochlorite, then rinsed six times in distilled water. Seedlings were used when roots were 20 to 25 mm in length.

Pathogen isolates. Many species share the anamorph name *F. solani* but, within the *F. solani* complex, there are seven distinct heterothallic groups called mating populations (MPs) that act as biological species (30). These isolates have been called by their teleomorph *N. haematococca*, and what is called *F. solani* in this article refers to *N. haematococca*. *F. solani* f. sp. *pisi*, pathogenic on pea; *F. solani* f. sp. *cucurbitaceae* (*N. haematococca* MPI), pathogenic on cucurbits; and *Phoma pinodella* (L. K. Jones), T409 cultures were maintained and processed as described previously (17,18).

CWE preparation. A fresh batch of compost was prepared for each experiment, and experiments reported here reflect results from 22 independent batches produced over a 2-year period (April 2010 to April 2012). A 18.9-liter brewer (www.simplicitea.com) was filled with well water. The following ingredients were added, and the mixture was maintained at 26°C for 18 h, 40 min: 250 ml of Alaskan humus (Earthfort; www.earthfort.com/), 250 ml of worm castings (Acme Worm Farm, Tucson AZ), 15 ml of rock phosphate dust (Peaceful Valley; www.groworganic.com), 57 g of fish hydrolysate (Earthfort), 28 g of Algamin (Cold Processed Kelp; Peaceful Valley), 28 g of organic Turf Pro

(Earthfort); and 15 ml of Compost Tea Catalyst (Peaceful Valley). The presence of a mixed population of bacteria, fungi, and protozoa was confirmed by direct microscopic examination of representative samples, and pH (5.2 ± 0.6) was measured prior to application. Filtered samples were obtained by sequential passage of CWE samples through Whatman 52 filter paper, a Nalgene 0.45- μ m syringe filter, and an Acrodisc 0.2- μ m syringe filter.

Root growth and infection assays. For each set of experiments, a 15-ml sample of water or CWE was placed into the bottom of a growth pouch (Mega International, St. Paul, MN) using a syringe. Five seedlings were placed into each pouch, and roots were inoculated uniformly from base to tip with a 0.2-ml spore suspension (10^6 spores/ml) and left in a horizontal position for 15 min. The position of each root tip was marked on the cellophane pouch at the time the experiment was initiated. Pouches then were maintained at 24°C, in an upright position, for up to 28 days. Shoot and root growth, morphology, and lesion development over time were measured nondestructively by direct observation. All values represent at least 30 replicates (five seedlings per pouch) in at least three independent experiments, unless otherwise indicated. Lesion development and localization on seedlings grown in pouches and in pots with sand or soil were documented based on the presence of a tan, brown, or black section of root with internal fungal hyphae, as described (17). Pot experiments were monitored over a 4-week period after inoculation; roots were removed for inspection when aboveground symptoms developed (wilt and chlorosis) or at the end of the 4-week period. Numbers of healthy versus infected seedlings were analyzed by contingency tables, performing Fisher's exact tests using IBM SPSS Statistics for Windows (version 20.0; IBM Corp., Armonk, NY) Fisher's exact test for pairwise comparison analysis between treatments was performed using JMP (version 10; SAS Institute Inc., Cary, NC).

Fungal growth assays. A radial growth inhibition assay was used to measure fungal growth in the presence of CWE or filtered CWE, as described (43). Mycelial plugs were placed at one edge of 35-mm plates containing 1 ml of Martin's peptone-glucose agar medium (28) mixed with 200 or 300 μ l of CWE or filtered CWE, and the diameter of hyphal extension was measured. Values represent means and standard deviations from three replicate plates in two independent experiments.

Identification of predominant culturable species on roots exposed to CWE. Roots with extended border cell sheaths were agitated in water to disperse microbial populations, and dilution plating was used to isolate predominant species. Single-colony isolates were subjected to rDNA analysis. Total DNA was extracted as described (42). Bacterial 16S rDNA was amplified from the purified DNA using Denville Taq DNA polymerase (Fisher Scientific, Pittsburgh, PA) and primers 10F and 1507R and subjected to a two-stage polymerase chain reaction, as described (41). The partial 16S rDNA sequences were assembled for each bacterial isolate from two overlapping sequences and used to identify sequences with highest identities among the National Center for Biotechnology GenBank.

RESULTS

Inhibition of pea root infection in growth pouches with CWE. When inoculated with *F. solani* spores, $\geq 93\%$ of Little Marvel and Alaska pea roots developed visible lesions within 24 h (Table 1). Despite uniform inoculation of spores over the entire root surface, the initiation of infection was localized to the site which was the region of elongation at the time of inoculation, and lesions remained localized to this ≈ 1 -cm section of root (Fig. 1A and B) (1). As described in previous studies (17,18), in most inoculated roots ($>90\%$), the apical region (≈ 0.5 cm from the apex) housing the root cap and apical meristems escaped infection despite the presence of the pathogen within adjacent tissue. In the

TABLE 1. Impact of compost water extract (CWE) on infection of pea roots by *Fusarium solani* and *Phoma pinodella*^y

Treatment ^z	Number of seedlings		Infected (%)
	Noninfected	Infected	
Growth pouch			
'Alaska' pea			
Water	129	0	0 a
CWE	124	0	0 a
<i>F. solani</i>	2	127	98 b
CWE + <i>F. solani</i>	121	3	2 a
'Little Marvel' pea			
Water	30	0	0 a
CWE	30	0	0 a
<i>F. solani</i>	2	28	93 b
CWE + <i>F. solani</i>	30	0	0 a
'Alaska' pea			
Water	10	0	0 a
CWE	10	0	0 a
<i>P. pinodella</i>	0	10	100 b
CWE+ <i>P. pinodella</i>	9	1	10 a
Sand ('Alaska' pea)			
Water	40	0	0 a
CWE	40	0	0 a
<i>F. solani</i>	0	40	100 b
CWE + <i>F. solani</i>	40	0	0 a
Soil ('Alaska' pea)			
Water	20	0	0 a
CWE	20	0	0 a
<i>F. solani</i>	0	20	100 b
CWE + <i>F. solani</i>	11	9	45 c

^y Values reflect results from at least 10 replicate samples for each treatment. Percentages of infection with the same letter are not significantly different according to Fisher's exact test ($P < 0.05$). Plants grown in sand or soil were monitored for 4 weeks after inoculation.

^z CWE = compost water extract.

absence of damage to the meristems, root growth continued in parallel with control roots despite the localized infection. New infections did not develop sequentially within new regions of elongation as they emerged, presumably due to activation of a systemic resistance response by the initial infection (17). In roots in which the meristem region was invaded (Fig. 1B, white arrow), root growth ceased within 16 to 24 h after infection.

In control samples, in the presence of CWE alone, no obvious phenotypic changes were evident in pea seedlings (Fig. 1C). However, development of lesions on inoculated seedlings of both tested cultivars was reduced from ≥ 93 to 2%; no significant differences between cultivars was observed. A similar reduction in frequency of infection, from 100 to 10%, occurred in CWE-treated seedlings inoculated with *P. pinodella* (Table 1). The protection was retained over a 4-week period in moist growth pouches maintained at 25°C, conditions highly favorable to disease development in pea (Table 1; Fig. 2D).

CWE used as a drench for roots of pea seedlings grown in sand, as in growth pouches, resulted in 100% protection (Table 1). Whereas 100% of Alaska seedlings inoculated with *F. solani* developed lesions, no infections occurred in seedlings treated with CWE at the time of inoculation, for up to 4 weeks after inoculation. When CWE was used as a drench for seedlings grown in a heavy clay soil, partial protection occurred in response to CWE (Table 1). Whereas 100% of inoculated seedlings developed root infections and ceased growth within 3 weeks in the absence of CWE, 45% developed infection in roots of seedlings drenched with CWE at planting.

Root growth and development in the presence of CWE. One explanation to account for the inhibition of root infection is that CWE contains nutrients or growth stimulants triggering changes in plant development that alter the normal coordination of spore germination and root susceptibility to infection. If so, then altered morphology and growth rate could be predicted to occur in pea and other species treated with CWE. This was tested by measuring rate of root growth, fresh weight, and lateral root development in pea, corn, and cucumber seedlings treated with CWE for 3 weeks. No significant changes were detected in any of the test parameters (Supplemental Tables S1 and S2). The results did not support the hypothesis that altered root development in response to CWE underlies pea root resistance to infection.

Increased pea root infection in the presence of filtered CWE. An alternative explanation for the results is that CWE contains a soluble metabolite triggering a systemic defense response in pea. If so, then filtered CWE would be predicted to inhibit infection as well as unfiltered samples. The results did not support this prediction. In control experiments, filtered CWE added to pouches resulted in no visible changes in growth, development, or morphology in pea seedlings (Fig. 2A). However, lesion development in the presence of filtered CWE was increased from 3 to 100% of inoculated seedlings (Table 2). Moreover, there was a change in the nature of infection which has not been observed among previous studies (17,18,21,22,40). Instead of a single infection initiated only in the region of elongation and remaining localized at that site (Fig. 1B), lesion development was initiated within each new region of elongation that emerged as growth progressed (Fig. 2B). The root tip region housing the apical meristem and root cap escaped infection; therefore, root growth and development continued as in uninoculated control samples.

Cucumber root infection in the presence of CWE. Some compost extracts have been shown to inhibit disease by direct inhibition of fungal growth (43). Inhibition of spore germination or hyphal growth could account for the observed changes in pea root infection in response to CWE. If so, then a similar effect might be expected to occur in a related fungal pathogen infecting a different plant host. Inoculating cucumber roots with spores of *F. solani* f. sp. *cucurbitacae* resulted in 100% infection in the growth pouch assay (Table 3). In the presence of CWE, however,

unlike in pea inoculated with *F. solani* f. sp. *pisi* in the presence of CWE, percentage of infection was reduced by only 38% (Table 3).

Fungal growth inhibition in response to CWE. To examine the possibility that a species-specific inhibition of fungal growth is a factor in the divergent results, responses of the *Fusarium* isolates infecting pea or cucumber to CWE were compared directly using a radial growth assay (43). The diameters of hyphal growth in control plates for both fungi were 30 ± 1 and 29 ± 1 mm, respectively, and the results were not significantly different between the two isolates. In the presence of CWE, growth for *F. solani* f. sp. *pisi* was reduced by 100% to 0 mm and *F. solani* f. sp. *cucurbitacae* was reduced by 97% to 1 ± 1 mm. The differences between isolates were not significantly different. The inhibitory effect was lost when CWE was filtered to remove microbial populations: Both isolates exhibited radial growth of 30 ± 1 mm in the presence of filtered CWE. The results did not support the hypothesis that the differences in protection resulted from differential toxicity of CWE to the fungal isolates under the conditions used.

Border cell trapping on root surfaces in CWE-treated pea but not cucumber. Pea roots grown in pouches with CWE exhibited a distinctive appearance not seen in previous studies: a prominent aggregation of border cells and other material was found to form a sheath surrounding the elongating roots (Fig. 3). The phenotype was evident without microscopic analysis within 24 h after adding CWE, and remained stable over the period of

Fig. 1. Impact of compost water extract (CWE) on pea root infection by *Fusarium solani*. **A**, Water control. Arrow denotes the site of the root tip at the time of inoculation; black triangle denotes the region of elongation at the time of inoculation. **B**, Pea roots were inoculated uniformly with *F. solani* spores and maintained in growth pouches at 25°C for 13 days. Localized lesion development is evident at the site that was the region of elongation at the time of inoculation. Most roots do not become infected at the root tip containing the root cap and apical meristems; therefore, root growth continues at the same rate as controls. Infection at the tip (white arrow) results in cessation of growth within 24 h. **C**, Pea roots grown in CWE alone reveal no changes in root growth or development, compared with control samples grown in water. **D**, Pea roots inoculated uniformly with *F. solani* spores in the presence of CWE develop no lesions. Photos show results 2 weeks after inoculation. Scale bar: 1 cm.

Fig. 2. Impact of filtered compost water extract (CWE) on pea root infection by *Fusarium solani*. Arrow denotes the site of the root tip at the time of inoculation; black triangle denotes the region of elongation at the time of inoculation. **A**, Pea roots grown in the presence of filtered CWE and maintained in growth pouches at 25°C for 14 days. **B**, Pea roots grown in the presence of filtered CWE and *F. solani*. Pea roots infected with *F. solani* are blackened due to repetitive infections initiated in the region of elongation but continue to grow because the root tip region escapes infection. Scale bar: 1 cm.

TABLE 2. Impact of filtered compost water extract (CWE) on infection of pea roots by *Fusarium solani*^z

Treatment	Number of seedlings		Infected (%)
	Noninfected	Infected	
Water	30	0	0 a
CWE	30	0	0 a
<i>F. solani</i>	0	30	100 c
<i>F. solani</i> + CWE	26	4	13 b
Filtered CWE	30	0	0 a
<i>F. solani</i> + filtered CWE	0	30	100 c

^z Values for each treatment reflect results from 30 replicates for each treatment. Percentages of infection with the same letter are not significantly different according to Fisher's exact test ($P < 0.05$).

TABLE 3. Impact of compost water extract (CWE) on infection of cucumber roots by *Fusarium solani*^z

Treatment	Number of seedlings		Infected (%)
	Noninfected	Infected	
Water	44	6	6 a
CWE	46	4	4 a
<i>F. solani</i>	0	50	100 c
<i>F. solani</i> + CWE	19	31	62 b

^z Values reflect results from a total of 50 replicates for each treatment. Percentages of infection with the same letter are not significantly different according to Fisher's exact test ($P < 0.05$).

observation. Border cells are produced only by the root cap, and disperse into the environment in response to free water. Even under conditions in which border cells are present on the root cap periphery, the surface of the region of elongation remains free of border cells (Fig. 4A). In the presence of CWE, however, the region of elongation in pea roots instead was covered in a continuous sheath that extended into the regions of lateral root emergence and did not disperse readily in water (Fig. 4B). Microscopic examination revealed that the adhering sheath appeared to consist of border cells interspersed with bacteria. Among single colonies isolated from sheath material dispersed from roots in response to vigorous agitation in water, *Acinetobacter* spp. (3) were identified as the predominant, culturable bacteria.

In contrast with pea roots, border cells of cucumber roots treated with CWE detached from the cap periphery as the root elongated, and no extended sheath was detected.

Fig. 3. Persistent mucilaginous sheath of material on pea roots grown in compost water extract. A granular matrix surrounding the root, well past the meristematic region and root cap at the apex, is apparent at low magnification. Scale bar: 5 mm.

DISCUSSION

We report here that an aerated CWE applied in early development is correlated with protection of pea seedlings against infection by *F. solani* and *P. pinodella* in growth pouches, even under warm, moist conditions highly conducive to root disease development. In cucumber inoculated with *F. solani*, <40% reduction in infection frequency occurred in response to CWE, even though CWE was equally inhibitory to mycelial growth of the cucumber and pea pathogens tested. Microscopic examination

Fig. 4. Border cell sheath extending past the region of elongation on pea roots inoculated with *Fusarium solani* in the presence of compost water extract (CWE). **A.** On roots of seedlings grown in water, a few border cells can be seen near the surface. **B.** On roots grown in CWE, a continuous sheath of border cells is evident within 24 h after inoculation, and the aggregate remains adhered to the root surface after 14 days (shown here) in moist growth pouches maintained at 25°C. Scale bar: 1 mm.

revealed a marked difference in the response of cucumber and pea seedlings to CWE that has not been reported in previous studies: The protection against infection in pea roots was correlated with the presence of an extended sheath of border cells interspersed with bacteria covering the primary site of susceptibility to *F. solani* and *P. pinodella*—the region of elongation. Border cells are programmed to separate from root tips, and comprise the bulk of organic matter released from young seedlings (11,21). Recent studies have revealed that border cells function in defense of roots in a manner parallel to that of mammalian white blood cells, which form mucilaginous extracellular traps comprised of extracellular DNA and antimicrobial proteins (22,40). Border cells provide a highly effective defense that protects the apical region housing the root meristems from infection and injury and also may be important for the maintenance of beneficial rhizosphere organisms including protozoa (39). Border cells of most species disperse into the environment as the root elongates, leaving the surface of the newly synthesized region of elongation accessible to colonization. It will be of interest in future studies to examine the possibility that the presence or absence of this sheath on roots treated with CWE underlies the fact that regions of elongation in pea but not cucumber were protected from infection.

In this study, the border cells within extended sheaths on CWE-treated roots were found to be coaggregated with bacterial populations in which *Acinetobacter* spp. were predominant. This genus is widely distributed in soil, water, sewage, rhizosphere, and compost (6,15). *Acinetobacter* spp. can be selected from soil and water under conditions similar to those used for preparation of the test CWE, including the use of vigorously aerated mineral-rich medium maintained for 24 to 48 h at 30 to 40°C and pH 5.5 (3). Recent studies suggest potential for these bacteria as a biological control agent for control of *Ralstonia solanacearum* wilt on tomato (44). Remarkably, some *Acinetobacter* spp. trigger coaggregation with other microbial species (27,37). It will be of interest to explore the possibility that microbial coaggregation properties interface with the “trapping” capacity of border cells, and that this plays a role in the observed protection of susceptible regions of pea roots by an extended border cell sheath. If this early rhizosphere modification can be shown to remain stable and exert protection long enough to harvest a crop, then further studies to define why it happens in pea but not cucumber, and is so effective in sand but not soil, will be warranted.

We described the use of a rapid, reproducible assay to measure direct effects of compost extracts on root structure and function in

early stages of development. Water and mineral uptake, as well as susceptibility of plants to soilborne pathogens and symbionts, are processes controlled primarily by specialized tissues within the apical regions of young roots (1,10,17,21). Root–rhizosphere associations established early in development may play a key role in plant health (9,35). Our results suggest that it may be possible to use the growth pouch assay to discern differences in effectiveness of specific compost preparations on crop species early in seedling development to evaluate possible underlying mechanisms for the differences and, thereby, assist in engineering plant growth media that optimize the benefits of compost applications.

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