Transmission of *Pantoea ananatis*, Causal Agent of Center Rot of Onion, by Tobacco Thrips, *Frankliniella fusca*

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ABSTRACT

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Center rot of onion, caused by *Pantoea ananatis*, was first reported on onion in Georgia in 1997 and has continued to reduce yields and cause postharvest losses. In a previous study, we developed a nondestructive assay that demonstrated an association between *P. ananatis* and approximately 10% of the tobacco thrips, *Frankliniella fusca*, surveyed. In this study, we report that all strains of *P. ananatis*, isolated from surface-sterilized, crushed thrips, were pathogenic when inoculated onto greenhouse-grown onion plants. Furthermore, when 6 to 12 thrips harboring populations of *P. ananatis* of 1×10^3 CFU ml⁻¹ or greater were placed on healthy onion seedlings to feed, disease transmission occurred in 52% of the plants challenged. Incubation periods ranged from 4 to 9 days. Bacteria isolated from symptoms typical of those associated with center rot were characterized and identified as *P. ananatis*. In contrast, an equal number of plants remained healthy for up to 28 days after being exposed to the same number of tobacco thrips that were identified as being free of *P. ananatis*.

Additional keywords: *Allium cepa*

Center rot of onion, caused by *Pantoea ananatis* (Serrano) Margaret, was first described in Georgia in 1997. The disease was devastating and accounted for 100% loss in some fields (15). In addition to onions, the bacterium has been reported to infect cantaloupe, *Eucalyptus*, honeydew melons, muskmelons, pineapple, and Sudan grass (1,4-6,26,27,36). It also has been associated with the condition known as graywall of tomato (28). Since 1997, *P. ananatis* has been responsible for sporadic losses in onion in Georgia, but it has been particularly severe in certain late-maturing cultivars. Symptoms generally include white streaks with water-soaked margins running the length of the leaf. Over time, the streaks may darken to a light brown or gray color. Symptoms first appear as one or two infected leaves in the center of the plant. As the disease develops, severely infected plants may wilt. Unfortunately, the invasion of bulb tissues does not appear to be related to the degree of foliar disease severity. Asymptomatic, mildly diseased,

and severely diseased plants are all prone to postharvest rots associated with *P. ananatis*. By itself, *P. ananatis* does not cause an extensive amount of damage, and based on results from growth on CVP medium (7), it does not have pectinolytic enzymes. However, infected bulbs frequently are colonized by secondary microbes that liquefy tissues and produce a foul odor. Consequently, center rot is not only an important disease in the field, but also is of great consequence as part of a postharvest disease complex.

Our goal is to develop management strategies that reduce losses caused by center rot. One approach to control plant diseases is to identify and eliminate the source of inoculum (32). To date, we have identified seed (33) and weeds (13) as potential sources of inocula. In previous work, we demonstrated an association between tobacco thrips *Frankliniella fusca* and *P. ananatis* as a member of its internal microflora, and described a nondestructive sampling method to identify thrips with internal populations of bacteria (37). In this study, we provide evidence for thrips transmitting virulent strains of *P. ananatis* to onion. Although thrips transmit viruses such as *Tomato spotted wilt virus* (2), to our knowledge this is the first report of members of the order Thysanoptera serving as a vector of a plant pathogenic bacterium.

MATERIALS AND METHODS

Collection of tobacco thrips. Adult tobacco thrips were collected from onion plants at the University of Georgia, Blackshank Research Farm in Tifton, on 17, 19, 20, and 30 April and 2 May 2001, and on 29 January, 6 February, 6, 12, 14, 19, and 27 March, and 15 April 2002. Adult tobacco thrips also were collected from peanut blossoms from the Rigdon Farm on 20 September 2001 and at the Blackshank Farm on 7 August and from peanut leaves at the Blackshank Farm on 14 August 2002.

Identification of positive thrips. Peanut leaflets were collected from thrips-free plants grown in the greenhouse or in growth chambers and processed as previously described (37). Briefly, leaflets were soaked in 10% sodium hypochlorite for 30 s and then transferred to 70% ethanol for 30 s. After rinsing in sterile PBST (phosphate-buffered saline, 0.01 M K₂HPO₄, 0.85% NaCl, and 0.02% Tween 20) at pH 7.0, leaflets were placed in labeled 1.5-ml microcentrifuge tubes. Adult thrips were surface-sterilized using the same procedures and placed individually into tubes to feed on peanut leaflets. After 24 h, thrips were removed and maintained in new, sterile microcentrifuge tubes and labeled accordingly, i.e., matching the corresponding tube containing the leaflet from which they were removed. Sterile PBST (1.0 ml) was added to the tubes containing peanut leaflets. Tubes were vortexed for 30 s, and 10⁻²-fold serial dilutions (1:9) were made in PBS. Aliquots of 0.1 ml were spread onto tryptic soy broth agar (TSBA) plates and incubated at 28°C for 48 h. As determined previously (37), thrips from leaflets with populations of *P. ananatis* of 1×10^3 CFU ml⁻¹ or greater were selected as positive. To verify the surface sterilization of thrips, 1.0-ml samples from five replicates of 10 thrips processed as above were plated onto TSBA. After the surface rinse was removed and plated, thrips were placed in another 1.0-ml tube of PBST, triturated as described previously (37), and the resulting suspension plated on TSBA. Bacterial colonies were counted after 48 h incubation at 28°C. Numbers of bacterial colonies isolated from external surfaces of thrips bodies and from crushed thrips were compared.

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Isolation and cultivation of *P. ananatis* from surface-sterilized thrips. A subset of adult tobacco thrips ($n = 16$) were surface-sterilized, identified as positive for carrying populations of *P. ananatis* by the peanut leaf assay described above, and crushed in 1.0 ml of PBST. The resulting suspensions were used to prepare serial dilutions (1:9) in PBST. Aliquots (0.1 ml) from the resulting suspensions were spread on the surface of TSBA plates. Plates were incubated at 28°C for 5 days, and colonies were enumerated. Single colonies of *P. ananatis* were selected, purified by sequential transfers on TSBA, and stored in 15% glycerol at -72°C as strains obtained from thrips. These strains were used to inoculate onion plants grown in the greenhouse, as previously described (13,15).

Transmission studies. A subset of adult tobacco thrips was surface-sterilized and identified as positive or negative for carrying populations of *P. ananatis* by the peanut leaf assay. Onions were grown in the greenhouse as previously described (13,15). For the transmission studies, 10-cm-diameter plastic pots containing 6- to 12-week-old onions were carefully placed into clear, plastic, 3.78-liter jars to avoid damaging leaves. Jars were tightly capped with rubber bands and 15 × 15 cm pieces of lightweight, polyester material with a

knit too fine for thrips to escape. Depending on the number of positive thrips detected, 6 to 12 thrips were placed on individual onion plants. A similar number of thrips testing negative for *P. ananatis* were placed on separate plants to serve as a negative control. Thrips testing negative for *P. ananatis* were used in the transmission studies as negative controls. Again, depending on the number of positive thrips detected, two to five plants were used in each trial, with a corresponding number of plants used as negative controls. Jars containing test plants were maintained in the laboratory at approximately 27°C under plant-growth light units (Helios, Percival Manufacturing Co., Boone, IA). Transmission trials were repeated five times. Thrips collected from onion plants were used in trials one through three, and when onions were unavailable, thrips collected from peanut plants were used for trials four and five.

Characterization and identification of bacteria. Bacteria from crushed thrips, from peanut leaflets from the disk assay, or from symptomatic plants after thrips feeding were isolated onto plates of TSBA. Suspect colonies were identified as previously described (13,15). Briefly, bacterial colonies were characterized by the presence of a yellow pigment and morphology on TSBA, Gram-reaction, utilization of glucose in an oxidative and fermentative manner, fatty acid profile, indole reaction, and nitrate reductase reaction (13). In addition, PCR was conducted using primers PanITS1 and EC5, described previously (13), which amplify specific DNA segments of the 16S-23S rDNA internal transcribed spacer (ITS) region of *P. ananatis*. Finally, strains were inoculated onto onion seedlings and pathogenicity was confirmed as previously described (13,15).

RESULTS

Isolation of *P. ananatis* from surface-sterilized, crushed thrips. Populations of *P. ananatis* recovered from surface-sterilized, crushed thrips were similar to what was recovered previously (37). In a subset of thrips ($n = 16$), *P. ananatis* populations ranged from 1.1×10^3 CFU ml⁻¹ to

1.3×10^5 CFU ml⁻¹, and the mean was 1.64×10^4 CFU ml⁻¹. Colonies recovered from surface-sterilized, crushed thrips were yellow, gram negative, utilized glucose as a facultative anaerobe, positive for indole, and negative for nitrate reductase. These characteristics are typical for *P. ananatis* and help differentiate it from most strains of similar species such as *P. stewartii* and *P. agglomerans*. In addition, thrips strains had a fatty acid profile typical of *P. ananatis*, and their DNA from the ITS region amplified with the *P. ananatis* primers. All strains of *P. ananatis* isolated from thrips ($n = 7$) produced typical center rot symptoms when inoculated onto greenhouse-grown onion seedlings. Onion plants inoculated with sterile water remained healthy.

Transmission studies. Tobacco thrips successfully transmitted *P. ananatis*, and typical center rot symptoms developed in 52% of the plants challenged in four out of the five trials (Fig. 1). Of those plants developing center rot, the mean incubation period was 6.75 days from the time plants were first exposed to thrips (Table 1). All plants challenged with thrips deemed negative for *P. ananatis* remained healthy up to 28 days after exposure to thrips, at which time tests were terminated. Colonies recovered from onion plants with typical center rot symptoms were yellow, gram negative, utilized glucose as a facultative anaerobe, positive for indole, and negative for nitrate reductase. In addition, they had a fatty acid profile typical of *P. ananatis*, and their DNA from the ITS region amplified with the *P. ananatis* primers.

DISCUSSION

We previously demonstrated an association between *P. ananatis* and the internal microflora of tobacco thrips (37). In this study, we demonstrated that the bacteria recovered from surface-sterilized, crushed thrips were pathogenic to onion. Furthermore, we demonstrated that thrips, identified by the peanut leaf assay as harboring populations of *P. ananatis*, served as a vector of this plant-pathogenic bacterium and succeeded in transmitting the disease to 52% of the plants challenged in 80% of

Fig. 1. Transmission of *Pantoea ananatis* to onion, *Allium cepa*, by tobacco thrips, *Frankliniella fusca*. **A**, Side-by-side comparison of plants challenged by tobacco thrips identified either as lacking (left) internal populations of *P. ananatis* or harboring (right) populations greater than 1×10^3 CFU ml⁻¹. **B**, Higher magnification of plant on right, to which tobacco thrips transmitted the bacterium and which displays typical center rot symptoms.

Table 1. Results of transmission of *Pantoea ananatis* to onion, *Allium cepa*, by surface-sterilized tobacco thrips, *Frankliniella fusca*

Trial	Source ^b	+ Thrips ^a transmission data			- Thrips ^a transmission data		
		Thrips ^c (no.)	Transmission (%)	Days ^d (no.)	Thrips ^c (no.)	Transmission (%)	Days ^d (no.)
1	O	9	50	7	9	0	28
2	O	6	0	28	6	0	28
3	O	6	50	7	6	0	28
4	P	12	60	9	12	0	28
5	P	6	100	4	6	0	28

^a + or - Indicates whether or not *P. ananatis* was detected in the peanut leaflet disk assay.

^b Source of thrips from O (onion) or P (peanut).

^c Values indicate how many adult thrips were placed on each onion plant.

^d Indicates either number of days until symptoms developed or, if no symptoms developed, number of days plants were maintained until the test was terminated.

the five trials attempted. In contrast, disease never developed the negative controls, i.e., onion plants infested with thrips deemed negative for the bacterium. These results further support the peanut leaf disk assay (37) as a nondestructive technique for identifying thrips that harbor virulent populations of *P. ananatis*. Peanut leaves were used in the assay primarily on the basis of convenience, because a thrips colony, maintained at our research station for use in tomato spotted wilt studies, already existed on peanut plants. Furthermore, peanut grows faster than onion, and detached peanut leaves are easier to disinfect, fit inside the plastic chambers, and have greater structural integrity when bacteria are washed off the surface.

Ingress and infection are crucial events in the life cycle of a plant pathogen; thus the identification of a specific infection court and transmission of a pathogen may have implications for the development of disease management strategies. There is no evidence for direct penetration of intact plant tissues by bacteria. Consequently, natural openings and wounds are important to bacterial ingress into plants (19,31). Closely related phytopathogenic bacteria such as *P. stewartii* and *P. tracheiphila* are known to overseason in the gut of, and are vectored by, corn flea beetles and cucumber leaf beetles, respectively (20,38). In addition, saprophytic strains of an *Erwinia* species were reported to inhabit and proliferate in the hindgut of western flower thrips, *Frankliniella occidentalis* (8,9). The authors never fully identified the bacterium to species, but suggested it was closely related to *Enterobacter agglomerans*. The taxonomy of this species is in flux, and the bacterium has been placed into the *Enterobacter agglomerans*-*Erwinia herbicola* complex. Some have classified the group into the genus *Pantoea* (24,30), which is the convention that we follow in this paper. In any account, *P. agglomerans* has been reported not only in association with thrips but also with grasshoppers (10,23), termites (25), beetles (3), aphids (18), and flies (21,22). There is disagreement within the scientific community as to whether these relationships are transient (21) or are examples of facultative symbionts (18,22). The majority of these studies do not go into depth regarding the population dynamics of the bacterium and therefore are inconclusive as to the exact relationship between insect and bacterium.

In addition to its close relatives, *P. ananatis* also has been reported as an inhabitant of the gut of insects. It has been reported from brown planthoppers (*Nilaparvata lugens*) (34) and mulberry pyralids (35). This is not surprising since the genus *Pantoea* is classified in the family Enterobacteriaceae, which contains *Escherichia coli*, the quintessential gut bacterium. However, none of the reports indicated that the insects served as a vector or that the

bacteria caused a plant disease. Rather, the studies dealt with the insect and the bacterium either as a symbiont or as an entity that reduced the hardness of the insect.

In this paper, we report the first instance of thrips serving as a vector for a plant-pathogenic bacterium. Transmission by thrips could explain a number of observations we have made regarding the epidemiology of center rot of onion. For example, in some field plots we observed a primary disease gradient, which was present in two separate years (12). Although gradients could be explained by several factors, such as transmission by mechanical equipment (16) or a local source of inoculum such as weeds (16,17), similar disease gradients also were observed in pepper fields where plants were infected with tomato spotted wilt (14), which also is transmitted by thrips. A gradient would not likely exist if the inoculum was seedborne or wind-blown from a great distance.

Another observation that could be explained by the thrips transmission theory is that center rot of onion is unaffected by spraying with fixed-copper bactericides. In lab surveys, *P. ananatis* is just as sensitive to copper compounds as *Pseudomonas viridiflava* (39), which causes bacterial streak of onion but is controlled quite effectively with copper bactericides (11). This discrepancy could be explained if *P. viridiflava* relied on epiphytic colonization of onion leaves and ingress through natural openings such as stomata. Under those circumstances, *P. viridiflava* would come into contact with a protective barrier of copper compounds sprayed on onion foliage. On the other hand, if *P. ananatis* was transmitted by thrips, the bacterium would be less likely to be exposed to bactericides in the infection court, as those tissues would not be covered by a pesticide applied in a conventional manner. Thrips colonize onions by feeding on succulent, growing tissues near the basal meristem of leaves. In the primary area of thrips feeding, the base of the leaf whorl, the leaves are folded upon one another creating several tightly wound or coiled layers. It is difficult if not impossible to protect this area with surface-protectant bactericides. It is likely that thrips feeding in this area create wounds that become contaminated with feces containing *P. ananatis*. Also, it is common for free water to flood and congest the tissues of the onion whorl. The presence of free water would enhance bacterial infection and movement up the leaf. Water congestion and other predisposing factors may increase the transmission rate that we observed under laboratory conditions. Also, the transmission rate in the field may be higher than what we observed in the laboratory. However, even at a transmission rate of 52% with an average of eight thrips per plant, the transmission rate is high enough to cause concern and

could account for disease levels observed in the field.

Finally, the appearance of the first symptoms of center rot and the development of disease over time may correspond to populations of thrips and the damage caused by thrips. Todd et al. (29) reported that seasonal fluctuations occurred in thrips populations and that peak populations of *F. fusca* adults in peanuts occurred 10 to 20 days after planting in April. This time period would correspond with peak levels of incidence of center rot of onion. However, further work is required to determine if the patterns of thrips' populations in peanut also exist in onion and are significant to development of center rot epidemics.

A number of other questions need to be answered before successful control measures can be developed. For instance, are other thrips species capable of harboring and transmitting *P. ananatis*? What life stage(s) of the thrips acquires the bacterium? Does the bacterium have a transient or permanent relationship with thrips? Does *P. ananatis* have a detrimental effect on tobacco thrips fecundity and longevity? Are the preferred location of tobacco thrips feeding and the infection court for *P. ananatis* the same location? Finally, do thrips play a role in secondary spread if the primary inoculum comes from seed or weeds?

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