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Biological Control of the Phytoparasitic Nematode *Mesocriconema xenoplax* on Peach Trees[1]

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Abstract: Seven fluorescent *Pseudomonas* spp. capable of inhibiting reproduction of *Mesocriconema xenoplax* have been isolated from soil sites that suppress both nematode multiplication and Peach Tree Short Life (PTSL). One of these seven strains, *Pseudomonas* sp. BG33R, inhibits *M. xenoplax* multiplication in vivo and egg hatch in vitro. *Mesocriconema xenoplax* populations on peach seedlings inoculated with BG33R and planted into soil-solarized field plots remained at or below the economic threshold for nematicide treatment in South Carolina for nearly 18 months. Soil solarization alone induced a shift toward a microbial community that was suppressive to nematode multiplication. Additionally, five Tn5 mutants of BG33R, lacking the ability to kill eggs, have been generated. The Tn5 insertion site in each mutant has been cloned and sequenced. DNA sequence analysis has revealed a high degree of homology to several genes of interest because of their potential involvement in the production of the egg-kill factor. These Tn5 egg-kill negative mutants also no longer produce protease or salicylic acid while producing nearly twice the amount of fluorescent siderophore as the wild type parent.

Key words: bacteria, biological control, *Mesocriconema xenoplax*, peach, *Prunus persica*, rhizobacteria, rhizosphere, ring nematode.

The migratory ectoparasitic ring nematode, *Mesocriconema xenoplax*, is a major factor in Peach Tree Short Life (PTSL), a syndrome that results in premature mortality of peach trees in the southeastern United States (Nyczepir et al., 1983). Peach tree short life and the associated ring nematode are most common in sandy loam soils and on sites where peaches or other stone fruits have been grown previously. The primary control method for PTSL has been the application of pre- and post-plant nematicides. However, since the early 1970s many effective nematicides have been shown to be hazardous to human health, prompting their removal from the market and leaving cultural practices and ring nematode-tolerant rootstocks as the primary method of PTSL control (Reighard et al., 1996). As a consequence, peach tree losses due to PTSL have increased to nearly three times the level experienced when the most effective nematicides were available (Miller, 1988, 1994; Miller, pers. comm.). In recent years, a potential multipurpose rootstock (Guardian™), which provides greater tolerance to the ring nematode than the commercial standard, Lovell, has been identified (Beckman et al., 1996; Okie et al., 1994; Westcott and Zehr, 1991).

An alternate approach to ring nematode control can be found in the use of microbial biocontrol agents. Soilborne microbes have been proposed as biocontrol agents for fungal pathogens since the mid-1970s (Schippers et al., 1987a, 1987b), yet only recently have investigators studied their potential to control plant-parasitic nematodes (Becker et al., 1988; Oostendorp and Sikora, 1989; Racke and Sikora, 1985; Sikora, 1988; Zavaleta-Mejia and Van Gundy, 1982). For example, *Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp., *Pasteuria penetrans*, and several fungal species all have been shown to reduce damage to plants caused by a wide variety of plant-parasitic nematodes (Becker et al., 1988; Jafee, 1992; Kerry and Bourne, 1996; Stirling, 1991).

The majority of nematode biocontrol research has focused on endoparasitic nematodes that feed after entering the root. Control of nematodes with this type of feeding habit is exceedingly difficult for root-colonizing microbes. In contrast, the ring nematode feeds on the surface of roots, exposing all phases of its life cycle to the rhizosphere environment. Consequently, control of this nematode using rhizosphere-inhabiting microbes is particularly attractive.

In our search for microbial biocontrol agents of *M. xenoplax*, attention was focused on the fluorescent pseudomonads because of this group's abundance in the rhizosphere (Kluepfel, 1993; O'Sullivan and O'Gara, 1992) and its demonstrated biological control activity against root-infecting microorganisms (Cook, 1993; O'Sullivan and O'Gara, 1992; Schippers et al., 1987a, 1987b), including plant-parasitic nematodes (Becker et al., 1988; Cronin et al., 1997; Hackenberg et al., 2000; Kerry, 2000; Kloepper et al., 1992; Stirling, 1991).

Pseudomonas sp. BG33R, isolated from a peach orchard site suppressive to PTSL, was demonstrated to suppress ring nematode reproduction in field soil under greenhouse conditions and to inhibit egg hatch of the ring nematode in vitro (Kluepfel et al., 1993; Westcott and Kluepfel, 1993). Bacteria have been shown to affect nematode egg hatch by a variety of mechanisms including production of toxins, and lipolytic, proteolytic, or chitinolytic enzymes (Parker et al., 1988). For example, *Bacillus thuringiensis* produces a number of exotoxins that have been shown to kill eggs of a ruminant nematode and of *Meloidogyne* spp. (Bottjer et al., 1985; Prasad et al., 1972). Other control mechanisms include the production of compounds generally toxic to nematodes, such as ammonia, cyanide, hydrogen sulfide, or volatile fatty acids (Kerry, 1990). In addition, at least one of the large variety of antibiotics produced by fluorescent pseudomonads, 2,4-diacetylphloroglucinol (Fravel, 1988; Keel, 1992; Thomashow and Weller,

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1991), has been shown to reduce juvenile mobility and enhance egg hatch of the potato cyst nematode, *Globodera rostochiensis* (Cronin et al., 1997). When this enhanced egg hatch occurs away from the host roots, the juvenile is left in a vulnerable position—often succumbing to starvation before an acceptable food source is found.

Solarization field trials with Pseudomonas *sp. BG33R*: To enhance survival and efficacy of BG33R in the field, we have combined the use of preplant solarization with BG33R inoculation of trees at time of planting. Solarization is the hydrothermal heating of soil accomplished by covering moist soil with clear polyethylene plastic during the summer months. Although heating appears to account for the majority of microbial killing, non-thermal factors such as light, water potential, and accumulation of antagonistic volatile compounds may be involved in the microbial alterations observed at depths of up to 90 cm (Katan and DeVay, 1991).

Numerous reports have shown that the biotic communities in solarized soil are significantly altered, both quantitatively and qualitatively. Typically, populations of Gram-negative bacteria (including fluorescent pseudomonads) plummet while many of the spore-forming bacteria and thermo-tolerant fungi and actinomycetes appear to be unaffected (Gamliel and Katan, 1991; Katan, 1996). A number of weeds and soilborne pests, including populations of plant-parasitic nematodes, also are significantly reduced in solarized soils.

Our intent in these solarization field trials was to significantly reduce or alter the indigenous microbial community with solarization so as to diminish competition, elevate rhizosphere populations, increase survival, and enhance biological control activity of the introduced microorganism *Pseudomonas* sp. BG33R.

In 1995, we initiated our first soil solarization test at the U.S. Department of Agriculture (USDA) Southeastern Fruit and Tree Nut Research Laboratory in Byron, Georgia. In this trial we examined changes in the microbial community in four different soil treatments—solarized, solarized plus BG33R, non-solarized, and non-solarized plus BG33R. Solarization of the orchard site prior to peach tree planting resulted in a significant alteration of the soilborne microbial community as compared to non-solarized soil as revealed by gas chromatographic analysis of fatty acids extracted directly from the soil. The altered microbial populations in the solarized plots, combined with addition of the biological control agent, *Pseudomonas* sp. BG33R at the time of planting, contributed to a significant reduction in the *M. xenoplax* population density as compared to non-solarized plots inoculated with BG33R and non-solarized plots that did not receive BG33R applications. Ring nematode populations in both non-solarized and solarized plots inoculated with BG33R were significantly reduced as compared to both BG33R inoculated and non-inoculated plots that had not been solarized. In addition, ring nematode populations were found to be significantly lower in solarized plots that were subsequently inoculated with BG33R than plots that were only solarized. This enhanced reduction in ring nematode populations in plots where BG33R was introduced was observed to decrease with time. This may be the result of the fact that BG33R was applied only at the time of planting and 8 months later.

The suppressive effect of solarization on ring nematode populations was long lasting. In fact, ring nematode numbers remained at or below detection limits for nearly 5 months after removal of the plastic in solarized plots. Up to 17 months after solarization, ring nematode populations were significantly lower in solarized plots as compared to non-solarized plots. During this period of time, the nematode population was at or below economic nematicide treatment thresholds as recommended for South Carolina peach growers. Twenty-four months after initial solarization, ring nematode populations in solarized plots were no longer different from those in non-solarized plots. Control of the nematode populations for 17 months post solarization/inoculation was similar to the ring nematode control previously reported using methyl bromide fumigation (Sharpe et al., 1989).

A second solarization trial was established at the same USDA field station in 1998. From June through August 1998 temperatures under the plastic ranged from 33.3 °C to 48 °C at a 10-cm depth. At a depth of 30 cm, a high of 37.2 °C was reached during this same period. Through October 1998, temperatures ranged from 35 °C to 41 °C at a depth of 10 cm. Nine weeks after the commencement of solarization, we observed that fluorescent pseudomonad populations had dropped below detection limits in solarized plots while total aerobic bacterial populations in solarized vs. unsolarized soils differed little over the solarization period. The effects of each of these four soil treatments on ring nematode populations were similar to those observed in the first solarization field trial described above.

In the second solarization trial, we also established a micro-jet irrigation system in the orchard through which we delivered the biocontrol agent, BG33R, at monthly intervals (6 to 8 times/year and at the time of planting) at a rate of approximately 3 liters of 10^7 cells/ml/tree. We hypothesize that by delivering BG33R at regular intervals to trees established in solarized soils, population levels and the resulting effectiveness of the introduced biocontrol agent will be enhanced. Initial data from both trials support this line of reasoning. If this hypothesis is confirmed, a practical approach to long-term integrated control of plant-parasitic nematodes would be realized.

Molecular examination of Pseudomonas *sp. BG33R*: We have cloned and sequenced the Tn5 transposon insertion site in each of five BG33R Tn5 mutants that lack the ability to kill ring nematode eggs in vitro (Wechter

et al., 2001). When these sequences were compared to the NCBI DNA sequence database, several interesting homologies were revealed. For example, the Tn5 insertion site in BG33R mutant 1122 yielded a significant match to the *rtp*A gene from *Pseudomonas tolaasii* at both the nucleotide level and amino acid level (Murata et al., 1998). This is significant because *rtp*A is a homolog of *lem*A, *rep*A, *apd*A, and other sensor kinases involved in two-component regulation of secondary metabolites, such as antibiotics, in numerous bacterial genera. Toxin production in *P. tolaasii*, as well as siderophore and pyoverdine production in *P. fluorescens*, has been shown to be under the control of similar two-component regulators (Hrabak and Willis, 1992; Liao et al., 1996). The sensor kinase gene in mutant 1122, as well as the other gene homologies discovered in the other four egg-kill minus mutants, will be useful as we move toward identification and characterization of the egg-kill factor produced by BG33R.

Biochemical characterization of the egg-kill factor: We have devised a purification strategy that results in a 50-fold increase in purity of the egg-kill factor while still retaining its biological activity (Leverentz, 1998; Leverentz et al., 1995). Further HPLC purification of this factor using acidified organic solvent extractions and polar-column elution conditions have facilitated collection of a single fraction found to contain salicylic acid. Interestingly, none of the Tn5 egg-kill minus mutants possessed the salicylic acid peaks detected during analysis of the BG33R wild-type strain. Commercial preparations of purified salicylic acid did not kill eggs of the ring nematode. However, salicylic-treated eggs were dramatically altered in their development. The involvement of salicylic acid in the BG33R egg-kill phenotype is currently being examined in our laboratory.

Conclusions

The use of rhizosphere-inhabiting microbial biocontrol agents for management of such migratory ectoparasitic nematodes as *M. xenoplax* offers a viable option for the control of plant-parasitic nematodes in the absence of chemical nematicides. Here we have shown that pre-plant solarization followed by repeated inoculation of peach trees affords effective control of *M. xenoplax*. Because PTSL symptoms are not manifested until 3 to 5 years post planting, it is necessary for the biological control agent to maintain elevated populations over a period of years. Repeated application of BG33R through the irrigation system will be a cost-effective and simple approach to maintaining the required, long-term effective populations of BG33R in the peach rhizosphere.

We have cloned and sequenced five genes from BG33R that are involved in production of the egg-kill factor. We are now preparing to examine their in-situ expression during BG33R growth in the peach rhizosphere. In addition, we are continuing our efforts to biochemically characterize this water-soluble, extracellular egg-kill factor. By taking this comprehensive approach (i.e., ecological, biochemical, and genetic analysis) in the investigation of BG33R's egg-kill phenotype, we anticipate moving closer to the development of a viable commercial product.

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