8-2007

PEACH TREE ROOT DEMOGRAPHY AND SOIL MICROBIAL CHARACTERISTICS IN PEACH REPLANT SOILS

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PEACH TREE ROOT DEMOGRAPHY AND SOIL MICROBIAL CHARACTERISTICS IN PEACH REPLANT SOILS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Plant and Environmental Science

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by
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August 2007

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ABSTRACT

Peach replant disorder (PRD) is a serious problem for peach growers throughout the United States. PRD refers to the pattern of reduced growth and yield observed in young peach trees planted on soil previously cropped with peaches. The conventional treatment for PRD, pre-plant soil fumigation with methyl bromide (a broad-spectrum biocide) adequately controls the disorder. This suggests that PRD results from harmful interaction(s) between the soil biotic community and peach roots. However, the specific causal agents of PRD are still unknown.

Methyl bromide is a known contributor to ozone depletion, and is only available to U.S. growers through Critical Use Exemptions. Therefore, the task of determining the specific cause of PRD has become increasingly important.

The overall objective of this research was to provide new insight into causes and potential management options for PRD. Our specific objectives were: (1) to determine whether prunasin-degrading, cyanide-producing bacteria were present in replant soils and could inhibit young peach tree growth, (2) to study fine root dynamics in treated and untreated replant soils using minirhizotrons, and (3) to compare the ability of traditional and non-traditional replant treatments to improve tree growth on a replant site.

One experiment was based on the theory that prunasin (a cyanogenic glycoside found in peach tissues) provides a substrate for cyanide production in

rhizobacteria, causing the growth reductions observed in PRD sites. We isolated prunasin- and amygdalin-degrading bacteria from the rhizosphere of seven-year-old peach trees. These isolates were identified and screened for cyanide production. Peach seedlings were inoculated with a prunasin-degrading, cyanogenic isolate in greenhouse experiments. The isolate was effective at colonizing the rhizosphere of seedling peaches, but no negative growth effects were observed.

Our field experiment compared tree growth and fine root (<1 mm diameter) dynamics of replanted peach trees under four PRD management strategies (methyl bromide fumigation, Telone C-17 fumigation, soil solarization, or systemic pre-kill of previous peach trees using glyphosate stem injections) and an untreated control, and two peach rootstocks (Guardian® and Lovell). Methyl bromide, soil solarization, and to a lesser extent Glyphosate and Telone C-17, increased stem diameter, decreased fine root production and mortality, increased time roots remained white, and increased fine root lifespan. Additionally, we observed differences in fine root dynamics between rootstocks.

While many questions remain concerning PRD and its management, we have gained valuable insight into the bacterial community of our replant site and the fine root dynamics as affected by soil treatments and rootstock selection. It is likely that a single treatment approach for PRD management will not sufficiently replace methyl bromide, but a multifaceted approach may be a viable option for the future.

DEDICATION

I dedicate this thesis to my wife Stephanie whose unending patience, encouragement, and love sustained me through this journey.

ACKNOWLEDGMENTS

There are many people who contributed to this thesis and I am indeed grateful to them all. My co-advisors, Dr. Wells and Dr. Reighard, made this work possible for me in everyway. Dr. Wells, thank you for your sound guidance, thorough teaching, constant accessibility, and the many resources you furnished for me. Thank you Dr. Reighard for your pomological expertise, steady encouragement, and for providing the finances and facilities that allowed me to perform this work.

I am very grateful to my other committee members, Dr. Kluepfel and Dr. Riley, for sharing their knowledge and technical expertise, and for the use of their labs. Additionally, I appreciate the contributions of the many research technicians and students. This work would not have been completed without all of your help.

I thank my wife Stephanie, my parents, and my very large and loving family for their constant patience and support. I love you all. My final and utmost thanks go to God, because through Him all things are possible.

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CHAPTER 1

PREFACE

Peach [*Prunus persica* (L.) Batsch] is an important fruit crop for both South Carolina and the United States. The 2003 peach crop was valued at approximately $30 million in South Carolina and over $400 million nationally (National Peach Council, 2004).

Peach replant disorder (PRD) is a serious problem for peach growers throughout the United States. PRD refers to the pattern of reduced growth and yield observed in young peach trees planted on soil previously cropped with peaches (Koch, 1955; Eayre et al., 2000). Trees grow slower and yield less on replant sites than they do on non-replant sites. Although, the specific causal agents of PRD are not known, pre-plant soil fumigation with methyl bromide, the conventional treatment for PRD, adequately manages the syndrome (Gur and Cohen, 1989). Methyl bromide is a broad-spectrum biocide that is highly effective for managing most soil-borne pests, pathogens and weeds (Chellemi, 2002). This suggests that the disorder results from harmful interaction(s) between the soil biotic community and peach roots (Traquair, 1984; Eayre et al., 2000).

Methyl bromide has been targeted by the Montreal Protocol as a contributor to ozone depletion. As methyl bromide fumigation is phased-out, the task of determining the specific cause of peach replant disorder has become increasingly important.

After reviewing PRD literature, we chose two primary research areas, presented in this thesis as chapters 3 and 4. The areas were: (1) to characterize a bacterial component of the peach rhizosphere that may contribute to PRD, and (2) to examine the effects of multiple PRD management treatments on the growth of replanted peach trees. The overall objective of this research was to provide new insight into causes and potential management options for PRD. Our specific objectives were: (1) to determine whether prunasin-degrading, cyanide-producing bacteria were present in replant soils and could inhibit young peach tree growth, (2) to study fine root dynamics in treated and untreated replant soils using minirhizotrons, and (3) to compare the ability of traditional and non-traditional replant treatments to improve tree growth on a replant site.

CHAPTER 2

LITERATURE REVIEW

The following discussion provides a detailed synopsis of published literature relevant to our research. It begins with the history and current state of PRD research. Next, potential PRD treatments and the effects of rootstocks on PRD are described. The "rhizosphere" where plant-microbial interactions occur is defined in the fourth section, and the fifth section contains a review of prunasin-degrading rhizobacteria and their potential role in PRD. The final section reviews the science of root observation, introduces minirhizotron technology, and defines various measurement parameters relevant to fine root development and demography.

Peach Replant Disorder

Peach tree replant disorder (PRD) has plagued peach growers for more than a century and has been described in nearly all peach growing areas of North America (Mai and Abawi, 1981). It is defined as a general decline in the growth and productivity of peach trees replanted in an old peach orchard (Koch, 1955; Eayre et al., 2000). The intensity of PRD symptoms is variable and differs according to rootstock, orchard, cropping history, and region (McKenry, 1999). PRD has been studied for many years without conclusive elucidation of the causal agent(s). A multitude of potential causes have been described, ranging from nutrition and poor soil structure to pathogens and phytotoxic chemicals such

as cyanide (Yadava, 1980; McKenry, 1999; Eayre et al., 2000). It is likely, however, that PRD is not the result of a single causative agent, but is instead a complex interaction of multiple factors. One author noted that, “Any event which acts to injure roots or interfere with their development may contribute to replant problems” (Zehr, 1979). In most studies, the application of a broad-spectrum soil fumigant such as methyl bromide provides adequate management of PRD symptoms (Wensley, 1956; Traquair, 1984; Eayre et al., 2000), suggesting that the disorder results from a deleterious interaction between the soil biotic community and peach roots.

Replant disorders in crops other than peach have also been described. The most significant of these are apple and grape replant disorders (Yadava, 1980; Westcott et al., 1986). Apple, grape and peach replant disorders are similar in several regards. All three disorders present symptoms of reduced growth and productivity, are effectively managed by using a broad-spectrum soil fumigant, and have incompletely-described etiologies (Mazzola, 1998; Westphal et al., 2002).

The causes of apple replant disorder appear to vary by region. In New York State, actinomycetes were discovered to be the primary causal agent (Westcott *et al.* 1986), whereas in Washington State, fungi were the dominant agent (Mazzola, 1998; Westphal et al., 2002). Some of the methyl bromide alternatives studied for the control of apple replant include soil fumigation with 1,3-dichloropropene or metam sodium (Otto and Winkler, 1993; Mazzola et al.,

2002), mulching of the rooting zone with black plastic (Jensen and Buszard, 1988), manipulation of the soil microbial community structure through cultural methods (Mazzola et al., 2002), and selection of tolerant rootstocks (Rumberger et al., 2004; Yao et al., 2006). Additionally, soil pasteurization has shown some ability to manage the disorder, indicating that soil solarization may have merit as a management alternative (Mazzola et al., 2002).

Multiple causal agents have been implicated in grape replant disorder. These include the presence of fungal organisms such as *Fusarium*, fluorescent pseudomonad bacteria, and the absence of adequate endomycorrhizal fungal colonization (Westphal et al., 2002). As in peach, the presence of remnant roots (old roots remaining in the orchard soil after their parent trees have been removed) has been suggested as a possible cause of grape replant problems, but contradictory research exists on this subject (Brinker and Creasy, 1988).

One PRD theory implicates the compound prunasin, a cyanogenic glycoside found in peach tissues, as the cause of reduced tree growth. Prunasin is composed of a sugar moiety and a cyanohydrin group (Figure 1). When prunasin is degraded by the enzyme β-glucosidase, the sugar moiety is cleaved and the cyanohydrin is degraded to release the respiratory inhibitor hydrocyanic acid (cyanide) (Gleadow and Woodrow, 2002). The ability to degrade cyanogenic glycosides and release cyanide is a trait that has been observed in soil bacteria (Bakker and Schippers, 1987). Previous authors have suggested

that remnant roots are a latent source of prunasin and subsequently cyanide (Israel et al., 1973; Gur and Cohen, 1989).

Replant Control Strategies

A variety of strategies have been employed to manage PRD. These include soil replacement, soil fumigation with a variety of chemicals, soil solarization, killing remnant roots with systemic herbicides, and breeding for resistant rootstocks (Yadava, 1980; McKenry and Buzo, 1997; McKenry, 1999). However, few strategies have proven consistently effective, with the exception of planting “virgin” fields (fields never planted with *Prunus* species), extensive fallow periods, and fumigation with methyl bromide or 1,3-dichloropropene.

Methyl Bromide

Methyl bromide is a broad-spectrum biocide that is highly effective at controlling most soil-borne pests, pathogens and weeds (Chellemi, 2002; Schneider et al., 2003). It is also considered the single best control strategy for PRD. Methyl bromide use is thought to contribute to the depletion of the atmospheric ozone layer (Eayre et al., 2000), and as a result, the availability of methyl bromide in the United States has been gradually reduced since 1991 by the Montreal Protocol and the U.S. Clean Air Act.

On January 1, 2005, methyl bromide was officially phased-out, however users of methyl bromide who currently have no feasible alternative may apply for Critical Use Exemptions (CUE) through the Environmental Protection Agency

(EPA) (McLean, 2005). Orchard replant disorders, including PRD, have been included in CUEs thus far, and these CUEs have been approved through 2007. CUE nominations being filed by the US in 2007 would allow for the continued use of methyl bromide through 2009. Information regarding the methyl bromide phase-out and CUEs may be found on the EPA website (http://www.epa.gov/ozone/mbr). Because methyl bromide is only available through CUEs and must be re-nominated each year, the evaluation and implementation of replacement strategies for PRD management are extremely important to the peach industry.

Soil fumigation with methyl bromide has been widely used in high-value horticultural crops since its introduction in the 1960s (Chellemi, 2002). Crops that have depended on methyl bromide fumigation include tree fruits, grapes, strawberries, solanaceous vegetables (i.e. tomatoes and peppers) and ornamental nursery crops (Duniway, 2002). Several factors have contributed to the widespread use of methyl bromide. The most obvious of these is the highly effective nature of the fumigant. In addition, methyl bromide is reasonably easy to apply, effective in a wide range of environmental conditions, and relatively affordable (Duniway, 2002). With the Montreal Protocol's phase-out of methyl bromide the need to identify methyl bromide alternatives for agriculture is urgent.

Many chemical and non-chemical replacements have been proposed, but only a few promising alternatives have been identified. The fumigants 1,3-Dichloropropene, chloropicrin and metam sodium show potential as chemical

alternatives to methyl bromide (Ibekwe et al., 2001; Rieger et al., 2001; Duniway, 2002; McKenry, 2003). Breeding for host resistance, cover cropping, addition of organic amendments, inoculation with plant growth-promoting rhizobacteria (PGPR) and soil solarization have shown potential as non-chemical methyl bromide alternatives (Rieger et al., 2001; Chellemi, 2002; Schneider et al., 2003). The benefits obtained using these methods vary from location to location and from crop to crop, but none provide the same level of management as methyl bromide. Combinations of treatments have shown favorable results, and it appears that an integrated approach will be necessary to replace methyl bromide (McKenry, 1999; Chellemi, 2002; Lopez-Medina et al., 2003; Schneider et al., 2003).

Methyl bromide has a particularly strong effect on soil microbial populations. While many soil fumigation treatments cause changes in the structure of soil microbial communities, methyl bromide typically has the greatest impact (Ibekwe et al., 2001). For example, a comparison of 1,3-dichloropropene, methyl isothiocyanate (the toxic breakdown product of metam sodium), chloropicrin, and methyl bromide demonstrated that methyl bromide-treated soils had the least microbial diversity following treatment. All treatments resulted in a shift in the bacterial community to a primarily Gram-positive composition (Ibekwe et al., 2001). The extent to which this restructuring of the soil microbial community is responsible for methyl bromide's powerful PRD control is unknown.

Solarization

Soil solarization may hold the most promise of all the non-chemical methyl bromide alternatives tested. Soil solarization is the process of using passive solar heating to reduce soilborne pathogen and weed populations. Solarization is accomplished by covering moist soil with clear plastic sheeting (usually polyethylene) during periods when solar radiation is highest (e.g. summer). Maximum soil temperatures under the plastic usually range from 37 to 60 °C, and are lethal or sub-lethal to a variety of soil organisms (Katan et al., 1976; Katan, 1980; Rieger et al., 2001; Pinkerton et al., 2002; Stevens et al., 2003). Effective solarization durations range from as little as four weeks in high light climates to more than six weeks in regions with lower light levels (Stevens et al., 2003). The beneficial effects of solarization often last as long as two growing seasons (Stapleton and DeVay, 1982; Stevens et al., 2003). Because solarization is an appealing non-chemical disease management strategy, it has been studied extensively in a variety of cropping systems over the last 25 years.

Steam pasteurization of soil has long been used to control soilborne pests, but it is impractical for use in large field situations such as orchards (Katan, 1980; Pullman et al., 1981). The use of mulching materials, both organic and inorganic, to improve plant growth has a similarly long history (Stapleton and DeVay, 1986). Katan *et al*. (1976) were the first to combine soil heating with mulching to control pathogens and weed populations. In a field planting of eggplant, they applied drip irrigation under polyethylene sheet mulching in the summer prior to planting.

This technique reduced *Verticillium* wilt by as much as 95%, improved plant growth and yield, and controlled weeds (Katan et al., 1976).

Various experiments have demonstrated the ability of solarization to control fungal and bacterial pathogens, as well as plant parasitic nematodes (Pinkerton et al., 2000; Ghini et al., 2003). Solarization can significantly alter soil microbial communities and reduce weed seed survival by as much as 100% (Peachey et al., 2001; Kluepfel et al., 2002). Gram-negative bacterial populations (e.g. *Pseudomonas* spp. and *Agrobacterium* spp.) are particularly vulnerable to the high temperatures associated with solarization, and most plant pathogenic bacteria belong to this group of prokaryotes (Kluepfel et al., 2002). Plant pathogenic fungi susceptible to soil solarization include *Fusarium* spp. and *Verticillium* spp. (Katan et al., 1976; Stapleton and DeVay, 1986). While plant pathogens are typically suppressed by solarization, beneficial microorganisms such as mycorrhizae and plant growth-promoting rhizobacteria (PGPR) are frequently unaffected or recover rapidly following treatment (Stapleton and DeVay, 1984; Stapleton and DeVay, 1986).

Many soil saprophytes, including Gram-positive bacteria (e.g. *Bacillus spp*.), some fungi, and actinomycetes, show greater thermotolerance and have the ability to survive solarization because of their spore-forming ability (Pinkerton et al., 2002). Many of these organisms also produce antibiotic compounds that are detrimental to other soil organisms (i.e. pathogens) (Stapleton and DeVay, 1984; Greenberger et al., 1987). As the proportion of plant pathogens and pests

in the soil environment is decreased and the proportion of plant growth-promoting organisms is increased, the soil may become “disease-suppressive.” This is one explanation for the extended benefit (> 1 growing season) observed following soil solarization.

Other mechanisms by which solarization improves crop productivity are less obvious. The release and accumulation of volatile compounds under the plastic sheeting may be antagonistic to a variety of organisms (Stapleton and DeVay, 1984). Solarization-induced changes in soil structure, penetration resistance and porosity may improve soil water relations and plant root development (Stapleton and DeVay, 1986; Ghini et al., 2003). A recent study in Brazil demonstrated that solarization significantly reduced soil penetration resistance and that this effect persisted more than 8 months post-treatment (Ghini et al., 2003). Mineral nutrition is also affected by solarization: concentrations of soluble nutrients, such as ammonium and nitrate, generally increase following treatment (Katan, 1980; Stapleton and DeVay, 1986; Ghini et al., 2003).

Telone C-17

Telone C-17 (Dow Agrosciences LLC, Indianapolis) is composed of 1,3-dicloropropene (1,3-D) and 17% v/v trichloronitromethane (chloropicrin). Telone is a widely used soil nematicide, and chloropicrin is a strong fungicide. The treatment is a liquid formulation that is shank-injected to a depth of >25 cm at the recommended rate 327 L/hectare (McKenry, 1999; Rieger et al., 2001). Both

chemicals have activity beyond their primary target group. For example, 1,3-D is known to be active against some fungal and bacterial plant pathogens, chloropicrin has some marginal nematicidal activity, and both give low levels of weed management (Mai and Abawi, 1981; Duniway, 2002).

Each chemical is commonly included on lists of methyl bromide alternatives, but individually neither compound is as effective as methyl bromide alone (Ibekwe et al., 2001; Duniway, 2002). The combination of these two compounds has occasionally resulted in pathogen management similar to those provided by methyl bromide in strawberry production (Rieger et al., 2001; Duniway, 2002). In a New York study of apple replant disease, 400 L/hectare of Telone C-17 altered the rhizobacterial community structure, but did not improve tree growth (Rumberger et al., 2004). Even so, Telone C-17 and Telone C-35 (35% chloropicrin) are receiving considerable attention as chemical alternatives to methyl bromide in replant situations.

Glyphosate Root Killing

The use of glyphosate herbicide to systemically kill remnant roots of a previous orchard before replanting has been studied as a replant control strategy in California (McKenry, 1999). Remnant roots are woody roots, either fragmented or whole, that remain in the orchard soil after the above-ground portions of the parent tree have been removed. As a matter of practicality, the vast majority of a tree's small roots remain in the orchard soil after the bole of the tree is removed. These roots may remain alive in the soil environment long after

the removal of the crown and trunk of the tree. For example, *Prunus* roots have been shown to remain alive for two years after tree removal (McKenry and Buzo, 1996). The longevity of these roots provides a long-term refuge for rhizosphere-inhabiting microbes. Particular attention has focused on nematode populations persisting on remnant roots, but it is logical that bacterial and fungal rhizosphere inhabitants may persist equally well. Remnant root longevity is one explanation of why extended pre-plant fallow periods alleviate replant disorder symptoms (McKenry, 1999).

Glyphosate [N-(phosphonomethyl)glycine] is a broad spectrum systemic herbicide that is effectively translocated in most plants (Weller and Skroch, 1983). Glyphosate is translocated to active meristems (root tips) of the deepest of roots where it causes tissue mortality (Weller and Skroch, 1983; McKenry, 1999).

McKenry (1999) demonstrated that glyphosate can effectively kill remnant roots of *Juglans* spp. and *Prunus* spp. (McDonald, 1992; McKenry and Buzo, 1996; 1997; McKenry, 1999). This was accomplished by injecting 1 mL concentrated glyphosate (41% active ingredient) for every 5 cm of stem diameter into the tree stem. Trees were then left in place for at least one month to allow all parts of the tree to die, prior to removal. In a peach study, stem applications of glyphosate were shown to kill 95% of Nemaguard roots and 60% Lovell roots (McKenry, 1999), substantially reducing the number of living remnant roots in

orchard soil. Methyl bromide and 1,3-D have both been shown to kill 99% of remnant roots to a depth of at least 4 feet (McKenry, 1999).

Glyphosate root kill provides a simple and inexpensive means to terminate remnant roots and reduce the rhizosphere-microbes that they maintain. For example, the number of root-knot nematodes on the roots of Lovell seedlings was reduced by 95% within 60 days of the application of glyphosate to the seedlings (McKenry et al., 1998). Using glyphosate in this manner, it is possible to reduce the fallow period required before replanting. However, it is recommended that an 18-month fallow period follow the glyphosate application to achieve the full benefit of the treatment (McKenry, 1999). If widely effective, the use of a systemic herbicide for replant management would be extremely desirable, since glyphosate is easy to inject, safe for workers, readily obtainable, and relatively affordable.

Rootstocks

Rootstock selection is critical to the overall success of an orchard. A variety of factors are used to determine which rootstock is most appropriate for a given orchard site in order to best meet the grower's production goals. Regional climate, orchard microclimates, soil type, orchard history, cultural practices, scion selection, and pathogen communities are some of the factors considered when selecting a rootstock. Nemaguard, Halford, and Lovell were the predominate peach rootstock varieties planted in the southeastern U.S. prior to the release of Guardian®.

Peach rootstock breeding and selection has been a primary research focus in peach-growing areas for many years. Many breeding programs have concentrated on producing rootstock cultivars resistant to various nematode species (Okie et al., 1994a; Reighard, 2000; Reighard, 2003). Lesion nematodes (*Pratylenchus spp.*), root-knot nematodes (*Meloidogyne spp.*), and ring nematode (C*riconemoides xenoplax*) are the more important nematode pests of peach and have been the primary breeding and selection targets for over 30 years (Dozier et al., 1984; Okie et al., 1994b; Lu et al., 1998; Lu et al., 1999; Nyczepir and Beckman, 2000; Nyczepir and Pinochet, 2001; Lynch et al., 2003; Blenda et al., 2006).

Considerable research has also focused on peach tree short life syndrome (PTSL). PTSL is an important disease complex in the southeastern United States that reduces productive lifespan and causes premature mortality of peach trees (Okie et al., 1994b; Wilkins et al., 2002). Parasitism by ring nematode is thought to be one of the primary factors contributing to PTSL (Harber et al., 1992; Okie et al., 1994a; Olien et al., 1995; Wilkins et al., 2002).

For many years, Lovell was the preferred peach rootstock in the southeastern U.S. because of the relatively high survival rates it conferred to scions on PTSL sites (Brittain and Miller, 1976; Ritchie and Clayton, 1981). However, Lovell is susceptible to root-knot nematodes, making it less desirable in locations with high root-knot nematode populations (Okie et al., 1994b; Nyczepir and Beckman, 2000). Nemaguard rootstock is resistant to root-knot nematodes, but the scions

of trees budded to Nemaguard are much more susceptible to PTSL than those budded to Lovell (Nyczepir et al., 1999). The lack of an acceptable rootstock for sites with both root-knot nematodes and PTSL led to the development of Guardian® peach rootstock. Guardian's® resistance to root-knot nematodes and tolerance of PTSL conditions have made it the preferred rootstock choice in the Southeast. A detailed account of the development of Guardian® is available from Okie *et al*. (1994a).

The development of PRD tolerant peach rootstock genotypes has received less consideration than PTSL, probably due to the disorder's ambiguity and the option of avoiding replant problems by planting in "virgin" soils. Little information exists on how different peach rootstock varieties perform in replant situations, and none of the currently available rootstocks show particular tolerance to the disorder. In apple replant disorder (ARD), tolerant rootstocks have been identified and were found to differ from traditional ARD susceptible genotypes in their rhizobacterial community structure (Rumberger et al., 2004). Systematic development of PRD tolerant rootstocks is dependent on better understanding of PRD's etiology.

Guardian® and Lovell peach rootstocks were evaluated in the current field experiment. These two rootstocks are similar in their cultural habits, and they influence their scions in comparable ways. Our research allows the direct comparison of fine root parameters of Guardian® and Lovell rootstocks, and represents the first time that such a comparison has been made. Also, it

provides a means to study the response of each rootstock to several soil treatments in a replanted orchard.

Rhizosphere

One of the primary objectives of this research is to investigate the influence of soil microbial communities on peach root dynamics, particularly in replant situations. The majority of plant-microbial interactions occur in a region known as the rhizosphere. Therefore, this section gives a brief overview of the rhizosphere concept. The term “rhizosphere” was first used by Lorenz Hiltner (1904) in his description of the interactions between legume roots and soil bacteria. Today, the rhizosphere is understood in a much broader sense. It can be defined as the ecologically complex region of soil directly adjacent to and surrounding the root, usually extending only a few millimeters from the root surface (Curl and Truelove, 1986; Campbell and Greaves, 1990). Roots influence the rhizosphere through the exudation of a diverse array of substances including amino acids, sugars, enzymes, and organic acids (Dakora and Phillips, 2002). Rhizosphere inhabitants may include fungi, nematodes, and bacteria; the structure of the community is determined, in large part, by the plant.

Control of the rhizosphere does not entirely belong to the plant. Microorganisms can influence the types and amounts of root exudates and can affect root system morphology through the production of plant growth regulators and plant toxins (Kluepfel, 1993; Yang and Crowley, 2000; Persello-Cartieaux et al., 2003).

Occupation of the nutrient-rich rhizosphere is intensely competitive. Many bacterial species are highly-effective colonizers of this niche and have the ability to defend their occupation through a variety of mechanisms including antibiotic and siderophore production and the induction of systemic acquired resistance (SAR) in the host plant (Kapulnik and Okon, 2002; Persello-Cartieaux et al., 2003). Gram-negative bacteria, notably *Pseudomonas spp*., are some of the most competent colonizers of the rhizosphere (Kluepfel, 1993; Lugtenberg et al., 2001).

Prunasin and Peach Roots

Prunasin (D-mandelonitrile β-D-glucoside) is a cyanogenic glycoside found in most species in the genus *Prunus*. Prunasin, or its metabolic derivatives, have been implicated by many researchers as a possible causal factor in PRD (Traquair, 1984; Gur and Cohen, 1989; Yadava and Doud, 1980).

Cyanogenic glycosides are a diverse group of compounds found within at least 100 angiosperm families (Harborne, 1998). The production of cyanogenic glycosides, from which hydrogen cyanide (HCN) is released through the enzymatic process of cyanogenesis, is thought to have primarily evolved as a mechanism of plant defense from herbivory (Jones, 1972). Most cyanogenic species require severe tissue disruption to initiate significant breakdown of the cyanogenic glycosides to HCN. Since HCN is detrimental to plant tissues, it is theorized that prunasin is spatially separated from its catabolic enzyme, β-

glucosidase, by compartmentalization (Swain and Poulton, 1992; Reilly and Okie, 1985).

The suggestion that peach roots may play a role in PRD stems from the high number of remnant roots remaining in the field after uprooting previously planted trees. Remnant roots may remain in the soil for many years, and the release of HCN is likely to be a gradual process as these roots disintegrate (Gur and Cohen, 1989). Peach root extracts have been shown to inhibit respiration of root tips, retard peach tree growth, cause premature leaf chlorosis, necrosis, and abscission, act as competitive inhibitors of nitrate reductase, and reduce the overall size of the root system (Israel *et al*, 1973; Gur and Cohen, 1989; Reilly et al, 1986). Israel *et al*. (1973) reported that root bark had the highest concentration of HCN of tested tissues, and Gur and Cohen (1989) stated "medium sized" lignified peach roots were a "rich source of HCN."

Since cyanogenesis rarely occurs in non-disrupted tissues, it is likely that healthy, non-injured roots do not leach prunasin or its derivatives. However, through the natural degradation of sloughed fine roots, some HCN may enter the soil, assuming that prunasin is stored in fine roots. The passive leaching of prunasin from living roots to soil has not been documented. However, if prunasin was exuded from peach roots, it would be available for metabolism by rhizosphere bacteria.

Cyanide Production by Rhizobacteria

Cyanide production by rhizobacteria can be both beneficial and harmful to crop plants: beneficial by protecting the plants from soil pathogens or suppressing competing weeds and harmful by inhibiting root respiration and impairing nutrient uptake. As biocontrol agents, rhizobacteria can both protect plants from other soil microbial disease agents and/or suppress the growth of competing weed species. Rhizobacteria that provide control of soilborne pathogens are referred to as plant growth-promoting rhizobacteria (PGPR) (Maurhofer et al., 1994; Walsh et al., 2001). Rhizobacteria that suppress weed growth fall into the category of deleterious rhizobacteria (DRB) (Kremer and Souissi, 2001; Kremer and Kennedy, 1996).

DRB, although beneficial when weeds are their targets, can also be destructive to economically valuable crops. DRB are considered minor plant pathogens that inhabit both the rhizosphere and root apoplast, but not the vascular system (Nehl et al., 1996). Although they rarely result in plant mortality, DRB can reduce crop yield, lower root and shoot growth, increase browning and root discoloration, and cause root hair deformation (Berggren et al., 2001; Bakker and Schippers, 1987; Nehl et al., 1996). Phytotoxin (e.g. cyanide) production by DRB is thought to be a primary mechanism by which they inhibit plant growth (Alstrom and Burns, 1989). Other mechanisms include production of phytohormones such as indole acetic acid (IAA), nutrient competition with the

plants themselves and inhibition of mycorrhizal development (Nehl et al., 1996; Barazani and Friedman, 1999).

DRB require a substrate to produce cyanide. The most commonly described substrate for cyanide production is the amino acid glycine (Bakker and Schippers, 1987; Owen and Zdor, 2001). However, cyanogenic glycosides (e.g. prunasin) are also suitable substrates for bacterial cyanide production (Knowles, 1976; Owen and Zdor, 2001). Both glycine and cyanogenic glycosides occur in root exudates (Knowles, 1976; Owen and Zdor, 2001; Bakker and Schippers, 1987).

Gur and Cohen (1989) investigated the potential link between cyanide production by DRB using cyanogenic glycosides from peach tissues as a cause of PRD. They extracted prunasin-degrading “bacilli” from oven-dried peach roots and demonstrated their ability to release cyanide *in vitro* when provided with amygdalin. They did not identify the bacteria to species, nor did they reintroduce them into the peach rhizosphere. Additionally, the experiment established that when oven-dried peach roots were added to non-peach soil, cyanide was released and growth of young peach trees was inhibited. The primary injury caused by cyanide release was “a severe reduction in the root system” (Gur and Cohen, 1989). We decided to pursue this theory in our system and build on the findings of Gur and Cohen by identifying isolates to species and reintroducing the bacteria to seedling peach roots to observe their potential to inhibit growth.

Root Observation

Historically, the greatest challenge to studying root systems has been the difficulty of observing living roots non-destructively *in situ*. Measurement of root growth parameters has relied on destructive methods such as root system excavation, soil coring, and root in-growth cores (Johnson et al., 2001). These techniques provide good estimates of root length density at a single point in time, but they provide little information root production, turnover and longevity. Such measurements require repeated observations of individual roots through time (Majdi, 1996; Johnson et al., 2001). The development of rhizotrons (root observation laboratories) and minirhizotrons provide a method to overcome the limitations of destructive root sampling techniques.

Minirhizotron systems consist of a minirhizotron tube, a fiber-optic color video camera, a camera control unit, and a digital video recorder. A minirhizotron tube is a clear tube inserted in the ground within a plant's root zone. Tubes are typically round and may be constructed of a variety of materials, such as glass, acrylic, and butyrate (Withington et al., 2003; Johnson et al., 2001). A miniaturized fiber-optic camera is inserted into the minirhizotron tube, a video feed is sent to the digital video recorder through the video control unit, and video footage of roots is recorded on digital videotape. The root images acquired from minirhizotrons may then be analyzed using specialized software.

Minirhizotrons allow researchers to monitor individual roots at regular time intervals from birth to death. They provide access to a variety of parameters that

are not otherwise measurable: rates of root production and mortality, root lifespan, and the phenology of root growth and development (Majdi, 1996). Improvements in minirhizotron technology have fostered the new research area of fine root demography and have greatly increased our understanding of fine root dynamics.

Data collection from minirhizotrons is accomplished through multiple video sampling dates spaced at regular intervals during a study. A two-week sampling interval is common. Data collected for each root include the date of first appearance (birth date), length, diameter, color, and date of disappearance (mortality date).

Fine Root Longevity

Fine root lifespan is highly variable. Root lifespans as short as 14 d have been observed in apple (Head, 1966), while root lifespans greater than 600 d have been recorded for Norway Spruce (Majdi and Kangas, 1997). While the influence of biotic and abiotic factors on fine root longevity has been measured in a number of species, many questions remain unanswered. Factors that influence root longevity include soil moisture, soil temperature, mycorrhizal colonization, nutrient availability, root herbivores, and pathogens (Hendrick and Pregitzer, 1993; Eissenstat et al., 2000; Yanai and Eissenstat, 2002). The research community is only beginning to understand the mechanisms that control root longevity.

In some ways, fine roots may be compared to leaves. Leaves and fine roots are both ephemeral organs that function in resource acquisition. Plants typically control leaf longevity, but it is not known whether plants have the same level of control over root longevity (Yanai and Eissenstat, 2002).

The idea that soil fauna (mammals, arthropods and microbes) have a powerful impact on fine root longevity is a relatively new concept in root ecology (Wells and Eissenstat, 2001; Eissenstat and Yanai, 1997; Wells et al., 2002; Dawson et al., 2003). Root herbivores and pathogens can exert strong feeding pressure on nutrient-rich fine roots (Blossey and Hunt-Joshi, 2003). The soil application of an organophosphate insecticide increased the median lifespan of peach fine roots by 46-125 d compared to an untreated control (Wells et al., 2002). A reduced density of roots following insecticide treatment may reflect a decreased need for root replacement due to reduced root herbivory (Dawson et al., 2003). Similar results would be expected when pathogens or other deleterious microbes are removed from the soil. Soil sterilization with methyl bromide is one way to reduce microbes and arthropods in the soil profile. In the absence of these organisms, we would expect root longevity to be increased.

Experimental Overview

Based on the literature reviewed above, we designed a two-fold approach to study PRD. First, we investigated the theory that prunasin plays a role in PRD through its breakdown product, cyanide. Second, we used minirhizotrons to study PRD where it occurs, in the root zone of replanted peach trees.

The crux of the prunasin theory is that the enzymatic breakdown of prunasin from remnant peach roots releases cyanide in concentrations toxic to the fine roots of newly planted peach trees. Following Gur and Cohen (1989), we speculated that remnant roots do not necessarily provide reserved prunasin, but instead provide refuge for populations of prunasin-degrading rhizobacteria to persist through fallowing and replanting of an orchard. These rhizobacteria would then consume prunasin from root exudates, dead fine roots, and remnant woody roots in quantities high enough to release toxic levels of cyanide. By definition, these rhizobacteria would then be classified as DRB of peach.

To test this hypothesis, we examined the rhizosphere bacterial community in a South Carolina peach orchard to determine whether it contained bacteria capable of evolving cyanide from prunasin. We then identified cyanogenic rhizobacterial isolates and used the most strongly cyanogenic isolate as inoculum in greenhouse experiments to determine whether its presence reduced the root and shoot growth of peach seedlings. This work expanded on that of Gur and Cohen (1989) by identifying cyanogenic rhizobacteria to species and reintroducing them into the peach rhizosphere.

In a concurrent field experiment, minirhizotrons were installed beneath replanted peach trees of two rootstocks and exposed to four PRD management treatments that targeted the rhizosphere microbial community. The trees were grafted onto two different, commercially relevant peach rootstocks, Guardian® and Lovell. We then observed fine root growth throughout the orchard to

compare root growth characteristics between untreated trees and trees in each PRD treatment. We also observed differences in fine root dynamics between the two rootstocks and the interactions between PRD treatments and rootstocks.

The overall objective of this research was to provide new insight into the causes and potential management options for PRD. Our specific objectives were: (1) to determine whether prunasin-degrading, cyanide-producing bacteria were present in replant soils and could inhibit young peach tree growth, (2) to study fine root dynamics in treated and untreated replant soils using minirhizotrons, and (3) to compare the ability of traditional and non-traditional replant treatments to improve tree growth on a replant site.

CHAPTER 3

CYANOGENIC RHIZOBACTERIA ISOLATED FROM PEACH ROOTS

Introduction

Peach replant disorder (PRD) refers to the pattern of reduced growth and yield observed in young peach trees [*Prunus persica* (L.) Batsch] planted on soil previously cropped with peaches (Koch, 1955; Eayre et al., 2000). Pre-plant soil fumigation with methyl bromide adequately controls PRD, suggesting that it results from harmful interaction(s) between peach roots and soil fauna (Traquair, 1984; Eayre et al., 2000). However, the specific etiology of PRD remains unknown. As methyl bromide is phased out (McLean, 2005), the task of determining the specific cause of PRD and its management have become important research goals.

One theory suggests that prunasin (D-mandelonitrile β-D-glucoside), a cyanogenic glycoside (CG) found in peach tissues, may play a crucial role in the disorder. Prunasin is composed of a sugar moiety and a cyanohydrin (Figure 3.1). When prunasin is degraded by the enzyme β-glucosidase, the sugar moiety is cleaved, the cyanohydrin is degraded, and the potent respiratory inhibitor cyanide (hydrocyanic acid) is released (Gleadow and Woodrow, 2002). Amygdalin (D-mandelonitrile 6-*O*-*β*-D-glucosido-*β*-D-glucoside) has the same molecular structure as prunasin, but with an additional sugar moiety (Harborne, 1998). While prunasin is located in all peach tissues, amygdalin is restricted to seed tissue (Reilly and Okie, 1985).

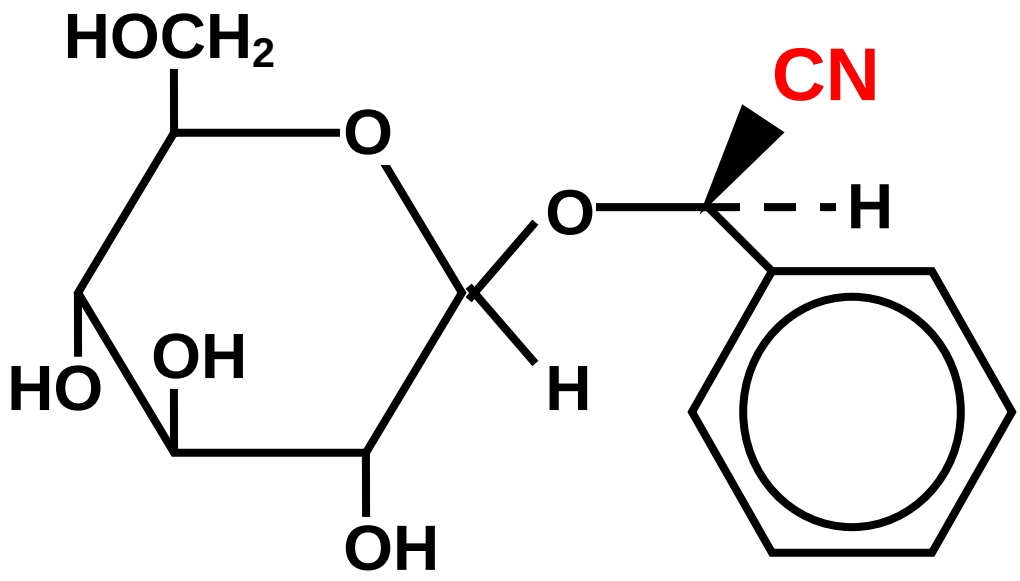

Figure 3.1 Molecular structure of prunasin.

Previous authors have suggested that remnant peach roots remaining in orchard soil after tree removal are a latent source of prunasin and subsequently, cyanide (Israel et al., 1973; Gur and Cohen, 1989). The ability to degrade CGs and release cyanide has been observed in some deleterious rhizobacteria (DRB) (Bakker and Schippers, 1987). Prunasin-degrading rhizobacteria may release cyanide when feeding on decomposing peach roots, thereby inhibiting root growth of new peach trees on replant sites. This is the first study to isolate prunasin-degrading bacteria from the peach rhizosphere of a replant site and to reintroduce the bacteria into seedling peach rhizosphere *in vitro*.

DRB are minor pathogens that inhabit the plant rhizosphere and endorhizosphere, but not the vascular system (Nehl et al., 1996). While they rarely kill plants directly, deleterious rhizobacteria can reduce yields, impair root and shoot growth, promote root discoloration, and cause root hair deformation (Bakker and Schippers, 1987; Nehl et al., 1996; Berggren et al., 2001). Phytotoxin production is thought to be the primary mechanism of plant growth inhibition by deleterious rhizobacteria (Alstrom and Burns, 1989).

The objectives of this research were to: 1) determine whether bacteria capable of evolving cyanide from prunasin were present in a South Carolina peach orchard, 2) identify cyanogenic rhizobacterial isolates, and 3) use the most strongly cyanogenic isolate as inoculum in greenhouse experiments to determine whether its presence would reduce the root and shoot growth of peach seedlings.

Materials and Methods

Isolate Selection

Rhizosphere Extraction.

In April 2002, bacterial isolates capable of degrading prunasin (D-mandelonitrile β-D-glucoside) and/or amygdalin (D-mandelonitrile 6-*O*-*β*-D-glucosido-*β*-D-glucoside) were obtained from peach rhizosphere soil by selection on M9 carbon source utilization medium. Amygdalin is similar in structure and cyanogenic potential to prunasin. As it is significantly lower in cost, we were interested in its potential to be used interchangeably with prunasin in future experiments.

Fine roots (<2 mm diameter) were obtained from the top 20 cm of soil beneath 10 randomly-selected seven-year-old 'Redglobe' peach trees grafted on Guardian® or Lovell rootstock. The trees were growing in a Cecil sandy loam soil at the Musser Fruit Research Center near Clemson, SC.

All fine root material was pooled into a single sample and lightly shaken by hand to remove loosely-adhered soil. Approximately 1.0 ± 0.05 g (fresh weight) of fine roots were removed and placed into a 30 ml Nalgene® centrifuge tube (product # 3119-0030; Nalge Nunc International, Rochester, NY) with 10 mL of phosphate buffering saline (PBS) at pH 7.2. The tube was agitated at 300 rpm for 15 minutes on an orbital shaker to separate rhizosphere material from roots. Root segments were removed, and the resulting solution was centrifuged at

10,800 x *g* for 15 min to pellet rhizosphere material [modified from Ong (2001)]. The rhizosphere pellet was resuspended in 1 mL PBS, and the resulting solution was serially diluted.

In May 2002, the experiment was repeated a second time using the same rhizophere extraction procedure. In addition, four soil cores (1 inch x 8 inch soil core) were taken from a fallow field location approximately 18 m from the nearest peach tree. The cores were combined into a single bulk sample, and approximately 1.0 ± 0.05 g of this bulk sample was extracted using the rhizosphere procedure extraction described above.

Isolation of Prunasin- and Amygdalin-Degrading Rhizobacteria

In the April 2002 experiment, M9 liquid medium (Eisenstadt et al., 1994) was prepared and dispensed in 2 mL aliquots into 5-mL tubes (product # 14-956-1D; Fisher Scientific). Stock solutions (10 $mg \cdot mL^{-1}$) of prunasin (product # M 0636; Sigma-Aldrich Co.), amygdalin (product # A 6005; Sigma-Aldrich Co.) and glucose were prepared and filter-sterilized through a 0.2 μm Sterile Acrodisc® 13 mm filter (product # 4602; Gelman Sciences).

The tubes of M9 media were amended with one of six different carbon source treatments: 0.025% prunasin, 0.025% amygdalin, 0.050% amygdalin, 0.025% glucose, 0.050% glucose, and a no-carbon-source negative control. Glucose served as a positive control for bacterial growth. Five tubes of each carbon source treatment were prepared by adding appropriate amounts of stock solution to the M9 media.

Samples (25 µL) of each rhizosphere dilution, 10^{-1} through 10^{-5}, were added to one tube of each amended medium type. Inoculated tubes were placed on an orbital shaker at 28 °C in the dark, and bacterial growth was assessed at 24, 48 and 72 hours post-inoculation by comparing the clarity of the amended media to that of the negative control. Cloudy media indicated bacterial growth.

In the May 2002 experiment, M9 liquid cultures were replaced by solid M9 agar plates (20 mL of media per plate), and the number of carbon source treatments was reduced to four: 0.025% prunasin, 0.025% amygdalin, 0.025% glucose, and a negative control. Culturing rhizosphere or bulk soil extract directly onto solid media simplified the isolation procedure, and results from the April experiment indicated that a 0.025% carbon source level was sufficient for culturing the isolates.

One M9 plate was prepared per treatment, and 100 µL of the 10^{-2}, 10^{-3}, and 10^{-4} serial dilutions were streaked for single colonies on each plate. The plates were then incubated at 28 °C, and bacterial growth was assessed visually at 24, 48, and 72 h post-inoculation.

Colony Selection

In the April 2002 experiment, single colonies were selected from the amended liquid M9 media cultures. The 10^{-3} dilutions were selected for plating, as they were the most dilute cultures with observable bacterial growth.

Twenty-five μL of media from the negative control, 0.025% prunasin, and 0.025% amygdalin tubes were plated for single colonies on 3 separate King's medium B (KB) plates (Smibert and Krieg, 1994) and incubated at 28 °C for 72 h. The 0.025% glucose treatment was not plated because of the nearly universal ability of microorganisms to metabolize glucose.

Individual colonies growing on each plate were selected and streaked onto trypticase soy broth agar (TSBA) plates (Cote and Gherna, 1994). The single colony isolates were labeled according to the carbon source on which they were selected and were sub-cultured on TSBA three times to ensure pure culture.

In the May 2002 experiment, solid M9 agar plates streaked with the 10^{-4} rhizosphere or bulk soil solution were scraped after 72 hours; colonies were suspended in 300 μL of PBS and serially diluted. Samples (100 μL) of the subsequent 10^{-3}, 10^{-4}, and 10^{-5} dilutions were plated on TSBA plates.

Single colonies were selected from the TSBA plates, sub-cultured onto new TSBA plates, and labeled according to the original carbon source from which they were selected. The isolates were sub-cultured three times on TSBA to ensure pure cultures. After all isolates from the first and second experiments were growing successfully in pure culture, they were placed into long-term -80 ºC storage (Gherna, 1994).

Isolate Screening for HCN Production.

Preparation of Picrate Papers

Filter papers saturated with picric acid and Na_2CO_3 (picrate papers) are commonly used for qualitative assessment of cyanide production (Lorck, 1948; Harborne, 1998; Kremer and Souissi, 2001). Cyanide reacts rapidly with picrate to form a red-brown pigment commonly referred to as 'isopurpuric acid' (Williams and Edwards, 1980).

To prepare picrate papers, Whatman No. 1 filter paper was cut into 2 cm^2 squares and dipped into a 1.2% saturated picric acid solution (product # RC586016; VWR Scientific). Papers were air-dried, saturated with 10% Na_2CO_3, air-dried again, and stored with desiccant, at room temperature and in the dark until used.

Screening for HCN Production

Seventeen individual rhizosphere or bulk soil isolates were grown in pure culture on KB media prior to screening for cyanide production. Isolates were tested for their ability to produce cyanide on 4 different media: unaltered KB medium, KB supplemented with 0.44% glycine, M9 media with 0.05% prunasin, and M9 with 0.05% amygdalin. Many *Pseudomonas* species, a common group of rhizosphere inhabiting bacteria, have demonstrated cyanide production on KB media and KB media amended with glycine (Kremer and Souissi, 2001). *Pseudomonas fluorescens* strain CHA0, which produces cyanide in pure culture,

was used as a positive control (Voisard et al., 1989; Laville et al., 1992; Maurhofer et al., 1994). Forty petri plates (35 x 10 mm product # 08-757-11YZ; Fisher Scientific) containing 3 mL of solid media were prepared for each treatment.

Isolates were individually streaked onto two replicate plates of each medium. Negative controls were created by streaking replicate plates with a clean, sterile loop. A single 2 cm^2 picrate paper was affixed to the lid of each petri plate with double-sided tape (Lorck, 1948). Plates were incubated at 28 °C and photographed at 4, 12, 24, and 48 h after inoculation. Following the technique of Kremer and Souissi (2001), color changes in the picrate paper from bright yellow to light brown, brown, or dark brown indicated weak, moderate, or strong cyanogenic potential, respectively (Figure 3.2).

Isolate Identification

All prunasin- and amygdalin-degrading isolates were identified using gas chromatography-fatty acid methyl ester analysis (FAME) following the procedure of Sasser (1990). Analysis and naming of isolates were performed using the Sherlock Microbial Identification System (MIS) (MIDI Inc., Newark, DE).

First Greenhouse Experiment

In April 2003, a greenhouse experiment was performed to assess the ability of the most strongly cyanogenic isolate, *Arthrobacter globiformis* strain 1P, to reduce the growth and performance of peach seedlings. To facilitate the re-

isolation of this strain from the rhizosphere of inoculated seedlings, the isolate was mutated for rifamipicin resistance. This antibiotic was selected because of the low occurrence of rifamipicin resistance in native soil bacterial populations (D. Kluepfel, personal communication).

Rifampicin resistance was established in *A. globiformis* strain 1P using the slant plate method with a rifamipicin concentration of 100 $\mu g \cdot ml^{-1}$ (Eisenstadt et al., 1994). The new, resistant isolate was named *A. globiformis* strain 1PRif^r. Screening of the mutated isolate for cyanide production on prunasin and amygdalin indicated that rifampicin resistance had no effect on the isolate's cyanogenic potential.

Inoculum Preparation

Four inoculum treatments were prepared for inoculation of peach seedlings (see below). The first consisted of *A. globiformis* strain 1PRif^R inoculum grown in standard trypticase soy broth (TSB). This treatment was referred to as the Arthro treatment. The second consisted of inoculum grown in TSB amended with 0.05% amygdalin to induce the bacteria's CG-degradation pathway (Artrho Primed). The third treatment involved inoculation with *Pseudomonas fluorescens* strain BG33R (Wechter et al., 2001), a non-cyanogenic, non-pathogenic, rifampicin-resistant rhizosphere bacteria that served as an inoculated control (BG33R). In the fourth treatment, sterile deionized water was used as an uninoculated control (Control).

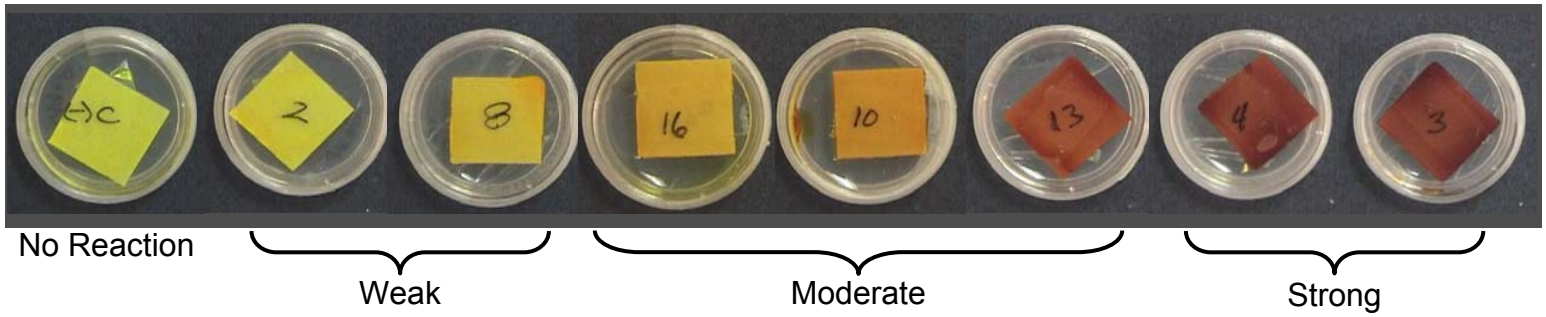

Figure 3.2 Range of picrate paper color change in response to cyanide (48 hr exposure).

The bacterial isolates for the three inoculated treatments were grown in 2 L aliquots of TSB at 28 ºC. After 24 h, the solutions were centrifuged at 10,800 x *g* for 15 minutes to precipitate bacterial cells. The cells were resuspended to a concentration of 1 X 10^8 colony-forming units (cfu) per mL in 2 L of sterile water.

Seedling Preparation

Seeds of Lovell peach rootstock were cold-stratified for 60 days at 4 °C beginning in December 2003. Ninety-seven germinating seeds with 1 to 3 cm radicals and no lateral roots were selected for use in the experiment. Seeds were planted in 5 cm Deepots™ (Hummert International, Earth City, MO) filled with steam-pasteurized river sand and fertilized weekly with 40 mL of one-quarter strength Hoagland's solution (product # H 2395; Sigma-Aldrich Co.). Sixty days later, 72 seedlings of uniform size were selected for inoculation.

Seedling Inoculation

Before inoculation, the seedlings were removed from their containers and the sand media was gently shaken from their roots. The bareroot seedlings were then soaked in 2 L of their assigned inoculum solution for 15 min. Seedlings were immediately repotted into 7.6 L Treepots™ (Hummert International, Earth City, MO) with new pasteurized sand, and 50 mL of inoculum was poured over the soil surface to fully drench the media.

Inoculated seedlings were randomly arranged on the greenhouse bench; they were not irrigated for 2 d following inoculation to prevent leaching of

bacterial cells during root colonization. Each seedling was fertilized weekly with 50 mL of one-quarter strength Hoagland's solution until harvest.

During the experiment, midday light levels inside the greenhouse were approximately 1400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, with a relative humidity of 30% and an average daily air temperature of 24.4 +/- 0.18ºC.

Experimental Design and Measurement

The experiment used a completely randomized design with four inoculation treatments and 3 sampling dates (42, 56, and 84 days post-inoculation). There were six replicates per harvest date yielding a total of 72 experimental units. Root colonization was assessed by re-isolation and subsequent dilution plating of the bacteria from a 1 g sample of the seedling roots. Dilution plating was performed on rifampicin-amended TSBA plates to exclude organisms from the ambient environment. Leaf area was measured with the LI-3100C leaf area meter (LI-COR Biosciences, Lincoln, NE). Total root system length of each plant was analyzed using the WinRhizo root scanning system (Regent Instruments Inc., Quebec, Canada). Root dry weight, shoot dry weight, and shoot height were also recorded.

Second Greenhouse Experiment

In February 2004, the greenhouse experiment was conducted a second time, incorporating several modifications based on the results of the first experiment. Lovell peach seedlings were prepared in the same manner as

before, and three inoculum treatments were applied. The first consisted of inoculation with *A. globiformis* strain 1PRif^{R} (Arthro). The second consisted of Arthro inoculation followed by weekly 100 mL soil drenches of a 500 ppm (equivalent to 110 µmol cyanide per application) amygdalin solution (Arthro + Drench). Amygdalin drenches were employed to maintain a high level of CG in the rhizosphere, as may occur in replant soils when remnant peach roots decay. The third treatment consisted of sterile deionized water as an uninoculated control (Control).

In this experiment, seedlings were not barerooted prior to inoculation. Instead, potted seedlings were randomly assigned to treatment groups and 150 mL of inoculum was poured on the soil surface of each, taking care to avoid cross-contamination. Seedlings were not irrigated for 4 d following inoculation to prevent leaching of bacterial cells during root colonization. Fertilization was applied at 50 ppm nitrogen in the irrigation water once per week with Peters Professional 20-20-20 soluble fertilizer (The Scotts Company, Marysville, OH).

The experiment used a completely randomized design with three inoculation treatments and a single harvest date, 84 days after inoculation. Root colonization was assessed in 12 randomly selected seedlings per treatment. Shoot height, shoot dry weight, and root dry weight were recorded for each seedling as described previously (24 replicates).

Statistical analyses for both greenhouse experiments were performed using PROC GLM in SAS version 8.0 (SAS Institute, Cary, NC). For each

experiment, the treatment main effect was assessed using analysis of variance. When necessary, values of dependent variables were transformed prior to analysis to satisfy normality and homogeneity of variance assumptions. When the treatment main effect was significant, dependent multiple comparisons were made between treatment groups using Fisher's LSD procedure ($\alpha = 0.05$).

Results

Isolation of Prunasin- and Amygdalin-Degrading Rhizobacteria

Multiple species of prunasin- and amygdalin-degrading bacteria were isolated from the rhizosphere of 'Redglobe' peach trees and from bulk orchard soil (Table 3.1). A total of ten different bacterial species were isolated which could use either prunasin or amygdalin as a sole carbon and nitrogen source.

Rhizosphere isolates of *Arthrobacter globiformis*, *Micrococcus luteus*, *Microbacterium saperdae*, and *Cellulomonas cartae* were selected on prunasin. Of these, *A. globiformis* demonstrated the most abundant growth on the carbon source utilization medium. Rhizosphere isolates of *A. globiformis*, *A. ilicis*, *Cellulomonas fimi*, and *Stenotrophomonas maltophilia* were selected on amygdalin, with *A. globiformis* again exhibiting the most prolific growth.

Extracts from bulk orchard soil also yielded prunasin- and amygdalin-degrading bacteria. *A. globiformis* and *Photobacterium leiognathi* were selected on prunasin, and *A. globiformis*, *C. cartae*, *Serratia liquefaciens*, and *Nocardia asteroides* were selected on amygdalin.

Table 3.1 Species of bacteria isolated from peach and non-peach soil on prunasin and amygdalin. Species identified using FAME analysis and the Sherlock Microbial Identification system (MIS).

Isolate ID	MIS Similarity Index	Peach Root Rhizosphere		Bulk Soil	
		Prunasin	Amygdalin	Prunasin	Amygdalin
Arthrobacter globiformis	0.90	X	X	X	X
Arthrobacter ilicis	0.73		X		
Cellulomonas cartae	0.87	X			X
Cellulomonas fimi	0.73		X		
Microbacterium saperdae	0.76	X			
Micrococcus luteus	0.61	X			
Nocardia asteroides	0.63				X
Photobacterium leiognathi	0.48			X	
Serratia liquefaciens	0.68				X
Stenotrophomonas maltophilia	0.76		X		

A. globiformis was the only species isolated from all combinations of site and carbon source. *C. cartae* was isolated from rhizosphere soil on prunasin-amended media and from bulk soil on amygdalin-amended media. All other species were isolated from one site/carbon source combination only.

Three of the 10 bacterial isolates were strongly cyanogenic on both prunasin- and amygdalin-amended M9 media: *A. globiformis*, *A. ilicis*, and *C. cartae* (Table 3.2). The positive control, *Pseudomonas fluorescens* CHAO, was strongly cyanogenic on KB media with and without added glycine, but none of the orchard isolates produced detectable cyanide on these media.

Greenhouse Experiments

In the first experiment, inoculation with Arthro, Arthro Primed and BG33R resulted in successful colonization of peach seedling rhizospheres under greenhouse conditions. The Arthro and Arthro Primed treatments produced significantly greater root colonization than BG33R and the control when averaged across all harvest dates [Figure 3.3(A); Table 3.3]. The extent of root colonization generally increased with time across all treatments (Table 3.3).

Despite significant root colonization in the Arthro and Arthro Primed treatments, most seedling growth parameters were unaffected by the inoculation treatments (see Appendix Table A-2). The single exception was root dry weight. Seedlings inoculated with Arthro and Arthro Primed had significantly higher root dry weights than those treated with BG33R on harvest date 3, although only Arthro differed significantly from the control (Figure 3.4).

Table 3.2 Isolate cyanide production.

Isolate Number	Isolate FAME ID	Location*	Cyanide Production by Culture Medium			
			KB	KB + Glycine	M9 + Prunasin	M9 + Amygdalin
Negative Control	no culture	--	--	--	--	--
Positive Control	*Pseudomonas fluorescens* CHAO	--	Strong	Strong	--	--
1P	*Arthrobacter globiformis*	PR	--	--	Strong	Strong
3A	*Cellulomonas fimi*	PR	--	--	Strong	Weak
4A	*Arthrobacter ilicis*	PR	--	--	Strong	Strong
8P	*Micrococcus luteus*	PR	--	--	Weak	--
9A	*Stenotrophomonas maltophilia*	PR	--	--	Strong	Moderate
10A	*Arthrobacter globiformis*	PR	--	--	Strong	Strong
11A	*Serratia liquefaciens*	BS	--	--	Strong	--
12A	*Arthrobacter globiformis*	BS	--	--	Strong	Strong
13A	*Nocardia asteroides*	BS	--	--	--	--
14P	*Arthrobacter globiformis*	BS	--	--	Strong	Strong
15P	*Photobacterium leiognathi*	BS	--	--	Moderate	--
16P	*Microbacterium saperdae*	PR	--	--	Weak	Weak
17P	*Cellulomonas cartae*	PR	--	--	Strong	Strong
18A	*Cellulomonas cartae*	BS	--	--	Strong	Strong

* Extraction location: either peach tree rhizosphere (PR) or bulk soil (BS).

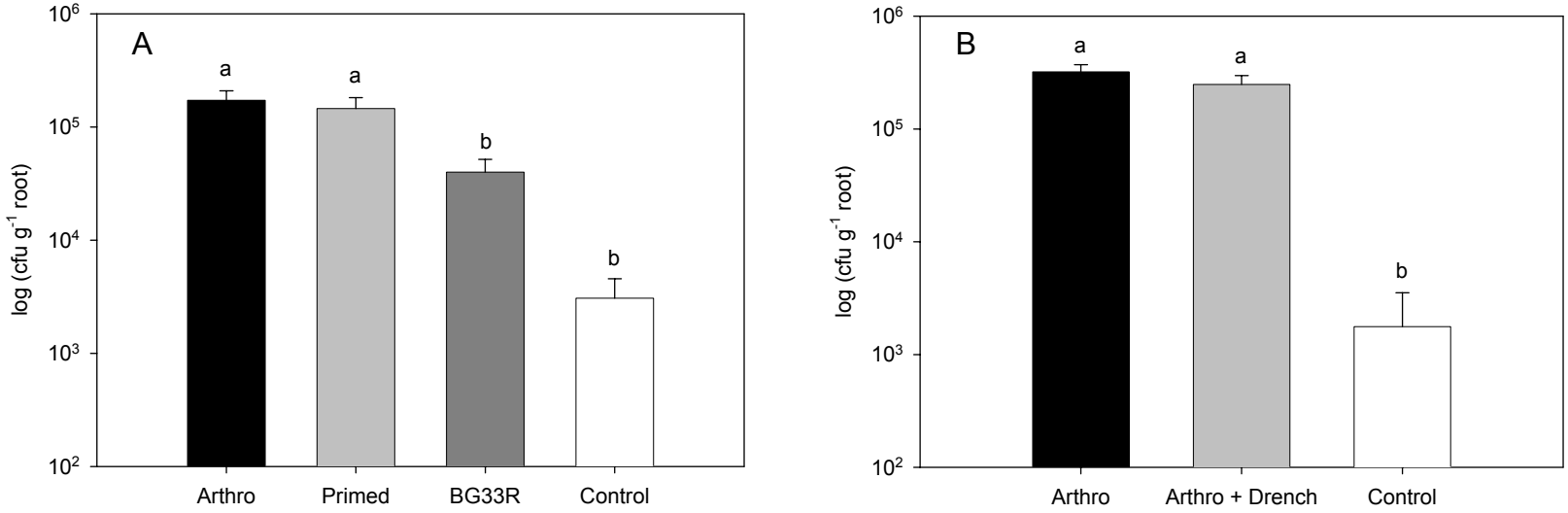

Figure 3.3 (A) Mean root colonization level by treatment in the first greenhouse experiment. Different letters within harvest date indicate significant differences at $P < 0.05$; n = 18 (SAS PROC GLM). (B) Second greenhouse experiment mean root colonization level by treatment. Error bars represent ± 1 standard error. Different letters indicate significant differences at $P < 0.05$; n = 12 (SAS PROC GLM).

Table 3.3 First greenhouse experiment: ANOVA table for bacterial colonization of Lovell roots.

Source	df	SS	MS	*F* Value	*P*-value
Treatment	3	3.56×10^{11}	1.19×10^{11}	10.9	<.0001
Harvest Date	2	8.51×10^{10}	4.25×10^{10}	3.89	0.0259
Treatment*Harvest Date	6	1.20×10^{11}	2.00×10^{10}	1.82	0.1097

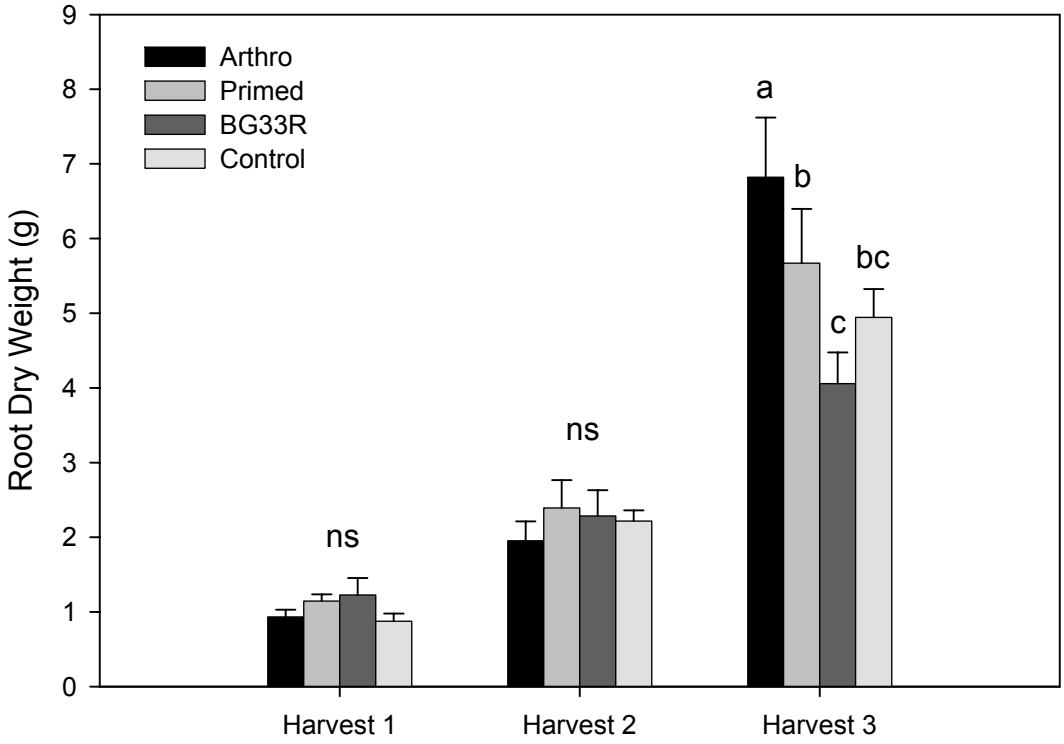

Figure 3.4 Mean root dry weight by treatment on each harvest date. Error bars represent ± 1 standard error. Different letters within harvest date indicate significant differences at $P < 0.05$; n = 6 (SAS PROC GLM) and (ns) represents no significant differences between treatments.

In the second experiment, Arthro isolates again successfully colonized peach seedling rhizospheres. Root colonization levels in the Arthro and Arthro + Drench treatments were more than 100 times greater than those in the control treatment [Figure 3.3(B)] and were comparable to levels observed in the first experiment. Despite high levels of root colonization and the repeated addition of amygdalin to the Anthro + Drench pots, no treatment differences were observed in any plant growth parameters (Table 3.4; $P < 0.05$).

Discussion

We performed a series of experiments to investigate the possibility that rhizobacterial cyanogenesis is a plausible mechanism for peach replant disorder. Previous authors have proposed that (1) orchard soil bacteria are capable of producing cyanide through prunasin degradation and (2) bacterial cyanogenesis reduces the growth and yield of young peach trees (Patrick, 1955); (Gur and Cohen, 1989). Our results support the first hypothesis, and provide some insight into the second.

Patrick (1955) demonstrated that peach root tip respiration was inhibited when peach root extract or amygdalin were acted on by unsterilized peach orchard soil. The author determined that cyanide release was responsible for the reduction in root tip respiration. When the same orchard soil was sterilized in an autoclave, cyanide production and reduced root tip respiration were not observed. A “large number” of soil microorganisms, both bacterial and fungal, were isolated from the orchard soils on agar with either peach root extract or

Table 3.4 Second greenhouse experiment: mean of growth parameters. Each value is the mean of 24 experimental units ± 1 standard error. No significant differences at $P < 0.05$ (SAS PROC GLM).

Treatment	Shoot Height (cm)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root:Shoot
Arthro	48.6 ± 1.32	15.6 ± 0.79	14.5 ± 1.37	0.90 ± 0.06
Arthro + Amygdalin	48.5 ± 0.99	15.1 ± 0.66	15.2 ± 1.53	1.01 ± 0.12
Control	46.6 ± 0.87	14.0 ± 0.87	14.0 ± 1.32	1.06 ± 0.13

amygdalin as the nutrient source. Cyanide production by the isolates was confirmed when picrate papers were added to the cultures, however, the microbes were not identified (Patrick, 1955). Additionally, Gur and Cohen (1989) extracted prunasin-degrading bacilli from oven-dried peach roots and demonstrated their ability to release cyanide *in vitro*.

This is the first study to identify prunasin- and amygdalin-degrading bacteria in the peach rhizosphere. The detection of multiple cyanide-producing isolates from rhizosphere and bulk soil suggests that the ability to metabolize cyanogenic glycosides is common among soil and rhizosphere. Cyanogenic isolates represented a number of genera which belong to two bacterial classes: *Actinobacteria* and *Proteobacteria*.

A. globiformis and *A. ilicis* (syn. *Corynebacterium ilicis*) were isolated from rhizosphere and bulk soil and produced high levels of cyanide when grown on both prunasin and amygdalin. *Arthrobacter* species are aerobic, Gram-positive bacteria with the distinctive feature of a rod-coccus growth cycle. They are coryneform members of the *Actinobacteria* and commonly inhabit the soil and rhizosphere (Keddie et al., 1986). They are known for their ability to degrade a wide range of organic chemicals, including many pesticides (Hagedorn and Holt, 1975; Mahaffee and Kloepper, 1997; Smalla et al., 2001; Turnbull et al., 2001a; Turnbull et al., 2001b). However, this is the first report of an *Arthrobacter* species degrading cyanogenic glycosides and generating cyanide.

The rhizospere isolate *C. fimi* was strongly cyanogenic on prunasin and weakly cyanogenic on amygdalin. Like *Arthrobacter*, this isolate is a coryneform *Actinobacteria*, are aerobic, Gram-positive, and non-motile (Stackebrandt and Keddie, 1986; Schumann et al., 2001). *C. fimi* is known to degrade cellulose and chitin (Mayer et al., 2006). *Cellulosimicrobium cellulans* (syn. *C. cartae*) was a rhizosphere and bulk soil isolate that was strongly cyanogenic on both glycosides. Until recently, this species was classified as member of the genus *Cellulomonas*, and has many of the same attributes as *Cellulomonas spp*., including cellulolytic activity (Schumann et al., 2001). CG degradation and cyanogenesis are not well documented for either *C. fimi* or *Cellulosimicrobium cellulans*.

Three additional Gram-positive species were isolated from various soil or rhizosphere samples. All were aerobic and belong to the class *Actinobacteria*. *Micrococcus luteus* was isolated from peach roots and produced little cyanide on prunasin and none on amgdalin. *Micrococcus spp*. are closely related to the genus *Arthrobacter* and form micrococci (Kocur, 1986). *Microbacterium saperdae* was a rhizosphere isolate that produced low levels of cyanide on both glycosides. Some *Microbacterium* have been identified as potential plant growth-promoting rhizobacteria (PGPR) and may reduce nematode populations (Zinniel et al., 2002). The bulk-soil isolate, *Nocardia asteroides*, produced no cyanide when grown on prunasin or amygdalin amended media. *Nocardia spp*. produce aerial mycelium and *Nocardia asteroides* strains are abundant in soil, most being

soil saprophytes (Goodfellow and Lechevalier, 1989). Little information is available about CG degradation or cyanogenic potential for any of these three species. Our results indicate that these isolates have very low cyanogenic potential when provided CG's *in vitro*, and are not likely to contribute to PRD symptoms.

Serratia liquefaciens was a bulk-soil isolate that produced high levels of cyanide on prunasin, but not on amygdalin. *Serratia spp.* are aerobic, Gram-negative, motile rod bacteria from the class *Proteobacteria*. They have been described as both facultative plant pathogens and plant growth promoting rhizobacteria (PGPR) (Kalbe et al., 1996; Someya and Akutsu, 2005). Several species have demonstrated antibacterial and antifungal activity in the rhizosphere. For example, a root isolate of *Serratia liquefaciens* from oilseed rape (*Brassica napus*) inhibited the growth of *Verticillum dahliae* and *Rhizoctonia solani* fungi *in vitro* (Kalbe et al., 1996). Antibiotic (pyrrolnitrin) activity and chitinase production were the primary mechanisms of fungal inhibition by this species. Although Kalbe *et al*. (1996) screened the *Serratia* isolates for cyanide production, none was detected.

Stenotrophomonas maltophilia was a rhizosphere isolate strongly cyanogenic on prunasin and moderately cyanogenic on amygdalin. *S. maltophillia* is an aerobic, Gram-negative member of the *Proteobacteria* (Berg et al., 2005). Inzuna *et al*. (2002) isolated *S. maltophillia* from the roots of potato (*Solanum tuberosum*) and screened the isolate for production of cyanide,

production of hydrolytic enzymes, suppression of trichodorid nematodes, and other functional traits. They did not observe cyanide production by the isolate; however it did produce cellulases, chitinases, and proteases. The authors also found that the isolate was suppressive to fungi (*Rhizoctonia solani*) *in vitro*, and suppressed trichodorid nematodes by 74.4% in soil (Inzuna et al., 2002).

Photobacterium leiognathi, isolated from the bulk soil, was moderately cyanogenic when provided prunasin, but was acyanogenic on amygdalin. *P. leiognathi* is an aerobic, Gram-negative, luminescent member of the *Proteobacteria*, and is most commonly found in marine environments (Thyssen and Ollevier, 2005).

No orchard isolate produced cyanide when provided with the amino acid glycine, a common root exudate and cyanide precursor. Glycine has been used in multiple studies to demonstrate the ability of bacterial species (usually *Pseudomonas* spp.) to produce cyanide *in vitro* (Bakker and Schippers, 1987; Kremer and Souissi, 2001; Owen and Zdor, 2001). However, the rhizobacteria isolated here clearly generated cyanide by a metabolic pathway separate from that of glycine metabolism.

Pseudomonas species, particularly fluorescent pseudomonads, are the most commonly reported cyanogenic bacteria (Bakker and Schippers, 1987; Kremer and Souissi, 2001; Owen and Zdor, 2001). They are also known to be frequent colonizers of the rhizosphere (Lugtenberg et al., 2001; Kapulnik and Okon, 2002). Thus, it was surprising that no *Pseudomonas* species were

isolated in the present study. It is possible that rhizosphere pseudomonads were not present at the study site, but this is unlikely given the regularity with which they are found in rhizosphere communities. It is more likely that pseudomonads at the site lacked the metabolic pathways necessary for prunasin and amygdalin degradation.

A. globiformis effects on seedling growth. The strongly cyanogenic isolate *A. globiformis* was capable of colonizing the rhizosphere of peach seedlings under greenhouse conditions. Interestingly, colonization levels obtained with *A. globiformis* were significantly greater than those of the known peach rhizosphere inhabitant, *Pseudomonas fluorescens* BG33R (Wechter et al., 2001).

Despite the establishment of *A. globiformis* populations in peach rhizospheres, root and shoot growth were generally unaffected. An exception occurred in Experiment 1 when inoculation caused a modest increase in root dry weight on the third harvest date. This could be attributed to plant growth regulator (PGR) production, particularly auxin production, by the bacterial isolate. PGR production by rhizosphere bacteria has been documented in other studies (Kalbe et al., 1996; Kapulnik and Okon, 2002; Garcia De Salamone et al., 2005), and auxin production by our isolate could have accounted for the increased root dry weight observed.

We considered the possibility that seedling root systems did not produce CGs in sufficient quantity to stimulate bacterial cyanogenesis. In the second experiment, we therefore added high levels of exogenous amygdalin to create

conditions favorable for bacterial cyanogenesis. However, even with added amygdalin, no effect of *A. globiformis* on plant growth was observed.

Results suggest that *A. globiformis* was not metabolizing CGs under our experimental conditions, perhaps preferring more abundant and/or easily-metabolized root exudates. Alternately, peach root metabolism may have been resistant to the effects of cyanide produced by *A. globiformis*. In either case, our results do not support an important role for *A. globiformis* in peach replant disorder. If *A. globiformis* preferentially utilizes carbon sources other than CGs, then it is unlikely to generate large quantities of cyanide under field conditions, even when CGs are present. If peach root metabolism can proceed in the presence of cyanide (at least in moderate concentrations), then CG-degrading rhizobacteria will have little effect on peach root growth.

Cyanide inhibits aerobic respiration by complexing with the iron in cytochrome oxidase (Salisbury and Ross, 1992). Various researchers have recorded a wide range of cyanide concentrations inhibitory to root growth. Kremer and Souissi (2001) demonstrated significant root growth reduction in several herbaceous weed species at concentrations ranging from 12.5 to 100 µmol cyanide. Potato root respiration was reduced by 40% when exposed to as little as 5 µM potassium cyanide (KCN) (Bakker and Schippers, 1987). In their PRD research, Gur and Cohen (1989) found that a total of 39 mmol KCN applied over 22 weeks reduced peach seedling and almond seedling dry weight to 50.4% and 27.4% of the control, respectively.

In our second greenhouse experiment, 12 weekly applications of 500 ppm amygdalin were applied to Lovell seedlings. If all of the amygdalin molecules were completely hydrolyzed, 110 µmol of cyanide would be released into the rooting zone of the seedlings, and the total for the 12 applications would be approximately 1.32 mmol cyanide. This value is considerably higher than the root inhibiting concentrations used by Kremer and Souissi (2001) and Bakker and Schippers (1987) above, but is lower than the concentrations that reduced peach and almond seedling growth for Gur and Cohen (1989).

Clearly, additional research is necessary to determine whether there is a link between the presence of CG-degrading rhizobacteria and peach replant disorder. However, the lack of any effect of *A. globiformis* on peach seedling growth indicates that high levels of colonization by a known CG-degrader does not necessarily impair plant performance.

Conclusion

Our results show that the ability to degrade prunasin and amygdalin is widespread among bacterial genera from rhizosphere and bulk soil in a South Carolina peach orchard. The most strongly cyanogenic of our rhizosphere bacterial isolates, *A. globiformis,* successfully colonized the rhizosphere of young peach seedlings. However, its presence had no negative effects on seedling growth, suggesting that it was not cyanogenic under our experimental conditions or that the peach seedlings were resistant to the effects of cyanide. In either case, our results do not offer strong support for a role of *A. globiformis* in peach

replant disorder. However, the full extent of the link between CG-degrading rhizobacteria and PRD remains to be investigated.

CHAPTER 4

PEACH TREE ROOT DEMOGRAPHY IN A REPLANT SITE

Introduction

Peach replant disorder (PRD) is a serious problem for peach growers throughout the United States and occurs when young peach trees are planted on a site previously planted with peaches. The young trees grow more slowly and produce lower yields than they do on non-replant sites. Although the specific causal agents of PRD are unknown, methyl bromide fumigation adequately manages the problem (Gur and Cohen, 1989), suggesting that the disorder results from harmful interaction(s) between the soil microbial community and peach roots. As methyl bromide fumigation is phased-out because of its negative environmental impacts, identification of PRD's specific cause has become an important research goal.

Root-microbe interactions occur below the soil-line, making their observation difficult. Most root studies have relied on relatively insensitive, destructive measurements, such as root system dry weight and total length (Nehl et al., 1996). However, non-destructive techniques that allow the observation of roots *in situ* can provide a better understanding root development and turnover (Berggren et al., 2001).

Minirhizotron techniques are a method for non-destructive fine root (<1 mm diameter) observation. Minirhizotrons are transparent tubes inserted into the root zone against which fine roots grow and through which a time series of

photographs can be made with a below-ground fiber optic camera system (Johnson et al., 2001). These photographs can be analyzed using commercially available image analysis software to obtain information on root production, growth and mortality.

Previous minirhizotron research indicates that soil fauna can have a dramatic impact on fine root lifespan (Kosola et al., 1995; Eissenstat et al., 2000; Wells et al., 2002) and that treatments targeted to suppress soil biota result in increased fine root longevity. In the present study, we evaluated four PRD management strategies that targeted different soil microbe groups: three direct soil treatments and one indirect soil treatment. The direct treatments included soil fumigation with methyl bromide, soil solarization, and soil fumigation with 1,3-dicloropropene + chloropicrin (Telone C-17). The indirect treatment was systemic pre-kill of the previous peach trees with stem injections of glyphosate. The direct soil treatments had the potential to directly kill or suppress soil fauna, while the glyphosate treatment indirectly affected soil microbe populations by destroying one of their nutrient sources, remnant peach roots.

Methyl bromide is a broad-spectrum biocide that is highly effective at controlling most soil-borne pests, pathogens and weeds (Chellemi, 2002; Schneider et al., 2003). It is also considered the single best control strategy for PRD.

Soil solarization is the process of using passive solar heating to reduce or eliminate soil-borne pathogen and weed populations by covering moist soil with

clear polyethylene sheeting during periods when solar radiation is highest (e.g. summer). Solarization has been shown to significantly alter soil microbial communities (Peachey et al., 2001; Kluepfel et al., 2002).

Telone C-17 is composed of 1,3-dicloropropene (1,3-D) + 17% chloropicrin. The chemical 1,3-D is a widely used soil nematicide, and chloropicrin is a fungicide. The treatment is a liquid formulation typically shank injected to a depth of > 25 cm. Both chemicals have activity beyond their primary target group. For example, 1,3-D is known to be active against some fungal and bacterial plant pathogens, while chloropicrin has some marginal nematicidal activity, and both give low levels of weed control (Mai and Abawi, 1981; Duniway, 2002).

The use of glyphosate herbicide to systemically kill remnant roots of the previous orchard before replanting has been studied extensively as a PRD control strategy in California (McKenry, 1999). Remnant roots are woody roots, either fragmented or whole, that remain in the orchard soil after the above-ground portions of the tree have been removed.

In addition to soil treatment, rootstock selection may contribute to PRD management. Resistance or tolerance to PRD soils would be a highly desirable rootstock trait, and such traits have been identified in other systems, particularly in apple (Yao et al., 2006). We chose Lovell and Guardian® rootstocks for this research. These are common commercial rootstocks in the southeastern U.S., and Guardian® is replacing Lovell because of its combination of peach tree short

life tolerance and root-knot nematode resistance. Both are similar in cultural habit and influence their scions in comparable ways, but Guardian® is known to be more vigorous than Lovell (Reighard et al., 1996; Beckman et al., 1997). Our work represents the first direct observation of fine root dynamics in these rootstocks and provides a means to assess their relative performance under treated and untreated replant conditions.

The primary objective of our study was to provide new insight into causes and potential management options for PRD. Specifically, we studied fine root dynamics of peach in treated and untreated replant soils using minirhizotrons and compared the ability of traditional and non-traditional replant treatments to improve tree growth on a replant site.

Materials and Methods

The experiment was conducted in a replanted peach orchard at the Musser Fruit Research Center near Clemson, SC. Soil at the site was an Appling Sandy Loam. In the spring of 2002, a planting of seven-year-old 'Redglobe' peach trees was removed. The original planting consisted of ten 36.6 m rows with 6 m between the rows and 5.2 m between trees in each row. Row length and between-row spacing was maintained during subsequent treatment application and replanting. The in-row spacing for our experiment was 3.3 m.

Prior to replanting, each orchard row received one of five treatments: trunk injections of glyphosate to pre-existing trees (Gly), soil solarization (Sol), methyl

bromide (MBr) soil fumigation, Telone C-17 (Tel) soil injection, and an untreated control.

Preplant Treatments

On May 2, 2002, pre-existing trees in two rows were killed using the herbicide glyphosate [N-(phosphonomethyl) glycine] prior to their removal from the field (McKenry, 1999). Horizontal notches (4-6 cm long, 0.5 cm deep) were cut into the trunk approximately 5-10 cm below each scaffold limb, and 1 mL of 41% glyphosate (Eraser, Control Solutions, Inc., Pasadena, TX) was sprayed into each notch. Within 5-10 seconds of application, the tree had absorbed most of the product. After 11 days, aboveground portions of treated trees had undergone considerable leaf fall. On the fourteenth day, any scaffold limb that still exhibited green leaves was re-treated as before. All treated trees were dead at the time of removal on July 24, 2002.

Soil solarization was applied to two orchard rows beginning on June 5, 2002. Prior to treatment application, rows were cultivated and irrigated to field capacity. They were then covered with 3 m-wide clear, plastic sheeting (4 mil Nursery Clear Film, GroSouth of Georgia, Inc.) whose edges were secured by burying them in 20-cm deep furrows at the row edges (Figure 4.1). The plastic remained in place until January 7, 2003.

Air and soil temperatures (7.5 cm depth) were recorded every fifteen minutes in one Sol and one Control row (Watchdog Temperature Logger, Spectrum Technologies, Inc.). The average monthly soil temperatures in August

were 29.8° C in the Control row and 38.0° C in the Sol row. The highest soil temperature recorded in the Sol row was 52.4°C on August 7.

Methyl bromide (Brom-O-Gas, Great Lakes Chemical, West Lafayette, IN) was applied to two orchard rows in November 2002. Rows were cultivated and covered in plastic sheeting as described above. Six 0.68 kg cans of MBr were placed beneath the plastic sheeting on each row and punctured to release their contents. The resulting MBr application rate was 4.76 kg per row (474.3 kg per hectare). Plastic sheeting was removed 12 days after MBr application.

Telone C-17 (DowAgroSciences, Indianapolis, IN) was applied to two orchard rows November 15, 2002. The rows were cultivated prior to application, and the product was applied using a Reddick shank-fumigation rig (Reddick Fumigants, Williamston, NC). The rig was fitted with 28 cm long fumigation shanks and was calibrated with water to deliver 4.17 L of Telone C-17 per row (374 L per hectare).

Orchard Planting

On January 23, 2003, 120 'Redglobe' peach trees (budded June 2002) were replanted on the treated site: 12 trees per row spaced 3.3 m apart in the row. Half of the trees were budded onto one-year-old Lovell (L) rootstock and half were budded onto one-year-old Guardian® (G) rootstock. Stem diameters of the trees were measured at 8 cm above the bud union. At planting, trees on G rootstock had an average stem diameter of 9.4 ± 0.3 mm and those on L had an average stem diameter of 7.8 ± 0.3 mm (see Appendix Table A-3). Subsequent

Figure 4.1 Application of solarization plastic.

stem diameter measurements were taken on three dates: June 10, 2003, September 19, 2003 and February 25, 2004.

Irrigation tubing and micro-sprinklers were installed in March 2003. Irrigation was applied at a rate of approximately 2.5 cm per week during the growing season in the absence of adequate rainfall. Each tree was fertilized in April with approximately 0.45 kg of 10-10-10 fertilizer. Calcium nitrate (15.5-0-0) was applied at 0.45 kg per tree in June. All fertilizer applications were evenly distributed within a 0.5 m radius of the trunk. Herbicide applications (glyphosate, 18 ml L^{-1}) and hand weeding were used to maintain adequate weed control in the rows.

Minirhizotron tubes constructed from cellulose acetate butyrate were installed beneath 60 of the experimental trees at the time of planting. Tubes were 70 cm in length (50 cm viewable) with an outer diameter of 6 cm and were marked with three vertical transects of twenty-eight 14 x 18 mm windows. Bottoms of the tubes were sealed with acrylic plugs. Tops were wrapped in black electrical tape and covered with white aluminum cans to exclude light and debris while minimizing radiant heating.

Tubes were located on the southwest side of every other tree and were flanked by two trees of the same treatment and rootstock. Each tube was installed approximately 0.6 m from the stem of the nearest tree at an angle of 30° from vertical. There were 12 tubes per treatment, six for each treatment by rootstock combination.

Bi-weekly observations of fine root growth were made with a miniaturized video camera (BTC-100X, Bartz Technology Company, Santa Barbara, CA) beginning in February 2003 and continuing through the end of the growing season. Still images of each minirhizotron window were obtained using Cleaner software (Terran Interactive, Montreal, Canada), and root growth data were collected using RootTracker software (Duke University Phytotron, Durham, NC). Parameters recorded for each root included date of appearance, date of browning, date of disappearance, length, diameter, and depth. Date of browning was defined as the first date on which the root exhibited brown pigmentation. A root was classified as dead when it (1) disappeared or (2) appeared black, ragged and shriveled. In a small number of cases, fine roots underwent pronounced radial expansion and appeared to be developing into woody structural roots. The first date on which marked radial expansion became apparent was noted.

Statistical Analysis

The experiment was arranged as a randomized block split-plot design with soil treatment as the whole plot factor and rootstock as the subplot factor. The effects of treatment, rootstock and treatment by rootstock interactions on growth parameters were evaluated using SAS PROC MIXED (SAS Institute, Cary, NC), and means were separated using Fisher's least significant difference (LSD).

The individual and interactive effects of treatment, root diameter, and root depth on root survivorship and browning were estimated with Cox proportional

regression (SAS PROC PHREG; Wells and Eissenstat, 2001). Roots of all cohorts produced during the first growing season were combined for these analyses.

Results

Stem diameter

Nineteen months post-transplant, trees grown in MBr-treated and Sol soils had slightly greater stem diameters than trees in the other treatment groups, although this difference was not significant (P = 0.2383; Table 4.1; Figure 4.2). Rootstock had a significant effect on stem diameter, with scions budded onto G rootstock having a mean stem diameter 5 mm greater than those budded onto L (Table 4.1; Figure 4.3).

Fine root production, mortality and standing crop

PRD treatments altered rates of root production, mortality and standing crop on several dates. The general trend was for Control trees to have greater root production, mortality and standing crop (total root length present per tube on a given date) than all other treatments, and this effect was particularly apparent in Control G trees. Root production on Control trees was significantly higher than on all other trees on one date: May 13, 2003 (P = 0.054; Table 4.2; Figure 4.4). Similar trends were present on additional dates.

Root mortality in trees treated with MBr, Sol and Tel was significantly lower (P < 0.1) than that of Control trees on three dates: May 27, June 26, and

Table 4.1 Split-plot ANOVA for final mean stem diameters (α = 0.1; SAS PROC MIXED).

Source	df	*F* Value	*P*-value
Treatment	4	1.96	0.2383
Rootstock	1	6.93	0.0098
Treatment by rootstock	4	1.59	0.1826

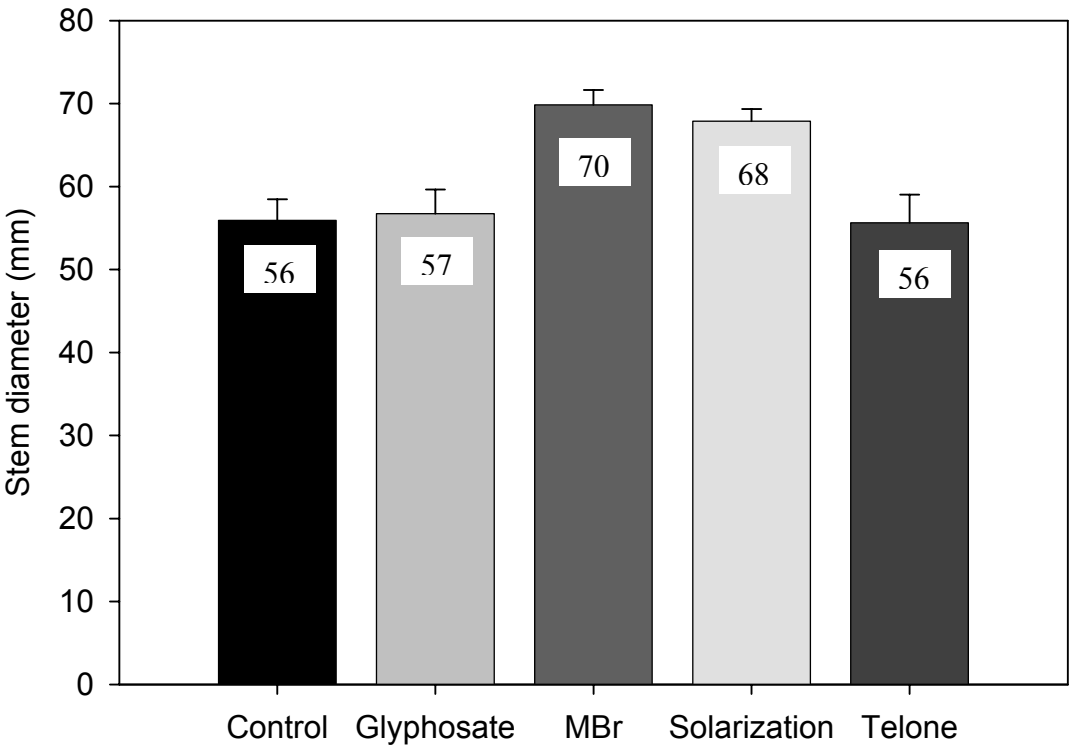

Figure 4.2 Mean stem diameters of replanted peach trees from each treatment on August 30, 2004. Error bars represent ± 1 standard error. No significant treatment differences; *P* = 0.2383; n = 2 for treatment main effect (SAS PROC MIXED).

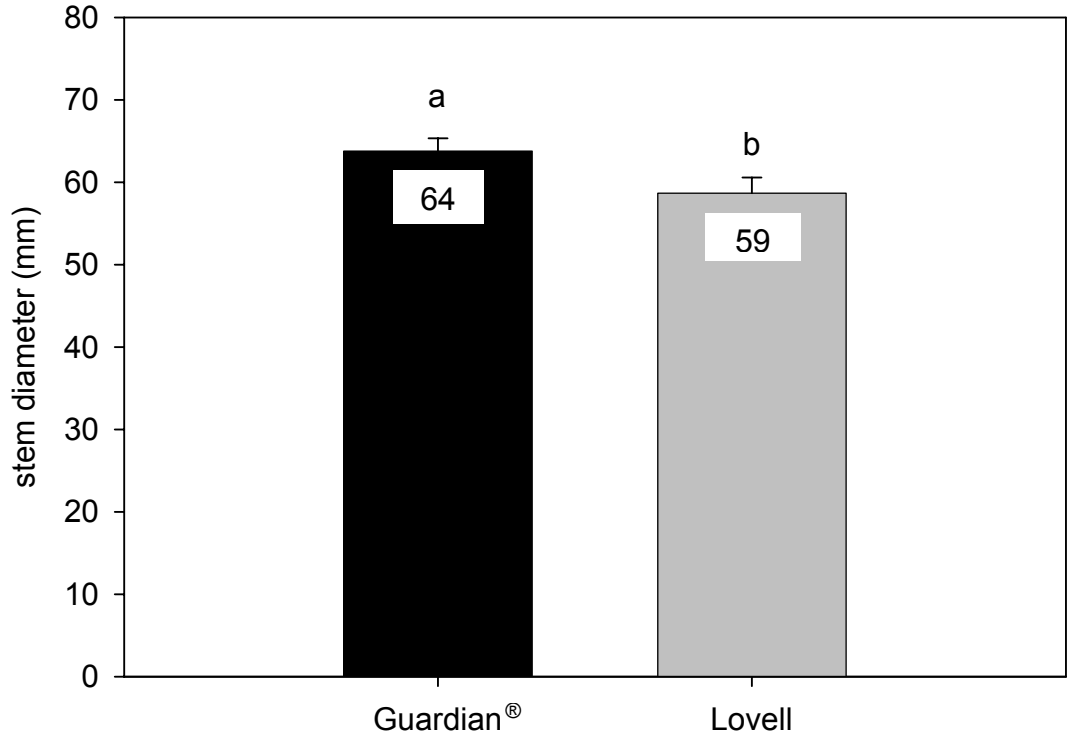

Figure 4.3 Mean stem diameters of replanted peach trees from each rootstock on August 30, 2004. Error bars represent ± 1 standard error. Different letters represent significant differences at $P < 0.05$ level; n = 30 for rootstock effect (SAS PROC MIXED).

Table 4.2 Split-plot ANOVA for root production. Dates with significant effects presented (α = 0.1; SAS PROC MIXED)

Source and date	df	*F* Value	*P*-value
Treatment			
May 13	4	4.98	0.054
June 26	4	1.63	0.299
July 9	4	2.28	0.222
October 22	4	0.83	0.559
Rootstock			
May 13	1	0.19	0.661
June 26	1	3.27	0.077
July 9	1	0.51	0.477
October 22	1	6.53	0.014
Treatment by rootstock			
May 13	4	1.44	0.237
June 26	4	1.14	0.349
July 9	4	5.59	0.001
October 22	4	0.58	0.679

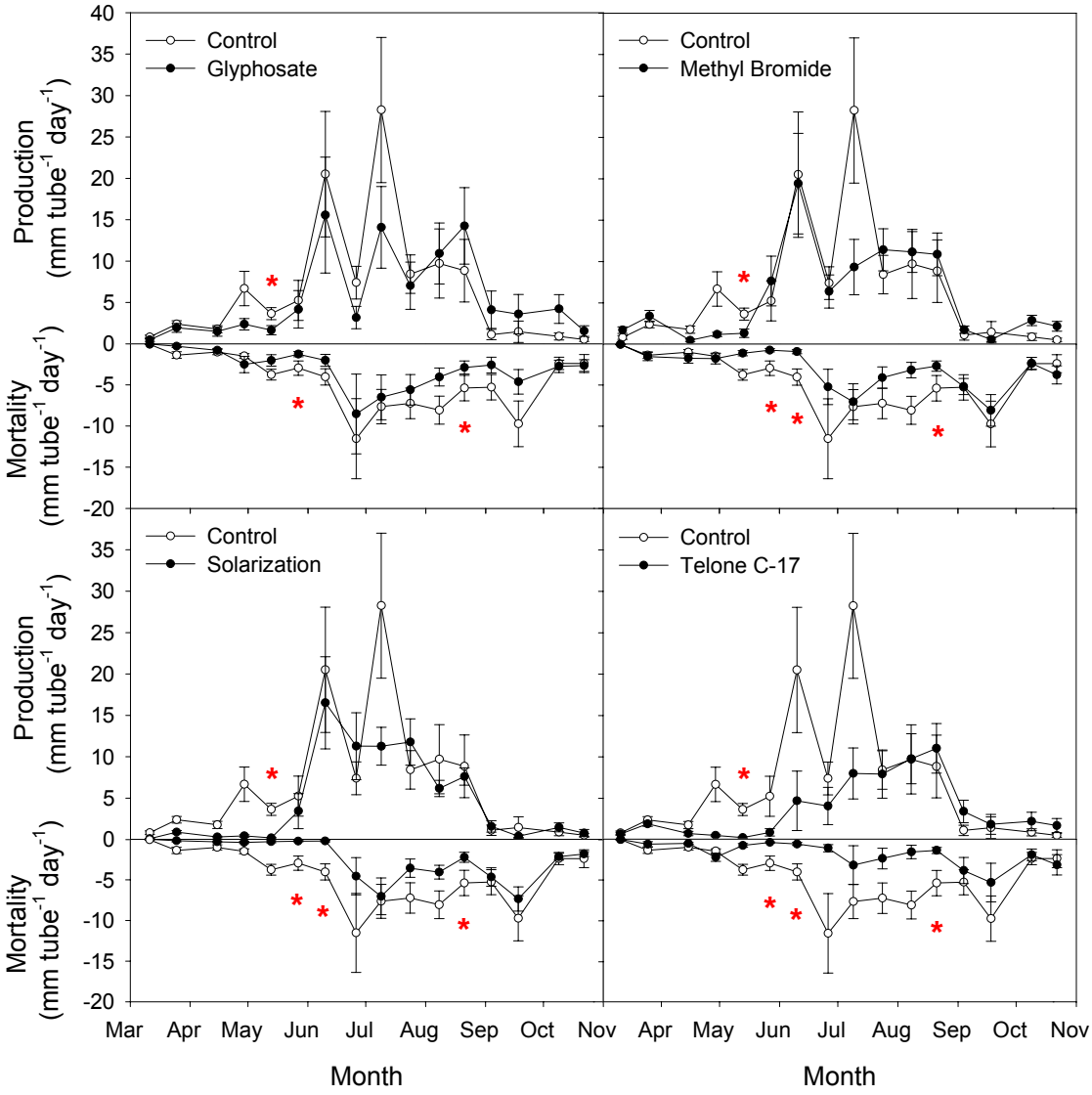

Figure 4.4 Root production and mortality from March 2003 to October 2003. Error bars represent ± 1 standard error. Asterisks (*) indicate treatment different from control at $P < 0.1$ level; n = 2 (SAS PROC MIXED).

August 21 (Table 4.3; Figure 4.4). Gly tree root mortality was significantly less than Control tree root mortality on May 27 and August 21 (Figure 4.4).

Standing root crop was not affected by treatment on any date ($P < 0.1$; Table 4.4; Figure 4.5). However, there was a trend toward lower standing crop in the Gly and Tel treatments throughout the summer (Figure 4.5).

Rootstock influenced root production but had no effect on root mortality or standing crop. The rate of root production was greater for L trees than G on two dates. On June 26, L produced an average of 8.4 mm roots tube^{-1} day^{-1} while G produced an average of 4.5 mm roots tube^{-1} day^{-1} ($P < 0.1$; Table 4.2). On October 22, L and G averaged 1.98 mm roots tube^{-1} day^{-1} and 0.73 mm roots tube^{-1} day^{-1}, respectively. This trend of L trees producing more root length than G trees was observed on all but four of the seventeen sample dates. A notable exception occurred for the Control G trees on July 9, as highlighted below.

We observed a large flush of root production in Control G trees on July 9. Control G trees had an average root production rate of 46.7 mm roots tube^{-1} day^{-1} on this date, more than double the production of any other treatment combination ($P = 0.001$; Table 4.2; Figure 4.6). Higher rates of root mortality and a larger standing root crop were subsequently observed in Control G trees throughout the rest of the growing season. For example, Control G trees had significantly more roots than Control L trees on four dates from July 9 through September 18 ($P < 0.1$; Table 4.4; Figure 4.7). Mortality rates were also

Table 4.3 Split-plot ANOVA for root mortality. Dates with significant effects presented (α = 0.1; SAS PROC MIXED)

Source and date	df	*F* Value	*P*-value
Treatment			
March 25	4	1.63	0.324
April 15	4	0.75	0.601
May 27	4	4.75	0.059
June 10	4	3.80	0.088
July 24	4	1.71	0.164
August 8	4	2.70	0.153
August 21	4	3.37	0.016
September 18	4	0.54	0.717
October 9	4	0.13	0.961
October 22	4	0.35	0.836
Rootstock			
March 25	1	1.12	0.295
April 15	1	0.03	0.855
May 27	1	0.04	0.841
June 10	1	0.17	0.686
July 24	1	0.01	0.940
August 8	1	1.06	0.308
August 21	1	0.59	0.446
September 18	1	0.36	0.552
October 9	1	0.02	0.895
October 22	1	0.52	0.474
Treatment by rootstock			
March 25	4	2.23	0.081
April 15	4	2.15	0.091
May 27	4	0.22	0.927
June 10	4	0.17	0.954
July 24	4	2.63	0.045
August 8	4	3.41	0.016
August 21	4	3.84	0.009
September 18	4	2.15	0.090
October 9	4	2.13	0.093
October 22	4	2.18	0.086

Table 4.4 Split-plot ANOVA for root standing crop. Dates with significant effects presented (α = 0.1; SAS PROC MIXED)

Source and date	df	*F* Value	*P*-value
Treatment			
July 9	4	1.72	0.280
August 21	4	0.29	0.876
September 4	4	0.17	0.946
September 18	4	0.11	0.973
Rootstock			
July 9	1	0.10	0.751
August 21	1	0.18	0.672
September 4	1	0.30	0.585
September 18	1	1.07	0.306
Treatment by rootstock			
July 9	4	2.22	0.082
August 21	4	2.10	0.096
September 4	4	2.38	0.066
September 18	4	2.28	0.076

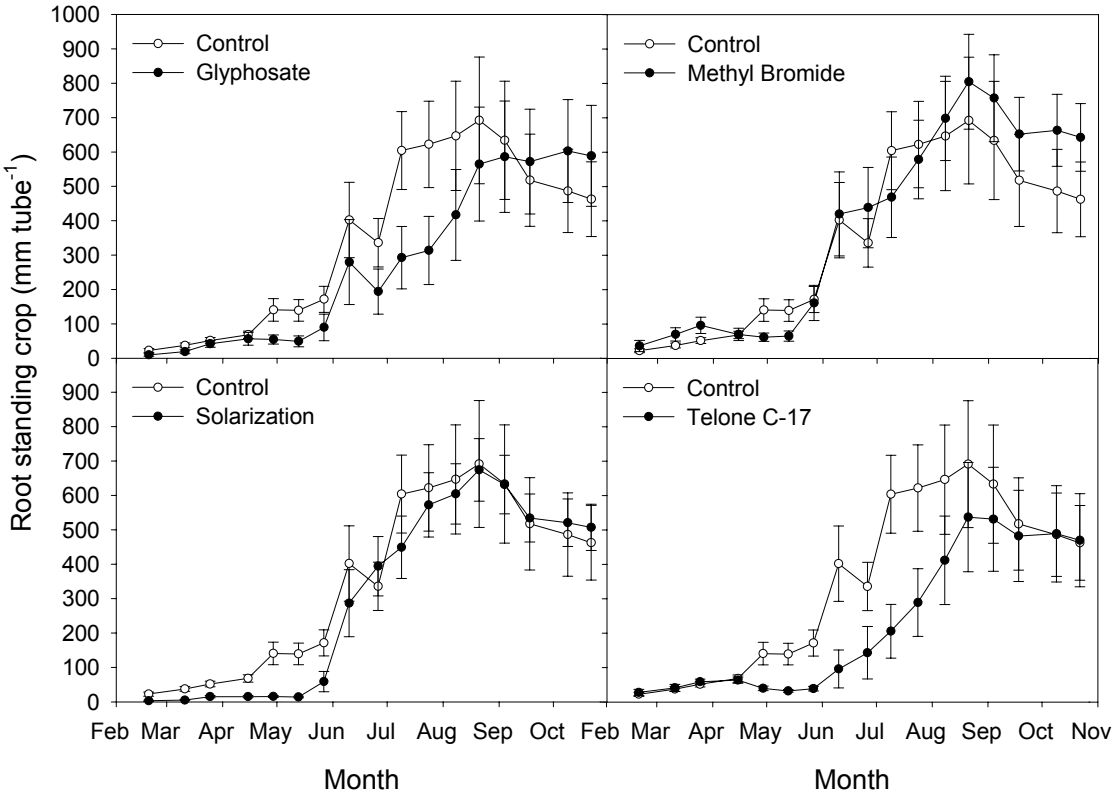

Figure 4.5 Standing root crop of each treatment compared to control. Error bars represent ± 1 standard error. No significant treatment differences; $P < 0.1$; n = 2 (SAS PROC MIXED).

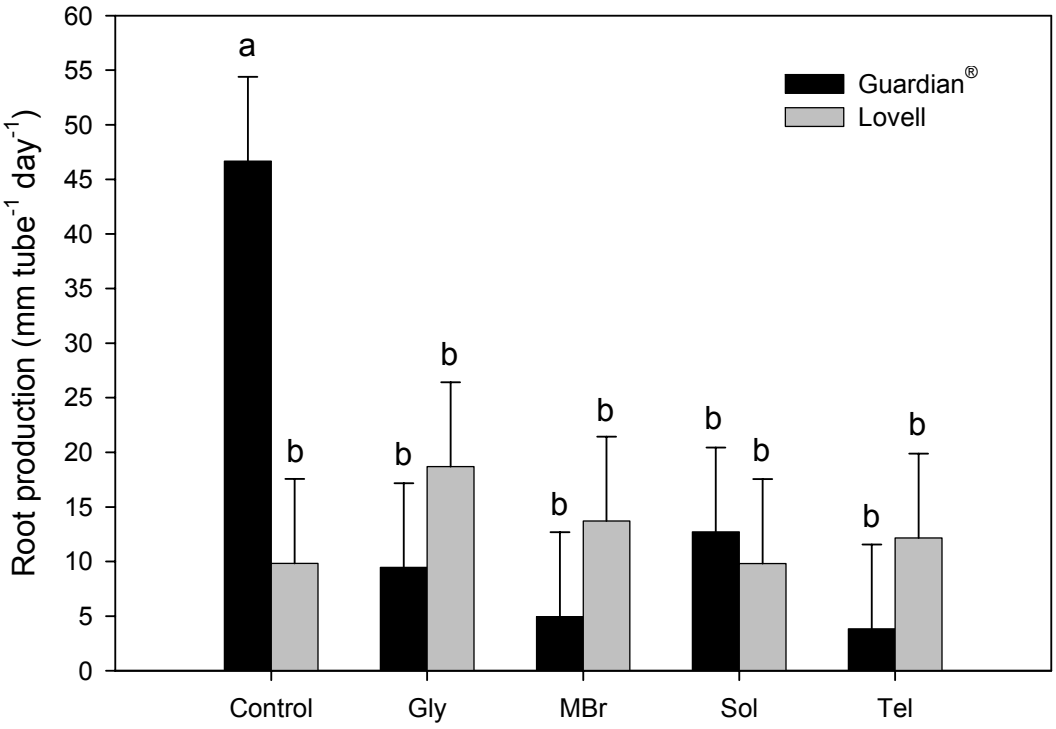

Figure 4.6 Effects of treatment and rootstock on root production on July 9, 2003. Error bars represent ± 1 standard error. Different letters represent significant differences within date at P = 0.001; n = 6 for interaction effects (SAS PROC MIXED).

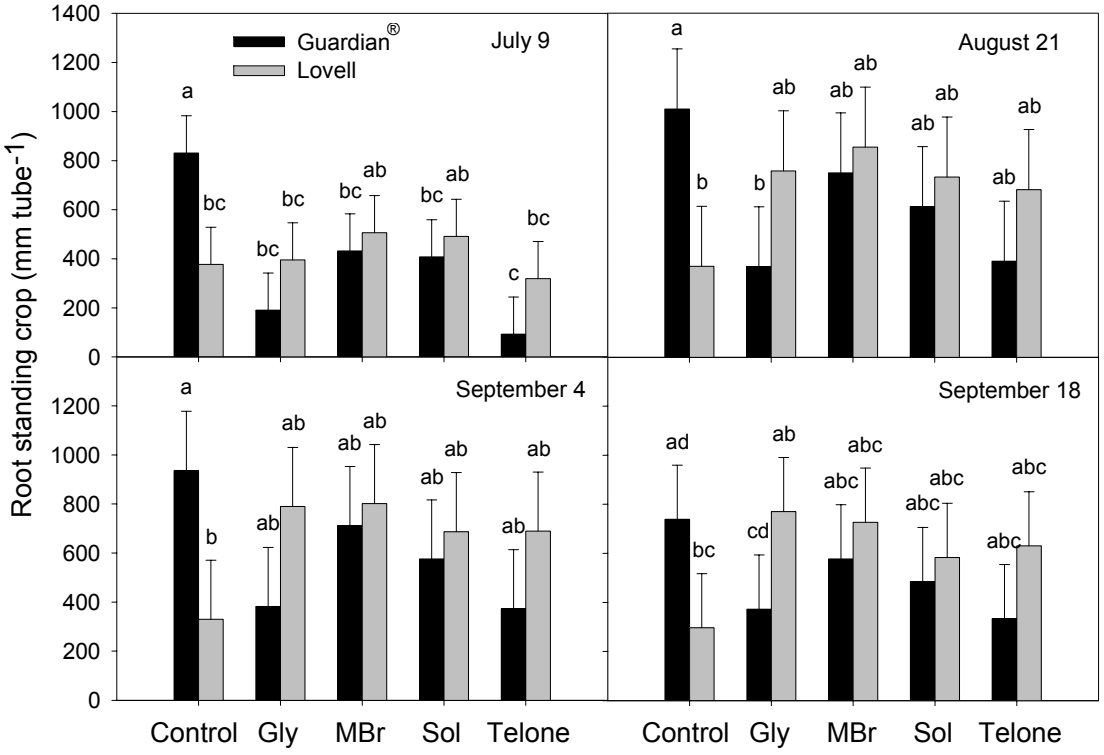

Figure 4.7 Effects of treatment and rootstock on standing root crop on four dates in 2003. Error bars represent ± 1 standard error. Different letters represent significant differences within date at $P < 0.1$ level; n = 6 (SAS PROC MIXED).

significantly higher for Control G trees than for most other combinations from July 24 through August 21 ($P < 0.1$; Figure 4.8).

In all treatment groups other than Control, L trees tended to have a higher standing crop than G trees. This effect was only significant for the Gly treatment on September 18.

Methyl bromide

Root production and standing crop in MBr trees were generally the same as in Control trees, but root mortality rate tended to be lower. Early in the growing season, MBr trees tended to have the highest root mortality rate, regardless of rootstock ($P < 0.1$; Table 4.3; Figure 4.8). On March 25, MBr-treated L trees had the highest mortality rate: significantly higher than MBr G trees and all other combinations except Control trees and Tel L trees ($P < 0.1$; Figure 4.8). MBr-treated G trees had the highest mortality rate on April 15 and were significantly higher than MBr L trees, both Sol rootstocks, and Tel G trees. After April, root mortality in MBr trees was essentially the same as in the other pre-plant treatments.

Solarization

The effects of the Sol treatment on root production, mortality and standing crop were very similar to those of MBr. Sol trees had higher root production than Controls on one date and lower root mortality than Controls on three dates ($P < 0.1$; Figure 4.4). Sol trees did not differ from MBr for any of the root

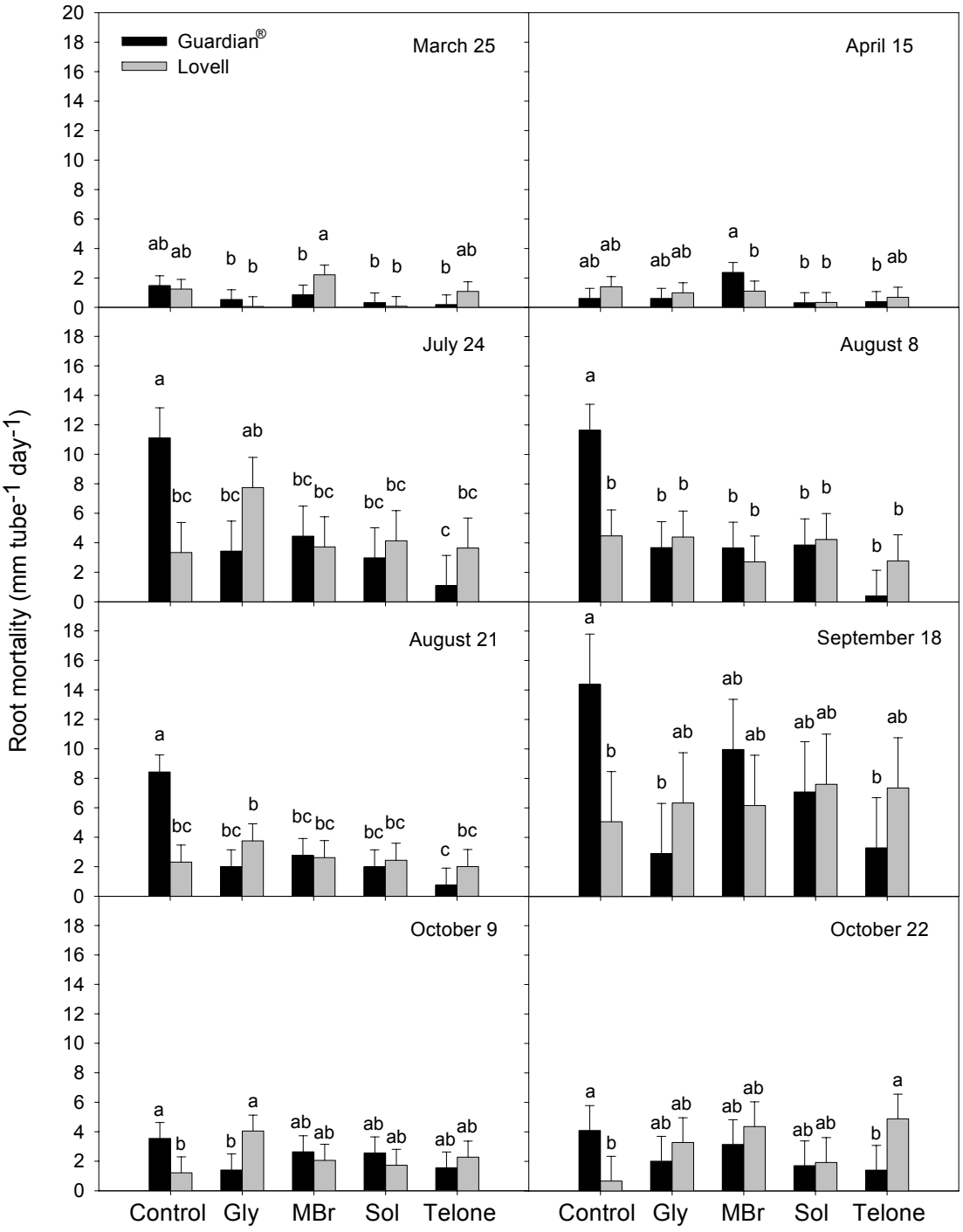

Figure 4.8 Effects of treatment and rootstock on fine root mortality on eight dates in 2003. Error bars represent ± 1 standard error. Different letters represent significant differences within date at $P < 0.1$ level; n =6 (SAS PROC MIXED).

growth parameters, nor was root growth in Sol G trees different from that in Sol L trees.

Telone C-17 and Glyphosate

Tel-treated trees generally had the lowest root production, mortality and standing crop throughout the season. Root production in Tel trees peaked in late summer, rather than early summer as it did in the other treatment groups (Figure 4.4). Root production and mortality in Gly trees were slightly less than those of Control trees throughout the experiment, but this difference was not significant (Figure 4.4).

Fine root lifespan

All treatments significantly reduced the risk of fine root mortality when compared to control [$P < 0.05$; Table 4.5(A)]. MBr had the greatest effect, increasing the median lifespan by 28 d. Sol and Tel increased median lifespan by 27 d and 26 d, respectively. Gly had the smallest effect, increasing median lifespan by only 13 d (Figure 4.9).

The effect of rootstock on root lifespan was marginally significant ($P = 0.0772$), with G roots having a slightly lower risk of mortality than L roots [Table 4.5(A)]. Root diameter and depth both influenced root survivorship significantly [Table 4.5(A)]. Larger diameter roots lived longer, as did roots located on the top half of the tube (0 – 21.7 cm depth).

Table 4.5 Results of proportional hazards regression for (A) root survivorship and (B) root browning (α = 0.1; SAS PROC PHREG).

A) Survivorship

Variable	df	Wald Chi-square	*P* > Chi-square	Risk ratio
Glyphosate	1	18.54	< 0.0001	0.867
Methyl bromide	1	162.43	< 0.0001	0.669
Solarization	1	112.22	< 0.0001	0.690
Telone C-17	1	88.61	< 0.0001	0.700
Rootstock (G)	1	3.12	0.0772	0.960
Diameter	1	269.10	< 0.0001	0.398
Depth	1	260.32	< 0.0001	1.444

B) Browning

Variable	df	Wald Chi-square	*P* > Chi-square	Risk ratio
Glyphosate	1	0.33	0.5659	1.023
Methyl bromide	1	22.09	< 0.0001	0.838
Solarization	1	9.32	0.0023	0.888
Telone C-17	1	0.510	0.4388	1.032
Rootstock (G)	1	24.78	< 0.0001	1.134
Diameter	1	133.34	< 0.0001	1.546
Depth	1	25.09	< 0.0001	1.140

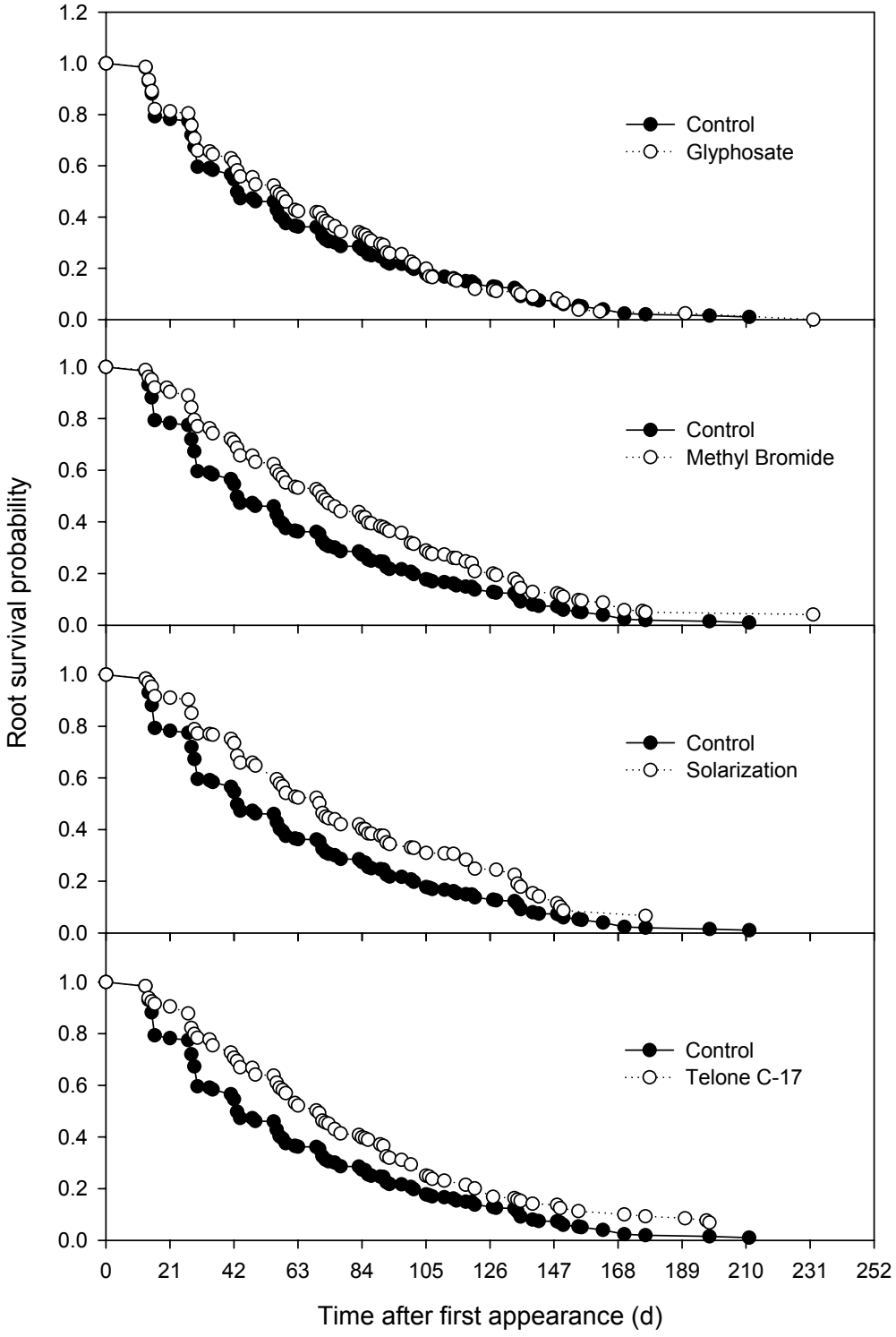

Figure 4.9 Survival probabilities of roots from each treatment compared to the control significant at $P < 0.05$ (SAS PROC PHREG).

MBr and Sol treatments significantly reduced the risk of fine root browning compared to control [$P < 0.05$; Table 4.5(B); Figure 4.10]. White roots in MBr-treated and solarized soils were less likely to become brown than those in control soil. The risk of browning for roots in the Gly and Tel treatments did not differ from control. The rootstock effect on root browning rate was also significant [$P < 0.05$; Table 4.5(B), Figure 4.11]. The risk of browning for Lovell roots was approximately 87% that of Guardian® roots [Table 4.5(B)].

Pruning fresh weights (12 months after transplant) and final shoot and root fresh weights (destructive harvest 23 months after transplant) did not differ significantly among treatment groups or rootstocks (see Appendix Table A-5).

Discussion

Replanted peach trees grown in the MBr-treated and Sol soils were visibly more vigorous at the end of the first growing season than trees in the control and other two treatments (Figure 4.12). This observation was supported by comparing the stem diameters of the trees. While not significantly different, trees of the MBr and Sol treatments had average stem diameters at least 10 mm greater the other treatments at the end of the study (Figure 4.2).

A recurring issue with this experiment was that the main plot factor was replicated only twice, giving us little statistical power when testing for treatment effects. Despite the lack of significance, there did seem to be a positive above-ground growth response to MBr and Sol treatments.

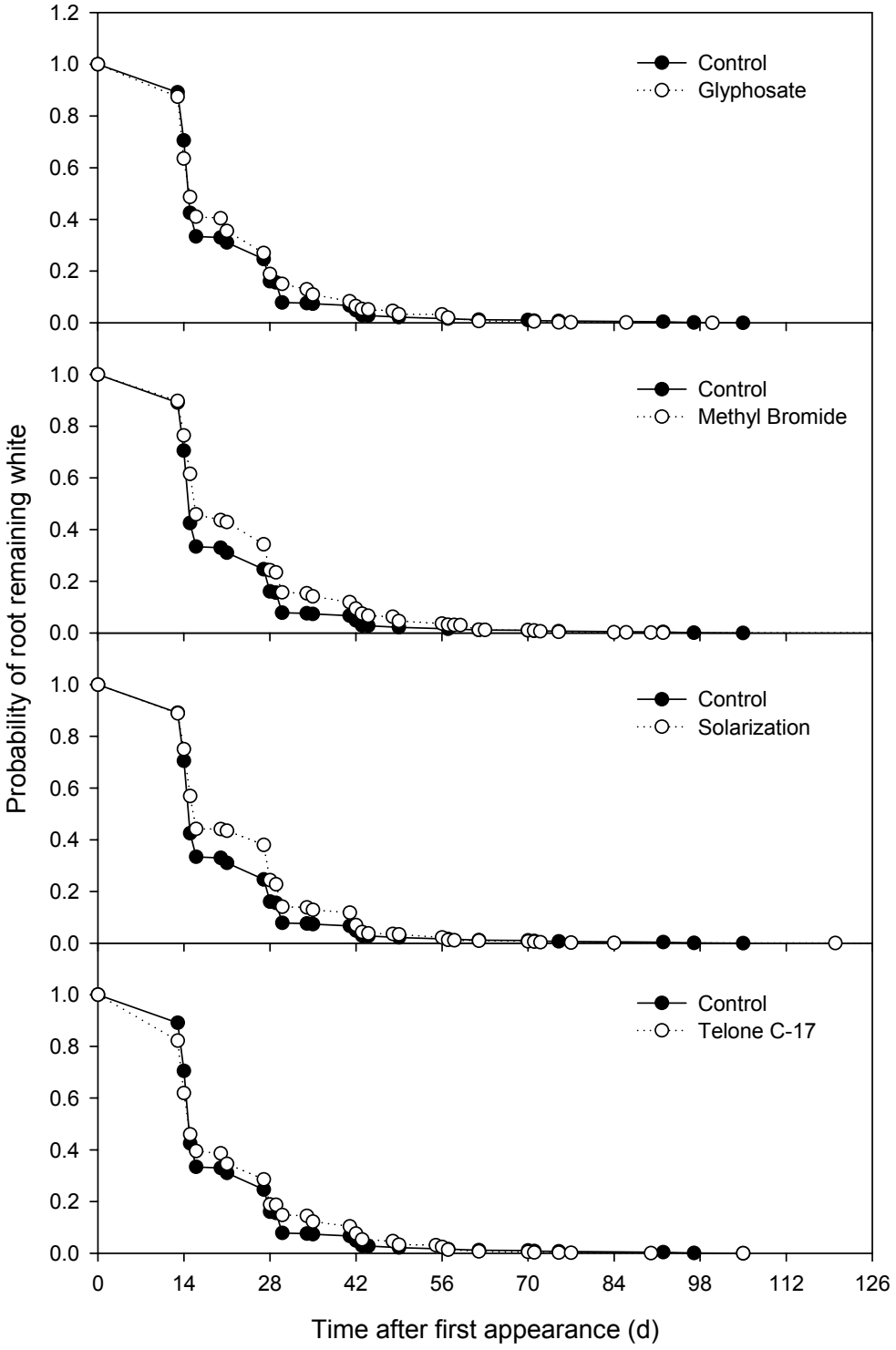

Figure 4.10 Browning probabilities of roots from each treatment compared to the control significant at $P < 0.05$ (SAS PROC PHREG).

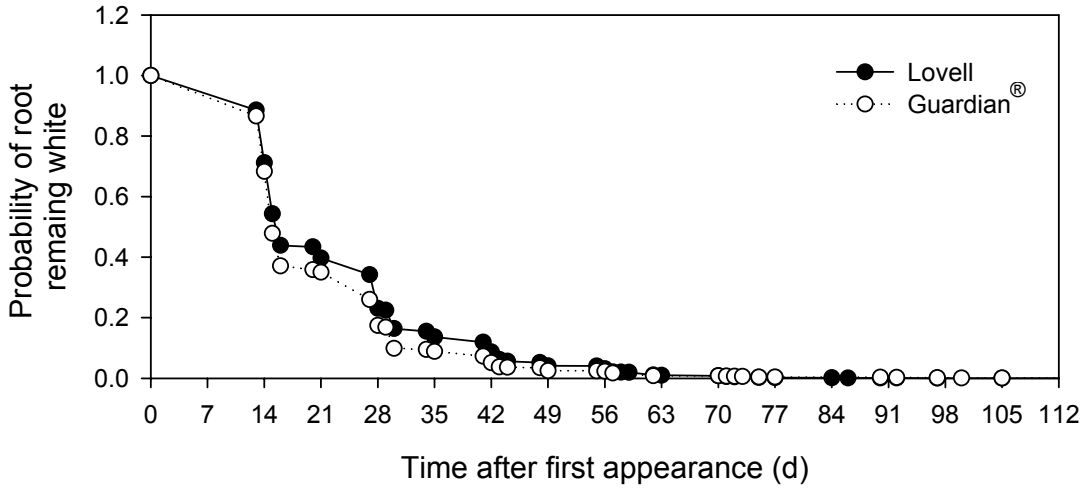

Figure 4.11 Browning probability of Guardian® roots as compared to Lovell roots significant at $P < 0.05$ (SAS PROC PHREG)

Figure 4.12 Visible growth responses to treatments at the end of the first growing season (September 2003).

A positive growth response to MBr treatment was expected. The extent of the growth response to Sol treatment was surprising, but not unprecedented. Stapleton and DeVay (1982) observed height increases of 25% and fresh weight increases 42% for peach seedlings grown in solarized soil.

There are many possible explanations for the Sol effect. One possibility is that the heating process destroyed or suppressed deleterious soil organism(s) in a manner similar to MBr. This is plausible, given the documented ability of soil solarization to control plant pathogenic organisms (Pinkerton et al., 2000; Ghini et al., 2003).

Another explanation is altered soil nutrient availability. There were no treatment differences among soil nutrients tested, but a test for nitrogen availability was not performed. A large influx of plant available nitrogen is common after solarization (Katan, 1980; Ghini et al., 2003) and could have accounted for some of the growth increase. Because the scope of this experiment was one growing season, it is unclear how long the Sol effect would have persisted. Effects have been known to last longer than two growing seasons in some systems (Stevens et al., 2003).

At the conclusion of the experiment, rootstock had a significant effect on stem diameter. Guardian® trees were on average 5 mm larger in stem diameter than Lovell trees, although this is probably a carry-over effect from the beginning of the experiment when the newly-planted Guardian® trees averaged 1.6 mm larger than the Lovell trees. The greater vigor of our Guardian® trees compared

to Lovell trees is consistent with earlier research (Beckman et al., 1997). Similarly, Yao *et al*. (2006) observed that an apple replant 'tolerant' apple rootstock conferred more trunk diameter growth than trees on two other rootstocks.

Fine root production, mortality and standing crop

Control trees in our experiment tended to produce more fine roots, have more roots present at any point in time (standing crop), and exhibit higher rates of root mortality than trees from other treatment groups. This high rate of root turnover may reflect greater pathogen/herbivore pressure in untreated soils; therefore trees may have produced roots at a higher rate to compensate for the high rate of root loss to disease and/or herbivory.

Many differences in root dynamics between control and treated trees appeared to be related to a surge in root production for Control G trees on July 9 (Figure 7). The reason for this flush in production is unknown, as are the physiological events underlying most pulses of fine root production and mortality. Aside from the July 9 event and its lingering effects, L trees tended to produce more fine roots than G trees.

Fine root lifespan

All the soil treatments were associated with increased peach fine root longevity to varying degrees. The magnitude of longevity increase was similar for the three direct soil treatments (MBr, Sol, and Tel). The increase in root

longevity increase due to Gly, an indirect soil treatment, was approximately 50% of the other three treatments. This observation was not surprising, as the three direct soil treatments all had the potential to directly kill deleterious soil fauna. The Gly treatment relied on indirectly affecting soil microbe populations by altering their nutrient source, remnant peach roots, and waiting for the populations to decline. Despite the widely different modes of action for the three direct soil treatments, they had similar effects on fine root longevity. This result suggests that the organism(s) responsible for root mortality in the Control soil may be similarly susceptible to all three treatments.

Similar results have been recorded when using insecticides and fungicides. For example, Wells *et al*. (2002) observed that soil application of a broad-spectrum organophosphate insecticide increased the median lifespan of peach fine roots by 46-125 d compared to an untreated control. Additionally, Eissenstat *et al*. (2000) found that the suppression of oomycete fungi through a fungicide drench increased fine root longevity in sugar maple (*Acer saccharum* Marsh.), and a combined treatment of that fungicide and an insecticide more than doubled the median lifespan compared to untreated trees. Our MBr treatment in particular behaved similar to the combination treatment above, suppressing multiple components of the soil biota leading to increased fine root longevity.

One notable difference between our research and that of the studies above is that the entire rooting zones of the trees were treated instead of only the area surrounding each individual minirhizotron. As a result, whole-tree carbon

allocation was likely affected by the treatments and may have contributed to the above-ground differences observed.

G fine roots had only slightly longer lifespans than L roots. This result may be related to the greater vigor of G trees, which may have had greater carbohydrate reserves available for the maintenance of fine roots. Similar research conducted for apple replant disease found that rootstock genotype had the greatest influence on fine root longevity, surpassing the effects of pre-plant soil fumigation with Telone C-17, compost soil amendments, and replant position (Yao et al., 2006).

Fine root browning

The development of brown root pigmentation is commonly observed through minirhizotrons and is thought to represent a distinct shift in the root's function as an organ (Comas et al., 2000). Fine roots in MBr and Sol treatments remained white longer than in Control, Tel and Gly, indicating that those two treatments created a more favorable environment for roots to remain physiologically active. Wells *et al*. (2002) found comparable results when arthropods were excluded from the root zone. In both of these cases, it is clear that soil organisms can have an impact on the rate of root pigmentation.

Additionally, G roots became brown earlier than L roots. The earlier development of pigmentation in G roots may have provided a mechanism against root herbivory, although there is little research to support this idea. To our

knowledge, this is the only study that demonstrates genotypic variation in root pigmentation rates within a given species.

Conclusion

Direct and indirect soil treatments designed to alleviate PRD symptoms provided some benefit to replanted peach trees in this experiment. MBr and Sol treated soils provided the greatest growth promotion compared to untreated soils, although the method of the growth promotion remains unclear. While Sol provided nearly equivalent results to MBr in our experiment, there are some questions as to the duration of the effect. The additional labor and materials required may make the widespread use of solarization impractical in large orchard settings. Our research demonstrates that MBr and Sol, and to a lesser extent Gly and Tel, increased stem diameter, decreased fine root production and mortality, increased the time a root remained white (its most physiologically active state), and increased fine root lifespan.

There is also evidence from our research that rootstock genotype may play a role in PRD management and further investigation into this possibility is merited. It is likely that a single treatment approach for PRD management will not be sufficient in the absence of methyl bromide. However, a multifaceted approach similar to that developed for apple replant management may be a viable option for PRD management in the future.

APPENDIX

Table A-1 Total cyanogenic glycoside (CG) content of different Lovell peach tissues. CG content determined by elution of color pigments from picrate papers and measurement of the colored solution's absorbance at 510 nm on a spectrophotometer following the method of Bradbury *et al*. (1999). Seed value is the mean of two samples ± 1 standard error. White root and brown root values are the mean of four and eight samples ± 1 standard error, respectively. Roots were all less than 2 mm in diameter. CG in seed is amygdalin, while CG in roots is prunasin as reported by Reilly and Okie (1985).

Source Tissue	Cyanogen Content (ppm g^{-1} fresh tissue)
Control (no tissue)	0
Seed	480 ± 30
White Roots	307 ± 16
Brown Roots	165 ± 17

Table A-2 First greenhouse experiment: mean of growth parameters for each harvest date. Each value is the mean of six experimental units ± 1 standard error. *Root Dry Weight was significant on Harvest Date 3. All other parameters non-significant at $P < 0.05$ (SAS PROC GLM).

	Control	Arthro	Arthro Primed	BG33R
Harvest Date 1				
Shoot Height (cm)	16.2 ± 0.97	17.5 ± 0.48	16.2 ± 0.73	18.7 ± 1.15
Shoot Dry Weight (g)	0.96 ± 0.07	1.06 ± 0.09	1.19 ± 0.09	1.24 ± 0.17
Root Dry Weight (g)	0.88 ± 0.10	0.93 ± 0.10	1.15 ± 0.09	1.23 ± 0.23
Root:Shoot Ratio	0.90 ± 0.06	0.88 ± 0.06	0.97 ± 0.06	0.97 ± 0.08
Harvest Date 2				
Shoot Height (cm)	24.3 ± 1.66	24.9 ± 1.79	27.3 ± 1.82	29.3 ± 1.44
Shoot Dry Weight (g)	2.71 ± 0.32	2.33 ± 0.19	2.59 ± 0.34	3.24 ± 0.39
Root Dry Weight (g)	2.22 ± 0.15	1.95 ± 0.26	2.39 ± 0.37	2.29 ± 0.34
Root:Shoot Ratio	0.85 ± 0.06	0.82 ± 0.07	0.99 ± 0.22	0.71 ± 0.06
Harvest Date 3				
Shoot Height (cm)	26.5 ± 2.24	28.4 ± 2.41	28.0 ± 1.86	28.4 ± 1.66
Shoot Dry Weight (g)	4.10 ± 0.70	4.35 ± 0.75	4.09 ± 0.74	3.34 ± 0.43
*Root Dry Weight (g)	4.94 ± 0.38	6.82 ± 0.80	5.67 ± 0.73	4.06 ± 0.42
Root:Shoot Ratio	1.35 ± 0.19	1.69 ± 0.23	1.47 ± 0.13	1.35 ± 0.24

Table A-3 Pre-plant fresh weight and stem diameter means for 'Redglobe' peach trees budded onto Guardian® and Lovell rootstocks. Measured on January 7, 2003. Each value is the mean of sixty individual trees ± 1 standard error.

Rootstock	Fresh Weight (g)	Stem Diameter (mm)
Guardian®	107.4 ± 7.1	9.37 ± 0.32
Lovell	71.2 ± 5.7	7.79 ± 0.32

Table A-4 Mean cumulative change in stem diameter from the pre-plant stem diameters (January 7, 2003) to three sample dates. Each treatment value is the mean of 12 observations representing two experimental units (n = 2) ± 1 standard error. Each rootstock value is the mean of 30 experimental units (n = 30) ± 1 standard error. Each treatment by rootstock value is the mean of 6 observations (n = 6) ± 1 standard error. No significant differences at $P < 0.05$ (SAS PROC MIXED).

	Change in Stem Diameter (mm)		
	9/19/2003	2/25/2004	8/30/2004
Treatment			
Control	21.6 ± 1.98	23.1 ± 2.13	46.8 ± 3.08
Glyphosate	24.4 ± 2.17	26.0 ± 2.45	51.2 ± 3.37
Methyl Bromide	31.3 ± 2.13	32.9 ± 2.19	61.3 ± 2.67
Solarization	30.9 ± 1.31	33.0 ± 1.50	60.0 ± 1.39
Telone C-17	21.8 ± 3.00	24.0 ± 3.39	51.0 ± 4.98
Rootstock			
Guardian®	25.5 ± 1.55	27.5 ± 1.73	54.1 ± 2.44
Lovell	26.4 ± 1.57	28.1 ± 1.64	54.1 ± 2.12
Treatment by rootstock			
Control*Guardian®	22.0 ± 1.85	23.8 ± 2.41	47.8 ± 3.73
Control*Lovell	21.1 ± 3.71	22.4 ± 3.75	45.9 ± 5.25
Gly*Guardian®	24.8 ± 3.75	26.1 ± 4.14	51.2 ± 5.45
Gly*Lovell	23.9 ± 2.57	26.0 ± 3.06	51.3 ± 4.43
MBr*Guardian®	29.3 ± 3.69	31.7 ± 3.91	61.7 ± 4.10
MBr*Lovell	33.3 ± 2.16	34.1 ± 2.31	61.0 ± 3.81
Sol*Guardian®	29.9 ± 1.77	31.8 ± 1.80	57.8 ± 1.67
Sol*Lovell	31.9 ± 2.00	34.1 ± 2.47	62.0 ± 1.99
Tel*Guardian®	21.5 ± 4.82	24.2 ± 5.74	52.2 ± 9.21
Tel*Lovell	22.0 ± 4.01	23.8 ± 4.18	49.8 ± 4.87

Table A-5 Pruning fresh weight means and final shoot and root fresh weight means for replanted 'Redglobe' peach trees. Trees pruned on February 3, 2004. Destructive harvest of shoots and roots performed in December 2005. Each treatment value is the mean of 12 observations representing two experimental units (n = 2) ± 1 standard error. Each rootstock value is the mean of 30 experimental units (n = 30) ± 1 standard error. Each treatment by rootstock value is the mean of 6 observations (n = 6) ± 1 standard error. No significant differences at $P < 0.05$ (SAS PROC MIXED).

	Pruning Fresh Weight (kg)	Shoot Fresh Weight (kg)	Root Fresh Weight (kg)
Treatment			
Control	0.50 ± 0.10	9.23 ± 1.06	6.50 ± 0.90
Glyphosate	0.59 ± 0.12	8.92 ± 1.08	7.02 ± 1.09
Methyl Bromide	1.03 ± 0.17	14.00 ± 1.33	13.40 ± 1.39
Solarization	0.98 ± 0.13	13.80 ± 0.85	11.10 ± 0.62
Telone C-17	0.65 ± 0.18	8.99 ± 1.56	7.92 ± 1.20
Rootstock			
Guardian®	0.76 ± 0.09	11.3 ± 0.88	9.20 ± 0.86
Lovell	0.74 ± 0.10	10.7 ± 0.84	9.27 ± 0.79
Treatment by rootstock			
Control*Guardian®	0.61 ± 0.14	10.71 ± 1.58	6.89 ± 1.17
Control*Lovell	0.38 ± 0.14	7.75 ± 1.27	6.12 ± 1.45
Gly*Guardian®	0.68 ± 0.20	9.04 ± 1.66	7.38 ± 1.77
Gly*Lovell	0.48 ± 0.14	8.78 ± 1.53	6.59 ± 1.38
MBr*Guardian®	0.90 ± 0.20	14.67 ± 1.95	13.18 ± 2.41
MBr*Lovell	1.16 ± 0.29	13.27 ± 1.96	13.68 ± 1.63
Sol*Guardian®	0.90 ± 0.21	12.55 ± 1.09	10.34 ± 0.86
Sol*Lovell	1.04 ± 0.15	14.95 ± 1.20	11.89 ± 0.83
Tel*Guardian®	0.71 ± 0.30	9.44 ± 2.77	8.20 ± 2.19
Tel*Lovell	0.59 ± 0.24	8.54 ± 1.72	7.63 ± 1.24

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