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Effect of pre-plant soil fumigants on *Agrobacterium tumefaciens*, pythiaceous species, and subsequent soil recolonization by *A. tumefaciens*

L.E. Yakabe, S.R. Parker, D.A. Kluepfel*

USDA/ARS Crops Pathology and Genetics Research Unit, 1 Shields Avenue, University of California, Davis, CA 95616, USA

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ABSTRACT

Paradox (*Juglans hindsii* × *J. regia*), the dominant rootstock used in the California walnut industry, is susceptible to crown gall caused by *Agrobacterium tumefaciens*. In practice, soil fumigation has been a common pre-plant management strategy for crown gall, but even the industry standard, methyl bromide (MeBr), results in inconsistent disease control. To examine MeBr efficacy and identify potential alternatives, combinations of 1,3-dichloropropene (1,3-D), chloropicrin, iodomethane, dazomet, and metam-sodium were examined. Except for 1,3-D alone, all treatments reduced *A. tumefaciens* and *Phytophthora cactorum* populations below detection limits. MeBr eliminated *A. tumefaciens* populations in buried gall tissue, but a combination of 1,3-D and chloropicrin (TC35) did not. An additional 280 kg/ha of chloropicrin in addition to TC35 eliminated *A. tumefaciens* populations in buried gall tissue. Of the treatments tested, TC35 was the best alternative to MeBr given its efficacy on soil populations of *A. tumefaciens* and *P. cactorum* and potential suppressiveness to soil recolonization by *A. tumefaciens*. MeBr reduced general aerobic bacterial populations below detection limits producing a temporary biological vacuum. *A. tumefaciens* reintroduced in soils treated with MeBr and TC35 reached significantly higher populations than in non-fumigated soil. However, *A. tumefaciens* populations in TC35-treated soil were 100-fold lower than MeBr-treated soil 110 d after reintroduction. Increased recolonization rates resulting in higher subsequent soil populations could be a mechanism underlying the observed inconsistent crown gall control after MeBr application.

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1. Introduction

California is responsible for 95% of United States walnut (*Juglans regia*) production, and 54% of worldwide production (Flint, 2003). Release of the hybrid walnut rootstock, Paradox (*Juglans hindsii* × *J. regia*), during the mid 1990s changed the industry. Not only was the rootstock vigorous, but for the first time growers had a precocious rootstock with some resistance to several species of *Phythophthora* (Flint, 2003). Paradox also expanded the range of soils on which walnut trees could be successfully grown (Flint, 2003). Consequently, Paradox developed into the dominant rootstock used by California walnut growers. Despite the success of Paradox, this rootstock is susceptible to crown gall, caused by *Agrobacterium tumefaciens* which reduces both yield and orchard life (Flint, 2003). Crown gall control is complicated by the ability of *A. tumefaciens* to effectively colonize and persist in soil in the absence of a host and cause disease at low populations (Agrios, 2005).

Crown gall incidence in commercial orchards has been attributed to several factors including wounding during harvest and handling of transplants, post-plant crown wounding, and inoculum transfer from soil (Ogawa and English, 1991; Ramos, 1998; Flint, 2003). The use of inoculum-free seed and transplants in nursery and orchard sites with low disease pressure are important first steps in managing crown gall. For this reason, the walnut industry has relied upon pre-plant soil fumigation in nurseries and orchards as the primary management strategy.

Paradox is recommended for use in areas with a history of *Phytophthora* problems even though this rootstock only has limited resistance to certain species. More than 14 *Phytophthora* species are associated with root or crown rots in walnuts (Ramos, 1998). *Phytophthora cactorum* causes a crown rot which, depending on environmental conditions and age of host tissue, will cause girdling, loss of thriftiness, and sometimes death as early as the first year (Flint, 2003). Consequently, the walnut industry also relies on prudent water management and pre-plant fumigation to manage root and crown rots caused by *Phytophthora*. While water management can help reduce disease incidence, pre-plant fumigation is highly recommended to reduce populations of all *Phytophthora* species to facilitate establishment of young trees.

* Corresponding author. Tel.: +1 530 752 1137; fax: +1 530 754 7195.
E-mail address: dakluepfel@ucdavis.edu (D.A. Kluepfel).

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Several studies have examined the use of pre-plant fumigation in perennial tree crops which require management for more than 30 years (Zehr et al., 1976; McKenry, 1987; Sharpe et al., 1989; Utkhede et al., 2001; Mazzola and Mullinix, 2005; Bhat et al., 2006). Few have examined the direct effects of soil fumigation on *A. tumefaciens* (Munnecke and Ferguson, 1960; Dickey, 1962; Utkhede and Smith, 1990; Raio et al., 1997). Observations by nursery operators and walnut growers suggest fumigation with methyl bromide (MeBr) is inconsistent in reducing crown gall incidence and, in some cases, actually increases crown gall incidence (Ramos, 1998; Flint, 2003). Deep et al. (1968) quantified the post fumigation increase in crown gall incidence on cherry 'Mazzard' indicating a 26.3% ($n = 100$ trees) increase in crown gall incidence after a 336 kg/ha treatment with 67% MeBr and 33% chloropicrin as compared to an untreated control. University of California Cooperative Extension Farm Advisors report that crown gall incidence has become an increasing problem despite MeBr alternative applications (Ramos, 1998; Flint, 2003). Fumigation failures have not been investigated to determine if fumigant material, improper fumigant application, infested plant material, encroachment of the pathogen from non-treated soil, or some other reason is responsible for the lack of efficacy. Moreover, the loss of MeBr has necessitated an evaluation of alternative fumigants.

Here we report on the effects of MeBr and MeBr alternatives on populations of *A. tumefacians* and *P. cactorum* in soil microcosms. Soil microcosms are well controlled environments used to determine fumigant efficacy and reduce confounding effects brought about by inefficient chemical delivery and contamination from unrelated sources. Since *Phytophthora* species have been extensively used in fumigation studies of many annual crops, the efficacy data collected in these experiments on *P. cactorum* also provided a means to evaluate the appropriateness of the microcosm assay system as a first screen for potential fumigation treatments (Duniway, 2002). Additionally, this study examines the ability of *A. tumefaciens* to colonize soil after fumigation in an attempt to elucidate factors contributing to observed inconsistencies in fumigation. Ultimately, the goal of this study is to provide the nursery and walnut industry with reliable integrated management options for the control of crown gall and *Phytophthora* root and crown rots.

2. Materials and methods

2.1. Microcosms

Laboratory microcosms were used to evaluate fumigation efficacy on *A. tumefaciens*, *P. cactorum*, and general aerobic bacterial populations. Microcosms consisted of one quart glass Mason jars (Ball, Muncie, IN) of approximately 950 ml capacity. The soil used in these experiments was collected from the surface layer of a commercial walnut orchard mapped as Yolo silty clay loam in Yolo County, California (Andrews, 1972). The soil had a mean particle size distribution of 33% sand, 47% silt, and 20% clay. The pH was 7.0 and the organic C content was 0.99%. Soil moisture content was assessed by drying a known mass of soil in a microwave oven (1.35 kW Dual Wave, General Electric, Louisville, KY) for 15 min. After drying in the microwave, the soil was weighed and percent moisture calculated. After infestation, soil moisture was adjusted by either incorporating sterilized water or allowing the soil to dry further in a clean laminar flow hood. Soil moisture was allowed to equilibrate for at least 24 h before fumigants were applied.

2.2. Inoculum preparation and soil inoculation

Wildtype *A. tumefaciens*, EC1, and its rifampicin resistant mutant, EC1R, were used in the fumigation experiments. Except for growth in the presence of rifampicin, EC1R expressed growth patterns and antibiotic resistance profiles indistinguishable from wildtype EC1. Antibiotic resistance profiles were assessed by swabbing cells of the isolates onto the surface of trypticase soy broth agar (TSBA). Antibiotic disks (Sensi-disk, Becton, Dickson and Company Sparks, MD) of ampicillin (10 μg), bactitracin (10 μg), carbenicillin (100 μg), cefoxitin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), minocycline (30 μg), nalidixic (30 μg), neomycin (30 μg), novobiocin (30 μg), penicillin (10 μg), rifampicin (5 μg), streptomycin (10 μg), tetracycline (30 μg), tobramycin (10 μg), trimethoprin (5 μg), and vancomycin (30 μg) were subsequently placed on the surface of the agar. The inoculated TSBA was incubated at 27 °C for 48 h then observed for zones of growth inhibition. Antibiotic profiles were considered to be the same if zones of inhibition appeared around the same antibiotics for both bacterial isolates. Five *P. cactorum* isolates were used in these experiments. Isolates sm3337, sm3471, and sm3398 were walnut isolates from the collection of S.M. Mircetich (Bhat et al., 2006). Isolates gb1100 and gb4025 were from the collection of G.T. Browne and were originally isolated from almond and pear, respectively (Bhat et al., 2006). All of these isolates were collected in the Central Valley of California.

A 3 μL loop of a 48 h culture of *A. tumefaciens* grown on trypticase soy broth agar (TSBA) was used to inoculate 2 L of trypticase soy broth. The 2 L culture was incubated at 28 °C on a rotary shaker (200 rpm) for 48 h. The bacterial suspension was pelleted at 5468 g for 10 min. The resulting pellets were washed twice using sterilized water. The final suspension of washed cells was used to either infect walnut 'Paradox' trees or infest soil.

To generate gall tissue, 'Paradox' walnut trees were wounded by cutting a 2 cm T-shaped incision into the crown and pipetting 100 μl of EC1 inoculum into the wound. The inoculum concentration was approximately 1×10^8 CFU/mL as determined photospectrometrically (OD_{600}). The inoculated wounds were wrapped with parafilm and galls developed over a three month period. Gall material ranging from 4 to 10 cm in diameter and connected crown segment were collected for fumigation experiments.

EC1R inoculated soil was used in fumigation experiments as described below. All cells harvested from the 2 L culture were used to inoculate approximately 70 kg soil. The inoculum was fully incorporated into the soil by using a clean hoe. Mixing was conducted outside of a laminar flow hood.

A 5 mm-diameter plug of 7-d-old V8 agar (Miller, 1955) culture of each *P. cactorum* isolate was used to separately inoculate 500 ml batches of 20% V8 liquid medium. These cultures were incubated at 22 °C for 14 d. Cultures were shaken daily to aerate the medium and disperse inoculum. Soil inoculum was prepared by washing the culture in sterile water and then emulsifying the cultures in sterile water for 2 min in a blender. The presence of mycelia, sporangia and oospores of *P. cactorum* was confirmed by microscopy. The resulting slurry was used to inoculate the soil used in the soil fumigation experiments as described below. The inoculum was fully incorporated into the soil using a clean hoe. Mixing was conducted outside of a laminar flow hood.

2.3. Microcosm fumigation experiments

Microbial inoculum was introduced into either autoclaved soil (soil autoclaved for 60 min thrice over a 72 h period) or native untreated soil. Both sterilized and native soils were infested with *A. tumefaciens* and *P. cactorum* as described above. After thorough incorporation of the inoculum, soil moisture content was adjusted to 12% (w/w) by drying in a laminar flow hood. Nine hundred grams of soil were subsequently packed into the microcosms at a density of 1 g/mL and fumigated. Fumigation treatments were as follows: 1)