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

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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 *Phytophthora* (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.
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; McKenney, 1987; Sharpe et al., 1989; Utokhed et al., 2001; Mazzola and Mullinix, 2005; Bhat et al., 2006). Few have examined the direct effects of soil fumigation on Agrobacterium tumefaciens (Munnecke and Ferguson, 1960; Dickey, 1962; Utokhed 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. tumefaciens 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 (Dunaway, 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 and the organic C content was determined. 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), bacitracin (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), trimethoprim (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 fumigant treatment, ‘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 × 10⁸ CFU/mL as determined photo-spectrometrically (OD₅₆₀). The inoculated wounds were wrapped with paraffin 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)
378 kg/ha 1,3-dichloropropene (1,3-D), 2) 378 kg/ha 1,3-D and 351 kg/ha chloropicrin (TC35), a mixture with the same proportion of 1,3-D and chloropicrin as Telone C35 (Dow AgroSciences, Indianapolis, IN), 3) TC35 plus an additional 280 kg/ha chloropicrin (TC35 + pic), 4) 224 kg/ha methyl iodide, 224 kg/ha chloropicrin, and 168 kg/ha 1,3-dichloropropene (iodo + pic + 1,3-D), 5) 701 L/ha Vapam (Amvac, Los Angeles, CA), a formulation of metam-sodium with 42% active ingredient, 6) 224 kg/ha Basamid (Ceris, Columbia, MD), a granular formulation of dazomet with 99% active ingredient, 7) 448 kg/ha methyl bromide (MeBr), and 8) untreated control. Fumigants, with the exception of MeBr and Basamid, were added by injection using a gas tight, glass syringe (Hamilton Co, Reno, NV). MeBr was injected directly from the storage cylinder via a stainless steel tube and needle. A flow meter was used to monitor MeBr injection quantities. Injections occurred at the surface of the soil profile in the center of the soil surface. Basamid granules were fully incorporated throughout the soil profile along with 100 ml sterile water. Untreated controls consisted of infested soil left untreated. After fumigants were applied, the microcosms were inverted for 5 min to aid in fumigant distribution, returned to soil left untreated. After fumigants were applied, the microcosms were sealed with gas permeable film and returned to an environment more conducive to bacterial replication. All soil was adjusted to 18% (w/w) soil moisture using sterile water and packed into microcosms at a density of 1 g/mL. One gram of sterilized soil infested with EC1R (2.1 × 10⁷ CFU/g) prepared by the aforementioned method was used to inoculate each of the microcosms. The microcosms were sealed with gas permeable film and vigorously shaken by hand for 1 min to fully incorporate the inoculum. Microcosms were assayed as described below for A. tumefaciens and aerobic bacterial populations prior to inoculation, immediately after inoculation, and at various intervals up to 110 d to determine colonization rates. Two trials were conducted. Each trial had six replications per treatment.

2.6. Pathogen enumeration from soil

Microbial populations were quantified by dilution plating. For all assays, microcosms were vigorously shaken by hand for 2 min prior to sampling. 10 g of soil was suspended in 10 ml sterile water and vortexed for 60 s. Ten-fold serial dilutions were generated using sterile water. For aerobic bacteria and A. tumefaciens, 10 μl of these dilutions were then spread onto Difco trypticase-soy agar (TSA) amended with 100 mg/L cyclohexamide and 100 mg/L rifampicin-residing bacteria on the agar and bacterial populations per g soil were calculated. For pythiaceous quantification, 1 ml of the soil dilution was plated onto CMA-PARP (Jeffers and Martin, 1986). Colonies on CMA-PARP were counted after 5 d incubation in the dark at approximately 22 °C. Colonies forming on CMA-PARP from sterilized soil were counted as P. cactorum colonies when verified morphologically using previously published descriptions (Erwin and Ribeiro, 1966). Colonies from native soil were grouped as “pythiaceous species” when verified morphologically as a Pythium or Phytophthora species using previously published descriptions (Erwin and Ribeiro, 1966; Dick, 1990).

2.7. Soil community profile

Sixty days after fumigation, three replicates of soil fumigated with MeBr, TC35, and untreated native soil were dilution plated on TSA. Bacterial colonies from each microcosm were collected by first selecting colonies with differing morphologies. Additional colonies were picked until a total of 10 colonies were picked from each microcosm by first drawing a line randomly across the plate and then picking colonies touching that line. Colonies were identified using Fatty Acid Methyl Extraction analysis or 16S rDNA sequence analysis (Weisburg et al., 1991; Sasser, 1990). Sequencing was performed at the University of California, Davis Division of Biological Sciences Automated DNA Sequencing Facility (Davis, CA). DNA sequencing data was analyzed using the University of Michigan 16S rRNA sequence database (http://rdp.cme.msu.edu/index.jsp).

2.8. Statistical analysis

In soil fumigation experiments, populations of P. cactorum, A. tumefaciens, and total aerobic bacteria were analyzed at each sampling time. Population values were transformed using PROC RANK to meet the assumptions for parametric analysis. The transformed data were subjected to analysis of variance (ANOVA) with the
PROC GLM procedure in SAS (SAS Institute, Inc., Cary, NC). Trial, treatment, and the interaction of trial and treatment were used as factors. Main treatment effects were separated using Tukey’s w procedure. In colonization experiments, populations of *A. tumefaciens* and aerobic bacteria were analyzed within each sample point. Propagule numbers were transformed using PROC RANK to meet assumptions for parametric analysis and subjected to ANOVA. Trial, treatment, and the interaction between trial and treatment were used as factors. Main effects were separated using Tukey’s w procedure when appropriate with a significance threshold of $P \leq 0.05$.

### 3. Results

#### 3.1. Fumigant effects

Before treatment, autoclaved soil was infested with 62 CFU/g soil and $7 \times 10^2$ CFU/g soil of *P. cactorum* and *A. tumefaciens*, respectively. Populations of these microorganisms were reduced below detection limits 5, 30, and 60 d following all fumigation treatments except when 1,3-D was applied alone (Figs. 1a and 2a). Similar results were obtained in microcosms filled with infested native soil. Before fumigation, native soil supported 1.4 $\times 10^2$ CFU/g soil and 2.2 $\times 10^2$ CFU/g soil of pythiaceous species and *A. tumefaciens*, respectively. Populations of these organisms were reduced below detection limits and remained below these limits for at least 90 d post fumigation with all fumigation treatments except when 1,3-D was applied alone (Figs. 1b and 2b).

Five days after fumigation with 1,3-D, *P. cactorum* and pythiaceous populations were reduced by 97% and 94% in autoclaved and native soils, respectively, while *A. tumefaciens* populations were reduced by 85% and 99% in autoclaved and native soils, respectively (Figs. 1 and 2). *P. cactorum* populations after 5 d averaged 1.4 CFU/g soil and declined below detection levels at 60 d post fumigation (Fig. 2a). Sixty days after 1,3-D fumigation, *A. tumefaciens* populations in sterilized soil declined from a high of $1.6 \times 10^3$ CFU/g soil 30 d post fumigation to $9.3 \times 10^2$ CFU/g soil 60 d post fumigation (Fig. 1a). *A. tumefaciens* populations rebounded in native soils, increasing to $4.5 \times 10^3$ CFU/g soil 60 d after fumigation (Fig. 1b). When 1,3-D was combined with chloropicrin, populations of the target organisms were reduced below detection limits.

Virulent *A. tumefaciens* was detected in gall tissue in all non-fumigated replications. Virulent *A. tumefaciens* was also detected in soil surrounding these galls in six of the eight non-fumigated microcosms. Virulent *A. tumefaciens* was not detected in galls or soil surrounding the galls fumigated with MeBr or TC35 + pic. In contrast, *A. tumefaciens* was detected in galls in three of the six microcosms fumigated with TC35. The galls in which *A. tumefaciens* was not detected were reduced to soft, decomposed tissue with occasional strands of lignified plant tissue. *A. tumefaciens*, however, was detected in soil surrounding the decomposed tissue. In total, virulent *A. tumefaciens* was detected in soil in five of the six TC35-treated microcosms. Data are summarized in Table 1.

Pre-treatment populations of aerobic bacteria were $3.3 \times 10^6$ CFU/g in native soil (Fig. 3). Five days after fumigation, aerobic bacteria were reduced by 45% and 85% in autoclaved and native soils, respectively, while *A. tumefaciens* populations were reduced by 85% and 99% in autoclaved and native soils, respectively.
Survival of Virulent Agrobacterium tumefaciens in Gall Tissue. Detection frequencies of A. tumefaciens in gall tissue and soil surrounding gall tissue in soil microcosms left untreated or fumigated with methyl bromide (448 kg/ha) (MeBr), 1,3-dichloropropene (378 kg/ha) and chloropicrin (351 kg/ha) (TC35), or 1,3-dichloropropene (378 kg/ha) and chloropicrin (631 kg/ha) (TC35 + pic).

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A. tumefaciens Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeBr</td>
<td>0/6</td>
</tr>
<tr>
<td>TC35</td>
<td>3/6</td>
</tr>
<tr>
<td>TC35 + pic</td>
<td>0/6</td>
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<tr>
<td>Non-fumigated control</td>
<td>16/16</td>
</tr>
</tbody>
</table>

Significantly different from non-fumigated controls as determined by chi-square analysis using a Bonferroni adjusted significance value (P < 0.016).

Fig. 3. Fumigation Effects on Aerobic Bacteria Populations. Fumigation effects on aerobic bacteria in native soil as determined by dilution plating. Treatments were evaluated within a given sample time and should not be compared across time. Different letters indicate statistically significant differences as determined by Tukey’s w tests. *1,3-D – 1,3-dichloropropene (378 kg/ha), TC35 – 1,3-dichloropropene (378 kg/ha) and chloropicrin (351 kg/ha), TC35 + pic – 1,3-dichloropropene (378 kg/ha) and chloropicrin (631 kg/ha), iodo + pic + 1,3-D – methyl iodide (224 kg/ha), chloropicrin (224 kg/ha), and 1,3-dichloropropene (186 kg/ha), Vapam – Vapam (701 kg/ha), Basamid – Basamid (224 kg/ha), and MeBr – methyl bromide (448 kg/ha).

3.2. Soil colonization

A. tumefaciens was introduced into native soil previously 1) autoclaved, 2) fumigated with MeBr, 3) fumigated with TC35, or 4) untreated. Initial A. tumefaciens populations (i.e. time 0) in all treatments was approximately 1.3 × 10^8 CFU/g soil (Fig. 4a). Three days after inoculation, A. tumefaciens populations in MeBr-treated soils (7.8 × 10^7 CFU/g) were significantly higher than populations in TC35 and untreated soils (8 × 10^5 CFU/g and 3.6 × 10^6 CFU/g, respectively), and remained significantly higher throughout the 110 d sample period (Fig. 4a). A. tumefaciens populations in TC35-treated soils also were significantly greater than native soil populations throughout the study. A. tumefaciens populations in autoclaved soil were similar to TC35-treated soils 49 d post inoculation. From 71 through 110 d post inoculation, autoclaved soil populations were statistically greater than both TC35-treated and native soils (Fig. 4a).

Table 2

| Aerobic Bacteria Post Fumigation. Aerobic bacteria isolated on trypticase soy broth agar amended with 100 mg/mL cyclohexamide 60 d after native soil was left untreated or fumigated with methyl bromide (448 kg/ha) (MeBr) or 1,3-dichloropropene (378 kg/ha) and chloropicrin (351 kg/ha) (TC35). |

<table>
<thead>
<tr>
<th>Treatment</th>
<th># isolates</th>
<th>TC35</th>
<th># isolates</th>
<th>Methyl Bromide</th>
<th># isolates</th>
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<td></td>
<td>Arthrobacter globiformis</td>
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</tr>
<tr>
<td>Bacillus megaterium</td>
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<td>Bacillus megaterium</td>
<td>4</td>
<td>Bacillus sp.</td>
<td>8</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
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<td>Bacillus subtilis</td>
<td>1</td>
<td>Bacillus amylolique</td>
<td>1</td>
</tr>
<tr>
<td>Brevibacillus choshinensis</td>
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<td>Brevibacillus choshinensis</td>
<td>1</td>
<td>Bacillus subtilis</td>
<td>3</td>
</tr>
<tr>
<td>Cellulomonas fimri</td>
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<td></td>
<td></td>
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<td>Kocuria kristina</td>
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<tr>
<td>Streptomyces sp.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>23</td>
<td>TOTAL</td>
<td>23</td>
<td>TOTAL</td>
<td>30</td>
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</tbody>
</table>
Native
TC35
Autoclaved

**Fig. 4.** Soil Colonization. Population levels of A. tumefaciens EC1R as determined by plating on TSBA + rif and b) aerobic bacteria as determined by plating on TSBA + cyc in native soils treated with methyl bromide (MeBr), chloropicrin and 1,3-D (TC35), autoclaved, or untreated (native). Treatments were evaluated within a given sample time and should not be compared across time.

### 4. Discussion

MeBr has been the standard pre-plant soil fumigant for both nursery and commercial walnut production in California. Unfortunately, there have been numerous accounts of inconsistent control of crown gall after MeBr soil fumigation. Interestingly, given the magnitude of the problem, few studies have directly examined the effects of MeBr or MeBr alternatives on soil populations of A. tumefaciens (Munnecke and Ferguson, 1960; Dickey, 1962; Raio et al., 1997). Studies evaluating fumigant efficacy only by disease incidence do not account for disease resulting from asymptomatic infections or contaminated soil on transplants. Direct examination of soil populations of A. tumefaciens would have eliminated these confounding effects. Reported here are the direct effects of MeBr and alternative soil fumigants on A. tumefaciens and pythiaceous species populations in soil microcosms.

This study indicates MeBr, as well as the alternatives tested, is effective in eliminating pythiaceous species. 1,3-D was less effective than the other treatments tested, but still significantly reduced pythiaceous populations. These findings are consistent with other soil fumigation studies evaluating the effect of MeBr and alternatives on pythiaceous species (Noling and Becker, 1994; Dunway, 2002; French-Monor et al., 2007). As a side note, the effects of Vapam in these experiments agree with the field results of Raio et al. (1997) who found a much lower rate of Vapam (100 L/ha) reduces A. tumefaciens populations below detection limits. The similarity of our results and published field studies support the utility of the microcosm used in our assay system.

MeBr fumigation eliminated A. tumefaciens populations in both soil and crown gall tissue. These results disagree with previously published studies of Dickey (1962) who reported a 489 kg/ha treatment with 98% MeBr and 2% chloropicrin (MC-2) injected 15 cm deep into a field plot did not reduce A. tumefaciens soil populations at depths of 8, 23, and 38 cm. Soil populations of A. tumefaciens were reduced by 99% at these same depths only when MC-2 was applied at a rate of 976 or 1464 kg/ha. Additionally, Dickey (1962) reported A. tumefaciens populations in tomato tissue buried in soil were not reduced after treatment with 1464 kg MC-2/ha. Munnecke and Ferguson (1960) also found MeBr at 489 kg/ha to be ineffective in eliminating A. tumefaciens from tomato tissue buried in soil contained in polyethylene bags. In disagreement with the findings of Dickey (1962), Munnecke and Ferguson (1960) reported A. tumefaciens could be eliminated at MC-2 rates of 976 kg/ha or greater. In part, this discrepancy may be explained by the differences in the A. tumefaciens detection assays used in each study. While Dickey (1962) assayed A. tumefaciens by direct plating, Munnecke and Ferguson (1960) used a tomato tumor formation bioassay, which could be less sensitive resulting in a lower lethal MeBr dosage estimation. This concern was illustrated by Lippincott and Heberlein (1965) who developed a bioassay using primary pinto bean leaves which required 1 × 10^5–1 × 10^6 cells to reliably initiate a tumor.

The lack of A. tumefaciens control in the studies of Dickey (1962) and Munnecke and Ferguson (1960) also may be explained by the limited exposure of the target organism to the fumigant. Efficiency of chemical delivery is dependent on diffusion distance, which is dependent on soil texture, organic matter, moisture content, and application method. Finer soil textures require higher rates of fumigant to achieve the same efficacy in coarser texture soils due to the increase in pore space (Cohen and Martin, 2008). In the experiments conducted by Dickey (1962), soil texture was coarser than the soil used in the experiments reported here. Organic matter also can absorb fumigants requiring a higher fumigant rate in high organic soils to achieve desired pathogen control (Cohen and Martin, 2008). Fumigants are also impeded in high moisture soils. Soil moisture of <12% (w/w) is recommended for optimal fumigant diffusion (Cohen and Martin, 2008). Soil moisture reported by Dickey (1962) was 23%, which would dramatically inhibit movement of MeBr though the soil profile as compared to the 12% moisture level used in our experiments. Additionally, and perhaps most importantly, interpretation of the Dickey (1962) results are confounded since their direct plating method employed Patel's medium, a medium shown decades later to support robust growth of many soil-borne bacterial genera other than A. tumefaciens (Brisbane and Kerr, 1983). This will result in dramatically overestimating the survival of A. tumefaciens populations leading to the conclusion that MeBr offered limited control of A. tumefaciens. In experiments reported by Munnecke and Ferguson (1960), the soil was composed of 50% Canadian peat moss with a soil moisture content of 30% (w/w). The moist soil with high organic content would dramatically reduce fumigation efficacy (Cohen and Martin, 2008). Unfortunately, fumigant injection site relative to target inoculum also was not reported by either Dickey (1962) or Munnecke and Ferguson (1960).

In our experiments, MeBr reduced the general aerobic bacterial population below detection limits for at least five days post fumigation creating a temporary “biological vacuum”. This empty niche could be colonized readily by many bacteria, including A. tumefaciens, introduced on or in infested soil, transplants, or resident populations surviving improper fumigation. Dickey (1962) observed A. tumefaciens populations were higher in soil adjacent to fumigated galls in comparison to soil adjacent to non-fumigated galls while Deep and Young (1965) documented an increase in crown gall diseased trees. The authors suggested that fumigation with MeBr and chloropicrin eliminates competitors and allows A. tumefaciens to
proliferate to higher populations and therefore cause more disease. The soil recolonization experiments conducted in this study quantified an increase in A. tumefaciens in fumigated soils as well. A. tumefaciens populations were maintained at 100-fold greater levels in MeBr-treated soils as compared to A. tumefaciens populations in native soil controls. Whether soil recolonization by A. tumefaciens explains the observed variable control of crown gall by MeBr is unknown, however, such a hypothesis should be considered.

Similar to populations in MeBr-treated soil, A. tumefaciens populations in autoclaved soil were higher than in native soil. A. tumefaciens populations in autoclaved soil, however, did not dramatically increase within a short period of time as observed in the MeBr treatment. This is likely due to chemical and physical alterations of the soil as a result of autoclaving (Jenneman et al., 1986; Alef and Nannipieri, 1995, Trevors, 1996).

The MeBr alternatives examined here reduced soil populations of A. tumefaciens and P. cactorum while exhibiting a limited effect on general aerobic bacterial populations. This is significant considering intact aerobic populations, such as those in native and TC35-treated soils may impede subsequent soil colonization by A. tumefaciens. Recolonization by A. tumefaciens in TC35-treated soil was suppressed in comparison to recolonization in MeBr-treated soil. On the other hand, A. tumefaciens remained. The decreased ability to recolonize TC35-treated soil in comparison to native soil. While aerobic bacterial populations in TC35-treated soils were not statistically different from untreated controls, the increased ability of A. tumefaciens to recolonize may be facilitated by a change in the bacterial community composition. The surviving populations after MeBr and TC35 treatment consisted mainly of Gram-positive bacteria while untreated controls contained Streptomyces and several Gram-negative bacterial genera. Many studies examining concentrations of soil enzymes, respiration, nitrification, and community profiles generated by PLFA, FAME, and DGGE also demonstrate that fumigation significantly alters soil microbial communities (Griffiths et al., 2000; Elliot and Des Jardin, 1981; Stromberger et al., 2005; Klose et al., 2006; Hoshino and Matsumoto, 2007; Yamamoto et al., 2008). van Elsas et al. (2007) investigated the colonization of Escherichia coli O157:H7 in chloroform fumigated soils in conjunction with measurements of microbial complexity as determined by PCR-DGGE and PLFA. Their findings also support the hypothesis that soils with reduced biotic complexity are more conducive to subsequent microbial invasive colonization and establishment.

The increased vulnerability of fumigated soil to invasive microorganisms may be problematic if the introduced microorganism was a plant pathogen. Alternatively, it may provide the opportunity for a grower to establish a biocontrol agent or plant-beneficial microbial community which otherwise may be difficult to establish in native soils. Further investigations exploring the decreased microbial complexity of fumigated soils may offer additional disease management strategies.

Of the MeBr alternatives examined, TC35 was the most efficient at reducing soil populations of A. tumefaciens and P. cactorum. While 1.3-D applied alone was not as effective in controlling A. tumefaciens and P. cactorum, it is a reliable treatment for lesion nematode (Noling and Becker, 1994; Elmore et al., 2007), another major pest of walnuts. The addition of chloropicrin to 1,3-D in TC35 eliminates A. tumefaciens and pythiacious species. Additionally, a resident general aerobic bacterial population remained in the treated soil potentially inhibiting A. tumefaciens recolonization. Gall tissue fumigated with TC35 was highly decomposed and often only strands of lignified tissue remained. The softer gall tissue was fully colonized by fungi. TC35 treatment seemingly increased gall decomposition resulting in the release of A. tumefaciens into adjacent soil. TC35 fumigation eliminated A. tumefaciens populations in soil, but not in gall tissue, suggesting TC35 does not efficiently penetrate gall material. Additional chloropicrin, such as the 280 kg/ha in the TC35 + pic treatment, was needed to eliminate A. tumefaciens from gall material. Based on the data presented here, TC35 is an effective alternative to MeBr soil fumigation for the control of A. tumefaciens and Pythiaceous species in soil. In sites with a history of high crown gall incidence, fumigation with TC35 + pic combined with extensive gall removal from the soil should be considered. In conjunction with prior reports on 1,3-D efficacy on lesion nematode, TC35 is a candidate for inclusion into an integrated pest management program controlling all three major plant pathogens in the California walnut industry.

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