Plant foliar disease suppression mediated by composted forms of paper mill residuals exhibits molecular features of induced resistance

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Abstract

\textit{Arabidopsis thaliana} grown in soil from field plots amended with composted forms of paper mill residuals (PMR) exhibited reduced symptoms of bacterial speck caused by \textit{Pseudomonas syringae pv. tomato} (Pst) compared with plants grown in soil from field plots amended with a non-composted PMR or non-amended soils. Similar results were obtained with tomato (\textit{Lycopersicon esculentum} Mill.). No relationship between foliar disease suppression and plant nutrition or stature was observed. In \textit{Arabidopsis}, the reduction of foliar disease symptoms ranged between 34 and 65\%, depending on the type of composted PMR amendment, and was associated with reduced \textit{Pst} titers \textit{in planta}. An \textit{Arabidopsis} \textit{npr1} defense mutant and a \textit{NahG} transgenic line, both of which exhibit disrupted systemic acquired resistance, were also disrupted in their suppression of \textit{Pst} disease symptoms in composted PMR treatments. \textit{Arabidopsis} grown in soil amended with composted PMR also displayed an increased expression of pathogenesis-related defense genes prior to pathogen inoculation. We conclude that plants grown in soils with composted PMR-amendments were more resistant to disease caused by \textit{Pst} due to the induction of plant defenses, similar to systemic acquired resistance. The identity of the PMR elicitor(s) is as yet unknown, but was shown to be heat labile.

Keywords: Paper mill residuals; Organic matter; Soil amendments; Compost; Disease suppression; \textit{Arabidopsis thaliana} (L.) Heynh; \textit{Lycopersicon esculentum} (Mill.); \textit{Tomato}; \textit{Pseudomonas syringae pv. tomato} (Okabe) Young; Dye and Wilkie; Bacterial speck; Benzothiadiazole; Systemic acquired resistance (SAR); Non-expressor of PR-1; \textit{npr1}; Salicylate Hydroxylase; \textit{NahG}

1. Introduction

From improved soil structure to increased plant nutrition, soil organic matter influences a wide array of environmental and agronomic characteristics and is, therefore, a crucial component of any sustainable agriculture system [5]. Management of soil organic matter with the addition of organic amendments, whether in a composted or raw form, is not a revolutionary concept and has probably existed in some rudimentary form for as long as agriculture itself. Numerous studies have demonstrated the effectiveness of composted organic amendments on various plant diseases, especially against soil-borne pathogens in container systems [14,25,38,40,43,44,55,56,57,60,74]. Nevertheless, our scientific understanding of the processes by which organic amendments influence the outcome of plant disease is still marginal, owing in part to the inherent complexities of soil and the organic material itself.

Previous work with compost amended potting mixes or light-colored peat mixes demonstrated the suppression of foliar disease symptoms caused by \textit{Colletotrichum orbiculare} (Berk. and Mont.) Arx. and \textit{Pseudomonas syringae pv. maculicola} on cucumbers (\textit{Cucumis sativus} L.) and
A. thaliana (L.) Henyh., respectively, relative to plants grown in dark peat mixes conducive to disease [73,74]. Molecular and biochemical assays associated an increase in two key plant defense enzymes, β-1,3-glucanase [73] and peroxidase [74], with reduced foliar disease symptoms following pathogen inoculation. These results suggest that the compost amended potting mixes may have potentiated plant defenses to create an increased state of resistance similar to systemic acquired resistance [73,74]. However, the authors did not rule out other confounding factors, such as differences in plant nutrition or the effects of specific biological control agents added to many of the pine bark composts [73], and lacked the proper tools to pursue the issue further.

Induced resistance is a ‘state of enhanced defensive capacity’ triggered by specific contact stimuli whereby the plant’s active defenses are potentiated against subsequent pathogen challenge [67]. The resistance responses are usually systemic [52], but localized forms also exist, and are effective against a broad range of pathogens [51,61]. Induced resistance can be triggered by exposure of plants to virulent, avirulent, or non-pathogenic microbes [61,67], or artificially by various chemical agents like salicylic acid, 2,6-dichloro-isonicotinic acid (INA), or benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) [61].

Of particular interest to us are the various soil-borne, nonpathogenic microorganisms that are referred to as plant growth-promoting rhizobacteria (PGPR), which are capable of stimulating plant defenses [47,48,67]. Several of these PGPR incite plant defenses through the production of siderophores [36,37,39] or salicylic acid [16,37]. A comparative study of culturable microflora from the rhizosphere of tomato plants grown in different organic amendments found a significant increase in the incidence of bacteria antagonistic to several soil pathogens in in vitro assays [15]. These antagonistic effects corresponded to an increase in the percentage of siderophore-producing bacteria present. Similar changes in populations of fluorescent pseudomonads were observed in soils suppressive to bacterial wilt (Ralstonia solanacearum (Smith) Yabuuchi et al.) of tomato as compared to soils conducive to disease [53]. Boehm et al. [7] also documented an increase in the number of bacterial isolates capable of suppressing Pythium ultimum Trow, in a cucumber seedling assay, in disease suppressive peat mixes compared to peat mixes conducive to disease. Substrate availability was determined to play a crucial role in sustaining the efficacy of these bacterial isolates to suppress P. ultimum [6,7]. These reports demonstrate that it is possible to enrich the soil environment for organisms with biological control potential through the addition of organic amendments.

Our research is focused on elucidating the mechanisms of foliar disease suppression associated with soils from field plots amended with two composted forms of paper mill residuals (PMR) [62]. A series of field experiments was initiated in 1998 to study the effects of PMR-derived soil amendments on various aspects of soil and plant health [17,62]. PMR (sludges), the by-products of local paper mills, are an ideal source of organic matter for the production of soil amendments, due to their consistency relative to other sources of organic matter and their local abundance [5].

2. Materials and methods

2.1. Soil collection and PMR amendment characterization

Soil (Plainfield loamy sand) was collected on October 10, 1999, September 4, 2000 and May 12, 2001 from experimental plots at the University of Wisconsin’s Hancock Agricultural Research Station (Hancock, WI, USA) that were annually amended each spring (late March to early April) with either a high (H) or low (L) rate of (a) non-composted PMR (PMR H, 44.8 dry Mg/ha; PMR L, 22.4 dry Mg/ha), (b) PMR composted alone (PMRC H, 78.4 dry Mg/ha; PMRC L, 38.1 dry Mg/ha), or (c) PMR composted with bark (PMRBC H, 78.4 dry Mg/ha; PMRBC L, 38.1 dry Mg/ha). Soil from non-amended, control plots was also collected. The amendments’ chemical characteristics are described in Table 1 and further detailed, along with soil characteristics, in Foley and Cooperband [17] and Stone and colleagues [62]. Soil samples were collected arbitrarily from the top six inches of the experimental plots using a spade, while carefully removing plant and other large debris by hand, following the end of

Table 1

<table>
<thead>
<tr>
<th>Amendment</th>
<th>Solids (g kg⁻¹)</th>
<th>Ash (g kg⁻¹)</th>
<th>Total nitrogen (g kg⁻¹)</th>
<th>Total carbon (g kg⁻¹)</th>
<th>NH₄-N (mg kg⁻¹)</th>
<th>NO₃-N (mg kg⁻¹)</th>
<th>Saltsb (S m⁻¹)</th>
<th>pH</th>
<th>C/Nc</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMR</td>
<td>227.0</td>
<td>366.1</td>
<td>15.5</td>
<td>296.9</td>
<td>66.4</td>
<td>4.0</td>
<td>0.29</td>
<td>7.0</td>
<td>19.2</td>
</tr>
<tr>
<td>PMRC</td>
<td>414.0</td>
<td>676.4</td>
<td>12.3</td>
<td>144.8</td>
<td>76.5</td>
<td>0.3</td>
<td>0.16</td>
<td>7.9</td>
<td>11.7</td>
</tr>
<tr>
<td>PMRBC</td>
<td>370.2</td>
<td>356.8</td>
<td>13.8</td>
<td>322.4</td>
<td>16.6</td>
<td>4.1</td>
<td>0.15</td>
<td>8.0</td>
<td>23.4</td>
</tr>
</tbody>
</table>

a Amendments include non-composted PMR, composted paper mill residuals (PMRC) and paper mill residuals composted with bark (PMRBC).
b Electrical conductivity.

c Ratio of carbon to nitrogen.
the growing season between the months of August and October. Soil samples were then stored at room temperature in 30.5 cm × 91.4 cm plastic gusseted bags with the ends held loosely shut by clothespins. For growth chamber experiments, replicate soil samples were pooled by treatment and slightly moistened with distilled water (10 ml/l of soil) prior to use in bioassays.

2.2. Arabidopsis bioassay

Seeds of the wild type, npr1 [9], jar1 [59], and ein2 [20] mutant lines, and a NahG transgenic line [18] of A. thaliana ecotype Columbia, were sown directly into 3.8 cm diameter pots of soil from the various treatments. Plants were cultivated at 20°C under a 9 h photoperiod (=300 µE/m²/s provided by 90 W cool white fluorescent lights) and 65% RH in controlled environment chambers at the University of Wisconsin-Madison Biotron. Plants were watered every other day and fertilized every fourth day with half-strength Hoagland’s nutrient solution [21]. A 300 µM solution of BTH (Syngenta, formerly Novartis, Research Triangle Park, NC, USA) was applied as an aerosol to the aerial portions of 4 week-old plants grown in non-amended soil. Nutrient analysis was carried out at the University of Wisconsin Plant and Soil Analysis Laboratory (Madison, WI, USA) on 5 week-old foliar tissue collected prior to inoculation.

Five week-old plants were inoculated with a bacterial suspension of P. syringae pv. tomato strain DC3000 (2 × 10⁷ cfu/ml; prepared from an overnight culture grown at 28°C in King’s B medium [31] amended with rifampicin (50 mg/l)) in 10 mm MgSO₄ and 0.01% (vol/vol) Tween20, and sprayed onto leaf surfaces using a RL Flomaster, Model 1998, home and garden sprayer (Root-Lowell Manufacturing Co., Lowell, MI, USA). Additional plants were mock inoculated with 10 mM MgSO₄ and 0.01% Tween20. Plants were maintained in a chamber at 85% RH thereafter. Four to five days after inoculation, disease severity was assessed as the number of lesions per leaf area (cm²) for all leaves.

2.4. Gene expression analysis

Prior to inoculation, leaf tissue was collected from five Arabidopsis plants per treatment, frozen in liquid nitrogen and stored at −80°C for future gene expression analysis. Tissue samples were pooled (in equal quantities) by treatment and homogenized using a mortar and pestle. Total RNA was extracted from homogenized tissue samples using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). RNA was treated twice with RQ1 DNase (Invitrogen) prior to cDNA synthesis using 200 units of SuperScript II reverse transcriptase per 5 µg of total plant RNA (Invitrogen) as suggested by the manufacturer. Subsequent PCR reactions consisted of five units of Taq polymerase (Promega, Madison, WI, USA), 500 nm of each deoxynucleoside triphosphate, 500 nm of each oligonucleotide primer, 1 µl of cDNA from 5 µg of RNA and 1× reaction buffer (Promega) in a total volume of 20 µl. Amplifications were performed in a Stratagene Robo40cycler (Stratagene, La Jolla, CA, USA). Primers were designed from sequences within the GenBank database (http://www.ncbi.nlm.nih.gov) for the following genes: hevein-like protein (Hel), plant defensin (Pdf1.2), A. thaliana vegetative storage protein (Atvsp), and PR-1 (see Table 2 for sequences). PCR products were separated by electrophoresis on 1.5% agarose gels in 1× TBE buffer, extracted using QIAquick gel extraction columns (Qiagen, Valencia, CA, USA), cloned into pGEM-T (Promega), and sequenced (University of Wisconsin Biotechnology Center, Madison, WI, USA) to verify primer specificity.

For northern analysis, 5 µg of total RNA was separated by electrophoresis on a 1.0% agarose/formaldehyde gel (2.2 M formaldehyde and 1× MOPS buffer (0.2 M MOPS, 0.5 M sodium acetate, and 0.01 M EDTA)) in 1× MOPS buffer. RNA was transferred overnight in 10× SSPE (1.5 M sodium chloride, 0.1 M sodium phosphate, and 0.01 M EDTA) to a MagnaGraph nylon transfer membrane.
Table 2

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR1</td>
<td>5′-ttc ttc cct cga aag ctc aa-3′</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>(M90508)</td>
<td>5′-ctc ttc cct cga aag ctc aa-3′</td>
<td>Hybridizations</td>
</tr>
<tr>
<td>BGL2</td>
<td>5′-ttg tct tact cag gag ctc ggt gct-3′</td>
<td>RT-PCR, Northern</td>
</tr>
<tr>
<td>(M90509)</td>
<td>5′-cat act aca cgc atgg aag gc-3′</td>
<td>Hybridizations</td>
</tr>
<tr>
<td>Pdfl.2</td>
<td>5′-cat ggc tta ggt tgc ttc ca-3′</td>
<td>RT-PCR, Northern</td>
</tr>
<tr>
<td>(AY063779)</td>
<td>5′-aaga gtt tgc tgg gaa gc-3′</td>
<td>Hybridizations</td>
</tr>
<tr>
<td>Atvsp</td>
<td>5′-ctc ctc gaa ttc aac acc at-3′</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>(D85190)</td>
<td>5′-gga aag tct cgc tag aa-3′</td>
<td>Hybridizations</td>
</tr>
<tr>
<td>Hel</td>
<td>5′-cct gga tgc tta gtc ctc ca-3′</td>
<td>RT-PCR, Northern</td>
</tr>
<tr>
<td>(AF370536)</td>
<td>5′-tag cca aaa cca tgc gct tc-3′</td>
<td>Hybridizations</td>
</tr>
<tr>
<td>PR1 (858)</td>
<td>5′-ace tct tgt agg tgc tct tc-3′</td>
<td>Northern</td>
</tr>
<tr>
<td>(M90508)</td>
<td>5′-gtc ctc gaa tgc aac acc at-3′</td>
<td>Hybridizations</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5′-tta gga gaa tct gaa gga gc-3′</td>
<td>Northern</td>
</tr>
<tr>
<td>(X51576)</td>
<td>5′-ttc ctc gtt gaa gac cca ca-3′</td>
<td>Hybridizations</td>
</tr>
</tbody>
</table>

 Primer sequences are shown in lowercase, whereas GenBank numbers are shown in uppercase. Application refers to the method of hybridization: RT-PCR for reverse transcription-polymerase chain reaction, Northern for Northern blots.

2.5. Statistical analysis

Experimental units in each independent experiment were arranged in a completely randomized design. Treatment effects were assessed using analysis of variance in the PROC GLM procedure of SAS (SAS Institute, Cary, NC, USA). Fisher’s protected LSD test at $P = 0.05$ was used to compare treatment means.

3. Results

Plants grown in soils amended with composted forms of PMR were more resistant to foliar diseases caused by *Pst*. In disease assays, using soils collected in 1999, both *Arabidopsis* and tomato plants exhibited reduced disease symptoms caused by *Pst* when grown in soils amended with composted forms of PMR (PMRC or PMRBC), compared with results in non-amended soils (control) or soil amended with a non-composted PMR (Table 3). In *Arabidopsis*, the reduction of disease symptoms ranged, on average, from 34 to 65% depending on the soil amendment and was consistent over several independent experiments. Similarly, results with tomato demonstrated a reduction of foliar symptoms associated with PMRC and PMRBC amended soils (62 and 47%, respectively) relative to plants grown in non-amended soil or soil amended with PMR (Table 3).

Plants exhibited few differences in stature or nutrient content when grown in soils amended with PMR. Soil amendment type did not influence shoot biomass (Table 3) or the number of rosette leaves per plant in *Arabidopsis* (data not shown) under the experimental conditions used. However, tomatoes (cv. M82) grown in soil amended with PMRC exhibited nearly a 50% reduction in leaf area and shoot biomass compared to those grown in the other soil treatments (Table 3). Experiments employing a second tomato cultivar, Moneymaker, lacked these probable allelopathic effects (data not shown).

To assess potential nutrient effects, *Arabidopsis* foliar tissues were sampled for nutrient analysis prior to inoculation with *Pst*. Of the macro- and micro-nutrients measured, significant differences in the levels of phosphorus, potassium, and magnesium in plant tissues were observed among soil treatments ($P < 0.05$; Table 4). No statistical differences in the levels of calcium, sulfur, zinc, boron, manganese, iron, copper, aluminum, or sodium were observed among treatments (data not shown). Similar results were obtained in a replicated experiment. Because insufficient quantities of tissue were available, nitrogen levels in *Arabidopsis* were not determined. Most differences in plant nutrient content were observed between plants grown in amended versus non-amended soils, while nutrient content was similar among plants grown in the amended soil treatments (Table 4). Overall, these differences in plant nutrient content did not influence plant growth, or correspond to the reduced symptoms of *Pst*. No signs indicative of nutrient stress were ever observed on plants.

An increased level of pathogenesis-related gene expression in *Arabidopsis* was associated with composted forms of PMR. The expression of several *Arabidopsis* defense genes was assessed by RT-PCR just prior to inoculation. The results demonstrated an increase in the expression of PR-1 in plants grown in soils amended with PMRC or PMRBC as compared to plants grown in PMR or non-amended (control) soils (Fig. 1). The increase in PR-1 expression between the non-composted and composted PMR treatments was similar to the effect seen between the control and BTH treatments. Little treatment effect was observed among the expression of several genes encoding a jasmonate-inducible vegetative storage protein (*Atvsp*) [3,47], an ethylene-inducible hevein-like protein.
(Hel) with antifungal activity [47,49], and a plant defensin (Pdf1.2) that encodes a small protein with antifungal activity that is inducible by ethylene and jasmonate [45,47].

Northern analysis demonstrated a similar, but varied, pattern of defense gene expression across independent experiments (Fig. 2). Plants grown in soils amended with PMRC exhibited gene expression patterns similar to plants treated with BTH, with the expression of the pathogenesis-related genes PR-1 and β-1,3-glucanase (BGL2), and the plant defensin Pdf1.2 consistently greater relative to the other soil treatments. In most cases, foliar induction with BTH elevated defense gene expression to a level higher than that observed for the PMRC soil treatment.

Defense gene expression varied among experiments for plants grown in soils amended with PMR or PMRBC (Fig. 2). In most cases, the expression level for those defense genes analyzed in the PMRBC soil treatment was equal to or greater than observed for the PMR soil treatment across experiments. The expression of BGL2 was elevated in the PMRBC soil treatment when compared to the non-amended control and PMR soil treatment at 8 and 16 months, while at 18 months it was similar to those same treatments. Overall, the non-amended control, PMR, and

Table 3
Severity of disease symptoms of bacterial speck caused by P. syringae pv. tomato on Arabidopsis and tomato

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Arabidopsis</th>
<th>Tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disease severity (%)</td>
<td>Relative reduction (%)</td>
</tr>
<tr>
<td>Control</td>
<td>49.1 a living</td>
<td>–</td>
</tr>
<tr>
<td>PMR H</td>
<td>32.4 b</td>
<td>34.0</td>
</tr>
<tr>
<td>PMRBC H</td>
<td>17.3 cd</td>
<td>64.7</td>
</tr>
<tr>
<td>PRMC H</td>
<td>19.1 c</td>
<td>61.0</td>
</tr>
<tr>
<td>PMR L</td>
<td>34.5 b</td>
<td>29.9</td>
</tr>
<tr>
<td>BTH</td>
<td>7.5 d</td>
<td>84.7</td>
</tr>
<tr>
<td>LSD0.05</td>
<td>9.9</td>
<td>0.43</td>
</tr>
</tbody>
</table>

a Plants were grown in a non-amended soil (control), or a soil amended with low (L) or high (H) rates of either non-composted PMR, composted paper mill residuals (PMRC) or paper mill residuals composted with bark (PMRBC). A second set of plants grown in non-amended soil was treated with BTH 1 week prior to inoculation. Soils were collected from field plots on October 10, 1999, and were stored for 3 months prior to the beginning of these experiments.

b Percentage of leaves with disease symptoms.

c Reduction of disease severity relative to control plants grown in a non-amended field soil.

d Average number of lesions per cm² of leaf area at 5 days post-inoculation.

e Values followed by the same letter are not statistically significant by Fisher’s protected LSD test at P ≤ 0.05.

Table 4
Nutrient analysis of Arabidopsis leaf tissue prior to inoculation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nutrient</th>
<th>Arabidopsis</th>
<th>Tomato</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% P</td>
<td>% K</td>
<td>% Mg</td>
</tr>
<tr>
<td>Control</td>
<td>0.66 a</td>
<td>2.54 a</td>
<td>1.14 b</td>
</tr>
<tr>
<td>PMR H</td>
<td>0.58 bc</td>
<td>2.96 ab</td>
<td>0.63 c</td>
</tr>
<tr>
<td>PMRBC H</td>
<td>0.52 c</td>
<td>2.91 bc</td>
<td>0.57 cd</td>
</tr>
<tr>
<td>PRMC H</td>
<td>0.42 d</td>
<td>2.56 a</td>
<td>0.43 d</td>
</tr>
<tr>
<td>PMR L</td>
<td>0.51 c</td>
<td>2.86 bc</td>
<td>0.68 c</td>
</tr>
<tr>
<td>BTH</td>
<td>0.62 ab</td>
<td>2.67 bc</td>
<td>1.12 b</td>
</tr>
<tr>
<td>LSD0.05</td>
<td>0.08</td>
<td>0.38</td>
<td>0.16</td>
</tr>
</tbody>
</table>

a No statistical differences in the percentage of Ca, S, Zn, B, Mn, Fe, Cu, Al, or Na were observed among treatments (data not shown).

b Plants were grown in a non-amended soil (control), or soil amended with low (L) or high (H) rates of either non-composted PMR, composted paper mill residuals (PMRC) or paper mill residuals composted with bark (PMRBC). A second set of plants grown in non-amended soil was treated with BTH 1 week prior to the collection of leaf tissue. Soils were collected from field plots on October 10, 1999, and were stored for 3 months prior to the beginning of these experiments.

c Values followed by the same letter are not statistically significant by Fisher’s protected LSD test at P ≤ 0.05.
BTH treatments exhibited the most consistent patterns of gene expression across experiments.

Disruption of NPR1, and expression of the NahG transgene in Arabidopsis inhibited the disease suppressive effects of composted forms of PMR. To address the importance of several biochemical pathways implicated in induced resistance in foliar disease suppression mediated by the composted forms of PMR, several Arabidopsis mutants were grown on non-amended soils and soils amended with either PMRC or PMRBC. Wild type plants exhibited a reduction in the disease severity of Pst when grown in soils amended with PMRC and PMRBC relative to the non-amended control, whereas npr1 plants did not (Fig. 3). Among jar1 and ein2 plants, no differences in disease severity were observed across soil treatments (Fig. 3). However, jar1 and ein2 plants were severely stunted relative to wild type plants (data not shown). In addition, jar1 plants exhibited symptoms of damping-off across all treatments, and succumbed to symptoms in the non-amended soil. Damping-off is a symptom typical of plant diseases caused by soil-borne oomycetes.

Disease assays utilizing a NahG transgenic Arabidopsis line demonstrated that plants unable to accumulate salicylic acid due to the expression of NahG were unresponsive to
soils amended with composted forms of PMR (Table 5). Bacterial growth of Pst in planta was also examined in the same bioassay. Four days following inoculation, bacterial titers averaged 50% less in wild type plants grown in PMRBC or PMRC compared to wild type plants grown in non-composted PMR. The NahG plants did not show any significant differences in disease severity or bacterial titer among treatments.

The disease suppressive effect of PMR amendments was heat labile. In a first step toward determining the nature of the eliciting factor(s) in composted PMR amendments, non-amended field soil was autoclaved and amended (10% w/v) with either a PMRBC amendment (collected in the spring of 2000) or an autoclaved PMRBC amendment for use in the Arabidopsis-Pst disease assay (Fig. 4). Wild type Arabidopsis exhibited a 60% reduction in disease severity caused by Pst when grown in an autoclaved soil amended with PMRBC as compared to plants grown in an autoclaved soil amended with autoclaved PMRBC. No reduction in disease severity caused by Pst when observed when the Arabidopsis npr1 mutant was grown in autoclaved soil amended with autoclaved PMRBC. The foliar disease suppression imparted by the PMRBC amendment in autoclaved soil was comparable to the foliar disease suppression associated with soil from the PMRBC-amended field plots (Table 3), demonstrating that the use of autoclaved soil did not diminish the ability of the PMRBC amendment to reduce disease symptoms associated with Pst.

The suppressive effect of PMR amendments diminished over time and varied with serial additions. Relative to the non-amended controls, the compost amended field soils collected in 1999 consistently suppressed foliar disease in the Arabidopsis-Pst system for about 18 months when stored at room temperature (Fig. 5). Disease symptoms were reduced 40–80% in composted PMR treatments (PMRC and PMRBC) relative to the non-amended controls. PMR treatments only reduced disease symptoms 20% or less relative to the controls, with one exception. Overall, PMRC reduced disease severity more than PMRBC. After 21 months of storage, all disease suppressive activity was lost in the 1999 amended soils, as determined with the Arabidopsis-Pst disease assay.

Following the loss of foliar disease suppression activity in field soils collected in 1999, soils collected in 2000 and 2001 were assessed for their potential to suppress foliar symptoms of bacterial speck (Fig. 6). Only the PMRC treatment soil collected in 2000 was able to suppress significantly foliar disease symptoms caused by Pst on Arabidopsis to an extent similar to the BTH treatment. This suppressive effect was also reflected by a reduction in bacterial titer in planta. To date, no other amendment derived from PMR that we have tested has been capable of suppressing foliar disease symptoms in the Arabidopsis-Pst disease assay.

4. Discussion

Our previous field experiments documented the suppression of symptoms of foliar brown spot (causal agent P. syringae pv. syringae) of snap bean and angular leaf

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Table 5
Bacterial titer and severity of disease symptoms of bacterial speck caused by P. syringae pv. tomato on wild type (WT) and NahG Arabidopsis lines.

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Genotype</th>
<th>Disease severityb</th>
<th>Bacterial titer Log cfu/g ± SDc</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMR H</td>
<td>WT</td>
<td>80.3 a</td>
<td>8.12 ± 0.04 a</td>
</tr>
<tr>
<td>PMRBC H</td>
<td>WT</td>
<td>55.4 b</td>
<td>7.86 ± 0.11 b</td>
</tr>
<tr>
<td>PMRC H</td>
<td>WT</td>
<td>45.0 c</td>
<td>7.77 ± 0.07 b</td>
</tr>
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<td>PMRBC H</td>
<td>NahG</td>
<td>77.0 a</td>
<td>9.48 ± 0.07 a</td>
</tr>
<tr>
<td>PMRC H</td>
<td>NahG</td>
<td>78.3 a</td>
<td>9.26 ± 0.05 a</td>
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<tr>
<td>PMRC H</td>
<td>NahG</td>
<td>70.2 a</td>
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<td>PMRC H</td>
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<td>LSD0.05</td>
<td>8.4</td>
<td>0.24</td>
</tr>
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</table>

a Plants were grown in soil amended with a high (H) rate of either non-composted PMR, composted paper mill residuals (PMRC), or paper mill residuals composted with bark (PMRBC). Soils were collected from field plots on October 10, 1999, and were stored for 6 months prior to the beginning of these experiments.

b Percentage of leaves with disease symptoms 4 days post-inoculation.

c Bacterial titer was determined 4 days after inoculation.

Values followed by the same letter are not significantly different by Fisher’s protected LSD test at P ≤ 0.05.

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Fig. 4. Effect of autoclaving on foliar disease suppression activity associated with the 2000 PMRBC amendment. The PMRBC amendment was collected May 28, 2000 and stored for 6 months prior to the beginning of this experiment. Wild type Arabidopsis and npr1 mutant were grown in autoclaved field soil amended (10% w/v) with autoclaved (1 h at 121 °C) or non-autoclaved PMRBC amendment. Values represent the mean percentage of total leaves exhibiting symptoms of bacterial speck per plant 5 days after inoculation. Bars represent the standard deviation of n = 8 replicate plants per treatment. Means with the same letter are not significantly different by Fisher’s protected LSD test at P ≤ 0.05.
Fig. 5. Stability of foliar disease suppression activity associated with the 1999 amended field soils, as assessed using the Arabidopsis-Pst DC3000 disease assay. Plants were grown in a non-amended soil (control), or a soil amended with a high (H) rate of either non-composted paper mill residuals (PMR), composted paper mill residuals (PMRC) or paper mill residuals composted with bark (PMRBC). Values shown are means converted to represent the percentage of disease severity relative to the control for each time point. Disease severity was rated as the percentage of leaves with symptoms of bacterial spot 5 days after inoculation; control means are 20.8, 49.1, 17.0, 63.4, 56.2, and 67.6% for month 1, 3, 8, 16, 18, and 21, respectively. Treatment means (n = 8 plants) within a single time point with the same letter are not significantly different by Fisher’s protected LSD test at P ≤ 0.05; n.s. = no significance.

Fig. 6. Foliar disease suppression activity associated with the 1999, 2000, and 2001 amended and non-amended field soils, following 23, 12 and 4 months of storage, respectively, as assessed using the Arabidopsis-Pst DC3000 disease assay. Plants were grown in a non-amended soil (control), or a soil amended with a high (H) rate of either non-composted paper mill residuals (PMR), composted paper mill residuals (PMRC) or paper mill residuals composted with bark (PMRBC). BTH treatment was applied to plants grown in 2001 non-amended field soil. (a) Values shown are mean disease severity of n = 8 plants rated as the percentage of leaves with symptoms of bacterial speck 4 days after inoculation. (b) Mean in planta titer of Pst DC3000 at 4 days post-inoculation in n = 5 plants per treatment. Treatment means across years with the same letter are not significantly different by Fisher’s protected LSD test at P ≤ 0.05; bars represent the standard deviation.
spot (causal agent *P. syringae pv. lachrymans*) and anthracnose (causal agent *Colletotrichum lindemuthianum*) of cucumber grown in soil amended with composted PMR [62]. We have further documented this phenomenon here in replicated experiments with the same field soils showing foliar disease suppression in a controlled environment on two unrelated plants, tomato and *Arabidopsis*, using *P. syringae pv. tomato* as the pathogen. Using the *Arabidopsis-Pst* disease model system, we demonstrated that the foliar disease suppression observed with the composted forms of PMR was an induced form of resistance, exhibiting several characteristic features of SAR.

In plants, induced forms of systemic resistance can be broadly categorized into SAR [51,65] and ISR [47,48], based on several characteristics (reviewed in Refs. [61,67]). ISR is potentiated by PGPR, of which the best characterized are several species of *Pseudomonas* that cause no visible damage to the plant’s root system [67]. Classical SAR, as induced by a biotic factor, requires some sort of necrotic symptom on the plant’s aerial surfaces. SAR is associated with a salicylic acid-dependent pathway [18,35,69] and the induction of pathogenesis related (PR)-proteins [65,71]. ISR, based mostly on the interaction between *A. thaliana* and *Pseudomonas fluorescens* WCS417r, is salicylic acid-independent, does not involve the induction of PR-genes or other defense related genes [23,47,48], and appears to rely on jasmonate-and ethylene-dependent pathways [47]. However, few ISR systems have been characterized to this extent, and some plant-PGPR interactions exhibit features of SAR [16,39,67], implying that the origin of the eliciting agent is not as critical in the classification of induced resistance phenomena as the biochemical responses incited within the plant [61,67]. In addition, SAR is effective across a wide array of plant species, whereas there is some demonstrated specificity in the ability of strains of PGPR to induce systemic resistance at the plant species and genotype level [68]. To date, this type of specificity has not been documented in SAR systems.

We present evidence for the induced expression of two SA-dependent, pathogenesis-related genes, *PR-1* and β-1,3-glucanase (*BGL2*) prior to challenge inoculation in plants grown in soils amended with composted PMRs (PMRC and PMRBC; Figs. 1 and 2). Even though the gene expression data were not replicated at each timepoint (since replicate plant RNA samples were pooled by treatment in each experiment), qualitative differences among treatments were consistent in replicated experiments over time, with a few exceptions. Plants grown in the PMRC treatment showed consistent elevated levels of expression of *PR-1* and *BGL2* relative to the non-amended control and the non-composted PMR treatment. Plants grown in the PMRBC treatment did not differ from the non-amended control or non-composted PMR treatments in the expression of *PR-1*. However, an elevated level of *BGL2* was associated with PMRBC treatment relative to the control or the PMR treatment until after 16 months, coinciding with the loss of foliar disease suppression in the PMRBC treatment (Figs. 2 and 5). This discrepancy in the expression of *PR-1* and *BGL2* in plants grown in PMRC and PMRBC treatments may represent a possible dose response, since the PMRBC treatment generally was not as effective as the PMRC treatment at suppressing foliar symptoms caused by *Pst* on *Arabidopsis* (Fig. 5).

The disruption of foliar disease suppression in *Arabidopsis* plants with an *npr1* mutation or the *nahG* transgene when grown in PMRC-and PMRBC-amended soils implies that this phenomenon is an inducible host defense response similar to SAR [9,47]. In comparison, a functional *NPR1* was also important for ISR [47], while the disruption of salicylic acid signaling by the *nahG* transgene was not [47,48]. Whether PMR-mediated foliar disease suppression is independent of ethylene or jasmonate regulated plant defenses needs to be explored further, since there is evidence that the action of ethylene and jasmonate together can stimulate salicylic acid action [35]. A synergistic effect on the expression of *PR-1* and osmotin (*PR-5*) was documented in tobacco plants exposed to combinations of either salicylic acid and methyl jasmonate, or ethylene and methyl jasmonate [72]. An increased level of expression of *Pdf1.2* was associated with the PMRC and BTH treatments as compared to the control and PMR treatments, similar to *PR-1*, in replicate experiments at 8 months and 18 months (Fig. 2), suggesting a possible role for ethylene and jasmonate-dependent host responses in foliar disease suppression mediated by composted PMR amendments. We have no explanation for the weak expression of *Pdf1.2* at 16 months (which was also observed in a replicated hybridization using the same RNA sample) or the increased expression of *Hel* in PMRC and BTH treatments at 18 months, except that these inconsistencies may reflect other gross changes occurring within the soil over time that are influencing the plant.

We were unable to use *Arabidopsis* mutants to test the importance of ethylene- and jasmonate-dependent defenses in PMR-mediated foliar disease suppression, since *jar1* and *ein2* plants either succumbed to symptoms of damping-off or were severely stunted. The difficulties incurred while growing the *jar1* and *ein2* mutants in field soil are not surprising. Disruptions in the jasmonate and ethylene signaling pathways often lead to susceptibility to various opportunistic oomycetes [19,32,58,70].

There are other examples of improved plant defense responses in relation to foliar disease suppression mediated by composted materials, but these are based on studies using only potting mix systems. Zhang et al. [73,74] showed that several formulations of a disease suppressive, pine bark compost were able to increase the levels of the plant defense-related enzymatic activities of peroxidase and β-1,3-glucanase compared to a peat-based potting mix conducive to disease. These increases in enzyme activities were only observed following inoculation of the plant with the pathogen. In addition, the disease suppression observed
in several experiments appeared to be more associated with a biological control agent that was added to the compost during its preparation, than the compost itself [73]. It is also possible that the increases in enzymatic activity and disease suppression could be attributed to plant nutritional status, which would also be systemic. Another possibility is that the amendments utilized by Zhang et al. [73,74] ‘primed’ the plant’s defenses to respond more rapidly and to higher levels when elicited during pathogenesis. The priming of host defenses has been documented in plants through low exposures to chemical elicitors, such as salicylic acid [13,27,28,41,42,54], methyl jasmonate [29] and β-aminobutyric acid [75]. Priming of Arabidopsis defenses with BTH [33] and β-aminobutyric acid [75] was still dependent on NPR1.

Another source of composted pulp and PMR was also investigated for its ability to suppress crown and root rot of tomato, caused by Fusarium oxysporum f.sp. radicis-lycopersici [46]. Several induced cytological responses indicative of a potentiated host response to F. oxysporum occurred when plants were grown in a peat moss potting mix amended with composted pulp and PMR. No apparent cytological changes were observed prior to pathogen introduction in non-compost or compost amended potting mixes [46]. This also suggests a possible priming effect on host defenses.

One possible confounding effect that might explain the apparent disease suppression observed in our experiments is differences in plant nutrient status. However, nutrient analysis of Arabidopsis tissue revealed little variation among PMR treatments (Table 4). Detected differences in plant nutrient status were generally between the non-amended and amended treatments, which would be expected because of the improved nutrient content, water holding capacity, and soil structure imparted to the soil by the amendments.

Even though we were unable to assess nitrogen content, due to the small stature of Arabidopsis, no differences in biomass or leaf number among soil treatments was ever observed in experiments using Arabidopsis. With the exception of the allelopathic interaction observed when tomato cv. M82 was grown on PMRC, there also were no treatment effects on biomass in tomato (Table 3). Elevated nitrogen content was found to increase the susceptibility of tomato plants to Pst [22]. Through chemical characterization of the amendments (Table 1) and amended soils [17], we also know that there is an increase in plant-available nitrogen in soils amended with PMR and PMRC amendments. Assuming nitrogen content would be highest in plants grown in PMR and PMRC treatments [17]; the decreased disease severity associated with PMRC and PMRBC treatments, and similarity in disease severity between the PMR and non-amended control seem to rule out nitrogen as a factor.

Experiments using autoclaved amendments demonstrated that the potentiating effect of PMRBC is heat labile and independent of the field soil (Fig. 4). Additionally, in the Arabidopsis npr1 mutant, foliar disease suppression associated with growth in autoclaved soil amended with PMRBC was disrupted. However, it is inconclusive whether this potentiating effect is biotic or abiotic, since many abiotic substances are also heat labile. A better approach to this question would be to use irradiation, which should ‘sterilize’ the amendment while preserving its physical and chemical composition. Zhang et al. [73] found that the disease suppressive activity of a fortified pine bark potting medium was also heat labile, but oddly, a fermented water extract (compost tea) from the same medium that also suppressed foliar disease was still active even following autoclaving and passage through a 0.2 μm membrane filter.

Successive disease assays over a 21-month period demonstrated the longevity of foliar disease suppression in amended soils originally collected in 1999 when stored at room temperature (Fig. 5). However, the stability of foliar disease suppression in fields amended with composted forms of PMR is unknown. Field soils collected annually following reaplication of amendments over 3 consecutive years were assessed for foliar disease suppression. From the soils collected in 2000, only the 12-month old PMR-amended soil exhibited foliar disease suppression (Fig. 6), which corresponded to the suppression of angular leaf spot of cucumber caused by P. syringae pv. lachrymans in field experiments [62]. Interestingly, the soil amended with PMRBC in 2000 failed to show any foliar disease suppression activity in Arabidopsis bioassays (Fig. 6) or in field experiments [63], even though the PMRBC amendment itself demonstrated foliar disease suppression activity when amended to autoclaved soils (Fig. 4). It is possible that the foliar disease suppression associated with the 2000 PMR amendments was not as durable as in the 1999 amendments or may have been residual of the 1999 PMR amendments. No foliar disease suppression was associated with amended soils in 2001 (Fig. 6) or 2002 (data not shown) in our experiments or in field experiments (L.R. Cooperband and D. Rotenberg, personal communication). However, all amended soils described in these experiments and others [62], regardless of amendment type, suppressed root diseases caused by soilborne oomycetes (L.R. Cooperband and D. Rotenberg, personal communication). These observations agree with those of Krause et al. [34] who found that less than 10% of composts tested in a potting mix system were able to suppress foliar diseases, suggesting that foliar disease suppression mediated through composts is a less frequent phenomenon than the suppression of diseases caused by soilborne oomycetes [62].

Why is foliar disease suppression so difficult to generate and maintain with organic amendments? PMR are heterogeneous mixtures of wood pulp, paper fillers and microbial biomass recovered during wastewater purification and are chemically composed of cellulose, hemicellulose, lignin, and other complex carbohydrates, in
addition to a vast assortment of other complex organic compounds, nutrient elements, trace elements, and complex fatty acids [1,12]. Any number of these chemical components could directly elicit a SAR-like response in plants, or indirectly following some sort of biologically driven chemical modification. In addition, numerous examples exist of microorganisms capable of inducing systemic forms of resistance in plants [67], so it is possible that the differences in foliar disease suppression observed among these serially amended soils were due to the presence or absence of specific microorganisms, or the availability of substrates that support these microorganisms [6,7,24]. Finally, because the field soils were serially amended, it is quite possible that over time changes in soil physicochemical properties [17] disrupted chemical or biological conditions required for the maintenance or reestablishment of foliar disease suppression.

Hoitink and Boehm [6,24] postulated that biological control in soil microbial communities is a substrate-dependent phenomenon. That is, microorganisms capable of biological control through mechanisms such as antibiosis, competition, parasitism (or predation) or induced resistance, have specific nutritional requirements that must be met in order to sustain populations and physiological states necessary to maintain microbial activities that impart biological control. However, our data, in addition to other anecdotal observations, beckons a different interpretation of Hoitink and Boehm’s [6,24] substrate-dependent hypothesis, beyond providing the essential ‘carbon’ requirements necessary for growth and persistence of specific microorganisms.

We hypothesize that the SAR activity associated with the composted PMR amendments was an indirect result of the biological degradation of polycyclic aromatic hydrocarbons (PAHs) or other complex molecules by various microorganisms in the soil and plant rhizosphere, producing chemical intermediates, such as salicylic acid, capable of eliciting an induced resistance response in plants. Chemical analysis of the PMR produced during wastewater treatment is performed quarterly by Stora Enso North America, the paper company that produced the non-composted PMR amendments used in this research. The polycyclic aromatic hydrocarbon naphthalene and the aromatic hydrocarbons p-isopropyl toluene and xylene were often present in PMR at varying levels (data not shown). Other researchers have also documented the presence of aromatic hydrocarbons (including PAHs) in PMR and other municipal sludges [1,2,4]. However, none of the PMR amendments or parental PMR used to generate the composted PMR amendments for this research were directly tested for the presence of PAHs or other aromatic hydrocarbons.

The differences in foliar disease suppression activity observed between composted and non-composted PMR amendments could be due to the sorption or modification of PAHs during the composting process. For example, when added to soil, researchers have found that a significant proportion of PAHs, such as naphthalene, are sorbed to the various organic fractions of the soil where they are protected from further degradation [26,50]. Therefore, it is possible that PAHs in composted PMR amendments were sorbed to the various organic fractions or modified during the composting process, protecting the PAHs from further degradation. In contrast, the PAHs in the non-composted PMR amendments were not sorbed or modified, making them more sensitive to volatilization or rapid biodegradation by a broad array of soil microorganisms under a variety of environmental conditions [10,11,64]. This hypothesis predicts that the loss of foliar disease suppression in soils amended with composted PMR was not due to the loss of substrate responsible for growth or persistence of any particular microorganism, per se, but due to the depletion of PAHs. Once the PAHs were degraded, no secondary intermediates of PAH degradation, such as salicylic acid, would be produced and foliar disease suppression would be lost, but the PAH degrading microbial population could potentially remain unchanged. It is well documented that plant defenses can be induced with the application of salicylic acid or other chemical elicitors of SAR to plant roots directly or as a soil drench [16,30,36,48,63,66]. In addition, some aromatic compounds, including salicylic acid, decompose when heated [8], another potential explanation for why foliar disease suppression was lost when the amendment was autoclaved (Fig. 4).

In summary, we have presented data demonstrating the suppression of two plant foliar diseases caused by *P. syringae pv. tomato* in soils amended with composites derived from PMR. These results corroborate similar findings from field experiments [62]. In *Arabidopsis*, foliar disease suppression was associated with the potentiation of plant defenses prior to pathogen inoculation, disrupted by plants expressing an *npr1* mutation or a * NahG* transgene, and destroyed upon autoclaving. Our data suggest that PMR-mediated foliar disease suppression is an induced plant defense response that shares molecular features most similar to SAR. Since the eliciting factor(s) responsible for this phenomenon is yet to be identified, it is important for future research to focus on chemical factors, in addition to microbial factors, that correspond to these disease suppression phenomena.

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