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Induced systemic resistance against three foliar diseases of *Agrostis stolonifera* by (2R,3R)-Butanediol or an isoparaffin mixture

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Abstract
Induced systemic resistance (ISR) is a type of plant defense mechanism typically activated by non-pathogenic root-associated microorganisms and systemic priming of gene expression in response to subsequent pathogen challenge. ISR was found to be activated by PC1, a mixture of food-grade synthetic isoparaffins, and (2R,3R)-butanediol, a volatile organic compound produced by bacteria. In controlled environment tests, application of PC1 or (2R,3R)-butanediol to the soil reduced the diseased leaf area of *Agrostis stolonifera* by 20 to 40% for the fungal pathogens, *Microdochium nivale*, *Rhizoctonia solani* or *Sclerotinia homoeocarpa* compared to the water control. In *A. stolonifera*, expression of the jasmonate synthesis-related genes, *AsAOS1*, encoding an allene oxide synthase, and *AsOPR4*, encoding a 12-oxo-phytodienoic acid reductase, and expression of a pathogenesis-related protein gene, *AsGns5*, encoding an acidic β-1,3-glucanase, were primed for increased expression by PC1 or (2R,3R)-butanediol when *M. nivale* was inoculated seven days later. However, the compounds differed in their ability to induce expression prior to pathogen challenge. PC1 induced *AsAOS1* expression upon treatment, whereas (2R,3R)-butanediol induced expression of *AsOPR4* and *AsGns5* upon treatment. These results indicate that both (2R,3R)-butanediol and PC1 can produce ISR in *A. stolonifera* but may do so through different mechanisms.

Keywords: bentgrass, ISR, pathogenesis-related protein, priming, SAR

Introduction

Among the mechanisms that plants use to defend themselves from pathogen attack is induced resistance (Métraux et al., 2002). During induced resistance, a biological or chemical stimulus is recognized by the plant setting in motion a signal transduction pathway that activates resistance responses, such as programmed cell death and defense gene expression (Edreva, 2004). One form of induced resistance is induced systemic resistance (ISR), which is produced in roots by non-pathogens, like beneficial soil-borne plant growth-promoting rhizobacteria (PGPR) and beneficial soil-borne plant growth-promoting fungi (PGPF), and is transmitted through the signal molecules of ethylene (ET) and jasmonic acid (JA) (Vallad and Goodman, 2004).

Most of the research in ISR has been done in dicotyledonous plants, but ISR by PGPR has been recorded for several monocotyledonous plants. In *Oryza sativa* (rice), bacterial strains PF1 and FP7 of *Pseudomonas fluorescens* reduced disease caused by the fungus, *Rhizoctonia solani* (Nandakumar et al., 2001). In *Pennisetum glaucum* (pearl millet), application of the PGPR, *Bacillus pumilis* strains T4, SE34 and INR7, *Bacillus amyloliquefaciens* IN937a or *Bacillus subtilis* IN937b suppressed disease caused by the fungus, *Sclerospora glaucum* (Niranjan Raj et al., 2003). In *Agrostis stolonifera* (creeping bentgrass), *P. fluorescens* strain HP72 suppressed disease caused by *R. solani* (Suzuki et al.

2003). A volatile organic compound (VOC) secreted by some PGPR, (2R,3R)-butanediol, has been found to promote plant growth as well as induce resistance in dicotyledonous plants (Ryu et al., 2004); however, no published studies have been done in monocotyledonous plants with this substance for its effect on inducing resistance or affecting gene expression.

Activation of ISR is associated with gene priming, in which plant defense genes are expressed more rapidly and strongly after pathogen attack (Conrath et al., 2006). However, some defense genes are induced, in which there is increased expression after treatment but without pathogen challenge, rather than primed during ISR. For example, expression of genes for a neutral osmotin-like protein (*PR-5d*), extensin and expansin was induced systemically in roots and upper leaves after root treatment of *Lycopersicon esculentum* (tomato) with the PGPF, *Trichoderma hamatum* 382, which is believed to activate ISR (Alfano et al., 2006).

In monocotyledonous plants, transcripts related to ET and/or JA signalling pathways have been induced or primed after treatment with beneficial fungi, bacteria, and insects associated with ISR (Djonovic et al., 2007; Kanno et al., 2005; Muyanga et al., 2005). In *O. sativa*, genes encoding two β-1,3-glucanases, *Gns4* and *Gns5*, were found to be induced after treatment with the planthopper, *Sogatella furcifera* (Kanno et al., 2005). In *Triticum aestivum* (wheat), a gene encoding a subtilisin-like protein was upregulated after seed treatment with *P. aeruginosa* (Muyanga et al., 2005). In *Zea mays* (maize), genes encoding an allene oxide synthase (*ZmAOS*), hydroperoxide lyase (*ZmHPL*), and 12-oxo-phytodienoic acid reductase (*ZmOPR-7*) were primed by root treatment with the PGPF, *Trichoderma virens*, followed by inoculation with the anthracnose fungus, *Colletotrichum graminicola* (Djonovic et al., 2007). Such genes are promising candidates for being induced or primed after treatment with (2R,3R)-butanediol.

Recently, a new organic compound, PC1, developed by the Lubricants Division of Research and Development of the petroleum company, Petro-Canada (Mississauga, ON, Canada), was found to affect turfgrass diseases in the field (Hsiang et al., 2009). PC1 is a mixture of food-grade synthetic isoparaffins and food-grade emulsifiers. It is a clear, colourless liquid at room temperature with a carbon number distribution of approximately 16 to 35 atoms. PC1 did not appear to have a direct effect on *Colletrotrichum orbiuclare*, a fungal pathogen of *Nicotiana benthamiana*, but rather primed gene expression indicating that PC1 is involved in ISR (Cortes-Barco et al., in press).

In order to better understand the possible mode of action of (2R,3R)-butanediol and PC1 in a monocotyledonous plant, this study evaluated the interaction between the fungal pathogens *Microdochium nivale* (causal agent of pink snow mold), *Rhizoctonia solani* (causal agent of brown patch) and *Sclerotinia homoeocarpa* (causal agent of dollar spot), and the turfgrass, *A. stolonifera* treated with (2R,3R)-butanediol or PC1. The effect of (2R,3R)-butanediol and PC1 on disease inhibition was assessed, and expression of ISR-related marker genes in *A. stolonifera* was measured after treatment with (2R,3R)-butanediol or PC1 and subsequent challenge by *M. nivale*. In addition, direct antimicrobial activity of PC1 was investigated against a wide range of fungal pathogens of *A. stolonifera*.

Materials and Methods

Plant material and treatments

Agrostis stolonifera cv. Penncross was grown in GA-7 Magenta boxes (Magenta Corp., Chicago, IL, USA) filled with 100 g of dry sand and 20 ml autoclaved tap water to give 17% moisture (w/w). Approximately 0.2 g of *A. stolonifera* seed was placed into each box. Boxes were tightly capped and incubated for 10 to 15 d at room temperature under fluorescent lights at 50 μmol/m^2/s. After the grass had grown, the sand was injected directly with 10 ml 100 μM (2R,3R)-butanediol (Sigma, Oakville, ON, Canada), or 10 ml 10% or 20% (v/v) PC1 (Petro-Canada) per box using a pipette. Control plants were likewise treated with distilled water or 100 μM (2S,3S)-butanediol (Sigma).

Disease assessment

Inoculum for *M. nivale*, *R. solani* and *S. homoeocarpa* was prepared by incubating five strains of each pathogen on autoclaved wheat bran for 2 to 3 weeks. The inoculum for each pathogen was dried and chopped into small particles with a grain mill and applied at a rate of 0.01 g/cm^2 (0.025 g/vial) on the leaf blade surfaces of *A. stolonifera* 7 d after treatment. Ratings were taken at 7, 10, and 15 d post inoculation to estimate the disease level in terms of mycelial growth and yellowing of the turfgrass leaves. Experiments were conducted in a complete randomized design with three replicates per treatment. Each experiment was repeated three times. An analysis of variance with SAS Proc GLM at P=0.05 was used to estimate significant differences among the treatments. When significant treatment effects were found, means were separated by the test of Least Significant Difference (LSD, P=0.05).

PC1 antimicrobial activity test

Fifteen fungal pathogens of *A. stolonifera* and other turfgrasses (*Bipolaris hawaiiensis*, *Colletotrichum cereale*, *Curvularia* sp., *Gaeummanomyces graminis*, *Laetisaria fuciformis*, *Ophiosphaerella korrae, Magnaporthe grisea, M. nivale*, *Pythium aphanidermatum*, *R. solani, R. cerealis*, *R. zeae*, *S. homoeocarpa*, *Typhula ishikariensis* and *T. incarnata*), were grown on potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD, USA). These were then inoculated onto PDA amended with PC1 at 1%, 5%, 10%, or 20% (v/v), using four 5-mm-diameter mycelial plugs per plate. Control plates of PDA without PC1 were likewise inoculated. The inoculated plates were incubated at room temperature for 4 d, except for *T. ishikariensis* and *T. incarnata* which were incubated at 10ºC. To calculate inhibition, colony diameters were measured at 1, 4, 7 and 10 d of incubation, except for *P. aphanidermatum*, which was measured at 24 h, and for *T. incarnata* and *T. ishikariensis* which were measured at 24 d. Growth inhibition for each growth period (0 to 4 days, 4 to 7 days, and 7 to 10 days) was calculated separately as follows: (growth on control plate - growth on amended plate) / (growth on control plate). These growth inhibition values were used in SAS (SAS Institute, Cary, NC, USA) Proc Probit to calculate EC_{50} values (the concentration required to inhibit diameter growth by 50%). An example of the SAS program statements is available upon request to the second author.

Primer design

To obtain putative homologs of ISR genes in *Agrostis*, previously published ISR-induced or ISR-primed gene sequences were downloaded from the NCBI GenBank NR database (http://www.ncbi.nlm.nih.gov/). With these sequences, BLASTN or a BLASTX searches were then conducted against the NCBI GenBank NR database limited by *Agrostis* or Liliopsida, or against Gene Index databases of the Computational Biology and Functional Genomics Laboratory (http://compbio.dfci.harvard.edu/index.html) targeting sequence data from *Hordeum vulgare* (barley), *O. sativa*, *Saccharum officinarum* (sugarcane), *Sorghum bicolor* (sorghum), and *T. aestivum*. The top matches for each gene from the different species were then aligned with the program MUSCLE (Edgar, 2004). Highly conserved regions in the multiple alignments were examined, and potential primer sequences were assessed with the program GeneRunner (Hastings Software, Hastings, NY, USA) for desired attributes.

Two putative constitutive genes were chosen for study, an elongation factor and an ubuiquitin gene. Primers for the translation elongation factor *1α* gene, *AsEF-1α*, were designed after multiple alignment of the *O. sativa* EF-1α sequence, Locus Os03g08020 (Caldana et al. 2007), and matching sequences from GenBank: *O. sativa* (Os03g08010, Os03g08050, Os03g08060, C98198), and *A. stolonifera* (DV860573, DV866631, DV865570, DV867571). The forward primer AsEF_F122 was designed in a conserved region among these EF-1α sequences, and the reverse primer AsEF_R562 was designed in the 3'UTR region of the *A. stolonifera* sequence DV860573 that was unique among the sequences examined, to produce a predicted RT-PCR product size of 422 bp (Table 1).

Primers for the second putative constitutive gene, *AsUBI-3*, were designed based on alignment of an UBI-3 sequence from *L. esculentum* (X58253, Rotenberg et al., 2006), and matching *Agrostis capillaris* sequences from GenBank (DV855391, CN816064). The forward primer AcUBI3_F217 was designed in a conserved region between DV855391 and X58253 that was divergent from CN816064,

the homolog with lower similarity to X58253. The reverse primer AcUBI3_R449 was designed in a conserved region between all the ubiquitin sequences tested to produce a predicted RT-PCR product size of 232 bp (Table 1).

Primers were also designed for genes related to ISR, such as the jasmonate synthesis-related genes, *AsAOS1*and *AsOPR7*, as well as a pathogenesis-related protein gene, *AsGns5*. For amplification of *AsAOS1*, primers were designed from an alignment of AOS sequences from *Z. mays* (AY488135), *O. sativa* (AY055775), *T. aestivum* (AY196004) and *H. vulgare* (AJ250864). Also included were the *A. capillaris* and *A. stolonifera* AOS sequences, DV855200, DV859558, DV854759, DV856232, and DV867400, which were found to have 100% identity in the overlapping region, and therefore were combined into a tentative consensus sequence. Forward primer AsAOS_F78 and reverse primer AsAOS_R520 were in conserved regions between all AOS sequences tested plus the consensus *Agrostis* sequence but were divergent from the *A. capillaris* sequence, DV856173, and *ZmHPL* (NM_001111785), to amplify a predicted RT-PCR product size of 437 bp (Table 1).

For amplification of *AsOPR7*, primers were based on the alignment of *ZmOPR-7* (AY921644, Djonovic et al., 2007), *ZmOPR-5* (NM_001112439), *ZmOPR-4* (NM_001105831), *ZmOPR-3* (NM_001112360) and *ZmOPR-2* (NM_001112435) from *Z. mays*, and OPR7-like sequences from *O. sativa* (AB040743), *A. stolonifera* (DY543700), *A. capillaris* (DV853567) and *A. capillaris* (DV857969). Forward primer AsOPR_F125 was designed in a conserved region between all the OPR sequences, while the reverse primer AsOPR_R615 was designed in a conserved region between AY921644 and DY543700, but was divergent from all the other OPR sequences. The primers were designed to amplify a predicted RT-PCR product size of 490 bp (Table 1).

The *Agrostis* sequences (DV852857 and DV865103) that were homologs of the *O. sativa* sequences, *OsGns4* (U72250) and *OsGns5* (U72251), were short and only aligned in the region where *OsGns4* and *OsGns5* were identical. Therefore, primers were designed directly based on *OsGns4* and *OsGns5*. Primer AsGns5_F1085 was designed in a region of *OsGns5* that was divergent from *OsGns4*, and primer AsGns_R2085o1598 was designed in a highly conserved region between U72250, U72251, DV852857 and DV865103, and β-1,3-glucanase sequences from *Avena sativa* (CN815155), *Festuca pratensis* (AJ295946), *T. aestivum* (TC259743), *H. vulgare* (TC130990) and *Z. mays* (TC370721). The primers were designed to produce a predicted RT-PCR product size of 562 bp.

Relative RT-PCR

Agrostis stolonifera leaf tissue (0.2 g) was harvested at designated periods and immediately frozen at -80ºC. For SAR, leaf tissue was harvested at 0, 24, 48, 72, 120 and 168 HPT. For ISR, leaf tissue was harvested at 0, 72, 168, 192, 240, 288 and 336 HPT. Total RNA was isolated from 0.2 g frozen tissue of *A. stolonifera* using TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) and oligo (dT) primer with total RNA following the manufacturer's instructions. Transcript levels were determined by relative RT-PCR, which involves co-amplification of the gene of interest with a constitutively expressed control gene used as a standard for comparison of band intensity (Dean et al., 2002).

RT-PCR following Dean et al. (2002) were done in 15 ml reaction volumes with 2 ml cDNA, 0.75 units *Tsg* polymerase (Biobasic, Toronto, ON, Canada), 10x *Tsg* polymerase buffer, 2 mM dNTPs, and 2.5 mM Mg^{2+}, with 1.0 mM of each primer (Table 1). Amplification conditions consisted of 1 cycle at 94ºC for 3 min followed by 30 cycles of 94ºC for 30 s; 55ºC to 70ºC depending on primer pair for 1 min; and 72ºC for 1 min, with a final extension period of 10 min at 72ºC. The annealing temperatures were 55ºC for *AsEF-1α* primers; 62ºC for *AsAOS1* primers*,* 65ºC for *AsOPR7* primers and 70ºC for *AsGns5* primers, all co-amplified with *AsUBI-3* primers. All reactions were carried out in an Eppendorf AG 22331 Master Cycler (Eppendorf, Hamburg, Germany).

To confirm that the relative expression values were within the number of cycles that gave accurate quantification, relative RT-PCR was also done with 25 cycles, which is 5 cycles fewer than that used for quantification. The expression pattern was then compared to that obtained with 30 cycles to show that the patterns of expression were not significantly different for each gene.

The RT-PCR products were separated in 1% TAE agarose gels containing 1% ethidium bromide. Bands were visualized with an UV transilluminator and saved as TIFF electronic image files, and used in NIH Image (Scion Corporation, Frederick, MD, USA) for quantification. In each lane, the band intensities were determined for both the gene of interest and the constitutive control, *AsUBI-3*. The relative expression for the gene of interest was calculated by taking a ratio of its band intensity over the band intensity of *AsUBI-3*, to standardize for the amount of RNA. The identity of the RT-PCR products was confirmed by direct sequencing of the respective RT-PCR product at Laboratory Services Division (University of Guelph, Guelph, ON, Canada). To confirm the identity of each sequenced RT-PCR product, a BLASTX search of the NCBI GenBank NR database (http://www.ncbi.nlm.nih.gov/) was done.

Results

Disease assessment after (2R,3R)-butanediol and PC1 treatments

The chemical, (2R,3R)-butanediol, significantly suppressed mycelial growth and necrosis caused by *M. nivale* at 5, 7 and 10 DPI (Table 2). By 10 DPI, the diseased area increased to between 94 and 98% in plants treated with water or (2S,3S)-butanediol, but in (2R,3R)-butanediol-treated plants, it was only 8% diseased area (Table 2). Disease caused by *R. solani* was also significantly suppressed by (2R,3R)-butanediol compared to plants treated with water or (2S,3S)-butanediol at 5, 7 and 10 DPI (Table 2). By 10 DPI, disease levels in plants treated with water or (2S,3S)-butanediol reached 55-65%, while diseased area in the (2R,3R)-butanediol-treated plants remained near 3%. Treatment with (2R,3R)-butanediol also significantly suppressed mycelial growth and necrosis caused by *S. homoeocarpa* (Table 2). By 10 DPI, the diseased area caused by *S. homoeocarpa* in the (2R,3R)-butanediol-treated plants was 8% compared to 80-99% in the plants treated with water or (2S,3S)-butanediol (Table 2).

PC1 at 10% and 20% treatments showed significant suppression (P=0.05) of *M. nivale*, *R. solani* and *S. homoeocarpa* in terms of mycelial growth and necrosis, compared to the inoculated control, with 20% PC1 showing greater suppression for *M. nivale* and *R. solani* than 10% PC1 (Table 3). By 10 DPI, the diseased area in water-treated plants was 93-96%, while it was 46-70% and 33-66% in the 10% and 20% PC1-treated plants, respectively, across the three diseases. One difference between the effects of PC1 and (2R,3R)-butanediol was that disease levels in PC1-treated plants increased from 7 to 10 DPI (Table 3), unlike (2R,3R)-butanediol-treated plants, which did not show an increase in diseased area from 7 to 10 DPI (Table 3).

PC1 antimicrobial activity

The direct antifungal activity of PC1 was tested against 15 fungal pathogens of turfgrasses, especially *A. stolonifera* (Table 4). Growth of *M. grisea*, *Curvularia* sp., *P. aphanidermatum*, *S. homoeocarpa*, *L. fuciformis*, *B. hawaiiensis*, *G. graminis* and *T. incarnata* was enhanced by 1% PC1, and growth of *P. aphanidermatum* was enhanced by 5% PC1 (data not shown). During the first four days of growth, only *C. cereale* showed an EC_{50} value below 5% PC1, and an isolate of *Curvularia* showed an EC_{50} value below 15% PC1 (Table 4). All other isolates showed EC_{50} values above 15% PC1. Growth rates of *L. fuciformis*, *R. solani* and *S. homoeocarpa* were not strongly inhibited even by the highest amount of PC1 tested (20%), and these mycelia were projected to grow well even on 100% PC1 (Table 4). Over time, the effects of PC1 on growth diminished, and radial growth of the fungal pathogens on 20% PC1 was as fast as the unamended control after 10 days (Table 4), implying that the inhibitory component had been dissipated or degraded. Although PC1 did not totally inhibit growth of fungal pathogens,

some changes in appearance of the cultures on agar were observed in some fungal pathogens. The most common was a decrease in white aerial hyphae and more pigmentation in the colonies.

Expression of *AsUBI-3* as a constitutive control for relative RT-PCR

Ubiquitin 3 (*AsUBI-3*) was selected as a potential constitutively expressed control gene because UBI-3 genes have been shown to be least variable in real-time RT-PCR analysis of virus-induced gene silencing of the phytone desaturase (*PDS*) gene in *L. esculentum* and *N. benthamiana* (Rotenberg et al., 2006). To confirm that *AsUBI-3* was a constitutively expressed gene suitable for normalizing data of the RT-PCR experiments, expression of *AsUBI-3* was compared to that of another reported constitutive gene for translation elongation factor 1*α* (Sturzenbaum and Kille, 2001). The expression of these genes appeared to be constitutive as the expression patterns of *AsUBI-3* and *AsEF1α* were constant to each other after treatment with (2R,3R)-butanediol followed by inoculation with *M. nivale* 7 days later (data not shown). The *AsUBI-3* RT-PCR product was used as it had a size that was compatible with the co-amplification of the genes of interest.

Gene expression after (2R,3R)-butanediol treatment

Sequence analysis of the RT-PCR product of *AsAOS1* confirmed that it was most similar to *OsAOS1*, and the RT-PCR product of *AsGns5* was most similar to *OsGns5*. However, sequencing of the RT-PCR product for *AsOPR7* demonstrated that the gene obtained was a much closer homolog of *ZmOPR-4* than *ZmOPR-7*, and therefore the gene was re-named *AsOPR4* for the remainder of the study. This was unexpected because although the forward primer was in a conserved region between *ZmOPR*-7 and *ZmOPR-4*, the reverse primer was only a partial match with *ZmOPR-4* (11 out of 21 nt), but was a 100% match with *ZmOPR-7*.

No change of expression of *AsAOS1* over pre-treatment levels was observed in the plants prior to inoculation with *M. nivale* (Fig. 1). After *M. nivale* inoculation, a rapid increase in *AsAOS1* expression was seen in the plants treated with 100 μM (2R,3R) butanediol, peaking at 288 HPT. No change in the expression of *AsAOS1* was observed in the plants treated with (2S,3S) butanediol plus water after inoculation. This shows that *AsAOS1* was primed but not induced after (2R,3R) butanediol application.

Treatment with (2R,3R)-butanediol resulted in increased expression of *AsOPR4* compared to both controls prior to pathogen challenge, but there was no change in the expression level in the controls during that period (Fig. 1). After inoculation with *M. nivale*, a rapid increase of expression was only observed in the (2R,3R)-butanediol-treated plants. This indicates that *AsOPR4* was induced and primed after (2R,3R)-butanediol application.

A continuous increase from no detectable expression was observed for *AsGns5* after treatment with (2R,3R)-butanediol (Fig. 1). After inoculation with *M. nivale*, expression of *AsGns5* in the (2R,3R)-butanediol-treated plants further increased peaking at 288 HPT. No detection of the expression of *AsGns5* was found for control treatments after *M. nivale* inoculation, indicating that *AsGns5*, like *AsOPR4*, was induced and primed after (2R,3R)-butanediol application.

Gene expression after PC1 treatment

Unlike (2R,3R)-butanediol, treatment with 10% PC1 resulted in transcripts of *AsAOS1* increasing prior to inoculation while expression did not change in the water controls (Fig. 1). Prior to inoculation with *M. nivale*, no significant increase in AsOPR4 expression was detected after PC1 treatment compared to the water treatment (Fig. 1). This shows that *AsAOS1* was being induced and primed by PC1.

No significant increase in either AsOPR4 or AsGns5 expression was detected after PC1 treatment compared to the water treatment, prior to inoculation (Fig. 1). After inoculation with *M. nivale*, a rapid increase in expression in PC1-treated plants was observed, but there was no change in expression was detected in the control indicating that AsOPR4 and AsGns5 were primed, but not induced, by PC1 treatment.

Discussion

There is relatively little information about ISR in monocotyledonous plants, although it can be induced by PGPRs as in dicotyledonous plants (Métraux et al., 2002). For example, *O. sativa* was more resistant to *R. solani* after application of the PGPR, *P. fluorescens* strains PF1 and FP7 (Nandakumar et al., 2001), and *P. glaucum* was more resistant to *S. glaucum* after application of the PGPR, *B. pumilis* strains T4, SE34 and INR7, *B. amyloliquefaciens* IN937a and *B. subtilis* IN937b (Niranjan Raj et al., 2003). The only previous putative demonstration of ISR activity in *A. stolonifera* is the application of the PGPR *P. fluorescens* strain HP72, which suppressed *R. solani* (Suzuki et al., 2003). This study shows that (2R,3R)-butanediol suppressed pink snow mold, brown patch and dollar spot of *A. stolonifera* by 90 - 99% in controlled environment tests, which is the first evidence of the effect of (2R,3R)-butanediol on inducing disease resistance of a monocotyledonous plant. Previous studies on the effect of (2R,3R)-butanediol on controlling diseases examined the dicotyledonous plants, *Arabidopsis thaliana* and *Nicotiana tabacum* (Hee et al., 2006; Ryu et al., 2004). There is no report on the fungicidal activity of (2R,3R)-butanediol, perhaps because of the high cost of making amended media with this chemical, but preliminary tests showed no volatile fungicidal activity, even at concentrations ten times higher than used here (data not shown). The amount of (2R-3R)-butanediol applied to each Magenta box was approximately 90 μg, which is much lower than turfgrass disease fungicides such as propiconazole. In the field, propiconazole is applied at a rate of 7.3 g per 100 m^2 (Hsiang et al. 2007), which is equivalent to approximately 1 mg for the soil surface area in a Magenta box.

PC1 was also effective at reducing diseases of *A. stolonifera*. However, at the two concentrations tested, PC1 was less effective than (2R,3R)-butanediol, suppressing the three diseases by 50 – 90%. One possible mechanism of disease suppression by PC1 is direct antifungal activity. However, PC1 seems to have fungistatic effects rather than direct fungal toxicity, since very high concentrations (30 to 50% PC1) were required for fungal growth inhibition, and the inhibitory activity was quickly lost after a few days of incubation. The only difference after 10 d of incubation was in the appearance of the colonies rather than the growth rates. Some mycelia appeared water soaked and shiny with more pigmentation. Since PC1 is a mixture of food-grade synthetic isoparaffins, it is possible that it behaves similarly to food-grade essential plant oils that produce some degenerative changes in hyphal morphology. For instance, mycelium of *Penicillium digitatum* appeared degraded with large vesicles inside the cell walls after exposure to essential oils, but essential oils did not seem to affect the growth rate of *P. digitatum* on agar plates (Soylu et al., 2005). The lack of strong antimicrobial activity against any of the fungal pathogens grown on PC1, implied that PC1 acted through other means such as by affecting host plant resistance. This would also be consistent with PC1 being applied to the soil, while the pathogen inoculum was applied to the leaves and thus not directly exposed to the chemical. As experiments with *N. benthamiana* indicated that PC1 was involved in ISR (Cortes-Barco et al., in press), the expression of *A. stolonifera* genes potentially related to ISR (*AsAOS1*, *AsOPR4* and *AsGns5*) was assessed in plants treated with butanediol or PC1.

Allene oxide synthase and 12-oxo-phytodienoic acid reductase genes were chosen as candidates, because treatment of *Z. mays* roots with the PGPF, *T. virens*, produced ISR, and induced the JA-regulated genes, *ZmAOS* and *ZmOPR-7* (Djonovic et al., 2007). The allene oxide synthases catalyze the formation of allene oxide from lipoxygenase-derived fatty acid hydroperoxides, and allene oxide is a precursor of JA in plants (Song et al., 1993). The 12-oxo-phytodienoic acid reductases catalyze the reduction of double bonds adjacent to an 0X0 group in a,b-unsaturated aldehydes or ketones (Kohli and Masey. 1998). This can produce a diverse group of jasmonates that are involved in the JA pathway (Feussner and Wasternack, 2002). Genes related to JA synthesis would be logical candidates for induction or priming during ISR as the ET/JA pathway regulates ISR (Okubara and Paulitz, 2005).

Induction of *AsAOS1* was observed after application of PC1 but not (2R,3R)-butanediol, but both treatments primed *AsAOS1* expression after inoculation with *M. nivale*. Djonovic et al. (2007)

showed that *ZmAOS* was primed in *Z. mays* treated with *T. virens*, increasing strongly after 24 HPI of *C. graminicola*. While primers were designed to amplify *AsOPR7* (the homolog of *ZmOPR-7* in *A. stolonifera*), the sequence of the RT-PCR product from *A. stolonifera* demonstrated that the gene under study was a homolog of *ZmOPR-4*. *ZmOPR-4* and *ZmOPR-7* are paralogs in a large gene family in corn, and it is likely that the primers which were based on *ZmOPR-7* better matched the OPR-4 homolog than the OPR-7 homolog in the 12-oxo-phytodienoic acid reductase gene family of *A. stolonifera*. Induction of *AsOPR4* expression was observed after (2R,3R)-butanediol treatment but not after PC1 treatment. However, *AsOPR4* expression increased more strongly after *M. nivale* challenge for both treatments, indicating that *AsOPR4* was being primed to respond more strongly to pathogen attack. Zhang et al. (2005) showed that *ZmOPR-4* was not induced after wounding, treatment with JA, ET, or abscisic acid or fungal infection, making the results with (2R,3R)-butanediol surprising. However, Zhang et al. (2005) did not evaluate priming in their studies. Therefore, it is possible that some of the treatments used by Zhang et al. (2005) could have primed expression of *ZmOPR-4*, like (2R,3R)-butanediol and PC1 did for *AsOPR4* in *A. stolonifera*. Also, as this is the first study of an 12-oxo-phytodienoic acid reductase gene in *A. stolonifera*, it may be that the gene family and its regulation is different in *A. stolonifera* compared to *Z. mays*.

In many plants, wounding and herbivore attack also result in ISR since they activate signals involved in the JA signalling pathway (Walters et al., 2003). In *O. sativa*, infestation by the planthopper, *S. furcifera*, resulted in ISR against *M. grisea* and also induction of two β-1,3-glucanases, basic *OsGns4* and acidic *OsGns5* (Kanno et al., 2005). β-1,3-glucanases can hydrolyze β-1,3-glucans, one of the major components of the cell walls of many fungi (Wessels and Sietsma, 1981). Studies in dicotyledonous plants have shown that β-1,3-glucanases are involved in defence against fungal pathogens (Leubner-Metzger and Meins, 1999). A role for β-1,3-glucanase genes in ISR is supported by the protection provided by the PGPR, *P. fluorescens* isolate 1, in *Eleusine corocana* (ragi) against *Pyricularia grisea*, which induced β-1,3-glucanase protein along with other defense proteins (Radjacommare et al., 2004). In *A. stolonifera*, induction of *AsGns5* was observed after (2R,3R)-butanediol treatment, similar to the response to the planthoppers in *O. sativa* (Kanno et al., 2005). In contrast, there was no induction of *AsGns5* due to PC1 treatment. Both the (2R,3R)-butanediol- and PC1-treated plants showed priming of *AsGns5* after inoculation with *M. nivale*.

The results obtained from the gene expression analyses demonstrate that PC1 exhibits ISR activity, since it is priming genes very similarly to (2R,3R)-butanediol. As PC1 was applied directly to the soil, it may be taken directly by the plant through the roots and priming gene expression, or it may be affecting some aspect of the soil and/or rhizosphere microorganisms resulting in priming of gene expression in the leaves.

Although both (2R,3R)-butanediol and PC1 appeared to activate ISR and both primed gene expression, there were differences in their ability to induce gene expression. PC1 induced *AsAOS1*, but (2R,3R)-butanediol did not, whereas (2R,3R)-butanediol induced *AsOPR4* and *AsGns5* but PC1 did not. This indicates that the chemicals act differently to affect resistance and activate ISR, but the mode of action of both chemicals remains to be determined.

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Table 1 Primers used for the amplification of constitutive and ISR-related genes for ISR assessment in *A. stolonifera*.

Target	Gene name	Primer name	Primer pair	Product size
Constitutive	*AsEF1*	AsEF_F122	F: 5'-AAGAACGGTGATGCTGGT-3'	422 bp
		AsEF_R562	R: 5'-AAATCCCCTAACATGACTACC-3'	
Constitutive	*AsUBI3*	AsUBI-3_F217	F: 5'-CAGGACAAGGAGGGCATC-3'	232 bp
		AsUBI-3_R449	R: 5'-TTCCTGAGCCTGGTGACC-3'	
ISR	*AsAOS1*	AsAOS_F78	F: 5'-GGCGCTGGAGAAGATGGA-3'	437 bp
		AsAOS_R520	R: 5'-GGACGTGGCCTTGGTGAC-3'	
ISR	*AsOPR4*	AsOPR_F125	F: 5'-CAACGACCGCACCGACGAG-3'	490 bp
		AsOPR_R615	R: 5'-GAGGAAGGGGTAGTCGGTGTA-3'	
ISR	*AsGns5*	AsGns5_F1085	F: 5'-CCTGCAGGCCCTCAGCGG-3'	562 bp
		AsGns_R1598	R: 5'-GCCACCCGCTCTCCGACA -3'	

Table 2 Effect of ISR-inducing agent (2R,3R)-butanediol on disease caused by *Microdochium nivale*, *Rhizoctonia solani* or *Sclerotinia homoeocarpa* on *Agrostis stolonifera*. Plants were grown in Magenta boxes for up to 15 days, and treated with 10 ml of water (inoculated control), 100 µM (2R,3R)-butanediol or (2S,3S)-butanediol (inactive isomer). At 7 days post treatment, plants were inoculated with wheat bran inoculum of *S. homoeocarpa*, *M. nivale* or *R. solani* at a rate of 0.01g/cm2. Inoculated plants were incubated at room temperature under fluorescent lights (50 µmol/m^2/s) for 15 days. Ratings were taken at various intervals, evaluating the percent diseased area.

		Percent area affected[1]		
Pathogen	**Treatment**	**Day 5**	**Day 7**	**Day 10**
M. nivale	Inoculated control	65.0 a	76.6 a	98.0 a
M. nivale	(2S,3S)-butanediol	65.0 a	70.0 a	94.6 a
M. nivale	(2R,3R)-butanediol	0 b	8.0 b	8.0 b
M. nivale	LSD (*P*=0.05)	8.1	12.0	4.8
R. solani	Inoculated control	55.0 a	63.3 a	65.0 a
R. solani	(2S,3S)-butanediol	38.3 a	48.3 a	55.0 a
R. solani	(2R,3R)-butanediol	2.3 b	3.3 b	3.3 b
R. solani	LSD (*P* =0.05)	13.6	12.9	10.5
S. homoeocarpa	Inoculated control	65.0 a	88.3 a	99.3 a
S. homoeocarpa	(2S,3S)-butanediol	51.6 a	77.0 a	80.0 a
S. homoeocarpa	(2R,3R)-butanediol	5.3 b	8.0 b	8.0 b
S. homoeocarpa	LSD (*P* =0.05)	6.7	12.0	41.0

[1] Data are means pooled from three separate experiments with a total of nine replications. An analysis of variance with SAS procedure GLM at *P*=0.05, was used to test for significant differences among the treatments. Means followed by a letter in common are not significantly different according to a test of Least Significant Difference (LSD, *P*=0.05).

Table 3 Effect of PC1 on disease caused by *Microdochium nivale*, *Rhizoctonia solani* or *Sclerotinia homoeocarpa* on *Agrostis stolonifera*. Plants were grown in Magenta boxes for up to 15 days, and treated with 10 ml of water (inoculated control) or PC1 at 10% or 20%. At 7 days post treatment, plants were inoculated with wheat bran inoculum of *S. homoeocarpa*, *M. nivale* or *R. solani* at a rate of 0.01g/cm2. Inoculated plants were incubated at room temperature under fluorescent lights (50 µmol/m^2/s) for 15 days. Ratings were taken at various intervals, evaluating the percent diseased area.

		Percent area affected[1]		
Pathogen	**treatment**	**Day 5**	**Day 7**	**Day 10**
M. nivale	Inoculated control	61.6 a	73.3 a	96.6 a
M. nivale	PC1 at 10%	17.0 b	28.3 b	46.6 b
M. nivale	PC1 at 20%	10.0 b	17.0 c	33.3 c
M. nivale	LSD (*P* = 0.05)	9.3	11.2	12.8
R. solani	Inoculated control	50.0 a	70.0 a	93.3 a
R. solani	PC1 at 10%	32.3 b	45.0 b	65.0 b
R. solani	PC1 at 20%	10.0 c	23.3 c	50.0 c
R. solani	LSD (*P* = 0.05)	6.8	6.6	8.8
S. homoeocarpa	Inoculated control	57.6 a	71.6 a	96.6 a
S. homoeocarpa	PC1 at 10%	25.0 b	46.6 b	70.0 b
S. homoeocarpa	PC1 at 20%	18.6 b	40.0 b	66.6 b
S. homoeocarpa	LSD (*P* = 0.05)	6.5	13.7	9.4

[1] Data are means pooled from three separate experiments with a total of nine replications. An analysis of variance with SAS procedure GLM at *P*=0.05, was used to test for significant differences among the treatments. Means followed by a letter in common are not significantly different according to a test of Least Significant Difference (LSD, *P*=0.05).

Table 4 Effect of PC1 on growth of fungal pathogens of *Agrostis stolonifera*. Values reflect the percentage (vol/vol) at which PC1 inhibits fungal growth by 50% (EC_{50}). Colony diameters were measured at 4, 7 and 10 d of incubation, except for *Pythium aphanidermatum* (at 24 h) and *T. incarnata* and *T. ishikariensis* (at 24 d).

	EC_{50} values (% PC1, vol/vol) [1]		
Fungal pathogen	0 to 4 d	4 to 7 d	7 to10 d
Bipolaris hawaiiensis	51.2	>100	>100
Colletotrichum cereale	4.8	12.5	>100
Curvularia sp.	14.1	30.0	>100
Gaeummanomyces graminis	16.1	28.4	>100
Laetisaria fuciformis	>100	>100	>100
Leptosphaeria korrae	54.2	>100	>100
Magnaporthe grisea	15.1	26.4	>100
Microdochium nivale	18.1	21.0	>100
Pythium aphanidermatum	25.9	>100	>100
Rhizoctonia cerealis	15.3	24.4	>100
Rhizoctonia solani	>100	>100	>100
Rhizoctonia zeae	19.2	26.4	>100
Sclerotinia homoeocarpa	>100	>100	>100
Typhula incarnata	-	-	>100
Typhula ishikariensis	-	-	>100

[1] Data are means pooled from three separate experiments with a total of 12 replications per combination of fungal pathogen by concentration of PC1 used.

Figure Legend

Fig. 1 Relative RT-PCR of *AsAOS1, AsOPR4* and *AsGns5* in *A. stolonifera* after treatment with 100 µM (2R,3R)-butanediol, 100 µM (2S,3S)-butanediol or water, or treatment with 10% PC1 or water. The quantity of mRNA levels for each gene was determined relative to the expression of *AsUBI-3*. Means are shown with standard error bars that were calculated based on four replications. To determine if each gene was primed after inoculation, plants were inoculated with wheat bran inoculum of *M. nivale* at 168 HPT.

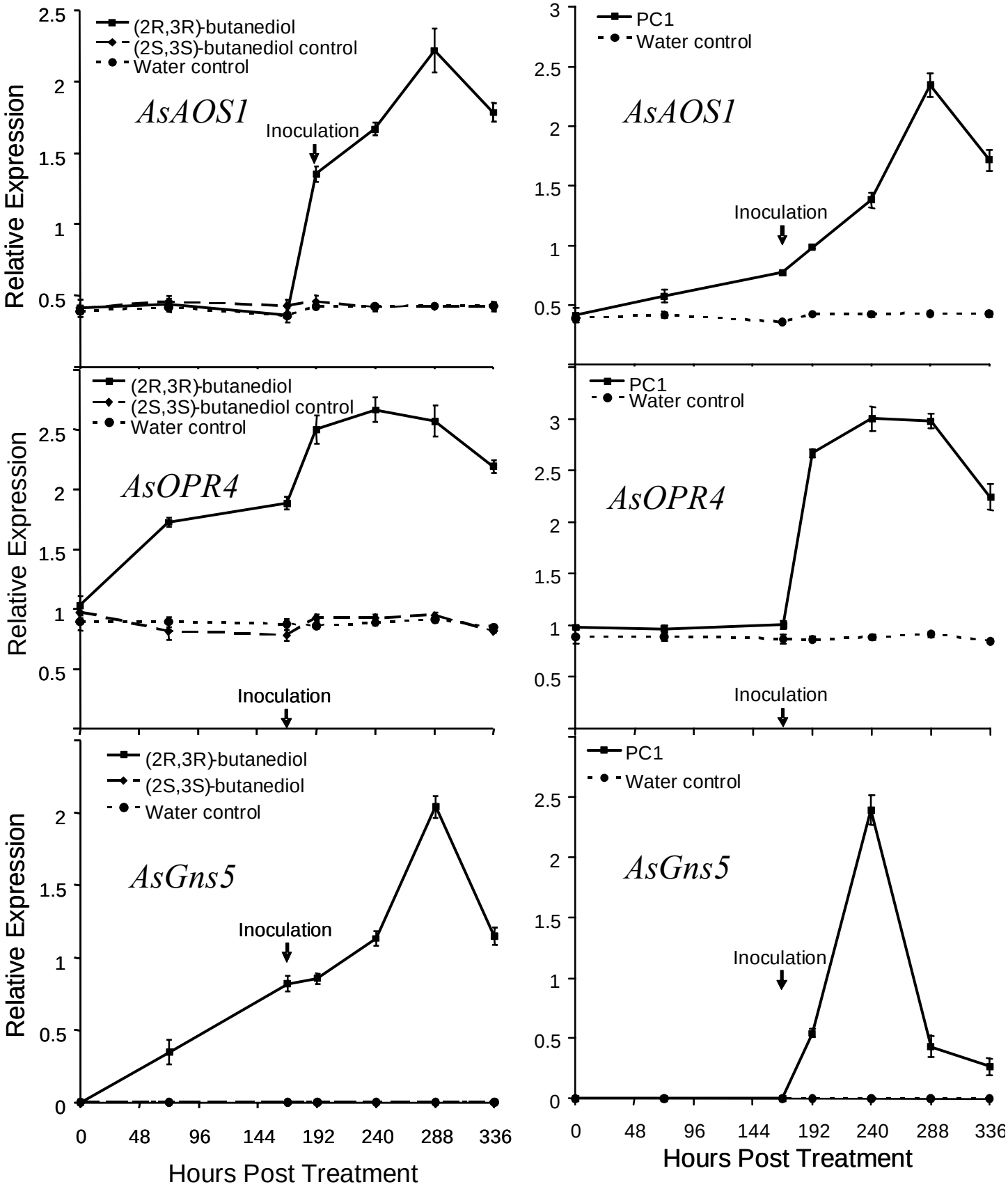
(2R,3R)-butanediol
(2S,3S)-butanediol control
Water control
AsAOS1
Inoculation
Relative Expression
PC1
Water control
AsAOS1
Inoculation
(2R,3R)-butanediol
(2S,3S)-butanediol control
Water control
AsOPR4
Inoculation
Relative Expression
PC1
Water control
AsOPR4
Inoculation
(2R,3R)-butanediol
(2S,3S)-butanediol
Water control
AsGns5
Inoculation
Relative Expression
PC1
Water control
AsGns5
Inoculation
0 48 96 144 192 240 288 336
Hours Post Treatment
Hours Post Treatment

Figure 1