RESEARCH/INVESTIGACIÓN

INDUCTION OF HOST-PLANT RESISTANCE IN CUCUMBER BY VERMICOMPOST TEA AGAINST ROOT-KNOT NEMATODE

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ABSTRACT

Mishra, S., K.-H. Wang, B. S. Sipes, and M. Tian. 2018. Induction of host-plant resistance in cucumber by vermicompost tea against root-knot nematode. Nematropica 48:164-171.

Split-root experiments and quantitative real time PCR were used to examine if vermicompost tea (VCT) drenching could induce host-plant resistance against root-knot nematodes (*Meloidogyne incognita*). Two greenhouse trials were conducted where cucumber (*Cucumis sativus*) roots were split into half; one part of the roots was drenched with VCT, and the other part received *M. incognita* inoculum. Control plants were drenched with water. Root penetration by *M. incognita* was lower in plants drenched with VCT as compared to the control ($P \leq 0.05$) in both trials. Quantitative real time PCR was used to measure relative expression of defense-related genes, *CHIT-1*, *PAL-1*, *β-1,3-Glucanase*, *LOX-1* and *PR-1* on cucumber. Plants were: 1) drenched with VCT 2 days prior to *M. incognita* inoculation, 2) inoculated with *M. incognita* only, or 3) received no VCT or nematodes as the control. Plants drenched with VCT showed an up-regulation of *CHIT-1* at 2 days, *PAL-1* at 2 and 8 days, *LOX-1* at 2 and 5 days, and down-regulation of *PR-1* at 0 and 5 days after *M. incognita* inoculation. Up-regulation of *CHIT-1*, *PAL-1* and *LOX-1* by VCT drenching supported the hypothesis that VCT imitated Induced Systemic Resistance (ISR) on cucumber.

Key words: chitinase, induced systemic resistance, *Meloidogyne incognita*, RT-qPCR, split-roots

RESUMEN

Mishra, S., K.-H. Wang, B. S. Sipes, y M. Tian. 2018. Inducción de la resistencia de la planta huésped en el pepino por té de vermicompost contra el nematodo agallador. Nematropica 48:164-171.

Se usaron experimentos de raíz dividida y PCR cuantitativa en tiempo real para examinar si el empapado con té de vermicompost (VCT) podría inducir resistencia de la planta huésped contra los nematodos agalladores (*Meloidogyne incognita*). Se llevaron a cabo dos ensayos de invernadero donde las raíces de pepino (*Cucumis sativus*) se dividieron en dos mitades; una parte de las raíces se empapó con VCT, mientras que la otra parte recibió inóculo de *M. incognita*. Las plantas de control se empaparon con agua. La penetración de la raíz de *M. incognita* fue menor en las plantas empapadas con VCT en comparación con el control ($P \leq 0.05$) en ambos ensayos. La PCR cuantitativa en tiempo real se usó para medir la expresión relativa de genes relacionados con la defensa, *CHIT-1*, *PAL-1*, *β-1,3-Glucanasa*, *LOX-1* y *PR-1* en pepino. Las plantas fueron 1) empapadas con VCT 2 días antes de la inoculación de *M. incognita*, 2) solo inoculadas con *M. incognita*, o 3) no recibieron VCT ni nematodos como control. Las plantas empapadas con VCT mostraron una regulación positiva de *CHIT-1* a los 2 días, *PAL-1* a los 2 y 8 días, *LOX-1* a los 2 y 5 días, y regulación a la baja de *PR-1* a los 0 y 5 días después de la inoculación. La

regulación positiva de *CHIT-1*, *PAL-1* y *LOX-1* mediante el empapamiento de VCT respaldaba la hipótesis de que el VCT imitaba la resistencia sistémica inducida (ISR) en el pepino.

Palabras clave: *Meloidogyne incognita*, quitinasa, raíces divididas, resistencia sistémica inducida, RT-qPCR

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are obligate plant-parasitic nematodes that infect more than 3,000 plant species and can cause significant yield loss on different vegetable crops (Sasser and Freckman, 1987; Sikora and Fernandez, 2005; Moenset *et al*., 2009;). For many years, the use of synthetic chemicals have been a successful strategy for management of plant-parasitic nematodes. However, due to the deleterious effects of nematicides on humans and the environment, many nematicides have been withdrawn from the market (Thomason, 1987). As a result, growers are exploring alternative strategies for the control of plant-parasitic nematodes. Much research has focused on organic soil amendments, biological control, naturally occurring nematicides, and plant breeding for nematode resistance (Oka *et al*., 2000). However, plant resistance to *Meloidogyne* spp. is limited and is especially rare in cucurbit crops. Only a wild species of cucurbit, *Cucumis metulifer*, is resistant to *Meloidogyne* spp. Deakin *et al*. (1971) noted that incorporating *M. incognita* resistance from *C. metulifer* into *C. melo* through conventional breeding was unsuccessful due to the interspecific incompatibility for hybridization and fruit set. Alternatively, plants have several endogenous defense genes against pathogen infection. These genes could be induced by biotic and abiotic agents through mechanisms such as Induced Systemic Resistance (ISR) (Siddiqui and Shaukat, 2002) or Systemic Acquired Resistance (SAR) (Chinnasri *et al*., 2006), and are worth exploring for nematode control.

Several commercial biopesticides or chemicals are available to induce ISR or SAR, naturally occurring soil bacteria with capability to stimulate plant growth known as plant-growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1981), have been found to induce ISR against several plant pathogens (Van Peer *et al*., 1991). Pathma and Sakthivel (2013) reported a wide range of PGPR species in straw and goat-manure based vermicompost. Thus, integrating vermicompost into a cucurbit cropping system could offer one approach to induce host-plant resistance against *M. incognita*.

Vermicomost tea (VCT) is an aqueous extract of vermicompost (VC) containing numerous bioactive molecules as well as microbial populations derived from VC (Edwards *et al*., 2006). Research has shown that VCT drenching suppresses root-knot nematodes (Edwards *et al*., 2007). However, mechanisms on how VCT drenching suppresses root-knot nematode are unknown. Potential mechanisms on how VCT could suppress root-knot nematodes include: 1) expression of extracellular enzymatic activities from rhizobacteria, 2) induction of host-plant resistance, 3) initiation of competitive exclusion and microbial antagonism, and 4) enhancement of plant tolerance by promoting plant growth. This research examined the induction of host-plant resistance by VCT drenching only.

Siddiqui and Shaukat (2002) and Adam *et al*. (2014) induced host-plant resistance on tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*) against *Meloidogyne* spp. by introducing *Pseudomonas* and *Bacillus* through split-root experiments. Adam *et al*. (2014) demonstrated the induction of ISR against *M. incognita* in tomato by introducing *Bacillus subtilis* to one side of a split root system and showed a 51% reduction of egg masses on the other side of the split roots. Siddiqui and Shaukat (2002) applied *Pseudomonas aeruginosa* strain IE-6st and *P. fluorescens* strain CHAO to half of a split-root system achieving 42% and 29% reduction in penetration by *M. javanica* on the other half of the split-tomato roots, respectively.

Another approach to demonstrate induction of ISR is to quantify expression of defense-related genes. Expression of these genes can be measured by quantitative real-time polymerase chain reaction (qRT-PCR). Some of the common defense genes known to be responsible for inducing host-plant resistance include *CHIT-1*, *β-1,3-glucanase*, *PR-1*, *PAL-1* and *LOX-1* encoding for chitinase, glucanase, pathogenesis-related protein 1, phenylalanine ammonia-lyase, and lipoxygenase protein 1, respectively (Alizadeh *et al*., 2013).

Khan *et al*. (2004) reported that enzymes such as chitinase and glucanase caused eggs to be vacuolated and became transparent, suggesting hydrolysis occurred in the eggs. Alizadeh *et al*. (2013) found the primed expression of defense related genes such as *CHIT-1*, *β-1,3-Glucanase*, *PAL-1*, *PR-1,* and *LOX-1* in stems of cucumber plants treated with *Pseudomonas* spp., *Trichoderma harzianum*, and their combination followed by inoculation of *Fusarium oxysporum* f. sp. *radices cucumerinum*.

Specific objectives of this study were to: 1) determine if VCT could induce host-plant resistance against *M. incognita* in cucumber, and 2) detect expression of defense-related genes in cucumber following drenching with VCT.

MATERIALS AND METHODS

Vermicompost tea preparation

Vermicompost was initiated in a 1.8 m-long × 0.6 m-wide wooden bin layered with plastic tarp at Poamoho Experiment Station in March 2013 with approximately 100 g of commercial mix of red wiggler (*Eisenia foetida*) and blue worms (*Perionyx excavatus*) (Waikiki Worms Company, Honolulu, HI). To ensure the consistency of vermicompost prepared, the worms in the bin were specifically fed leaves of lettuce (*Lactuca sativa*) and kale (*Brassica oleracea* var. sabellica), and skin of papaya (*Carica papaya*) every week. Uncured vermicompost used for the experiment was freshly collected from the worm bin immediately before each experiment by removing earthworms from the vermicompost (VC). Vermicompost tea (VCT) was prepared by soaking VC in water at 1:10 (v/v) ratio, aerated for 24 hr using 2.5 W Elite 800 air pumps (Rolf C. Hagen Inc., Montreal, Canada). VCT was filtered using a kitchen strainer to separate solids from the liquid prior to application.

Nematode inoculum

Eggs of *M. incognita* were obtained from 'Orange Pixie' tomato (*Solanum lycopersicum*) where the nematodes were cultured under greenhouse conditions with sterile sand and soil mix for approximately 3 months. Eggs were extracted using NaOCl and centrifugal flotation methods (Hussey and Barker, 1973). Eggs extracted were placed on 60.3-µm pore screens to allow egg hatch for 2 wk. Hatched second-stage juveniles (J2) were then counted and used as inoculum.

Split-root experiment

Two split-root trials were conducted in Gilmore greenhouse at the University of Hawaii at Mānoa, Honolulu, HI, to determine if drenching of VCT prepared from uncured VC could induce host-plant resistance against *M. incognita* (Fig. 1). Average ambient temperature in the greenhouse was 23.8°C. 'Bush Champion' cucumber (*Cucumis sativus*) (W. Atlee Burpee and Company, Warminster, PA) seeds were placed in the middle of two conjoint plastic pots (5.5×5.5×8 cm3) filled with sterile sand: soil (1:1 v/v) mix. Ten days after seed sowing, roots were examined to ensure growth on both sides of the conjoint pots. One part of the root system was drenched with VCT prepared from uncured VC, or with water at 50 ml/plant. Vermicompost tea was prepared using the protocol described earlier. Three days after VCT drenching, the undrenched part of the root system was inoculated with 200 J2 of *M. incognita* delivered in 1-ml aliquots by pipette. A total of six plants were arranged in a completely randomized design with 3 replications in Trial I. The split-root experiment was repeated once with 5 replications arranged in a completely randomized design in Trial II. One week after inoculation, roots from the inoculated side were collected, weighed, and stained with acid fuchsin to determine penetration by *M. incognita* (Byrd *et al*., 1983).

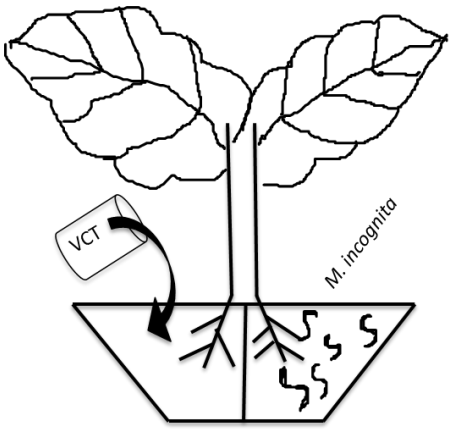

Fig. 1. Cucumber roots split into two conjoined pots. One side of the cucumber root was drenched with vermicompost tea (VCT) prepared from uncured vermicompost while the other side was inoculated with *Meloidogyne incognita*.

Gene expression experiment

'Bush Champion' cucumber (W. Atlee Burpee & Company, Warminster, PA) seedlings were raised in peat moss: vermiculite (2:1 v/v) mix for 2 wk and transplanted into individual sterile sand: soil (1:1 v/v) mix contained in 8.5-cm tall and 9.5-cm-d plastic pots. Cucumber plants were: (1) drenched with VCT prepared from uncured VC followed by *M. incognita* inoculation = VC(+) RK(+), (2) not drenched with VCT but inoculated with *M. incognita* = VC(-) RK(+), or 3) not drenched with VCT and not inoculated with *M. incognita* = VC(-) RK(-). VCT was prepared using the protocol described earlier. Seedlings drenched with VCT received 50 ml/plant at 2 days after transplanting. Seedlings that received *M. incognita* were inoculated with 500 J2/plant 2 days after drenching with VCT. Each treatment was destructively sampled at 0, 1, 2, 5, and 8 days after inoculation with *M. incognita*. Two plants were sampled for each treatment combination. Thus, a total of 30 plants were assayed.

To evaluate expression of defense-related genes, cucumber leaves (first true leaf from each plant) were sampled at the designated sampling dates. Leaf samples from the two replicated plants were combined and flash frozen in liquid nitrogen and stored at -80°C until assay. Each treatment sample was ground with mortar and pestle in liquid nitrogen and total RNA was extracted using QiagenRNeasy plant mini kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Extracted RNA was treated with Ambion DNA-free kit (Thermo Fisher Scientific, Waltham, MA) to remove genomic DNA from the sample. The DNA-free total RNA was converted into cDNA using the Invitrogen SuperScript II reverse transcription kit (Invitrogen, Corp., Carlsbad, CA).

Five genes of interest (*CHIT-1*, *β-1,3-glucanase*, *PR-1*, *PAL-1* and *LOX-1*) and a reference gene in cucumber (*EF 1-α*) were quantified using qRT-PCR for their expression in each cucumber sample. *CHIT-1*, *β-1,3-glucanase*, *PR-1*, *PAL-1* and *LOX-1* encoded for chitinase, glucanase, pathogenesis-related protein 1, phenylalanine ammonia-lyase, and lipoxygenase protein 1, respectively. *EF 1-α* encodes for elongation factor gene in cucumber. The qPCR primer sets used for the five targeted genes were: *EF 1-a*, forward 5′-CTGTGCTGTCCTCATTATTG-3′ and reverse 5′-AGGGTGAAAGCAAGAAGAGC-3′; *CHIT-1*, forward 5'-TGGTCACTGCAACCCTGACA-3' and reverse 5'-AGTGGCCTGGAATCCGACT-3′; CACS, forward 5′ -TGGGAAGATTCTTATGAAGTGC-3′ and reverse 5′ -CTCGTCAAATTTACACATTGGT-3′; *β-1, 3-Glucanase*, forward 5′ -TCAATTATCAAAACTTGTTCGATGC-3′ and reverse 5′-AACCGGTCTCGGATACAACAAC-3′; *PAL-1*, forward 5′-ATGGAGGCAACTTCCAAGGA-3′ and reverse 5′- CCATGGCAATCTCAGCACCT-3'; *PR-1*, forward 5'-TGCTCAACAATATGCGAACC-3′ and reverse 5′-TCATCCACCCACAACTGAAC-3′; *LOX-1*, forward, 5′-CTCTTGGGTGGTGGTGTTTC-3′ and reverse 5′-TGGTGGGATTGAAGTTAGCC-3′ (Shoresh *et al*., 2005; Wan *et al*., 2010; Migocka and Papierniak, 2011; Alizadeh *et al*., 2013). PCR products for *CHIT-1*, *β-1,3-glucanase*, *PR-1*, *PAL-1*, *LOX-1* and *EF-1-α* were expected to be between 100–200 bp.

PCR reactions (20 µl per well) were carried out in 96-well plates. Each reaction mixture contained 2 µl of diluted cDNA, 10 µl of SYBR Green mix (AB Applied Biosystems, Foster, CA) and 8 µl primers mix (Biolegio 1 pmol). Quantitative real time PCR was performed using an iCyclerThermal Cycler (BioRad Laboratories, Inc., Philadelphia, PA) at 95°C for 10 min. followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. The cycle threshold (Ct) values of three replicates of each sample were obtained.

Statistical analysis

For the split-root experiment, all data were subjected to one-way analysis of variance (ANOVA) using the general linear model procedure in Statistical Analysis System (SAS Institute Inc., Cary, NC). Prior to analysis, nematode data were log-transformed [log10 (x+1)] wherever appropriate to normalize the data distribution. Untransformed arithmetic means are presented. For gene expression in cucumber, relative expression level was determined by calculation of the fold change ($2^{-\Delta\Delta Ct}$) (Asai *et al*., 2014). All data obtained after the fold change calculation were subjected to 3×5 (treatment × days after inoculation) factorial analysis of variance using SAS. When interaction between treatment and days after inoculation occurred, treatment means by days after inoculation were separated

using Waller-Duncan *k*-ratio (*k*=100) t-test wherever appropriate.

RESULTS

Split-root experiment

VCT prepared from uncured VC drenched on one side of the roots suppressed root penetration of *M. incognita* on the other side of the roots compared to the water control ($P < 0.05$, Fig. 2A). VCT drenching reduced numbers of *M. incognita*/root system by 80.0% and 71.4% in Trial I and Trial II, respectively. No effect of VCT drenching on cucumber root growth was observed in either trial (Fig. 2B).

Gene expression of plant defense genes

VC(+)RK(+) showed an increased expression of *CHIT-1* at 2 days after inoculation (dai) but not thereafter compared to the no VC controls VC(-)RK(-) and VC(-)RK(+) (Fig. 3A). Inoculating the plants with the nematode alone, the VC(-)RK(+) treatment, did not induce *CHIT-1*. VC(+)RK(+) caused an increased expression of *PAL-1* at 0, 2, and 8 dai compared to the control (Fig. 3 B). However, no up-regulation of *PAL-1* was detected at 5 dai in VC(+)RK(+).VC(-)RK(+) did not induce *PAL-1*. *β-1,3-glucanase* was not expressed in any of the treatments, thus data are not shown. VC(-)RK(+) showed an increased expression of *LOX-1* at 8 dai, whereas VC(+)RK(+) resulted in increased expression of *LOX-1* at 2 and 5 dai (Fig. 3C). Expression of *PR-1* was not up-regulated by VC(+)RK(+) at any time point but was down-regulated at 0 and 5 dai (Fig. 3D). Inoculating plants alone up-regulated *PR-1* at 1 and 8 dai compared to the VC(-)RK(-). In fact, VC(+)RK(+) reduced the expression of *PR-1* at 2 dai compared to VC(-)RK(+) (Fig. 3D).

DISCUSSION

Split-root experiment

The split-root experiment confirmed that VCT prepared from uncured VC can induce host-plant resistance against infection by *M. incognita* on cucumber. Reduction in root penetration by *M. incognita* on cucumber plants was not due to direct interference of VCT on *M. incognita* because VCT and nematodes were spatially separated. This result is similar to suppression of *M. javanica* penetration in tomato roots by rhizobacteria applied to only half of a split-root system as demonstrated by Siddiqui and Shaukat (2002). This result is also consistent with field experiments where cucumber plants drenched weekly with the same VCT prepared from uncured VC had lower root-knot nematode population densities compared to that in the no VCT drenching throughout a cucumber crop (Mishra *et al*., 2016). Interestingly, this field experiment showed that the VCT would not suppress the population densities of root-knot nematodes if the cucumber plants were only drenched every 2 wk or once a month (Mishra, 2016). The transient gene expression of *CHIT-1* in the gene expression experiment partially explained

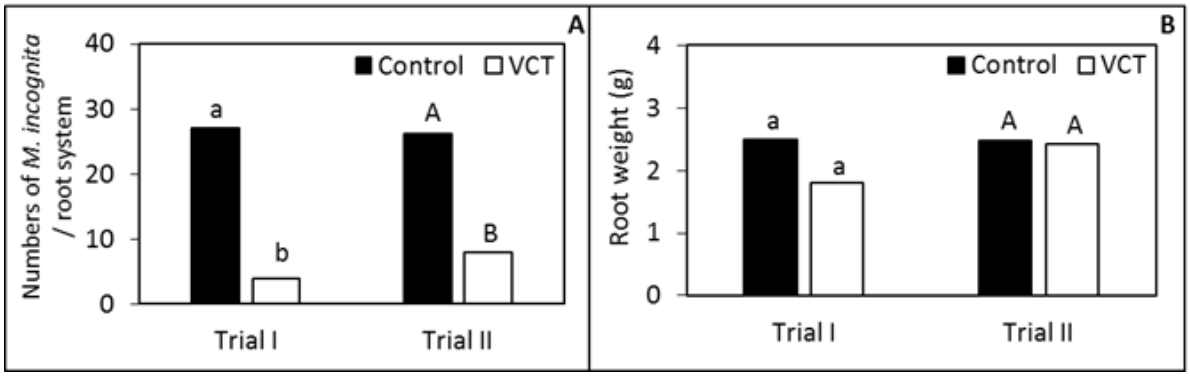

Fig. 2. Effect of vermicompost tea (VCT) on: A) number of *Meloidogyne incognita* per plant and B) root weight of cucumber roots 7 days after nematode inoculation in Trial I and Trial II of a split-root experiment. Means are average of 3 replications in Trial I, and 5 replications in Trial II. Nematode abundance data were log-transformed, log(x+1), prior to analysis of variance. Columns followed by same letter(s) are not different according to Waller-Duncan *k*-ratio (*k*=100) t-test.

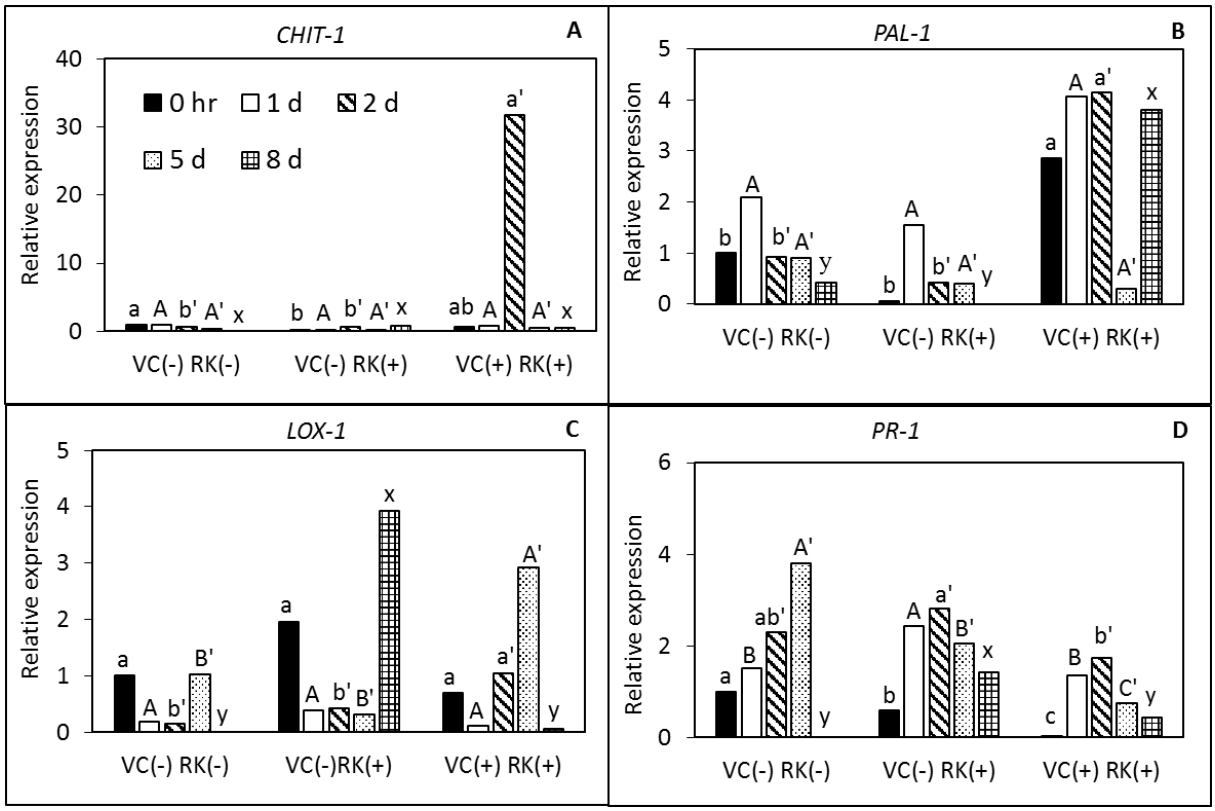

Fig. 3. Effects of vermicompost tea (VCT) drenching on relative expression of A) *CHIT-1*, B) *PAL-1,* C) *LOX-1*, and D) *PR-1* genes in leaves of cucumber plants at 0, 1, 2, 5, and 8 days after inoculation with *Meloidogyne incognita*. Means (n=3) for each time point followed by different letter(s) are significantly different according to Waller-Duncan *k*-ratio (*k*=100) t-test.

why frequent drenching of VCT is needed to suppress root-knot nematode infection.

Gene expression experiment

VCT from uncured VC induced expression of *CHIT-1*, *PAL-1,* and *LOX-1* genes temporarily, but not *β-1,3-glucanase* after the cucumber plants were challenged with *M. incognita*. Several reports have shown that colonization of rhizosphere by PGPRs leads to up regulation of defense-related genes after challenged with pathogen (Conrath *et al*., 2002; Shoresh *et al*., 2005). In particular, *CHIT-1* and *PAL-1* were only induced by drenching of VCT after nematode inoculation. Inoculating *M. incognita* alone VC(-)RK(+) failed to induce *CHIT-1* and *PAL-1* in cucumber plants. This suggested that VCT induced ISR and resulted in the temporal expression of *CHIT-1* and *PAL-1*. These two genes were previously confirmed to be ISR related genes (Shoresh *et al*., 2005). It is sensible to assume that VC(+)RK(+) also induced expression of *LOX-1* as lipoxygenaseis, the first enzyme synthesized in the biosysthesis pathway of jasmonic acid (Melan *et al*., 1993), the phytohormone that regulates ISR (Spoel and Dong, 2012). However, it is perplexing that *LOX-1* was expressed in VC(-)RK(+) without the presence of VCT at 8 dai.

Induction of *CHIT-1* by VCT was most obvious among the genes tested, increasing 30-fold compared to the control, VC(-) RK(-), in the leaves of cucumber at 2 dai or 5 d after VCT drenching, but not thereafter. This temporary gene expression of ISR is similar to that reported by Yedida *et al*. (1999) where they found increased chitinase and peroxidase activities by *T. harzianum* within 48 and 72 hr after inoculation, but not thereafter. When examining if ISR induced by VCT drenching on cucumber plants over a longer period, Mishra *et al*., 2017 showed that drenching VCT prepared from uncured VC of the same feedstock used in this study at 2-wk intervals did not suppress reproduction of *M. incognita* at 2.5 months after nematode inoculation.

Results from this gene expression experiment verified that induction of host-plant resistance by VCT from uncured VC is not due to the induction of SAR. This was shown by the down-regulation of *PR-1* gene by VCT drenching. *PR-1* is a gene commonly expressed when SAR is induced (Spoel and Dong, 2012). *PR-1* is anticipated to be expressed when a pathogen infects a plant (Conrath *et al*., 2006), as shown by the temporary expression of this gene at 1 and 8 d after *M. incognita* inoculation on VC(-) RK(+) plants.

In conclusion, VCT prepared from uncured VC induced ISR as shown by split-root and the gene expression experiment. However, most of the induction of host-plant resistance genes disappear within 1 wk after drenching. Due to the narrow window of expression of these plant defense genes, *M. incognita* can still infect VCT drenched plants and complete its life cycle over time. Thus, frequent drenching of VCT at least at 1-wk intervals could provide more consistent suppression of *M. incognita* using VCT drenching solution prepared from uncured VC.

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