

Thermo-stability, dose effects and shelf-life of antifungal metabolite-containing supernatants produced by *Xenorhabdus szentirmai*

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Abstract Entomopathogenic nematodes in the genus *Steinernema* are associated with *Xenorhabdus* spp. bacteria. When steinernematid colonise an insect host the nematode-bacterium association overcomes the insect immune system and kills the host within 48 h. *Xenorhabdus* spp. produce secondary metabolites that are antifungal to protect nematode-infected cadavers from fungal colonization. The concentrated, or cell-free metabolites of *X. szentirmai* exhibit high toxicity against various fungal plant pathogens and show potential as natural bio-fungicides. In the current study, we determined 1) thermo-stability, 2) dose-response, and 3) shelf-life of antifungal metabolites of *X. szentirmai* against *Monilinia fructicola* (cause of brown rot of peach and other stone fruit) and *Glomerella cingulata* (cause of anthracnose). Thermo-stability was determined by autoclaving bacterial culture broths (121 °C and 15 psi for 15 min) and measuring fungal growth on in potato dextrose agar (PDA) containing 10% of the supernatants. Autoclaving had no impact on the antifungal activity of the secondary metabolites.

Over a test period of 9 months, the activity of both extract types did not decline when stored at 4 or 20 °C. A dose-response study (10, 20, 40, 60, 80 and 100% supernatant-containing metabolite) using both phytopathogens demonstrated that a greater dose of supernatant increased antifungal activity. The antifungal-metabolite containing supernatant of *X. szentirmai* has potential as a bio-fungicide. These results demonstrate the metabolite(s) are thermo-stable, they have a long shelf-life and require no stabilizing formulation, even at room temperature.

Keywords *Xenorhabdus szentirmai* · Entomopathogenic nematodes · Biological control · Bio-fungicide · *Monilinia fructicola* · *Glomerella cingulata*

Introduction

Entomopathogenic nematodes (EPNs) in the families Steinernematidae and Heterorhabditidae are used for augmentative biological control of insects. Different species of EPNs have mutualistic and obligate associations with bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) that reside in the gut of infective juvenile (IJ) nematodes (Hazir et al. 2003; Lewis and Clarke 2012). The IJs enter the host insect through natural openings (oral cavity, anus, and spiracles) or, in some cases, through the intersegmental membranes. After penetrating the hemocoel, the IJs release their symbiotic bacteria (Shapiro-Ilan et al. 2017). Inside the host, the mutualistic bacteria colonise the insect, kill the host, and digest host tissues, allowing the IJs to feed. The mutualistic bacteria not only provide nutrition to

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the nematode hosts, but also degrade the host's tissues and protect the insect cadavers against secondary microbial invaders by producing immune-suppressive and antibiotic compounds (Dowds and Peters 2002; Shapiro-Ilan et al. 2015). Typically there are two to three generations in a cadaver, but if nutrients are scarce, there may be only one generation. Once nutrients are exhausted, the second stage nematodes develop into the IJ stage where they reinitiate the symbiosis by sequestering bacterial cells. The IJs subsequently exit the host cadaver in search of new hosts to infect (Shapiro-Ilan et al. 2017).

Before the new generation of IJs emerge, the nematode-killed host remains in the soil or on the soil surface for 7–15 days or longer, depending on the nematode species and environmental conditions. During this period, the nematode-killed host would be at risk of contamination by opportunistic bacteria and fungi. A variety of small molecules are produced by the *Xenorhabdus* and *Photorhabdus* bacteria to protect the cadaver from additional bacterial and fungal colonization (Boemare and Akhurst 2006; Brachmann et al. 2006; Hinchliffe et al. 2010).

Xenorhabdus spp. produce secondary metabolites including linear and cyclic peptides such as xenocoumacin, xenorhabdin, indoles, cabanillasin that have antimicrobial activity (Maxwell et al. 1994; Boemare and Akhurst 2006; Bode 2009; Houard et al. 2013; Lewis et al. 2015). *Photorhabdus* spp. produce the antimicrobials 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3-diol, 3,5-dihydroxy-4-isopropyl-stilbene and the β -lactam carba-penam (Hu et al. 2006; Bode 2009; Lewis et al. 2015). The anthraquinone pigments and the trans-stilbenes were determined as antibacterial, whereas trans-stilbenes and trans-cinnamic acid (TCA) were found to be antifungal (Boemare and Akhurst 2006; Bock et al. 2014; Hazir et al. 2016). Some of these antimicrobial compounds attracted substantial interest for pharmaceutical purposes (Webster et al. 2002; Bode 2009; Fang et al. 2011, 2014).

These antimicrobial compounds may also have applications in agriculture. One potential use for metabolites of *Photorhabdus* and *Xenorhabdus* is to use them as natural bio-fungicides. The concentrated or cell-free metabolites of *Photorhabdus* spp. and *Xenorhabdus* spp. have been tested for toxicity against various fungal plant pathogens, and all of those species studied demonstrated inhibitory effects on these fungi (Chen et al. 1994; Webster et al. 2002; Shapiro-Ilan et al. 2009, 2014; Fang et al. 2011, 2014; San-Blas et al. 2012; Bock et al. 2014; Hazir et al. 2016).

Xenorhabdus szentirmaii is mutualistically associated with the EPN *Steinernema rarum*, and cell-free broth (centrifuged and filtered) containing the secondary metabolites of *X. szentirmaii* showed great potential for use as natural bio-fungicides (Hazir et al. 2016). Centrifugation alone may not be ideal to generate a product of commercial interest because inevitably some live cells may still exist in the supernatant. An active compound without living cells is preferable and might either be obtained by filtration or by heat-killing the bacteria cells. The objective of this study was to assess the properties and stability of these metabolites in filtrated or heat-inactivated broth (after centrifuging), which has not previously been investigated. Clearly this is important if they are to be stored and/or used under field conditions. Thus, defining the stability characteristics of the secondary metabolites is critical and necessary for their future application. Specifically, we aimed to determine 1. thermo-stability, and 2. dose-response, and 3. shelf-life of *X. szentirmaii* antifungal metabolites.

Material and methods

Isolation of *Xenorhabdus szentirmaii* and production of the bacterial supernatant

The mutualistic bacteria of *S. rarum* (strain17c + e) were isolated from the hemolymph of 36 h nematode-exposed *Galleria mellonella* (Lepidoptera: Pyralidae) larvae according to Kaya and Stock (1997). A drop of hemolymph was streaked onto NBTA (nutrient agar with 0.004% (w/v) triphenyltetrazolium chloride and 0.025% (w/v) bromothymol blue) and the plates were incubated at 25 °C for 48 h in the dark. A typical reddish bacterial colony-type was selected from the culture (unlike other species of *Xenorhabdus*, *X. szentirmaii* cells produce red colonies on NBTA). The cell morphology and the phase of the bacteria were determined using a light microscope (1000X) and a simple catalase test (Akhurst 1980; Boemare and Akhurst 2006). The stock culture of *X. szentirmaii* was prepared as described by Hazir et al. (2016) and stored at -80 °C until required. As needed, the bacterial cells were taken from the frozen stock cultures and transferred directly to fresh NBTA. The growth of the bacteria and colony morphology were checked after 48 h incubation to ensure that there was no contamination.

To obtain culture supernatant including secondary metabolites, a loop of bacteria from the NBTA colony

was transferred to 100 ml TSBY (Tryptic Soy Broth (Difco, Detroit, MI) + 0.5% yeast extract Sigma, St. Louis, MO) in an 300 ml Erlenmeyer flask. The liquid culture was incubated for 24 h at 25 °C on a rotary shaker at 130 rpm. Subsequently, 5 ml of the bacterial suspension was transferred to 1000 ml TSBY and the culture incubated on a rotary shaker at 130 rpm for 24 h at 25 °C in the dark (Shapiro-Ilan et al. 2014; Hazir et al. 2016). To obtain a cell-free supernatant, the bacterial culture was transferred to multiple 50 ml Falcon tubes and centrifuged at 10,000 rpm for 20 min at 4 °C. The centrifuged supernatant was filtered through a 0.22 µm Millipore filter (Thermo scientific, NY) (Houard et al. 2013) and poured into the 50 ml sterile centrifuge tubes (Coming, NY) which were maintained at 4 °C for up to two weeks prior to use.

Cultures of phytopathogens

Isolates of *Monilinia fructicola* (cause of brown rot on stone fruit) and *Glomerella cingulata* (cause of anthracnose) were obtained from peach trees located at the USDA-ARS research station in Byron, GA, and from diseased pecan leaves in Albany, GA, respectively. Both cultures were grown on potato dextrose agar (PDA) at 25 °C with a 12 h photoperiod. Prior to use, cultures were grown by transferring a 5 mm agar plug to the center of a fresh plate of PDA and incubating for 7 days. Cultures were refrigerated for up to 2 weeks prior to use. In all cases, 5 mm plugs were selected from the outer, actively growing region of the colony.

Thermo-stability of metabolites

Two methods were used to exclude living cells from supernatants containing the bacterial metabolites: autoclaving and filtration, which was previously used and thus acted as a control (Hazir et al. 2016). Metabolite thermo-stability is a necessity if cells are to be killed with heat. To determine the thermo-stability of the anti-fungal compounds produced by *X. szentirmaii*, 900 ml of a bacterial supernatant in TSBY was sub-divided into 300 ml aliquots and treated as follows:

A. Autoclaved metabolite: The 300 ml was placed directly in an autoclave (Model 522LS, Getinge, Rochester, NY) in a 2000 ml flask and sterilized at 121 °C and 15 psi for 15 min.

B. Filtrated metabolite: The 300 ml aliquot was passed through a 0.22 µm Millipore filter (Thermo scientific, NY) as previously described (Hazir et al. 2016).

C. Autoclaved-and-filtrated metabolite: The final 300 ml aliquot was first autoclaved as described above and allowed to cool to room temperature (25–26 °C). The cooled sample was filtrated through a 0.22 µm Millipore filter as described above.

Autoclaved, filtrated and autoclaved-and-filtrated samples were incorporated into potato dextrose agar (PDA) at 10% (v/v) (that is, the samples were each used to make up 10% of the volume of the PDA). The sample was added to the media when it had cooled to 45–50 °C, mixed thoroughly, and poured into 9 cm Petri-plates (~15 ml/plate). A 5 mm diameter mycelia plug from a colony of *M. fructicola* or *G. cingulata* culture plates grown as described above was placed in the center of each Petri-plates containing 10% bacterial culture media using a transfer tube (Fang et al. 2011; Hazir et al. 2016). Control plates of PDA with 10% sterile TSBY (no amendment) were included. Trans-cinnamic acid (TCA) was used as a positive control due to the high level of fungal suppression previously observed with this metabolite (Bock et al. 2014; Hazir et al. 2016). The stock solution of TCA (99% purity, Sigma, St. Louis, MO) was prepared with 12.7 g dissolved in 100 ml ethanol (96%) (Hazir et al. 2016), and incorporated into PDA at 5% (v/v). The plates were incubated at 25 °C and the diameter of the colony (cm) measured after 7 days using a ruler. Two perpendicular measurements were made on each plate. Taking the mean diameter, colony area was calculated as πr^2 . The area of the 5 mm plug in the middle of the plate was not included in the measurement (i.e., the area of the plug was subtracted from the total area of the plug + fungal growth) (Fang et al. 2011; Hazir et al. 2016). For all vegetative growth assays, nine replicate plates were used for each treatment and the experiments were repeated twice (a total of three trials).

Metabolite dose-response experiments

Different doses (10, 20, 40, 60, 80 and 100%) of supernatant containing metabolite of *X. szentirmaii* were tested for inhibition of growth of *M. fructicola* and *G. cingulata*. For each dose (10, 20, 40, 60, 80 and 100%), filtrated supernatant was incorporated on a v/v basis into autoclaved PDA as described for the thermo-

stability test (for example, for 10% we incorporated 100 ml supernatant into 900 ml distilled water and added PDA (39 g according to manufacturer direction) to prepare 1000 ml PDA medium, and so on). As described above, a 5 mm diameter mycelia plug from a colony of *M. fructicola* or *G. cingulata* was transferred to the center of each dose-treatment plate (Hazir et al. 2016). Control plates were included as follows: PDA with the same quantity of sterile TSBY (no supernatant); a TCA treatment control incorporated into PDA at 5% (v/v) as described above. Plates were incubated at 25 °C and the diameter of the colony (cm) was determined after 7 days. The area of fungal growth was determined as described above. There were nine replicate plates for each treatment and the experiments were repeated once (a total of two trials).

Metabolite shelf-life experiments

Autoclaved or filtrated supernatant was added to sets of nine sterile 50 ml Falcon tubes. One set of tubes was kept at room temperature in the lab (23–24 °C), the second set was placed in the refrigerator (+4 °C) and the third set was placed in a freezer (−20 °C). All tubes were wrapped in aluminum foil. Once a month for 9 months, a sample tube was taken from each set of stored tubes and incorporated at a rate of 10% (v/v) into PDA (the lowest concentration of supernatant used in the dose-response experiment described in the previous section). Agar plugs with mycelia of *M. fructicola* were transferred from the cultures as described above. Control plates comprised PDA (no amendment). Vegetative growth of the fungus was measured after 7 days. The plug in the middle of the plate was not included in the measurement, and colony area calculated as described above. Nine replicate plates were used for each treatment and sample date; the experiment was performed once (one 9 month period).

Statistical analyses

For each experiment, analysis of variance (ANOVA) was used to explore effects of heat, filtration and any dose-response, respectively (SAS 2002). Experiments for each phytopathogen (*M. fructicola* and *G. cingulata*) were analyzed independently. Data from repeated experiments were combined and repeat experiment was treated as a block effect. If the treatment effects were significant, treatment differences were further explored using Tukey's HSD test ($\alpha = 0.05$). Fungal

growth was square-root transformed to satisfy assumptions of normality prior to analysis (Southwood 1978; Steel and Torrie 1980). For the shelf-life experiment, treatment effects over the 9-month duration were explored using linear regression analysis. The goodness-of-fit of the regression models was determined based on the F- and P-values, residuals and the coefficient of determination (R^2). Subsequently, the treatments were compared using a general linear model to explore homogeneity of intercepts and slopes. Individual hypothesis tests were performed to compare treatments.

Results

Thermo-stability of metabolites

Differences in anti-fungal activity were detected among treatments in the thermo-stability experiments for both *M. fructicola* and *G. cingulata* ($F_{4,84} = 2225.84$, $P < 0.0001$ for *M. fructicola*, and $F_{4,84} = 2324.31$, $P < 0.0001$ for *G. cingulata*). In both experiments, all metabolite treatments suppressed fungal growth relative to the non-treated control. Also, in both experiments, the positive control (TCA) caused complete fungal suppression (Figs. 1 and 2). For *M. fructicola*, the autoclaved treatment suppressed growth more compared to the autoclaved-and-filtrated treatment but was not different from the filter (only) treatment (Fig. 1). Growth of *G. cingulata* after exposure to the autoclaved metabolite treatment was reduced compared to both the filtrated and the autoclaved-and-filtrated treatments, which were not different from each other (Fig. 2).

Metabolite dose-response experiments

All metabolite treatments, regardless of dose, resulted in significant suppression of fungal growth relative to the non-treated control for both phytopathogens ($F_{7,135} = 12,447.9$, $P < 0.0001$ for *M. fructicola*, and $F_{7,135} = 2034.19$, $P < 0.0001$ for *G. cingulata*) (Figs. 3 and 4). The 10% metabolite dose caused the least suppression of *M. fructicola*, followed by the 20% dose. There was no growth detected at doses of metabolite of 40% to 100%, or the positive control (TCA) (Fig. 3). In contrast, suppression of growth of *G. cingulata* increased sequentially with each higher concentration of metabolite, and TCA caused the highest level of suppression (Fig. 4). In both experiments, there was a

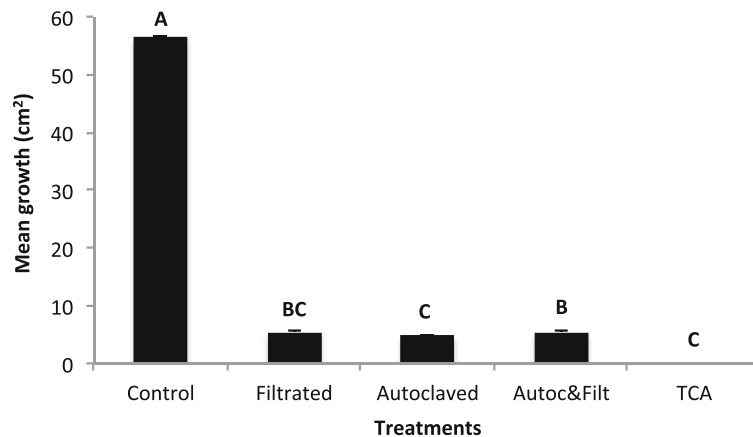


Fig. 1 Mean vegetative growth of *Monilinia fructicola* on potato dextrose agar following treatments of filtrated, autoclaved, autoclaved-and-filtrated (Autoc&Filt) supernatant of *Xenorhabdus szentirmaii* and *trans*-cinnamic-acid (TCA). Controls had no

amendment. Treatment effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$). The whiskers on the bars indicate standard errors of the means

negative linear relationship between metabolite dose and fungal growth, indicating higher concentrations caused greater inhibition: $Y = -0.78X + 3.8$ ($r^2 = 0.79$, $P < 0.0001$) for *M. fructicola*; $Y = -0.78X + 3.8$ ($r^2 = 0.96$, $P < 0.0001$) for *G. cingulata*.

Metabolite shelf-life experiments

Regression analysis demonstrated that in most cases there was a linear relationship between measured growth of *M. fructicola* and time for the control, and for the supernatant that had been stored for both the filtrated and autoclaved treatments (Table 1 and Fig. 5). Only the

models for the autoclaved treatment stored at room temperature, and the filtrated treatment stored in the refrigerator were not significant ($F = 3.4$ and 2.9 , $P = 0.07$ and 0.09 , respectively). All other treatments showed a significant linear relationship ($F = 5.5$ – 59.8 , $P < 0.0001$ – 0.02 , respectively), although the relationships were very weak to moderate ($R^2 = 0.08$ – 0.50). However, the nature of the regression was variable. The control treatment (which contained no metabolite) showed a slight decline in growth over time, as did the filtrated treatment stored in the refrigerator. Why the control showed a slight decline in growth is not known, but may indicate a slight loss in viability in the fungus that was not detected in relation to

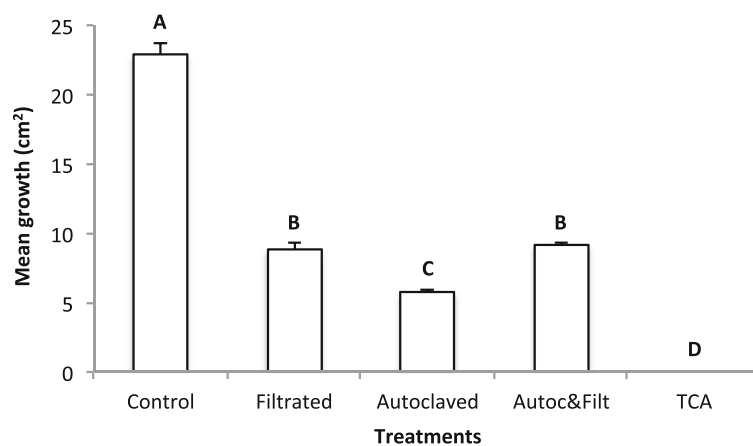
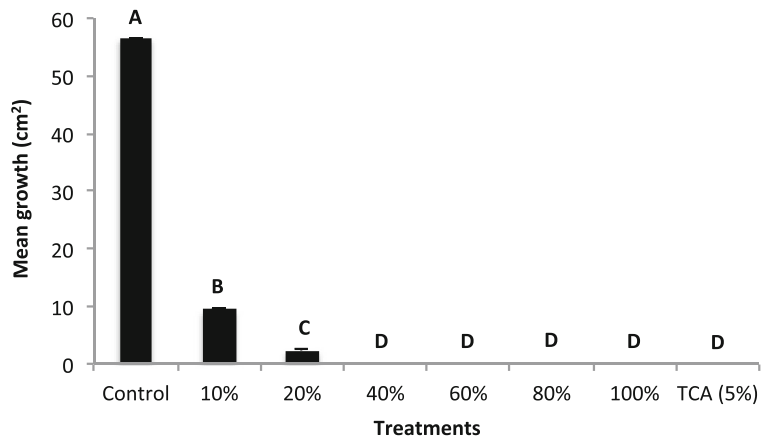


Fig. 2 Mean vegetative growth of *Glomerella cingulata* on potato dextrose agar following treatments of filtrated, autoclaved, autoclaved-and-filtrated supernatant of *Xenorhabdus szentirmaii* and *trans*-cinnamic-acid (TCA). Controls had no amendment.

Treatment effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$). The whiskers on the bars indicate standard errors of the means

Fig. 3 Mean vegetative growth of *Monilinia fructicola* on potato dextrose agar containing different rates of *Xenorhabdus szentirmaii* supernatant and 5% *trans*-cinnamic-acid (TCA). Controls had no amendment. Treatment effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$). The whiskers on the bars indicate standard errors of the means



the metabolite treatments, or some other aspect of the isolate of the fungus used affecting its growth over the 9-month duration of the study. All other treatments showed a slight positive trend to increasing growth with time (possibly indicating a very slight loss in activity), but differences in growth of *M. fructicola* were numerically exceedingly small among treatments, and all treatments clearly had much reduced growth at all sample times compared to the control.

The analysis for homogeneity of the regressions demonstrated the slopes were not parallel ($F = 16.1$, $P < 0.0001$). Individual hypothesis tests between treatments effects on growth of *M. fructicola* over time and the control demonstrated that all treatments had reduced growth ($F = 8.0$ – 78.8 , $P < 0.0001$ – 0.005 , Table 2). There were differences in growth among most treatment comparisons ($F = 6.1$ – 36.5 , $P < 0.0001$ – 0.01), except between the autoclaved treatment stored at room temperature and the filtrated treatment stored in the refrigerator ($F = 0.8$, $P = 0.4$), the autoclaved treatment stored at room temperature and the autoclaved treatment stored

in the refrigerator ($F = 0.0$, $P = 1.0$), the autoclaved treatment stored at room temperature and the autoclaved treatment stored in the freezer ($F = 0.3$, $P = 0.6$), and the autoclaved treatment stored in the refrigerator and the filtrated treatment stored in the freezer ($F = 0.8$, $P = 0.4$). But in all cases among treatments, any differences in growth of *M. fructicola* or trends were numerically small over the duration of the experiment.

Discussion

The secondary antifungal metabolites of *X. szentirmaii* showed good thermo-stability and did not decay as demonstrated by the shelf-life experiment, when material was stored under different temperatures regimes. Not unexpectedly, the efficacy of the antifungal metabolites of *X. szentirmaii* was dose-dependent. Thus standardization of concentrations will be a critical factor in deploying these materials.

Fig. 4 Mean vegetative growth of *Glomerella cingulata* on potato dextrose agar containing different rates of *Xenorhabdus szentirmaii* supernatant and 5% *trans*-cinnamic-acid (TCA). Controls had no amendment. Treatment effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$). The whiskers on the bars indicate standard errors of the means

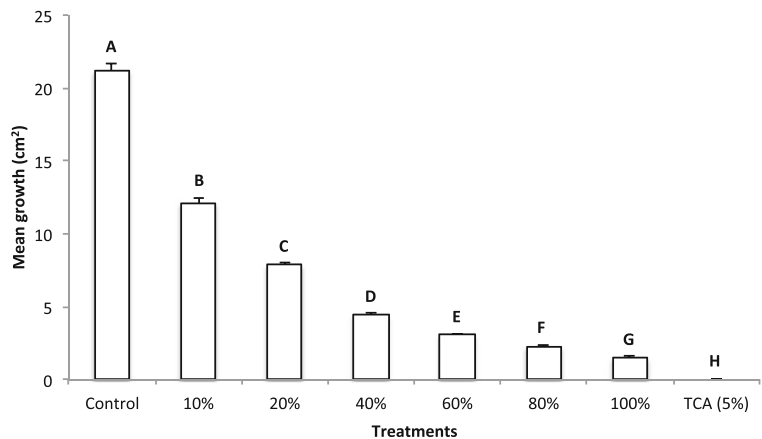


Table 1 Linear regression solutions for the relationship between storage time and vegetative growth of *Monilinia fructicola* on potato dextrose agar containing filtrated or autoclaved supernatant

of *Xenorhabdus szentirmaii* kept at different temperatures over a period of nine months. The control had no metabolite amendment. Treatment effects were assessed monthly

Treatment	β_0 (SE) ^a	β_1 (SE) ^b	F-value (<i>P</i> -value) ^c	R ^{2d}	
Control	56.6 (0.67)	−0.88 (0.11)	59.8 (<0.0001)	0.50	
Autoclave	Freezer	15.8 (0.65)	0.37 (0.11)	11.0 (0.002)	0.15
	Fridge	16.1 (0.64)	0.26 (0.11)	5.5 (0.02)	0.08
	Room temp	14.5 (0.79)	0.25 (0.14)	3.4 (0.07)	0.05
Filtrate	Freezer	10.5 (1.09)	0.44 (0.19)	5.5 (0.02)	0.08
	Fridge	15.3 (1.03)	−0.30 (0.18)	2.9 (0.09)	0.05
	Room temp	9.13 (0.97)	0.94 (0.17)	32.5 (<0.0001)	0.35

^{a-d} β_0 (SE) is the intercept and standard error of the intercept, respectively, and β_1 (SE) is the slope and standard error of the slope, respectively for the linear regression. R² is the coefficient of determination for the regression solution, and the F-value (and *P*-value) is the F-statistic for the regression analysis model, and the probability it is significant

Use of environmentally safe biological control products are preferable for a healthy and sustainable agriculture. Hence much research has been conducted to identify new and effective control agents (Solter et al. 2017). As a result of these studies many organisms and/or their secondary metabolites have been identified and their efficacy has been tested on different pathogens and pests (Glare et al. 2017). However, only a few of those that are identified have the characteristics that allow them to be developed as

commercially viable products in agribusiness. The main hurdles to developing a new biocontrol agent are 1) difficulties in mass production, 2) the high cost of production, 3) limited tolerance to environmental factors such as high and low temperatures, desiccation, humidity etc., 4) limited shelf-life, and 5) demand by end users etc.

In the process of commercialization of a good biocontrol agent, it is necessary to overcome all these obstacles. Preservation of activity during storage of compounds is

Fig. 5 The relationship between mean vegetative growth of *Monilinia fructicola* on potato dextrose agar containing filtrated (F_) or autoclaved (A_) supernatant of *Xenorhabdus szentirmaii* stored at different temperatures and storage time over a period of nine months. The control had no amendment. Treatment effects were assessed monthly. See Table 1 for regression solutions and Table 2 for a comparison of treatments

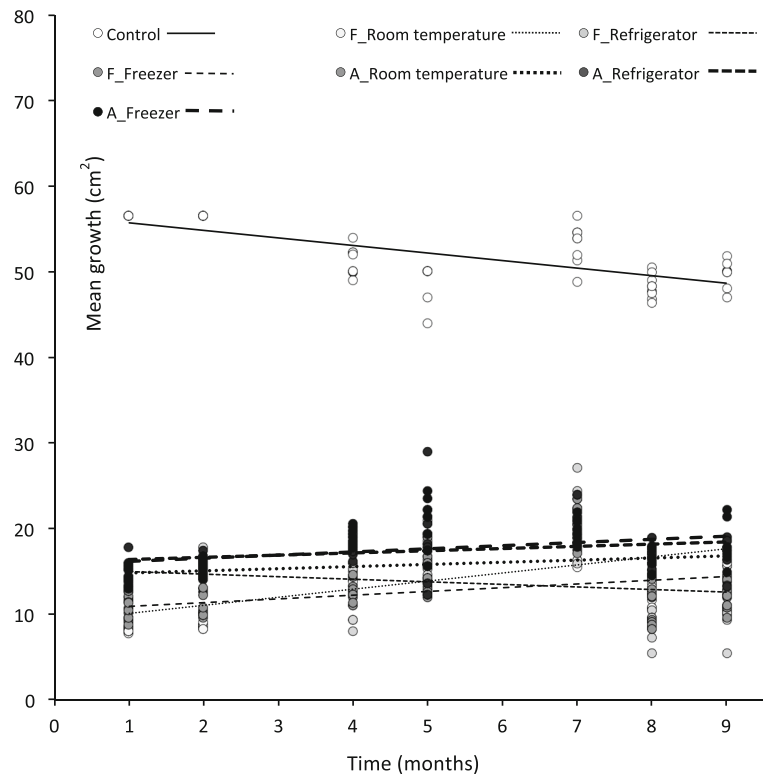


Table 2 Pairwise comparison of different treatments based on the linear relationship between storage time and vegetative growth of *Monilinia fructicola* on potato dextrose agar containing filtrated (F_) or autoclaved (A_) supernatant of *Xenorhabdus szentirmaii* kept at different temperatures over a period of nine months. The control had no metabolite amendment. Treatment effects were assessed monthly

Contrast ^a	F-value ^b	P-value
Control vs F_Room temp	78.8	<.0001
Control vs F_Fridge	8.0	0.005
Control vs F_Freezer	41.0	<.0001
Control vs A_Room temp	30.2	<.0001
Control vs A_Fridge	30.6	<.0001
Control vs A_Freezer	36.9	<.0001
F_Room temp vs F_Fridge	36.5	<.0001
F_Room temp vs F_Freezer	6.1	0.01
F_Room temp vs A_Fridge	11.2	0.0009
F_Room temp vs A_Freezer	7.9	0.005
A_Room temp vs F_Fridge	0.8	0.4
A_Room temp vs F_Freezer	7.1	0.008
A_Room temp vs A_Fridge	0.0	1.0
A_Room temp vs A_Freezer	0.3	0.6
F_Fridge vs F_Freezer	12.8	0.0004
F_Fridge vs A_Freezer	10.5	0.001
A_Fridge vs F_Freezer	0.8	0.4
A_Room temp vs F_Room temp	11.4	0.0008

^a Homogeneity of intercepts and slopes of the regression solutions were compared using a general linear model with hypothesis tests subsequently performed individually using ‘contrast’ statements in SAS (SAS 2002). Analysis of homogeneity of the regressions demonstrated the slopes were not parallel ($F = 16.1$, $P < 0.0001$)

^b F-value (and P-value) represents the F-statistic of the GLM analysis, and the probability it is significant

essential for practical use in pest and disease control and is a pre-requisite for success. In this study we demonstrated that the antifungal activity of *X. szentirmaii* supernatant was stable even when autoclaved at 121 °C and 15 psi for 15 min. This shows that the bioactive secondary metabolites are particularly thermo-stable.

Shelf-life is a crucial factor. The supernatant containing the metabolites of *X. szentirmaii* did not lose its antifungal activity over a period of 9 months, even at room temperature without any additional stabilizing formulation. No pretreatment appeared to drastically alter survival over 9 months either, and all were numerically similar when compared to the control treatment. There were some just-perceptible trends with time, but not sufficient to be of relevance to shelf life. This is in

contrast to some other biocontrol agents, especially living organisms that have a limited formulation and shelf-life which restrict their usage and market values (Lacey et al. 2015). For example, entomopathogenic nematodes cannot survive more than a month as formulated products at room temperature (Shapiro-Ilan et al. 2017). They need a specific formulation method and storage at low temperatures (5–10 °C) to prolong shelf-life. Furthermore, EPNs lose their survival and infectivity gradually depending on their decreasing lipid reserves (Selvan et al. 1993) and generally they are not used if older than six month.

Photorhabdus bacteria are known to produce the antifungal molecule TCA, which also exhibited good thermostability. In this study, there was no significant difference in the activity of autoclaved or none autoclaved TCA. To the best of our knowledge *Xenorhabdus* bacteria do not produce TCA. But they produce a number of compounds which have antifungal properties including bicomutin A, B, C, nematophin, xenocoumacins, xenorhabdins I, II, III, IV, V, xenorxide and cabanillasin (Houard et al. 2013; Lewis et al. 2015). One of these antifungal metabolites, bicomutin A, has been identified from *X. budapestensis* and *X. szentirmaii* (Boszormenyi et al. 2009). It should be determined whether bicomutin A or another unidentified bioactive antifungal compound(s) is produced by *X. szentirmaii*. If the substance can be obtained as a pure chemical, similar to the availability of TCA, a much lower quantity would be required for the suppression of various phytopathogens. Hazir et al. (2017) showed that the combined application of low doses of TCA plus supernatant of *X. szentirmaii* or TCA plus various fungicides or *X. szentirmaii* supernatant plus various fungicide exhibited synergistic effects on suppression of various phytopathogens.

Furthermore, supernatant (undiluted) of 24 h old cultures of *X. szentirmaii* sprayed directly on the leaves of six different plant species, tomatoes (*Solanum lycopersicum*), eggplants (*Solanum melongena*), peppers (*Capsicum annum*), peaches (*Prunus persica*), pecans (*Carya illinoensis*) and tobacco (*Nicotiana tabacum*) resulted in no observable phytotoxic effect (Hazir et al. 2016). It is important to demonstrate early that putative biocontrol products do not have a phytotoxic effect.

In conclusion, supernatant of *X. szentirmaii* cultures have potential to be part of a new generation of antifungal products that can be used against various phytopathogens. We have demonstrated here that they have some desirable characteristics from the viewpoint of

commercialization: they are thermo-stable, they have a long shelf-life and require no stabilizing formulation, even at room temperature. There is potential for mass production. The bacteria can be grown in large fermenters and all media can be autoclaved directly to kill living bacterial cells in the supernatant, with no harm to bioactivity. Such a method would provide straightforward and inexpensive production for commercial applications, which would be useful for competitive pricing and capability to satisfy demand.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research involving animal and human rights The research did not involve human participants and/or animals.

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