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Comparison of Powder and Liquid Forms of Antifungal Metabolites Produced by *Xenorhabdus szentirmaii*, the Symbionts of Entomopathogenic Nematodes, against Gray Mold *Botrytis cinerea*

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ABSTRACT

***Xenorhabdus* spp. bacteria are known to produce antifungal compounds that are highly efficacious against important plant pathogens such as *Botrytis cinera.* Generally, centrifuged and filtered supernatant or growth cultures are used to test effects of secondary metabolites of *Xenorhabdus bacteria* against different phytopathogens. We hypothesized that turning the bacterial supernatant into powder will increase the antifungal effects of the bioactive metabolite. Therefore, as a first step, we investigated and compared the effects of powder and liquid forms of antifungal metabolites of *X. szentirmaii* against *B. cinerea.* The powdered form of the supernatant was obtained using spray drying technology. The different doses of the powdered supernatant and their liquid equivalents were compared via in vitro assays. Our data indicated that the antifungal activity of the liquid *Xenorhabdus* supernatant was stronger than the powdered form in *in vitro* assays. We posit that during the pulverization process, some of the antifungal compounds in cell-free supernatants were either degraded or evaporated as the supernatants were subjected to high processing temperatures and pressure of the spray drying process. It is also possible that the powdered form of the supernatant did not dissolve well in PDA, so, the antifungal compound had limited contact with the tested fungal pathogen. Future studies should extract and purify the bioactive compound/s present in the supernatants of these bacteria and test their efficacy in ppm doses as powdered forms of these compounds have longer shelf-life and can be easily formulated compared to liquid supernatants.**

Keywords: Antifungal activity, Secondary metabolites.

INTRODUCTION

Certain fungi and oomycetes pose a major threat to food security worldwide. They are responsible for a wide range of debilitating diseases on important plants and crops, causing economic losses estimated to exceed $1 billion dollar (Strange and Scott, 2005). *Botrytis cinerea* (gray mold), for instance, is a necrotrophic phytopathogenic fungi that infects and damages fruits, flowers, storage organs, shoots and leaves of numerous horticultural plant species such as strawberries, grapes, tomatoes, celery, beans, lettuce, etc. This fungus produces toxins and cell-wall-degrading enzymes that damage host cells, which causes plant decay or death (Williamson *et al*., 2007). Accordingly, as with all fungal pathogens, *B. cinerea* is controlled by the repeated application of synthetic chemical fungicides, periodic removal of infected plant material, and regulation of environmental conditions such as temperature and humidity. However, chemical fungicides are highly toxic and are not easily biodegraded in nature, so, there are residues of these compounds on fruits

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and vegetables meant for consumption. More importantly, overuse of chemical fungicides can lead to pesticide resistance (Haggag and Mohamed, 2007; Williamson *et al*., 2007; Rupp *et al*., 2017). This has necessitated the search for safer eco-friendly alternatives from secondary metabolites of bacteria, fungi and plants with antifungal potential (Keller *et al*., 2005; Arif *et al*., 2009; Coleman *et al*., 2011). The bacteria in the genus *Xenorhabdus* are a rich source of novel bioactive secondary metabolites (Dreyer *et al*., 2018).

Xenorhabdus spp. are enteric bacteria found in the intestines of *Steinernema* spp. nematodes. Together, the nematode-bacteria complex infects and parasitizes soil dwelling insects. In this mutualistic relationship, *Steinernema* spp. nematodes provide *Xenorhabdus* bacteria with transportation and safety between insect hosts while *Xenorhabdus* spp. produce toxic secondary metabolites and enzymes that induce insect death, digest insect tissues, and hinder the invasion of rival microorganisms (Griffin *et al*., 2005; Boemare and Akhurst, 2006; Lacey and Georgis, 2012). The secondary metabolites produced by the bacteria have antibacterial, antifungal, antiviral, antiprotozoal etc. activities that have captured the interest of scientists searching for new and novel agricultural and pharmaceutical compounds (Bode, 2009; Dreyer *et al*., 2018). Several studies have been conducted to investigate the biological applications of the compounds present in supernatants of *Xenorhabdus* bacteria against important plant pathogens such as *B. cinera* (Fang *et al*., 2011, 2014; Adlig and Gulcu, 2019). These studies have demonstrated that the secondary metabolites present in Cell-Free Supernatants (CFS) or cell cultures of *Xenorhabdus* bacteria are highly effective against *B. cinerea.* Success in field application, however, would require the application of large quantities of bacteria supernatant. Spray drying is a widely used industrial process for the transformation of liquids to dry pharmaceutical, industrial, and food products. This technology helps to prolong shelf life of easily perishable products (Woo and Bhandari, 2013; Sosnik and Seremeta, 2015). Kulkarni *et al*. (2017) used this method to transform CSF of *Photorhabdus* spp. bacteria into powdered form. They reported an increase in the insecticidal and acaricidal effect of metabolites present in powdered forms of supernatant of *Photorhabdus luminescens* bacteria, enteric symbionts of *Heterorhabditis* spp. nematodes, as compared to liquid supernatant. We hypothesize that turning the bacterial supernatant into powdered form will increase the antifungal effects of the bioactive compounds in *Xenorhabdus szentirmaii* supernatant. Therefore, as a first step, we investigated and compared the effects of powder and liquid forms of antifungal metabolites of *X. szentirmaii* against *B. cinerea.*

MATERIALS AND METHODS

Preparation of Powder and Liquid Forms of *Xenorhabdus szentirmaii* Metabolites

Xenorhabdus szentirmaii (DSM 16338 type strain) was used in this study. Stock cultures of bacteria were prepared and stored in 20% glycerol at -80℃ (Boemare and Akhurst, 2006). *Xenorhabdus* bacteria can change spontaneously from nematodes-associated and antibiotic- producing Phase I to undesirable Phase II variants when cultured *in vitro*. Therefore, Phase 1 was used throughout this study and this was monitored by the bacteria`s characteristic colony morphology and dye absorbing ability on NBTA (Nutrient agar-bromothymol blue-tetrazolium chloride) agar and with catalase test (Akhurst, 1980; Boemare and Akhurst, 2006). An overnight culture of bacteria was prepared by inoculating a single colony from NBTA agar into 20 mL nutrient broth (Merck, Germany) and incubated the suspension at 28℃ and 250 rpm in a rotary incubator. Afterwards, 3

mL of this overnight culture was transferred to 300 mL nutrient broth and incubated at 28°C and 125 rpm for six days (Furgani *et al*., 2008). After this time, the bacterial supernatant was obtained by centrifuging cell cultures at 10℃, 3,700 rpm for 20 minutes. The supernatant was collected and autoclaved at 121℃ for 15 minutes and stored at 4℃ for use in subsequent experiments (Hazir *et al*., 2016, 2018; Donmez-Ozkan *et al*., 2019).

The spray-drying method as described in Kulkarni *et al*. (2017) was used to turn bacterial supernatant into dry powder. Briefly, 3% Gum Arabica was added to the supernatant in order to eliminate the resulting hydroscopic structure. Gum Arabica was allowed to completely dissolve in supernatant by stirring with a magnetic stirrer at room temperature. After the complete dissolution of the Gum Arabica in the supernatant, samples were dried and reduced to the nanoparticle level in a spray-dryer device (Bakon B15 mini spray dryer) located in the Traditional and Complementary Medicine Research Center of Duzce University, according to the parameters in Table 1. The working principle of the spray dryer is as follows: First, the supernatant solution was broken into small droplets using an ultrasonic assisted atomizer. Then, these fine droplets were directed into a hot air-assisted vacuum chamber where, through continuous heat and mass transfer in the chamber, moisture was extracted from the particles and the dry powder was collected in the cyclone chamber (Kulkarni *et al*., 2017).

From 300 mL of bacterial supernatant 3580 mg powder was obtained using the spray-dryer device. This powder is believed to consist of secondary metabolites in the supernatant. Consequently, the ppm value of dry powder was calculated with the formula: Solute (3,580 mg)/Solvent (0.3 L.)= 11,933 ppm.

Table 1. Defining parameters for downsizing cell free supernatant by spray dryer.

Parameters	Value
Inlet temperature	120 (Set)–119 (℃)
Outlet temperature	80 (℃)
Compression rate	9 (L min^{-1})
Aspiration rate	75 (%)
Feed-flow rate	5 (mL min^{-1})

Effects of Powder and Liquid Forms of Bacterial Supernatant on Vegetative Growth of *Botrytis cinerea*

Botrytis cinerea (isolate Dzc-1) was obtained from Dr. Nedim Altin`s phytopathogenic fungus collection at Department of Plant Protection, Faculty of Agriculture, Duzce University. The fungus cultures were maintained on Potato Dextrose Agar (PDA) (Merck, Germany) and stored at 25℃ (Adlig and Gulcu, 2019).

Efficacy of different doses of the powdered supernatant that yielded final concentrations of 120 (398 ppm), 240 (796 ppm), 480 (1,592 ppm) and 600 mg (1,988 ppm) were tested. The liquid equivalent of the powdered doses was also prepared and tested. The amounts of tested liquid supernatants incorporated into 500 mL PDA media were 10 mL (2%), 20 (4%), 40 (8%) and 50 mL (10%) and it is estimated that each have 398, 796, 1,592, and 1,988 mg of dried supernatant, respectively. All treatment groups to be tested were mixed into the prepared PDA media before autoclaving. Autoclaved media at 121^{o}C for 15 min. were poured into 9 cm Petri dishes and allowed to cool. The control group had no powder or liquid supernatant. Then, at the center of each prepared Petri dish, an 8 cm mycelium plug of *B. cinerea* was placed with a transfer tube and the plates were incubated at 25℃ for five days. After five days, the vegetative growth diameter (cm) of the colony was recorded using a ruler; two perpendicular measurements of the diameter of fungal growth area was measured and the average was recorded. This was used to calculate vegetative growth area using the formula $A= \pi r^2$ (Fang *et al*., 2011). The diameter of the inoculated mycelium was

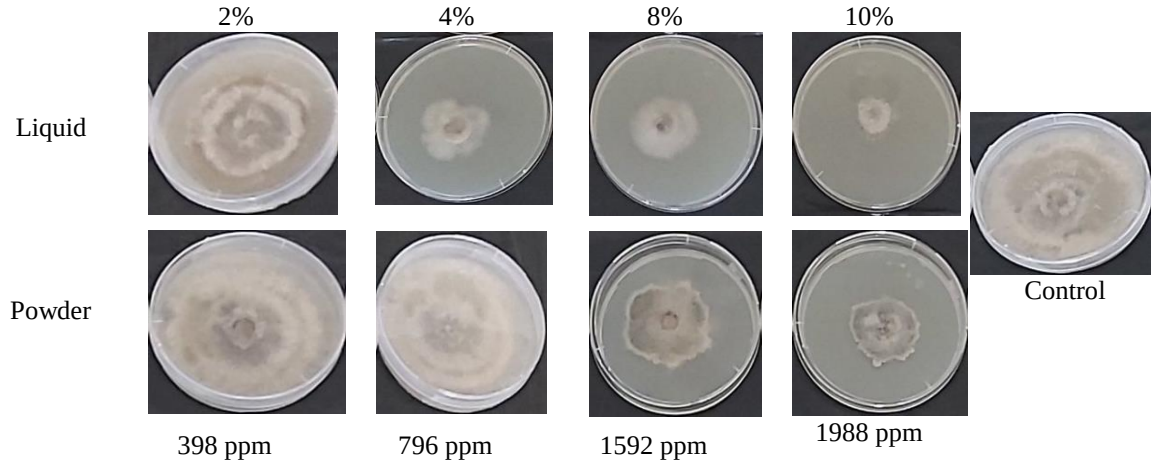

Figure 1. Comparison of antifungal effect of liquid and powder forms of *Xenorhabdus szentirmaii* supernatant against *Botrytis cinerea*.

not included in the calculations. Each treatment had 12 replicates and the experiments were conducted twice.

Statistics

Area of vegetative growth was analyzed using one-way ANOVA (SPSS 22.0). Data from repeated trials were pooled and trial was considered as a block effect. If a significant treatment effect was detected in the ANOVA (α= 0.05), a means separation was performed using Tukey's HSD. Non-transformed means of vegetative growth are presented in the results.

RESULTS AND DISCUSSION

Effects of Powder and Liquid Forms of Bacterial Supernatant on Vegetative Growth of *Botrytis cinerea*

According to the results, the liquid form of *X. szentirmaii* supernatant added to the agar media showed better suppressive effects on the vegetative growth of *B. cinerea* compared to the powdered form (Figure 1). Comparison of 398 ppm powder form with 2% liquid showed that the cell-free liquid form of bacterial supernatant exhibited significantly more antifungal activity than powder form (F= 28.28; t= 13.70; P< 0.0001). Mean growth of *B. cinerea* on PDA media amended with powder and liquid form were 54 and 32 cm^2, respectively (Figure 2). The mean vegetative growth area of fungal pathogen at 4% liquid form was 9.9 cm^2, whereas, the mean growth area was 52 cm^2 when 796 ppm powder form was incorporated into PDA. Statistically significant difference was observed between liquid and powder forms (F= 5.67; t= 55.01; P< 0.05) (Figure 2). Although the liquid form of supernatant showed numerically better inhibition on fungal growth when compared with the powder form, no significant difference was observed between 1592 ppm (19.8 cm^2) vs 8% (8.8 cm^2) (F= 0.898; t= 17.11; P> 0.05), and 1988 ppm (15 cm^2) vs 10% (7.4 cm^2) (F= 10.52; t= 5.58; P> 0.05) treatments (Figure 2). Mean vegetative growth area in the control group was 55 cm^2.

This is the first study to compare the effects of powder and liquid forms of *Xenorhabdus szentirmaii* supernatant against a fungal pathogen. The data indicated that the antifungal activity of the liquid form of *X. szentirmaii* supernatant was stronger than the powdered form in *in vitro* assays. Like the current study, Kulkarni *et al*. (2017) used the same method on *Photorhabdus* bacteria, a close relative of *Xenorhabdus* spp. and noted that the use of powdered matter from supernatants significantly increased biological activity of the supernatant against mites and

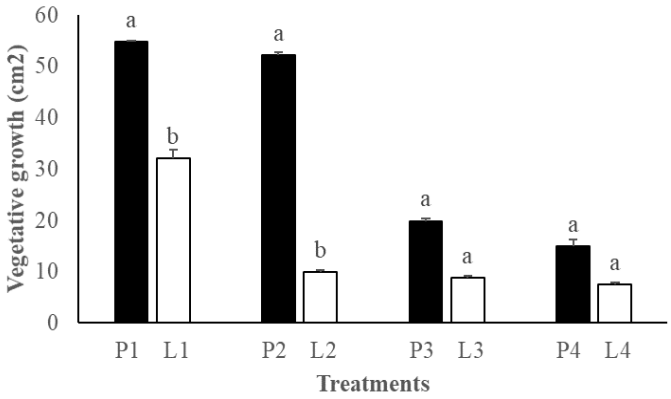

Figure 2. Colony area (cm^2) of *Botrytis cinerea* on potato dextrose agar media amended with powder and liquid form of *Xenorhabdus szentirmaii* supernatant suspensions. Different letters above bars indicate statistical significance (independent t-test, α= 0.05). Black bars indicate Powder form (P) and White bars indicate Liquid form (L) of *Xenorhabdus szentirmaii* supernatant. P1: 398, P2: 796, P3: 1592, P4: 1988 ppm; L1: 2, L2: 4, L3: 8, L4: 10%.

aphids compared to liquid supernatant obtained from growth cultures. They applied different concentrations (ranging from 0.0001 to 105 ppm) of the powdered form obtained from CFS on an inert surface such as borosilicate glass petri dish, then, transferred mites and aphids to petri dish. From the results of their experiments, Kulkarni *et al*. (2017) posited that the increase in bioactivity was due to the ease of penetration of the powder compared to secondary metabolites in the supernatant. The general approach to date has been to test the effects of secondary metabolites against different organisms using centrifuged and filtered supernatant or growth cultures of *Photorhabdus* and *Xenorhabdus* spp. (Boszormenyi *et al*. 2009; Fang *et al*., 2011, 2014; San Blas *et al*. 2012; Hazir *et al*., 2016; Adlig and Gulcu, 2019). For instance, Adlig and Gulcu (2019) examined the effects of different concentrations (2%, 5%, 7%, and 10%) of supernatants of *Xenorhabdus* bacteria on the vegetative growth area of *B. cinerea*. They observed that 10% bacterial supernatant had the highest antifungal activity. Fang *et al*. (2011) reported that the supernatant of *X. bovienii* inhibited the vegetative growth of *B. cinerea* and *Phytophthora capsici* by more than 98%. In another study, they observed that *X. nematophila* suppressed more than 90% of the vegetative growth of *B. cinerea* and *P. capsica* (Fang *et al*., 2014). Our study showed that the *X. szentirmaii* exerted a strong antifungal effect on *B. cinerea*. This antifungal effect increased with the applied concentration (2, 4, 8, and 10%) tested, and the highest antifungal activity was at 10%. After examining the antifungal effects of *X. bovienii*, *X. nematophila*, *X. cabanillasii*, *X. szentirmaii*, *P. temperata*, *P. luminescens* (VS) and *P. luminescens* (K22) against different phytopathogens, Hazir *et al*. (2016) stated that, in general, supernatants of *Xenorhabdus* bacteria were more efficacious against fungal phytopathogens than supernatants of *Photorhabdus* bacteria. In addition, Hazir *et al*. (2016) included an antifungal compound, Trans-Cinnamic Acid (TCA), which was identified in supernatants of *Photorhabdus* bacteria (Bock *et al*. 2014). Hazir *et al*. (2016) observed that the powdered form of TCA is highly efficacious against plant fungal phytopathogens. This shows that pure and powdered forms have a higher bioactivity at lower doses compared with bacterial supernatants, which has different secondary metabolites. Data obtained in our study, on the contrary, indicated that the antifungal activity of the powdered *Xenorhabdus* supernatant was weaker than the liquid form. Chacón-Orozco *et al*. (2020) recently assessed the antifungal effects of Cell-Free Supernatants (CFS) of 16 strains of *Xenorhabdus* and *Photorhabdus* bacteria on mycelium growth of *Sclerotinia*

sclerotiorum. Amongst the strains assayed, *X. szentrimaii* had the highest fungicidal effect, so, they further asssessed the effects of Volatile Organic Compounds (VOCs) of this bacteria on the fungus mycelium and sclerotium inhibition. They reported that *X. szentrimaii* generates an unidentified VOC that inhibits *S. sclerotiorum* growth or its consequent production of sclerotia. We point that during the pulverisation process, the antifungal compounds or volatiles in CFS were either degraded or lost their antifungal activities as the supernatants were subjected to high processing temperatures and pressure for 180 minutes cycling period. Also, it has been determined that the operating conditions such as inlet air temperature, outlet air temperature and atomization pressure of the spray dryer are important factors in the pulverisation process that can affect the chemical, structural, functional and physical properties of nanopowders (Koca, *et al*., 2015; Correa-Filho *et al*., 2019; Sun *et al*., 2019). It is also possible that the powdered form of the supernatant did not dissolve well in PDA, so, the antifungal compound had limited contact with the tested fungal pathogen. Upcoming studies should consider alternative methods such as freeze drying, or optimize operating conditions used in the spray drying process to obtain powdered forms of supernatants of *Photorhabdus* and *Xenorhabdus* bacteria.

CONCLUSIONS

As conclusion, the liquid form of *X. szentirmaii* supernatant showed better suppressive effects on the vegetative growth of *B. cinerea* compared to the powdered form. This is the first study to compare the effects of powder and liquid forms of *X.* szentirmaii. Moreover, future studies should extract and purify the bioactive compound/s present in the supernatants of these bacteria and to test their efficacy in ppm doses as powdered forms of these compounds have longer shelf-life and can be easily formulated compared to liquid supernatants.

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مقایسه شکل‌های پودری و مایع متابولیت های ضد قارچ تولید شده توسط *Xenorhabdus szentirmaii*، همزیست‌های نماتدهای حشره‌پاتوژن، برعلیه قارچ خاکستری Botrytis cinerea

ب. گلکو

چکیده

گونه Xenorhabdus باکتری ها به تولید ترکیبات ضد قارچی معروف هستند که در برابر پاتوژن های مهم گیاهی مانندBotrytis cinera بسیار موثراند. به طورکلی، مواد سانتریفیوژ و فیلتر شده رویی (supernatant) یا کشت های رشد برای آزمایش اثرات متابولیت های ثانویه Xenorhabdus bacteria بر علیه پاتوژن های گیاهی مختلف استفاده می شود. در این پژوهش، چنین فرض کردیم که تبدیل مایع رویی باکتری به پودر، اثرات ضد قارچی متابولیت زیست فعال را افزایش می دهد. بنا بر این، در گام نخست، ما اثرات پودر و شکل‌مایع متابولیت‌های ضد قارچی X. szentirmaii را در برابر B. cinerea بررسی و مقایسه کردیم. شکل پودری مایع رویی با استفاده از فناوری خشک کردن اسپری (spray drying) به دست آمد. دوزهای مختلف مایع رویی پودر شده و معادل های مایع آنها با سنجش های آزمایشگاهی (درون شیشه‌ای) مقایسه شد. داده های ما حاکی از آن بود که در سنجش آزمایشگاهی، فعالیت ضد قارچی مایع رویی Xenorhabdus قوی تر از حالت پودری بود. فرض ما بر این است که در طول فرآیند پودر کردن، برخی از ترکیبات ضد قارچی موجود در موا رویی بدون سلول یا تجزیه شده یا تبخیر شده‌اند، زیرا مواد رویی در معرض دماها و فشار بالا در فرآیند خشک‌کردن با اسپری قرار می‌گیرند. همچنین ممکن است شکل پودری مواد رویی به خوبی در PDAحل نشده و بنا بر این مواد ضد قارچ تماس محدودی با پاتوژن های قارچی آزمون شده داشت. از آنجایی که فرم های پودری این ترکیبات ماندگاری بیشتری دارند و در مقایسه با مایع رویی به راحتی فرموله می شوند، در پژوهش های آینده، باید ترکیب(های) زیست فعال موجود در مواد رویی این باکتری ها را استخراج و خالص کرده و کارایی آنها را در دوزهای پی‌پی‌ام آزمایش کرد.