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Supplementing biocontrol efficacy of *Bacillus velezensis* against *Glomerella cingulata*

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ABSTRACT

This research was initiated with the objective of supplementing the biocontrol activities of metabolites produced by *Bacillus velezensis* AR1 isolate via the addition of Neem oil (N), sulfur solution (SS) and mixture of N and SS (NS). The AR1 supernatant could inhibit the growth of *Glomerella cingulata* by 50% at 50% concentration rate. Application of the 50% supernatant concentration with NS_{500} and NS_{300} dilution rates have optimized the relative inhibition capacity of the supernatant by 64.3 and 63.5% respectively. The supernatant and its mixture with NS_{300} successfully suppressed lesion development on the peach fruits. Therefore, the use of AR1 and its co-application with the NS_{300} have promising potentials to control peach fruit diseases caused by *G. cingulata*.

1. Introduction

A search for non-toxic and organic products has become a worldwide concern, which demands target-oriented solutions in agricultural research and production systems. Fungal plant pathogens are known to be the most important microbial agents causing serious diseases resulting in economic losses in agriculture annually [1]. *Glomerella cingulata* (Stoneman), the sexual stage (teleomorph) while the more commonly referred to an asexual stage (anamorph) is called *Colletotrichum gloeosporioides*, is a significant problem worldwide, causing anthracnose and fruit rotting diseases on hundreds of economically important hosts [2]. For instance, reports of [33] on peach fruits; [14,24] on strawberries; [4,29] and [32] on mangoes and others indicated the severity of the pathogen on various plants. Suppressing this pathogen has been performed by intensive use of fungicides; namely, folpet, copper hydroxide and mancozeb [12]; Prochloraz, benomyl, chlorothalonil, and captafol used for protection of leaves [10]. However, the regular use of fungicides can potentially pose a risk to the environment, particularly if residues persist in the soil or migrate offsite and enter waterways [19,38]. This, in turn, can have adverse effects on soil organisms and potentially pose a risk to the long-term fertility of the soil [17,18]. To reduce these problems, the use of biocontrol agents has been considered as a realizable alternative to manage plant diseases [11]. Studies indicated that *Bacillus* species such as *B. subtilis, B. cereus, B. amyloliquefaciens* [9,37]; *B. velezensis* [8,28]; and [13] and other *Bacillus species* [20,36] produce various secondary metabolites that effectively suppress phytopathogenic fungi. As a result, research on biological control of plant pathogens has received much attention in recent years, as a means of increasing crop production by avoiding many problems related to chemical control. However, biocontrol agents have been blamed for their non-selectiveness and incapability to suppress phytopathogens at a satisfactory level due to several biotic and abiotic factors [5]. Thus, we found that the use of compatible supplementary sources with biocontrol agents can improve the extent of phytopathogen suppression to a reliable level. So far, the antifungal efficacy of *Azadirachta indica* (Neem) leaf and seed oil has been reported on various pathogenic fungi, namely, *G. cingulata* [21]; *Aspergillus flavus, Alternaria solani* and *Cladosporium sp*. [34]; *Fusarium sp. Rhizopus sp. Curvularia sp*. and *Aspergillus sp* [31]. Similarly, sulfur has been used in colloidal and solution forms against *Phacidium infestans Karst* (snow blight) and *Lophodermium pinastri Chev* [26]; micro and nanoparticles of sulfur against *Alternaria alternata, Àspergillus niger, Candida albicans, Fusarium graminearum, Penicillium notatum* [27]; *Fusarium solani* and *Fusarium* wilt disease fungi [16]. However, as to our knowledge, the application of a neem oil and sulfur solution mixed with bacteria culture filtrate has not been reported. Therefore, in our in-vitro study, we investigated the effect of *Bacillus velezensis* AR1 strain; and content modified neem oil (N) and sulfur solution (SS) as the sources of supplementary material against the growth of *Glomerella cingulata*. In addition, the role of the combined treatment in preventing peach fruit lesion formation and decay due to infection by the test pathogen was investigated.

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2. Material and methods

2.1. Bacteria and media

Composite soil samples were collected from beach area soils of Muan district, Republic of Korea, and brought to the Chonnam National University, soil microbiology laboratory. Samples were sieved to separate rock debris and plant tissues as well as faunal biomass and used for bacterial single colony isolation. Tenfold diluted aliquots were spread on a TSA media and incubated at 28° C for 5–7 days. Bacterial colonies were identified and tested for their biocontrol activities. A pure culture of the bacterial strain isolated as *B. velezensis* AR1 was maintained in 50% glycerol at −80° C.

2.2. Dual culture activities of the treatments

The pathogenic fungi, *Glomerella cingulata* (KACC 40299), obtained from Korea Agriculture Culture Collection Seodundong, Suwon, Gyunggi Province, Korea) was used in the in-vitro antagonism assays. AR1 was inoculated by line streaking, while N, SS, and NS were applied by paper disc loading methods on PDA medium. Briefly, 1–3 µl of each N, SS and NS stock solutions were dissolved in 1 ml deionized distilled water to make 1000, 500 and 300 dilution rates, respectively; and then 25 µl of the diluted samples were loaded on the paper disc for the antifungal assay. The test fungi plugs were placed 4 and 3 cm apart from the AR1 treatment and the other treatments, respectively, and all cultures were incubated at 26 °C. Growth of pathogenic fungi was evaluated after 7 days of inoculation using the following formula:

$[(R - r)/R \times 100]$ where r is the distance of fungal growth from the point of inoculation to the colony margin and R is the distance between the points of inoculation of the fungal colony and the control treatments. All the experiments were repeated three times (unless mentioned otherwise) and data presented as the mean.

2.3. Chitinase and β-1,3-Glucanase activity assay

Samples were prepared from bacteria culture filtrate for the assay. Briefly, 50 µl of each sample was centrifuged at 12,000 rpm for 10 min and the supernatants were used for the assay. Chitinase activity was then assayed according to the method of [23]; using colloidal chitin as substrate. β-1,3-Glucanase activity (EC 3.2.1.6) was assayed using laminarin (Sigma, St Louis, MO) as a substrate using the method of [30] and [35]. The chitinase and β-1,3-Glucanase activities were measured using a spectrophotometer at wavelengths of 420 and 550 nm, respectively, and calculated from their respective standard curves.

2.4. Antifungal assay of AR1 cell-free culture filtrate

AR1 was grown in black and white (BW) broth at 30° C for 7 days with shaking at 170 rpm. Briefly, the black media constitutes 5 g $MgSO_4$, 125 g chitin powder, 6.25 g gelatin, 665 ml power chitin (water fertilizer containing micronutrients), 10 g $CaCl_2$, 20 g KCl and 250 ml distilled water. Then 4 ml of the black media was mixed with 2.2 g urea, 2.5 g KH_2PO_4, 0.3 g K_2SO_4, 5 g sucrose and 0.2 g yeast extract to make 1-L (L) BW Media. In our first assay, the culture broth was centrifuged at 6000 rpm at 4° C for 20 min and the supernatant collected was then filtered serially through Whatman filter paper No.6 followed by syringe filtering (using sterile 0.2 µm). However, we found that this process is laborious, time-consuming and even many cells were seen growing on agar plates. Thus, to avoid these problems, wool chlon was dissolved to 1000 ppm working solution and checked for the rate (0, 15, 25, 35, 45, 50, 60 and 70 ml of the working solution per liter of culture filtrate) at which cells could be removed. Various bacteria have different resistance levels, but the AR1 strain could resist up to 60 ml of working solution per liter then disappeared at 70 ml. The wool chlon solution was added to the culture filtrate and kept for 24 h before application. The filtrate was mixed with autoclaved PDA with final concentrations of 10%, 30%, and 50%. A mycelium plug of the fungi was placed in the center of the PDA plate and incubated at 25° C in the dark. Mycelia growth was measured after 7 days using the same formula mentioned above. Each assay was carried out in triplicate.

2.5. Preparation and application method of neem oil

The treatment neem oil, employed in this manuscript, was prepared from the composition of the real neem oil, rotenone, tween-80 and ethanol. Briefly, we used 100 ml neem oil; 100 ml rotenone; 600 ml tween-80; and 200 ml ethanol to prepare 1 L stock solution. Then from the stock solution, 0.1, 0.2 and 0.3 ml were taken and used to make 100 ml media diluted at 1000 (N_{1000}), 500 (N_{500}) and 300 (N_{300}) rates, respectively, to test their antifungal activities. Potato-dextrose agar (PDA, Difco) was autoclaved at 121° C for 20 min and when the temperature cooled to 45–50° C neem oil was added at the rates mentioned above. The agar was then poured into Petri dishes and kept for 48 h. to fully dry. A mycelium plug of the fungi was then placed at the center of the PDA plate and incubated at 25° C in the dark. The growth inhibition activities of the treatment were calculated after 7 days using the method mentioned above. Each assay was carried out in triplicate.

2.6. Sulfur solution preparation method and its antagonistic activity

In the process of sulfur solution (SS) preparation all materials used were of mineral origin. In general, sulfur powder (99.9% pure yellow sulfur made of brimstone powder), rock (phyllite) powder, ochre (loess), NaCl and NaOH were used. Briefly, we first mixed 25 kg of sulfur powder with 50 L of first stage distilled water in a clean container followed by addition of 20 kg NaOH. At this stage foaming and heating can occur so gradual and continuous string is needed. Then 0.5 kg ochre followed by 0.5 kg rock powder were slowly added and continuously stirred. Finally, 1.5 kg NaCl was added and stirred smoothly. At the end, 32 L of second stage distilled water was added and thoroughly stirred until all the components were well dissolved. The well dissolved upper part of the solution was then collected and used for the intended objectives. Finally, antifungal activity was assayed using the same amount, procedure and dilution rates of SS_{1000}, SS_{500} and SS_{300} with that of neem oil mentioned above. The growth inhibition activities of the treatment were calculated after 7 days using the method mentioned above. Each assay was carried out in triplicate.

2.7. Effect of mixed neem oil and sulfur solution on G. cingulata

The neem oil (obtained from Green focus co.Ltd. Gyeongi province, Republic of Korea) and sulfur solution prepared as mentioned earlier were equally mixed after checking their separate assay results, to evaluate whether they inhibit the growth of the pathogenic fungi when mixed or not. As such, using the same dilution rates of 1000 (NS_{1000}), 500 (NS_{500}) and 300 (NS_{300}) and amount indicated above, we added half of neem oil and half of sulfur solution into the PDA. Then the same inoculation and calculation methods mentioned above were used. The growth inhibition activities of the treatment were calculated after 7 days using the method mentioned above. Each assay was carried out in triplicate.

2.8. In-vitro assay on peach fruit

From the treatments and their combinations mentioned above the best performing, 50% AR1 cell-free culture filtrate concentration and mixed neem oil and sulfur solution were selected for this experiment. The *G. cingulata*, obtained from the Korean agricultural culture collection (KACC), was grown for 7 days on potato-dextrose agar (PDA, Difco) plates. The cultures were flooded with sterilized distilled water and the resulting spore suspensions (10^6 ml^{-1}) were used to inoculate peach