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Isolation of Antagonistic Rhizosphere Bacteria Toward Phytophthora capsici Induce Phytophthora Blight in Pepper (Piper nigrum)

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Vietnam has been the largest exporter of pepper globally in recent years. However, the quick death disease caused by *Phytophthora capsici* is spearing rapidly, causing notable damage to many concentrated cultivation areas. The regular application of chemical pesticides to combat the diseases at pepper farms has increased the certain environmental problems. To reduce pesticide usage, biological methods for controlling *P. capsici* have been implied. In this study, rhizosphere bacterial strains were isolated from pepper plantations, and their chitinolytic and phytophthora antagonistic activities were evaluated. The chitinolytic activity was conducted on an agar medium supplemented with chitin (CM), and the antagonistic activity was done using a dual culture inhibition assay. As the result, 46 strains (signed NH1 - NH46) were isolated based on morphological distinctions in the CM medium. Out of the 46 isolated strains, 8 strains including NH7, NH10, NH11, NH27, NH31, NH32, NH33, and NH46, which accounted for 17 % of the isolates, showed high chitinolytic activity. In the dual culture assay, the strain NH7 showed the highest effectiveness that inhibiting the *P. capsici* mycelial growth with an antagonistic distance of 20.33 mm, followed by three strains NH27, NH32, and NH46 antagonistic distances of 13.67 – 15.33 mm. These strains were further identified as *E. cloacae, S. flaveus*, *K. pneumoniae*, and *B. amyloliquefaciens* through 16S rRNA sequencing. A phylogenetic tree showed a closed connection between the antagonistic isolated strains and antagonistic bacteria reported previously.

1. Introduction

Black pepper (*Piper nigrum* L.) has been deemed the “king of spices”. Its unique spicy flavor makes some dishes become tastier, and easily reserved as well. This kind of spice has various good effects on our health. Black pepper has become the main export agricultural product of Vietnam in recent year. Pepper began to be introduced to our country and became popular during the postwar period in the 17th century (Ravindran, 2020). Vietnam is currently the world leader in pepper production. The central production areas are from Quang Tri to the Central Highlands, Southeast, and Phu Quoc Island (Thuy et al., 2012). Vietnam pepper production was always high and was expected to increase next year, accounting for 47 % of global production. New plantation area reached 16 % annually since 2011 and relatively high in the last 3 y (18 % - 28 %). The total pepper area of Vietnam was 140,000 ha, and production reached 287,000 t in 2019 (Thuy et al., 2012).
Black pepper cultivation is not always going smoothly. Diseases on pepper have arisen and caused significant harm to many centralized production areas of Vietnam. Several pathogens comprising viruses, bacteria, fungi, and nematodes cause disorders in pepper’s normal metabolic pathway (Kang et al., 2022). Phytophthora blight caused by *Phytophthora capsici* is the most alarming disease that causes severe yield losses of about 2 % in pepper cultivation areas and 15 – 20 % production per year (Nguyen et al., 2020). The disease affects plants at any growth stage and the damping-off syndrome kills seedlings within 5 d of infection.

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The pathogen also causes crown, leaf, and fruit blight, wilting of the whole plant and dark purplish discoloration of the stem (Babadoost et al., 2015). Blight control is dependent on cultural practices, utilization of resistant varieties, pesticide application, and combined with water management. These methods might not deliver significant results, as well as damage the environment even the food chain, and human survival (Hoang et al., 2021). Biological control has become the better method due to its eco-friendly feature and has become an important strategy to manage soil-borne diseases and reduce the application of chemical pathogens suppression methods (Hoang and My, 2021). Many microorganisms have been reported to suppress the growth of *P.* capsici including *Streptomyces* spp., *Bacillus* spp., *Trichoderma* sp., *Paenibacillus* spp., *Aspergillus* sp. The mechanisms of control include the production of antibiotics and lytic enzymes, physical or chemical interference, competition, induction of host resistance, hyperparasitism, and predation (Ozyilmaz, 2020). In recent years, rhizosphere bacteria have gained interest as biocontrol agents because of their abilities to colonize the rhizosphere of plants and their beneficial effects on plant growth (Khatun et al., 2018). In this study, bacterial strains from the pepper rhizosphere soil with a strong antagonistic potential to *P. capsici* were isolated, identified, and archived for further studies.

2. Materials and method

2.1 Materials

Soil samples were collected from the rhizosphere of healthy plants at pepper plantations in the main production areas in Dong Nai province, Vietnam. The plantations had not been sprayed with pesticides less than 30 d. *Phytophthora capsici* strain was received from the Department of plant Biotechnology, Ho Chi Minh City of Biotechnology Center. *Bacillus subtilis* strain was received from the Department of Biotechnology, HCMUT-VNU.

2.2 Chitin solution 1 % w/v preparation

The chitin powder was prepared from the shrimp shell using the method of base and acid treatment. The shrimp shell after washing several times to eliminate impurities was treated with NaOH 4 % at 70 – 75 °C for 4 h. The shell was treated with HCl 8 % at room temperature for 16 h and followed by water washing and centrifugation. The treated shrimp shell was ground into a powder that served as the chitin source. Gradually added 1 g chitin powder to 20 mL of concentrated HCl and kept at 4 °C overnight and stir vigorously. The mix was added to 200 mL of ice-cold ethanol 95 %, left overnight at 4 °C, and stirred rapidly. Precipitate was received using centrifugation at 4,000 rpm/min at 4 °C for 20 min, and then rinsed with sterile water until chitin colloidal evolved neutral (pH 7.0). The last volume was adjusted to 100 mL with 50 mM phosphate buffer (pH 6.5) (Dai et al., 2011).

2.3 Isolation of bacterial strains on chitin medium

A volume of 100 µL of ten-fold serial dilutions (10^{-6} – 10^{-10}) of each soil samples with sterile water was spread on Chitin Media (CM). CM components included chitin 0.5 %, yeast extract 0.01 %, KH_2PO_4 0.1 %, Na_2HPO_4 0.2 %, NaCl 0.05 %, $MgSO_4.7H_2O$ 0.05 %, NH_4Cl 0.1 %, KNO_3 0.05 %, $CaCl_2.2H_20$ 0.05 %, agar 2 %, and adjust pH to 7). After 5 d incubation at 30 °C, the strains that appeared halo zones around colonies were selected and checked for further experiments (Sophearenth et al., 2013).

2.4 Evaluation of the chitinolytic activity of bacterial isolates

The diffusion method was used to evaluate the chitinolytic activities of isolates following by Dinh et al. (2018). Created a 9 mm diameter well in the center of a CM media Petri dish. Bacterial density was acquired by measuring the OD value (at OD 600 nm) in a spectrophotometer and controlling this value of all specimens to 1. Applied 100 µL of cultured broth into the well, incubated at 37 °C, 3 replicates for each isolate. *B. subtilis* was used as a positive control and fresh LB-broth media as a negative control. Stained the agar dish with Lugol, observed, and measured the halo diameter on the dish after 120 h of incubation. The diameter of the halo was used to evaluate the chitinolytic activity of isolates.

2.5 *In vitro* evaluation of *Phytophthora capsici* antagonistic ability

Evaluating *P. capsici* antagonistic activity on Chitin Potato Dextrose Agar Media (CP) was taken from 4 d old PDA plate of *P. capsici* and placed at the center of the new CP plate. After 24 h of incubation, created 3 wells on the plate, 30 mm from the center on this CP plate. The bacterial culture LB broth at OD value = 1 was filled into the well (100 µL), 3 replicates for each strain. A similar agar disc of 4 d old cultured with *P. capsici* was placed at a fresh CP center and was used as a negative control and *B. subtilis* had been used as a positive control (Chung et al., 2008).

All the Petri plates were incubated at room temperature. After 5 d of incubation, antagonistic activity was acquired by measuring the radius (R) of the *P. capsici* colony in the direction of the antagonist *colony* (R2) and the radius of the *P. capsici* colony in the control plate (R1). The antagonistic activity was calculated as in Eq(1) (Zhao et al., 2022).

$$R = R1 - R2 \quad (1)$$

2.6 Identification of bacterial strains, phylogenetic tree, and data processing

Identification based on 16S rRNA sequence and BLAST program for bacterial strains which showed the chitin degradation and suppression of *P. capsici*. The nucleotide sequences of 16S rRNA were compared with the 16S rRNA genes which published on NCBI using the BLAST. Phylogenetic tree based on 16S rRNA sequences with MEGA X software using UPMA method. All the data in study were processed using the one-way analysis of variance method (ANOVA) and post-hoc analysis by DUNCAN test.

3. Results

3.1 Isolation of bacterial strains on chitin medium

There are 46 obtained isolates (NH1 – NH46) growing and creating halo zones around their colonies on the CM medium. They were distinguished by morphological characters. Most of the obtained isolates showed blight white, small, protruding, and smooth colonies. The colonies of three strains NH10, NH27, and NH46 were slightly different from the others. There was a morphology transformation from a milk-white, small and protruding colony to a colony spreading over an agar surface with more bacterial biomass. NH27 was observed at the time later in the culturing period with the small, wrinkled, rough, and ivory-colored colony. NH46 also had its colony spreading over the plate surface with the wrinkled biomass.

3.2 Evaluation of chitinolytic activity of bacterial isolates

The chitin break-down ability of the 46 isolated strains was evaluated on the CM medium. Out of 46 strains, 8 strains (17 %) showed a decent ability to break down chitin (Table 1). The strains had chitin degradation diameters from 24 mm to 50 mm, NH46 strain showed the maximum chitin degradation activity with the largest halo zone diameter (50 mm) which was significantly different from other strains ($P \leq 0.05$). NH27 strain was the smallest of the eight chosen strains (24 mm). The positive control showed strong chitinase activity (70 mm) and the negative control did not appear in any halo zone on the plate.

Table 1: The chitinolytic activity of bacterial isolates

Isolates	Mean Diameter* (mm)
NH7	26^{b}
NH10	25^{bc}
NH11	26^{bc}
NH27	24^{c}
NH31	31^{c}
NH32	25^{bc}
NH33	34^{d}
NH46	50^{e}
Bacillus subtilis (+)	70^{f}
(-)	0^{a}

3.3 Evaluation of Phytophthora *capsici* antagonistic ability *in vitro*

The antagonistic ability to *P. capsici* of eight isolates (NH7, NH10, NH11, NH27, NH31, NH32, NH33, and NH46) showing high chitinolytic degradation was assessed using a dual culture technique on CP medium. Four bacterial isolates including NH7, NH27, NH32, and NH46 inhibited the mycelial growth of pathogens on CP accounting for 50 % of total chitinolytic bacteria (Table 2 and Figure 1). The NH7 strain demonstrated the

best capacity for the growth inhibition of hyphae with a resistance distance of 20.33 mm, which was statistically different from the other 3 strains. strains NH27, NH32, and NH46 had no difference in their ability to inhibit the growth of mycelium with resistance distances of 13.67 mm and 15.33 mm.

Table 2: Phytophthora capsici antagonistic activity of bacterial isolates

Strains	Antagonistic activity R (mm)			Mean antagonistic activity $\bar{R}$ (mm)
	1st	2nd	3rd	
NH7	19	21	21	20.33[a]
NH27	15	17	14	15.33[b]
NH32	14	12	15	13.67[b]
NH46	15	14	12	13.67[b]

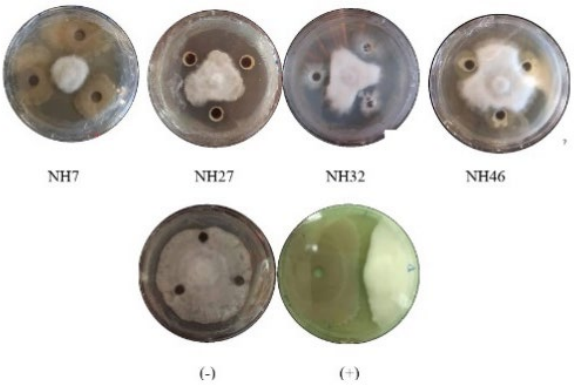

Figure 1: Soil bacterial isolation on CM media

3.4 Identification of bacterial strain

Four bacterial strains (NH7, NH27, NH32, NH46) not only had high chitinolytic activity but also inhibited the growth of *P. capsici.* Gram staining results showed that strain NH7 and NH32 belonged to the group of gram-negative *cocci*, strain NH27 with filaments belonged to the gram-positive group, and strain NH46 belonged to the group of gram-positive *Bacilli*.

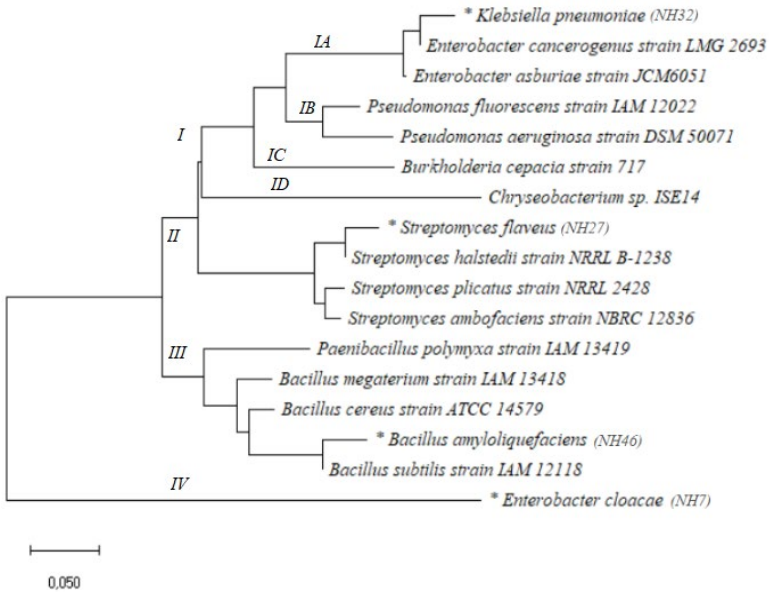

Figure 2: Phylogenetic tree based on isolated strains 16S rRNA gene sequence

Identification results based on 16S rRNA sequences showed that strains NH7, NH27, NH32, and NH46 were identified as *Enterobacter cloacae*, *Streptomyces flaveus, Klebsiella pneumoniae, Bacillus amyloliquefaciens*. The phylogenetic tree in Figure 2 showed the four isolates and the reference strains divided into 4 different groups. The strain NH32 belonged to group IA had a close genetic relationship with strains of *Enterobacter* sp., strain NH27 belonged to group II to other *Streptomyces* strains, strain NH46 belonged to group III had high genetic similarity with strain *B. subtilis*, and strain NH7 belonged to strain NH7 belonged to group 4 with low genetic similarity with the remaining strains.

4. Discussion

The mechanisms for biological control of diseases could include competition for infection sites, nutrients, and spaces, parasitism on pathogens, destruction of fungal pathogens by the action of lytic enzymes (chitinase and β-1,3-glucanase), uncharacterized antifungal factors and many other metabolites produced by rhizobacteria (Rosier et al., 2018). The biocontrol activities of antagonistic bacteria could be related to their chitinase production (Zhang et al., 2016). This was a reason to explain chitin amendment of soil to stimulate the growth of chitinolytic microorganisms, increase the biocontrol efficacy and stimulate the expression of plant defense protein (Shao et al, 2018). Volatile factors formed in chitin-amended have been demonstrated to suppress chlamydospore formation such as NH_3 release (Prigigallo et al., 2021). For all reasons above, isolation of antagonistic bacteria was conducted with the selective media which contains chitin as the main carbon source.

As for the research of Toh et al. (2016), isolated strain *E. cloacae* had been reported with the highest antagonism against *P. capsici* mycelia with the percentage of inhibition up to 47.63 % and produced clear zones in spore germination test with radius measurements of 10 – 17 mm because of its produced volatile bioactive compounds (Toh et al., 2016). Prigigallo et al. (2021) reported that volatile compounds such as ammonia produced by *E. cloacae* were able the prevention of *Pythium* which caused disease in cotton. The increase of biological control was also shown in the release of ammonia during chitin degradation which subsequently reduces mycelia spreading (Prigigallo et al., 2021). It could be predicted that the use of selective media containing chitin was reasonable for *E. cloacae* isolation. During the bacterial growth, *E. cloacae* could utilize chitin provided, producing NH_3 as a volatile active ingredient inhibiting *P. capsici*.

Based on the phylogenetic tree, *S. flaveus* had very close relationship with *S. halstedii* whose culture broth suppressed the growth of *P. capsici* due to their low molecular faction (≤ 10 kDa) (Joo, 2005). A manumycin-type antibiotic from *S. flaveus* showed strong antifungal activity against *P. capsici* (Minh et al., 2015).

NH32, *K. pneumoniae,* be in a relative correlation with *E. cancerogenus* which was reported as a phytophthora blight pathogen agent. *K. pneumoniae* was also a member of the family *Enterobacteriaceae* and known nitrogen-fixing bacterium, able to convert atmospheric nitrogen into ammonium. Subsequently, *K. pneumoniae* was considered to be a biological control agent, not only able to inhibit *P. capsici* but also to promote plant growth (Sophereth et al., 2013). *K. pneumoniae* belonged to the group of bacteria that cause disease in humans, so evaluation was needed to confirm the safety of the strain.

B. amyloliquefaciens was found to be a potential biocontrol agent for controlling the plant pathogen *P. capsici* (Zhang et al., 2016). The *in vitro* test demonstrated this strain to have antifungal properties with high efficiency and broad-spectrum. This bacterium could promote the growth of pepper seed, solubilize phosphate and produce indole acetic acid (IAA) and ammonia.

5. Conclusion

In summary, the present study successfully isolated four bacterial strains that had a good inhibitory effect *P. capsici* growth suppression including *E. cloacae, S. flaveus, K. pneumoniae and B. amyloliquefaciens* which were potential strains to use as biological control agents for the rapid death on Pepper. These strains could have other beneficial properties that needed further research.

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