Vietnam Journal of Science and Technology ***63*** *(2) (2025) 249-261*

doi:10.15625/2525-2518/18307

Fungal antagonistic activity of rhizobacteria isolated from black pepper

Thi Huyen Trang Trinh[1], Van Bon Nguyen[2], Manh Dung Doan[2], Dinh Minh Tran[2], Mai Huong Le[3,*], Anh Dzung Nguyen[2,*]

[1]*Department of Science and Technology, Tay Nguyen University, 567 Le Duan Str., Buon Ma Thuot, Dak Lak, Viet Nam*

[2]*Institute of Biotechnology and Environment, Tay Nguyen University, 567 Le Duan Str., Buon Ma Thuot, Dak Lak, Viet Nam*

[3]*Institute of Natural Products Chemistry, 18 Hoang Quoc Viet Str., Cau Giay, Ha Noi, Viet Nam*

*Email: *nadzung@ttn.edu.vn*

Received: 5 May 2023; Accepted for publication: 3 July 2023

Abstract: Decline disease associated with *Fusarium* fungus is serious damage to black pepper and other crops. Biocontrol using rhizosphere bacteria is a sustainable and eco-friendly solution to manage this disease. From the collection, 44 isolates were screened *Fusarium* antagonistic acitivity. The results showed that *Fusarium* mycelium growth inhibition activity ranged from 12.50 to 66.25 %, and six potent isolates were selected to evaluate the *Fusarium* antagonistic activity on black pepper seedlings in the greenhouse. These rhizobacterial isolates significantly affected the growth and the rate of *Fusarium* disease of the seedlings in the greenhouse. The results found the most potential isolate to be *Bacillus subtilis* RB.CJ41. Furthermore, the *Fusarium* antagonistic activity of RB.CJ41 was investigated by hydrolysis enzymes and chemical compounds by GC/MS method. It found that chitinase, protease, and beta-glucanase contributed to almost the inhibition. Nineteen major volatile compounds were detected and identified by the GC/MS method. Some volatile compounds produced by RB.CJ41 are antifungal compounds derivatized from Benzoic acid, Triazole, Bromoacetyl, Pyrazole, and Acetamide. Since the *Fusarium* antagonistic activity, the *Bacillus subtilis* RB.CJ41 is a promising bacterial strain to apply for the sustainable production of black pepper.

Keywords: *Bacillus subtilis*, black pepper, *Fusarium*, Fungal antagonism, Rhizobacteria.

Classification numbers: 1.2.1, 1.3.2

1. INTRODUCTION

Fusarium is a large genus of filamentous fungi, widely distributed in soil and associated with plant pathogens. The *Fusarium* diseases are the most common for many crops, such as barley, tomato, potato, chili, banana, coffee, black pepper, etc. Some the *Fusarium* species may synthesize mycotoxins harm to the health of humans and livestock [1 - 5]. Therefore, they have a con-

siderable economic impact on all countries' agriculture, food security, food industry, and human health. The *Fusarium* diseases are a significant impediment to food plant production and are very difficult to control, especially soil-borne diseases caused by *F. oxysporum*, and *Fusarium solani* [5 - 10].

Black pepper (*Piper nigrum* L.) is one of the most important spices in the world and is cultivated in tropical countries such as India, Viet Nam, Malaysia, Sri Lanka, Indonesia, etc. Of these, Viet Nam is one of the largest producers and exporters of black pepper, producing around 250,000 - 280,000 tons annually, contributing to nearly 40 % of black pepper productivity in the world market [11]. However, the production of black pepper in Viet Nam has faced serious diseases such as quick wilt and slow decline. Quick wilt and yellowing (slow decline) diseases are serious limitations to black pepper cultivation, resulting in yield reduction and a large area of black pepper loss [12 - 15]. The infection combination of *Fusarium* and *Meloidogyne* sp. is the main cause of the yellowing disease [14]. Nowadays, using chemical fungicides is the most common approach for the disease management of black pepper. However, this way harms the farmer's health and the sustainable environment and reduces product quality due to toxicity pollution. Thus, controlling the diseases is one of black pepper production's most prominent challenges [12, 14].

Biological control has been a component of an integrated pest management strategy that is a more sustainable approach to managing agricultural pests and diseases of crops such as chili, lettuce, tomato, coffee, etc. [16 - 23]. In the disease management of black pepper, endophytic and rhizosphere microorganisms are widely applied to prevent damage caused by quick wilt and yellowing diseases. Endophytic bacteria and rhizobacteria could suppress the pathogenic fungi and nematodes and possess bioactivities such as nitrogen fixation, IAA biosynthesis, and phosphate solubilization, promoting the growth of crops. In addition, endophytic and rhizosphere bacteria may produce extracellular enzymes (chitinases, proteases, and glucanases) and chemical compounds (volatile substances and antibiotics) against pathogenic fungi and nematodes [24 - 29]. However, almost previous studies have focused on using biocontrol to manage *Phytophthora* and *Meloidogyne* nematodes for black pepper. This study aims to isolate and screen *Fusarium* antagonistic rhizobacteria from random farms in the Central Highland and then find out the mechanism of their antagonism against the *Fusarium* fungus.

2. MATERIALS AND METHODS

2.1. Materials

A collection of 269 rhizobacteria isolates, Fungus *Fusarium oxysporium* F.TNU02 (LC707983) were isolated from diseased roots of black pepper kept in Institute of Biotechnology and Environment, Tay Nguyen University (TNU). The black pepper seedlings were Vinh Linh variety used for bioassay in the green house.

2.2. Methods

2.2.1. In Vitro Fusarium Antagonism by the Rhizobacteria

In vitro, the *Fusarium* antagonism was tested following the method described by Ngo *et al.* [26]. The tests were conducted on PDA plates in triplicates. The plates were incubated at 28 ^{o}C for 5 days. The antagonistic activity was evaluated by comparing mycelium growth in the control and the treatment groups. The radial growth of fungal mycelium was measured, and the percentage of growth inhibition was calculated as follows:

$$\text{Rate of mycelium growth inhibition (\%)} = [(D1 - D2)]/D1] \times 100$$

where D1 = diameter of the fungus mycelium grown on the control disk (cm) and D2 = diameter of the fungus mycelium grown on the treated bacteria disk (cm).

2.2.2. Bioassay in the Greenhouse

Six potent rhizobacteria selected from *in vitro* and *in vivo* tests were used for *Fusarium* antagonistic activity in the greenhouse. The rhizobacteria were cultivated in LB medium for 72 h at 25 ^{o}C with a shaking speed of 150 rpm, and adjusted to 10^7 CFU/mL. The evaluation of the *Fusarium* antagonism by the rhizobacteria was conducted in a greenhouse in conditions: humidity of 80 %, temperature of 25 - 30 ^{o}C, and light intensity of 2000 - 3000 lx. The six potent rhizobacteria and two control groups were used for this experiment. The experiment had 8 plots in triplicates under randomized complete block design (RCBD). The seedlings were planted in sterilized artificial compost pots and irrigated with 10 mL bacterial suspension (10^7 CFU/mL) except for negative and positive control. After three months, the soil and roots were collected from the pots of the experiment in the greenhouse. 10g soil was ground and put into 90 mL WA medium (0.1 g agar in 1 L water). Then, it was diluted to 10^2, 10^3. After that, 1 mL of the soil suspension was inoculated on PPA medium (15 g peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 1 g tetracycline chloride, 1 g streptomycin sulfate, 0.12 g neomycin sulfate, 1 L water, pH:6.0) for 5 d at room temperature. The *Fusarium* colony was identified by the key (No.632.4/S459) described by Seifert [30]. The growth data on the black pepper seedlings, rate of *Fusarium* infection roots, and rate of fatality were also observed; mean values were calculated from six plants.

2.2.3. Biosynthesis of enzymes by strain RB.CJ41 and antagonistic action against Fusarium oxysporium F.TNU02

The strain RB.CJ41 was grown in LB medium supplemented with 1% casein (1), or 1 % chitin (2), or 0.1 % β-glucan (3), or 10^7 spores of *Fusarium oxysporium F.TNU02* (4) as inducers to promote the production of protease, chitinase, or β-glucanase. And a control had only RB.CJ41 in LB medium (5). The strain was cultivated at 30 ^{o}C for 5 d and 150 rpm. Then, the culture was centrifuged at 13,000 × *g* and 4 ^{o}C for 5 min to remove the bacterial biomass and collect the supernatant used as crude enzymes. The activity of protease, chitinase, and β-glucanase was determined as following procedures:

- *Chitinase assay:* The cells were separated by centrifugation at 13,000 × *g* and 4 ^{o}C for 5 min. The supernatant was used to measure the chitinase activity by Imoto method [31].

- *Protease assay:* The rhizobacteria were grown in LB medium and added 1 % casein for 5 d, at 37 ^{o}C with a shaking speed of 150 rpm. After that, the culture was centrifuged at 13,000 *x g* and 4 ^{o}C for 5 min. Anson's methods used the supernatant as crude protease [32].

- *β - glucanase assay:* The rhizobacteria were cultured in LB medium supplemented with 0.1 % β-glucan at 37 ^{o}C, 200 rpm for 4 d. After that, the culture was centrifuged at 13,000 *x g*, and 4 ^{o}C for 5 min, and the supernatant was used as crude enzymes. Enzyme activity was measured by the reducing sugar produced, determined by the method described by Miller [33].

The fungus was grown on PDA medium and added to 0.5 mL of the supernatant as activated crude enzymes or the crude enzymes were deactivated by boiling water for 15 min. Each test was replicated on 5 plates. The rate of mycelium growth inhibition (%) was calculated in section 2.3.

The correlation index R between the enzymatic activity and antagonistic activity against *Fusarium oxysporium* F.TNU02 was determined by SPSS 16.0 software.

2.2.4. PCR amplification, sequencing, and phylogenetic analysis of the 16S rRNA gene

The genomic DNA of each isolate was extracted and amplified by PCR. A nearly full-length segment of 16S rRNA gene nucleotides was amplified in a 100 µL reaction tube using universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTT ACGACTT-3′). The 16S rRNA gene was amplified by iCycler thermal cycler (Bio-Rad, Hercules, California, USA) with the schedule: 94 ^{o}C for 5 min, repeated in 30 cycles of denaturation at 95 ^{o}C for 30 s, annealing at 55 ^{o}C for 30 s, and extension at 72 ^{o}C for 2 min. The amplified products were then collected by electrophoresis on agarose gel (1.5 % w/v). The target bands in the agarose gel were cut out and purified using a QIA quick PCR purification (Promega Co., USA). Sequencing reactions were performed in a CEQ8000 Genetic Analysis System (Beckman Coulter Inc., USA) using a CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc., USA). The sequences (1300 - 1400 bps) were compared to known sequences in the DDBJ/Genbank/EMBL databases using BLAST (https:// blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the taxonomic positions of the rhizobacteria isolates. A phylogenetic tree was built by MEGA version 6.0 software using Kimura two-parameter method [34].

2.2.5. GS/MS analysis

The supernatant was purified by solid-phase extraction using the QuEChERS method. The analysis procedure was conducted by GC (Thermo Trace GC Ultra, USA) and ITQ900 (Thermo, USA). GC/MS was equipped with a TG-SQC capillary column (30 m × 0.25 mm × 0.25 µm). A carrier gas, Helium gas (99.999 %) was used at a constant flow rate of 1 mL/min, and 1µL of an injection volume was employed (a split ratio of 10:1). The injector temperature was maintained at 250 °C, the ion-source temperature was also set 230 °C, the oven temperature was programmed to 70 °C (isothermal for 2 min), with an increase of 15 °C/min to 280 °C, ending with a 10 mins isothermal at 280 °C. MS data were acquired at 70 eV, a scanning interval of 0.5 s, and fragments from 50 to 650 Da. The compounds were identified using data from the Mass Spectra Library (NIST 17.L and Wiley).

2.2.6. Data analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests using SAS 9.1 software. $\alpha \leq 0.05$ was considered to be significant.

3. RESULTS AND DISCUSSION

3.1. In vitro *Fusarium* antagonistic activity by rhizobacteria

From the roots of samples collected from three provinces in the Central Highland, Viet Nam, 269 rhizobacteria were isolated and characterized the morphology of their colonies and cells. It showed the diversity of rhizosphere bacteria of black pepper due to the diversity of soil, ecological system, and plant varieties. The *in vitro Fusarium* antagonist activity screening tests were conducted on the plates for 7 days, as shown in Fig. 1. The results (Table 1) showed that 44 isolates among 269 isolates possessing antagonism against *Fusarium* fungus.

*Table 1***.** *In vitro* screening *Fusarium* antagonistic activity of the rhizobacteria isolates.

No.	Isolates	*Fusarium* mycelium growth inhibition (%)	No.	Isolates	*Fusarium* mycelium growth inhibition (%)
1	RB.DC6	12.50 ± 0.07^{u}	24	RB.BH14	12.50 ± 0.04^{u}
2	RB.DC16	43.75 ± 0.05^{c}	25	RB.KN4	25.00 ± 0.03^{o}
3	RB.CP5	17.50 ± 0.17^{s}	26	RB.KN5	23.75 ± 0.04^{p}
4	RB.CP12	37.50 ± 0.07^{h}	27	RB.KN6	23.75 ± 0.04^{p}
5	RB.CP15	26.25 ± 0.01^{n}	28	RB.KN9	23.75 ± 0.04^{p}
6	RB.CP17	18.75 ± 0.04^{r}	29	RB.KN10	15.00 ± 0.02^{t}
7	RB.CS21	12.50 ± 0.07^{u}	30	RB.CJ1	15.00 ± 0.01^{t}
8	RB.CS33	12.50 ± 0.05^{u}	31	RB.CJ2	12.50 ± 0.07^{u}
9	RB.EK2	66.25 ± 0.02^{a}	32	RB.CJ3	12.50 ± 0.06^{u}
10	RB.EK3	17.50 ± 0.00^{s}	33	RB.CJ4	42.50 ± 0.07^{d}
11	RB.EK4	31.25 ± 0.03^{k}	34	RB.CJ10	12.50 ± 0.01^{u}
12	RB.EK5	35.00 ± 0.01^{j}	35	RB.CJ12	41.25 ± 0.06^{e}
13	RB.EK6	31.25 ± 0.02^{k}	36	RB.CJ13	12.50 ± 0.00^{u}
14	RB.EK7	23.75 ± 0.00^{p}	37	RB.CJ27	47.50 ± 0.06^{b}
15	RB.EK8	25.00 ± 0.01^{o}	38	RB.CJ32	15.00 ± 0.00^{t}
16	RB.EK9	40.83 ± 0.01^{f}	39	RB.CJ41	43.75 ± 0.03^{c}
17	RB.EK10	21.25 ± 0.02^{q}	40	RB.DS1	35.00 ± 0.09^{j}
18	RB.EK11	30.00 ± 0.01^{l}	41	RB.DS22	12.50 ± 0.00^{u}
19	RB.EK12	18.75 ± 0.02^{r}	42	RB.DS28	25.00 ± 0.07^{o}
20	RB.EK13	36.25 ± 0.00^{i}	43	RB.DS32	23.75 ± 0.05^{p}
21	RB.EK14	36.25 ± 0.01^{i}	44	RB.DL3	37.50 ± 0.01^{h}
22	RB.EK15	40.00 ± 0.03^{g}	45	Control	0.0^{u}
23	RB.EK17	27.50 ± 0.03^{m}	-	-	-
	P				<0.01
	CV%				0.41

The tests were conducted on PDA plates in triplicates. The plates were incubated at 28 ^{o}C for 5 d. The antagonism activity was evaluated by comparing mycelium growth in the control and the treatment groups. The data were analyzed via the simple variance (ANOVA) then Duncan′s multiple range test at $p = 0.01$ was evaluated. Values in the same column with different letters are significantly different. CV - Coefficient of Variation.

Their values of *Fusarium* mycelium growth inhibition ranged from 12.50 to 66.25 %. The highest activity was RB.EK2 isolate. From these results, there were six potential isolates selected to be RB.EK2, RB.DC16, RB.CJ4, RB.CJ12, RB.CJ27 and RB.CJ41 isolate with activity from 41.25 to 66.25 % (Table 1). Compared to previous studies, our study's activity is the same as those others, even though higher. Ann (2012) isolated rhizobacteria and evaluated the antagonism of pathogenic fungi causing the diseases of black pepper. The *in vitro Fusarium solani* antagonistic activity of six potent rhizobacteria was 15 % to 25 %, lower than in this study [12]. Kota *et al.*

isolated seven bacterial strains from soil and seawater and evaluated *Phytophthora* and *Fusarium* antagonistic activity *in vitro*. The *Phytophthora* and *Fusarium solani* cause quick wilt and slow decline in black pepper in Malaysia. The results showed that *Bacillus amyloquefaciens* pp3 inhibited up to 50 % of the growth of *Fusarium* fungus [6]. Morsy *et al.* reported that *Bacillus* sp could inhibit 34 % of the growth of *Fusarium solani* caused by wilt disease of tomato [21]. Wang *et al.* showed *Bacillus licheniformis* CHM1 had an inhibition of 51.61 % against *Fusarium oxysporum* [22]. In recent, Khalifa *et al.* isolated 17 *Pseudomonas* strains from the rhizosphere of chickpea, and found that these rhizobacteria inhibited from 4.66 to 33.03 % of the growth of *Fusarium oxysporium* causing wilt disease of chickpea plant [9].

3.2. Effect of the potential rhizobacteria on the growth of black pepper seedlings in the green house

Usually, endophytic and rhizosphere microorganisms have multiple activities, such as pathogen fungal antagonism, anti-bacteria, anti-nematodes, nitrogen fixation, IAA production, and phosphate solubilization [17 - 24].

Table 2. Effect of the potential rhizobacteria on the growth of black pepper in greenhouse.

Treatments	Diameter of the shoot (mm)	Plant height (cm/plant)	Fresh biomass (g/plant)	Leaf number/ Plant	Leaf area (cm^2/plant)
RB.EK2+*Fusarium*	3.17 ± 0.05^{ab}	55.57 ± 4.80^{a}	17.24 ± 4.63^{ab}	11.67 ± 0.71^{a}	36.12 ± 4.51^{b}
RB.DC16+*Fusarium*	3.17 ± 0.05^{ab}	48.67 ± 7.12^{ab}	15.95 ± 2.59^{ab}	11.67 ± 1.88^{a}	38.25 ± 4.91^{ab}
RB.CJ4+*Fusarium*	3.30 ± 0.03^{a}	53.27 ± 7.53^{a}	18.17 ± 2.39^{a}	12.00 ± 1.91^{a}	40.12 ± 2.81^{ab}
RB.CJ12+*Fusarium*	3.30 ± 0.06^{a}	52.27 ± 10.54^{ab}	19.38 ± 3.35^{a}	11.33 ± 1.23^{a}	41.37 ± 2.72^{a}
RB.CJ 27+*Fusarium*	3.03 ± 0.24^{c}	49.67 ± 6.15^{ab}	18.35 ± 4.17^{a}	12.33 ± 0.45^{a}	$38.73 \pm 4{,}50^{ab}$
RB.CJ 41+*Fusarium*	3.23 ± 0.07^{ab}	54.63 ± 6.46^{a}	19.80 ± 2.46^{a}	12.33 ± 1.42^{a}	37.38 ± 0.56^{ab}
Without *Fusarium*	3.17 ± 0.14^{ab}	52.97 ± 7.77^{ab}	17.24 ± 2.68^{ab}	10.67 ± 0.59^{ab}	40.07 ± 4.34^{ab}
With *Fusarium*	3.10 ± 0.11^{ab}	42.73 ± 5.75^{b}	12.69 ± 0.65^{b}	8.67 ± 0.55^{b}	36.13 ± 0.13^{b}
CV%	3.59	13.74	16.17	11.45	8.21
P	< 0.01	< 0.05	< 0.05	< 0.01	< 0.05

A bioassay in the Greenhouse was conducted following the method presented in section 2.4. The data were analyzed via the simple variance (ANOVA) then Duncan's multiple range test at $p = 0.01$ was evaluated. Values in the same column with different letters are significantly different. CV - Coefficient of Variation.

The impact of the rhizobacteria on the growth of black pepper was shown in Table 2. The diameter of the shoot, plant height, fresh biomass, number of leaves, and leaf area of the black pepper in the plots treated with the rhizobacteria was significantly higher than in the control (with *Fusarium* or without *Fusarium*) at $p < 0.05$ and 0.01. Among the six isolates, the RB.EK2 and RB.CJ41 showed better effects than the others because these isolates possess higher bioactivities. The plant height and fresh biomass of the plots treated the RB.EK2 and RB.CJ41 were 27 - 56 % higher than the control with the *Fusarium* and 3 - 17 % higher than those without the *Fusarium*.

The six potential rhizobacteria also strongly affected the growth of roots and the rate of root disease (Table 3). The results showed that the length, weight, and number of roots in the treatment were significantly higher than the control with *Fusarium* treatment. Although all plots treated the

rhizobacteria and *Fusarium*, the roots' growth data were the same as the control without *Fusarium* treatment at $p < 0.05$ and $p < 0.01$. In particular, the rate of root disease in control with *Fusarium* was 8.23 %, whereas others were lower, only from 1.63 to 5.88 %. In the rhizobacteria group, using RB.CJ41 isolate was 2.06 %, the lowest rate of root disease. It means that using rhizobacteria not only promotes the plant's growth but also protects the roots from *Fusarium* attack due to a decrease in the density of *Fusarium* in soil. The results (Table 3) indicated that the density of *Fusarium* in the soil treated with the rhizobacteria was lower by approximately 10 times than the control with *Fusarium*.

Table 3. Effect of rhizobacteria treatment on the growth and disease rate of the roots.

Treatments	Length of root (cm)	Weight of root (g/plant)	Number of roots/plant	Rate of root disease (%)	*Fusarium* (10^3 CFU/g soil)
RB.EK2+*Fusarium*	21.81 ± 1.41^{ab}	1.35 ± 0.44^{ab}	8.00 ± 1.54^{ab}	$3.42 \pm 0.004^{c-e}$	0.83
RB.DC16+*Fusarium*	19.34 ± 0.64^{bc}	1.33 ± 0.20^{ab}	7.33 ± 0.25^{ab}	$5.88 \pm 0.007^{b-d}$	1.21
RB.CJ4+*Fusarium*	22.48 ± 2.62^{a}	1.39 ± 0.38^{ab}	8.00 ± 0.43^{ab}	4.10 ± 0.006^{c}	0.91
RB.CJ12+*Fusarium*	21.45 ± 1.00^{ab}	1.34 ± 0.17^{ab}	10.00 ± 0.25^{a}	$3.41 \pm 0.008^{c-e}$	1.29
RB.CJ 27+*Fusarium*	21.23 ± 1.74^{ab}	1.39 ± 0.99^{ab}	8.67 ± 1.95^{ab}	4.28 ± 0.007^{bc}	0.89
RB.CJ 41+*Fusarium*	22.68 ± 0.90^{a}	1.66 ± 0.26^{a}	9.67 ± 0.58^{ab}	2.06 ± 0.002^{de}	0.78
Without *Fusarium*	23.19 ± 1.58^{a}	1.61 ± 0.22^{ab}	7.67 ± 1.38^{ab}	1.63 ± 0.006^{e}	0.08
With *Fusarium*	18.58 ± 0.59^{c}	1.14 ± 0.25^{b}	7.00 ± 0.78^{b}	8.23 ± 0.014^{a}	8.90
CV%	7.09	19.39	17.23	1.42	-
P	< 0.01	< 0.05	< 0.05	< 0.01	-

The data were analyzed via the simple variance (ANOVA) then Duncan′s multiple range test at $p = 0.01$ was evaluated. Values in the same column with different letters are significantly different. CV - Coefficient of Variation.

The impact of effective microbes on black pepper's growth and disease resistance has been also reported [35 - 36]. Based on the 16S rDNA gene sequence analysis, the three potential strains were further identified as the *Bacillus amyloliquefaciens* RB.EK2, *Bacillus subtilis* RB.CJ 27, and *Bacillus subtilis* RB.CJ41 The 16s rRNA gene sequence of these identified strains was submitted in DDBJ/EMBL/Genbank with the accession numbers of the LC705187 (*Bacillus amyloliquefaciens* RB.EK2), LC705188 (*Bacillus subtilis* RB.CJ 27), and LC602155 (*Bacillus subtilis* RB.CJ41) (see Fig.S1 supplement).

3.3. Effect of the bacterial extracellular enzymes on the *Fusarium* inhibition

The cell walls of *Fusarium* fungus contain chitin, α-1,3-glucan, β-1,3-glucan, and protein (Schoffelmeer *et al.* [37]). Therefore, chitinase, protease, and β-1,3-glucanase play an important role in the antifungal activity of the bacteria. The results shown in Table 4 indicated that inducers such as casein, chitin, and β-glucan play very important roles in inducing the production of extracellular enzymes. In this case, using the *Fusarium* spore in the culture medium stimulated three enzymes protease, chitinase, and β-glucanase. The roles of the extracellular enzymes in the growth inhibition of *Fusarium* are clear. The *Fusarium* mycelium growth inhibition *in vitro* was from

18.3 to 20.8 % at the No.1-4 test and decreased to 3.3 % at No.5 test. The enzymatic effect on *Fusarium* antagonistic activity was also demonstrated in No. 5 experiment of Table 4. In this experiment, strain RB.CJ41 was cultivated in LB medium without inducers such as chitin, casein, or the *Fusarium* spores. Therefore, there was no chitinase, protease, and β-glucanase in the supernatant, resulting in *Fusarium* mycelium growth inhibition being only 3.3 %. The role of chitinase, glucanase and protease in fungal antagonistic activity has been reported. Figueroa-López *et al.* [38] screened three *Bacillus* strains from maize which possessed chitinase, glucanase, and protease. These strains were very potential for *Fusarium verticillioides* antagonism. Ngo *et al.* isolated and selected six potent endophytic bacterial strains from black pepper. They exhibited chitinase, protease, and β-glucanase activities and showed strong *Phytophthora* inhibition in vitro and greenhouse [26].

Table 4. Relationship between the enzymes of strain RB.CJ41 and *Fusarium* inhibition.

No.	Medium content	Enzyme activity			*Fusarium* mycelium growth inhibition (%)
		Protease (U/ml)	Chitinase (U/ml)	β-glucanase (U/ml)	
1	LB+casein+RB.CJ41	0.36 ± 0.03	-	-	18.3[a]
2	LB+chitin+RB.CJ41	-	3.96 ± 0,08	-	18.3[a]
3	LB+β-glucan+RB.CJ41	-	-	0.41 ± 0.04	20.8[a]
4	LB+*Fusarium* spore+ RB.CJ41	0.15 ± 0.01	1.32 ± 0,05	0.29 ± 0.02	20.8[a]
5	LB+RB.CJ41	-	-	-	3.3[b]

The data were analyzed via the simple variance (ANOVA) then Duncan′s multiple range test at p = 0.01 was evaluated. Values in the same column with different letters are significantly different.

The correlation (R) indexes between the enzymatic activities and the fungal antagonistic activity were calculated. It found positive correlations between chitinase, protease, β-glucanase, and *Fusarium oxysporium* F.TNU02 antagonism with R = 0.515, R = 0.680, and R = 0.640 ($P < 0.05$, $n = 25$). Some recent works also reported these findings, but these are not the same results. The chitinase, protease, and β-1,3 glucanase were strongly inhibited mycelium growth of *Fusarium oxysporum* and determined the R index to be 0.991, 0.703, and 0.644 [39]. Ngo *et al.* reported that the correlation index (R) between chitinase and *Phytophthora* antagonism was determined to be 0.32, but the negative correlation index with protease (R= - 0.23) [26]. The chitinase from *B. subtilis* NPU 001 exhibited an antifungal activity of the *Fusarium oxysporium* [40].

3.4. Identification of antifungal compounds from RB.CJ41 by GC/MS

Volatile compounds produced by microbes play an important role in fungal antagonistic activity. Khan *et al*. [41] reported that *B. subtilis* 30VD-1 possessed *Fusarium* antagonistic activity by its chitinase and some compounds such as phenol, 1-decanol, 1-dodecanol, and benzaldehyde. Chaurasia *et al.* also indicated that *Bacillus subtilis* (NRRL B-30408) isolated from the rhizosphere of tea plants produced some volatile compounds which had a strong impact on the mycelium and spores of *Fusarium oxysporum* [42].

To identify the volatile compounds produced by the Bacillus subtilis RB.CJ41, this strain was cultivated in LB medium supplemented with the Fusarium spores. The supernatant of the culture

was used to identify antifungal compounds by GC/MS. Based on GC/MS analysis, 19 major volatile compounds were detected and identified (Table 5).

*Table 5***.** Profile of volatile compounds produced by *Bacillus subtilis* RB.CJ41 detected by GC/MS.

No	Chemical compounds	RT (min)	Area (%)
1	4,5-Bromoacetyl benzocyclobutene	3.15	5.35
2	Octanoic acid, 2-methyl-	3.38	7.83
3	4-Ethylbenzoic acid, cyclopentyl ester	3.77	7.94
4	Pyrazole-4-carboxaldehyde, 1-methyl-	4.31	2.36
5	Acetamide, N-(5-benzofuroxanyl)-	4.52	6.00
6	Pyrazinecarboxamide	5.00	2.06
7	Benzeneacetaldehyde	5.31	8.81
8	Benzene,1-chloro-3,5-bis(1,1-dimethylethyl)-2-(2-propenyloxy)-	5.63	10.21
9	1H-Pyrrole, 1-(2-furanylmethyl)-	6.93	2.08
10	Piperidine, 1-methyl-	6.98	3.17
11	Benzeneacetic acid	7.60	3.55
12	Vitamin B1	8.02	1.02
13	3-Aminothiophenol, N,S-diacetyl-	10.20	1.45
14	5-Hydroxy-2-indolecarboxylic acid	10.48	8.76
15	2-(4'-Methylphenyl)-1,2,4-triazolo[1,5-a]pyridine - 1-oxide	10.71	1.32
16	Uric acid (Thermolysis Product)	11.98	1.45
17	Glycyl-L-proline	12.34	10.06
18	Cyclo(L-prolyl-L-valine)	12.81	1.64
19	Bromocriptine	13.64	1.06

The spectra of GC/MS of the identified compounds were presented in the supplementary section (Fig. S2 – Fig. S22). The results indicated that some main volatile compounds produced by the *Bacillus subtilis* RB.CJ41 are antifungal compounds such as compound 1, 5, 6, 7, 8, 10, 11, 15. They are derivatized from benzoic acid, triazole, pyrazole, and acetamide. Benzoic derivatives were previously reported with potential antifungal effects against various fungi, including *Candida albicans*, *Cochliobolus lunatus*, *Aspergillus niger* and *Pleurotus ostreatus* [42 - 44]. 1,2,4-Triazole with heterocyclic rings was reported to show broad activities such as fungicidal, insecticidal, herbicidal, and bactericidal effects [45]. Pyrazole ring were found as new therapeutic antifungal agent related to phenylpyrazole system [46]. Synthesised acetamide derivatives also showed antibacterial and antifungal activity [47]. However, these compounds have not been reported to show the *Fusarium oxysporium* antagonistic activity. LC/MS was detected but, there was no fungal antagonist chemical compounds identified.

4. CONCLUSIONS

From the rhizobacteria collection of black pepper, it found a rhizobacteria *Bacillus subtilis* CJ41 has great potential for *Fusarium* antagonism. The mechanism action of the *Fusarium* an-

tagonism is mainly hydrolysis enzymes such as chitinase, protease, β-glucanase and volatile compounds. The volatile compounds produced by RB.CJ41 are potent antifungal compounds to be benzoic acid, triazole, pyrazole, and acetamide derivatives. Additionally, strain CJ41 promotes growth and decreases the root disease rate caused by *Fusarium* in green house. Therefore, the *Bacillus subtilis* CJ41 is a potent biological agent for biocontrol and sustainable production of black pepper.

Supplementary Materials: The following supporting information can be downloaded at: www., Figure S1-S22.

Author Contributions: Conceptualization, methodology: Anh Dzung Nguyen, and Mai Huong Le; software, validation, formal analysis, investigation: Thi Huyen Trang Trinh, Van Bon Nguyen, Manh Dzung Doan, Dinh Minh Chan; writing and editing: Anh Dzung Nguyen. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a grant from B/2017-2019, and a part of NDT.61.HU/19.

Data Availability Statement: Not applicable.

***Conflicts of Interest*:** The authors declare no conflict of interest.

REFERENCES

1. Wegulo S. N., Baenziger P.S., Hernandez N.J., Bockus. W. W., Hallen A. H. - Management of *Fusarium* head blight of wheat and barley, Crop Prot. **73** (2015) 100-107. https://doi.org/10.1016/j.cropro.2015.02.025
2. Shahnazi S., Meon S., Vadamalai G., Ahmad K., Nejat N. - Morphological and molecular characterization of *Fusarium* spp. associated with yellowing disease of black pepper (*Piper nigrum* L.) in Malaysia, J Gen Plant Pathol. **78** (2012*)* 160-169. https://doi.org/ 10.1007/s10327-012-0379-5
3. Aïcha B., Nisserine H. K., Abdeslem S. M., Hadjira B., Lalia B. S., Mebrouk K., and Jamel-Eddine H. - Antagonist effect of nonpathogenic *Fusarium oxysporum* strain Fo47 on *Fusarium oxysporum* f. sp. radicis-lycopersici the cause of fusarium crown and root rot in tomato cultivation, Advances in Environmental Biology **8**(10) (2014) 50-56.
4. Musoli C. P., Pinard F., Charrier A., Kangire A., Kabole C., Ogwang J., Bieysse D., Cilas C. - Spatial and temporal analysis of coffee wilt disease caused by *Fusarium xylarioides* in *Coffea canephora*, Eur. J. Plant Pathol. **122** (2008) 451-460. https://doi.org/ 10.1007/s10658-008-9310-5
5. Antonissen G., Martel A., Pasmans F., Ducatelle, R., Verbrugghe E., Vandenbroucke V., Li S., Haesebrouck F., Van I.F., Croubels S. - The impact of *Fusarium Mycotoxins* on human and animal host susceptibility to infectious diseases, Toxins **6** (2014*)* 430-452. https://doi.org/10.3390/toxins6020430
6. Kota M.F., Husaini A.A.S.A., Lihan S., Hasnain M., Hussain M., Roslan H.A. - In vitro antagonism of *Phytophthora capsici* and *Fusarium solani* by bacterial isolates from Sarawak, Malays. J. Microbiol. **11** (2) (2015) 137-143.
7. SolehaS., Muslim A., Suwandi S., Kadir S., Pratama R. – The identifcation and pathogenicity of *Fusarium oxysporum* causing acacia seedling wilt disease, J. For. Res. **33** (2022) 711-719. https://doi.org/10.1007/s11676-021-01355-3.

8. Saengchan C., Sangpueak R., Le T.T., Phansak P., Buensanteai N. - Induced resistance against *Fusarium solani* root rot disease in cassava plant (*Manihot esculenta* Crantz) promoted by salicylic acid and *Bacillus subtilis*, Acta Agric Scand B Soil Plant Sci. **72** (1) (2022) 516-526. https://doi.org/10.1080/09064710.2021.2018033.
9. Khalifa M.W., Rouag N., Bouhadida M. - Evaluation of the antagonistic effect of *Pseudomonas* rhizobacteria on *Fusarium* wilt of chickpea, Agriculture **12** (2022) 429. https://doi.org/10.3390/agriculture12030429.
10. Ann Y. C - Rhizobacteria of pepper (*Piper nigrum*) and their antifungal activities, Afr. J. Microbiol. Res. **6** (19) (2012), 4185-4193. https://doi.org 10.5897/AJMR12.583.
11. Vietnam Pepper Association (VPA). *Report*; VPA: Ho Chi Minh, 2020 (in Viet Nam).
12. Kueh T. K., Gumbek M., Wong T. H., Chin S. P. - A field guide to diseases, pests and nutritional disorders of black pepper in Sarawak, Agricultural Research Centre Semongok, Department of Agriculture Kuching. Lee Ming Press, Sarawak, 1993.
13. Duarte M. L. R., Albuquerque F. C. - *Fusarium* disease of black pepper in Brazil, In: Sarma YR, Premkumar T (eds) Black pepper diseases, National Research Centre for Spices, Calicut, 1991, pp. 39-54.
14. Thuy T. T. T., Chi N. T. M., Yen N. T., Anh L. T. N., Te L. L., Waele D. D. - Fungi associated with black pepper plants in Quang Tri province (Vietnam), and interaction between *Meloidogyne incognita* and *Fusarium solani*, Arch. Phytopathol. Plant Prot. **46** (4) (2013) 470-482. https://doi.org/10.1080/03235408.2012.744621.
15. Sitepu D., Mustika I. - Diseases of black pepper and their management in Indonesia, In: Ravindran PN (ed.) Black pepper, Piper nigrum, Medicinal and aromatic plants-industrial profiles, vol 13. Black pepper, *Piper nigrum*, Harwood, Amsterdam, 2000, pp. 297-305.
16. Jayapala N., Mallikarjunaiah N. H., Puttaswamy H., Gavirangappa H., Ramachandrappa N. S. - Rhizobacteria *Bacillus* spp. induce resistance against anthracnose disease in chili (*Capsicum annuum* L.) through activating host defense response, Egypt. J. Biol. Pest Control. **29** (1) (2019) 1-9. https://doi.org/10.1186/s41938-019-0148-2.
17. Hyder S., Gondal A. S., Rizvi Z. F., Ahmad R., Alam M. M., Hannan A., Inam-ul-Haq M. - Characterization of native plant growth promoting rhizobacteria and their anti-oomycete potential against *Phytophthora capsici* affecting chilli pepper (*Capsicum annum* L.), Sci. Rep. **10** (1) (2020) 1-15. https://doi.org/10.1038/s41598-020-69410-3.
18. Kaushal M., Mandyal P., Kaushal R. - Field based assessment of *Capsicum annuum* performance with inoculation of rhizobacterial consortia, Microorganisms **7** (3) (2019) 89. https://doi.org/10.3390/microorganisms7030089.
19. Meena M., Swapnil P., Divyanshu K., Kumar S., Tripathi Y.N., Zehra A., Upadhyay R.S. - PGPR-mediated induction of systemic resistance and physiochemical alterations in plants against the pathogens: Current perspectives, J. Basic Microbiol. **60** (10) (2020) 828-861. https://doi.org/10.1002/jobm.202000370.
20. Yadav D. R., Adhikari M., Kim S. W., Kim H. S., Lee Y. S. - Suppression of *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. lactucae and growth promotion on lettuce using bacterial isolates, J. Microbiol. Biotechnol. **31** (9) (2021**)** 1241-1255. https://doi.org/ 10.4014/ jmb.2104.04026.
21. Morsy E. M., Abdel-Kawi K. A., Khalil M. N. A. - Efficiency of *Trichoderma viride* and *Bacillus subtilis* as biocontrol agents gainst *Fusarium solani* on tomato plants, Egypt J Phytopathol. **37** (1) (2009) 47-57.

22. Wang H., Wen K., Zhao X., Wang X., Li A., Hong H. - The inhibitory activity of endophytic *Bacillus* sp. strain CHM1 against plant pathogenic fungi and its plant growth-promoting effect, Crop Prot. **28** (8) (2009) 634-639. https://doi.org/10.1016/j.cropro.2009.03.017.
23. Nguyen D. N., Wang S. L., Nguyen A. D., Doan, M. D., Doan C. T., Tran M. D., Nguyen V. B. - Potential application of rhizobacteria isolated from the Central Highland of Viet Nam as an effective biocontrol agent of Robusta coffee nematodes and as a bio-fertilizer, Agronomy **11** (9) (2021) 1887. https://doi.org/10.3390/agronomy11091887.
24. Lau E. T., Tani A., Khew C. Y., Chua Y. Q., San H. S. - Plant growth-promoting bacteria as potential bio-inoculants and biocontrol agents to promote black pepper plant cultivation, Microbiol. Res. **240** *(*2020) 126549. https://doi.org/10.1016/j.micres.2020.126549.
25. Paul D., Lade H. - Plant-growth-promoting rhizobacteria to improve crop growth in saline soils: a review, Agron Sustain Dev. **34** (4) (2014**)** 737-752. https://doi.org/10.1007/s13593-014-0233-6.
26. Ngo V. A., Wang S. L., Nguyen V. B., Doan C. T., Tran T. N., Tran D. M., Tran T. D, Nguyen A. D. - *Phytophthora* antagonism of endophytic bacteria isolated from roots of black pepper (*Piper nigrum* L.), Agronomy **10** (2) (2020) 286. https://doi.org/10.3390/agronomy10020286.
27. Trinh T. H. T., Wang S. L., Nguyen V. B., Tran M. D., Doan C. T., Vo T. P. K., Huynh V. Q., Nguyen A. D. - A potent antifungal rhizobacteria *Bacillus velezensis* RB.DS29 isolated from black pepper (*Piper nigrum* L.), Res. Chem. Intermed. **45** (2019**)** 5309-5323. https://doi.org/10.1007/s11164-019-03971-5.
28. Nguyen A. D., Wang S. L., Trinh T. H. T., Tran T. N., Nguyen V. B., Doan C. T., Huynh V. Q., Vo T. P. K. - Plant growth promotion and fungal antagonism of endophytic bacteria for the sustainable production of black pepper *(Piper nigrum L.)*, Res. Chem. Intermed. **45** (2019**)** 5325-5339. https://doi.org/10.1007/s11164-019-03972-4.
29. Nguyen D. S., Nguyen T. H., Trinh T. H. T., Tran T. D., Nguyen V. T., Hoang V. C., Nguyen A. D. - Combined application of rhizosphere bacteria with endophytic bacteria suppress root diseases and increases the productivity of black pepper (*Piper nigrum* L.), Agriculture **11** (1) (2020**)** 15. https://doi.org/10.3390/agriculture11010015.
30. Seifert K. - *Fuskey: Fusarium interactive key* (No.632.4/S459), Agriculture and Agri-Food Canada, 1996, pp. 65.
31. Imoto T., Yagishita K. - A simple activity measurement of lysozyme, Agric. Biol. Chem. **35** (1971) 1154-1156. https://doi.org/10.1271/bbb1961.35.1154.
32. Anson M. L. - The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin, J. Gen. Physiol. **22** (1) **(**1938**)** 79. https://doi.org/10.1085%2Fjgp.22.1.79.
33. Miller G. L. - Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. **31** (3) (1959) 426-428. https://doi.org/10.1021/ac60147a030.
34. Kimura M. - A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, J. Mol. Evol. **16** (1980), 111–120. https://doi.org/10.1007/BF01731581
35. Kodithuwakku R. D., Wijekoon W. M. R. W. B., Kumari I. S., De Silva D. P. P. - Efficacy of single and combined application of *Trichoderma* spp. and *Pseudomonas fluorescens* along with bio-fertilizer (*Arbuscular Mycorrhizae*-AM) on the growth of nursery plants of black pepper (*Piper nigrum* L.), SLJFA **2** (1) (2016) 69-72.

36. Dastager S. G., Deepa C. K., Pandey A. - Potential plant growth-promoting activity of *Serratia nematodiphila* NII-0928 on black pepper (*Piper nigrum* L.), World J. Microbiol. Biotechnol. **27** (2) (2011) 259-265. https://doi.org/10.1007/s11274-010-0454-z.
37. Schoffelmeer E. A. M., Kli F. M., Sietsma J. H., Cornelissen B. J. C. - The cell wall of *Fusarium oxysporum*, Fungal Genet. Biol. **27** (1999) 275-282. https://doi.org/10.1006/fgbi.1999.1153.
38. Figueroa L. A. M., Cordero R. J. D., Martínez A. J. C., López M. M., Lizárraga S. G. J., Félix G. R., Maldonado M. I. E. - Rhizospheric bacteria of maize with potential for biocontrol of *Fusarium verticillioides*, Springerplus **5** (1) (2016) 330. https://doi.org/10.1186/s40064-016-1780-x.
39. Rathore R., Vakharia D. N., Rathore D. S. - In vitro screening of different *Pseudomonas fluorescens* isolates to study lytic enzyme production and growth inhibition during antagonism of *Fusarium oxysporum* f. sp. *cumini*, wilt causing pathogen of cumin, Egypt J. Biol. Pest Control **30** (1) (2020) 1-8. https:// doi.org/10.1186/s41938-020-00259-4.
40. Chang W. T., Chen M. L., Wang S. L. - An antifungal chitinase produced by *Bacillus subtilis* using chitin waste as a carbon source, World J. Microbiol. Biotechnol. **26** (5) (2010) 945-950. https://doi.org/10.1007/s11274-009-0244-7.
41. Khan N., Martínez H. P., Ice T. A., Maymon M., Humm E. A., Nejat N., Hirsch A. M. - Antifungal activity of *Bacillus* species against *Fusarium* and analysis of the potential mechanisms used in biocontrol, Front. Microbiol. **9** (2018*)* 2363. https://doi.org/10.3389/fmicb.2018.02363.
42. Chaurasia B., Pandey A., Palni L.M.S., Trivedi P., Kumar B., Colvin N. - Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structure deformation in pathogenic fungi in vitro, Microbiol. Res. **160** (1) (2005**)** 75-81. https://doi.org/10.1016/j.micres.2004.09.013.
43. López A., Ming D. S., Towers G. H. N. - Antifungal Activity of Benzoic Acid Derivatives from Piperlanceaefolium, J. Nat. Prod. **65** (1) (2002) 62-64. https://doi.org/10.1021/np010410g.
44. Berne S., Kovačič L., Sova M., Kraševec N., Gobec S., Križaj I., Komel R. - Benzoic acid derivatives with improved antifungal activity: Design, synthesis, structure–activity relationship (SAR) and CYP53 docking studies, Bioorg. Med. Chem. **23** (15) (2015) 4264-4276. https://doi.org/10.1016/j.bmc.2015.06.042.
45. Tang R., Jin L., Mou C., Yin J., Bai S., Hu D., Wu, J., Yang S., Song B. - Synthesis, antifungal and antibacterial activity for novel amide derivatives containing a triazole moiety, Chem. Cent. J. **7** (2013) 30. https://doi.org/10.1186/1752-153X-7-30
46. Farag A. M., Mayhoub A. S., Barakat S. E., Bayomi A. H. - Synthesis of new N-phenylpyrazole derivatives with potent antimicrobial activity, Bioorg. Med. Chem. **16** (8) (2008) 4569-4578. https://doi.org/10.1016/j.bmc.2008.02.043.
47. Ozkanli F., Dalkara S., Caliş U., Willke A. - Synthesis of some N-arylazole acetamide derivatives and their anticonvulsant and antimicrobial activities, Arzneimittel-forschung **44** (8) (1994) 920-924. PMID: 7945533.