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ISOLATION AND SELECTION OF INDIGENOUS ANTIFUNGAL MICROORGANISMS AGAINST PATHOGENIC FUNGI OF PEPPER PLANT IN TAY NGUYEN

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SUMMARY

The pathogenic fungi often cause huge impacts on agricultural crops, and occupy over 80% of plant diseases. *Fusarium oxysporum* and *Rhizoctonia solani* are fungal pathogens that can lead to rapid development of plant diseases on important crops in Tay Nguyen (e.g., pepper, coffee, rubber, cashew). Therefore, the study of microorganisms with bioactivity against these pathogens is essential to control plant diseases. In this study, we isolated microorganisms from rhizospheres of pepper in Tay Nguyen and screened beneficial microbes against two pathogenic fungi using agar well diffusion assay. Obtained results showed that there are different about isolated microbial density between samples collected from diseased and healthy pepper. The bacterial population is higher in rhizosphere region of healthy pepper than in those of diseased plants. In contrast, fungal density is lower in rhizosphere region of healthy plants than in those of diseased ones. From isolation plates, we selected and purified 391 strains including 236 bacteria, 149 actinomycetes and 6 fungi for screening antifungal activity. Out of isolated microorganisms, 44 strains (36 bacteria, 6 actinomycetes, and 2 fungi) showed antagonistic activity against at least one of two pathogens (*F. oxysporum* and *R. solani*), of which 15 isolates showed activity against both fungi. Identification of isolates with highest activity using the 16S rRNA gene sequences showed bacterial strains belonged to different species *Enterobacter ludwigii*, *Pseudomonas fulva*, *Bacillus subtilis*, whereas 2 actinomycetes belonged to the genus *Streptomyces*: *Streptomyces* sp. and *Streptomyces diastatochromogenes*. Identification of the isolated fungus based on morphological characteristics and the 18S rRNA gene sequence revealed that this strain belonged to species *Penicillium oxalicum*. Our study revealed the potential of the indigenous microorganisms in preventing and controlling plant-pathogenic fungi.

Keywords: *Antifungal activity, Fusarium oxysporum, pepper plant, plant diseases*, *Rhizoctnia solani*

INTRODUCTION

Tay Nguyen is one of the important regions of agricultural production in Vietnam, especially for long-term industrial plants such as rubber, pepper, cashew, coffee. Pepper in Tay Nguyen accounts for 43.3% of the country's area and 47.4% of the country's yield, and Vietnam has become the biggest pepper exporter in the world (Vu Nang Dung, 2015). However, Vietnam is characterized by hot and humid climate, large annual rainfall amount. These are favourable conditions for the growth of microorganisms, typically pathogenic fungi causing damages to crops, which leads to great losses to agricultural production. Pepper's diseases (e.g., root and stem rot, leaf yellow) are often caused by different pathogenic fungi such as *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani*, *Phytophthora*, etc. (Plant Protection Department, 2007). These plant's diseases have increased the cost of preventing and treating pepper's diseases.

In order to control diseases caused by plant pathogenic fungi, pesticides and plant protection chemicals are widely used in Vietnam. However, using of pesticides and plant protection products often lead to increasing the cost of agricultural production, land degradation, and problems of food safety (Ministry of Natural Resources and

Environment, 2010). Disadvantages of the use of chemical products in preventing and controlling plant's diseases have promoted the development and use of biological products to control plant pathogenic fungi. The use of antifungal microorganisms to control plant pathogenic fungi is not only highly effective, and safe, but also may significantly reduce the amount of chemicals used. In this study, we isolated and selected of indigenous antifungal microorganisms against to fungal pathogens of pepper plant (*F. oxysporum and R. solani*) to enhance the effective of control of pepper's pathogenic fungi, and contribute to increasing pepper's productivity and yield, meet domestic demand and export.

MATERIALS AND METHODS

Materials

Soil samples were collected from the pepper soil and root region in Tay Nguyen including diseased plants (leaf yellow, root rot) and non-diseased plants. Soil samples were taken at a depth of 5 - 30 cm, then placed in sterile polypropylene bags and kept at 4^{o}C. The samples were taken to the laboratory for isolation of microorganisms and store at -20^{o}C for further studies.

F. oxysporum and *R. solani* strains were derived from the collection of Mientrung Institute for Scientific Research.

Methods

Isolation of microorganisms

The soil sample (10 g) after removal of the waste was homogenized in a sterile porcelain mortar, then diluted until the concentration of 10^{-6}. The diluted sample (100 µL) at concentrations of 10^{-4}, 10^{-5}, 10^{-6} was spread on petri dishes contained the MPA medium (for bacteria), ISP4 medium (for actinomycetes), and Czapek-Dox (for fungi). The petri dishes were incubated at 30^{o}C - 37^{o}C in 1 - 3 days for bacteria and actinomycetes, and at 28^{o}C - 30^{o}C in 7 days for fungi (Harigan, McCance, 1966).

Assessment of antifungal activity of isolates

Assessment of antifungal activity of isolated bacteria and actinomycetes

Culture solutions of bacteria (24 h) and actinomycetes (48 h) were centrifuged for 30 s to remove biomass, and 100 µL of solution was put into wells (6 mm) on agar plates containing pathogenic fungi (*F. oxysporum, R. solani*). The plates were kept in fridge in two hours to diffuse culture solution in agar. Plates are incubated at 30^{o}C - 37^{o}C for 1 - 3 days. The antifungal activity was determined by inhibition zone diameter (D) minus well diameter (d) (Balouiri *et al.*, 2016).

Assessment of antifungal activity of isolated filamentous fungi

Antifungal activity of isolated filamentous fungi was determined based on the method described by Imtiaj and Lee (2008). Isolated filamentous fungi and fungal pathogen were implanted symmetrically on the agar plates, in which the pathogenic fungus was implanted before one day. After 7 days of incubation at 28^{o}C - 30^{o}C, the colony diameter of pathogenic fungus on the agar plate containing isolated fungi (R2) and without isolated fungi (R1) was measured. Antifungal activity was calculated by the formula: PIMG (%) = (R1 - R2)/R1 × 100.

Identification of high antifungal isolates

Antifungal bacteria and actinomycetes were identified based on 16S rRNA sequences. The bacterial and actinomycete DNA were extracted according to the method described by Sambrook and Russell (2001). The 16S rRNA genes for bacterial and actinomycete strains were amplified using primer pairs 27F/1492R (Lane, 1991) and 27F/1525R (Sambrook, Russell 2001) respectively. The PCR reaction was performed in volume of 25 µL including 5 µL of Taq buffer, 1 µL dNTPs, 0.3 µL of *taq*-polymerase, 1 µL of each primer, 1 µL template DNA, and 15.7 µL nuclease free water. The PCR conditions included an initial denaturation at 94^{o}C for 5 min, followed by 30 cycles (98^{o}C for 60 s, 55^{o}C for 50 s, 72^{o}C for 1.5 min), and elongation at 72^{o}C for 7 min. The PCR products were sequenced on sequencer ABI PRISM 3100. The obtained DNA sequences were removed poor quality ends using BioEdit software v.2.7.5. The high-quality sequences in our study were blasted to sequences in the GenBank database (NCBI) to find their highest similarity sequences, then these sequences were aligned using the ClustalW algorithm. The phylogenetic tree of 16S rRNA sequences was created by the Neighbor Joining algorithm with 1000 bootstraps using MEGA software v.7.0.0.

Fungal strain was identified based on morphological characteristics at Institute 69,

Command Defending Ho Chi Minh Mausoleum. The morphological characters of colony were observed in different media (e.g., Czapek-Dox, PDA). The other characters such as spore, conidia were examined under the Olympus BX43 Microscope (Japan) (Frisvad, Samson, 2004). In addition, the identification of fungal strain was confirmed based on the 18S rRNA gene amplified using primer pairs Eukf/Eukr (Medlin *et al*., 1988). The PCR reaction was performed in volume of 25 µL including 5 µL of Taq buffer, 1 µL dNTPs, 0.3 µL of *taq*-polymerase, 1 µL of each primer, 1 µL template DNA, and 15.7 µL nuclease free water. The PCR conditions included an initial denaturation at 94^{o}C for 5 min, followed by 30 cycles (98^{o}C for 60 s, 58^{o}C for 50 s, 72^{o}C for 1.5 min), and elongation at 72^{o}C for 7 min.

RESULTS AND DISCUSSION

Isolation of microorganisms from soil samples in Tay Nguyen

The microbial density isolated from soil samples in Tay Nguyen was shown in table 1. The results showed that bacterial density was highest (10^5 - 10^6 CFU/g), followed by actinomycetes (10^4 CFU/g), and fungi (10^3 - 10^4 CFU/g). The microbial density in our study was similar to those was isolated and estimated in previous studies (Marinkovic *et al.,* 2012; Raynaud, Nunan, 2014). In the study, soil samples collected from rhizosphere region of diseased pepper plants contained a lower bacterial density than those from rhizosphere region of non-diseased pepper plants. However, fungal density was higher in rhizosphere region of diseased pepper plants than in rhizosphere region of non-diseased pepper plants. This finding indicates that plant pathogenic fungi may have a significant effect on the microbial population in rhizosphere soil. Mendes *et al.,* (2013) have also reported on the complex interactions between rhizosphere microorganisms with various factors, including the interaction of plant pathogenic microorganisms and microbes in rhizosphere regions.

Table 1. Microbial density isolated from soil samples.

Sample	Location	Origin	Bacteria (CFU/g)	Actinomycetes (CFU/g)	Fungi (CFU/g)
CMk	Cu M'gar, ĐakLak	Non-diseased pepper	2.4×10^6	5.4×10^4	1.3×10^3
CSk	Chu Se, Gia Lai	Non-diseased pepper	4.2×10^6	6.2×10^4	3.4×10^3
EHk	Ea H'Leo, ĐakLak	Non-diseased pepper	1.2×10^6	7.1×10^4	1.5×10^3
CM	Cu M'gar, ĐakLak	Diseased pepper	2.1×10^5	2.1×10^4	3.1×10^4
CS	Chu Se, Gia Lai	Diseased pepper	3.4×10^5	3.4×10^4	2.4×10^4
EH	Ea H'Leo, ĐakLak	Diseased pepper	1.7×10^5	1.7×10^4	2.7×10^4

From the isolation plates, the purified 236 bacterial strains, 148 actinomycete strains, and 36 fungal strains were selected for assessment of antifungal activity.

Antifungal activity of microbial strains isolated from soil sample in Tay Nguyen

Antifungal activity of isolates against pathogenic fungi (*F. oxysporum* and *R. solani*) was presented in tables 2, 3 and 4. Obtained results showed that 36 bacterial strains, 6 actinomycete strains and 2 fungal strains had antifungal activity against at least one of the pathogenic fungi. Among the 36 bacterial strains, 11 strains inhibited both fungal pathogens; 10 strains inhibited only *F. oxysporum*, and 15 strains showed antagonistic activity against only *R. solani*. Similarly, 2 actinomycetes among 6 actinomycete strains exhibited antagonistic activity against both fungal pathogens, 3 strains inhibited *F. oxysporum*, and remaining strain inhibited *R. solani*.

Only 2 fungal strains in this study showed antifungal activity, however, they exhibited antagonistic activity against both fungal pathogens. The fungal strain N_1CS_1trk showed strong inhibition against both pathogenic fungi. This strain grew strongly and overlapped the growth of fungal pathogens.

Table 2. Antifungal activity of bacterial strains.

No	Strain	Activity (D-d, mm)		No	Strain	Activity (D-d, mm)	
		F. oxysporum	*R. solani*			*F. oxysporum*	*R. solani*
1	VK_5CM_4. Cđk	-	10.43 ± 1.62	19	VK_6CM_6crk	9.25 ± 0.58	-
2	VK_1CM_5cđk	-	17.60 ± 1.69	20	VK_6CM_2cđ	11.94 ± 1.38	-
3	VK_2CM_5cđk	13.74 ± 1.95	-	21	$VK_5CM_{2,1}$ cr	10.1 ± 0.55	-
4	VK_5CM_5cđk	5.33 ± 1.25	10.02 ± 1.16	22	$VK_9CM_{2,1}$cr	15.07 ± 0.54	-
5	VK_6CM_5cđk	-	9.21 ± 1.25	23	$VK_{14}CM_{3,1}$ cr	-	22.32 ± 0.65
6	$CM_{5.7}$ cđk	8.84 ± 1.23	-	24	$VK1_5CM_{3,1}$ cr	-	17.3 ± 0.41
7	$CM_{5.10}$ cđk	17.01 ± 0.85	-	25	VK_4CM_1tl	-	8.19 ± 1.05
8	$CM_{5.19}$ cđk	8.98 ± 0.7	14.18 ± 1.23	26	VK_5CS_1trk	21.2 ± 0.62	23.0 ± 1.83
9	VK_2CM_6 cđk	-	8.26 ± 0.43	27	$VK_{10}CS_{3,2.}$ tr	15.04 ± 0.92	13.28 ± 0.84
10	VK_3CM_6 cđk	11.08 ± 0.61	9.3 ± 1.30	28	VK_1CS_1tđk	15.1 ± 0.54	-
11	$VK_{10}CM_6$ cđk	-	14.9 ± 1.21	29	VK_1EH_1 tđ	-	10.99 ± 0.58
12	$VK_{10}CM_4$crk	26.9 ± 1.62	24.1 ± 1.21	30	VK_1EH_2 cđ	-	15.68 ± 1.42
13	VK_3CM_5crk	-	12.95 ± 1.33	31	VK_2EH_2cđ	-	20.04 ± 1.89
14	VK_4CM_5crk	-	5.1 ± 0.68	32	VK_1CSk-vp17	-	12.68 ± 1.20
15	VK_5CM_5crk	22.31 ± 1.74	24.96 ± 1.38	33	CSkTi-B6	8.16 ± 1.34	14.94 ± 1.34
16	VK_6CM_5crk	9.0 ± 1.46	13.2 ± 0.82	34	CSkTi-vp10	-	9.75 ± 1.28
17	VK_2CM_6crk	4.17 ± 1.53	-	35	EHkTi-vp18	12.14 ± 1.27	14.0 ± 1.80
18	VK_4CM_6crk	11.31 ± 1.85	-	36	EHkTi-vp24	12.93 ± 1.85	13.7 ± 1.19

Table 3. Antifungal activity of actinomycete strains.

No	Strain	Activity (D-d, mm)		No	Strain	Activity (D-d, mm)	
		F. oxysporum	*R. solani*			*F. oxysporum*	*R. solani*
1	CS_{25} trk	17.26 ± 1.78	-	4	$CS_{3.27}$ tđ	15.98 ± 1.66	-
2	CS_{26} trk	16.12 ± 0.97	15.33 ± 1.15	5	$CM_{5.11}$ cđk	18.08 ± 1.72	16.07 ± 1.67
3	$CS_{3.2.3}$ tr	16.05 ± 1.07	-	6	$CM_{6.28}$ crk	-	10.06 ± 1.01

Table 4. Antifungal activity of fungal strains.

No	Strain	PIMG (%)	
		F. oxysporum	*R. solani*
1	N_1EH_3tđ	11.09 ± 1.34	18.06 ± 1.13
2	N_1CS_1trk	90.22 ± 4.34	20.63 ± 1.37

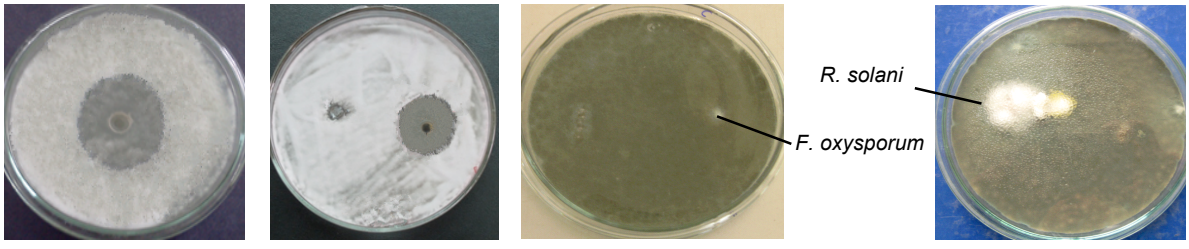

Figure 1. Antifungal activity of some microbial strains. Antifungal activity of the strain $CM_{5.11}$cđk against *F. oxysporum* (A) and *R. solani* (B). Antifungal activity of the strain N_1CS_1trk against *F. oxysporum* (C) and *R. solani* (D)

Identification of antifungal isolates

Identification of antifungal bacteria and actinomycetes

Three bacterial strains and two actinomycete strains inhibiting both pathogenic fungi *F. oxysporium* and *R. solani* were selected for identification based on 16S rRNA sequences. The results (Figure 2) showed that three bacterial strains in this study closed to different genera: $VK_{10}CM_4$crk showed highest similarity (100%) with *Pseudomonas fulva* 67 (FJ972539) on GenBank, whereas VK_5CS_1trk had highest similarity (99%) with *Enterobacter ludwigii* OS5.4 (KX242269), and VK_5CM_5crk exhibited the highest similarity (99%) with *Bacillus subtilis* G-13 (KJ139434). Two actinomycetes had the closest relationship to genus *Streptomyces*: $CS_{2.6}$trk showed the highest similarity (99%) with *Streptomyces* sp. YIM 30823 (AY237555) and $CM_{5.11}$cdk showed the highest similarity (99%) with *Streptomyces diastatochromogenes* WAJ62 (KU877594).

Previous studies have shown that many species in some genera such as *Pseudomonas*, *Bacillus*, *Enterobacter* have antifungal activity against *F. oxysporum* and *R. solani* (Paulitz *et al*., 2000; León *et al*., 2009; Hunziker *et al*., 2015). Species of the genus *Pseudomonas*, *Bacillus*, *Enterobacter* have produced a number of important antibiotic compounds that control plant diseases (Chernin *et al*., 1996, Ligon *et al*., 2000; Raaijmakers *et al*., 2002; Souto *et al*., 2004). Furthermore, *Streptomyces* has also been reported to be capable of producing a wide variety of antibiotic compounds that are resistant to many different pathogens. The antifungal activity of *Streptomyces* and its extracts against *F. oxysporum* and *R. solani* has also been reported in previous studies (Bordoloi *et al*., 2002; Quecine *et al*., 2008).

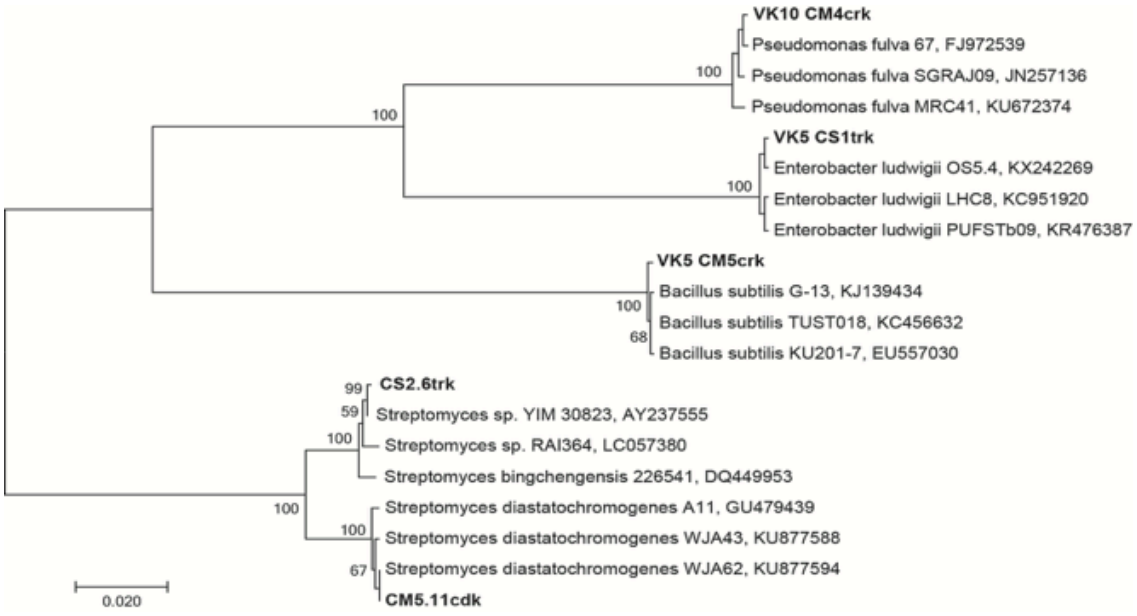

Figure 2. Phylogenetic tree of antifungal strains based on 16S rRNA sequences.

Identification of the fungus N_1CS_1trk

The fungus N_1CS_1trk was transplanted into Czapek-Dox, PDA, PSA media to observe the characteristics of colony. Results showed that the N_1CS_1trk had colony diameter of 4.5 - 5.5 cm after 5 days of incubation on Czapek-Dox medium (Fig. 3). The centre and margin of the colony were green, and the colony surface was velvet with the layers of spores covered. Upside-down of colonies was yellow. The spore of the N_1CS_1trk was examined under the microscope 40x showed that conidiophore was smooth (100 - 200 µm x 3.5 - 4.5 µm) and carried 2 - 3 verticils of metulae. Branches (10 - 20 µm x 3.0 - 3.5 µm) were closely spaced. Branch carried phialides in verticils of 5 - 10. The phialides were 9 - 15 µm x 3.0 - 3.5 µm. Conidia were ellipsoidal and smooth with 4.5 - 6.5 µm x 3.0

- 4.0 µm. These conidia were arranged into long chain 500 µm. These morphological characteristics are similar to those of *Penicillium oxalicum* Currie & Thoms. In addition, identification of $N1CS_1trk$ based on the 18S rRNA sequence also showed that the 18S rRNA sequence of N_1CS_1trk was similarity 100% with those of *P. oxalicum* SAR-3 (JQ349066) on GenBank.

Previous studies have also reported that *P. oxalicum* and some of its extracts exhibited antifungal activity to many plant fungal diseases, including *F. oxysporum* and *R. solani* (Yang *et al.,* 2008). In other studies, Sabuquillo *et al.,* (2005, 2006) studied and used *P. oxalicum* to prevent and control for plant fungal pathogens of tomato plant in greenhouse and fields (Sabuquillo *et al.,* 2005, 2006).

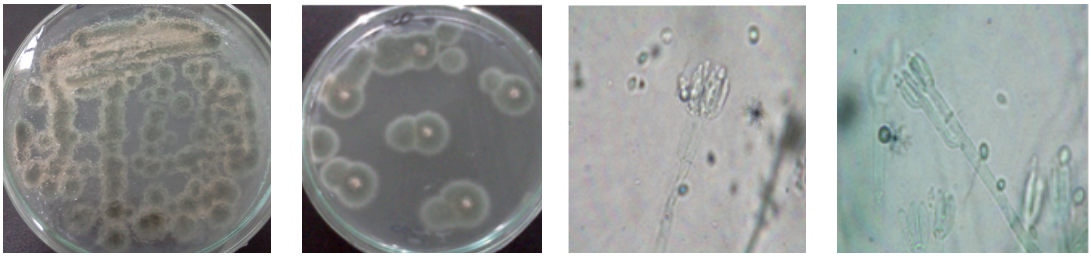

Figure 3. Colony of N_1CS_1trk on Czapek-Dox, PDA media and under microscope 40x.

CONCLUSION

In this study, we isolated 236 bacterial strains, 149 actinomycetes and 6 fungi from rhizospheres of pepper crop in Tay Nguyen, in which 36 bacterial strains, 6 actinomycetes and 2 fungi showed antifungal activity against at least one of two fungal pathogens (*F. oxysporum* and *R. solani*). Identification of selected strains based on morphological and molecular methods showed 3 selected bacterial strains belonged to *Enterobacter ludwigii*, *Pseudomonas fulva*, *Bacillus subtilis*, 2 actinomycetes belonged to the genus *Streptomyces*: *Streptomyces* sp. and *Streptomyces diastatochromogenes*, and the fungal strain belonged to *Penicillium oxalicum*.

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PHÂN LẬP VÀ TUYỂN CHỌN CÁC VI SINH VẬT BẢN ĐỊA KHÁNG NẤM BỆNH CÂY TIÊU Ở TÂY NGUYÊN

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TÓM TẮT

Các loại nấm bệnh thường gây ra các thiệt hại lớn đến nông nghiệp và trên 80% các loại bệnh thực vật được gây ra bởi các loại nấm. *Fusarium oxysporum* và *Rhizoctnia solani* là hai loại nấm gây bệnh cho các loại cây trồng quan trọng ở Tây Nguyên như hồ tiêu, cà phê, cao su, cây điều. Trong nghiên cứu này, chúng tôi tiến hành phân lập các vi sinh vật từ các mẫu đất trồng cây hồ tiêu ở Tây Nguyên và sàng lọc hoạt tính đối kháng với hai loại nấm bệnh (*F. oxysporum* và *R. solani*). Kết quả thu được cho thấy có sự khác biệt về mật độ vi khuẩn giữa các mẫu thu thập từ cây hồ tiêu bị bệnh và không bị bệnh. Mật độ vi khuẩn ở vùng rễ cây không bị bệnh cao hơn ở các cây bị bệnh. Ngược lại, mật độ vi nấm phân lập ở vùng rễ cây không bị bệnh lại thấp hơn so với các vùng rễ cây bị bệnh. Chúng tôi đã lựa chọn 391 chủng bao gồm 236 chủng vi khuẩn, 149 chủng xạ khuẩn và 6 chủng vi nấm để kiểm tra hoạt tính. Trong số các vi sinh vật phân lập, 44 chủng (36 chủng vi khuẩn, 6 chủng xạ khuẩn và 2 chủng nấm) có hoạt tính đối kháng với ít nhất một trong hai loại nấm gây bệnh,

trong đó 15 chủng có hoạt tính đối kháng với cả hai loại nấm gây bệnh. Kết quả định danh các chủng phân lập có hoạt tính cao nhất cho thấy 3 chủng vi khuẩn thuộc các chi khác nhau *Enterobacter ludwigii*, *Pseudomonas fulva*, *Bacillus subtilis*, trong khi hai chủng xạ khuẩn đều thuộc chi *Streptomyces* và chủng vi nấm phân lập thuộc loài *Penicillium oxalicum*.

Từ khóa: *Bệnh thực vật, cây hồ tiêu, Fusarium oxysporum, hoạt tính kháng nấm, Rhizoctnia solani*