TRƯỜNG ĐẠI HỌC SƯ PHẠM TP HỒ CHÍ MINH
TẠP CHÍ KHOA HỌC
KHOA HỌC TỰ NHIÊN VÀ CÔNG NGHỆ
ISSN: 1859-3100 Tập 14, Số 9 (2017): 160-169

HO CHI MINH CITY UNIVERSITY OF EDUCATION
JOURNAL OF SCIENCE
NATURAL SCIENCES AND TECHNOLOGY
Vol. 14, No. 9 (2017): 160-169

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ISOLATION AND SELECTION OF FUNGAL SPECIES FROM THE COMPOST WITH THE CELLULOLYTIC ACTIVITY AND RESISTANCE TOPATHOGENIC PHYTHIUM AND PHYTOPHTHORA CAPSICI FUNGI ON THE PEPPER PLANTS

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Received: 14/8/2017; Revised: 30/8/2017; Accepted: 23/9/2017

ABSTRACT

Pepper is one of the most exported crops in Vietnam. However, pepper farms are usually threatened with diseases, causing deaths over the large areas. The main features of pepper diseases are the abilities to spread rapidly and cause mass death, without recovery or prevention methods. Therefore, the research and production of biological products are essential to prevent, inhibit and resist to fungal diseases. This research focuses on identification of the mold species isolated from the compost, and investigation of their cellulolytic activity, inhibitory or resistant abilities to fungal diseases on the pepper tree (Piper nigrum), in order to produce biological products for fertilization. The results showed that, 2 strains (C1-1 and C1-2) of Aspergillus oryzae was isolated and identified had the fungal resistance to Phytophthora capsici and Pythium, which cause rapid death on the pepper. Therefore, these types of molds can be combined to produce biological products for practical use in agriculture.

Keywords: *Aspergillus oryzae*, resist, pepper, fungus, compost, inhibit.

TÓM TẮT

Phân lập, tuyển chọn các chủng nấm mốc từ phân compost có khả năng phân giải cellulose cao và hạn chế bệnh nấm Phythium, Phytophthora capsici trên cây tiêu

Hồ tiêu là một trong những nông sản có sản lượng xuất khẩu cao nhất Việt Nam. Tuy nhiên, những vườn trồng tiêu thường hay bị đe dọa bởi các loại dịch bệnh, gây chết trên diện rộng. Đặc điểm chính của dịch bệnh trên cây tiêu là khả năng lây lan nhanh và gây chết hàng loạt, không có cách phục hồi hoặc ngăn chặn khi dịch bùng phát. Vì thế, việc nghiên cứu và tạo ra những chế phẩm sinh học có khả năng phòng ngừa, ức chế và đối kháng với các loài nấm bệnh là vấn đề cần thiết. Đề tài tập trung, phân lập và định danh các chủng nấm mốc trong phân compost có khả năng phân hủy cellulose và kháng nấm bệnh trên cây tiêu để định ra hướng tạo chế phẩm sinh học kết hợp trong quá trình bón phân. Kết quả nghiên cứu đã phân lập và định danh được 2 chủng C1-1 và C1-2 thuộc loài Aspergillus oryzae có khả năng đối kháng với hai chủng nấm bệnh Phytophthora capsici và Pythium gây ra hiện tượng chết nhanh trên cây tiêu, có thể sử dụng kết hợp các chủng nấm mốc này để sản xuất chế phẩm sinh học dùng trong thực tiễn.

Từ khóa: *Aspergillus oryzae*, đối kháng, hồ tiêu, nấm mốc, phân compost, ức chế.

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1. Introduction

Vietnam is currently the world's largest pepper supplying nation, accounting for approximately 30.4% of global total pepper production. About 95% of Vietnam's pepper is exported to more than 80 countries and territories, which obtains an export turnover of more than $1 billion per year [1], [3]. However, the sustainability of pepper production, as other crops, is highly dependent on its own anti-pest ability. In addition to the attack of a root-knot nematode, six viruses and two insects, peppers are easily infected by five pathogenic fungi on their trunk, leaves, roots and seeds. In particular, rapid death due to *Phytophthora sp*., yellow leaves due to *Fusarium sp., Phythium sp*. and *Meloidogyne sp*. are the popular causes of the pepper garden recession [1], [4].

Currently, biological products mainly used in pepper plants by farmers are from *Trichoderma* fungi, *Bacillus subtilis*, etc. These products are capable of inhibiting the growth of fungal pathogens in the short term without effects on ecological environment, pepper quality as well as farmers' health [1], [3], [4]. In this study, fungal species with the resistance and growth inhibition abilities to pathogenic *Phythophthora capsici* and *Pythium* fungi, are isolated and identified from the compost created from the domestic solid waste. The success of this study is a basis for the preparation of specific biological products with wide applications in the pepper cultivation.

2. Research methods

2.1. Samples for the isolation of fungi

Compost was produced from the following components: dried cow dung, grass, vegetable waste and etc. during the 8-week aerobic incubation in the solid waste laboratory, in the Institute of Environmental Science, Engineering and Management, at Industrial University of Ho Chi Minh City [5], [6]. Microorganisms were isolated from the samples immediately after sample taking, and the following experiments were performed continuously during 6 weeks.

2.2. Methods for isolation of fungi

0.1 mL of diluted compost was pipetted into Czapek medium (30g Saccharose, 1.0g K_2HPO_4, 0.5g $MgSO_4$, 0.5g KCl, 0.01g $FeSO_4$, 3g $NaNO_3$, 20g Agar, 1000 ml distilled water; pH 5.0 - 5.5). The compost droplet was spread across the agar surface in Petri dishes by sterile glass spreaders. These Petri dishes were then placed upside down, sealed and kept at 37°C in the incubator chamber for 5-7 days [7], [8]. Based on the differences in colony morphology and color on the agar surface, mold species were selected and cultured repeatedly on new Petri dishes to obtain the pure lines, which were then stored on agar slants in test tubes.

2.3. Investigation of cellulose-degrading potentials

CMC medium was made from 3g $NaNO_3$, 1g K_2HPO_4, 0.5g $MgSO_4$, 0.5 KCl, 0.01g $FeSO_4$, 5g CMC, 20g agar, 1000mL distilled water, pH 7.0 – 7.4 and sterilized at 121^oC;

1.0 atm for 30 minutes, then poured into sterile petri dishes. A small amount of biomass of the isolates above was taken and implanted in the center of the agar plate by using an inoculation loop. Each species was repeated 3 times. After incubation at 37ºC for 5-7 days, small droplets of Lugol reagent on the agar surface would create a colorless bright hydrolysis halo zone around the colony [9], [10]. The diameter (d) of the colony and the diameter (D) of the hydrolysis halo were measured. The higher the D-d ratio was, the higher cellulose-degrading ability was.

2.4. Investigation of fungal resistance

The resistant activity of the isolated fungal strains to the pathogenic fungi was evaluated by co-culture method on PDA medium (200g potato extract, 20g glucose, 20g agar, 1000mL distilled water, pH 7, 0 - 7,4) [4], [12]. *Pythium* fungus was provided by the Open University of Ho Chi Minh City and *Phytophthora capsici* fungus was provided by the National Science and Technology Development Fund. Respectively, one pathogenic fungus was implanted in the center of each petri dish, together with one fungal strain isolated from the compost at the position of 3.0-5.0 cm away from the pathogenic fungus. Growth-inhibiting ability was determined after 4-to-7-day culture at 37 °C.

2.5. Fungal identification methods

Identification of isolated fungal strains relied on macroscopic and microscopic observations. Macroscopic observations included morphological characteristics, mycelia color and spore color on the medium seen by the naked eye. Microscopic observations included specimen preparation, fungal mycelia and spore imaging under the microscope.

Then, the results were compared with the classification system of ~~Dr.~~ Dang Vu Hong Mien (2015) to identify the name of mold species isolated from the compost [13].

3. Results

3.1. Results of fungi isolation

Table 1. *Isolation and evaluation of appearance frequency of fungal species*

Mark (No)	Appearance frequency	Macroscopic observations		Mark (No)	Appearance frequency	Macroscopic observations	
		Colony color and diameter	Spore color			Colony color and diameter	Spore color
C1-1	+++	White, 4cm	Light green	C1-13	+	White to dark brown, 5cm	Light brown
C1-2	+++	White, 2cm	Dark yellow	C1-14	+	White, 1.5cm	Opaque white
C1-4	++	Pale white, 5cm	Light brown	C1-15	+	Opaque white, 6cm	Very light brown
C1-5	+++	Milk white, 5cm	Black	C1-16	+	Milk white, 3.5cm	Light brown
C1-6	+++	White to yellow, 1.5cm	Black	C1-17	++	Pale white, spreading	Cinereous

						develop	
C1-7	+	Opaque white, spreading develop	Grey	C1-19	+	Milk white, 1cm * yellow medium	Opaque white
C1-8	+	White, 3cm	Light brown	C1-20	+	White, 1.5cm	Milk white
C1-9	+++	Gray white, 4cm	Grey blue	C1-21	+	Pale white, spreading develop	Light orange
C1-10	++	Milk white, 4.5cm	Blue	C1-22	+	White, 4.5cm	Opaque white
C1-11	++	Dark white, 5.5cm	Very pale blue	C1-23	+	White, spreading develop, 10cm	Pale blue

** The proliferation of these species excreted substances turning the medium to yellow.*

From 6 compost samples, 20 mold species were isolated, marked from C1-1 to C1-23. The appearance frequency of these species was divided into 3 levels: most frequent (+++), less frequent (++) and rare (+) appearance. The frequency of appearance of one species was evaluated based on the appearance times when compared to those of other species.

All fungal species had substrate mycelium with opaque color to opalescent color, embedding in the nutrient agar, among them, C1-19 made the medium become yellow. The colony diameter of these 20species varied from minimum of 1cm (C1-C19) to maximum of 10cm (C1-C23). The colonies of only 3 species (C1-7, C1-17 and C1-21) spread all over the agar surface, but did not develop into characteristic colony clusters. All 20 species produced fungal spores after 7-day culture with various colors: orange, brown, blue and black.

Regarding appearance frequency, 5 species most usually seen were C1-1, C1-2, C1-5, C1-6 and C1-9. Eleven species with low appearance frequency were C1-7, C1-8, C1-13, C1-14, C1-15, C1-16, C1-19, C1-20, C1-21, C1-22 and C1-23.

3.2. Investigation of cellulose-degrading activity

The cellulolytic activity was evaluated by measuring hydrolysis halo diameter of cellulase enzyme secreted from 20 isolated species, as illustrated in Table 2 and Figure 1.

Table 2. *The proportion of cellulolytic fungi isolated from the compost and their cellulose-degrading potentials*

Cellulolytic degree	Hydrolysis halo diameter (mm)	The number of fungal species	Percentage (%)
No	0	4	20
Weak	< 10	10	50
Average	10 – 14	4	20
Strong	15 – 20	2	10
Very strong	> 20	0	0

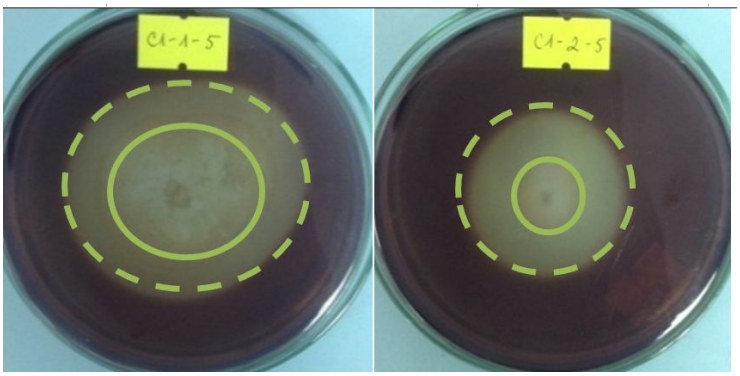


Figure 1. *Hydrolysis halos of C1-1 and C1-2 species*

The result showed that, 4 isolated mold species did not have cellulolytic activity, whereas, other species had this activity with weak to average degree. Among them, two highest cellulolytic species were C1-2 and C1-1 with the largest hydrolysis halos diameter of 16mm and 14mm, respectively. Therefore, 16 species in 20 isolated fungal species with cellulose-degrading ability can be used in fertilization to promote humus production.

3.3. Investigation of resistance ability to pathogenic Phytophthora capsici

The resistance ability to pathogenic fungi of 20 isolated species from the compost was investigated by co-culturing, in which *Phytophthora capsici* grew together with every isolated fungal species. This ability is divided into two types of anti-proliferative mechanisms: (1) Inhibiting activity: useful fungi inhibit the growth of pathogenic fungi, but do not kill them completely and (2) Resisting activity: useful fungi extirpate pathogenic fungi by creating resistance circle to prevent the proliferation of pathogenic fungi.

The result showed that 10 species had the resistance to pathogenic *Phytophthora capsici*. Among them, 9 species had inhibiting activity, and 1 species (C1-19) could create resistance circle to *Phytophthora capsici* with the circle diameter of 18mm (Figure 2). Hence, the resistance of these species to *Phytophthora capsici* is mainly based on the growth inhibition, which could be owing to the nutrient competition and inhibition of secondary products excreted from the growth of useful fungi to the development of pathogenic fungi.

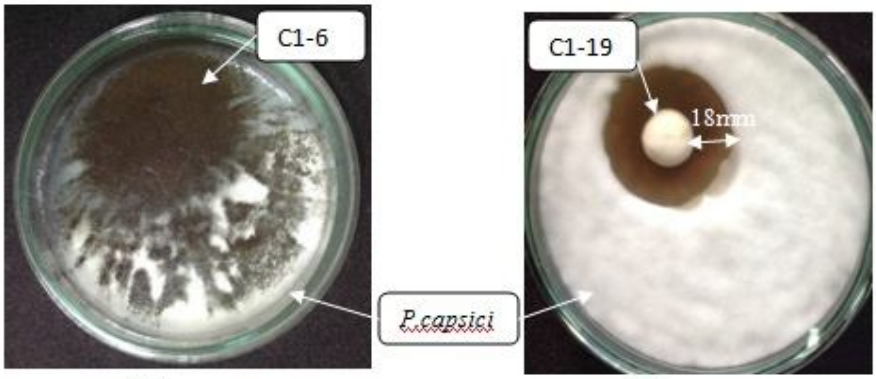


Figure 2. *The inhibition activity of C1-6 species to the proliferation of P.capsici (A) and the resistance activity (resistance circle)* of *C1-19 species to P.Capsici (B)*

3.4. Investigation of resistance to pathogenic Pythium

The experiments on the resistance to pathogenic *Pythium* were carried out similarly to the experiments of *Phytophthora capsici*. The result showed that 11 mold species in total could be resistant to the development of *Pythium*, among them, only C1-19 species could create the resistance circle and other species had inhibitory activity to the growth of *Pythium* (Figure 3).

The research on resistance to two popular pathogenic fungi – *Phytophthora capsici* and *Pythium* showed that 8 fungal species isolated from the compost could inhibit simultaneously the proliferation of these both pathogenic fungi, especially, C1-19 species could create resistance circle to both *Phytophthora capsici* and *Pythium*. The culturing period of these isolated species was 2 to 6 days enough to perform their resistance to the pathogenic fungi.

Figure 3.*The resistance circle of C1-19 species to Pythium*

In summary, the study on resistance to pathogenic *Phytophthora capsici* and *Pythium* showed 10 species (accounting for 50% of isolated fungal species) with inhibitory action to *Phytophthora capsici* and 11 species (55% of isolated species) with inhibitory activity to *Pythium*. Among them, 8 species (accounting for 40% of isolated species) prevented the growth and spread of both *Phytophthora capsici* and *Pythium*.

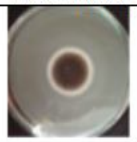
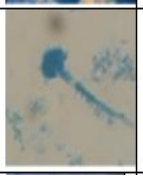
3.5. Identification results of isolated fungal species

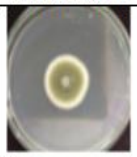
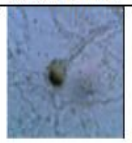
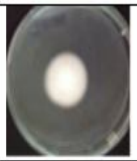
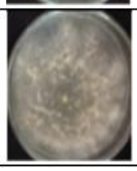
After experiments in the resistance of 20 isolated species, these mold species were identified by macroscopic and microscopic observations. The result was obtained by comparing macroscopic and microscopic observations, as illustrated in Table 3. The result showed that only 11 species were determined according to the classification system of ~~Dr.~~ Dang Vu Hong Mien (2015), other species were the coincidence.

The result showed that all mold species belonged to mesophilic group, with 8 species (72.7%) of *Aspergillus* genus, other species of *Amblyosporium*, *Coccidioides* and *Cladosporium* genus. Compared to the study of A. Anastasi *et al.*, 2005, the fungal community isolated from the compost comprised of 66 species belonging to *Aspergillus, Cladosporium, Acremanuon* and *Penicilium*, among them, lower than 50% were *Aspergillus* [5]. This difference can be owing to the components of the compost, which decide the microorganism system in the compost [6], [14].

With regard to the cellulolytic potential, the study of Khokhar *et al.*, (2012) isolated 17 fungal species except *Aspergillus*. Among them, 3 species had excellent cellulose-degradation ability, with the highest hydrolysis halo diameter of 9cm for *Penicilium* and *Trichoderma* after 7 days [7]. Pham Bich Hien *et al* (2011) isolated 2 species among 55 fungal species with the maximum cellulolytic halo diameter of 2.8cm, but could not identify the name of these 2 species [10]. Therefore, compared to previous studies, this research isolated and determined two cellulolytic fungal species (C1-1 and C1-2) belonging to *Aspergillus oryzae* with the halo diameter of 14cm and 16cm.

Table 3. *Identification results of fungal species isolated from the compost*

Strain	Macroscopic Observation	Microscopic Observation	Scientific Name
C1 - 5, C1- 6			*Aspergillus niger*
C1 - 1, C1- 2			*Aspergillus oryzae*
C1 - 4, C1 - 22			*Aspergillus fumigatus*
C1 - 9			*Aspergillus clavatus*

Strain	Macroscopic Observation	Microscopic Observation	Scientific Name
C1 - 10			*Aspergillus ochraceus*
C1 - 19			*Coccidioides spp.*
C1 - 20			*Cladosporium spp.*
C1- 21			*Amblyosporium spp.*

C1-19 species with high anti-proliferative ability to pathogenic *Phytophthora capsici* and *Pythium*was classified into *Coccidioides* genus. This is a hazardous genus because it causes coccidioidomycosis disease, commonly called as rift valley fever with serious consequences. Thus, *Coccidioide* fungiare only researched in laboratories with accreditation of biological safety, III grade. And therefore, they are not allowed to use for preparation of biological products in agriculture despite their high resistance to pathogenic fungi on crops, especially on pepper plants.

4. Conclusion

This study isolated 20 mold species in the compost incubated from municipal solid waste in aerobic conditions. Identification of 11 species among these 20 species showed that 8 species belonged to *Aspergillus* genus (72.7%), and other species belonged to *Amblyosporium, Coccidioides* and *Cladosporium* genus.

Among these 20 species, 16 species had cellulose-degrading ability with the minimum hydrolysis halos diameter of 2mm. Two species (C1-1 and C1-2) identified as *Aspergillus oryzae* had the highest cellulolytic activity with the hydrolysis halos diameter of 14mm and 16mm, respectively.

The results show that 8 fungal species isolated and identified from the compost had the growth inhibition and resistant ability to two pathogenic fungi *Pythium* and *Phytophthora capsici*. Although *Coccidioides sp.* (C1-19) had high anti-proliferative abilities to fungal pathogens, it would not be biologically safe enough to be utilized as

biological products. In addition, C1-1 and C1-2 species belonged to *Aspergillus oryzae* with high cellulolytic potential and resistant ability to these two pathogenic fungi.

In conclusion, compost produced from domestic solid waste is a large reservoir for fungal species. In the compost, *Aspergillus oryzae* (C1-1 and C1-2) can be used to produce biological products for fungal diseases on pepper plants due to its high anti-fungal characteristics. Finally, it is essential to extend this research to produce more environmentally friendly biological products with reasonable costs for agriculture and pepper industry in particular.

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