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Disease-reducing effects of antagonistic soil bacteria on Fusarium basal rot of shallot caused by *Fusarium oxysporum* in Vĩnh Châu, Sóc Trăng

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ABSTRACT

Fusarium basal rot caused by Fusarium oxysporum is one of the most important diseases of shallot (Allium cepa var. ascalonicum). This study aims at isolating soil bacteria from Vĩnh Châu (Sóc Trăng) and testing for their antagonistic and disease-reducing effects which could be used for biological control of the disease. A number of 224 isolates was collected and tested for their antagonistic effects on mycelial growth and conidia germination of the pathogen. Among those, three isolates exhibited the strongest effects. These isolates were tested for their disease-reducing effects under greenhouse conditions using two application methods, i.e., bulb coating and soil drenching. Two treatments using ATB-24 and ATA-33 (10^8 CFU/mL) showed the highest effects on disease incidence and severity; their effects remained through all assessment time points (until 65 days after planting). The results suggest the applicability of the ATB-24 and ATA-33 isolates as biological control agents for Fusarium basal rot of shallot.

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1 INTRODUCTION

Shallots (*Allium cepa* var. *ascalonicum*) have been commonly cultivated in Southeast Asia. It is an important vegetable crop in many Asian countries and widely used as an additive in food processing (Tashiro *et al.*, 1982). In Vietnam, shallots are cultivated largely at Vĩnh Châu-Sóc Trăng, Lý Sơn-Quảng Ngãi and the suburb of Hà Nội (Quách Nhị, 2009). Vĩnh Châu, Sóc Trăng has been well known for its tradition in shallot cultivation in Vietnam. However, the excessive use of fertilizers and chemicals results in higher susceptibility of shallots to diseases, which leads to shallot yield and quality losses (Đặng Thị Cúc, 2008). Fusarium basal rot (FBR) caused by *Fusarium oxysporum* is one of the most destructive diseases of shallots (Sintayehu *et al.*, 2014). It is widely distributed in most large cultivation areas around the world, especially in temperate and subtropical regions (Cramer, 2000). Application of chemicals is the most common means in disease management. Although this measure shows high efficacy and instant effects, the use of chemicals can lead to various adverse effects on environment and human. Moreover, it can result in emergence of novel chemical-resistant pathogens (Sigee, 2005).

A sustainable disease control measure is at requirement in order to reduce the use of chemicals as well as to promote sustainable and eco-friendly agriculture. From its sustainability and eco-friendliness, biological control using antagonistic bacteria is one of the promising measures that attract scientists' interest recently. There have been many studies on using antagonistic bacteria for controlling plant pathogens on various kinds of host plants such as root rot on cucumbers (*Pythium*

aphanidermatum), damping-off on sugar beet (*Rhizoctonia solani*) and leaf blight disease on corn (*Pantoea* sp.) (Moulin *et al.*, 1994; Thrane *et al.*, 2001; Javandira *et al.*, 2013). These are solid premises for applying antagonistic bacteria in FBR control, which is a promising solution for Vĩnh Châu in the progress of developing sustainable and less chemical-depended cultivation.

2 MATERIALS AND METHODS

2.1 Soil collection and isolation of soil bacteria

Soil samples were collected from uninfected shallot fields in the epidemic areas in Vĩnh Châu, Sóc Trăng. Ten grams of each soil sample were agitated in a sterile Erlenmeyer flask containing 100 mL sterile distilled water for 30 minutes. Then, the suspension was diluted 1000-fold with sterile distilled water. A volume of 50 µL of this dilution was spread on nutrient agar (NA) plates that contained 5 g peptone, 3 g beef extract, 5 g NaCl, 15 g agar and distilled water for 1 L medium, pH 6.8. After 24 hours of incubation at 28°C, based on colony morphology, well-separated individual colonies were isolated and transferred to new NA plates until single pure bacterial colonies were obtained. Bacterial isolates were stored in 100% glycerol at -20°C for following experiments.

2.2 Testing for inhibitory effects of soil bacteria on mycelial growth and conidial germination of *F. oxysporum* under laboratory conditions

2.2.1 Testing for inhibitory effects of soil bacteria on mycelial growth of F. oxysporum under laboratory conditions

Pathogen preparation

The virulent *F. oxysporum* was provided by the Plant Pathology Group of the Molecular Biology Laboratory, Biotechnology Research and Development Institute, Can Tho University. *F. oxysporum* was incubated on the potato dextrose agar (PDA) plate, which contained 250 g sliced potatoes, 20 g dextrose, 20 g agar, distilled water for 1 L medium for 7 days.

Dual culture technique

The test was conducted based on the dual culture technique described by Dhanasekaran *et al.* (2012). Bacterial isolates from 2-day-old NA cultures were dotted at four points which were 3 cm away from the center of the PDA plate, and each isolate was done in triplicate. The mycelium disc (diameter 0.5 cm) of 7-day-old PDA culture of *F. oxysporum* was placed at the center of the plates which were incubated at 28 ± 2°C for 7 days.

Data collection

The radii of inhibition zones around bacterial isolates were measured after 7-day incubation as described by Kim *et al.* (2008). Bacterial isolates which expressed inhibitory effects on mycelial growth of *F. oxysporum* were selected for the following experiment.

2.2.2 Testing for inhibitory effects of soil bacteria on conidial germination of F. oxysporum under laboratory conditions

Mixture preparation

The experiment was conducted as described by Rodríguez-Algaba *et al.* (2015). Conidia harvested from 7-day-old PDA culture of *F. oxysporum* was diluted with sterile distilled water to obtain the concentration at 5 x 10^3 conidia/mL, 150 µL of this suspension was mixed with 500 µL each of the 2-day-old NB cultures of the antagonistic bacterial isolates (10^9 CFU/mL). NB medium (NA without agar) was mixed with conidial suspension as a control. A volume of 50 µL of each mixture was incubated in 1.5 mL micro-centrifuge tubes in darkness in a shaker at 28 ± 2°C, 80 rpm for 2 days. The test was performed in triplicate.

Data collection

After 2 days of incubation, the conidial germination was determined by recording the number of conidia germinated under light microscopy (minimum 30 conidia studied per replication). The inhibitory effects on conidial germination were determined by comparing the number of germinated conidia of control to those of the bacterial mixtures.

2.3 Testing for disease-reducing effects of antagonistic bacterial isolates under greenhouse conditions

The experiment was arranged in a completely randomized design. For each antagonistic bacterial isolate, two application methods were tested, i.e. bulb coating and soil drenching, each at three cell density levels (10^9, 10^8, 10^7 CFU/mL). Shallot bulbs were treated with Score 250 EC (fungicide compound difenoconazole at concentration 23.2% W/W, Syngenta) (Nguyễn Thị Nguyệt, 2014) in the positive control and with sterile distilled water in the negative control.

Soil preparation and shallot cultivation

The mixture of soil with rice straws and husks at the mass ratio 2:2:1 was sterilized by autoclaving at 121°C, 1 atm in 30 minutes. Then, round pots (height 10 cm x diameter 17 cm) were used for containing the mixture. The disease-free shallot bulbs were provided via the Plant Protection Department in Sóc Trăng. The shallot bulbs were planted in a pot by burying their lower three-quarters into the ground after eliminating all old roots. Five bulbs were grown in each pot. The plants were watered daily and fertilized with recommended dose instructed by the Plant Protection Department in Sóc Trăng (Đặng Thị Cúc, 2011).

Inoculum preparation and inoculation method

Conidial suspension (10^7 conidia/mL) was harvested from 7-day-old PDA cultures of *F. oxysporum*. The experiment was divided into two cases which differed in inoculation time.

The first inoculation time point was at 30 days after planting shallot bulbs which was the most sensitive period of shallots with soil pathogens (Đặng Thị Cúc, 2011). Conidial suspension of *F. oxysporum* at concentration 10^7 conidia/mL was directly sprayed on the shallot root with the amount of 5 mL suspension per pot.

The second inoculation time point was before planting to simulate the situation in which shallot bulbs had already been infected before planting. Shallot bulbs were coated with *F. oxysporum* conidial suspension at concentration 10^7 conidia/mL (Stankovic *et al.*, 2007).

Preparation and application of antagonistic bacterial suspension

For each inoculation time point, there were two application methods (bulb coating and soil drenching) for antagonistic bacteria. Antagonistic bacterial isolates from 2-day-old NA culture were suspended in sterile distilled water and the suspensions were adjusted to three cell density levels of 10^9, 10^8, 10^7 CFU/mL by measuring the absorbance at 600 nm. For bulb coating, the shallot bulbs were dipped in bacterial suspensions at three cell density levels before planting (30 mL per 100 bulbs). For soil drenching, bacterial suspensions at three cell density levels were sprayed on the soil at 2 days before planting (5 mL per pot).

Data collection

The incidence of FBR was recorded 5 times at 7-day intervals from 30 days after planting (DAP) (37, 44, 51, 58, 65 DAP). Percent infected bulbs and disease severity were collected from the experiment. Percent infected bulbs was the number of infected bulbs over the total bulbs. Disease severity was recorded on a 0–4 scale, where 0 = no symptom, 1 = up to 10% rotted roots, 2 = more than 10 to 30% rotted roots with up to 10% rotted basal plates, 3 = completely rotted roots and more than 10 to 30% rotted basal plates, and 4 = completely rotted roots and more than 30% rotted basal plates (Sintayehu *et al.*, 2014). FBR severity scores were converted into percent severity index (PSI) according to the formula:

PSI = (sum of numerical rating x 100)/ (number of plants scored x maximum score on scale)

2.4 Data analysis

Mean radii of inhibition zones were calculated by Microsoft Excel. The data of greenhouse experiment were calculated using IBM SPSS Statistics v.16.0. Mean data of treatments were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test and all hypotheses were rejected at $P \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 Inhibitory effects of soil bacterial isolates on mycelial growth and conidial germination of *Fusarium oxysporum* under laboratory conditions

There were 224 bacterial isolates obtained from soil samples from Vĩnh Châu, Sóc Trăng. Inhibition zones were observed in 19 out of 224 bacterial isolates by using dual culture method as described in Section 2. Among these 19 isolates, 3 isolates showed the inhibition zone radii greater than 5 mm (Table 1). The biggest inhibition zone was observed in isolate ATB-24 with the radius 5.7 mm (Fig. 1). However, these 3 isolates expressed no inhibitory effects on conidial germination of *F.* oxysporum. They were obtained from healthy or less infected shallot fields. The presence of antagonistic bacteria in nature contributes to lower disease incidence observed at these sites (Nguyễn Đắc Khoa *et al.*, 2010).

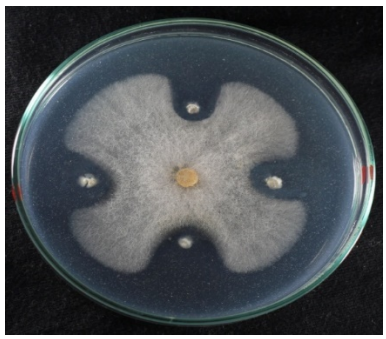

Fig. 1: The inhibition zone of ATB-24 against Fusarium oxysporum on PDA plate after 7 days

Table 1: The radii of inhibition zones of antagonistic bacterial isolates against Fusarium oxysporum after 7 days

No.	Isolate	Inhibition zone radius (mm)
1	VA 30	0,3
2	LH 4	0,4
3	K1P1 8	0,5
4	LH 2	0,6
5	CLA 16	0,7
6	LH 25	0,7
7	ATB 22	0,7
8	ATA 17	0,8
9	ATA 19	0,8
10	ATB 30	0,9
11	ATB 2	1,1
12	LH 15	1,2
13	CLA 14	1,2
14	ATB 10	1,3
15	ATA 34	1,7
16	LH 16	1,8
17	ATA 33	5,2
18	ATB 32	5,5
19	ATB 24	5,7

3.2 Disease-reducing effects of antagonistic bacterial isolates under greenhouse conditions

Three criteria were set up to select effective treatments in this experiment. Firstly, treatments needed to have high disease-reducing effects. Secondly, their effects were durable throughout five assessment time points. Finally, low cell density level was required. Disease-reducing effects would be evaluated through percent infected bulbs and PSI.

3.2.1 Inoculation before planting

For the case of inoculation before planting, the effective treatments that had significantly lower PSI from both application methods were shown in the Table 2. With bulb coating, three treatments ATA-33 at 10^9 CFU/mL, ATB-24 at 10^9 and 10^8 CFU/mL had the highest efficacy in disease reduction with significantly lower PSI compared to that of negative control. Their efficacy also maintained through five assessment time points from 37 DAP to 65 DAP. With soil drenching, three treatments ATA-33 at 10^9 CFU/mL, ATB-24 at 10^9 and 10^8 CFU/mL were still the most effective ones with the significantly lower PSI compared to that of negative control. However, only two out of three treatments were durable through five assessment time points. The treatment ATB-24 at 10^8 CFU/mL only had its efficacy in four out of five time points, from 44 DAP to 65 DAP.

With bulb coating, ATB-24 at 10^9 CFU/mL was the only treatment maintaining its efficacy through five assessment time points. Two remained treatments showed their efficacy with statistically significant low percent in an unstable way. ATA-33 at 10^9 CFU/mL had efficacy at four assessment time points except 58 DAP and ATB-24 at 10^8 CFU/mL at three assessment time points except 44 DAP and 58 DAP. With soil drenching, ATB-24 at 10^9 CFU/mL was also the only treatment maintaining its efficacy through all assessment time points. Two treatments ATA-33 at 10^9 CFU/mL and ATB-24 at 10^8 CFU/mL had a reverse pattern with their results in bulb coating. ATB-24 at 10^8 CFU/mL had efficacy at four assessment time points except 58 DAP and ATA-33 at 10^9 CFU/mL at three assessment time points except 44 DAP and 58 DAP.

Although the treatment ATB-24 at 10^9 CFU/mL expressed its significant low PSI and percent infected bulbs in both application methods at all assessment time points, it had a high cell density level, which leads to difficulties in incubation. Moreover, efficacy of ATB-24 at 10^8 CFU/mL and 10^9 CFU/mL showed no statistical difference at different assessment time points. Therefore, according to aforementioned criteria, ATB-24 at 10^8 CFU/mL was chosen as the most effective treatment for both application methods.

Percent infected bulbs of these effective treatments were shown in Table 3.

Table 2: Percent severity index of effective treatments when inoculating before planting

Application methods	Treatment	Percent severity index				
		37 DAP	44 DAP	51 DAP	58 DAP	65 DAP
Bulb coating	ATA-33 (10^9 CFU/mL)	5.45^{efg}	11.3^{c-f}	19.5^{ghj}	35.5^{b-f}	48.3^{f}
	ATB-24 (10^9 CFU/mL)	4.09^{g}	9.97^{f}	19.1^{hj}	32.2^{ef}	48.4^{f}
	ATB-24 (10^8 CFU/mL)	5.86^{c-f}	12.1^{c-f}	21.1^{d-j}	34.9^{b-f}	51.4^{def}
Soil drenching	ATA-33 (10^9 CFU/mL)	6.40^{b-f}	12.0^{c-f}	20.8^{e-j}	33.0^{def}	49.8^{ef}
	ATB-24 (10^9 CFU/mL)	5.01^{fg}	12.0^{c-f}	17.7^{j}	29.1^{f}	48.3^{f}
	ATB-24 (10^8 CFU/mL)	6.83^{a-e}	12.7^{b-e}	20.6^{f-j}	34.6^{c-f}	51.3^{ef}
	Negative control	8.32^{a}	15.6^{a}	26.6^{a}	43.1^{a}	67.4^{ab}
	Positive control	0.00^{h}	4.95^{g}	10.3^{k}	15.3^{g}	26.9^{g}

In the same column, means followed by the same letters are not significantly different at $P \leq 0.05$

DAP: days after planting

Table 3: Percent infected bulbs of effective treatments when inoculating before planting

Application method	Treatment	Percent infected bulbs				
		37 DAP	44 DAP	51 DAP	58 DAP	65 DAP
Bulb coating	ATA-33 (10^9 CFU/mL)	7.86^{bc}	15.7^{c-f}	27.6^{b-e}	46.2^{ab}	64.2^{cd}
	ATB-24 (10^9 CFU/mL)	5.59^{c}	12.8^{d}	24.5^{de}	40.8^{b}	60.1^{d}
	ATB-24 (10^8 CFU/mL)	8.51^{bc}	17.9^{a-d}	29.2^{b-e}	46.1^{ab}	67.8^{bcd}
Soil drenching	ATA-33 (10^9 CFU/mL)	8.90^{bc}	16.9^{a-d}	28.2^{b-e}	43.0^{ab}	62.4^{cd}
	ATB-24 (10^9 CFU/mL)	7.61^{bc}	15.2^{cd}	23.8^{e}	39.1^{b}	64.9^{cd}
	ATB-24 (10^8 CFU/mL)	8.74^{bc}	16.6^{bcd}	26.5^{cde}	44.1^{ab}	65.9^{bcd}
	Negative control	13.7^{a}	22.3^{a}	36.0^{a}	53.5^{a}	79.8^{ab}
	Positive control	0.00^{d}	6.62^{e}	15.9^{f}	23.9^{c}	40.0^{e}

In the same column, means followed by the same letters are not significantly different at $P \leq 0.05$.

DAP: days after planting.

3.2.2 Inoculation after planting 30 days

The effective treatments that had significantly lower PSI from both application methods were shown in the Table 4.

With bulb coating, three treatments ATA-33 at 10^9 CFU/mL and 10^8 CFU/mL, ATB-24 at 10^9 CFU/mL had the high efficacy in disease reduction with the significantly lower PSI compared to that of negative control. The treatment ATB-24 at 10^9 CFU/mL had its significantly low PSI through five assessment time points while two remained treatments only showed their efficacy from 44 DAP to 65 DAP. With soil drenching, three treatments ATA-33 at 10^9 CFU/mL, ATB-24 at 10^9 and 10^8 CFU/mL were the most effective ones with significantly lower PSI compared to that of negative control. The treatment ATB-24 at 10^9 CFU/mL was the only one having efficacy durable through all assessment time points. The treatment ATA-33 at 10^9 CFU/mL and ATB-24 at 10^8 CFU/mL showed their efficacy at four assessment time points except 37 DAP.

Table 4: Percent severity index of effective treatments when inoculating after planting 30 days

Application method	Treatment	Percent severity index				
		37 DAP	44 DAP	51 DAP	58 DAP	65 DAP
Bulb coating	ATA-33 (10^9 CFU/mL)	8.67^{abc}	13.0^{gh}	23.0^{b-e}	35.5^{c-e}	56.1^{cd}
	ATB-33 (10^8 CFU/mL)	8.88^{a}	14.6^{c-h}	24.0^{b-e}	38.0^{b-e}	56.6^{bcd}
	ATB-24 (10^9 CFU/mL)	6.59^{c}	13.5^{d-h}	21.7^{de}	32.9^{de}	52.7^{cd}
Soil drenching	ATA-33 (10^9 CFU/mL)	7.80^{abc}	13.1^{fgh}	22.6^{cde}	34.5^{de}	53.8^{cd}
	ATB-24 (10^9 CFU/mL)	6.69^{bc}	11.9^{h}	20.6^{e}	31.6^{e}	51.5^{d}
	ATB-24 (10^8 CFU/mL)	7.29^{abc}	13.3^{e-h}	22.8^{b-e}	35.1^{de}	53.5^{cd}
	Negative control	9.40^{a}	18.8^{a}	28.3^{a}	47.1^{a}	70.3^{a}
	Positive control	0.00^{d}	6.97^{i}	11.8^{f}	18.5^{f}	25.8^{e}

In the same column, means followed by the same letters are not significantly different at $P \leq 0.05$.
DAP: days after planting.

Percent infected bulbs of these effective treatments were shown in Table 5.

With bulb coating, two treatments ATA-33 at 10^9 CFU/mL and ATB-24 at 10^9 CFU/mL showed their efficacy in disease reduction with significant low percent infected bulbs at all assessment time points. The treatment ATA-33 at 10^8 CFU/mL showed its efficacy at only three assessment time points 44 DAP, 58 DAP and 65 DAP. At two remained time points, the treatment had insignificantly low percent infected bulbs compared to that of negative control. With soil drenching, ATA-33 at 10^9 CFU/mL was the only treatment expressing the efficacy unstably at three out of five assessment time points (44, 58 and 65 DAP) while two other treatments of ATB-24 at 10^9 and 10^8 CFU/mL expressed and maintained their efficacy through all assessment time points.

For inoculation after planting 30 days, the most effective treatments in corresponding with two application methods were different. With bulb coating, ATA-33 at 10^8 CFU/mL was the effective treatment with low PSI and percent infected bulbs maintained through many assessment time points. Moreover, its low cell density level was an advantage for later applications. Therefore, this treatment was chosen for the bulb coating method. With soil drenching, the treatment ATB-24 at 10^8 CFU/mL was the chosen treatment which satisfied aforementioned criteria.

Table 5: Percent infected bulbs of effective treatments when inoculating after planting 30 days

Application method	Treatment	Percent infected bulbs				
		37 DAP	44 DAP	51 DAP	58 DAP	65 DAP
Bulb coating	ATA-33 (10^9 CFU/mL)	9.72^{bcd}	16.7^{cd}	27.9^{bc}	45.6^{bc}	68.2^{bcd}
	ATB-33 (10^8 CFU/mL)	12.1^{a-d}	19.4^{bcd}	30.3^{abc}	46.0^{bc}	68.0^{bcd}
	ATB-24 (10^9 CFU/mL)	9.30^{bcd}	17.2^{bcd}	27.6^{bc}	42.1^{bc}	65.8^{cd}
Soil drenching	ATA-33 (10^9 CFU/mL)	10.4^{a-d}	18.4^{bcd}	30.1^{abc}	46.1^{bc}	70.1^{bcd}
	ATB-24 (10^9 CFU/mL)	8.01^{c}	14.6^{de}	25.2^{c}	38.6^{c}	59.7^{d}
	ATB-24 (10^8 CFU/mL)	8.98^{bc}	17.8^{bcd}	28.8^{bc}	43.1^{bc}	65.8^{cd}
	Negative control	14.1^{a}	27.8^{a}	38.3^{a}	60.4^{a}	84.2^{a}
	Positive control	0.00^{e}	9.93^{e}	16.0^{d}	25.7^{d}	37.9^{e}

In the same column, means followed by the same letters are not significantly different at $P \leq 0.05$.
DAP: days after planting.

All treatments of ATB-32 at three cell density levels with two application methods expressed no efficacy in disease reduction at both inoculation time points. Most effective treatments were of isolate ATB-24. Their antagonistic effects which lead to a reduction in disease incidence could be from one mechanism or from the combination of different mechanisms. In bacterial growth, they can excrete some kinds of metabolites such as antibiotics and other lytic enzymes as chitinase, glucanase, which can play partial role in suppressing plant pathogenic activities. In the study by Défago and Haas (1990), the combination of various metabolites of *Pseudomonas fluorescens* such as siderophore, phenazine, 2,4-diacetylphloroglucinol and cyanide (CN^-) was proved to contribute to the antagonistic effects against *Gaeumannomyces graminis* var. *tritici* and *Chalara elegans*. Besides, a previous study has found that various compounds from *Bacillus* strains with antifungal and antibacterial effects have been used in disease reduction on plants (Todorova and Kozhuharova, 2010). Many actinomycetes strains, particularly *Streptomyces* spp., can inhibit plant fungal pathogens by producing antifungal compounds. For example, the study by Prapagdee *et al.* (2008) showed that extracellular chitinase and β-1,3-glucanase produced by strain *Streptomyces hygroscopicus* had antagonistic effects on the growth of plant fungal pathogens *Colletotrichum gloeosporioides* and *Sclerotium rolfsii*. In the current study, the colony morphology of antagonistic bacterial isolates on NA showed the features which are expected to be actinomycetes. Identification of the antagonistic bacterial isolates is being carried out. Moreover, the competition of nutrients and space between the antagonist and the pathogen was also one of the most important mechanisms for suppression of plant disease. One of the best documented examples was the competition for iron in soil of *Pseudomonas putida* with *Fusarium* (Scher, 1986). The competition can lead to the suppression of pathogenic growth, resulting in disease reduction. Some antagonistic bacterial strains can also induce plant resistance against the progression of pathogenic strains (Hammerschmidt, 2007).

4 CONCLUSIONS

There were 224 bacterial isolates obtained from shallot fields Vĩnh Châu, Sóc Trăng. From these isolates, 19 isolates expressed antagonistic effects on *F. oxysporum* under laboratory conditions. Three isolates ATA-33, ATB-24 and ATB-32 showed highest inhibitory effects on mycelial growth, but none of them had the inhibitory effects on conidial germination of *F. oxysporum*. In greenhouse experiment, with the inoculation before planting, bulb coating and soil drenching with ATB-24 (10^8 CFU/mL) were the most effective treatments. However, with the inoculation after planting 30 days, with bulb coating, the effective treatment was ATA-33 at 10^8 CFU/mL and with soil drenching, it was ATB-24 at 10^8 CFU/mL.

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