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Potential of local *Bacillus* spp. isolates as wilt disease biocontrol agents for *Fusarium oxysporum* f. sp. *cepae* on *Allium x wakegi*

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Abstract. *Asrul. 2023. Potential of local* Bacillus *spp. isolates as wilt disease biocontrol agents for* Fusarium oxysporum *f. sp.* cepae *on* Allium x wakegi. *Biodiversitas 24: 4989-4997.* Implementation of biological agents for eradication and inhibition of plant pathogens is often ineffective due to its protracted consequences. The objective of this research was to examine the potential of local isolates of *Bacillus* spp. as a biocontrol agent in suppressing *Fusarium* wilt disease (*Fusarium oxysporum* f. sp. *cepae*) on wakegi onions (*Allium* × *wakegi* Araki). The research was conducted in a completely randomized design (CRD) with the treatment of rhizospheric bacterial isolates. The treatments included control (without isolate application) and seven bacterial isolates, namely KP17, KP5, DB9, DB12, DB18, DG4 and DG11. Each treatment was repeated five times and each replication consisted of 10 wakegi onion plants. The results showed that a total of 46 isolates were isolated from the rhizosphere of wakegi onion and seven isolates among them were selected as biocontrol agents. These isolates had similarities with *Bacillus* spp. in accordance with the colony morphology, physiology and biochemical characteristics. Among the isolates found, DB12 emerged as the isolate that had the potential to be developed as a biocontrol agent.

Keywords: *Bacillus* sp., biocontrol, *Fusarium oxysporum*, local isolate, rhizosphere, wakegi onion, wilt disease

INTRODUCTION

Wakegi onion (*Allium* × *wakegi* Araki) is one of the primary vegetable widely cultivated by farmers, especially in the Sigi Regency of Central Sulawesi. Wakegi onions are predominantly consumed as fried onions, while shallots are chiefly used as spice ingredient in cooking. Characteristically, shallots can flower in either lowlands or highlands, while wakegi onions cannot. Wakegi onion is the result of a natural interspecific cross between leek (*Allium fistulosum* L.) and shallot (*Allium cepa* L. *aggregatum* group) (Setyowati et al. 2013). The wakegi onion cultivars include 'Palasa', 'Tinombo', 'Palu Valley' (Central Sulawesi) and 'Sumenep' (Madura, East Java) (Sulistyaningsih et al. 2008).

One of the main obstacles in the development of wakegi onion cultivation in Sigi Regency is the presence of wilt disease/basal rot of stems, or moler disease caused by the fungus *Fusarium oxysporum* Schlechtend (Cramer et al. 2021). Foc (*Fusarium oxysporum* Schlechtend) fungus attacks can cause yield losses of up to 50% (Kalman et al. 2020) and are even capable of causing crop failure if the environment is fungi-favour (Prabowo et al. 2020). Local farmers have faced this challenge especially during the commencement of rainy season when the risk of pathogen attack is higher, so it can be handled immediately. Therefore, the presence of this disease could not be ignored, because even though the Foc attack is slow, once the onion gets infected it can cause plant death. In order to inhibit Foc fungus, farmers still predominantly practice applying precipitating pesticide residues that are detrimental to the environment and human health (Chethana et al. 2012; Suganda et al. 2019). Presence of pesticide residues on consumable vegetable and fruits is imperatively concerning due to its hazardous effects on health. Cultivation of Foc-resistant varieties is conventionally more practical, effective and ecological, however there are only few varieties that possess resistance ability against these pathogens.

The application of biological agents is currently drawing attention from farmers for its characteristic advantages namely, cost-effectiveness, safer option for farmers and the environment and sustains ecological balance. However, the biological agents historically applied have been specific, affecting their survivability and restricting their adaptability to develop in a new environment unlike their natural habitat. Therefore, it is imperative to develop control technology that is adaptive to the local ecosystem and sustainable based on the principle of 'from nature for nature' by utilizing antagonistic local isolates of rhizosphere bacteria (indigenous). This strategy not only facilitates local bacterial isolates to be comparatively more adaptive to survive but also provides better potential to suppress pathogens in their origin area than isolates introduced from other areas (Sarris et al. 2012). Utilizing local antagonist microbes to control pathogens in the area would provide relatively systemized results compared to antagonistic microbes introduced from outside.

One of the potential rhizospheric bacteria that could be recommended as a substitute for synthetic pesticides and widely used as a biological control agent is *Bacillus* spp.

This bacterial species are not only capable of inhibiting growth of various pathogenic soil-borne fungi and bacteria (Ramyabharathi and Raguchander 2014) but are also abundantly found in soil, water, air and decaying plant material (Basamma and Kulkarni 2017) that comprise several species namely, *B. amyloliquefaciens*, *B. cereus*, *B. pumilus*, *B. licheniformis* and *B. subtilis* (Ajilogba et al. 2013; Ann et al. 2015).

Bacillus spp. represses growth of pathogenic fungi through antagonistic mechanisms such as antibiosis (antibiotics), enzyme production, competition (space and nutrition), parasitism (bacteria moves and adheres to surface of germinated through polarity and secretes catalytic enzymes), induces systemic resistance in plants (through the jasmonic acid pathway) and siderophore production (Ruiz-Sanchez et al. 2016; Ali et al. 2020). This study aimed to investigate the potential of local isolates of *Bacillus* spp. as biological control agents to suppress *Fusarium* wilt disease in wakegi onions.

MATERIALS AND METHODS

Study area

This research was conducted from April to September 2021 and was categorized into two stages. The first stage was performed in the laboratory, that included isolation, hypersensitivity reaction test, characterization of rhizosphere bacterial isolates based on colony morphology, physiology and biochemistry, and inhibition power test. On the other hand, the second stage was conducted in the greenhouse, which included suppression tests of bacterial isolates against *Fusarium* wilt disease in wakegi onion plants.

Procedures

Research preparation

The tools used during the research were 0.9 mm hole sieve, microscope, LAF (Laminar Air Flow), autoclave, hand spray, hemacytometer, micropipette, test tube, Erlenmeyer flask, glass spatula, watch-glass and petri dish. The material in this research was *Bacillus* spp. from rhizosphere soil of wakegi onion plants, fungi *F. oxysporum* f. sp. *cepae*, healthy wakegi onion bulbs, sterile water, toothpicks, potato dextrose agar (PDA) and nutrient agar (NA) media and 75% alcohol.

Rhizosphere soil sampling

Soil samples were obtained from the rhizosphere layer of healthy wakegi onions located in Petobo Village (Palu City), Bulupontu/Sidera Village (Sigi Regency) and Guntarano Village (Donggala Regency). Soil samples were collected using a small shovel in the area around the plant roots. Next, the sample was wrapped in wet paper and kept in a plastic bag and stored in an ice flask to be brought to the laboratory.

Laboratory test

Isolation of Bacillus *spp. from the rhizosphere of wakegi onion plant*

Isolation of antagonistic bacteria was conducted using the serial dilution method (Gajbhiye et al. 2009; Munif et al. 2012; Butar-butar et al. 2018). Cultures were incubated at room temperature for 24 to 48 hours. The grown colonies were then purified using the same medium and tested for hypersensitivity reactions, colony morphology characterization, as well as physiological and biochemical properties testing.

Hypersensitivity reaction test

A hypersensitivity reaction test of antagonistic bacterial isolates to tobacco plants was done according to Asrul et al. (2013). Isolates that showed negative reaction (no symptoms of necrosis but the leaves remained green) in the inoculated tobacco leaf area were then tested for antagonists to determine their inhibitory power.

Characterization of rhizospheric bacterial isolates

Characterization of rhizosphere bacterial isolates was conducted according to the method proposed by Di et al. (2023). This characterization aimed to determine the genera of bacterial isolates isolated from the rhizosphere of the wakegi onion. Morphological characteristics of *Bacillus* spp. were observed visually on the seventh day after being rejuvenated on NA media. The characterizations included colony morphology (shape, color, margin, elevation, texture and optical properties), gram reaction, produce fluorescent pigment, facultative anaerobes, oxidation, catalase and growth at the temperature of 65°C. The observations were then compared with the morphological characteristics of the colonies previously described by Calvo and Zuniga (2010), Lu et al. (2018), Nurcahyanti and Ayu (2020), Prihatiningsih et al. (2020) and Di et al. (2023).

In vitro inhibition power test of local isolates

The inhibition power test of rhizospheric bacteria against FOC pathogenic fungi (laboratory collection) was conducted using dual culture technique according to Jimtha et al. (2016). The inhibitory activity was observed based on the presence of a clear zone formed around the bacterial colonies. Cultures were incubated at room temperature and antagonism interactions were observed at 7 days after inoculation (dai). Percentage of rhizospheric bacterial inhibition was calculated by the formula (Lamsal et al. 2012): $P = (R-r)/R \times 100\%$, where P = the percentage of antagonist inhibition power against pathogens, R = the maximum radius of the pathogenic colony stay away from the rhizosphere bacterial colony (cm), and r = the radius of the pathogenic colony opposite with the rhizosphere bacterial colony (cm).

Greenhouse test

Preparation of antagonist bacterial suspension

The preparation of the suspension was carried out aseptically by means of rhizospheric bacterial colonies on rejuvenation media, which were 48 hours old, were taken

using a loop needle and suspended in a tube containing 10 mL of sterile water. Furthermore, the suspension was dispensed into the mother liquor to calculate the density of colony population, which was done by the spread dish method. A series of culture tubes containing 9 mL of sterile distilled water were taken, and serial dilutions of 10^{-5}, 10^{-6} and 10^{-7} were made. A total of 1 mL from each dilution series was pipetted and grown on NA medium to which 100-ppm cycloheximide was added to restrict fungal growth. Each dilution series was replicated three times. Bacterial colonies that grown were also counted to determine their population density. Next, the suspension was diluted again to obtain a population density of 10^8 CFU/mL for further application.

Preparation of pathogenic fungal suspension

Preparation of conidial suspension of *F. oxysporum* f. sp. *cepae* was carried out by retrieving 1 Ose sample from the mushroom colony that was rejuvenated, then transferring it to 9 mL of sterile distilled water and homogenized using a vortex mixer to form a suspension. The formed suspension was used to calculated conidial density using a hemacytometer under a microscope at a magnification of 400 times. Next, the suspension was diluted to obtain fungal conidia density of 10^8 conidia/mL for application.

Fusarium *wilt suppression test*

The trial of rhizospheric bacterial suppression as a biocontrol agent against *Fusarium* wilt disease on wakegi onions was conducted in a greenhouse. The research was conducted in a completely randomized design (CRD) with the treatment of rhizosphere bacterial isolates. The treatment included control (without isolate application) and seven isolates, namely KP17, KP5, DB9, DB12, DB18, DG4 and DG11, a total of eight treatments were used for this test. Each treatment was repeated 5 (five) times and each replication consisted of ten wakegi onion plants.

Treatment application

The planting medium used was a mixture of sifted and sterilized soil and manure as basic fertilizer (2:1). The soil was sterilized by tyndallization in a drum with hot steam (temperature of 65°C) for three consecutive days. After the sterilization period was completed, the soil was allowed to cool, then put into a 3-kg polybag. Planting healthy wakegi onions was done by immersing seeds (bulbs) into the soil and in the afternoon.

Disease suppression test was conducted by growing wakegi onions until they reached 28 days of age, after which pathogens were applied. Suspension of pathogen *F. oxysporum* f. sp. *cepae* as much as 10 mL/bulb (density 10^8 conidia/mL) was poured into polybag planting holes. A suspension of rhizospheric bacterial isolates, as much as 20 mL/polybag (10^8 CFU/mL), was introduced into the soil in polybags one week before the inoculation of *F. oxysporum* f. sp. *cepae*. Furthermore, the infestation of isolate suspension was repeated every 7 days, with up to four total applications. The control treatment (without suspension of bacterial isolates) was used as a comparison.

Observation

Disease suppression was observed on the 56 (fifty-six) days after the infestation of biocontrol agents. The parameters were the incubation period and the percentage of disease incidence. The incubation period was observed from the time of pathogen was first inoculated until the initial symptoms of *Fusarium* wilt disease appeared. The intensity of disease was calculated from the onset of symptoms at an interval of 7 days. The percentage of disease incidence was calculated using the formula proposed by Shamyuktha et al. (2020) and Lilai et al. (2021).

Data analysis

The data obtained were then analyzed statistically by ANOVA (Analysis of Variance). If there was a significant difference, then the Tukey's HSD (Honestly Significant Difference) test was performed at the 5% level.

RESULTS AND DISCUSSION

Laboratory test

A total of 46 bacterial isolates were isolated from the rhizospheric layer of wakegi onion plant. The results of hypersensitivity reaction test showed that seven local isolates produced negative reactions. Negative reactions were observed in tobacco leaves injected with local isolate bacterial suspension, which did not cause water soaking and necrosis symptoms but remained green. The seven local isolates consisted of two isolates (KP17 and KP5) from Petobo Village (Palu City), 3 isolates (DB9, DB12 and DB18) from Bulupontu/Sidera Village (Sigi Regency) and two isolates (DG4 and DG11) from Guntarano Village (Donggala Regency). The results of negative reaction affirmed that the local isolate bacteria obtained from the rhizosphere of wakegi onion were not plant pathogenic, but might belong to the group of antagonistic bacteria. Mougou and Boughalleb-M'Hamdi (2018) reported that one of the antagonistic bacteria that reacted negatively to the hypersensitivity test is *Bacillus* spp.

The bacteria that were pathogenic reacted positively (compatible) in the hypersensitivity test and necrotic spots on the leaf area where the bacterial suspension was injected. The necrosis symptom is one of the characteristics of a hypersensitive reaction (Asrul et al. 2019). On the other hand, non-pathogenic bacteria reacted negatively (incompatible) by not changing the color of the leaves (remaining green), because necrosis was not visible. Hypersensitivity reactions are a mechanism of plant resistance triggered in response to pathogenic infections in the form of the rapid death of plant tissue cells to localize pathogens so that plants could overcome infections that have the potential to cause disease (Mougou and Boughalleb-M'Hamdi 2018).

Local isolate inhibition power test

The results of antagonist test of each isolate measured by observing the percentage of inhibition *in vitro* are depicted in Table 1.

The results of antagonist test on 7- day demonstrate that all local isolates of rhizosphere bacteria obtained could inhibit the growth of pathogenic mycelium from 43.28 to 67.66%. The highest inhibition power was found in DB12 isolates at 67.66% and the lowest was in KP5 isolates with an inhibition power of 43.28%. The DB12 isolate exhibited the highest effect and showed significant inhibition effect, causing more than 60% inhibition of mycelium growth in comparison to isolates KP17, KP5, DB9, DB18, DG4 and DG11 (<60%). Thus, strain DB12 was chosen to be developed because it had the best antifungal potential as indicated by a significant inhibition effect.

The inhibition test was conducted to obtain rhizosphere bacterial isolates that had the potential as biocontrol agents against plant pathogens. The results of *in vitro* inhibition power test using the dual culture method showed that all local isolates bacteria were able to inhibit the growth of Foc, but each isolate displayed different percentages of inhibition power. The difference in inhibition effect that occurred was probably due to the different strains of wakegi onion rhizosphere bacteria, which secreted distinct secondary metabolite compounds, both in production (volume) and type. According to Nega (2014), the production of secondary metabolites is strain-dependent, namely different strains of the same bacterial species could produce distinct products and types of metabolites so that the effect may be non-identical on the target pathogen. Even though the isolates are in the same species or genus, they are not necessarily the same strain because the phenotypic character could vary due to environmental effects (not static). Therefore, strain selection was a crucial element in the development of bacterial strains as biocontrol agents for plant diseases.

All local isolates of rhizospheric bacteria were able to form a clear zone on the PDA media (Figure 1A). Isolates of rhizospheric bacteria that germinate and can form clear zones are distinguished as isolates that have an antibiosis mechanism in suppressing the growth of pathogenic fungal colonies (Margani et al. 2018).

The inhibition power of local isolates on the growth of Foc mycelia was presumed to lead to an antibiosis antagonist mechanism. Antibiosis mechanism involves production of secondary metabolites in the form of antibiotics or antibiotic-like compounds, such as lytic enzymes, volatile compounds, siderophores and other toxic substances (Prihatiningsih et al. 2015). The antibiosis mechanism was characterized by forming a clear zone between isolates of rhizosphere bacteria and pathogens on solid media (PDA). The formation of clear zone signified the presence of an antibiosis mechanism of the isolate against pathogens (Margani et al. 2018). The presence of clear zone stipulated the presence of active secondary metabolites (antibiotics) secreted by isolates of rhizosphere bacteria that had surfaced in the PDA media (Abbas et al. 2019). Secondary metabolites produced by antagonistic bacterial strains demonstrated antifungal potential (Ali et al. 2020). The antifungal activity is exhibited with the formation of a clear zone produced by the activity of antibiotic compounds (Andriani et al. 2017).

Antibiotic production is a fundamental expedient in augmenting the competitiveness of rhizosphere bacteria such as *Bacillus* spp. under a restricted resource environment (Chalasani et al. 215). Antibiotics are secondary metabolites produced by an organism in small amounts that could eradicate or inhibit other microorganisms (Andriani et al. 2017). Toxic effects of antibiotic compounds could interfere with the normal growth process of pathogenic fungi such as deformations in the shape of mycelia (malformations), as well as inhibit the growth of mycelia and spore germination (Lamsal et al. 2012). The malformations in inhibition power test were indicated by the shortening of colonies (mycelia) that approached the rhizosphere bacterial isolates. This shortening of the colony was a form of abnormal growth in hyphae, which hampered mycelia development on artificial media (PDA) around the rhizosphere bacterial colony.

The inhibition of pathogenic fungi growth by rhizosphere bacterial isolates confirmed that isolated local isolates had the potential to produce several secondary metabolites such as antifungal compounds. Khedher et al. (2015) reported that the formation of a clear zone in an *in vitro* inhibition power test of mycelia growth of *Rhizoctonia solani* fungus indicated that *B. subtilis* bacteria produced antifungal compounds. Many antifungal compounds secreted by *Bacillus* spp. have been reported, including fengycin, bacilisin, lipopeptide (Caulier et al. 2018). These secondary metabolites played an important role in the control of plant pathogens (Jayaprakashvel et al. 2011).

Table 1. Locations of various bacterial isolates isolated from the rhizosphere of wakegi onion and the average inhibition power (%) of isolates against Foc

Locations	Isolates code	Inhibition power (%)
Petobo	KP17	48.82 ± 3.06
	KP5	43.28 ± 1.27
Bulupontu	DB9	51.47 ± 1.34
	DB12	67.66 ± 3.91
	DB18	49.70 ± 1.36
Guntarano	DG4	46.25 ± 2.76
	DG11	51.19 ± 4.02

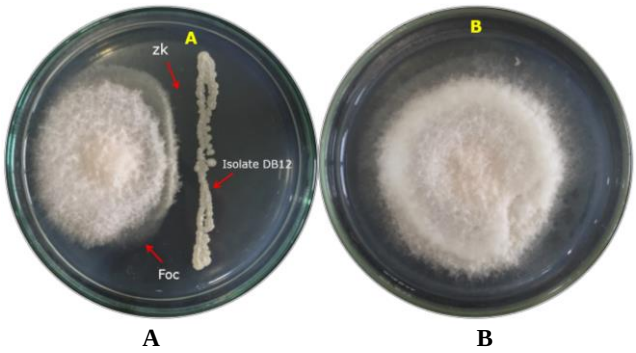

Figure 1. *In vitro* inhibition power test of DB12 isolate on the mycelium growth of *F. oxysporum. cepae*: clear/empty zone (zk) (A) and pathogen colony (B)

Secondary metabolite compounds secreted by rhizosphere bacterial isolates to suppress the pathogenic fungi mycelia growth on PDA media were hypothesized to be lipopeptide antibiotics compounds. Although the identity of antibiotic was not tested in the present research, the assumption was based on the ability of the lipopeptide to be heat-resistant and antifungal. In this research, the suspension of rhizosphere bacterial isolates obtained was heated prior to being used for the inhibition power test. Ruiz-Sanchez et al. (2016) reported that lipopeptide compounds such as iturin are antifungal and heat-resistant. Malviya et al. (2020) added that lipopeptides are stable at high temperature and pH, and demonstrated distinguished ability as biocontrol agents against various types of pathogens. Furthermore, it has also been reported that almost all bacteria species of *Bacillus* spp. produced antimicrobial compounds known as lipopeptides. *Bacillus* spp. are known to have a capsule-shaped structure containing a polypeptide of D-glutamic acid and are spore-producing bacteria (Puspita et al. 2017). *Bacillus subtilis* spores in the form of endospores could survive in extreme environmental conditions or were very tolerant of heat and drought (El-Gayar 2017; Asthana et al. 2016). Cyclic lipopeptides are categorized into three groups, namely iturin, fengycin and surfactin (Mieuwesteeg 2017)

The ability of local isolates of rhizosphere bacteria to inhibit pathogens exhibited that these isolates were able to act as biological control agents. A microbe is distinguished as a biological agent if it could inhibit the development and growth of other microbes (Bonaterra et al. 2022). Among isolates of rhizospheric bacteria found, isolate DB12 was the most effective bacteria strain, with the potential for inhibiting the growth of Foc pathogenic mycelium more than 60% *in vitro*. According to Noverizaa et al. (2004), a microbe is said to be antagonistic if it has inhibitory activity above 60% against pathogenic mycelium. Thus, the isolate produced an inhibition zone large enough to prevent physical contact with the pathogen.

Results revealed that DB12 isolate had the potential to be utilized and developed as a biological agent in controlling pathogens in plants. This strong inhibition power could be used as an indicator of the ability of DB12 isolates as a biocontrol agent to suppress the growth of pathogens in the field.

Characteristics of local isolates of bacteria

The macroscopic characterizations of all colonies of local isolates exhibited antibiosis activity on NA media are presented in Table 2.

Based on macroscopic observations, colonies of local isolates were round or circular shape, dull-white or slightly yellow in color, opaque optical properties, flat elevation and rough and dry texture with medium colony size (Figure 2A). The results of observations of local isolate colonies had similarities with the characteristics of *Bacillus* spp. bacteria described by Calvo and Zuniga (2010), Lu et al. (2018) and Di et al. (2023). Figure 2B depicts that the center of local isolates colony had a concentric ring-shaped circle and an irregular center border. These characteristics were found similar to *Bacillus* spp.

Table 2. The morphological characteristics of isolated colonies of rhizosphere bacterial

Characteristics	Isolates code						
	KP17	KP5	DB9	DB12	DB18	DG4	DG11
Shape	Round	Irregular	Irregular	Irregular	Round	Round	Round
Color	Dull-white	Beige	Dull-white	Dull-white	Beige	Beige	Beige
Edge	Jagged	Flat	Jagged	Jagged	Flat	Flat	Flat
Elevation	Flat	Flat	Flat	Flat	Flat	Flat	Flat
Texture	Fine	Rough and dry	Fine	Fine	Rough and dry	Rough and dry	Rough and dry
Colony size	Medium	Medium	Medium	Medium	Medium	Medium	medium
Appearance	Dull	Dull	Dull	Dull	Dull	Dull	Dull
Optical properties	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Slimy/not slimy	Not slimy	Not slimy	Not slimy	Not slimy	Not slimy	Not slimy	Not slimy

Table 3. Physiological and biochemical characteristics of local isolates of wakegi onion

Characteristics	Isolates code						
	KP17	KP5	DB9	DB12	DB18	DG4	DG11
Gram reaction	+	+	+	+	+	+	+
Fermentative (OF)	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Fluorescent Pigment	-	-	-	-	-	-	-
Growth at 65°C	+	+	+	+	+	+	+

Note: (+) = positive reaction/bacteria grow; (-): negative reaction/bacteria do not grow

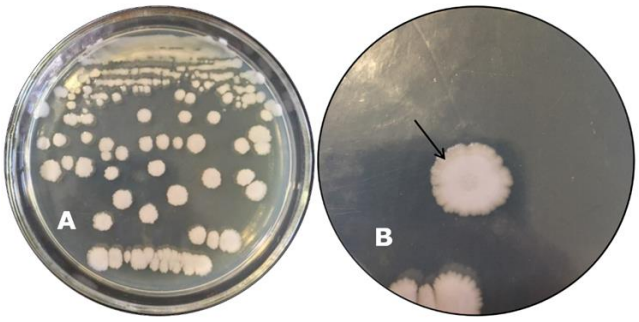

Figure 2. Macroscopic characteristics of bacterial isolates of DB12; (A) a single colony of local isolate; (B) concentric ring-shaped bacterial colony (arrow)

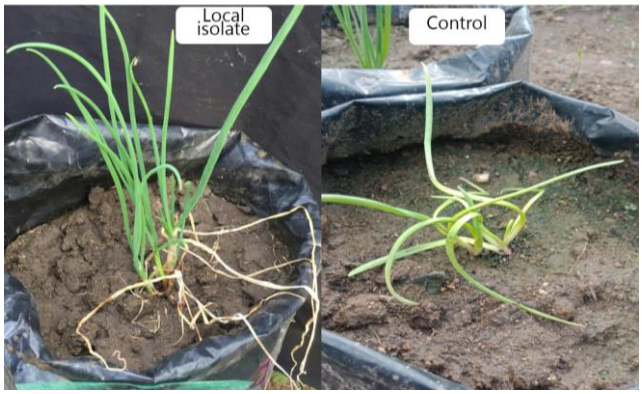

Figure 3. Suppression test of *Fusarium* wilt disease

Table 4. The average incubation period (dai), disease incidence in various local isolates of wakegi onion rhizosphere. The average incubation period, disease incidence and disease suppression effectiveness of various local isolates

Local isolates code	Incubation period (DAI)	Disease incidence (%)	Disease suppression effectiveness (%)
Control	5.45 ± 0.11a	98.57 + 1.91a	-
KP17	13.63 ± 0.22b	48.49 ± 3.81b	50.80
KP5	17.03 ± 0.45c	57.52 ± 3.41c	41.64
DB9	16.80 ± 0.14c	59.34 ± 5.51c	39.80
DB12	19.73 ± 0.31e	31.50 ± 2.52d	68.04
DB18	17.88 ± 0.19d	60.61 ± 7.72c	38.51
DG4	14.15 ± 0.13b	43.77 ± 6.81b	55.59
DG11	17.45 ± 0.46c	62.38 + 5.97c	36.71

Note: Numbers followed by the same letter and column show no significant difference based on the Tukey's HSD test at a significance level of 5; DAI = day after inoculation

Based on the physiological and biochemical characteristics, the local isolates were gram-negative bacteria, aerobic or fermentative anaerobic, with positive oxidation, positive catalase, did not produce fluorescent pigments and were able to grow at a temperature of 65°C (Table 3 and Figure 3). These characteristics were similar to the characteristics of *Bacillus* spp. bacteria that have been described by Nurcahyanti and Ayu (2020), Prihatiningsih et al. (2020) and Di et al. (2023). Based on the morphological characteristics of colonies as well as physiological and biochemical properties, the local isolates obtained were bacteria of the *Bacillus* genus.

Greenhouse test

The incubation period of *Fusarium* wilt disease was observed daily, from the beginning of the inoculation of pathogen until the infection occurred, which was expressed in the form of the first symptoms on the wakegi onion plant. Early visible symptoms were wilting of leaves which later became bent or twisted starting from the top and yellow coloration (chlorosis). The results of data analysis showed that the application of local *Bacillus* spp. isolates had a significant effect on the incubation period of *Fusarium* wilt disease (Table 4).

Table 4 depicts that application of local isolates resulted in a significantly longer incubation period than the control. In control, the incubation period required for the appearance of the first symptoms was 5.45 dai, while in the application of local isolates there was a delay in the appearance of disease symptoms with an incubation period between 8.18-14.28 dai. This indicates that local isolates protect plants from pathogen attack resulting in a slower incubation period compared to control. The DB12 isolate had the longest incubation period (19.73 dai) and was significantly different from all tested isolates.

The appearance of first symptoms of *Fusarium* wilt disease occurred more quickly in the control due to the absence of *Bacillus* spp. bacteria as a biocontrol. The absence of *Bacillus* spp. biocontrol agents caused the pathogen to infect plant roots quickly, resulting in a short incubation period before symptoms appeared. *F. oxysporum* is known to be able to grow and develop in the soil and infect plant roots quickly (Audenaert et al. 2021). The incubation period was longer than the control because the *Bacillus* spp bacteria applied were local isolates obtained from the rhizosphere of wakegi onions in the field. Local isolates are known to be able to adapt (pH, nutrition, temperature and humidity) to soil planted with wakegi onions in polybags. It was also observed that all bacteria applied were able to adapt, grow and develop well in polybags, which was indicated by a delay in the incubation period. The ability to adapt to the environment in artificial media accelerates microbial growth followed by an increase in the ability to produce toxins (Ram et al. 2019). In the field optimum environmental factors may support the ability of antagonists to suppress soil-borne pathogens. Santhanam et al. (2015) stated that the use of local antagonist microbes tended to have better adaptability in comparison to the isolates introduced from elsewhere. The delay in the incubation period of wilt disease on wakegi onions suggests that the bacteria applied were actively working to inhibit the development of pathogens. The activity that occurred was probably a form of the mechanism of antagonistic bacteria in antibiosis in

suppressing and inhibiting the growth of pathogens. According to Ali et al. (2020), antibiosis mechanism of *Bacillus* spp. played a key role in the biocontrol of plant diseases. The antibiotic activity of the *Bacillus* spp. biocontrol organisms occurred with the production of pathogen-inhibiting antibiotics arresting the incubation period. This is in the line with the statement by Marzaini and Mohd-Aris (2021) that antagonist mechanism of *Bacillus* spp. lead to the ability to produce antibiotics. The ability to produce antibiotics is a fundamental mechanism of *Bacillus* spp. bacteria in protecting plants against pathogen attacks.

Krzyzanowska et al. (2013) reported that *B. subtilis* is a bacterium commonly found in soil, especially in the rhizosphere area and it is an effective biocontrol agent against plant pathogens due to its ability to produce various kinds of antibiotics. *Bacillus subtilis* is known to produce lipopeptide compounds that are antibiotic and have broad spectrum, such as iturin, surfactin and fengycin (Ruiz-Sanchez et al. 2016; Płaza et al. 2013; Hashem et al. 2019). These compounds have demonstrated potential antagonistic activity against pathogenic bacteria and fungi in both *in vitro* as well as planta conditions to suppress disease incidence and severity (Ruiz-Sanchez et al. 2016; Lilai et al. 2021).

The results of data analysis exhibit that the application of local isolate bacteria had a significant effect on the incidence of *Fusarium* wilt disease (Table 4). In addition to delaying the incubation period, the application of local isolates was also very effective in reducing the incidence of *Fusarium* wilt disease. During observations 56 days after inoculation, the application of local isolates to suppress the incidence of disease was significantly different from the control. The average percentage of disease incidence in the application of local isolates was lower than that of control. In control, the disease incidence reached 98.57% (highest), while in the case of local isolates, the disease incidence ranged from 38.51 to 68.04%. This showed that the application of local isolates was significantly reduce the incidence of *Fusarium* wilt in wakegi onions in comparison to the control. Among the tested isolates, isolate DB12 had the lowest percentage of disease incidence and was significantly different from other test isolates (Figure 3).

The success of *Bacillus* spp. local isolates in reducing the percentage of *Fusarium* wilt disease incidence was in line with their ability to delay the appearance of symptoms indicated by a longer incubation period. Each time a delay in the incubation period was observed, there was generally a decrease in the percentage of disease incidence. *Bacillus* spp. bacteria especially DB12 isolate exhibited the ability to act as a biocontrol agent against pathogenic fungi because it was able to arrest the incubation period longer than the control, followed by the reduction in disease incidence to 31.50% lower over the control. This proves that *Bacillus* spp. local isolates had higher adaptability to survive and had a better chance of controlling pathogens in their place of origin than using isolates introduced from elsewhere.

The ability of *Bacillus* spp. to suppress Foc pathogens was presumed to be caused by an antibiosis mechanism by producing antibiotic compounds to counter the damaging effects of these pathogens. Arseneault and Filion (2017) reported that antibiosis is the most widely used mechanism of *Bacillus* spp. to control plant pathogens. *Bacillus* spp. bacteria were known to produce twelve major antibiotics including bacillomicin, mycobacillin, fungistatin, iturin, fengycin, plipastatin and bacillin (Ali et al. 2020).

Research on the use of antibiotics produced by bacteria belonging to the genus *Bacillus* spp. as a biocontrol against pathogens has been widely reported. Plaza et al. (2013) reported that surfactant antibiotic compounds secreted by bacterial strains belonging to the species *B. subtilis* could inhibit the mycelia growth of *Botrytis cinerea, Sclerotinia sclerotiorum, Colletotrichum gloeosporioides*, *Phoma complanata* and *Phoma exigua* var. *exigua.* Zalila-Kolsi et al. (2016) reported that iturin antibiotic compounds, fengycin, surfactin produced by strains of bacteria *Bacillus* spp. are able to suppress the growth of *Fusarium graminearum.* Similarly, strains of *B. subtilis* PCL1608 and PCL1612 could produce high levels of antibiotics, especially iturin A which served as the main mechanism in controlling *F. oxysporum* and *Rosellinia necatrix* (Hashem et al. 2016).

Bacillus spp. isolated from soil rhizosphere has been reported to be effective in controlling various fungal pathogens, including *Pythium* spp., *Botrytis* spp., *Phytophthora* spp., *Fusarium* spp. (Lamsal et al. 2012; Ali et al. 2020). Therefore, the low incidence of *Fusarium* spp. wilt disease indicates the ability of local isolates *Bacillus* spp. to produce antibiotics to suppress the growth of Foc pathogenic fungal colonies in the wakegi onion rhizosphere.

In conclusion, the isolates obtained from wakegi onion rhizosphere could inhibit the growth of *F. oxysporum* f. sp. *cepae in vitro* with an inhibition power between 43.28 and 67.66%. The results of colony morphology, physiological and biochemical characteristics revealed that bacterial isolates from the rhizosphere of wakegi onion belonged to the genus *Bacillus*. The DB12 isolate of *Bacillus* spp. had the best inhibitory ability, with the longest incubation period (19.73 DAI) and the lowest disease incidence (31.50%).

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