The inhibitory role of dark septate endophytic fungus Phialocephala fortinii against Fusarium disease on the Asparagus officinalis growth in organic source conditions

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ABSTRACT

Five of eight selected dark septate endophytic (DSE) fungal isolates had the ability to inhibit the growth of Fusarium oxysporum f.sp. asparagi in a dual culture test. We demonstrated that Asparagus officinalis inoculated with DSE fungi could survive and grow healthily without any typical disease symptoms after Fusarium disease challenge in inorganic or organic conditions in vitro. Phialocephala fortinii isolate CKG.I.11 most effectively promoted the growth of A. officinalis after Fusarium disease challenge either with inorganic or organic sources. Inoculation of P. fortinii CKG.I.11 in A. officinalis plant on the organic nursery setting decreased the severity of Fusarium disease and increased the A. officinalis growth compared to A. officinalis challenged with F. oxysporum f.sp. asparagi and not inoculated with P. fortinii CKG.I.11 so that P. fortinii effectively promoted A. officinalis growth in Fusarium disease challenge.

1. Introduction

Asparagus officinalis is a long-term perennial vegetable crop with high market value and low input in its production (Nahiyan and Matsubara, 2012). Demand for this crop is increasing worldwide every year, but several factors limit its production, with one of the main limiting factors being Fusarium disease (Nahiyan and Matsubara, 2012; Waśkiewicz et al., 2013; Matsubara et al., 2014). Fusarium disease significantly reduces A. officinalis production is mainly caused by Fusarium oxysporum f.sp. asparagi and Fusarium proliferatum (Reid et al., 2002; Nahiyan et al., 2011). Fusarium oxysporum f.sp. asparagi is the dominant pathogen of A. officinalis, especially in Japan (Nahiyan et al., 2011) causing root, stem, and crown rot (Elena, 2007, Nahiyan and Matsubara, 2012). This pathogenic fungus may persist during the asparagus-free period for at least 25 years (Elena, 2007). Controlling this disease using chemicals has an impact on environmental pollution and the disruption of agriculture ecosystems, but breeding for disease resistant plants requires high cost and a long time to develop (Pontaroli and Camadro, 2001). Therefore, an environmentally friendly and sustainable approach should be conducted for the control of this disease. One approach is to use dark septate endophytic (DSE) fungi as a biocontrol agent for F. oxysporum f.sp. asparagi.

DSE fungi are a group of endophytic fungi that usually have melanized hyphae, form dark colonies in agar media, and make symbiotic interactions with plants (Jumpnone and Trappe, 1998; Thommion et al., 1999; Jumpnone, 2001). They colonize living plant root tissues inter- and intracellularly but do not cause any typical disease symptoms in the plant (Jumpnone and Trappe, 1998; Knapn et al., 2012). Based on the definition and category of symbiosis by De Bary (1879), the symbiosis between DSE fungi and its host plants includes mutualism. Several studies have reported that DSE fungi have the potential as a biocontrol agent for pathogenic fungi such as Heteroconitrium chaetospira that suppressed Verticillium yellows in Chinese cabbage (Narisawa et al., 2006) and Verticillium wilt in eggplant (Narisawa et al., 2002). Khastini et al. (2012) reported that Veronaepis simplex suppressed Fusarium disease in Chinese cabbage. Other DSE fungal species such as Cadophora sp. effectively controlled Fusarium wilt on melon (Khastini et al., 2014), and Phialocephala sphareoides controlled Heterobasidion parviporum in Norway spruce (Terhonen et al., 2016). Tellenbach et al. (2013) demonstrated that Phialocephala europaea significantly reduced the growth of Pythophthora citricola in vitro by producing secondary metabolites compounds. However, regarding the control of Fusarium disease in A. officinalis caused by P. oxysporum f.sp. asparagi, the use of DSE fungi is still limited so that the focus of our research in this study was to...
select DSE fungus that suppresses Fusarium disease on A. officinalis. Therefore, further studies on the use of DSE fungi to control Fusarium disease especially caused by F. oxysporum f.sp. asparagi are needed, to obtain the most effective DSE fungus to suppress this disease. Based on previous research on the role of DSE fungi in suppressing plant diseases, we hypothesized that DSE fungi may have the potential to be used to suppress Fusarium disease in A. officinalis.

The role of DSE fungi related to its effect in promoting A. officinalis growth in organic conditions and the preferences for organic source use such as organic N and P have been reported in our previous study (Surono and Narisawa, 2017). Therefore, further studies are needed to investigate whether selected DSE fungal isolates from our previous study are able or not able to promote the host plant in Fusarium disease challenge with various nutrient sources, especially organic source. There are only a few reports related to the association between DSE fungi and vegetable plant, such as asparagus, especially in plant disease challenge.

The aim of this study was to investigate the ability of DSE fungal isolates from our previous study (Surono and Narisawa, 2017) to suppress Fusarium disease either in an inorganic or organic condition in vitro systems and in an asparagus nursery setting. Testing in organic conditions, it is expected that the most effective DSE fungus may also be used as a biocontrol agent of Fusarium disease for A. officinalis organic farming in the future. Organic asparagus cropping systems produce better spear quality than conventional systems with agro-chemical sources (Caruso et al., 2012). Asparagus officinalis as a host plant can be used as a valuable model to study microbial ecology and the biocontrol of pathogens because it is a low-input perennial crop and is a better spear quality than conventional systems with agro-chemical farming in the future. Organic asparagus cropping systems produce disease either in an inorganic or organic condition. Therefore, the utilization of DSE fungi has the potential to overcome pathogen attack and promote A. officinalis growth.

In organic A. officinalis cultivation, biocontrol agent to reduce the use of pesticides and decomposer to produce organic fertilizer to replace the use of inorganic fertilizers are needed (Rapejo et al., 1997; Kibblewhite et al., 2008; Monokrousos et al., 2008; Xu et al., 2014). The supply of organic fertilizer can be obtained from composting of A. officinalis biomasses such as twigs and leaves when thinning in winter and can be used other sources such as manure when the initial planting to harvest time is over a long period of cultivation (Monokrousos et al., 2008; Xu et al., 2014). The DSE fungal isolates of our previous study to be used in the present study are known to be potential as decomposers of soil organic matter (Surono and Narisawa, 2017) and if the present study is able to suppress Fusarium disease in A. officinalis, the DSE fungal isolates will play a significant role in promoting growth and production of A. officinalis organically in the future because they have a double ability as a growth promoter, decomposer of soil organic matter and Fusarium disease controller in A. officinalis.

2. Material and methods

2.1. Dark septate endophytic and pathogenic fungal material

Eight DSE fungal isolates were used that promote A. officinalis growth in previous studies, comprising P. fortinii isolates III.F1.18, CKG.I.1.1, CKG.I.10.3, CKG.I.11, CKG.II.10.1, CYA.II.14.3, CYA.II.17, and unidentified isolate sp. CKG.IV.10 (Surono and Narisawa, 2017). All DSE fungal isolates were obtained from Prof. Kazuhiko Narisawa. Fusarium oxysporum f.sp. asparagi NBRC 31382 used as a pathogen of A. officinalis in this study was obtained from NBRC (National Institute of Technology and Evaluation – Biological Resource Center, Japan). All DSE fungal and pathogen fungal isolates were grown on potato dextrose agar (PDA) media.

2.2. Antagonism between Fusarium oxysporum f.sp. asparagi and dark septate endophytic fungal isolates

An agar plug (5 mm diameter) of DSE fungal isolates was placed on PDA 6 cm distance away from F. oxysporum f.sp. asparagi. Due to the slow growth of DSE fungi, the fungi were firstly grown for 2 weeks, subsequently the fungal pathogen was grown in the same Petri dish as the DSE fungal isolates for dual culture testing. Fusarium oxysporum f.sp. asparagi was grown alone in a Petri dish as a control. The measurement finished when one of the control plates was fully covered with colony (Terhonen et al., 2016). After 2 weeks, the inhibition zone and inhibition of the radial growth of the pathogen were measured (Maciá-Vicente et al., 2008). The experiment was repeated three times. The radial colony growth of F. oxysporum f.sp. asparagi towards the antagonistic fungus (Ri) and that on a control (Rc) dish were measured and the colony growth inhibition was calculated using the formula: (Rc – Rr)/Rc × 100 (Lahiala and Hjiri, 2016).

2.3. Effect of dark septate endophytic fungal isolates to promote Asparagus officinalis growth in Fusarium disease challenge in the inorganic and organic source conditions

Experiments were conducted to determine the effects of DSE fungi inoculation on A. officinalis growth with Fusarium disease challenge in inorganic and organic conditions in vitro. The compositions of the media used for this test are shown in Table 1. The procedure for these treatments was in accordance with previous procedures in Surono and Narisawa (2017). Corn steep liquor (CSL; Nature Aid; Sakata Seed, Yokohama, Japan) used in the organic media treatment contained 3% nitrogen (N), 3% phosphorus (P) and 2% potassium (K). A. officinalis seeds were surface sterilized by immersion in 70% ethanol for 1.5 min and then a solution of sodium hypochlorite (1% available chlorine) for 3 min. The seeds were rinsed three times with sterilized distilled water, then dried overnight, and ready to be used in next experiment (Surono and Narisawa, 2017). Then, A. officinalis seedlings with or without DSE fungal inoculation were challenged with F. oxysporum f.sp. asparagi grown on water agar. A. officinalis seedlings were overlaid onto the fungal colony and incubated for 3 weeks in the same conditions (Khastini et al., 2012). There were three replicates for each treatment, including the control.

2.4. Effect of Phialocephala fortinii isolate CKG.I.11 to promote A. officinalis growth and suppress Fusarium disease in an organic nursery setting

This experiment was conducted to investigate the ability of P. fortinii isolate CKG.I.11 as the best promoter of A. officinalis growth in previous treatments to promote A. officinalis growth and suppress Fusarium disease in an organic nursery setting. The treatments consisted of 1) A. officinalis was not inoculated with P. fortinii CKG.I.11 and was not infected with F. oxysporum f.sp. asparagi as a control, 2) A. officinalis was

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Inorganic</th>
<th>Organic</th>
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<tbody>
<tr>
<td>KH₂PO₄</td>
<td>1.5 g</td>
<td>0 g</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>1.0 g</td>
<td>0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1.0 g</td>
<td>0 g</td>
</tr>
<tr>
<td>Corn steep liquor (CSL)</td>
<td>0 g</td>
<td>1 mL</td>
</tr>
<tr>
<td>Oatmeal</td>
<td>10 g</td>
<td>10 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 mL</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

* CSL contained 3% nitrogen (N), 3% phosphorus (P) and 2% potassium (K).
infected with *Fusarium oxysporum f.sp. asparagi*. 3) *A. officinalis* was inoculated with *P. fortinii* isolate CKG.I.11 and was infected with *Fusarium oxysporum f.sp. asparagi*, and 4) *A. officinalis* was inoculated with *P. fortinii* isolate CKG.I.11 and was not infected with *Fusarium oxysporum f.sp. asparagi*. Each treatment consisted of ten *A. officinalis* seedlings and three replications.

The experiment was conducted using organic soil from the Field Science Center (FSC), Ibaraki University, Japan. The soil properties of organic soil sample from FSC were pH 6.37 (slightly acidic soil), 4.31% C, 0.41% N and C/N 10.6. The organic soil samples were air-dried, sieved through a 5 mm mesh, autoclaved at 121 °C for 30 min, and re-autoclaved after 24 h using with same procedure. The *P. fortinii* CKG.I.11 inoculum was prepared as described by Usuki et al. (2002). Subsequently, 5% of *P. fortinii* CKG.I.11 inoculum was added and mixed with the sterilized soil samples in each treatment. Surface-sterilized *A. officinalis* seed was transplanted into pots contain 25 g soil. An inoculum of *Fusarium oxysporum f.sp. asparagi* was prepared in accordance with Khastin et al. (2014). Seedlings without fungal inoculum were as the controls. The procedure for surface sterilization of *A. officinalis* seed was same as previously (Section 2.3). All seedling treatments were incubated for 4 weeks at room temperature with an 18 h: 6 h (L: D) regimen and intensity of 180 mol m⁻² s⁻¹ and watered with distilled water every 3 days. After incubation for 1 week, the nursery seedling soil was supplemented with 0.1% corn steep liquor as a sole organic nutrient. Then, after incubation time for 2 weeks, treatments 2 and 4 were infected with *Fusarium oxysporum f.sp. asparagi*, then disease symptoms were observed every week using the disease index of Narisawa et al. (2004) until 8 weeks. The *A. officinalis* plants were harvested after 8 weeks of infection with *Fusarium oxysporum f.sp. asparagi* and oven-dried at 35 °C for five days, and the roots were used for recording DSE fungal colonization. The dry weight of the biomass of each treatment with *P. fortinii* CKG.I.11 or *Fusarium oxysporum f.sp. asparagi* was measured and was compared with that of the uninoculated controls (Surono and Narisawa, 2017).

2.5. Anatomical observations, assessment of root colonization, and re-isolation of DSE fungal isolate from Asparagus officinalis root tissues

To determine the effects and the colonization degree of selected DSE fungi on the roots of *A. officinalis* to inhibit *Fusarium* disease, 5-week-old asparagus seedling roots and 56-day-old asparagus seedlings roots with DSE fungal inoculation and *Fusarium* disease challenge in vitro and in a nursery with setting using organic soil media were washed, cross-sectioned, and stained with 50% acetic acid solution containing 0.005% cotton blue, before being examined using a light microscope (BX51; Olympus, Tokyo, Japan). The calculation of the percentage of DSE fungal colonization referred to the method described by Usuki et al. (2002). Stained *A. officinalis* roots were examined along grid lines and each grid cell was designated as either colonized or non-colonized (Narisawa et al., 2004).

The DSE fungal re-isolation was determined according to methods described by Narisawa et al. (2002). Inoculated DSE fungi were re-isolated from *A. officinalis* roots by washing with Tween 20 three times and sterile water three times, next the sterilized roots were dried overnight. Root segments were placed on 50% corn meal agar. Forty-five root segments were chosen randomly from each treatment. The frequency of re-isolation was calculated as the mean number of root segments colonized by the fungus (Narisawa et al., 2002).

2.6. Data analysis

The mean dry biomass of each treatment was calculated and analyzed using one-way ANOVA with R version 3.0.2 (The R Foundation for Statistical Computing Platform, Vienna, Austria). Differences among treatment means were detected using Tukey’s Honestly Significant Difference test (Tukey HSD).

3. Results

3.1. Antagonism between *Fusarium oxysporum f.sp. asparagi* and dark septate endophytic fungal isolates

Five of eight DSE fungal isolates inhibited the growth of *F. oxysporum f.sp. asparagi* (Fig. 1). The inhibition levels by *P. fortinii* isolates III.PI.I8, CKG.I.1.1, CKG.I.11, CKG.II.10.1, and unidentified isolate sp. CKG.IV.10 on *Fusarium oxysporum f.sp. asparagi* were 75.1%, 74.9%, 67.8%, 65.7%, and 79.0% (Table 2), respectively. The other three DSE fungal isolates (*P. fortinii* isolates CKG.I.10.3, CYA.II.14.3, and CYA.II.17) did not inhibit the growth of *Fusarium oxysporum f.sp. asparagi*.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Growth inhibition of <em>Fusarium oxysporum f.sp. asparagi</em> by dark septate endophytic fungal isolates in an antagonism assay using a dual culture method.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSE fungal isolate</td>
<td>Origin</td>
</tr>
<tr>
<td><em>Phialocephala fortinii</em> III.PI.I8</td>
<td>Rhabus sp. root</td>
</tr>
<tr>
<td><em>P. fortinii</em> CKG.I.1.1</td>
<td>Chamaecyparis obtusa root</td>
</tr>
<tr>
<td><em>P. fortinii</em> CKG.I.11</td>
<td>C. obtusa root</td>
</tr>
<tr>
<td><em>P. fortinii</em> II.10.1</td>
<td>C. obtusa root</td>
</tr>
<tr>
<td>Unidentified isolate CKG.IV.10</td>
<td>C. obtusa root</td>
</tr>
<tr>
<td><em>P. fortinii</em> I.10.3</td>
<td>C. obtusa root</td>
</tr>
<tr>
<td><em>P. fortinii</em> CYA.II.14.3</td>
<td>C. obtusa root</td>
</tr>
<tr>
<td><em>P. fortinii</em> CYA.II.17</td>
<td>C. obtusa root</td>
</tr>
</tbody>
</table>

* Values within columns followed by the same letter are not significantly different (P < 0.05) after Tukey’s Honestly Significant Difference test.
3.2. Inoculation of Asparagus officinalis with dark septate endophytic fungal isolates and Fusarium oxysporum f.sp. asparagi under inorganic and organic conditions in vitro

In the agar media with inorganic conditions, all DSE fungal isolates promoted *A. officinalis* growth with *Fusarium* disease challenge (Fig. 2). *Asparagus officinalis* grew without any disease symptoms and normally compared with control plants in which the leaves began yellowing (Fig. 3). All DSE fungal isolates suppressed *Fusarium* disease up to 100% in organic conditions (Table 3). The growth rates versus the control of *P. fortinii* isolates CKG.I.11, CKG.II.10.1, CKG.I.1.1, III.Pi.I8, CYA.II.17, CKG.I.10.3, and unidentified isolate CKG.IV.10 were 240%, 198%, 189%, 179%, 171%, 142%, 141%, and 127%, respectively. *Asparagus officinalis* inoculated with *P. fortinii* isolate CKG.I.11 had the highest dry weight compared with other DSE fungal treatments.

In the treatment using 0.1% CSL as an organic nutrient source, the control plants did not grow well and were stunted and showed appear any disease symptoms, whereas *A. officinalis* inoculated with *P. fortinii* isolates CKG.I.11, CYA.II.17. CKG.I.1.1 and CKG.I.10.1 could grow without any disease symptoms (Fig. 5) and suppressed *Fusarium* disease up to 100% in organic conditions (Table 3). Whereas, the disease suppression of *A. officinalis* inoculated with *P. fortinii* isolates CKG.I.10.3, CYA.II.14.3, III.Pi.I8, and unidentified isolate CKG.IV.10 were 93.3%, 72.2%, 70%, and 78.9%, respectively (Table 3). Compared with control plants, the growth rates of *A. officinalis* inoculated with *P. fortinii* isolates CKG.I.11, CYA.II.17, CKG.I.1.1, CKG.II.10.1, CKG.I.10.3, CYA.II.14.3, III.Pi.I8, and unidentified isolate CKG.IV.10 were 474%, 449%, 407%, 380%, 286%, 175%, 169%, and 204%, respectively (Fig. 4). In this treatment, *P. fortinii* isolate CKG.I.11 also gave the highest *A. officinalis* dry weight compared with other organic treatments (Fig. 4).
Table 3
The inhibition role of Phialocephala fortinii isolate CKG.I.11 on the disease severity and suppression of Fusarium disease in Asparagus officinalis in inorganic and organic conditions in the in vitro system.

<table>
<thead>
<tr>
<th>DSE fungal isolates</th>
<th>Disease severity (%)</th>
<th>Disease suppression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inorganic</td>
<td>Organic</td>
</tr>
<tr>
<td>Phialocephala fortinii III PI.1B + Fusarium oxysporum f.sp. asparagi</td>
<td>0a</td>
<td>30d</td>
</tr>
<tr>
<td>P. fortinii CKG.I.1.1 + F. oxysporum f.sp. asparagi</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>P. fortinii CKG.I.1 + F. oxysporum f.sp. asparagi</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>Unidentified isolate CKG.IV.10 + F. oxysporum f.sp. asparagi</td>
<td>0a</td>
<td>21.1c</td>
</tr>
<tr>
<td>P. fortinii CKG.I.10.3 + F. oxysporum f.sp. asparagi</td>
<td>0a</td>
<td>6.67b</td>
</tr>
<tr>
<td>P. fortinii CYA.II.14.3 + F. oxysporum f.sp. asparagi</td>
<td>0a</td>
<td>27.6d</td>
</tr>
<tr>
<td>P. fortinii CYA.II.17 + F. oxysporum f.sp. asparagi</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>F. oxysporum f.sp. asparagi</td>
<td>53.3b</td>
<td>100e</td>
</tr>
</tbody>
</table>

* Values within columns followed by the same letter are not significantly different (P < 0.05) after Tukey’s Honestly Significant Difference test.

3.3. Inoculation of Phialocephala fortinii to promote Asparagus officinalis and suppress Fusarium disease in a nursery setting using organic soil

Fusarium oxysporum f.sp. asparagi caused an increase in disease severity in A. officinalis. The length of incubation time with the highest severity at 56 days after inoculation of F. oxysporum f.sp. asparagi reached 44% (Fig. 6). Whereas A. officinalis treated with P. fortinii isolate CKG.I.11, the disease severity was lower even decreased during incubation from 42 to 56 days after inoculation with F. oxysporum f.sp. asparagi reached 1.33% (Fig. 6). The performance of A. officinalis treated with P. fortinii isolate CKG.I.11 appeared healthy and grew well compared with A. officinalis treated with F. oxysporum f.sp. asparagi without treatment with P. fortinii isolate CKG.I.11 (Fig. 7).

Inoculation of A. officinalis with P. fortinii caused enhancement of the growth in all treatments either with or without Fusarium challenge, and even P. fortinii was effective in Fusarium-challenged asparagus (Fig. 8). Phialocephala fortinii-inoculated asparagus increased dry weight significantly even though it was challenged with Fusarium disease compared with P. fortinii-uninoculated asparagus (Fig. 8). As well as treatments without Fusarium challenge, dry weight of A. officinalis increased in the P. fortinii inoculation treatments compared with uninoculated treatments (Fig. 9).

The colonization rate of P. fortinii in A. officinalis roots with Fusarium disease challenge in a nursery setting using organic soil reached 46.7%, whereas in the treatment with only inoculation with P. fortinii isolate CKG.I.11 without Fusarium disease challenge was higher and reached 53.3%. Fusarium oxysporum f.sp. asparagi colonization at the root of control plants reaches 100%, roots grew abnormally and tend to a dwarf. While F. oxysporum f.sp. asparagi colonization of A. officinalis inoculated with P. fortinii and infected with F. oxysporum f.sp. asparagi only reached 12.5%. Phialocephala fortinii isolate CKG.I.11 penetrated the cortical tissues of A. officinalis roots and formed microsclerotia-like forms (Fig. 9). The results of re-isolation of P. fortinii in A. officinalis seedling with and without Fusarium disease challenge in a nursery setting were 22.2% and 26.7%, respectively.

3.4. Anatomic observation of fungal colonization

Heavy colonization of P. fortinii isolate CKG.I.11 occurred in A. officinalis roots especially in epidermal cells in inorganic and organic source conditions in vitro, and it may protect A. officinalis roots from Fusarium disease (Fig. 9). However, the colonization pattern did not reach the vesicular systems of A. officinalis roots. Phialocephala fortinii isolate CKG.I.11 formed darker microsclerotia forms inside cortical tissues of A. officinalis roots in the treatment with Fusarium disease challenge than in treatment without Fusarium disease challenge in a nursery setting using organic soil (Fig. 9).

4. Discussion

We demonstrated that five of eight selected DSE fungal isolates had the ability to inhibit the growth of F. oxysporum f.sp. asparagi in dual culture tests (Table 2; Fig. 1). Slow-growing endophytic fungi including DSE fungi are known as antifungal producers and have the potential to be fungal pathogen inhibitors (Sieber, 2002; Mandiyam and Jumpomon, 2005; Maciá-Vicente et al., 2008; Terhonen et al., 2016). However, investigations on the ability of DSE fungi to inhibit fungal pathogen growth and to produce antifungal compounds are still limited. Antagonism between F. oxysporum f.sp. asparagi and DSE fungal isolates in this study may be because of biologically active compounds...
produced in media by these DSE fungi (Castillo et al., 2002) and had a detrimental effect on pathogen growth. Although they are the same species, *P. fortinii* isolates CKG.I.1.1, CKG.II.10.1, and unidentified isolate CKG.IV.10.3 did not inhibit the growth of *F. oxysporum f.sp. asparagi* in dual culture tests. These *P. fortinii* isolates may have the potential to induce *A. officinalis* to come resistant to *Fusarium* disease attack by other mechanisms when associated with plants. In the next treatment, *P. fortinii* also promoted *A. officinalis* growth in inorganic and organic source conditions (Fig. 2, Fig. 4). As in the previous study by Khastini et al. (2012), although there was no zone of inhibition at the point contact between DSE fungus *Veronaeopsis simplex* and *F. oxysporum* in dual culture tests, the DSE fungus suppressed *Fusarium* disease in Chinese cabbage. In contrast, Terhonen et al. (2016) reported that although the endophytic fungus *Cryptosporiopsis* sp. was extremely effective in inhibiting *Heterobasidion purpureum* growth in dual culture tests, this endophyte had detrimental effects on Norway spruce seedlings. However, in this study, we reported that *P. fortinii* isolates III.PI.18, CKG.I.1.1, CKG.II.10.1, and unidentified isolate CKG.IV.10.3 have the double ability to inhibit *F. oxysporum f.sp. asparagi* growth in agar medium and to promote *A. officinalis* growth during pathogen attack (Table 2, Table 3, Fig. 1, Fig. 2, Fig. 4). Therefore, these DSE fungal isolates have the potential to be used as a biocontrol agent for *F. oxysporum f.sp. asparagi*.

We demonstrated that *A. officinalis* inoculated with DSE fungi could survive and grow healthily without any typical disease symptoms with *Fusarium* disease in inorganic or organic nutrient conditions in vitro and promoted *A. officinalis* growth compared with the control plants. The effect of each treatment using DSE fungi was significant different from the control, but all DSE fungi were able to promote and protect *A. officinalis* growth in disease conditions. The other mechanism of *Fusarium* disease protection cannot be explained in detail from this study, but a previous study with dual culture tests showed that several DSE fungal isolates have the ability to suppress the growth of *F. oxysporum f.sp.*

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**Fig. 5.** Performances of *Asparagus officinalis* inoculated with DSE fungal isolates with *Fusarium oxysporum f.sp. asparagi* challenge on organic agar media. A) Control plant (with arrow to show yellowing effect on asparagus leaves), B) treatment with *Phialocephala fortinii* isolate CKG.I.11, C) treatment with *P. fortinii* isolate CYA.II.17, and D) treatment with *P. fortinii* isolate CKG.II.1.1 inoculation incubated at 23 °C with a 6-h: 8-h (L: D) photoperiod (180 mol m⁻² s⁻¹) for 3 weeks.

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**Fig. 6.** The inhibition role of *Phialocephala fortinii* isolate CKG.I.11 on the disease severity index of *Fusarium* disease in *Asparagus officinalis* in organic nursery setting. Mean values that differ significantly at each time point (*P* < 0.01) are indicated by asterisk after Tukey’s Honestly Significant Difference test, *n* = 10. Foa = *F. oxysporum f.sp. asparagi*, Pf = *Phialocephala fortinii*. Bars indicate standard deviation of means.
asparagus in agar media (Table 2). The compact colonization of DSE fungal hypha also occurred in A. officinalis roots in these treatments (Fig. 9). These abilities may make a contribution to Fusarium disease reduction in A. officinalis inoculated with DSE fungi. Other DSE fungal isolates such as P. fortinii isolate CKG.I.10.3 and CYA.II.17 that had no ability to reduce F. oxysporum f.sp. asparagi growth, but it could promote the growth of A. officinalis either in inorganic or organic source media, so there are may be other mechanisms whereby this fungus can improve the resistance of A. officinalis to Fusarium disease. In contrast, Schulz and Boyle (2005) stated that endophytic fungi may cause disease symptoms when inoculated with a non-original host plant, but our previous study (Surono and Narisawa, 2017) demonstrated that the selected DSE fungi did not produce any typical disease symptoms in A. officinalis as a non-original host plant. Furthermore, in the current study we demonstrated these DSE fungi also promoted A. officinalis growth in disease conditions. Reported previously, the selected DSE fungal isolates used in this study were originally from Chamaecyparis obtusa (Japanese cypress). He et al. (2002) reported that induced systemic resistance developed when A. officinalis inoculated with non-pathogenic fungi was challenged with F. oxysporum and caused a reduction in root lesions from 50% to 25%. Arbuscular micorrhizal fungi species Glomus intraradices increased Fusarium root rot tolerance in A. officinalis by indirectly induced resistance of A. officinalis roots to that disease before the pathogen colonization (Matsubara et al., 2003). In the previous study by Narisawa et al. (2002), P. fortinii suppressed Verticillium dahliae and protected eggplant from the attack by this fungal pathogen with colonization of eggplant root tissues. Jumpponen (2003) stated there are three mechanisms related to the role of DSE fungi in inhibiting fungal pathogens or minimizing the negative effect of pathogens in plant growth and performance. i.e., competition for plant photosynthates or for colonization sites, production of pathogen inhibitory compounds, and colonization of host plants by DSE fungi developed inducing plant defense responses to plant infection. We reported for the first time that P. fortinii isolate CKG.I.11 was the best isolate among other DSE fungal isolates to promote A. officinalis growth either with inorganic or organic nutrient sources in Fusarium disease in vitro. When
this isolate was applied to promote *A. officinalis* in a nursery setting with a disease challenge using organic soil media, and which consistently showed positive effects on *A. officinalis* growth with or without the pathogen infection. The disease severity of *A. officinalis* treated with *P. fortinii* isolate CKG.I.11 decreased in the period of 45–56 days of pathogen incubation time and *A. officinalis* that had shown symptoms of the disease grew normally again by producing new healthy shoots. It was indicated that *P. fortinii* isolate CKG.I.11 potentially protected *A. officinalis* from pathogen attack and promoted plant growth especially in an organic nursery setting.

Heavy colonization by DSE fungi occurred in *A. officinalis* roots inoculated with DSE fungal isolates and reached the cortical cells of roots. In the organic nursery setting, hyphae of *P. fortinii* isolate CKG.I.11 penetrated the cortical cells and formed microsclerotia inside the A. officinalis root both with or without pathogen attack, but in the presence of pathogen, the microsclerotia-like forms of *P. fortinii* isolate CKG.I.11 appeared darker (Fig. 9). Peterson et al. (2008) stated that colonization by some DSE did not invade the vascular cylinder or cause degradation of cortical and vascular parenchymal cell of plants. Heavy colonization indicated a protection pattern from DSE fungi for *A. officinalis*, so that it became tolerant to *Fusarium* disease. The heavy colonization in roots allowed the aboveground parts of *A. officinalis* to grow normally with higher dry weight either in inorganic or organic media compared with the control plant. There were no typical disease symptoms appearing on *A. officinalis*. In contrast to the effect of the interactions between fungal pathogens and plants, the interaction of mutualistic fungal endophytes by root colonization provides many beneficial effects for the plant, such as systemic protection against pathogen attack (Liu et al., 2007). Colonization by endophytic fungi fills out an ecological niche and left no space for fungal pathogens, and the process including penetration of epidermal and cortical cells without causing any disease symptoms in the plants, even it in the event of pathogen attack (Dutta et al., 2014). Endophytic fungi are generally considered to protect plants through rapid colonization and thus exhaust the limited available substrates, leaving nothing available for pathogens to grow (Pal and Gardener 2006). Colonization by endophytic fungi usually offers protection to plants in various ways, such as the production of pathogen inhibitor compounds, ecological niche occupations used by pathogens, or interference with cellular membrane pathogens by producing chitinase, cause cell death in pathogens (Ganley et al., 2008; Shittu et al., 2009; Waqas et al., 2014). This study showed that *P. fortinii* isolate CKG.I.11, both in *in vitro* treatment and in

Fig. 9. Image of *A. officinalis* roots challenged with *Fusarium oxysporum* f.sp. *asparagi* and treated with *Phialocephala fortinii* CKG.I.11 on inorganic and organic agar media in the *in vitro* system (A–C) and in the nursery setting (D–E). The image in (A) shows uninoculated roots under *Fusarium* disease challenge with *Fusarium oxysporum* f.sp. *asparagi* hyphae (arrows) as a control treatment, (B) *A. officinalis* roots colonized by *P. fortinii* CKG.I.11 hyphae (arrows) under *Fusarium* disease challenge in inorganic conditions, (C) *A. officinalis* roots colonized by *P. fortinii* CKG.I.11 hyphae (arrows) under organic conditions. DSE fungal colonization can be seen on the root surface and within epidermal cells (Ep) and the cortex (Co). Note the absence of DSE fungal colonization in the vascular cylinder (Ve). Bars = 20 μm. Images of *A. officinalis* seedling roots colonized by *P. fortinii* CKG.I.11 with (D) and without (E) *Fusarium* disease challenge in organic nursery setting. *Phialocephala fortinii* CKG.I.11 hypha formed in the cortical tissues (arrows) and formed microsclerotia-like form (asterisk). *Phialocephala fortinii* isolate CKG.I.11 formed darker microsclerotia forms inside cortical tissues of *A. officinalis* roots in the treatment with *Fusarium* disease challenge than in treatment without *Fusarium* disease challenge. Bars = 10 μm.
a nursery setting, actively colonized the roots of A. officinalis that have an impact on healthy asparagus growth and to protect it from pathogen attack.

In conclusion, *P. fortinii* isolate CKG.1.11 most effectively promoted the growth of *A. officinalis* with *Fusarium* disease either in inorganic or organic sources conditions in vitro and in a nursery setting using organic soil media. In the nursery setting, *P. fortinii* isolate CKG.1.11 suppressed *Fusarium* disease severity. Therefore, *P. fortinii* isolate CKG.1.11 has the potential for further testing in the field to determine the consistency of its ability to promote the growth of *A. officinalis* and to suppress *Fusarium* disease attack on *A. officinalis* organically.

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