The dark septate endophytic fungus *Phialocephala fortinii* is a potential decomposer of soil organic compounds and a promoter of *Asparagus officinalis* growth

Surono a,b,c, Kazuhiko Narisawa a,b,*

a United Graduate School of Agriculture, Tokyo University of Agriculture and Technology, 3–8–1 Harumi-cho, Fuchu-shi, Tokyo 183-8538, Japan
b College of Agriculture, Ibaraki University, 3–21–1 Chuou, Ami, Ibaraki 300–0393, Japan
c Indonesian Agency for Agricultural Research and Development, Jl. Ragunan No.29, Pasar Minggu, Jakarta 12540, Indonesia

**A R T I C L E   I N F O**

Article history:
Received 26 July 2016
Received in revised form
30 March 2017
Accepted 3 April 2017
Available online 4 May 2017

Corresponding Editor: Kevin K. Newsham

Keywords:
*Asparagus officinalis*
Corn steep liquor
Dark septate endophytic (DSE) fungi
Organic carbon
Phosphorus and nitrogen
*Phialocephala fortinii*

**A B S T R A C T**

There is limited information as to whether dark septate endophytic (DSE) fungi are able to degrade organic carbon, nitrogen and phosphorus compounds in soil and if these fungi have a significant role in nutrient cycles in nature, especially under organic nutrient conditions. In order to further knowledge in this area, 25 DSE fungi were isolated and tested for their promotion of *Asparagus officinalis* seedling growth. Three *Phialocephala fortinii* isolates were found to be most effective in increasing the growth of *A. officinalis* plants compared with uninoculated controls. These isolates had the ability to degrade all carbon and nitrogen compounds tested except for lignin. Using organic phosphorus and nitrogen sources, the three *P. fortinii* isolates were able to promote the growth of *A. officinalis* compared with control plants. The isolates were also able to promote the growth of *A. officinalis* seedlings on semi-organic and organic media. Our findings demonstrate that *P. fortinii* has a role in the promotion of *A. officinalis* growth under organic nutrient conditions, possibly by decomposing organic phosphorus and nitrogen compounds in soil.

© 2017 Elsevier Ltd and British Mycological Society. All rights reserved.

**1. Introduction**

More than 90% of all terrestrial plant species enter into symbiotic relationships either with mycorrhizal or endophytic fungi, including dark septate endophytic (DSE) fungi, in natural ecosystems (*Carroll, 1988; Shahollari et al., 2007; Behie et al., 2013*). Recently, DSE fungi have been shown to be abundant in natural forest ecosystems and have shown promise for application in agricultural systems, although their positive impact on plants in natural ecosystems needs to be studied further (*Hennon et al., 1990; Addy et al., 2000; Mahmoud and Narisawa, 2013*).

DSE are a group of endophytic fungi that generally have melanized hyphae, form dark colonies on agar media, and are able to colonize plant roots both inter- and intracellularly without causing disease symptoms (*Jumpponen and Trappe, 1998; Thormann et al., 1999; Jumpponen, 2001; Wilson et al., 2004; Diene et al., 2013; Knapp et al., 2015*). The role of DSE fungi in nature has been considered to be similar to that of mycorrhizal fungi (*Peterson et al., 2008; Della Monica et al., 2015*), although the mechanisms involved in nutrient transfer from DSE fungi to their hosts are still unclear. The well-known DSE fungus *Phialocephala fortinii* is able to enhance nitrogen (N) and phosphorus (P) uptake by pine. Although this phenomenon is amongst the better-known beneficial effects of DSE fungi (*Smith and Read, 1997; Jumpponen and Trappe, 1998*), more research is still needed to support the view that the fungus has mycorrhizal-like interactions with its host plants. *P. fortinii* is abundant in nature and generally has symbiotic relationships with several coniferous trees such as *Fusus sylvestris*, *Pinus contorta*, *Picea abies* and *Abies alba* (*Konner and Bergmann, 1995; Ahlich-Schlegel, 1997*). It has not yet been determined if *P. fortinii* can be isolated from other coniferous trees dominant in natural Japanese forests, such as *Chamaecyparis obtusa* and *Cryptomeria japonica*.

*P. fortinii* is abundant in natural forest ecosystems in conditions rich with organic matter. Therefore, along with other fungal decomposers, it may have a significant role in organic material...
decomposition in forest soils (Stoyke et al., 1992; Thorllmann et al., 1999; Menkis et al., 2005; Grünig et al., 2006). Caldwell et al. (2000) demonstrated that *P. fortinii* has the ability to degrade polymeric forms of carbon (C), N, and P such as cellulose, starch and protein. To support plant growth under organic conditions, it is important for DSE fungi such as *P. fortinii* to be able to degrade organic compounds to produce nutrients in forms available to its host plant. Information on the ability of DSE fungi to degrade organic polymeric forms of C, N and P is still limited, especially in relation to the use of organic media for promoting host plant growth. Recently, five DSE fungi, including *P. fortinii*, have been reported to decompose organic compounds (Caldwell et al., 2000; Menkis et al., 2004; Mandiyam et al., 2010). Therefore, like ectomycorrhizal and ericoid mycorrhizal fungi, *P. fortinii* may have the ability to retrieve N and P from plant litter, although research in this area remains limited (Caldwell et al., 1996; Jumpponen and Trappe, 1998; Lindahl and Tunlid, 2015).

*P. fortinii* has been reported to be a plant growth promoter in many studies (Jumpponen et al., 1998; Jumpponen and Trappe, 1998; Narisawa et al., 2002, 2004; Vohnik et al., 2005). However, some studies have found that *P. fortinii* has neutral or even negative effects on host plants (Wilcox and Wang, 1987; O’Dell et al., 1993; Fernando and Currah, 1996; Jumpponen and Trappe, 1998; Jumpponen, 2001; Vohnik et al., 2005; Grünig et al., 2008). Clearly, the effect of DSE fungi on host plants, either positive or negative, still needs to be elucidated (Grünig et al., 2008).

The role of *P. fortinii* in promoting plant growth under organic conditions and its preferences for organic N and P sources have yet to be reported. To date, only the DSE species *Heteroconium chaetospira, Pseudozimospora ibarakiensis* and *Scolecosbasidium humicola* have been reported to promote plant growth under organic conditions when amino acids such as leucine and valine are used as organic N sources (Usuki and Narisawa, 2007; Diene et al., 2013; Mahmoud and Narisawa, 2013). Therefore, further in vitro studies are needed to select DSE that have positive effects on host plants before these fungi are applied in the field under organic conditions.

In this study, we focused on *Asparagus officinalis* as a target host plant, because of the species’ increasing importance in agriculture (Zentkeler et al., 2012). *A. officinalis* is a perennial crop that can be cultivated for over 10 years and can be harvested continuously (Wang et al., 2010). Moreover, in *A. officinalis* cropping systems, a supply of organic fertilizer is needed to increase *A. officinalis* production (Espejo et al., 1997; Monokrousos et al., 2008; Xu et al., 2014). Therefore, the use of DSE fungi that can function either as plant growth promoters or as organic compound decomposers would be beneficial in such systems. Based on a previous report, *P. fortinii*, which may have the potential to be used to promote the growth of asparagus, can associate with *A. officinalis* (Yu et al., 2001). There are only a few reports relating to the association between DSE fungi and *A. officinalis*, in contrast to numerous reports of the associations between the plant species and arbuscular mycorrhizal fungi. *A. officinalis* plants are usually symbiotic with mycorrhizal fungi such as *Gleomus* spp. and *Ambispora granatensis* (Burrows et al., 1990; Mizonobe et al., 1991; Palenziula et al., 2011; Matsubara et al., 2014), which increase the absorption efficiency of P from soil (Xu et al., 2014).

The objectives of this study were to test (1) the ability of DSE fungi to promote *A. officinalis* growth, (2) the ability of DSE fungi to degrade C and N compounds, (3) the effect of DSE fungi on *A. officinalis* growth under organic N and P conditions, and (4) the effect of DSE fungi on *A. officinalis* growth under semi-organic and organic conditions.

## 2. Materials and methods

### 2.1. Isolation of dark septate endophytic fungi from plant roots in a natural environment

#### 2.1.1. Plant materials

Plant root sampling was carried out in May 2015. We collected 580 root samples of Japanese cypress (*C. obtusa*), 80 of wild Japanese raspberry (*Rubus* sp.), 40 of skervish (*Erigeron philadelphicus*), 80 of wild strawberry (*Fragaria inunae*), 104 of Japanese cedar (*C. japonica*), 70 of dandelion (*Taraxacum officinale*), 40 of phragmites (*Phragmites japonicus*), 42 of goldenrod (*Solidago sp.*), 40 of Japanese ginger (*Zingiber mioga*), 112 of wheat (*Triticum sp.*), and 40 of unidentified plant species. The 11 sites sampled were natural ecosystems close to the town of Ami (35°59′53″–36°04′29″ N, 140°12′17″–140°23′19″ E) and the cities of Tsuhiura (36°03′26″ N, 140°13′19″ E) and Kasumigaura (36°15′18″ N, 140°23′70″ E) in Ibaraki Prefecture, Japan.

#### 2.1.2. Surface disinfection and fungal isolation

The root samples were washed with tap water to remove debris and then cut into approximately 10 mm segments. The segment root samples were washed three times using 0.005% solutions of Tween 20 or polyoxyethylene (20) sorbitan monolaurate (Wako, Pure Chemical Industries, Ltd., Japan), followed by three rinses in sterilized distilled water using a vortex mixer (CM-1000; Tokyo Rikakikai, Tokyo, Japan). Root segments, air-dried overnight, were then plated into 50% corn meal agar medium (Table 1) in 90 mm plastic Petri dishes (with three segments in each dish). For the purposes of identification, single fungal colonies were grown at 23 °C on 50% corn meal malt yeast medium in 60 mm Petri dishes (Table 1).

### 2.2. Dark septate endophyte fungal screening

#### 2.2.1. Fungal isolates

DSE in this study were considered to be fungal isolates that were dark and slow-growing (growth rate < 3 mm per day), usually starting development after at least 7–14 days of incubation. All DSE fungal candidates isolated from roots collected from the natural environment (25 fungal isolates) were grown on oatmeal agar medium (Table 1) in 55 mm diameter Petri dishes. The fungi were incubated for three weeks at room temperature (approximately 23 °C).

#### 2.2.2. Selection of isolates

*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 placed on 1.5% water agar (15 g Bacto agar per 1 L H₂O) in Petri dishes. After 5–7 days, the axenically grown seedlings (three seedlings per plate) were transplanted onto growing fungal colonies on this medium. Seedlings transplanted onto non-inoculated medium were used as controls, and the whole set was placed into sterile culture bottles (As One Corporation, Osaka, Japan) and incubated for three weeks at room temperature with an 18 h: 6 h day/night cycle and a PPDF of 180 μmol m⁻² s⁻¹. There were three replicates for each treatment, including the control. Symptoms were evaluated on a scale of 0–3 (0: no visible symptoms; 1: light yellowing; 2: yellowing and late growth; 3: wilting or death). The aboveground parts of plants were harvested and oven-dried at 35 °C for five days, and the roots were used for recording DSE fungal colonization. The dry weight of the aboveground biomass of inoculated plants was measured and was compared with that of the uninoculated controls.

2.3. Identification of selected DSE fungi

2.3.1. Morphological identification

Slide cultures of Pablum agar were used to morphologically identify selected DSE fungal isolates, using light microscopy at 40 × magnification (Diene et al., 2013). To investigate the growth rates and the characteristics of colonies under different nutrient supplements, pure cultures of selected DSE isolates were grown at room temperature in 55 mm diameter Petri dishes on 50% cornmeal malt yeast extract agar media (see Table 1).

2.3.2. Molecular identification

DNA from the selected DSE fungal isolates was extracted using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer’s protocol. Universal primers ITS1F (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4R (5'-TCC TAT GAT TTA TGC CGC-3') were used to amplify the internal transcribed spacer (ITS) region of ribosomal RNA operons by polymerase chain reaction (PCR). Fifty microliters of PCR mixture containing 0.2 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 10 × Ex Taq buffer (TaKaRa Bio, Otsu, Japan) and 0.25 U Ex Taq DNA polymerase (TaKaRa Bio), and 50 ng DNA template. The reaction cycle consisted of initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 55 s and extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were sequenced using a model 3130x™ DNA sequencer (Applied Biosystems) and an ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Reaction Kit (Applied Biosystems). The primers used for sequence determination were ITS1F and ITS4R. The sequences were analyzed using MEGA version 5.05 and compared with similar DNA sequences retrieved from the DDBJ/EMBL/GenBank databases using the NCBI BLAST program (Mumford and Narisawa, 2013).

2.4. Degradation of selected carbon and nitrogen compounds by selected DSE fungi

Carboxymethyl cellulose (CMC) agar basal medium containing 200 mL CMC (as the cellulose source, 10 g CMC in 200 mL distilled water), 1 g KH₂PO₄, 0.5 g K₂SO₄, 0.5 g NaCl, 0.5 g FeSO₄, 1 g NH₄NO₃, 0.01 g MnSO₄, 20 g agar and 1 L distilled water was used to determine cellulose degradation activity (Janson and Coronel, 1986). The methods of Hutchison (1989) using modified Melin-Norkrans agar (MMN) as the basal medium were used to determine the abilities of the DSE isolates to degrade lignin, amylose, lipid, casamino acids, urea, pectin and gelatin. The MMN medium contained 1 g glucose, 2 g malt extract, 1 g yeast extract, 0.5 g KH₂PO₄, 0.25 g (NH₄)₂HPO₄, 0.15 g MgSO₄, 7H₂O, 0.05 g CaCl₂, 0.025 g NaCl, 0.012 g FeCl₃, 6H₂O, 15 g agar and 1 L distilled water. Chemicals were supplied by Wako Pure Chemical Industries, Ltd., Becton, Dickinson and Company and Sigma Chemical Company. Unless otherwise stated, all media were poured into 90 mm diameter plastic Petri dishes after autoclaving. Dishes were inoculated in triplicate and incubated at 23 °C in the dark for 2–3 weeks.

2.4.1. Carboxymethyl cellulose degradation

Three selected DSE fungal isolates were grown on CMC agar basal medium. After two weeks, the plate was flooded with aqueous 1% Congo red for 15 min. The plate was then flooded with 1% NaOH for 15 min. Cellulase activity was indicated by a clear zone around the colony.

2.4.2. Lignin

DSE fungal isolates were grown on basal MMN medium containing (NH₄)₂HPO₄ and containing 0.1 ml L⁻¹ guaiacol. After inoculation, if lignase is produced, a clear zone occurs around and beneath the colony against an otherwise purplish background.

2.4.3. Amylose

Fungal isolates were grown on basal MMN medium containing 2.0 g L⁻¹ soluble starch. After two weeks, Petri plates were flooded with a solution of iodine (5.0 g KI and 1.5 g l 100 mL⁻¹ water). A clear zone against an otherwise purplish background formed around the colony if amylose was produced.

2.4.4. Lipid

Isolates were grown on basal MMN medium containing 0.1 g L⁻¹ CaCl₂. Tween 20 was autoclaved separately and added to the autoclaved agar at 10 mL L⁻¹. Breakdown of Tween 20 caused by lipase production resulted in the precipitation of macroscopically visible crystals in the agar around and below the colony.

2.4.5. Casamino acids

DSE isolates were grown on basal MMN medium without (NH₄)₂HPO₄ and containing 5.0 g L⁻¹ of casamino acids. To each litre of medium, we added 2 ml of bromoresol purple (1.6 g in 100 mL of 95% ethanol). If casamino acids were broken down, then the medium turned from reddish grey (at pH 6.5) to purple (at pH > 6.8).

2.4.6. Urea

Isolates were grown on basal MMN medium without (NH₄)₂HPO₄ and containing 5.0 g L⁻¹ glucose, with phenol red added at 12 mg L⁻¹. The medium was made up to 900 mL with water and autoclaved. Separately, 20 g of urea was dissolved in 100 mL of water and aseptically filtered through a 0.45-μm Millipore filter. This was then added to the 900 mL of autoclaved agar to make up the volume to 1 L. Two or three drops of concentrated HCl were added and the agar was swirled until the medium just turned yellow in colour (pH 6.8). The medium was then poured into Petri dishes. After inoculation, if urease was produced, the medium turned red (pH > 8.2).

2.4.7. Pectin

Isolates were grown on basal MMN medium containing 5 g L⁻¹ citrus pectin. After two weeks, the plates were flooded for several hours with a 1% aqueous solution of hexadecyltrimethylammonium bromide. After the liquid was removed, a clear zone occurred around the colony against an otherwise opaque background if pectinase was produced.

2.4.8. Gelatin

DSE isolates were grown on basal MMN medium containing 120 g L⁻¹ of gelatin instead of agar. Gelatin was added to 900 mL of...
water, dissolved, and autoclaved separately from the remaining ingredients, which were dissolved in 100 mL of water. After autoclaving, the mixture of salts was added to the cooled gelatin just before pouring into Petri dishes. If gelatinase was produced by the isolate, liquefaction of the gelatin occurred around or beneath the colony.

2.5. Evaluation of the ability of selected DSE fungi to utilize organic and inorganic nutrient sources to promote Asparagus officinalis growth

Inoculation tests were conducted to determine the effects of selected DSE fungi on asparagus growth with various organic and inorganic nutrient sources (organic and inorganic P, organic N, and under semi-organic and organic conditions). The compositions of the media used for this test are shown in Table 2.

After incubating isolates at room temperature (c. 23 °C) for two weeks, 5–7-day-old A. officinalis L. cv. Welcome (Sakata seed, Yokohama, Japan) seedlings were transplanted onto each DSE fungal colony (three seedlings per dish). Seedlings transplanted onto uninoculated medium were used as controls. The dishes were placed into sterile culture pots (As One Corporation, Osaka) and incubated in a growth chamber at 23 °C for 7 days. The dishes were then divided into three groups: one group was used as a control and the other two were incubated at 23 °C for 3 weeks under a 12 h day/night cycle and a PPFD of 180 μmol m⁻² s⁻¹. There were five replicates for each treatment, including the control. Aboveground biomass of the media used for this test is shown in Table 2.

2.6. Anatomical observations

To determine the effects of selected DSE fungi on the roots of A. officinalis, 3-week-old asparagus seedling roots 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).

2.7. Data analysis

The mean dry aboveground biomass of plants in 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 determined using Tukey’s Honestly Significant Difference test.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Inorganic P</th>
<th>Organic N</th>
<th>Semi-organic N</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>1 g</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>1 g</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1 g</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CaSO₄</td>
<td>2.5 g</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-valine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-lysine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phytic acid</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sodium salts</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Corn steep liquor</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oatmeal</td>
<td>10 g</td>
<td>10 g</td>
<td>10 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
<td>15 g</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 mL</td>
<td>1000 mL</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

3. Results

3.1. Isolation of DSE fungi from plant roots collected from the natural environment

Twenty-five darkly-pigmented and slow-growing (growth rate < 3 mm per day) fungal isolates were obtained from 1282 root segments of various plant species in natural forest environments in Ibaraki Prefecture, Japan. We obtained 22 DSE fungal isolates from Japanese cypress roots (C. obtusa), two isolates from wild raspberry roots (Rubus sp.) and one isolate from skervish roots (E. philadelphicus). DSE fungi were not isolated from root samples of other plant species.

3.2. Screening of the ability of DSE fungal isolates to promote Asparagus officinalis plant growth and identification of selected isolates

Eleven DSE fungal isolates (III.Pi.I8, CKG.II.10.1, CKG.I, CKG.II.10.3, CYA.II.17, CYA.II.14.3, CKG.II.10.4, III.Ep.9, CKG.I.I, III.PI.I7 and CKG.IV.10) promoted A. officinalis growth without causing any typical disease symptoms (Fig. 1; Table 3). These isolates increased A. officinalis aboveground biomass by 14–53% compared with uninoculated control plants (Fig. 1). Another 14 isolates (CKG.II.10.2, CKG.II.6, CKG.I.I8, CKG.IV.12, II.PI.16, CKG.II.2.2, CKG.I.9, CKG.I, CKG.IV.1.2, CKG.IV.4, CKG.I.8, CKG.I, CKG.III.13 and CKG.I.9) reduced the aboveground biomass of A. officinalis, relative to uninoculated control plants (Fig. 1; Table 3).

The sources of the 25 isolates and their putative identities are shown in Table 3. Analyses of the ITS regions of seven isolates (III.Pi.I8, CKG.I.I, CKG.II.10.1, CKG.I, CKG.II.10.3, CYA.II.14.3, and CYA.II.17) that promoted asparagus growth showed them to have 99% homology with sequences of P. fortinii (Table 3). Two other DSE fungal isolates that promoted asparagus plant growth, CKG.II.10.4 and CKG.IV.10, were identified at the phylum level as members of the Ascomycota, III.PI.I7 was identified at the class level as Dothideomycetes, and III.Ep.9 was identified at the family level as a member of the Arthopyreniaceae.

P. fortinii isolated III.PI.I8 (from wild raspberry), CKG.II.10.1 and CKG.I.I (both from Japanese cypress) were the most effective in promoting A. officinalis biomass (Fig. 1). The mean dry weights of asparagus plants inoculated with isolates III.PI.I8, CKG.II.10.1, and CKG.I.I were 16.1 mg, 14.7 mg and 13.4 mg, equivalent to increases of 53.5%, 40.1% and 27.5%, respectively, over control plants, the mean dry weight of which was 10.5 mg. When inoculated onto asparagus seedlings, the three isolates of P. fortinii formed microsclerotia-like structures inside roots, with those formed by isolate CKG.II.10.1 being densely-packed in root cells.

P. fortinii isolates III.PI.I8, CKG.II.10.1 and CKG.I.II were selected for further studies into their effects on asparagus growth under organic conditions. On 2% malt extract medium, the colony growth rates of isolates III.PI.I8, CKG.II.10.1 and CKG.I.II were 2.59, 1.98 and 2.69 mm per day, respectively, at 23 °C. On 2% MEA, colonies of these isolates were velvety and dark blackish. The aerial hyphae of the three isolates had similar characteristics to P. fortinii, with numerous wart-like blisters.

3.3. Confirmation of the ability of selected DSE fungi to degrade selected carbon and nitrogen compounds

With the exception of lignin, P. fortinii isolates III.PI.I8, CKG.II.10.1 and CKG.I.II were capable of degrading all of the organic compounds tested, including cellulose, starch, lipid, casamino acids, urea, pectin and gelatin (Table 4).
3.4. Evaluation of the ability of DSE fungi to utilize organic and inorganic phosphorus in promoting asparagus plant growth

In media with phytic acid as the sole source of P, isolates III.Pi.I8, CKG.II.10.1 and CKG.I.11 significantly increased the growth of *A. officinalis* seedlings by 132%, 79% and 117%, respectively, compared with control plants (Fig. 2). In medium with calcium phosphate as a source of insoluble P, the dry weights of *A. officinalis* seedlings inoculated with *P. fortinii* isolates III.Pi.I8 and CKG.II.10.1 were significantly increased by 118% and 122%, respectively, compared with control plants, whereas the mean dry weight of plants inoculated with *P. fortinii* isolate CKG.I.11 was not significantly different from that of the controls (Fig. 2).

3.5. Evaluation of the ability of DSE fungi to utilize organic nitrogen sources in promoting asparagus plant growth

When supplied with L-leucine as the sole organic N source, *P. fortinii* isolates III.Pi.I8, CKG.II.10.1 and CKG.I.11 significantly increased the growth of *A. officinalis* seedlings by 132%, 79% and 117%, respectively, compared with control plants (Fig. 2).
With L-valine as the sole organic N source, *P. fortinii* isolates CKG.II.10.1 and CKG.I.11 significantly increased the dry weight of *A. officinalis* plants by 57% and 79%, respectively, compared with control plants (Fig. 3). *P. fortinii* isolate III.Pi.I8 had no effect on the dry weight of *A. officinalis* when organic N was supplied as L-valine (Fig. 3).

### 3.6. Evaluation of the ability of DSE fungi to promote asparagus plant growth under semi-organic and organic conditions

On a semi-organic medium, *P. fortinii* isolates III.Pi.I8, CKG.II.10.1 and CKG.I.11 increased *A. officinalis* growth by 81%, 72% and 82%, relative to control plants (Fig. 4). On an organic medium, the three isolates increased the dry weight of *A. officinalis* seedlings by 117%, 197% and 235% compared with control plants (Fig. 4). On the organic medium, the uninoculated control plants were unable to grow well compared with asparagus plants inoculated with the three *P. fortinii* isolates (Fig. 4).

### 3.7. Anatomical observations

Based on anatomical observations, the three *P. fortinii* isolates were able to colonize the epidermal and cortical root cells of...
A. officinalis seedlings without causing any typical disease symptoms. The colonization patterns of P. fortinii isolates were similar in all treatments, involving colonization processes in epidermal cells and the formation of dense fungal hyphae structures (Fig. 5).

4. Discussion

In this study, we succeeded in isolating P. fortinii from the roots of plants growing in natural ecosystems, and showed that the isolates promoted the growth of asparagus. Previous studies have isolated P. fortinii from the roots of P. sylvestris (Wang and Wilcox, 1985), P. contorta (O’Dell et al., 1993); Vaccinium vitis-idaea, Betula platyphylla var. japonica, Luetkea pectinata (Addy et al., 2000), P. abies, Betula pendula (Menkis et al., 2004), Fagus sylvatica, Calluna vulgaris, A. alba and Rhododendron sp. (Konnert and Bergmann, 1995; Ahlich-Schlegel, 1997; Rendell and Ennos, 2002; Grünig et al., 2008), but this is the first report of P. fortinii being isolated from the roots of C. obtusa (Japanese cypress) and Rubus sp. (wild...
Despite several DSE fungi being known for their ability to degrade C, N and P organic compounds, their ability in decomposing organic material is still poorly understood (Caldwell et al., 2000). The results of our study were similar to those described by Caldwell et al. (2000) in that P. fortinii had the ability to degrade organic compounds such as cellulose, starch and protein. Three selected DSE isolates (P. fortinii III.P.II.10.1, and CKG.L11) in this study were able to degrade cellulose, starch, lipid, casamino acid, gelatin, urea and pectin (Table 4). Until now, only five DSE species (P. fortinii, Phialophora finlandica, Leptodontium orchidicola, Periconia macrospinosa and Microdochium sp.) have been reported to degrade cellulose, starch and protein (Caldwell et al., 2000; Menkis et al., 2004; Mandyam et al., 2010). However, our study is the first to report that P. fortinii degrades lipid, casamino acids, urea and pectin. We assume, based on their degradation abilities, that the selected P. fortinii isolates are able to mineralize organic nutrient sources in soil into available forms that support asparagus growth. However, further research is needed to confirm this assumption. The ability of P. fortinii to degrade C and N organic compounds suggests that the fungus may have a role in supplying nutrients to plants through organic matter decomposition, although its role in decomposition processes in situ is still unclear (Jumpponen and Trappe, 1998; Thorrmann et al., 1999; Thorrmann, 2006). Endophytic fungi such as P. fortinii are present in living, senescent and decaying plant tissues and may thus act as pioneer decomposers in natural ecosystems (Müller et al., 2001; Snadjr et al., 2011; Hirose et al., 2013). Although some reports have stated that P. fortinii lives on the decaying roots of various plant species (Sieber, 2002; Menkis et al., 2004), the P. fortinii isolates studied here originated from healthy roots of Japanese cypress and wild raspberry.

We also report for the first time that P. fortinii isolates were able to promote plant growth using phytic acid sodium salt as a sole source of organic P. Asparagus plants inoculated with isolates CKG.L11, CKG.L10.1 and III.P.I8 had greater biomasses than control plants, and dense colonization by P. fortinii occurred in root epidermal cells. Although a previous study found that Phialocephala glacialis and Phialocephala turicensis are able to mineralize organic P in vitro (Della Monica et al., 2015), the ability of P. fortinii to mineralize organic P has not been previously reported. Therefore, P. fortinii isolates CKG.L11 and III.P.I8 may have substantial potential to promote A. officinalis growth under organic conditions using P from natural materials, such as leaves and wood debris. According to Della Monica et al. (2015), DSE fungi are efficient at releasing P from organic sources in an available form that can be utilized by plants. Although P is abundant in agricultural soils, it is mostly in an insoluble form, and it hence cannot be utilized directly by plants (Neumann and Romheld, 2002; Miller et al., 2010; Moe, 2013). In addition, P fertilizer use efficiency is only 30% because it is fixed in the soil (Sharma et al., 2013). Therefore, microbes such as DSE fungi that are capable of dissolving P sources that are otherwise unavailable to plants, such as calcium phosphate (Barrow and Osuna, 2002; Della Monica et al., 2015), might be used to convert inorganic phosphorus into an available form that can be absorbed by plants to increase growth and production (Oteino et al., 2015). Our findings are similar to that of Barrow and Osuna (2002), who reported that another fungal endophyte, Aspergillus ustus, promoted Atriplex canescens growth supplied with inorganic P. The ability of P. fortinii isolates to promote asparagus growth using calcium phosphate in vitro might be applied in organic asparagus farming systems to meet plant P nutrient demands through the use of natural inorganic P, such as rock phosphate. In addition to being able to promote A. officinalis growth using organic and inorganic P sources, the three P. fortinii isolates in this study were also able to promote the growth of A. officinalis on agar medium using the organic N sources L-leucine and L-valine. This is the first report that P. fortinii is able to promote asparagus growth using these amino acids as organic N sources. In previous tests, the three P. fortinii isolates were found to degrade all of the organic N compounds tested, such as gelatin, urea and casamino acid (Table 4), suggesting that they may mineralize organic nitrogen into available forms for plants. DSE fungi have the ability to supply N to plants from organic N forms (Upson et al., 2009; Mandyam et al., 2013), and several studies have stated that these fungi are able to promote plant growth through the use of amino acids as organic N sources (Usuki and Narisawa, 2007; Diene et al., 2013; Mahmoud and Narisawa, 2013). However, several DSE species, such as L. orchidicola, Helminthosporium velutinum, Pseudocercospora abelmoschi and Pseudosignigmaea sp., are unable to enhance host plant growth on media using leucine as an organic N source (Diene, 2009), suggesting that DSE species may have different preferences for the use of amino acids.

Studies of the associations between DSE fungi and agricultural plants under organic conditions in vitro and the application of the fungi in organic farming are still scarce. Sadowsky et al. (2012) stated that blueberry roots by DSE fungi was frequent, especially in loamy sand soils in organic fields, compared with colonization in conventional fields. Previous research conducted by Mahmoud and Narisawa (2013) succeeded in isolating the DSE species Scolobasidium humicola from organic fields and found that the fungus had the ability to promote tomato plant growth under organic N conditions in vitro. Our results similarly showed that A. officinalis inoculated with three P. fortinii isolates, grown on an agar medium supplemented with only 0.1% corn steep liquor as an organic nutrient source and without inorganic nutrients, grew better than control plants. A. officinalis inoculated with P. fortinii isolate CKG.L11 showed better growth than in other treatments. Similarly, when the amount of KH₂PO₄, MgSO₄.7H₂O and NaNO₃ in the agar medium was halved and 0.1% corn steep liquor was added to the medium, the P. fortinii isolate CKG.L11 also increased asparagus plant growth compared with control plants (Fig. 4). This indicated that selected DSE fungi supported asparagus growth effectively under organic conditions, and the improved efficiency of nutrient uptake from organic sources such as corn steep liquor caused an increase in asparagus growth. However, the mechanisms leading to growth increases in asparagus caused by inoculation with P. fortinii under organic and semi-organic conditions are not yet fully understood and require further investigation.

The colonization patterns of the three P. fortinii isolates in asparagus roots were similar in all treatments, with hyphae colonizing epidermal cells and forming microsclerotia-like structures (Fig. 5). However, the hyphae did not invade the vascular cylinder and did not cause any damage or typical disease symptoms. In previous studies of other DSE fungal species, DSE fungi have densely colonized root epidermal and cortical cells (Peterson et al., 2008; Andrade-Linares et al., 2011; Sadowsky et al., 2012; Diene et al., 2013; Mahmoud and Narisawa, 2013). The DSE fungi studied here formed dark brownish microsclerotia-like structures in the epidermal and cortical cells of asparagus plants (Fig. 5), a pattern similar to that reported by Cameron (1998) and Yu et al. (2001). In some cases, the hyphae of endophytic fungi have colonized the cortical cells of host plants and have exerted effects on plant growth (Dewan and Sivathamparam, 1988; Gasoni and Stegman De Gurfinkel, 1997; Vadassery et al., 2009). Microsclerotia-like structures of the selected DSE fungi were not present in the vascular cylinder tissues in the roots studied here, indicating that there was little potential for parasitism of asparagus plants (Yu et al., 2001; Peterson et al., 2008; Sadowsky et al., 2012).
We conclude that the *P. fortinii* isolate CGK.I11, originating from the roots of Japanese cypress, is consistently able to promote asparagus growth under organic conditions using either organic P or N sources or corn steep liquor as an organic plant nutrient source. A previous study by Usuki and Narisawa (2007) found that the DSE fungus *H. chaetospira* was able to facilitate growth by Chinese cabbage plants and that it densely colonized roots without causing any disease symptoms. *H. chaetospira* was hence considered to be a mycorrhizal fungus (Usuki and Narisawa, 2007). *P. fortinii* isolate CGK.I11 was similarly able to promote asparagus growth using organic N sources and to densely colonize asparagus roots (Figs. 3 and 5). Although the mechanism of P uptake facilitated by this fungus from the medium to the host plant has yet to be elucidated, we suggest that *P. fortinii* may promote asparagus plant growth by degrading soil organic N and P compounds and making them available to host plants. However, we cannot yet provide sufficient detail of the uptake mechanisms involved to be certain of the symbiotic role of *P. fortinii* with *A. officinalis*. Thus, further investigation of the association between *P. fortinii* isolate CGK.I11 and asparagus is necessary to determine the potential influence of the fungus on asparagus production and fertilizer use efficiency under organic conditions in the field.

Acknowledgments

This study was supported by the Council for Science, Technology, and Innovation (CSTI), Cross-ministerial Strategic Innovation Promotion Program (SIP), “Technologies for creating next-generation agriculture, forestry, and fisheries” (funding agency: Bio-oriented Technology Research Advancement Institution, NARO, Japan, No. 14537523). Two anonymous reviewers provided helpful comments on the manuscript.

References


