The Journal of Microbiology (2012) Vol. 50, No. 4, pp. 618–624
DOI 10.1007/s12275-012-2105-6

The Role of a Dark Septate Endophytic Fungus, *Veronaeopsis simplex* Y34, in Fusarium Disease Suppression in Chinese Cabbage

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(Received February 28, 2012 / Accepted May 8, 2012)

The soil-inhabiting fungal pathogen *Fusarium oxysporum* has been an increasing threat to Chinese cabbage (*Brassica campestris* L.). A dark septate endophytic fungus, *Veronaeopsis simplex* Y34, isolated from Yaku Island, Japan, was evaluated *in vitro* for the ability to suppress Fusarium disease. Seedlings grown in the presence of the endophyte showed a 71% reduction in Fusarium wilt disease and still had good growth. The disease control was achieved through a synergetic effect involving a mechanical resistance created by a dense network of *V. simplex* Y34 hyphae, which colonized the host root, and siderophore production acting indirectly to induce a resistance mechanism in the plant. Changes in the relative abundance of the fungal communities in the soil as determined by fluorescently labelled T-RFs (terminal restriction fragments), appeared 3 weeks after application of the fungus. Results showed the dominance of *V. simplex* Y34, which became established in the rhizosphere and out-competed *F. oxysporum*.

Keywords: dark septate endophytic fungus, *Veronaeopsis simplex* Y34, Fusarium disease, siderophore, T-RFLP

Introduction

Fusarium wilt, induced by pathogenic strains of *Fusarium oxysporum*, is a serious soil-borne disease in many crops of economic importance, such as Chinese cabbage. It causes stunted growth, chlorosis, loss of older leaves, wilting and even death (Jones *et al*., 1991). Different approaches can be used to prevent, mitigate or control Fusarium disease. Disease management options include crop rotation, soil sterilization and the use of resistant cultivars. Beyond good agronomic and horticultural practices, growers often rely on chemical pesticides, which can act as pollutants in the environment. Biological control methods have the potential to control the disease.

A variety of soil microorganisms including bacteria and fungi have demonstrated potential as biocontrol agents against Fusarium disease (Ogawa and Komada, 1984; Mousseaux *et al*., 1998; Pereira *et al*., 2007; Moretti *et al*., 2008). Among them, mycorrhizal fungi like *Gigaspora* sp. have been known to enhance the suppression of pathogens in plants (Machon *et al*., 2006; Hu *et al*., 2010). Chinese cabbage is a fast-growing plant and an important vegetable in Asia including China, Japan and Korea. This plant belongs to the Cruciferae, which contain thioglucoside compounds that have a negative effect on vesicular arbuscular mycorrhizal (VAM) fungi. There is also evidence of their involvement in the lack of VAM fungal colonization in most Brassicaceae (Ocampo, 1980; Smith and Read, 1997). Recently, Narisawa *et al*. (2000) reported that mycorrhizae-free Brassicaceous plant could be colonized by a dark septate endophyte (DSE), *Heteroconium chaetospirae*, which acts as a biocontrol agent.

DSE fungi are defined as conidial or sterile ascomycetous fungi that live in symbiosis with certain plants and colonize living plant root tissues intracellularly and intercellularly without causing any apparent negative effect, such as tissue disorganization, or forming any typical mycorrhizal structures (Jumpponen and Trappe, 1998). Despite an almost ubiquitous occurrence, their behavior in plant roots, and hence their impact on plant performance and fitness, are poorly understood. However, many studies have found that DSE fungi improve plant performance, including nutrient uptake and the ability to withstand adverse environmental conditions (Mandyam and Jumpponen, 2005). Furthermore, DSE fungi can protect host plants and increase their tolerance against pathogens directly and indirectly by producing antifungal metabolites, fungal parasitism or inducing plant systemic resistance (Samuel *et al*., 2000; Campanile *et al*., 2007). For these reasons, there is growing interest in the application of microorganisms, especially DSE fungi, as biological control and growth promoting agents in many crops (Thakuria *et al*., 2004).

We described *Veronaeopsis simplex* Y34 (MAFF240802), a DSE fungus isolated from a sub-tropical area of Yaku Island, Japan. The first report about *V. simplex* (Papendorf) was by Arzanlou and Crous who only provided information about its isolation from leaf litter of *Acacia karroo* in South Africa and its morphology (Arzanlou *et al*., 2007), but little information is available about utilization of *V. simplex* in plants. In a recent study, we observed the ability of this fungus to promote the growth of Chinese cabbage *in vitro*. Plants inoculated with *V. simplex* Y34 had greater biomass than untreated plants (Khastini *et al*., 2011). This study raised the prospect of using *V. simplex* for Fusarium disease suppression, since some DSE fungi capable of promoting plant growth also have the ability to suppress fungal pathogens

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(Narisawa *et al*., 1998).

Therefore, in the present study, we examined the characteristics, behavior and development of *V. simplex* Y34 in Chinese cabbage and propose a possible mechanism for Fusarium disease suppression *in vitro*. In addition, to obtain information about the relative abundance of the *V. simplex* Y34 and *F. oxysporum* communities present in the rhizosphere of Chinese cabbage, T-RFLP analysis of soil samples was performed.

Materials and Methods

Fungal isolate

Veronaeopsis simplex Y34 (MAFF240802) was isolated from a soil sample collected from wooded areas of Yaku Island, Japan (available from Dr. K. Narisawa). To prepare the inoculum, the isolate was grown on oatmeal agar [OMA; oatmeal, 10 g/L; and Bacto agar, 18 g/L] enriched with nutrients [$MgSO_4 \cdot 7H_2O$, 1 g/L; KH_2PO_4, 1.5 g/L; and $NaNO_3$, 1 g/L] in Petri dishes (90-mm diameter). The fungal pathogen *Fusarium oxysporum* (isolate number 301, also available from Dr. Narisawa), isolated from naturally infested soil at Ibaraki, Japan, was grown in the same medium. Pathogenicity tests carried out in a preliminary assay confirmed that the *Fusarium* isolate caused wilt disease.

Biocontrol assay of *V. simplex* Y34 against Fusarium disease *in vitro*

V. simplex Y34 was grown on OMA enriched with nutrients [$MgSO_4 \cdot 7H_2O$, 1 g/L; KH_2PO_4, 1.5 g/L; and $NaNO_3$, 1 g/L] in Petri dishes (55-mm diameter), and incubated for 3 weeks at room temperature (approximately 23°C). Chinese cabbage cv. Musou (*Brasica campestris* L.) (Takii seed, Japan) was used. The seeds were surface sterilized by immersion in a 70% solution of ethanol for 1 min, and a solution of sodium hypochlorite (1% available chlorine) for 5 min. Seeds then were rinsed three times with sterilized distilled water. Seeds were dried overnight and placed on 1.5% water agar (15 g of Bacto agar [Difco] and 1 L of H_2O) in Petri dishes. After 2 days, the axenically grown seedlings were transplanted onto growing fungal colonies on the medium. Seedlings transplanted onto non-inoculated medium were used as controls and the whole set placed into sterile culture bottles (CB-1, As One, Japan) and incubated for 2 weeks at room temperature with an 18 h:6 h (L:D) regime and intensity of 74 $\mu mol/m^2/s$. Seedlings grown for 2 weeks with and without the fungal isolate then were challenged with the 3 to 4-day-old *F. oxysporum* grown in water agar. Seedlings were overlaid directly onto the fungal colony and incubation continued for 2 weeks under the same conditions.

To analyze the fungal community associated with the disease suppression, 3-week-old *V. simplex* Y34-treated and untreated (control) seedlings were transplanted to sterile plastic pots containing 10^3 conidia/ml peat soil-compost mixture (1:1, w/w) and incubated for 3 weeks as mentioned above. The symptoms of disease were scored from 0 to 3 (0=no visible symptoms; 1=light yellowing; 2=yellowing and late growth; 3=wilting or death). Then, a disease index was calculated as the sum of the scores obtained as follows: in class 0 (×0), in class 1 (×10), in class 2 (×30), and in class 3 (×100). Finally, the sum was divided by the total number of plants (Narisawa *et al*., 2000).

Plants were harvested and oven-dried at 60°C. Dry weight was measured and compared with the control. At harvest time (3 weeks after planting), the root system was separated from the shoot and weighed after drying at 60°C. The root system was divided into two parts for determining dry weight and fungal colonization. The entire root system was removed from the agar, cleared in 10% KOH at 80°C for 20 min, and neutralized in 1% HCl at 80°C for 20 min and stained with 0.05% cotton blue in 50% acetic acid. The stained root was examined along grid lines to estimate the extent of *V. simplex* colonization. Each grid cell was designated as either colonized or non-colonized (Usuki *et al*., 2002). Root segments were then sectioned with a cryostat (Leica CM1800, Leica Microsystems GmbH, Germany), then the roots were mounted on a slide and observed under a light microscope (Olympus BX51).

Siderophore analysis: CAS-agar universal test plate

CAS-blue agar was prepared as described by Schwyn and Neilands (1997). Briefly 60.5 mg/L Chrome Azurol-S (CAS) was dissolved in 50 ml of distilled water, mixed with 10 ml of iron (III) solution (1 mM $FeCl_3 \cdot 6H_2O$, 10 mM HCl), and then 15 ml of agar was slowly added to 72.9 mg of hexadecyltrimethylammonium (HDTMA) in 40 ml of water. The resultant dark blue liquid was autoclaved at 121°C for 15 min. A mixture of 750 ml of distilled water, 15 g of agar, 30.24 g of PIPES, and a solution of 50% (w/w) NaOH to raise the pH was also autoclaved. The dye solution was finally poured and agitated, taking care to avoid foaming. Petri dishes (90 mm in diameter) were prepared with 30 ml of appropriate medium for culturing each strain.

After solidifying, half of the agar was removed and replaced by culture medium (PDA). The halves containing the culture medium were inoculated with *V. simplex* Y34. The inocula were placed far from the border between the two media. The plates were incubated at a suitable growth temperature for three weeks in the dark. The growth rates of the isolates were observed and expressed as the number of days required by the mycelia to cover the halves of Petri plates containing the culture medium. The CAS reaction rate was evaluated by measuring the advance of the color-change front in the CAS-blue agar, starting from the border between the two media. Inoculum-free CAS-agar was used as a control and no color change in the CAS-blue agar was observed.

Test for antagonistic activity

To test for direct antibiotic effects, an agar disc 0.5 cm in diameter completely covered by *V. simplex* mycelia was placed in the centre of an OMA plate and four agar discs of *F. oxysporum* were placed around it at equal distance after a 2-week incubation. The plates were incubated at room temperature and the zone of inhibition was observed.

Fungal community analysis in the rhizosphere of Chinese cabbage

Differences in *V. simplex* Y34 and *F. oxysporum* community structure associated with the disease suppression were monitored through terminal restriction fragment length polymorphism (T-RFLP) analysis of amplified ITS regions. Total DNA from soil samples (0.5 g wet weight) was isolated using an Isoil for Beads Beating kit (Nippon Gene, Japan). The ITS region was amplified by PCR from total genomic soil DNA with the primer QITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), labeled with quenching fluorescence and LR21 (5′-AC TTCAAGCGTTTCCCTTT-3′, region 424-393 in the *Saccharomyces cerevisiae* large subunit rRNA). The 5′-end fluorescence-labeled primer was purchased from J-Bio21 (Japan). The PCR mixture (30 µl) was prepared by combining 0.1 µg of template soil DNA, 1.0 µl of 10 pmol/µl primers, TaKaRa Ex Taq, dNTPs, and 3 µl of optimized 10× Ex buffer (TaKaRa Bio Inc.) in a PCR cycler. The PCR of ITS for T-RFLP profiling was carried out under the following conditions: 2 min at 95°C, followed by 30 cycles of 95°C (1 min), 59°C (1 min), and 72°C (1.5 min). T-RFLP was carried out as described previously (Nishizawa *et al.*, 2008) Aliquots (5 µl) of the amplified ITS fragment were separately digested with *Taq*I, *Hae*III, *Mbo*I, (TaKaRa Bio) according to the manufacturer's instructions. The precise lengths of T-RFs (terminal restriction fragments) from the amplified ITS fragments were determined on a 3130xl PE DNA Sequencer (Applied Biosystems). The purified T-RF DNA (2 µl) was mixed with 15 µl of Hi-Di formamide and 0.1 µl of DNA standard LIZ®500 (Applied Biosystems) for standardization. This mixture was then denatured at 96°C for 2 min and immediately chilled on ice prior to electrophoresis with the automated DNA sequencer in the GeneScan mode. The lengths of fluorescently labeled T-RFs were determined by comparison with internal standards using GeneMapper software (version 3.7; Applied Biosystems). T-RFLP profiling of the fungal community structure in soil samples was obtained with peaks ranging from 50 bases to 650 bases. The definition of T-RFs was peaks with a fluorescence threshold of more than 30.

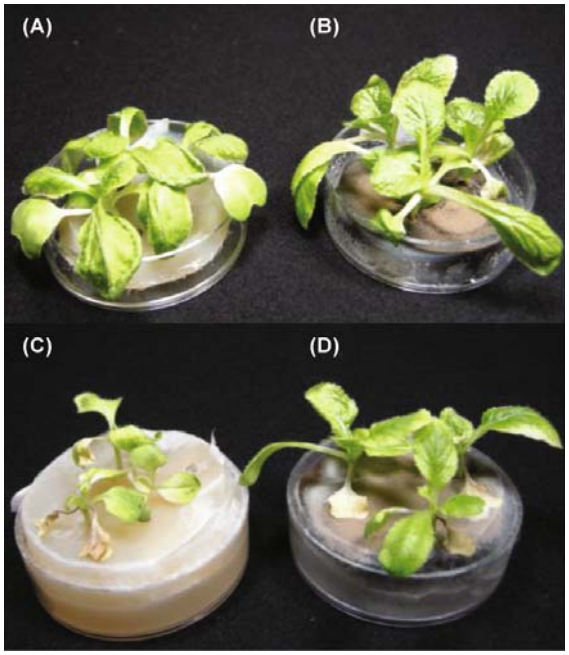

Fig. 1. The effect of *V. simplex* Y34 on Fusarium disease in Petri-dish-grown Chinese cabbage seedlings. Disease symptoms were assessed three weeks after transplanting. (A) Non inoculated plants; (B) *V. simplex* Y34-inoculated plants; (C) The foliage of plants inoculated with *F. oxysporum*. Seedlings were yellow and severely damaged; (D) The *V. simplex* Y34-treated seedlings did not show typical external symptoms and appeared to be healthy after being challenged with *F. oxysporum*.

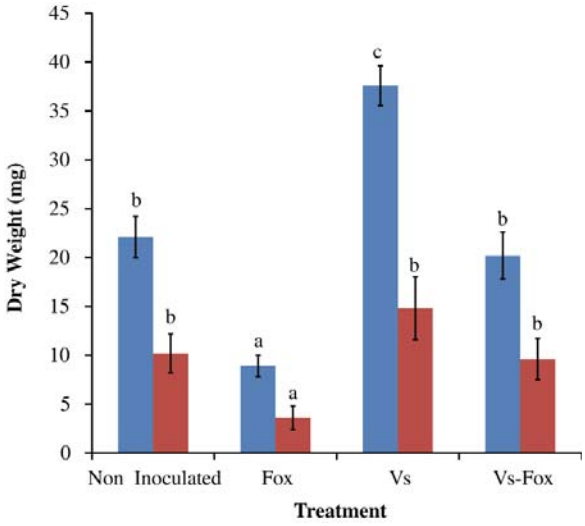

Fig. 2. The effect of *V. simplex* on suppression of Fusarium disease shown by shoot and root dry weight. Vs, *V. simplex* Y34-inoculated plant; Fox, *F. oxysporum*-inoculated plant and Vs-Fox, *V. simplex* Y34 challenged with *F. oxysporum*. Results were subjected to a one-way ANOVA; Values followed by the same letter are not significantly different at P<0.05 according to Tukey's least significant difference test.

Results

Effects of *V. simplex* Y34 on Fusarium disease

Figure 1 shows that *V. simplex* Y34 had a significant effect not only on the growth of Chinese cabbage but also in Fusarium disease suppression (P<0.05). The dry weight of *V. simplex* Y34-treated plants (shoots and roots) increased almost 2 fold compared to that of control plants. We found that the Y34-treated plants were more resistant to Fusarium disease as the isolate still had a positive effect when the plants were challenged with *F. oxysporum* (Fig. 2). The Y34-treated plants had significantly greater biomass, showed slight yellowing on the leaves and were more vigorous. Plants inoculated with *Fusarium* showed extremely inhibited growth, yellowing and wilting.

In the absence of *V. simplex* Y34, *F. oxysporum* caused severe disease in Chinese cabbage. The disease's progression appeared exponential with time as shown in Fig. 3. However, prior application of *V. simplex* Y34 caused a 71% reduction in disease.

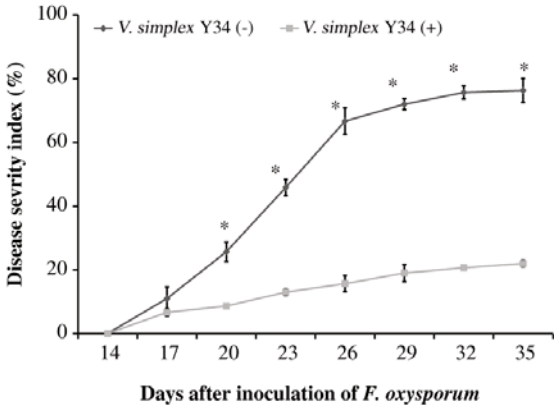

Fig. 3. Effect of *V. simplex* Y34 on the development of Fusarium disease in Chinese cabbage. Mean values that differ significantly at each time point (P=0.05) are indicated by an asterisk. Bars indicate standard errors of means.

Growth of *V. simplex* Y34 in the roots of Chinese cabbage

The roots of 4-week-old plants grown in medium with and without *V. simplex* Y34 were observed and the extent of root colonization was estimated. No fungal colonization of root cells was observed in control plants. The degree of root colonization by *V. simplex* Y34 was 66%. In the presence of *F. oxysporum*, this value was reduced insignificantly to 59% (P<0.05).

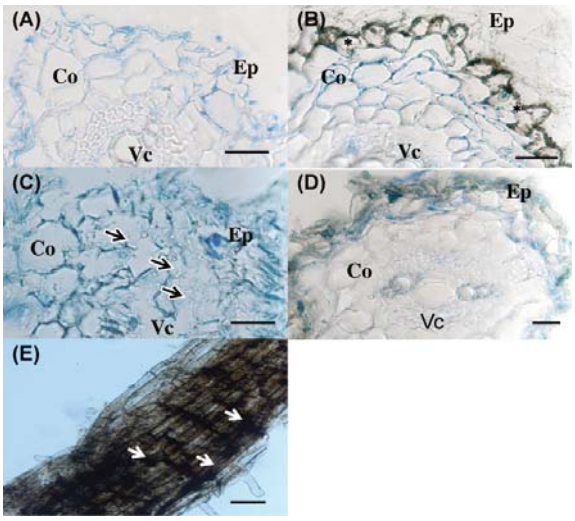

Fig. 4. Cross-section of a Chinese cabbage root stained with 0.005% cotton blue in 50% acetic acid. (A) root without fungal inoculation; (B) *V. simplex* Y34-treated Chinese cabbage. Fungal hyphae were seen on root surfaces within epidermal cells (*Ep*) and within cortical cells (*C*) Fungal colonization was restricted to epidermal and cortical cells (asterisk). The vascular cylinder (VC) was not invaded; (C) Heavy fungal colonization of *F. oxysporum* in the root. Fungal colonization by the pathogen reached the vascular cylinder (Vc) (arrows). Epidermal (Ep) and cortical (Co) cells were already colonized by fungal hyphae; (D-E) *V. simplex* Y34-treated Chinese cabbage roots challenged with *F. oxysporum*. Arrows show a dark coloring, cell wall appositions, and thickening of some epidermal (Ep) and cortical (Co) cells (asterisks). Bar=10 μm.

Light microscopy of semi thin cross sections of colonized Chinese cabbage roots showed that dense networks of *V. simplex* Y34 hyphae colonized epidermal cells (Fig. 4). Although the intercellular hyphae developed parallel to the longitudinal root axis, they were restricted to the cortical cells and not present in the vascular cylinder. In the roots of plants treated with the pathogen, *F. oxysporum* hyphae heavily colonized the root surface and the inside of lateral roots, not just in epidermal and cortical cells but also in the vascular cylinder. The *V. simplex* Y34-treated roots exposed to *F. oxysporum* showed a darkening and thickening of some epidermal cells, while in the cortex area, hyphae of the pathogen were rarely found.

Siderophore production and *in vitro* interaction of *V. simplex* Y34 with *F. oxysporum*

The universal assay described by Schwyn and Neilands (1997) is usually used for the detection of siderophores produced by different microorganisms (fungi and bacteria) in solid medium. *V. simplex* was incubated on CAS-agar plates and the color reaction was observed. The fungus turned the CAS medium purple (Fig. 5A).

We tested for direct antibiotic effects of *V. simplex* Y34 on *F. oxysporum in vitro* as shown in Fig. 5B. In agar plates, neither *F. oxysporum* nor *V. simplex* growth was affected by the presence of the other fungus, and there was no zone of inhibition at the point of contact between the two fungal colonies. This result indicated that the fungus did not show any direct antagonistic activity toward *F. oxysporum.*

Fungal community analysis in the Chinese cabbage rhizosphere

Soil DNA was prepared from the rhizosphere of Chinese cabbage that had been inoculated with either *V. simplex* Y34 (Vs), *F. oxysporum* (Fox) or *V. simplex* Y34 challenged with *F. oxysporum* (Vs-Fox) and analyzed by T-RFLP profiling. It is believed that the activity of *V. simplex* Y34 is not restricted to the root, as in the natural habitat, the DSE fungi

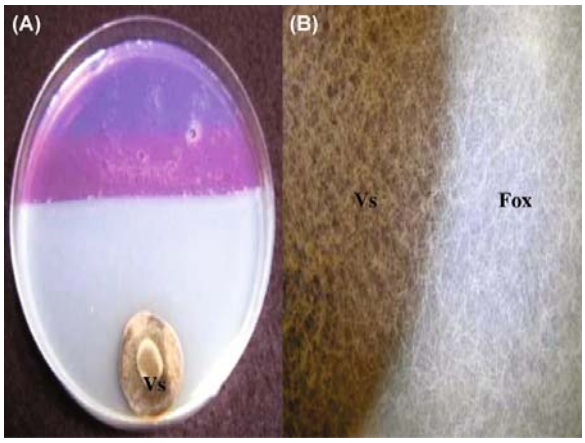

Fig. 5. (A) Siderophore test of *V. simplex* Y34 grown in CAS media; (B) Test of direct fungal antagonism. Agar plate co-cultivated with *V. simplex* (Vs) and *F. oxysporum* (Fox). Fungal cultures were grown for 2 weeks.

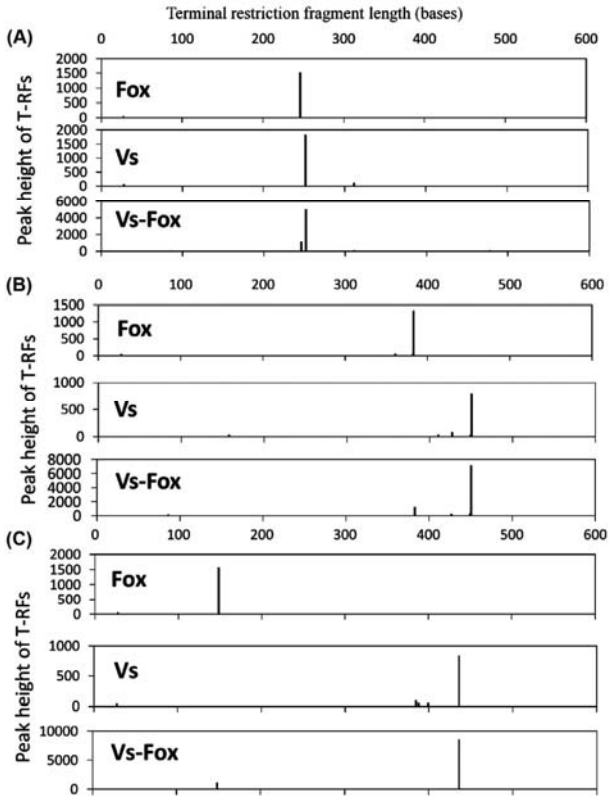

Fig. 6. T-RFLP profiling of treatment soil using the amplified fungal ITS gene as template. Fox, *F. oxysporum* inoculated soil; Vs, *V. simplex* Y34 inoculated soil and Vs-Fox, *V. simplex* Y34 challenged with *F. oxysporum*; (A) *Taq*I, (B) *Hae*III, (C) *Mbo*I. The x-axis indicates the terminal restriction fragment length between 0 and 650 bases, and the y-axis represents the peak height.

are widely distributed in the rhizosphere and rhizoplane of plant growing soil. The electropherograms obtained with *Taq*I, *Hae*III, and *Mbo*I are illustrated in Fig. 6. The resulting output was in the form of an electropherogram in which each terminal restriction fragment (TRF) is separated according to size (bp).

The profiling revealed one major peak T-RF each for Fox and Vs, with 246 bases and 251 bases respectively after digestion with *Taq*I. Similarly, single major T-RFs (384 bases and 450 bases) were detected in the *Hae*III-digested electropherogram, and single major T-RFs (148 bases and 435 bases) in the *Mbo*I digested one. Two major peaks were detected on the Vs-Fox electropherogram after digestion with *Hae*III, *Mbo*I, and *Taq*I with the same sizes of bases as seen for Vs and Fox in corresponding digestion profiles.

The peak and peak height showed the fluorescence intensity that corresponds to a specific fungal abundance in the rhizosphere. By analyzing the peak height, the fungal abundance in the rhizosphere that was inoculated with *V. simplex* Y34 alone showed a similar trend to that inoculated with *F. oxysporum* alone. While in the rhizosphere that was inoculated with *V. simplex* Y34 exposed to the *F. oxysporum*, the *V. simplex* Y34 population was higher (Fig. 6). These results suggested that even though both fungal communities were present, their relative abundance was different. This result strongly suggests that the *V. simplex* Y34 community suppresses the *F. oxysporum* community in the soil rhizosphere.

Discussion

Despite numerous studies, the utilization of DSE fungi for biological control purposes still has not been realized and these plant-microbe interactions remain poorly understood. This paper presents the first assessment of *V. simplex* Y34, a member of the DSE fungi, for Fusarium disease suppression in Chinese cabbage. *V. simplex* Y34 isolates were obtained from forest soil samples in the southwest of Yaku Island, Japan (Narisawa, 2008). The use of annual plants as bait for the DSE taxon, i.e., *H. chaetospira* and *P. fortinii*, is an effective method of recovering these species from woodland soils and the egg plant (*Solanum melongena* L.) was used as bait for the fungus. *V. simplex* is not very common in the soil but is often associated with the roots of plants, probably forming "symbiotic" relationships. This fungal species may have great potential as a biocontrol agent; however, its specific roles in ecosystems remain speculative. Only three isolates including the *V. simplex* Y34 isolate, exist worldwide. In our previous work, the ability of *V. simplex* Y34 to promote growth in Chinese cabbage was successfully confirmed for the first time. We compared the Y34 isolate with two other *V. simplex* isolates and found that it provided the best results related to plant growth (Khastini *et al.*, 2011).

Differences that emerged from measurements of growth parameters following the infection of Chinese cabbage with *V. simplex* Y34, and production of selected biochemical molecules, provided evidence of the potential of *V. simplex* Y34 as an agent for suppressing Fusarium disease. In the current study, increases in shoot and root weight were observed after inoculation with *V. simplex* Y34, with the extent of colonization being 66% (Fig. 2). When Chinese cabbage root was inoculated with *V. simplex* and challenged with *F. oxysporum*, the percent colonization of *V. simplex* Y34 was reduced insignificantly. For this reason, heavy colonization of *V. simplex* in plant roots inhibits the growth of *F. oxysporum* inside the root and also acts as an aid to blocking deleterious organisms from potential infection sites of.

The plants inoculated with *F. oxysporum* showed severe symptoms of disease with stunted growth, wilting and yellowing of leaves (Fig. 1). Some of the root epidermal and cortical cells turned a dark color, which indicated a host defense response. In DSE-treated host roots, a marked host reaction, mainly in epidermal and cortical cells, against an ingressive pathogen is common. This phenomenon was also observed in *Phialocephala fortinii*-treated Chinese cabbage post-inoculated with *Verticillium longisporum* (Narisawa *et al.*, 2004). The dense hyphal networks of *V. simplex* Y34 that formed surrounding the root might enhance mechanical resistance of plant tissue to the penetration of pathogens such as *F. oxysporum*, since the proportion of the root surface it can colonize is reduced in the presence of the *V. simplex* Y34 mycelium. In this context, competition for space or infection sites between *V. simplex* and *F. oxysporum* does occur

in Chinese cabbage roots.

V. simplex Y34 acted to control Fusarium disease through a synergetic effect involving mechanical resistance and siderophore production, where the fungus has an indirect role by triggering the plant's induced systemic resistance. Van Loon *et al.* (1998) defined induced systemic resistance (ISR) as a state of increased defensive capacity developed by plants when appropriately stimulated, through activation of latent resistance induced by diverse agents, including fungi. Induced systemic resistance in plants is attractive from this point of view, as *V. simplex* does not kill the pathogen directly with a toxic metabolite, but restricts the penetration of *F. oxysporum* into Chinese cabbage by activating the defense system of the plant.

Many studies have focused on the utilization of bacteria and fungi including *Pseudomonad*, *Trichoderma*, and *Gigaspora* species that belong to the AMF group for managing Fusarium disease. However, the utilization of *V. simplex* as a biocontrol agent has not been documented previously. Regardless of the organism used, an important criterion for the successful implementation of a biocontrol agent is knowledge about related mechanisms. The mechanisms by which *V. simplex* suppresses Fusarium disease are not completely understood. Several possibilities have been proposed based on fungi as biocontrol agents against Fusarium disease, such as the secretion of antibiotic and other secondary metabolites (Fere and Santamarina, 2010), fungal competition for nutrients (Serra-Witling *et al.*, 1996), competition for infection sites and root colonization (Mandeel, 2007), plant-induced resistance through synergy with plant growth elicitors (Amini, 2009) and inactivation of pathogenesis-related proteins such as chitinases and β-1,3-glucanases (Duijff *et al.*, 1999).

Here, to study the possible mechanism of disease suppression, we performed antibiosis assays, which revealed no secretion by *V. simplex* of antibiotics that inhibit the growth of *F. oxysporum.* Similar findings have been made by Kumar *et al.* (2009) in another root endophytic fungus, *Piriformospora indica,* and its interaction with Maize. The suppressive effect of *P. indica* on Fusarium disease in Maize occurs not through antibiotic production but via oxidative defense.

A factor almost certainly involved in the biological control of plant diseases is siderophore production by antagonistic organisms (Dutta *et al.*, 2006). The siderophore is a complex of low-molecular mass (1000 Da) compounds that can bind iron with high specificity and affinity, making the iron unavailable for other microorganisms and thereby limiting their growth. Siderophores produced by *V. simplex* Y34 were observed using CAS-blue agar. This assay is based on competition for iron between the ferric complex of the indicator dye Chrome Azurol S (CAS), and the siderophore produced by the microorganism (Milagres *et al.*, 1999). That *V. simplex* Y34 produced siderophores can be seen from the change in color of CAS blue agar to purple. This color is typical of fungi belonging to zygomycetes, basidiomycetes and deuteromycetes (Payne, 1994). *V. simplex* chelates Fe^{3+}, making it unavailable to *F. oxysporum,* and consequently, the growth of *F. oxysporum* is inhibited. Aside from that, *V. simplex* supplies iron to plants, stimulating growth. *V. simplex* Y34 exhibits the same characteristics as *Pialocephala fortinii*, which also belongs to the DSE group of fungi, and has the capacity to produce the siderophore iron-chelating agent, (Bartholdy *et al.*, 2001). It also was effective in suppressing Verticillium wilt (Narisawa *et al.*, 2002).

Analysis of the soil microbial community structure is likely essential for a better understanding of pathogen-biocontrol agent interactions. Ryder and Jones (1993) reported that the ability of the beneficial microorganism to maintain a sufficient population density in the rhizosphere for a sufficient length of time was critical for the success of any biocontrol method. Based on the idea above, the presence of *V. simplex* in the Chinese cabbage rhizosphere needed to be investigated. In this paper, we attempted a microbial analysis under controlled conditions. As shown in the T-RF electropherograms, digestion with *Taq*I, *Hae*III, and *Mbo*I clearly showed the difference between *V. simplex* Y34 and *F. oxysporum* relative abundance in the soil when challenged with each other. Because the *V. simplex* association is formed early in the development of the plants, they represent nearly ubiquitous root colonists that assist plants with the uptake of nutrients. This was observed by Usuki *et al.* (2007), who found that DSE fungi provided nitrogen to Chinese cabbage plants, thereby increasing the growth of inoculated plants. In return, the fungus obtained carbon from the plant. A high population of *V. simplex* Y34, as a dominant and beneficial microorganism for Chinese cabbage, was established to outcompete *F. oxysporum.* The T-RFLP assay developed in this study offers a good alternative for estimating the potential of *V. simplex* Y34 as an agent for Fusarium disease suppression through field application.

In conclusion, the effectiveness of *V. simplex* Y34 in a laboratory setting indicates that the fungus has potential as a biocontrol agent and merits further investigation.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (B) (No. 23380190) from the Japan Society for the Promotion of Science (to K. Narisawa).

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