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Pre-inoculation of cucumber roots with *Verticillium lecanii* (*Lecanicillium muscarium*) induces resistance to powdery mildew

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キュウリの根に *Verticillium lecanii* (*Lecanicillium muscarium*) を処理するとうどんこ病に対して抵抗性が誘導される

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Abstract

The ability of *Verticillium lecanii* (*Lecanicillium muscarium*; B-2) to induce host resistance and defense responses against subsequent challenge with cucumber powdery mildews was examined in a glasshouse. A root test showed that, after inoculation with *V. lecanii* blastspore on cucumber roots, induction of systemic resistance in those *V. lecanii* pre-inoculated plants engendered significantly fewer lesions and reduced disease severity compared with non-inoculated plants. Furthermore, in plants inoculated with *V. lecanii* blastspore on their roots, the fungi showed high colonizing ability on cucumber rhizoplane and inside root tissues. Nevertheless, activities of peroxidase (PO) and phenylalanine ammonia-lyase (PAL) increased slightly, but not significantly. In a leaf test of inoculation with *V. lecanii*, activity of PAL tended to increase when powdery mildew was inoculated, but the PO activity did not change significantly. These results indicated that an isolate of *V. lecanii* (B-2) potentially induced host resistance to cucumber and is an effective candidate for biological control of cucumber powdery mildews, even in inoculation into soil.

Key words; induced resistance; *Lecanicillium muscarium*; Powdery Mildew; *Verticillium lecanii*

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摘要

本論文では、*Verticillium lecanii*（*Lecanicillium muscarium*）B-2がキュウリのうどんこ病に対して抵抗性を誘導するか否か調べた。*V. lecanii*（B-2）の出芽型胞子の懸濁液（1×10^7/ml）を10ml もしくは50ml をキュウリの根部に処理すると、本葉におけるうどんこ病の発病程度が有意に減少した。さらに、*V. lecanii*（B-2）は根面および根部内における高い定着率を示した。しかし、これらの抵抗性が誘導された時のペルオキシダーゼ(PO)やフェニールアラニンアンモニアリアーゼ(PAL)の酵素活性は顕著な増加が認められなかった。

キーワード：うどんこ病, *Lecanicillium lecanii*, *Verticillium lecanii*, 誘導抵抗性

1. Introduction

Numerous reports have highlighted the remarkable potential of *Verticillium lecanii* (A. Zimmerm.) Vie'gas (Recently renamed to *Lecanicillium muscarium*) as a powerful biocontrol agent against several arthropod pests and some plant pathogens. It has a very wide host range: it is especially parasitic of aphids and whiteflies – two of the most common greenhouse pests (Askary et al., 1999; Drummonds et al., 1987; Koike et al., 2004; Spencer et al. 1981). This ability has been used for production of commercial preparations: Vertalec and Mycotal. *V. lecanii* also is an effective mycoparasite of several rust fungi, green mold and fungi casing root rot diseases (*Pythium ultimum*), as well as of some powdery mildew pathogens (Askary et al., 1997; Benhaumau & Brodeur, 2000; Koike et al., 2004; Spencer et al., 1981; Verhaap et al., 1996). Numerous studies have mainly investigated morphological and functional aspects underlying microbial interaction (Benhaumau & Brodeur, 2000). Therefore, whether *V. lecanii* can interact with host plants and whether the fungi can induce disease resistance and pathogen defense responses systemically is an issue that demands investigation.

Systemic acquired resistance (SAR), localized exposure of plants to certain avirulent microbes (He et al., 2002; Ryu et al., 2003; Shimamoto & Watanabe, 2004) or treatment with certain biotic or chemical agents (Cools & Ishii, 2002; Katz et al., 1998; Ryu et al., 2003) can render an entire plant resistant to further infection by virulent pathogens. In addition, SAR is associated with hypersensitive reaction (HR) and with increased expression of many defense response mechanisms, such as R gene-mediated resistance. It causes accumulation of pathogenesis-related proteins (PR protein) (Cools & Ishii, 2002; He et al.,2002; Juen et al., 2003; Katz et al., 1998; Koike et al., 2001; Meera et al., 1994, Ongena et al., 2000; Ryals et al.,1996). Subsequently, SAR reduces the size and number of lesions that develop after inoculation with virulent pathogens (He et al.,2002). The signal transduction pathway in SAR is shown to be dependent on salicylic acid (SA) (Juen et al., 2003; Ryals et al., 1996; Ryu et al., 2003). Another form of resistance, induced systemic resistance (ISR), is induced mostly by root-inhabiting fungi and endophytes, especially plant growth-promoting rhizobacteria and fungi (PGPR and PGPF) (Juen et al., 2003; Koike et al., 2001; Meera et al., 1994; Ongena, 2000) ; ISR is distinguished from SAR by its different signal pathway. In short, ethylene and jasmonic acid (JA) have a role in the signal pathway of ISR (Katz et al., 1998; Ryu et al., 2003). These mechanisms are particularly attractive as strategies for control of plant diseases, especially amid growing concerns over adverse impacts of conventional pesticides on the environment, and the capacity of pathogens to generate resistance to these compounds (He et al.,2002).

Management of powdery mildews in glasshouses has become a challenging research area in plant pathology. Because of their wide host range and ability to proliferate

abundantly under favorable environmental conditions that usually prevail in greenhouses; yields are reduced (Askary et al., 1997). At least one study has estimated *V. lecanii* strains' high colonization ability on leaf surfaces, along with their consequent ability to control powdery mildew on cucumber: spore suspensions (1×10^7 spores/ml) were sprayed on leaf surfaces (Koike et al., 2004). Some strains demonstrated a significant reduction of powdery mildew. This evidence suggested that these strains might be dominant in a phyllosphere microbial community before the pathogen arrives (Koike et al., 2004). Therefore, not only parasitism, production of antibiotics, and hydrolytic enzymes, but also competition (nutrients and space) might be mechanisms of pathogen inhibition (Benhamou & Brodeur, 2000). On the other hand, some strains of *V. lecanii* showed high ability of colonization on the tomato rhizoplane and reduced Verticillium wilt, especially the severity of internal symptoms (Koike et al., 2004). For that reason, in the rhizosphere, *V. lecanii* also might reduce the diseases by equivalent mechanisms on leaf surfaces.

This study is intended to evaluate whether *V. lecanii* that could reduce powdery mildews on leaf surfaces of cucumber have the ability to induce plant resistance by inoculation on leaf surfaces, and whether the fungi are effective to control powdery mildews by inoculation on the rhizosphere.

2. Materials and Methods

2.1. Growth and maintenance of fungi and plants

Two isolates of *V. lecanii* were studied: B-2 (*L. muscarium*), which was collected from green peach aphid in Obihiro (*L. longisporum*; Koike et al., 2004); and a commercial preparation of Vertalec (Koppert UK Ltd., Wadhurst, East Sussex, UK). Each isolate of *V. lecanii* was cultured on potato dextrose agar (PDA; 200 g of potatoes, 20 g of glucose, and 20 g of agar /l) in 9-cm petri dishes and stored at 4°C. Spore suspensions were obtained from 12-day-old shaken cultures (Potato Dextrose Broth; Becton, Dickinson and Co., USA) by filtering the culture medium through gauze. Suspensions were adjusted to 1×10^7 spores/ml using a hemocytometer.

Leaves of cucumber (*Cucumis sativus L*, cv. Hokushin) that had been intensely, but naturally, infected with *Sphaerotheca fuliginea* were collected. Colonized areas were scrubbed in petri dishes with a brush using distilled water. Suspensions were obtained by filtering through gauze. They were adjusted to 1×10^5 spores/ml using a hemocytometer. These spore suspensions were stored at 4°C in darkness for all experiments. *V. lecanii* cultures and *S. fuliginea* cultures were used within 3 days. Cucumber plants (*Cucumis sativus L*, cv. Hokushin) were grown in an incubator at 25°C for 25 days under 8D16 l for all experiments.

2.2. Control powdery mildew in a glasshouse by pre-inoculation of cucumber roots with *V. lecanii*

This experiment assessed B-2 for its ability to control cucumber powdery mildew. In previous studies at our laboratory, B-2 showed high colonizing ability on tomato rhizospheres and rhizoplanes, along with reduced severity of internal symptoms of Verticillium wilt (Koike et al.,2004).

First, the root systems of cucumber plants grown 25 days in individual pots were pre-inoculated with *V. lecanii* (1×10^7 spores/ml, 10 ml or 50 ml) or distilled water by pouring spore suspensions over the soil. Then, pre-inoculated plants were sprayed with a fine mist of *S. fuliginea* spore suspension (1×10^5 spores/ml) 24 h or 72 h after pre-inoculation. Following inoculation, plants

were kept at 25°C under natural light in the glasshouse. Lesions on each inoculated plant were counted 2, 3 and 4 weeks after inoculation. Diseased areas on all leaves were estimated visually in two categories: lesions appearing on leaves emerging during plant growth after spraying pathogen by airborne infection (called not-sprayed-occurring) as well as lesions on sprayed leaves (sprayed-occurring). A score was calculated using the percentage of leaf area covered by mildew (five disease classes): 0, for 0% mildew area per leaf; 1, < 10%; 2, (10-25%; 3, 25-50%; 4, 50-75%; and 5, 75-100%. The mildew index of sprayed-occurring = sum of scores of leaves sprayed with pathogen / number of leaves sprayed with pathogens. The mildew index of not-sprayed-occurring = sum of each score of leaves not-sprayed with pathogen / number of leaves not-sprayed with pathogen. Each test was performed in quadruplicate.

2.3. Colonization test on rhizoplanes and inside root tissues

Using the plants that had been used to examine the possibility of B-2 to control powdery mildew by inoculation in soil, as mentioned above, colonization ability of B-2 on rhizoplanes and inside root tissues was studied using dilution plate methods. Simultaneously, the possibility of spores of B-2 treated in soil to stick to leaves by splashing during plant watering was confirmed.

Roots were taken and washed to remove soil particles. These roots were rinsed with distilled water. First, we prepared rhizoplane dilutions. A 1.0×10^{-1} dilution of rhizoplane was prepared by shaking roots with sterile distilled water (45 ml) in a test tube, using a ultrasonic cleaner for 1 min. The root was removed to a Petri dish, which was used to prepare root tissue dilutions, and sterilized root surface by momentary dipping into 70% ethanol and subsequently into 5% sodium hypochlorite solution. After sufficient rinsing with sterile distilled water, the root was homogenized using a blender with sterile distilled water (10 ml/g). This was a 1.0×10^{-1} dilution of root tissue. Then 5 ml of each suspension was transferred to 45 ml sterile distilled water. This procedure was continued until a 10-fold dilution series was obtained ranging from 1.0×10^{-1} to 1.0×10^{-4}; 0.2 ml of each 1.0×10^{-3} and 1.0×10^{-4} dilution were dropped onto rose-bengal medium containing streptomycin sulfate, and incubated at 25°C in darkness for 8 days. Then colony-forming units of B-2 per gram of fresh weight of the roots were counted. Three replicates were done for each test.
The first leaf and the second leaf were measured similarly to root assays, as mentioned above.

2.4. Inoculation of cucumber roots with *V.lecanii* to evaluate induction of systemic disease resistance

In this study also, B-2 was examined. The 25-day-old cucumber plants were uprooted from their pots, and soil was washed off slowly with tap water. These naked roots were dipped in a spore suspension of B-2 (1×10^{7} spores/ml, 500 ml) or sterile distilled water for 30 min. Roots inoculated with B-2 were rinsed out lightly using tap water. Then each plant was put into a test tube with sterile distilled water and kept at 25°C under natural light in a glasshouse. Roots and stems, from the joint with the root to the cotyledon, were sampled at 0 h, 24 h, 48 h and 72 h after inoculation. All samples were kept in plastic packs (100×70 mm) and stored at -80°C. Each test was performed in quadruplicate.

2.5. PO and PAL assay

An acid peroxidase (PO) and a phenylalanine ammonia lyase (PAL) are putative biochemical markers for

SAR. The leaf samples in packs were shattered to pieces by hand. Then each sample (0.5 g) was homogenized with liquid nitrogen by grinding in a pestle, and mixed 4.5 ml of 50 mM Tris-HCl buffer (pH 8.8). The homogenate was centrifuged at 12,000 g for 5 min at 4°C. The protein content in crude extracts was determined using the Proteostain protein quantification kit (Dojindo Molecular Technologies, Inc.). The PO and PAL activities were measured at room temperature. Using guaiacol as a substrate, PO was assayed. The assayed mixture contained 3 ml of 5 mM guaiacol in 15 mM sodium phosphate buffer (pH 6.8), 5 mM H_2O_2. Assays were initiated by addition of the crude extract (100 μl) to the mixture and the change in optical density at 470 nm was measured for 1 min. The PO activity was calculated as ΔA_{470} nm mg-1 min-1.

Using L-phenylalanine as the substrate, PAL was assayed. The reaction mixture consisted of 500 μl of 0.2 %(w/v) L-phenylalanine in 50 mM Tris-HCl buffer (pH 8.8) and 500 μl of the crude extract. The mixture was incubated for 1 h at 40°C, and 100 μl of 0.1 N-HCl was added to stop the reaction. Formation of cinnamic acid was monitored at 268 nm. PAL activity was expressed as microgram-order cinnamic acid formation per milligram of protein. Each test was performed in quadruplicate.

2.6. Pre-inoculation of cucumber plants with V. lecanii and subsequent inoculation with pathogen to evaluate induction of systemic disease resistance

In a glasshouse under natural light (25±1°C,), suspensions of two isolates of *V. lecanii* (B-2, selected because of their ability of high colonization on cucumber leaf surfaces and of reduction of powdery mildew (Cools & Ishii, 2002) and Vertalec, selected because of its ability of low colonizing on cucumber leaf surfaces, for comparison with B-2, 1×10^7 spores/ml) were sprayed on leaf surfaces of the second leaves of cucumber plants using customized sprayers. For control, plants were sprayed with distilled water. The second leaves and third leaves of cucumber plants were collected immediately after pre-inoculation (0 h) and at designated time intervals thereafter (12 h, 24 h, 48 h, 72 h). On the other hand, the plants treated as described above were sprayed with the spore suspension of *S. fuliginea* (to the second and third leaves, 1×10^5 spores/ml) 24 h after pre-inoculation of *V. lecanii*. Similarly, the second and third leaves of cucumber plants were collected (0 h – immediately after pre-inoculation; 12 h; 24 h – immediately after pathogen inoculation; 48 h; and 72 h). Each sample was kept in a plastic pack and stored at -40°C in darkness. Each test was performed four times.

2.7. Relative, multiplex reverse transcription PCR

Using an RT-PCR system, the expression of three defense-related genes in the second leaves, which were treated B-2 and subsequent inoculation with pathogen, was examined. These were a PO encoding gene (*PO*), a gene encoding a PAL homologue (*PAL1*) and a pathogenesis-related protein 1 gene homologue (*PR1-1a*), a commonly used marker for SAR.

Samples of the second leaves (0 h, 24 h, and 48 h after inoculation of B-2) that had been frozen at -80°C were shattered by hand. Using a NucleoSpin® RNA Plant total RNA purification kit (Macherey-Nagel GmbH, Germany) according to the manufacturer's instructions, RNA was extracted from each sample. The RNA samples were quantified at 25 μg, using a spectrophotometer.

For analyses of *PO*, *PR1-1a*, and *PAL1* expression, multiplex reverse transcription PCR (RT-PCR) was used to simultaneously amplify transcripts of the target gene and the constitutively expressed reference gene, β-actin. The following primers were used: β-actin (forward,

5'-GGCCGTTCTGTCCCTCTAC-3'; reverse, 5'-CAGCTCCGATGGTGATGAC-3'), *PO* (forward, 5'-CTGACATTCTACTAGCCCTTGGTTCT-3'; reverse, 5'-TCTGCGTTTGGATTATTGTTTAGG-3'), *PR1-1a* (forward, 5'-GGCAGCCCAGACTTCTCAGC-3'; reverse, 5'-GCATCTCACTTTGGCACATCCTA-3') and *PAL1* (forward, 5'-ATAACGGTTTGCCTTCTAATCTT-3'; reverse, 5'-CATCCTGGTTGTGTTGCTCA-3'). Reactions were carried out with mRNA selective PCR kit (TaKaRa BIO Inc., Japan), again according to the manufacturer's instructions. Cycle numbers were 30 for all genes. Conditions for all reactions were the same, with an initiate RNA reverse transcribe at 45°C for 30 min, followed by cycles of 85°C for 1 min, 50°C for 1 min, and 72°C for 2 min (Cools & Ishii, 2002).

The PCR products were separated on a 0.2% agarose gel and visualized by ethidium bromide staining. Fluorescence values for target genes were measured using comparison with a fluorescence value of β-actin gene; obtained information was processed using a densitometer. Each test was performed three times.

3. Results

3.1. Control powdery mildew in glasshouse by pre-inoculation of cucumber roots with *V. lecanii*

Cucumber plants pre-inoculated with 50 ml of spore suspension of B-2 showed the lowest powdery mildew value in this experiment (Fig. 1). Comparing amounts of spore suspension, 50 ml was more effective than 10 ml for both sprayed-occurring and not-sprayed-occurring. Comparison of the period from pre-inoculation with B-2 to inoculation powdery mildew showed that both were effective, but plants 24 h post-B-2 inoculation were more successful than those of 72 h (not shown).

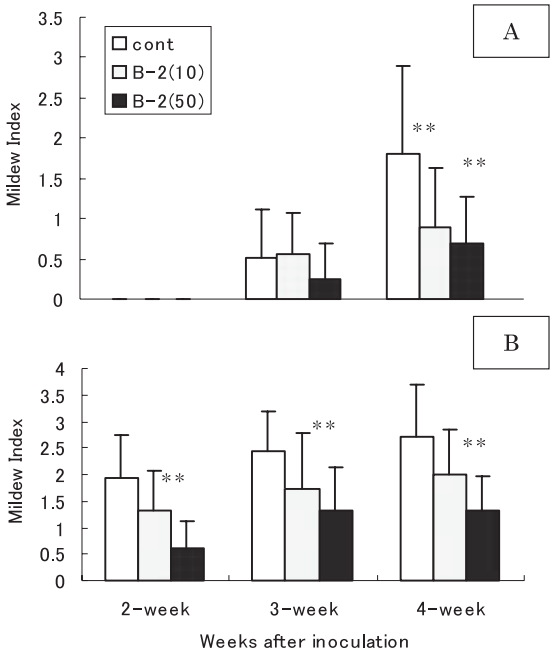

Fig.1 Powdery mildew value of non-sprayed pathogen leaves (A) and sprayed pathogen leaves (B), at 24h after B-2 (*Verticillium lecanii*) pre-inoculation of *V. lecanii* (B-2). ＊＊, significant difference from control, p = 0.01. (Scheffe's F test)

3.2. Colonization test on rhizoplanes and inside root tissues

The highest colonization on cucumber rhizoplane was shown by 50 ml of spore suspension of B-2 (Fig. 2): it was colonized on rhizoplanes less than 1.0×10^{3} spores/g in control roots that had been treated with distilled water. Without affecting plant vitality, B-2 was allowed to penetrate inside the root tissues and colonize.

3.3. PO and PAL activities in root tissue

Inoculation with B-2 on cucumber roots did not increase respective PAL and PO activities both in roots and stems compared with those of uninoculated control plants (Fig. 3). Very little difference existed between activities of plants inoculated with B-2 and distilled water.

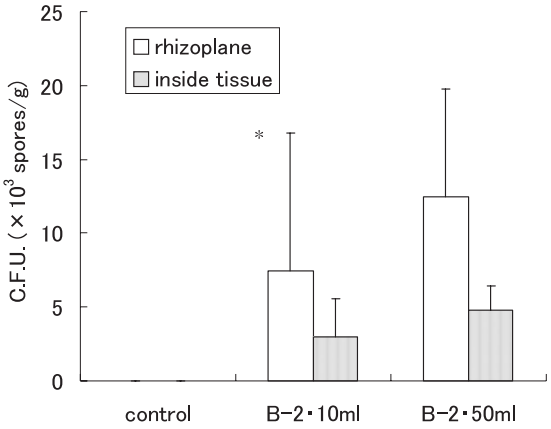

Fig.2. Colonization of *V. lecanii* on cucumber rhizoplane and inside the root tissues. *, significant difference from control, p = 0.05. (Scheffe's F test)

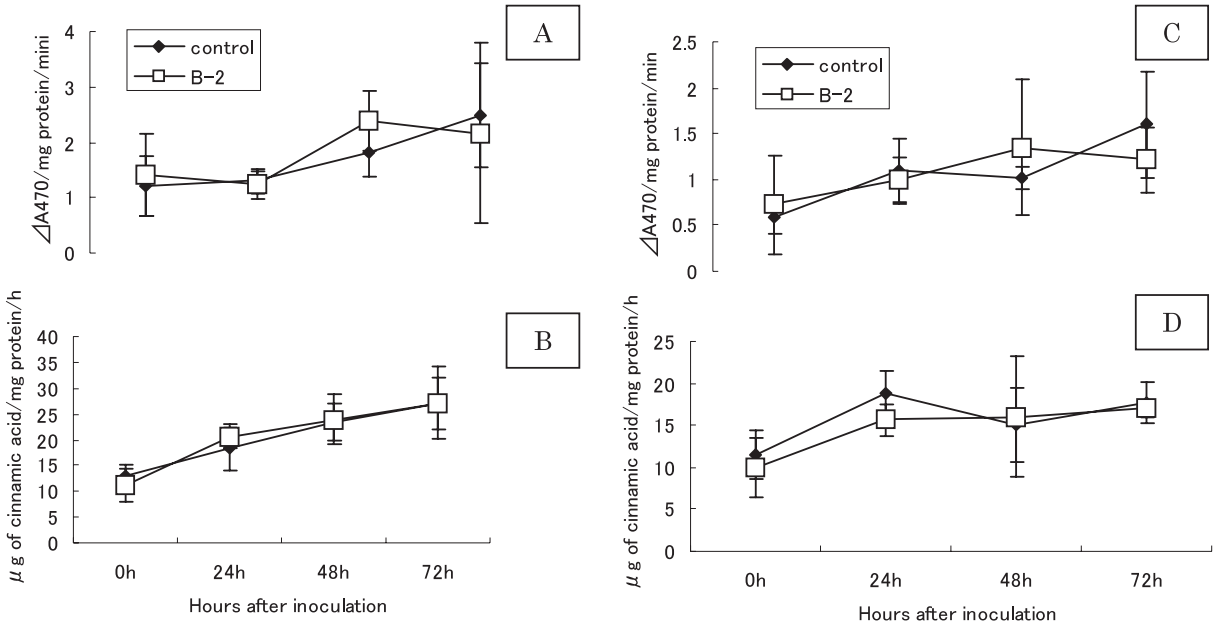

Fig.3. Induction of peroxdase (PO) (A,C) and phenylalanine ammonia-lyase (PAL) (B,D) activities in cucumber stems (A,B) and roots (C,D) inoculated with B-2 (*Verticillium lecanii*) . Standard errors are shown.

3.4. PO and PAL activities in leaf tissue

Inoculation with each of the two *V. lecanii* strains did not increase PO and PAL activities in the second and third leaves as compared with those of uninoculated control plants (Figs. 4A and 4B). In this case, PO activity of both second and third leaves inoculated with B-2 was almost always highest over the 3-day sample period for all treatments (Fig. 4A). Comparing the second leaves with the third leaves in plants inoculated with B-2, the PO activity was higher at all times in the second leaves than in the third leaves (Fig. 4C).

In response to powdery mildew challenge, although very little difference existed between plants inoculated with *V. lecanii* and control plants, PAL activity tended to increase: plants inoculated with B-2 showed 2.97-fold

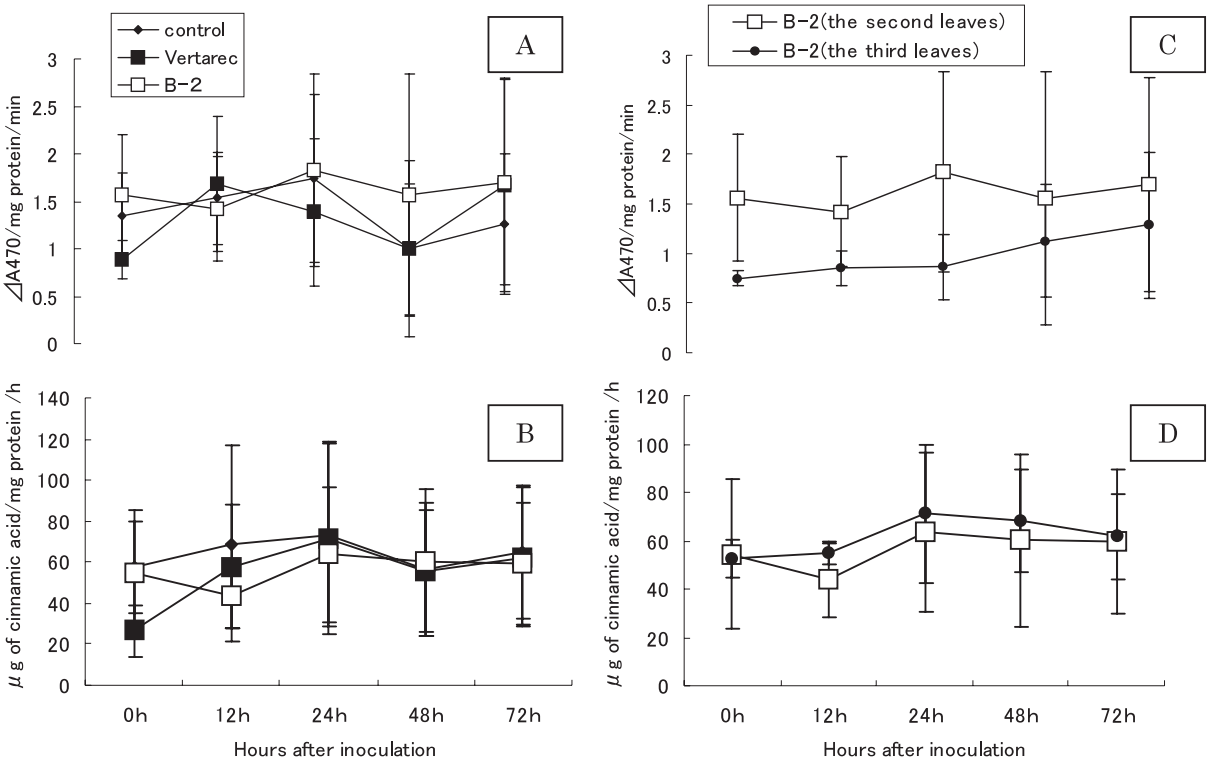

Fig.4. Induction of peroxidase (PO)(A,C) and phenylalanine ammonia-lyase (PAL)(B,D) activities in cucumber leaves inoculated with 2 isolates of *V. lecanii* (A,B). The second leaves and the third leaves of cucumber plants inoculated with B-2 (C,D). Standard errors are shown.

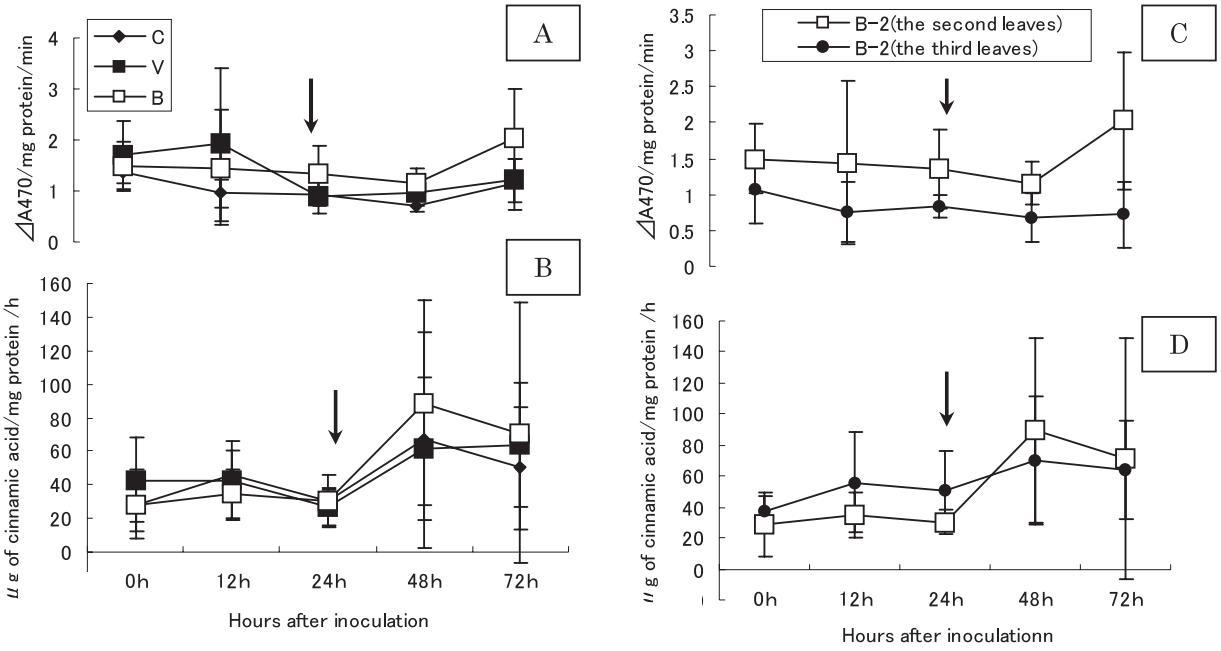

Fig.5. Induction of peroxidase (PO)(A,C) and phenylalanine ammonia-lyase (PAL)(B,D) activities in cucumber leaves inoculated with pathogen after pre-inoculation of 2 isolates of *V. lecanii* (A,B). The second leaves and the third leaves of cucumber plants inoculated with B-2 (C,D). Standard errors are shown. Arrow indicates the time of inoculation.

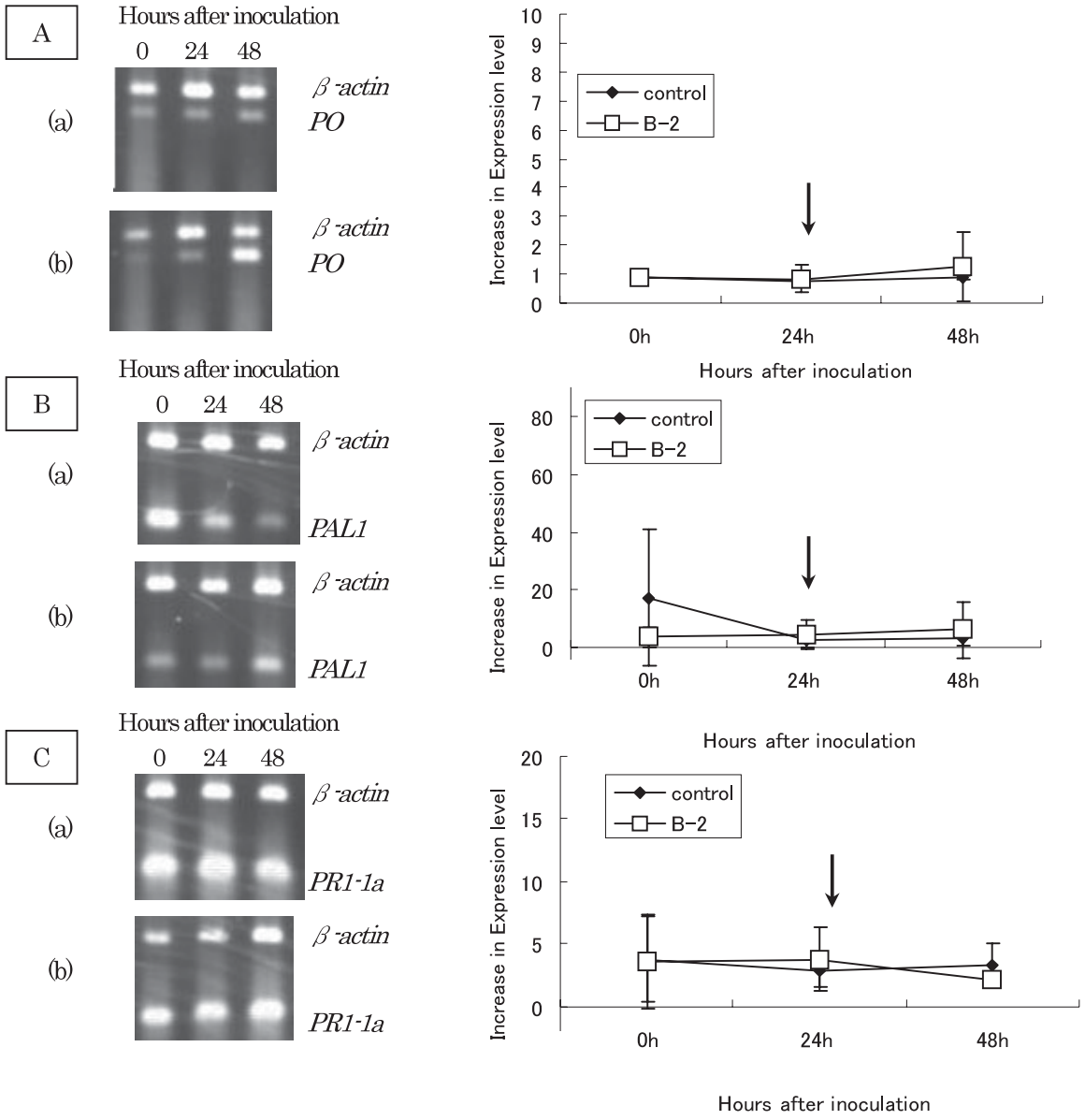

Fig.6. RT-PCR analysis of PO(A), PAL1(B) and PR1-1a(C) expressions in plants inoculated with pathogen after pre-inoculation with B-2(a) or DW(b). Gels shown are representative of one of the three replicates tested per treatment. Standard errors are shown. Arrow indicates the time of inoculation.

at h-after-inoculation with B-2 from 24 h to 48 h (Fig. 5B). The PAL activity of the second leaves inoculated with B-2 showed a sharper increase than that of the third leaves (Fig. 5D). No significant changes in PO activity were observed in the second leaves and the third leaves of all plants (Fig. 5A). Comparing the second leaves with the third leaves in plant-inoculated B-2, the PO activity was also higher at all times in the second leaves than in the third leaves (Fig. 5C).

3.5. Activation of PO, PAL1 and PR1-1a expression in inoculated plant leaf tissue

No significant differences were observed in activation of *PO*, *PAL1* and *PR1-1a* expression in the second leaves of cucumber plants inoculated with pathogen after pre-inoculation with B-2 (Fig. 6).

4. Discussion

This study revealed that *V. lecanii* suppressed cucumber powdery mildew. Application of *V. lecanii* maintained mildew severity below 20% in terms of infected area per leaf for 4 weeks after inoculation with *S. fuliginea*. Disease suppression appeared to be systemic: roots were treated with *V. lecanii* and the pathogen was challenged by leaf-inoculation, thereby separating the two spatially. *V. lecanii* did not recover from regions growing above the ground; *V. lecanii* also colonized root surfaces strongly and penetrated the root tissues. Moreover, the more strongly the fungi colonized root tissues, the more the disease abated, comparing the quantity of spore suspension of B-2. In the leaf test, comparing plants treated B-2 or Vertalec, enzyme activities tended to increase higher in B-2 treated plants. In previous studies at our laboratory, as mentioned above, B-2 showed a high ability to colonize cucumber leaves. In contrast, Vertalec showed no colonization ability on cucumber leaves (Koike et al., 2004), suggesting that a longer period of their association with plants was necessary for induction of resistance (Meera et al., 1994). On the other hand, growth promotion might be independent of root colonization ability (Koike et al., 2001), so that additional examination is needed of the ability of *V. lecanii* as a plant growth promoting fungi (PGPF) for cucumber.

Comparison was made of the mildew levels of plants inoculated with pathogen 24 h after pre-inoculation with B-2 with 72 h after pre-inoculation with B-2: although both were effective, the former was better at controlling disease. These results suggest that this biocontrol treatment had to be repeated to protect plants from pathogens over a long period. How often the treatments are required and how long the effect of this treatment persists remains unclear, so additional study is needed.

Verhaar et al.(1999) indicated that humidity conditions at the plant surface are probably the most important factor influencing germination, growth and survival of *V. lecanii* in this habitat. Consequently, high humidity conditions seemed to be of great importance to obtain good control. In commercial glasshouses, conditions are rarely favorable for good development of *V. lecanii*. Relative humidity during the daytime is low and temperatures can rise to high levels (Verhaar et al., 1996). In short, when *V. lecanii* spores are sprayed onto leaves in a water suspension, evaporation of the water carrier might be too rapid for germination in free water. Thereby, *V. lecanii* becomes dependent on the air humidity.

In this paper, however, *V. lecanii* showed satisfactory control of cucumber powdery mildew by inoculation of fungi to soil. Therefore, using this method of inoculation to soil, not to leaves, seems to solve problems of humidity in the case of cucumber powdery mildew.

Although plants inoculated with B-2 to their roots reduced lesions that developed after inoculation with powdery mildew, surprisingly, the activities of defense-related enzymes such as PO and PAL were not considerably enhanced; PO is involved in cross-linking extension molecules to form lignin (He et al., 2002). Increased lignin deposition is believed to play a role in barricading the pathogen from invading the plant through physical exclusion. In addition, PAL activity is associated with the biosynthesis of toxic metabolites, such as phytoalexins, phenols, lignins, and salicylic acid (SA), in plant defense pathways (He et al., 2002; Ryals et al., 1996). In addition, the biosynthetic pathway of SA appears to begin with the conversion of phenylalanine to trans-cinnamic acid catalyzed by PAL. Therefore, PAL accumulation in roots might reduce phenylalanine, which is necessary for several pathogens' growth and development (He et al., 2002). Accordingly, *PO* and *PAL1* and *PR1-1a* are categorized as SAR genes – known markers for SAR in cucumber: PO and PAL are marker enzymes for SAR. Elicitation

of induced resistance by pathogens is termed SAR and some other signal pathways have been reported. However, precise mechanisms of induced resistance are not yet clearly understood.

Especially in the root tests presented in this paper, the possibility exists that ISR was induced, not SAR. As mentioned above, ISR is distinguished from SAR by a different signal pathway, which is independent of SA accumulation. Marker enzymes (PO and PAL) might not be enhanced if the resistance induced by *V. lecanii* inoculated to soil is ISR. On the other hand, leaf tests showed PAL tended to increase on inoculating pathogens. One group of enzymes, including anionic peroxidase, is directly responsive to inducer. Another set of defense enzymes, including PAL, is not directly activated by exogenous inducer, but rather, is alerted to subsequent elicitor. This capacity has recently been defined as the "primed" state of a plant (Cools et al., 2002). Priming is reportedly an important component of SAR. Although PO activity was not changed, at least, increase of PAL activity in this study might be related to primed state.

Generally speaking, there is association SA with acid pathogenesis-related proteins (PR protein) (Shimamoto & Watanabe, 2004; Strange, 2003). In most cases, accumulation of SA causes accumulation of acid PR proteins (Jeun et al., 2003). However, in this study, expression of *PAL1* did not increase and expression of *PR1-1a* was not recognized.

To reiterate, little is known about specific mechanisms of induced host resistance. For example, contradictory results were obtained in attempts to associate ISR with PR protein accumulation, a marker often used for SAR expression (Ongena et al., 2000). The ISR induced by *Pseudomonas fluorescens* CHAO in tobacco is coupled with stimulation of PR protein synthesis, whereas such proteins did not accumulate in radish and *Arabidopsis-expressing* PGPR-mediated ISR (Juen et al., 2003; Ongena et al., 2000). The above-mentioned facts represent only some instances. In addition, induced systemic resistance was also expressed in cucumber upon challenge with PGPR prior to infection by the pathogen, but no specific mechanisms were suggested to explain the observed protective effect (Ongena et al., 2000), the cases of PGPF are as numerous as those of PGPR. In short, further work targeting other genes and enzymes is likely to be worthwhile . The possibility exists that induced resistance by inoculation with *V. lecanii* is associated with SA pathway and/or JA, ethylene pathway and/or new pathway, which have not been reported previously (Ryu et al., 2003). Cytological results demonstrate that the beneficial effect of *V. lecanii* in repressing *Pythium* ingress in root tissues relies on a strong antifungal activity associated with an induction of structural (formation of elongated wall appositions, resembling papillae) and biochemical (occlusion of some intercellular spaces by an amorphous material or phenolic compound) barriers in host tissues (Benhamou et al., 2001).

Although the exact mechanisms remain unclear, this is the first report describing the effect by which *V. lecanii* can suppress and control powdery mildew by inoculation to soil. Results suggest that *V. lecanii* is capable of inducing systemic protection against powdery mildew by root or leaf colonization, or by other means of triggering the host defense mechanism, which are based on systemic activation of natural plant defense mechanisms. A detailed investigation is under way to understand the exact mechanism of systemic resistance in cucumber using biocontrol agents. Further work is needed to identify mechanisms of host resistance by treating *V. lecanii*. Induced host resistance is considered to offer broad-spectrum resistance against many pathogens. *V. lecanii* also has an ability of plant colonization, penetration of root tissues, pathogen antagonism, antimicrobial activity, mycoparasitism, and stimulating plant defense systems. It

might therefore become a valuable alternative to current management of plant pathogens as well as a preferred method of management of cucumber powdery mildew in a glasshouse.

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