Induction of Disease Resistance in Tissue-Cultured Seedlings of Mountain Laurel after Treatment with Streptomyces padanus AOK-30

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(Received Sep. 27, 2004 / Accepted Nov. 29, 2004)

An endophytic actinomycete, Streptomyces padanus AOK-30, isolated from a potted mountain laurel (Kalmia latifolia L.) plant, was used to protect tissue-cultured seedlings of mountain laurel from Pestalotia disease and Rhizoctonia root rot. AOK-30, which has a broad antagonistic spectrum against various microbes, did not adversely affect the seedlings in glass flasks. A spore suspension of AOK-30 was spread on the surface of the rooting medium in glass flasks in which seedlings were growing. Ten days later, the 4th upper leaves were inoculated with Pestalotiopsis sydowiana and incubated for 20 days. In controls untreated with AOK-30, substrate mycelia of this fungus grew on all leaves and stems above and below the 4th leaves within 7–10 days after inoculation. Such growth resulted in the wilting death of 13 of 20 seedlings by the 20th day. In contrast, only the inoculated leaves and some neighboring leaves turned brown in 17 of 20 seedlings growing on medium treated with AOK-30. Thus, treatment of the medium surface with AOK-30 efficiently protects the seedlings from infection by P. sydowiana. The treatment of seedlings with AOK-30 in flasks before transplanting in soil and transplanting of untreated seedlings in soil mixed with suspension of AOK-30 significantly and marginally, respectively, slowed the expansion of root rot caused by Rhizoctonia sp. in seedlings in the cell trays.

INTRODUCTION

Agrochemicals are invaluable and effective for increasing food production to stave off hunger throughout the world, although they have fallen into disfavor because of associated environmental pollution and detrimental effects in a variety of nontarget organisms. Because of such disfavor, the use of microbe-based biological control agents has increased in agricultural importance as a replacement or supplement for agrochemicals.

In 1978 Hasegawa et al.2) isolated a new genus of actinomycete, Actinosynnema, from a grass blade. As far as we know, this report is the first to describe the isolation of an actinomycete from plant materials. In the 1990s, a number of research groups isolated several actinomycetes from surface-sterilized leaves and roots of a variety of plants, thereby showing the presence of endophytic actinomycetes. Since then, more reports have described the use of actinomycetes in biological control. Based on these reports, Kunoh1) assumed that a symbiotic actinomycete that is able to produce antibiotics in plants without any adverse effects on plant growth could be used as a biological control agent and that such an endophytic actinomycete could efficiently colonize in a plant when applied to tissue-cultured seedlings in axenic flasks, in which the applied actinomycete does not have to compete with other microbes. We therefore started to isolate endophytic actinomycetes from a variety of field-grown plants and successfully isolated a number of strains from rhododendron. An isolate of Streptomyces galbus R-5, which had the most intense antagonistic activity against a variety of microbes including Phytophthora cinnamomi and Pestalotiopsis sydowiana, major pathogens of rhododendron was selected as a potential biocontrol agent. Shimizu et al.12) spread a suspension of R-5 on the surface of the multiplication medium in glass flasks in which rhododendron seedlings were growing, and successfully induced seedling resistance against Pestalotia disease. Their success suggested a novel way for producing disease-resistant tissue-cultured seedlings without using agrochemicals.

Nishimura et al.13) isolated a strain of Streptomyces padanus AOK-30 (hereafter AOK-30) from potted mountain laurel by the method of Shimizu et al.11) and found that this strain also had broad antimicrobial activities similar to those of the R-5 isolate reported previously. In this paper, we describe the control of Pestalotia disease caused by P. sydowiana in seedlings of mountain laurel tissue-cultured in glass flasks after treatment of the medium surface with a spore suspension of AOK-30. The suppression of expansion of Rhizoctonia root rot in a cell-tray with transplanted mountain laurel seedlings treated with AOK-30 in different ways is also described.

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MATERIALS AND METHODS

Plants
Young seedlings of mountain laurel (Kalmia latifolia L. hybrid cultivar “Ostbo Red”) were grown by a tissue-culture technique in glass flasks in an incubator conditioned at 25°C with 12 hr illumination per day at 11.8 Wm⁻². A number of young seedlings without roots were aseptically separated and transferred to fresh rooting medium and incubated in the same chamber for a further one month. The resultant seedlings (10 to 12 clusters of seedlings per flask) were used for the following inoculation test.

Preparation of a spore suspension of Streptomyces padanus AOK-30 and treatment of rooting medium
A spore suspension of AOK-30 in 10% glycerol supplemented with DMSO was transferred from the stock culture to IMA-2 liquid medium in a glass flask and incubated on a rotary shaker (AT-12S, Thomas Sci. Inst. Co. Ltd., Tokyo, Japan) at 100 rpm and 30°C for 24 hr. A spore suspension (3–4 × 10⁶ cfu/ml) was prepared from this culture. One ml of the spore suspension was dropped and spread on the surface of the rooting medium in glass flasks with mountain laurel seedlings growing as described. In untreated controls, 1 ml of IMA-2 liquid medium was spread on the medium surface. The seedlings, treated and untreated with AOK-30, were incubated for an additional 10 days before inoculation of P. sydowiana.

Reisolation of AOK-30 from mountain laurel seedlings
Seedlings treated with AOK-30 were taken from one flask on designated days until the 14th day after inoculation. Each seedling was cut at intervals of every two nodes with a flame-sterilized razor blade in a laminar flow chamber. Each piece was placed on IMA-2 agar medium then incubated at 30°C for 14 days. The number of tested nodes from which AOK-30 grew was recorded every 4 days from the 2nd to 14th day after initiation of incubation.

Inoculation of mountain laurel seedlings with Pestalotiopsis sydowiana and evaluation of disease severity
Pestalotiopsis sydowiana (Bresadola) Sutton, a causal pathogen of Pestalotia disease of Ericacea (provided by the National Institute of Agrobiological Resources, stock no. 305755), was cultured on potato dextrose agar (PDA) at 25°C for 5 days. Mycelial disks (4-mm diameter) were then transferred onto the 4th upper leaves of the mountain laurel seedlings treated with AOK-30 in glass flasks as described previously (Fig. 2-A), followed by incubation for 20 days at 25°C and 11.8 Wm⁻² for 12 hr per day. In controls untreated with AOK-30, similar mycelial disks were placed on the 4th upper leaves. Twenty seedlings were used for the treated and untreated sections, respectively.

All seedlings were taken from the flasks at 20 days after inoculation with P. sydowiana, and growth of this fungus on leaves and stems was carefully observed with a dissecting microscope. Symptoms on the seedlings were observed with the naked eye and the number of seedlings in each of the following symptom categories was recorded: 1 = brownish symptom on inoculated leaves (Fig. 2-B), 2 = brownish symptom on the 3rd-5th leaves (Fig. 2-C), 3 = browning of entire seedling (Fig. 2-D), 4 = browning of inoculated and neighboring seedlings (Fig. 2-E). The degree of expansion of P. sydowiana mycelia from the inoculated leaf was assessed.

Inoculation of mountain laurel seedlings with Pestalotiopsis sydowiana and evaluation of disease severity.

Fig. 1. Reisolation of AOK-30 from nodes of seedlings treated with AOK-30 in flasks.
*Number of nodes from which AOK-30 grew / Total number of nodes tested.

Fig. 2. Inoculation of P. sydowiana on tissue-cultured seedlings of mountain laurel in flasks and subsequent development of mycelia on the seedlings. The arrow in each frame shows the inoculated leaf.
A. A mycelial disk of P. sydowiana was placed on the 4th upper leaf.
B. Whitish aerial mycelia grew on the inoculated leaf and the leaf turned brownish.
C. The 3rd and 5th leaves turned brownish due to covering with aerial mycelia extending from the inoculated leaf.
D. Almost all leaves and stems turned brownish due to active growth of aerial mycelia.
E. The inoculated and neighboring seedlings became entirely covered with aerial mycelia and wilted.
the inoculated leaves was compared between AOK-30-treated and -untreated seedlings by the χ² test using the distribution of the seedling numbers in the symptom categories.

**Dissemination of Rhizoctonia root rot in a cell tray with transplanted mountain laurel seedlings**

To examine the most efficient and effective method of treating seedlings with AOK-30 to induce disease resistance, seedlings were treated with AOK-30 as follows: i) seedlings in flasks of rooting medium were treated with a spore suspension of AOK-30 as described earlier and transplanted into soil in a cell tray after 10 days incubation in the flasks (described as flask treatment in Table 2), ii) seedlings were removed from flasks and immersed in 100 mg/l of indole butylic acid (IBA), a rooting accelerator, for 2 hr and then in a spore suspension of AOK-30 for 1 hr before transplanting (suspenion treatment in Table 2), and iii) seedlings were transplanted directly from flasks into a cell tray containing a soil mixed with 1 ml of an AOK-30 spore suspension (soil treatment in Table 2); the soil was a mixture of peat moss, perlite and vermiculite (8:1:1 in volume). The cell trays were 33 × 27.5 × 5.5 cm with 80 cells and individual cell volume was ca. 25 cm³.

*Rhizoctonia* sp., which causes root rot of Ericaceae plants, was grown on potato dextrose agar medium at 20°C for 5 days. Mycelial disks (4 mm in diameter) were punched from the margin of the colony with a cork borer. One disk was placed on the soil surface near the transplanted seedling in the center cell of the tray. In preliminary tests, one disk was placed on the soil surface near each seedling in each cell; however, because all seedlings developed serious symptoms within a few days because of rapid mycelial growth, the suppressive effects of the AOK-30 treatments on disease occurrence were too difficult to distinguish. Therefore, we changed the method to the above and evaluated effects of the treatments on dissemination of the disease within the cell tray.

All transplanted seedlings in the cell trays were grown in a transparent plastic tunnel in a glasshouse with daily water misting every 3 hr from 8 am to 5 pm. Disease severity was observed with the naked eye and the number of seedlings in each of the following categories was recorded (Table 2): 0 = no change, 1 = brownish symptom on less than 25% of leaves, 2 = brownish symptom on 25–49% of leaves, 3 = brownish symptom on 50–75% of leaves, 4= brownish symptom on more than 75% of leaves. Eighty seedlings were originally transplanted in each tray; however, the total numbers of observed seedlings in each tray were reduced during the 3-week incubation, because seedlings that had wilted or become discolored by causes other than *Rhizoctonia* root rot were removed immediately. Distributions of seedlings with light (0 and 1) and serious symptoms (2–4) within the treatment groups were compared with the controls by the χ² test.

**RESULTS**

When the rooting medium in the glass flask with mountain laurel seedlings was treated with AOK-30, the mycelia grew and sporulated to form white, powdery colonies on the medium surface within 2–3 days. Their growth did not cause any adverse effects in the seedlings.

As illustrated in Fig. 1, AOK-30 grew from the base to the 1st to 4th basal nodes of some seedlings by the 2nd day after the initiation of incubation. Colonization was so rapid that AOK-30 was reisolated from the top node of the seedlings by the 10th day.

Growth of inoculated *P. sydowiana* and visible symptoms in AOK-30-untreated and -treated seedlings are explained briefly, although the timing of seedling growth was not always synchronous in all test seedlings. When the 4th leaves of seedlings in rooting medium untreated with AOK-30 were inoculated with *P. sydowiana* (Fig. 2-A), whitish, aerial hyphae and substrate mycelia of the fungus actively grew on the inoculated leaves by the 3rd day after inoculation (Fig. 2-B). By the 7th day, the 3rd and 5th leaves covered with aerial hyphae turned brownish (Fig. 2-C). All leaves and stems of seedlings in the untreated controls turned brownish by the 20th day, and the entire seedlings wilted (Fig. 2-D). These symptoms also spread to some of the neighboring seedlings (Fig. 2-E). However,

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in AOK-30-treated seedlings, substrate mycelia of *P. sydowiana* were observed mainly on the 4th leaves, and the leaves turned brownish as shown in Figs. 2-B, C; they slowly grew to the upper or lower leaves (as in Fig. 2-D). Consequently, almost all non-inoculated leaves and stems remained green for 20 days (as in Fig. 2-B). Another interesting point was that stems of the seedlings turned reddish from the base to the top by the 5th day after the onset of AOK-30 treatment. Because AOK-30-untreated seedlings slowly turned red by the 20th day, regardless of the presence or absence of *P. sydowiana*, the color change was accelerated but not newly induced by AOK-30 treatment.

In AOK-30-untreated controls (Table 1), 13 of 20 fungus-inoculated seedlings and/or their neighbors were completely browned by the 20th day after inoculation. Only the upper and lower leaves browned in 6 of 20 seedlings, and in only one seedling, *P. sydowiana* stayed in the inoculated leaves until the 20th day after inoculation. These numerical data, which support the above-described observations, show that *P. sydowiana* quickly extended from the inoculated leaves to the entire seedling within 20 days if seedlings were not pretreated with AOK-30. In contrast (Table 1), in 9 of 20 AOK-30-treated seedlings, only the inoculated leaves turned brownish within 20 days. In eight seedlings, only upper and lower leaves browned. Three inoculated seedlings and/or their neighboring seedlings wilted entirely. According to the χ² test on distribution of the categorized seedling numbers within AOK-30-treated and -untreated sections, expansion of mycelia from the inoculated leaves was significantly lower in AOK-30-treated seedlings than -untreated seedlings (P<0.01).

The seedlings treated with AOK-30 in different ways were transplanted in cell trays with one mycelial disk of *Rhizoctonia* sp. at the center. As expected, after mycelia developed on the soil surface, the severity of symptoms in infected seedlings and the direction of spread within the tray varied from week to week, regardless of the AOK-30 treatment. However, some seedlings far from the inoculation site became wilted or discolored within 3 weeks by physiological causes probably resulting from mechanical damage during transplanting. Such seedlings were removed from the tray immediately, thus reducing the total number of observed seedlings by the end of the observation period.

In trays containing transplanted AOK-30-untreated or suspension-treated seedlings, more than 90% of seedlings were infected within 3 weeks after transplanting, and 70–75% of these showed the highest symptom category. However, in trays of flask or soil AOK-30 treatments, infected seedlings with the greatest severity were localized near the tray center, and 40–50% of seedlings towards the tray edge had no symptoms or a severity of 1 within 3 weeks.

Table 2 describes details of weekly disease development in an entire tray of different treatments. In AOK-30-untreated seedlings, some brownish seedlings (9 of 72) had severities of 1 to 4. At 2 weeks, 15 of 68 seedlings showed the category 4 symptom but 48 showed no symptoms. At 3 weeks, 48 of 68 seedlings had the highest rating, and only 15 of 68 (22.1%) were free of symptoms.

In contrast, only 5 of 79 seedlings pretreated with AOK-30 in flasks had symptoms ranging from 1 to 4 at week 1. The number of seedlings with symptoms increased (14 of 76) at 2 weeks, but 62 still had no symptoms. At 3 weeks, seedlings with highest disease rating increased in number (41 of 74), but 30 (40.5%) did not show any symptoms.

Seedlings immersed in an AOK-30 suspension before transplanting showed a similar tendency to untreated seedlings with regards to occurrence of symptoms: only 15 of 78 seedlings (19.2%) remained green at 3 weeks. This treatment did not effectively suppress spread of the disease.

In seedlings transplanted directly from flasks to AOK-30-amended soil, spread of the disease had a tendency similar to that seen in seedlings treated in flasks: 27 of 73 seedlings (37.0%) remained green at 3 weeks. Thus, direct mixing of AOK-30 with soil appeared as effective in suppressing root rot as flask treatment.

According to the χ² test on distribution of the categorized seedling numbers within treatments, dissemination of the disease in the soil treatment was significantly slower than that in control soil during the observation period of 3 weeks (P<0.01). However, dissemination of the disease in both the flask treatment and control was only marginally different at 3 weeks (P<0.05). Therefore, it was concluded that soil treatment was the most effective and efficient way for slowing progression of root rot within cell trays.

**DISCUSSION**

The endophytic presence of *Streptomyces* spp. might have some important roles in plant development and health, because they can affect plant growth either by nutrient assimilation or secondary metabolite production. Matsukuma et al. and Okazaki et al. reported that a variety of actinomycetes inhabit a wide range of plants as either symbionts or parasites. Sato et al. attempted to isolate endophytic actinomycetes from 27 species of 20 genera in 15 families of higher plants and found that all test plants harbored at least one species of actinomycetes. Thus, endophytic actinomycetes are distributed commonly in a wide range of higher plants. Shimizu et al. demonstrated that pretreatment of the medium surface with *S. galbus* R-5, an endophytic actinomycete, rendered tissue-cultured seedlings of rhododendron resistant to *P. sydowiana*. In this study, a similar result was obtained: pretreatment of the medium surface with *S. padanus* AOK-30 in flasks reduced the severity of *Pestalotia* disease in tissue-cultured seedlings of mountain laurel (Table 1). A further experiment showed that mixing a spore suspension of AOK-30 with soil was more effective than the flask treatment in suppressing the dissemination of *Rhizoctonia* in a
cell tray with transplanted with tissue-cultured seedlings (Table 2). For practical purposes, the soil treatment is simpler and more efficient than the flask treatment, because of possible contamination with other microbes when AOK-30 is added to axenic flasks.

As demonstrated in Fig. 1, AOK-30 could grow either on or in seedlings when it was spread on the medium surface. Based on results of Fig. 1, it is uncertain whether AOK-30 was colonized within or on the tissue-cultured seedlings. Shimizu et al. demonstrated by scanning electron microscopy that S. galbus R-5 entered leaves of tissue-cultured seedlings of rhododendron through their stomata, and in addition, Suzuki et al. presented electron micrographs showing colonization of this strain in the intercellular spaces of rhododendron leaves after stomatal penetration. Because rhododendron and mountain laurel are closely related within the same family, Ericacea, AOK-30 perhaps resides in the host plant, as does R-5, although this requires confirmation. Nevertheless, AOK-30 treatment is likely to render tissue-cultured seedlings of mountain laurel disease-resistant. Shimizu et al. demonstrated by HPLC analysis that when the surface of the tissue-culture medium of rhododendron seedlings was amended with R-5 (analogues of actinomycin X and fungichromin produced by R-5), all seedlings contained these antibiotics at concentrations higher than the minimum levels suppressive to mycelial growth of P. sydowiana. However, the disease was only suppressed in seedlings after treatment of the medium surface with R-5, but not by treatment with these antibiotics. Thus, they assumed that growth of R-5, an antibiotic producer, was essential for inducing disease resistance in tissue-cultured seedlings of rhododendron, and that production of antibiotics within the host plant might not be a key event for suppressing the disease in seedlings. Their assumption was supported by the observation of Suzuki et al. that wall appositions are newly formed barriers that influence of such stimuli. Furthermore, Kunoh et al. (unpublished data) obtained evidence by northern analysis of Arabidopsis (within the host range of R-5) seedlings treated with R-5; rhododendron seedlings in flasks intensely expressed the PDF1.2 gene, but only slightly expressed the PR-1 and PAL genes. Production of camelexin, a phytoalexin of Arabidopsis, was also remarkably enhanced by R-5 treatment. This suggests that systemic resistance associated with jasmonate could be induced by R-5 treatment in this plam. Thus, it is likely that disease resistance of tissue-cultured seedlings treated with endophytic actinomycetes might be ascribed to systemic resistance induced by their colonization within the host plants.

As discussed here, many questions remain unsolved concerning the mechanisms underlying this method of disease resistance induction in tissue-cultured seedlings of mountain laurel. Nevertheless, our previous works and the present study strongly suggest that endophytic symbiosis of actinomycetes might play an important role in protecting the host plants from diseases in nature.

ACKNOWLEDGEMENTS

We thank Dr. Beth E. Hazen for correction of the English and valuable suggestions. This work was partially supported by Grants-in-Aid for Scientific Research (A) (No. 10306004) from the Ministry of Education, Science, Sports and Culture of Japan to Hitoshi Kunoh in 2003.

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