

In vitro and *in vivo* Assay for Assessment of the Biological Control Potential of *Streptomyces* sp. KT

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Abstract

Rhizoctonia solani is fungal plant pathogen that infects many different host plants. Recently, biological control agents that are friendly to the environment and ecosystems have attracted much attention as an alternative to the use of chemical fungicide which have been used worldwide to control soil borne pathogens including *R. solani*. In this study, 53 strains of actinomycetes isolated from environmental soils, and antifungal activities of them were assessed by the dual culture assay. Strain KT showed strong inhibitory activities against 8 phytopathogenic fungi. A great suppressive effect on *R. solani* growth was observed in the inoculation test of plants using cucumber and chin-geng-sai. In addition, infection of *Bipolaris oryzae* also could be suppressed in the detached leaf assay using oats. As a result of genetic analysis, it was shown that KT was a species closely related to *Streptomyces lavenduligriseus* NRRL B-3173^T. However, as far as we know, there is no report for biological control agents using *S. lavenduligriseus*. This study suggests that the strain KT may useful as biological control agents to suppress various crop diseases.

Keywords: biological control, actinomycetes, plant pathogens

1. Introduction

Currently the world population is increasing more than 7 billion people and is projected to reach over 9.8 billion people by 2050 (United Nations, 2017). On the other hand, the arable land area per capita in the world continues to decrease, and it is predicted that in 2050 it will decrease to 30% compared to 1950 (FAO, 2012). Therefore, improvements of the crop yields must be necessary in limited agricultural land. In order to contribute to solve this problems, reducing the loss of crops in the cultivation steps is important. Plant pathogens are one of the main causes of losses of crop production and pesticides are an effective means to prevent losses in crop production.

Rhizoctonia solani is a very influential plant pathogen in crop production and infects many different host plants, causing damping-off, sheath blight, stem canker, and seed decay. As the disease of *Rhizoctonia* occurs in all parts of the world, many researches have been done since the discovery of *R. solani* in 1858 (Ogoshi, 1987). In case of rice, yield losses due to sheath blight disease caused by this pathogen was estimated to range from 5 to 10% in Asia. Moreover, the estimated yield losses increased up to 50% when susceptible cultivars were grown under suitable conditions for this pathogen. (Lee & Rush, 1983, Marchetti & Bollich, 1991, Savary, Willocquet, Elazegui, Castilla, & Teng, 2000). In potato, *R. solani* causes stem canker in some case, and marketable yield losses have been reported to be approaching 30% (Banville, 1989). In the case of cucumber fields, the average annual loss in the United States is approximately 7-9%, which equals to a loss of approximately 4-5 million dollars (Lewis & Papavizas, 1980). In recent years, up to 75% mortality in cucumber seedlings in the most affected greenhouse has been reported in countries where greenhouse cultivation is major agriculture (Al-Sa'di, Drenth, Deadman, De Cock, and Aitken, 2007). Therefore, cucumber damping-off is a serious problem not only in agricultural field but also in greenhouse cultivation (Al-Sa'di et al, 2007, Lewis & Lumsden, 2001). In Japan, it has been reported that *R. solani* K1 isolated in Kanagawa prefecture in 1986 causes stem rot in various crops such as rice, eggplant, cucumber, spinach, tomato (Asaka & Shoda, 1996). From these facts, controlling plant pathogens, especially controlling *R. solani* is an important issue for improving agricultural production.

Chemical fungicides have been used worldwide to control soil borne pathogens including *R. solani* (Parry, 1990). However, chemical fungicide application has some problems, such as environmental pollution, deteriorating

human health, and development of drug-resistant pathogens/insects (Clevo & Clem, 2001). Furthermore, current chemical agents are not completely effective and *Rhizoctonia* disease remains to be a persistent problem. Therefore, biological control agents that are friendly to the environment and ecosystems have attracted much attention as an alternative to the use of chemical fungicide.

In recent years, biocontrol of soil born disease, particularly using antibiotic activity of actinomycetes, has been put forward as an alternative to chemical control agents. Actinomycetes are filamentous soil bacteria and also known for their ability to produce many antibacterial/antifungal compounds (Sanglier, Haag, Huck and Fehr, 1996, Jiayue et al., 2016). They also secrete extracellular hydrolytic enzymes such as chitinases and glucanases, which can degrade components in the cell walls of plant pathogens (Castillo et al., 2016, Sakdapetsiri et al., 2016). Therefore, actinomycetes are promising group of root-colonizing microbes where they may influence plants by promoting growth and protecting them from diseases. However, there have been a few reports under unsterilized condition, a similar condition at open field. The objective of this study is to isolate an actinomycete that has a strong antifungal activity and bring out their potential ability to suppress the diseases caused by *R. solani* and other phytopathogens.

2. Method

2.1 Microorganisms

Actinomycete strain KT was isolated from the soil sample collected in Wakayama, Japan and incubated on PDA (PDA; BD Difco) at 24 °C for 7 days. Isolated colonies of the actinomycetes were preserved in glycerol suspension (15% v/v) as a stock at -30 °C. The plant pathogenic fungi [*Bipolaris oryzae* MAFF 305382, *Colletotrichum echinocloeae* MAFF 305460, *Colletotrichum orbiculare* MAFF 306685, *Fusarium oxysporum* MAFF 103038, *Fusarium solani* MAFF 235170, *Monilinia fructigena* MAFF 305640, *Phytophthora infestans* MAFF 236324, *Rhizoctonia solani* MAFF 235846), *Rhizoctonia solani* K-1] were incubated on potato dextrose agar (PDA) at 24 °C for 7 days before use.

2.2 Identification of Actinomycete Strain KT

Phylogenetic analysis based on 16S rDNA sequence and morphological observation were done as follows. Bacterial 16S rRNA genes were PCR-amplified with primers 9F (5'-GAGTTTGATCCTGGCTCAG) and 1510R (5'-GGCTACCTTGTTACGA). The 16S rRNA gene sequences determined were compared with those retrieved from DNA database of APORON DB-BA 11.0 (Techno Suruga Lab., Shizuoka, Japan) and GenBank/EMBL/DBJ using a BLAST homology search, and phylogenetic tree was constructed to ascertain the phylogenetic position of the actinomycete strain KT. Phylogenetic trees were generated by the neighbor-joining method. Gene sequencing and phylogenetic analysis were carried out at Techno Suruga Lab., Co., Ltd. (Shizuoka, Japan).

2.3 Dual Culture Assay for in vitro Inhibition of Mycelial Growth of Plant Pathogens

Antifungal activity of KT against plant pathogens was evaluated by using dual assay. An agar plug of 0.7-cm diameter from actively growing fungal mycelia was placed on the perimeters of the PDA plate at a distance of 1.5 cm, and then strain KT was inoculated on the opposite of the fungal mycelia. Plates were incubated for 7-14 days at 24 °C. The zone of inhibition was measured as halo area around the actinomycete strain KT, and inhibition index was determined by the following criteria; +: <20 cm², ++: 20-30 cm², +++: >30 cm².

2.4 Infection Control of Isolated Actinomycete Strain KT against *R. solani*

Cucumis sativus L. (cucumber) and *Brassica rapa* var. *chinensis* (qing-geng-cai) were used for infection control test. Seeds were soaked in 70% ethanol for 10 sec, and sterilized with 1% NaClO for 10 min, finally rinsed three times with sterile distilled water. Sterilized seeds were germinated under dark conditions for 3 days on water agar plate. The germinated seeds were then transferred to agripot containing modified Hyponex medium (Hyponex powder; 0.0375%, sucrose; 0.35%, agar; 0.8%, pH6.0), with 3 (cucumber) or 5 (qing-geng-cai) seeds in one pot. KT was cultured in PDB medium at 24°C, 1500 rpm (stirred culture) for 3 days and 200 µL of the culture broth was spread in the agripot on the day of transplant. Five days after the transplant, the infection assay was carried out by placing 0.7-cm-diameter agar plugs of plant pathogens onto the center of agripots. Biocontrol effect was assessed after the plants were grown in growth chamber for 15-20 days at 28°C, 12 h light/dark condition.

Seed and soil were not sterilized in the soil experiment, and 2 g of wet hyphae of KT were used in the experiment. Disease severity was evaluated according to the 0–5 indexes (0: no disease, 1: browning of root, 2: browning of stem, 3: wilting of plants, 4: lodging of plants, 5: plant death), and determined by the following equation;

$$\text{Disease index} = \frac{\Sigma(\text{score of disease} * \text{number of plants})}{\text{total number of plants}}$$

2.5 Detached Leaf Assay of Isolated Actinomycete Strain KT against *B. oryzae*

Avena sativa (oat) was used for detached leaf assay. Plants at 2 to 3 true leaf stage in pots were harvested and washed with distilled water. They were cut into uniform lengths of 1.5 cm and cut edge was sealed with paraffin. Sealed leaves were sterilized with 1% NaClO for 90 sec and rinsed three times with sterile distilled water. After sterilized, leaves were soaked in KT culture for 10 min. Then, leaves were placed in 12-well microplate with sterilized moistened gauze. For the detached leaf assay, mycelial plug (4 mm²) of *Bipolaris oryzae* from PDA medium was inoculated onto the upper surface of the *Avena* leaves pierced with needle of syringe followed by the incubation at 25 °C, 12-h light/dark conditions for 12 days. Disease severity was evaluated according to the leaf area covered by the fungus by the following 0–4 indexes (0: no disease, 1: hyphal formation around the agar pieces, 2: hyphal coverage of less than 50%, 3: hyphal coverage of 50-80%, 4: completely covered with hyphae).

3. Results

3.1 Dual Culture Assay for in Vitro Inhibition of Mycelial Growth of Plant Pathogens

Fifty-three actinomycete strains were isolated from environmental soil samples, and anti-fungal activities of them were assessed by the dual culture assay using *R. solani*. Among them, an isolate named KT showed strong suppressive effects on the growth of *R. solani*. The inhibitory effect for other phytopathogens was assessed in the same way as *R. solani*. Antifungal activity of the strain KT was assessed by measuring the inhibition zones present after incubation of dual culture with 8 fungi (Fig. 1). The strong antagonistic activities (+++) against *B. oryzae*, *C. echinocloae*, *C. orbiculare* and *F. oxysporum* were observed in the dual culture plates, and the inhibition zones were 42.1, 30.9, 37.5, 31.6 cm², respectively (Fig. 1 and Table 1). KT also showed moderate antagonistic activity (+ and ++) against other pathogenic fungi.

3.2 Identification of Actinomycete Strain KT

The actinomycete strain KT was isolated from a soil sample on the root of a tree collected at Wakayama, Japan. KT showed the filamentous growth and was gram-positive. It formed 2.0-3.0 mm white colony and aerial hyphae, but, did not form spores. The 16S rDNA sequence of actinomycete strain KT shows high sequence similarity to genus *Streptomyces*, and the closest similarity with that of *S. lavenduligriseus* NRRL B-3173^T (Accession No. NR043824) with 99.6% similarity (Fig.2).

3.3 Infection Control of *Rhizoctonia* Damping-off of Cucumber by Strain KT

As shown in Fig.3B, symptom of *R. solani* infection was observed on the third day after inoculation in the control experiment, and severe damage was observed from days 6 to 8. But in the KT treated pots, only a weak infection was observed as shown in Figs. 3A, B. Since the clear suppression of *R. solani* growth was observed in cucumber which belongs to Cucurbitaceae, we performed the same assay using other plant species, qing-geng-cai in the family of Brassicaceae, in order to reveal whether the effect depends on host plants. The first infection was observed on the day 7 in the control experiment, and it was 4 days later compared with that of cucumber, but the severe damage was observed from days 15 to 20. However, in the KT treated experiment, strong suppressive effect against the infection by *R. solani* was clearly observed (Figs. 4A, B). Since the remarkable inhibitory effect by KT against the infection of *R. solani* was observed in the Hyponex medium, a sterilized and nutrition-rich medium, the similar experiment was done using the unsterilized soil, which is similar to the actual field. As shown in Figs. 5,6, KT showed a noticeable inhibitory effect against the infection by *R. solani* and protected cucumber and qing-geng-cai from the infection even in the non-sterile soil.

3.4 Detached Leaf Assay of Isolated Actinomycete Strain KT against *B. oryzae*

As the clear suppressive effect against *R. solani* was observed, another important phytopathogenic fungus *B. oryzae*, which causes disease symptoms mainly in high value field crops in the family Poaceae, including rice, maize, wheat, was used. To assess whether actinomycete KT could control the plant pathogen *B. oryzae*, *A. sativa* (oat) was used for detached leaf assay. Oat leaves were treated with or without KT. It was observed that leaves with soaking treatment of KT culture inhibited the growth of the plant pathogen and showed almost no lesion on the leaves, while the leaves of non-treated plants were covered by the pathogens as show in Fig.7A (top panels). The KT-treated leaves showed little mycelial formation of *B. oryzae* even on the final day of the test, and the disease severity was also low (Fig.7B).

4. Discussion

There are a variety of microorganisms in the rhizosphere of plants. Interaction between plants and microorganisms influence on the plant growth in a positive or negative manner. Biological control agents are thought to be able to promote the growth of plants and suppress plant pathogens. Actinomycete is one of the potential biological control agents for plant disease and they are known to produce useful antimicrobial compounds.

Growth of 8 plant pathogens was inhibited in vitro assay by dual culture method, and strain KT had the strongest antifungal activity in the actinomycetes that were collected in our library. These results suggested the presence of antifungal metabolites which control wide range of plant pathogens. In this study, a novel strain *Streptomyces* sp. KT was evaluated as a biological control agent against cucumber damping-off, qing-geng-cai damping-off caused by *R. solani* and oat leaf blight caused by *B. oryzae*.

Disease suppressive potential of KT was tested by cucumber or qing-geng-cai infection control assay. The strain KT was able to protect the seedlings of cucumber or qing-geng-cai from *R. solani*. Infection control tests were performed on both Hyponex medium and unsterilized soil. Both tests showed successful suppression against plant pathogens. Previous studies often use culture medium and sterilized soil, but few reports use unsterilized soil. The experiments using unsterilized soil cannot avoid the influence of microorganisms present in the unsterilized soil, and thus, the focused microorganism is difficult to proliferate. However, KT could inhibit the growth of phytopathogenic fungi under the unsterilized soil condition. This result suggests that pretreatment with KT culture can reduce the infection of a plant pathogen even in the open fields.

In this study, we tried infection control tests on not only *Rhizoctonia* damping-off but also Brown spot (*B. oryzae*). *B. oryzae* is known to cause brown spot in the Poaceae family, and brown spot is diseases that cause important losses between 1-10%. (Sarvary et al., 2000, 2006) Disease area of brown spot is the third largest in Japan, following blast rice blight (neck rot) and sheath blight (Matsumoto et al., 2016). Control of brown spot is an important issue for the rice cultivation area. KT can significantly delay the infection of *B. oryzae* in the detached leaf assay. Therefore, KT may be useful as a biological control agent to manage many plant pathogens in view of the results of dual culture assay.

The results of phylogenetic analysis based on 16S rDNA sequences indicate that actinomycete strain KT belongs to the cluster of the genus *Streptomyces*, however, the strain KT showed different phylogenetic position with *S. lavenduligriseus* NRRL B-3173^T. Differences of 6 base in the 16S rDNA base sequence was observed between *S. lavenduligriseus* NRRL B-3173^T and strain KT. Among the difference of the segment, 2 bases are suggested to be caused by insertion/deletion, and the remaining 4 bases was clearly different. From these results, KT was identified as *Streptomyces* sp., which closely related to strain *S. lavenduligriseus* NRRL B-3173^T. *S. lavenduligriseus* is known to produces pentenomycin and polyene macrolides (Jiayue et al., 2016). Pentenomycin is antibacterial compound and is not effective against fungi. On the other hand, polyene macrolides show a broad antifungal spectrum, and inhibition of plant pathogens has been reported, which may suggest that the antifungal compound of KT has also a similar structure (Kim et al., 2012). As far as we know, there is no report for biological control agents using *S. lavenduligriseus*. Therefore, research on the use of strain KT is considered to contribute to the development of novel biological control agents in the future.

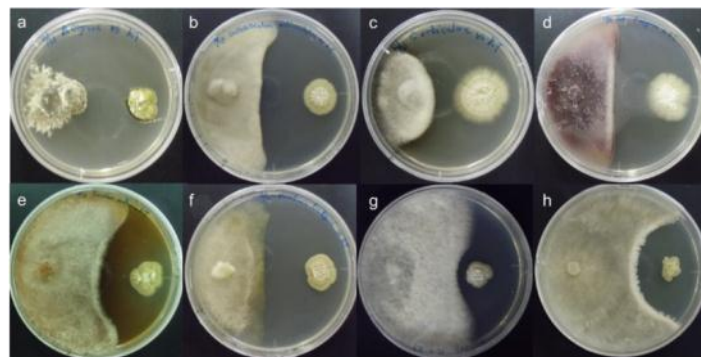


Figure 1. Dual culture assay of actinomycete strain KT (right) against plant pathogens (left). Strain KT and plant pathogens were inoculated on the PDA medium at the same time. (a) *Bipolaris oryzae*, (b) *Colletotrichum echinochloae*, (c) *Colletotrichum orbiculare*, (d) *Fusarium oxysporum*, (e) *Fusarium solani*, (f) *Monilinia fructigena*, (g) *Phytophthora infestans*, (h) *Rhizoctonia solani*

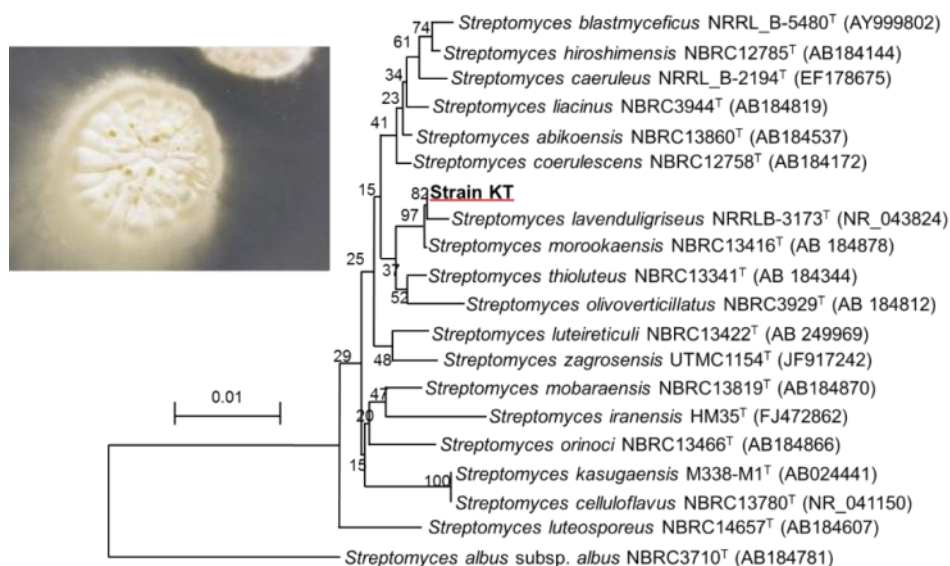


Figure 2. 16S rDNA gene sequences showing relationships between *Streptomyces* isolates and related *Streptomyces* type strain species. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets

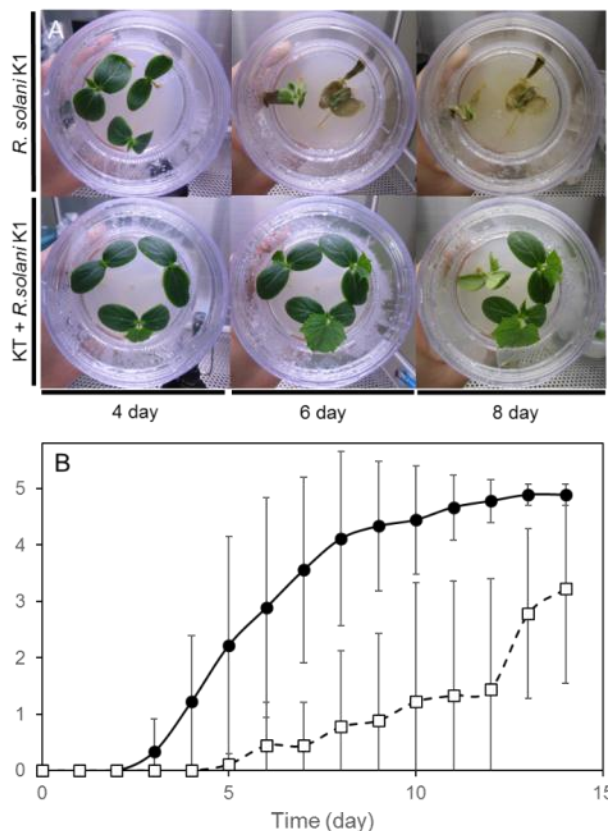


Figure 3. Infection control of strain KT against cucumber damping-off (*R. solani*). The photograph of infection control of *R. solani* by strain KT(A). Reduction of disease severity of cucumber damping-off by strain KT(B). Inoculated with only *R. solani* (closed circle). Inoculated with *R. solani* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-5 value. The same experiment was repeated three times, and the average value and standard deviation were shown

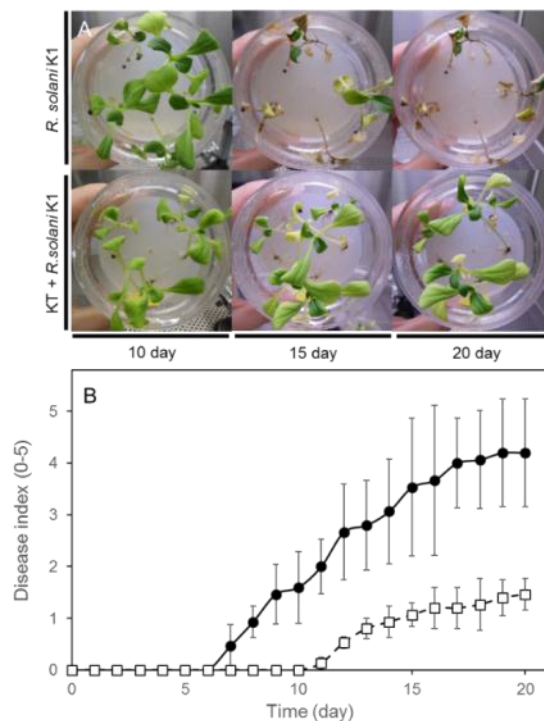


Figure 4. Infection control of strain KT against qing-geng-cai damping-off (*R. solani*). The photograph of infection control of *R. solani* by strain KT(A). Reduction of disease severity of qing-geng-cai damping-off by strain KT (B). Inoculated with only *R. solani* (closed circle). Inoculated with *R. solani* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-5 value. The same experiment was repeated three times, and the average value and standard deviation were shown

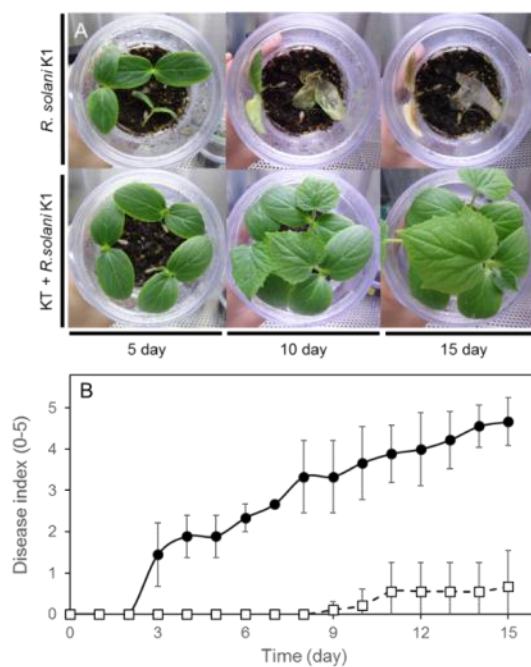


Figure 5. Infection control of strain KT against cucumber damping-off (*R. solani*) under unsterilized soil condition. The photograph of infection control of *R. solani* by strain KT (A). Reduction of disease severity of cucumber damping-off by strain KT (B). Inoculated with only *R. solani* (closed circle). Inoculated with *R. solani* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-5 value. The same experiment was repeated three times, and the average value and standard deviation were shown

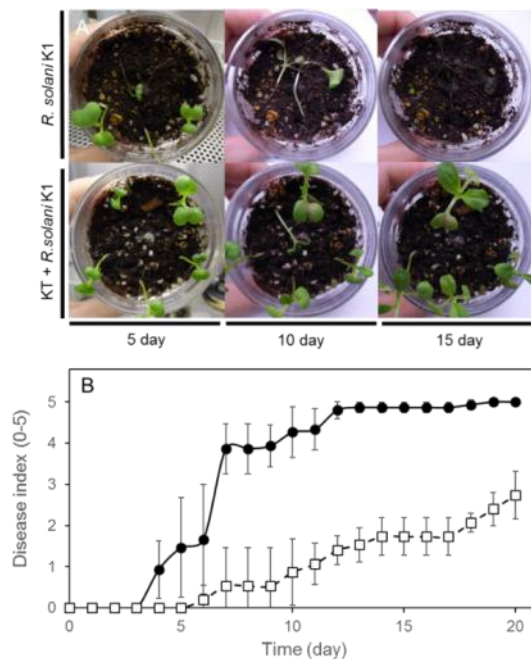


Figure 6. Infection control of strain KT against qing-geng-cai damping-off (*R. solani*) under unsterilized soil condition. The photograph of infection control of *R. solani* by strain KT (A). Reduction of disease severity of qing-geng-cai damping-off by strain KT (B). Inoculated with only *R. solani* (closed circle). Inoculated with *R. solani* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-5 value. The same experiment was repeated three times, and the average value and standard deviation were shown

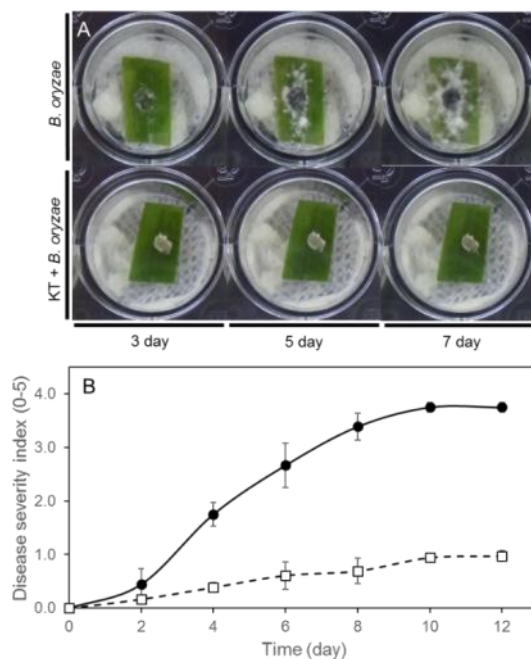


Figure 7. Detached leaf assay of strain KT against *Bipolaris* leaf blight. The top panels showed the result inoculated with *B. oryzae* and bottom panels showed that inoculated with *B. oryzae* and strain KT (A). Reduction of disease severity of Oat leaf blight (*B. oryzae*) by strain KT was clearly observed (B). Inoculated with only *B. oryzae* (closed circle). Inoculated with *B. oryzae* and strain KT (open square). Disease severity index was developed based on the time period during the test that seedlings became infected and was calculated on a 0-to-4 value (B). The same experiment was repeated three times, and the average value and standard deviation were shown

Table 1. Antifungal activity of actinomycete strain KT against plant pathogens on PDA.

Plant pathogens	Crops	Inhibition zone area (cm ²)	Inhibition index*
<i>Bipolaris oryzae</i>	rice	42.1±0.6	+++
<i>Colletotrichum echinocloae</i>	Japanese millet	30.9±0.5	+++
<i>Colletotrichum orbiculare</i>	watermelon	37.5±0.8	+++
<i>Fusarium oxysporum</i>	cabbage	31.6±0.5	+++
<i>Fusarium solani</i>	rice	18.6±0.1	+
<i>Monilinia fructigena</i>	apple	29.6±0.5	++
<i>Phytophthora infestans</i>	tomato	14.1±0.2	+
<i>Rhizoctonia solani</i>	lawn grass	11.7±0.2	+

(*inhibition zone +, < 20 cm²; ++, 20 - 30 cm²; +++, > 30 cm²)

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