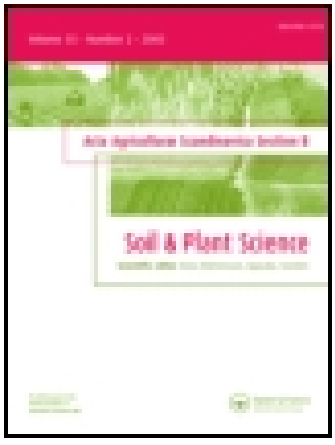

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Suppression of rice blast, cabbage black leaf spot, and tomato bacterial wilt diseases by Meyerozyma guilliermondii TA-2 and the nature of protection

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ORIGINAL ARTICLE

Suppression of rice blast, cabbage black leaf spot, and tomato bacterial wilt diseases by *Meyerozyma guilliermondii* TA-2 and the nature of protection

Mohsen Mohamed Elsharkawy[a], Mai Nakatani[b], Mitsuyoshi Nishimura[b], Tatsuyuki Arakawa[b], Masafumi Shimizu[b] and Mitsuro Hyakumachi[b]*

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This study was conducted to evaluate the efficacy of yeast strain TA-2 for controlling rice blast, cabbage black leaf spot, and tomato bacterial wilt diseases. Microscopic and phylogenetic analyses based on rDNA-internal transcribed region (ITS) and rDNA-D1/D2 sequences indicated that yeast strain TA-2 is *Meyerozyma guilliermondii*. Pretreatment with TA-2 by soil drenching significantly reduced the severity of black leaf spot disease caused by *Alternaria brassicicola* and leaf blast disease caused by *Magnaporthe oryzae*. Symptom development of tomato bacterial wilt caused by *Ralstonia solanacearum* in both soil drench and needle inoculation tests was significantly reduced in TA-2-pretreated plants under soil drenching. Disease severity and *R. solanacearum* growth were significantly reduced in tomato plants pretreated with yeast culture, cell suspension, or culture filtrate of TA-2 under soil drenching. TA-2 does not produce antibiotics. The present study indicates that disease suppression is systemic, as the roots were treated with TA-2 and the pathogens were inoculated onto leaves or stems, thereby separating the two spatially. *M. guilliermondii* TA-2 could become a promising natural antimicrobial agent against rice blast, cabbage black leaf spot, and tomato bacterial wilt diseases and might be useful as an eco-friendly control measure, contributing to sustainable agriculture.

Keywords: *Alternaria brassicicola*; induced systemic resistance; *Magnaporthe oryzae*; *Ralstonia solanacearum*; yeast

Introduction

Plants are under continuous attack by a wide range of potential microorganisms. Plant–microbe interactions have evolved to reflect both the nutrient acquisition strategies of microbes and the defense strategies of plants (Veronese et al. 2003). *Alternaria brassicicola* (Schw.) Wiltshire, the causal pathogen of black leaf spot, is a common and dangerous pathogen of crucifers worldwide; this disease causes significant losses in crucifer crop yield (80–100%) and quality (Muto et al. 2005). In addition, rice blast disease caused by *Magnaporthe oryzae* is one of the major causes of decreases in rice production worldwide (Taguchi et al. 2014). Bacterial wilt is an important disease of tomatoes and peppers, causing plant death and significant losses in yield up to 100% (Hayward 1991; Milling et al. 2011).

The chemical control of plant pathogens is becoming less acceptable due to the environmental hazards and the development of strains of plant pathogens that are resistant to pesticides (Wydra & Semrau 2005). In the last two decades, new eco-friendly and safe methods have been used for the control of plant pathogens, including induced systemic resistance, which is gaining public acceptance (Van Loon et al. 1998; Van Wees et al. 2008; Elsharkawy et al. 2012a, 2012b, 2013, 2014, 2015; Sudisha et al. 2013; Hassan et al. 2014).

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Recently, *Candida oleophila* strain I-182 was registered as a postharvest pesticide in the USA (Chand-Goyal et al. 1999), and other yeast species have been reported as effective biocontrol agents. Indeed, *Saccharomyces cerevisiae* and some bacterial isolates were reported to be effective against root rot pathogens under greenhouse conditions (Abd El-kader et al. 2012).

In this study, a yeast strain TA-2 was identified by microscopic and phylogenetic analyses and evaluated its ability to suppress cabbage black leaf spot, rice blast, and tomato bacterial wilt diseases. The nature of this protection is also discussed.

Materials and methods

Scanning electron microscopy observation

Yeast strain TA-2 was initially isolated from tomato roots in Gifu University, Japan. TA-2 was grown on soybean casein digest medium (SCD; Eiken, Tokyo, Japan) for 2 d at 30°C, and the organism was assessed using scanning electron microscopy (SEM), as described by Koilery and Keerthi (2012). An aliquot of 1.5 mL of yeast culture (YC) was centrifuged at 8000 rpm in a refrigerated centrifuge for 15 min. The pellet was washed with sterile saline at 50/100 and fixed at 4°C overnight in 0.5 mL of 2.5 % glutaraldehyde prepared in sterile saline. The pellet was washed repeatedly with saline, and dehydration was performed through a 70–100% acetone series and kept overnight in a desiccator. The particle was spread on SEM stubs, dried using a critical point dried apparatus, platinum coated, and observed under a SEM (JSM-820, Jeol, Ltd., Japan).

Molecular phylogenetic analysis

Yeast strain TA-2 was phylogenetically analyzed. The strain was maintained on SCD at 30°C for 2 d. *Penicillium chrysogenum* was used as out-group. Genomic DNA was extracted from a loopful of fresh TA-2 culture as described by Fernández et al. (2012). For DNA sequencing, the D1/D2 domain of the rDNA region was amplified using primers NL1 and NL4 (Table 1). The PCR reaction was performed with 1 × PCR buffer, dNTP mixture (containing 2.5 mM of each dNTP), 1 μM each primer, 1.25 U TaKaRa Taq (TaKaRa, Japan), and 50 ng genomic DNA. The PCR conditions were as follows: a denaturing step at 94°C for 1 min, 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, and a final extension step of 3 min at 72°C. The purified PCR products were sequenced with an Applied Biosystems 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions. The rDNA-internal transcribed region (ITS) sequence was edited using ChromasPro version 1.33 (Technelysium Pty Ltd.), and the nucleotide sequences were aligned using Clustal W (v1.7; Thompson et al. 1994) with minor manual adjustments. Phylogenetic trees were constructed with the neighbor-joining method, and the reliability of the branching orders was implemented in Clustal W using the bootstrap approach. TreeView Win16 (Page 1996) was used to draw trees for illustration purposes.

Table 1. List of primer used in molecular phylogenetic analysis.

Area	Primer name	Base sequence
rDNA-ITS	ITS1	TCCGTAGGTGAACCTGCGG
	ITS4	TCCTCCGCTTATTGATATGC
D1/D2	NL4	GGTCCGTGTTTCAAGACGG
	NL1	GCATATCAATAAGCGGAGG AAAAG

Preparation of TA-2 culture, cell suspension, and culture filtrate

TA-2 was grown on SCD at 30°C for 2 d, and colonies were collected with a cotton swab and suspended in sterilized distilled water (SDW). The suspension was diluted with SDW to a turbidity of OD600 nm = 0.5 (1.0×10^7 cell mL^{-1}) and used as the YC. For preparation of the cell suspension (CS) and culture filtrate (CF), TA-2 was grown in nutrient broth (NB) for 2 d at room temperature. CS was adjusted with NB to a turbidity of OD600 nm = 0.5 (1.0×10^7 cell mL^{-1}). CS was centrifuged for 10 min at 3000 rpm (room temperature), and the supernatant was used as CF.

Inoculation with **A. brassicicola** *and disease assessment*

Cultures of *A. brassicicola* were grown on a potato dextrose agar (Difco, Detroit, MI, USA) slant for 10 d. SDW was then added to prepare a spore suspension. A disease suppression test for cabbage black leaf spot disease was carried out as follows. First, 3-day-old cabbage plants (*Brassica oleracea* L.) grown in plastic pots (15 cm in diameter) filled with 700 g soil (Kanuma soil and Star bed mixed soil, 1:1) were treated with 10 mL of TA-2 YC by soil drenching. Three days later, the leaves were inoculated with 5 mL *A. brassicicola* spore suspension (1.0×10^6

conidia mL^{-1}). The plants were incubated for six more days at 25°C in a growth chamber under 12 h light (300 μmol m^{-2} s^{-1}): 12 h dark conditions before checking the disease severity on each plant. The experiments were repeated four times, and three individual plants were used per replicate.

The disease severity was assessed using the method described by James (1971): disease rate 0, leaves showing 0% leaf area infected; 1, leaves showing 33% leaf area infected; 2, leaves showing 67% leaf area infected; 3, leaves showing 100% leaf area infected.

$$\mathrm{DI} = \frac{1}{N}\sum_{i=0}^{3}\left(\frac{100}{3} i \times n_i\right)$$

where DI is disease severity index; N is total number of replicates; i is disease rate; *and* n_i is number of plants in each replicate.

Inoculation with M. oryzae *and disease assessment*

A culture of *M. oryzae* was maintained on oat meal agar (50 g oatmeal L^{-1}) and incubated for 2 wks at 25°C. A spore suspension of the pathogen was prepared by adding 2–3 mL SDW and collecting the spore suspension in small beakers. A disease suppression test for rice blast disease was carried out as follows. Seeds of commercial rice, *Oryza sativa* L. (cv. Koshihikari), were surface-sterilized with 70% ethanol for 1 min, soaked in NaOCl 20% for 5 min, and rinsed with SDW five times. The seeds were pre-germinated on autoclaved filter paper that was soaked with SDW and incubated for 3 d at 25°C. Sterilized plastic pots (6 cm in diameter) were filled with 150 g soil (Kanuma soil and Star bed mixed soil, 1:1), and four pregerminated rice seeds were sown 3–4 cm below soil surface of each pot. The plants were grown for 3 wks in growth chambers at 25°C under 75% relative humidity and a 12/12 light/dark cycle (cool fluorescent lamps at 300 μmol m^{-2} s^{-1}). The plants at three leaves stage were then separated into four groups: (1) pathogen alone, (2) pathogen plus benzothiadiazole (BTH), (3) pathogen plus TA-2, and (4) control (untreated). For the TA-2 or BTH treatment, TA-2 YC or 50 μM BTH was added to the pots (30 mL pot^{-1}); an equal amount of distilled water was added to the pathogen alone treatment and the untreated control treatment. For pathogen inoculation, a conidial suspension of *M. oryzae* (1×10^5 conidia mL^{-1}) with Triton X-100 (250 ppm) was sprayed onto the rice plants at 3 d after treatment. The plants were kept in a moist chamber at 25°C for 24 h under dark conditions (Zarandi et al. 2009) and then transferred and grown for 1 wk in growth chambers at 25°C under 75% relative humidity and a 12/12 light/dark cycle (cool fluorescent lamps at 300 μmol $m^{-2}s^{-1}$). The experiments were repeated six times, with four individuals rice plants per replicate. The disease severity was assessed using the method described by Taguchi et al. (2014).

Inoculation with Ralstonia solanacearum *and disease assessment*

Seeds of *Solanum lycopersicum* cv. 'Oogata-fukuju' were sown in a cell tray filled with cultured soil mix (Kureha, Tokyo, Japan) and grown at 28°C in a growth chamber under 12 h light (300 μmol m^{-2} s^{-1}): 12 h dark conditions. After 3 d, the plants were transferred to pots (9 cm in diameter) filled with the soil mix and grown for 2 wk at 28°C in a growth chamber under 14 h light (300 μmol m^{-2} s^{-1}): 10 h dark conditions. The tomato seedlings were treated with 30 mL of YC, CS, or CF of TA-2 by soil drenching and grown in the growth chamber under the same conditions. Five days after TA-2 treatment, the seedlings were inoculated with the pathogen. *R. solanacearum* isolate SUPP100 was cultured at 30°C on potato sucrose agar (PSA) medium containing 5 gl^{-1} $Ca(NO_3)\cdot 4\,H_2O$, 2 gl^{-1} $Na_2HPO_4\cdot 12\,H_2O$, and 5 gl^{-1} peptone for 48 h. The bacterial cells were collected by centrifugation at 7000 rpm for 10 min at 25°C and resuspended in DW to a final density of 1×10^7 cfu mL^{-1}. Two pathogen inoculation tests, a soil drench inoculation test and a needle inoculation test, were conducted. In the case of soil drench inoculation, 30 mL of the pathogen suspension was drenched into each pot containing seedlings that had been soil drenched with TA-2 YC, CS, or CF and grown for 14 d at 30°C in a greenhouse under natural light conditions. For needle inoculation, tomato seedlings pretreated with TA-2 YC by soil drenching were inoculated just above the cotyledons with a needle soaked in 1×10^7 cfu mL^{-1} of pathogen suspension. The disease severity, based on foliar symptoms of wilting, was monitored daily after inoculation. The experiments were repeated three times, and four individuals tomato per replicate were prepared.

Disease severity was assessed according to Hyakumachi et al. (2013) using the following scale: 0, healthy; 1, partial wilting of one lower leaf; 2, wilting of two to three lower leaves; 3, wilting of all but the top two to three leaves; 4, wilting of all leaves; or 5, dead. The disease severity was calculated using the formula:

$$(5\mathrm{A} + 4\mathrm{B} + 3\mathrm{C} + 2\mathrm{D} + \mathrm{E})/5\mathrm{N} \times 100,$$

where A = the number of plants with a score of 5; B = the number of plants with a score of 4; C = the

number of plants with a score of 3; D = the number of plants with a score of 2; E = the number of plants with a score of 1; N = the total number of plants.

Effect of YC, CS, and CF treatments of TA-2 on R. solanacearum *populations*

Fourteen days after soil drench inoculation of the pathogen, bacterial populations were analyzed in the stem tissues of tomato seedlings pretreated with soil drench of YC, CS, or CF of TA-2. Stem fragments (10 cm in length from near the root base) were excised from four plants. The samples were ground with DW in a mortar and pestle. The original solution and 10-fold serial dilutions of the homogenate were spread onto three plates of Hara–Ono medium (Hara & Ono 1983), and colonies were counted after 2 d of incubation at 30°C. The experiment was repeated three times with four individuals tomato plants per replicate.

Evaluation of antibacterial activity

SDW, NB medium, or CF of TA-2 (80 µL each) was dropped onto sterile thick paper disks (8 mm in diameter, produced by Advantec ® Japan); the disks were air dried for 1 min. A 10-ppm gentamicin disk (Gentamycin 10 sensing disk: Becton, Dickinson and Company, USA) was used as a positive control. After dispensing, 50 µL of *R. solanacearum* suspension, as described previously, was suspended in 50 mL of 1/3 NA medium and poured into 9-cm Petri dishes. The treated paper disks were placed in the NA culture medium and incubated for 48 h at 30°C. The evaluation of the antibacterial activity was based on the inhibition zone formed around the periphery of the disks.

Statistical analysis

The data were evaluated by an analysis of variance. A Tukey's studentized range test was used using EKUSERU-TOUKEI 2010 (Social Survey Research Information Co., Ltd). The means were compared at significance values of $P \leq 0.5$.

Results

Microscopic observations

Conventional microscopic observation was used for microbial characterization and showed that the cells of TA-2 are similar to those of yeasts. Electron micrography of TA-2 showed the characteristic single-cell morphology of *M. guilliermondii* (formerly known as *Pichia guilliermondii*, *C. guilliermondii*; Figure 1).

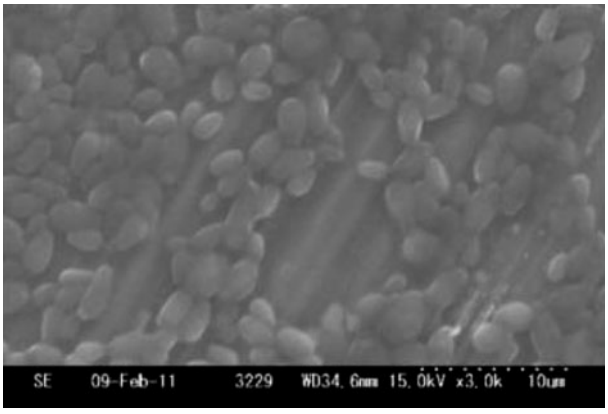

Figure 1. SEM image of yeast strain TA-2 cells (*M. guilliermondii*).

Molecular phylogenetic analysis

The different clusters on the phylogenetic trees were related to the taxa to which the TA-2 strain belongs to yeast (Figure 2). As shown in Figure 2, yeast strain TA-2 is similar to the *M. guilliermondii* cluster. The PCR amplification of isolated DNA using yeast-specific universal primers produced an amplicon of

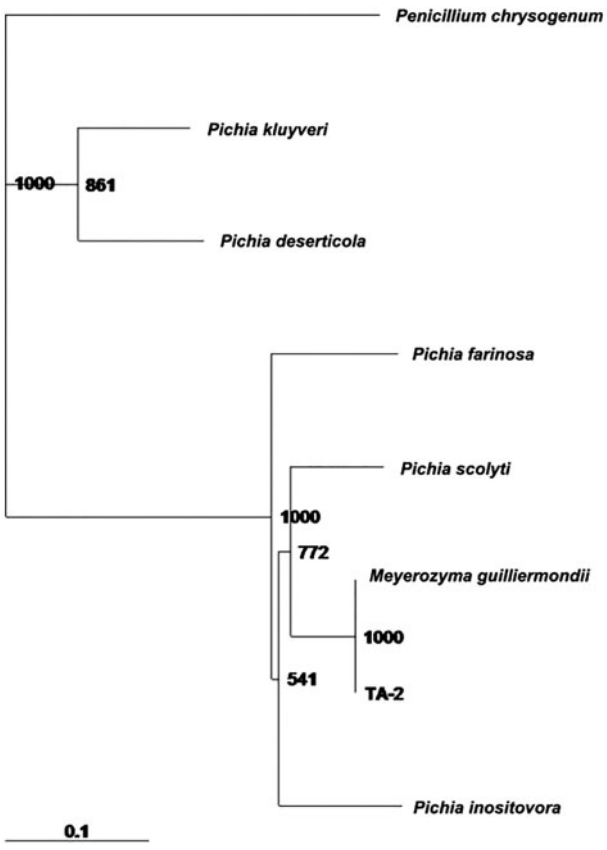

Figure 2. Neighbor-joining trees based on rDNA-ITS region sequence data. Numbers above branches are bootstrap values.

rDNA-ITS, which showed that the organism is *M. guilliermondii* (Figure 2). This finding was verified using the amplicon of the highly conserved rDNA-D1/D2 region with yeast-specific universal NL-1 (forward) and NL-4 (reverse) primers, which showed a high degree of similarity with *M. guilliermondii* (Figure 3).

Effect of TA-2 treatment on the severity of cabbage black leaf spot disease

TA-2 significantly reduced disease symptoms and caused a significant reduction in disease severity compared with the control. The suppressive effects of TA-2 YC against *A. brassicola* are shown in Figure 4.

Effect of TA-2 treatment on the severity of rice blast disease

Disease-suppressive activity of TA-2 against rice blast disease, caused by *M. oryzae*, was tested. The number of lesions in the control treatment was 10.0, whereas the number of lesions on TA-2 YC- and BTH-treated plants were 3.4 and 5.0, respectively. TA-2 significantly inhibited disease occurrence compared to the control. No significant difference was observed between the plants treated with TA-2 and BTH (Figure 5).

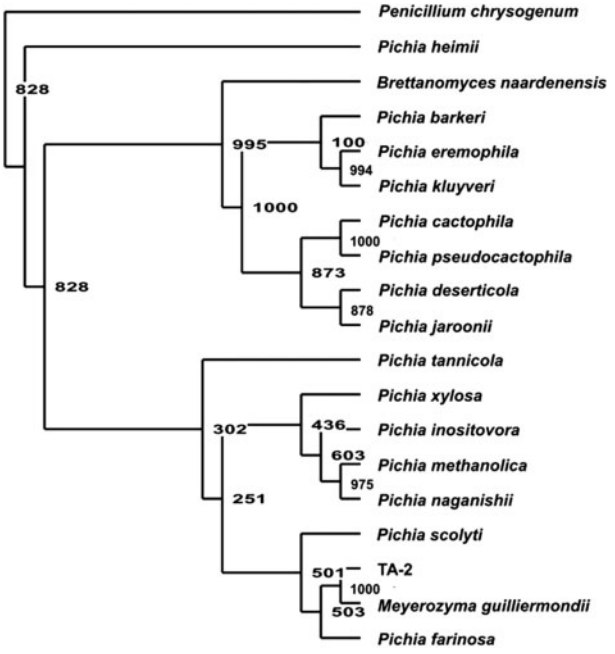

Figure 3. Neighbor-joining trees based on rDNA-D1/D2 region sequence data. Numbers above branches are bootstrap values.

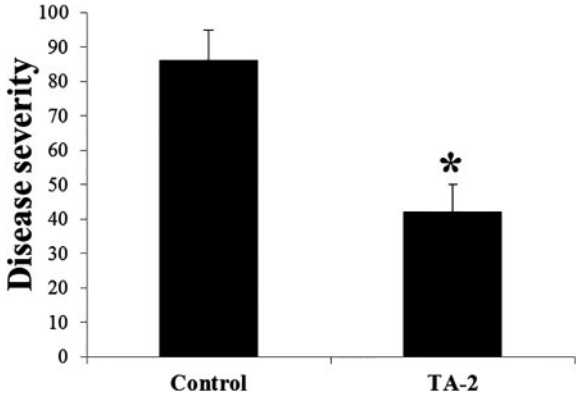

Figure 4. Induced suppression of disease symptoms of cabbage black leaf spot disease by yeast strain TA-2. Columns represent mean values. Bars represent standard error.
Note: Star (*) on bars indicates significant difference between control and inoculated samples ($P \leq 0.05$ independent Student's t test).

Effect of TA-2 treatment on the severity of tomato bacterial wilt disease

To investigate the potential activity of TA-2 for suppressing bacterial wilt in tomato, caused by *R. solanacearum*, tomato plants were treated with TA-2 YC, CS, and CF then challenge-inoculated with *R. solanacearum*. In a soil drench inoculation test under greenhouse conditions, TA-2 YC, CS, and CF treatments resulted in a significant reduction in tomato bacterial wilt disease compared to the control (Figure 6). Needle inoculation test in the growth chamber and TA-2 YC pretreatment by soil drenching caused a significant reduction in disease compared to the control (Figure 7). In the soil drench inoculation test, the growth of *R. solanacearum* in the stem tissues, moving from the initially inoculated root tissues, was significantly suppressed

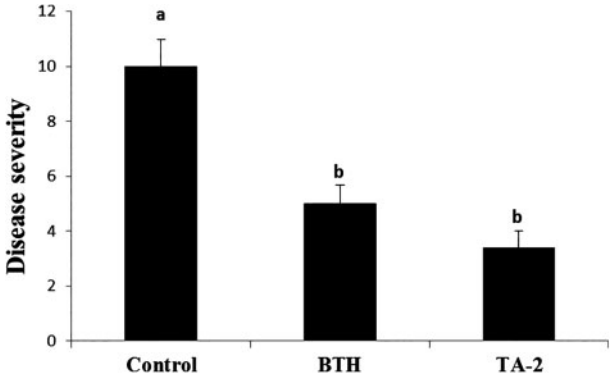

Figure 5. Induced suppression of disease symptoms of rice blast by TA-2 or benzothiadiazole (BTH) relative to nontreated control plants. Columns represent mean values. Bars indicate standard errors.
Note: Different letters indicate significant difference by Tukey's studentized range test at $P \leq 0.05$.

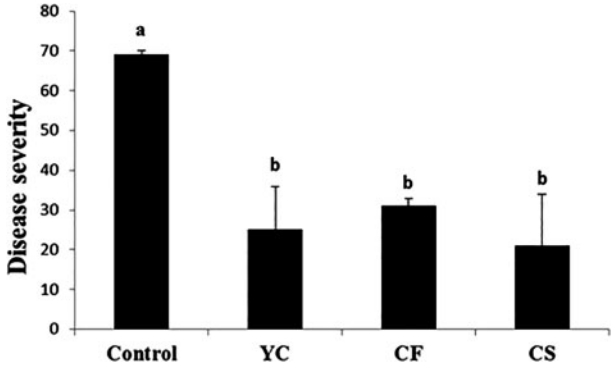

Figure 6. Induced suppression of disease symptoms of tomato bacterial wilt by yeast culture (YC), culture filtrate (CF), and cell suspension (CS) of TA-2. Columns represent mean values. Bars indicate standard errors.
Note: Different letters indicate significant difference by Tukey's studentized range test at $P \leq 0.05$.

in tomato seedlings pretreated with TA-2 YC, CS, or CF by soil drenching in comparison with DW-treated plants (Figure 8).

Evaluation of antimicrobial activity of TA-2 CF against R. solanacearum

The antimicrobial activity of TA-2 against *R. solanacearum* is shown in Figure 9. An inhibition circle was confirmed around gentamicin, whereas inhibition circles were not found around disks soaked with SDW, NB, and TA-2 CF. This implies that TA-2 CF does not exhibit antimicrobial activity against *R. solanacearum*.

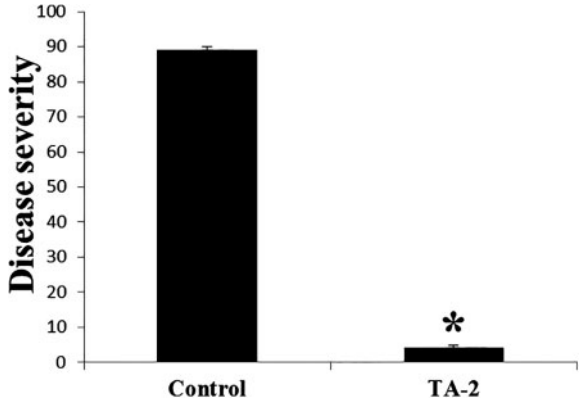

Figure 7. Induced suppression of disease symptoms of tomato bacterial wilt using a needle inoculation test of the pathogen. Columns represent mean values. Bars represent standard error.
Note: Star (*) on bars indicates significant difference between control and inoculated samples ($P \leq 0.05$ independent Student's t test).

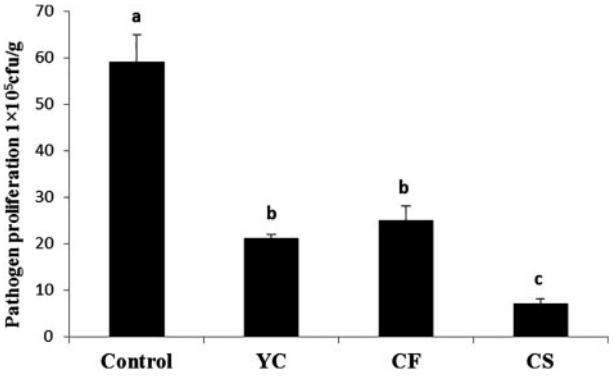

Figure 8. Comparison of growth inhibition of *R. solanacearum* in tomato plants treated with the yeast culture (YC), culture filtrate (CF) and cell suspension (CS) of TA-2 strain. Columns represent mean values. Bars indicate standard errors.
Note: Different letters indicate significant difference by Tukey's studentized range test at $P \leq 0.05$.

Discussion

The micrograph we obtained for yeast strain TA-2 in Figure 1 was similar to previous report on *M. guilliermondii* (Beresa et al. 2011). Scanning electron micrography, which is widely used for investigation of the surface structure of biological samples (Duckett & Ligrone 1995; Minoura et al. 1995), of yeast strain TA-2, showed the characteristic of single-cell morphology of *M. guilliermondii*. Yeast strain TA-2 was identified using PCR-based molecular diagnosis (Table 1). Recently, molecular phylogenetic analysis had been applied as a powerful tool for taxonomy, and sequencing of the ITS of the nuclear rRNA gene had been specifically used to

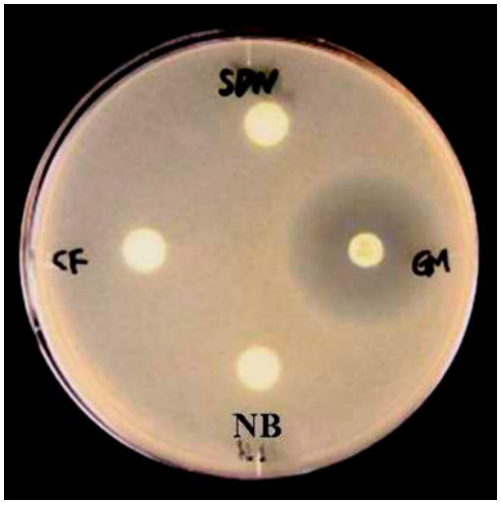

Figure 9. Antibacterial activity of culture filtrate (CF) of TA-2.
Note: Above: SDW, center left: CF, center right: gentamicin (G1) and under: NB.

identify and discriminate between 40 species of different yeast strains (Chen et al. 2001). The sequence analysis of the rDNA-ITS spacer region obtained in this study indicated that the yeast isolate was *M. guilliermondii* (Figures 2 and 3). All DNA sequences obtained shared significant similarities with *M. guilliermondii*, which is synonymous to *C. guilliermondii* or *P. guilliermondii* (Kurtzman et al. 2011). The partial sequencing of the rDNA-D1/D2 region further identified the organism as *M. guilliermondii*. Molecular identification based on the D1/D2 region of the rDNA gene is a reliable and robust means for identifying yeast isolates (Linton et al. 2007).

The present study revealed that diseases caused by *A. brassicola* and *M. oryzae* on the leaves of cabbage and rice and bacterial wilt on tomato were suppressed by TA-2 YC using soil drench treatment (Figures 4–6). The disease suppression appeared to be systemic, as roots were treated with TA-2 and the pathogens were inoculated on leaves or stems, thereby separating the two spatially. YC, CS, and CF of TA-2 were tested to characterize the disease-suppressive activity of TA-2 for the control bacterial wilt disease. When tomato roots were treated with TA-2 YC, CS, or CF followed by inoculation with *R. solanacearum* under a soil drench system, the development of wilt symptom and pathogen growth were significantly suppressed by all treatments (Figures 6 and 8). Additionally, TA-2 significantly reduced the severity of bacterial wilt under needle inoculation test (Figure 7). However, no antimicrobial effect was found for TA-2 CF against *R. solanacearum* (Figure 9). These results suggest that the disease suppression effect by TA-2 is due to the induction of systemic resistance. The mechanisms of action and efficacy of yeast against the molds are variable and include the production of cell wall lytic enzymes and the induction of resistance in the host plants (Urquhart & Punja 2002) and the production of toxic volatile compounds and killer toxins (Bruce et al. 2004). It is important to determine the substances that induce disease resistance. A sterilized solution of yeast successfully induced resistance against anthracnose in cucumber (Hisayoshi & Kazuhiro 2014). Thus, substances resistant to heat may be involved.

Several yeast species have been reported as active biological control agents and have been implemented in the postharvest field. Additionally, yeast cell wall extracts enhanced barley resistance to powdery mildew (Reglinski et al. 1994). Some isolates are also known for their ability to induce systemic resistance against different foliar pathogens. Considering the many excellent synthetic fungicides that confer resistance against cabbage black leaf spot, rice blast, and tomato bacterial wilt that are available, further studies should be conducted through field trials to determine whether the level of disease control by yeast treatment is acceptable to farmers.

Disclosure statement

No potential conflict of interest was reported by the authors.

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