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ISOLATION AND IDENTIFICATION OF CHARCOAL ROT DISEASE CAUSING AGENT IN SESAME (*SESAMUM INDICUM* L.) AND THEIR GROWTH INHIBITION BY *BACILLUS METHYLOTROPHICUS* KE2

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Abstract

Seedling emergence and plant growth of sesame (*Sesamum indicum* L.) are severely affected by charcoal rot disease. The aim of present study was to identify the disease causing agent of charcoal rot in sesame and prevent their growth by bacterial biocontrol agent. The infected stems of sesame plants were collected from the agricultural field and the disease causing agent, *Macrophomina phaseolina* NICS01 was identified in infected parts of plants by 18 rDNA sequencing and phylogenetic analysis. The biocontrol agent was isolated from Kimchi, a fermented Korean food and identified as *Bacillus methylotrophicus* KE2**.** The antagonism activity of *B. methylotrophicus* KE2 against *M. phaseolina* NICS01 was determined by *In vitro* study. The results of current study showed that the growth of *M. phaseolina* NICS01 was significantly inhibited by the effect of *B. methylotrophicus* KE2 and suggested that the application of bacterium, *B. methylotrophicus* KE2 could be a biocontrol agent to prevent the damage of charcoal rot disease.

Key words: *Bacillus methylotrophicus* KE2; Bio-control; *Macrophomina phaseolina* NICS01; Sesame.

Introduction

Sesame (*Sesamum indicum* L.) seed has rich source of fatty acids and antioxidants, so it is used as healthy and nutritive additive to prepare several foods. The yield of sesame has been affected by several abiotic and biotic factors. The seedlings emergence, plant growth and the yield of sesame are inhibited up to 40%, during the effect of destructive pathogens of *Fusarium oxysporum* and *Macrophomina phaseolina*, which cause wilt and charcoal rot disease (El-Bramawy & Wahid, 2009; Kumar *et al*., 2011). The dry root rot or charcoal root rot caused by *M. phaseolina* is endemic nature in tropical and temperate regions of the world and it infects over 400 plant species (Mihail & Taylor, 1995). *M. phaseolina* infected seedlings become brown to black based on their intensity of disease infection (Manjunatha *et al*., 2013).

The application of chemical fungicides (benomyl, carbendazim, thiabendazole, and thiram) can effectively control the pathogen growth, but the deposition of those fungicides in soil is toxic to other living organisms and pollutes the environment (Pimentel & Levitan, 1986; Hariprasad *et al*., 2011). In addition, 32% of food products in Korea are not suitable for consumption due to excess accumulation of pesticides (Kim *et al*., 2013). Alternatively, the treatment of beneficial micro-organisms on soil, seeds or plants can prevent the pathogen infection in crop plants (Radhakrishnan *et al*., 2013a; 2013b; Kang *et al*., 2015) and also helps to increase abiotic stress tolerance (Hashem *et al*., 2015) and while controls the weed growth (Radhakrishnan *et al*., 2017). Some of beneficial fungi including *Trichoderma viride* and *T. harzianum* were reported to control the *M. phaseolina* induced charcoal root rot disease in plants (Manjunatha & Naik, 2011). Plant growth promoting rhizobacteria (PGPR) support the plant growth and yield, and while prevent the pathogen attach. The number of bacterial species of *Bacillus* and *Pseudomonas* was identified and reported their bio-control activity against pathogens including *M. phaseolina* (Latha *et al*., 2001; Hariprasad *et al*., 2011; Hashem *et al*., 2017) by parasitism, nutrients competition and antibiosis processes (Alabouvette *et al*., 2009). There were no reports documented about the *Bacillus methylotrophicus* induced growth inhibition of *M. phaseolina* isolated from sesame. This study was aimed to identify the disease causing agent of infected sesame plants and find out their antagonistic organism (*B. methylotrophicus*) to prevent the pathogen growth.

Materials and Methods

Isolation and identification of charcoal rot disease causing agent in sesame: The disease infected (dark brown colour) stems of sesame plants were collected from the agricultural field of Miryang, Republic of Korea and washed with sterile water and sliced as small pieces (~1 cm). A surface sterilizing agent, 0.5% sodium hypochloride was used to remove other microbial flora on the stem. The upper layer of stem was scrapped and removed, and then their central part was kept over the

potato dextrose agar (PDA) in petri plates. The inoculated culture was incubated at 28 ± 2°C. The uniform growth of fungal (NICS01) mycelia around stem was observed within 2 days and it was subcultured for further use. For identification of charcoal rot disease causing agent, the isolated fungi was cultured in potato dextrose broth and genomic DNA of fungi was isolated by a kit prepared by solutions for genetic technologies, Daejeon, Republic of Korea and their 18S rDNA sequence was done by universal primers: ITS-1, 5'-TCC GTA GGT GAA CCT GCG G-3'; and ITS- 4, 5'-TCC TCC GCT TAT TGA TAT GC-3' (Radhakrishnan *et al*., 2013a). The obtained sequence was compared with similar sequence of ITS region of related fungi by BLAST search program (http://blast.ncbi.nlm.nih.gov). Selected *Macrophomina phaseolina* sequences were aligned using ClustalW, and a neighbor-joining tree was constructed using MEGA software.

Isolation and identification of *Bacillus methylotrophicus* KE2: Kimchi (fermented Korean traditional food) was used to isolate the beneficial bacteria for human and plants, and it was immersed in sterile saline water and inoculated on plates containing tryptic soy/agar medium and incubated for 48 h at 30 °C. The bacterial isolate, KE2 was identified on the basis of partial 16S ribosomal DNA (rDNA) sequence. The 27F primer (50-AGAGTTTGATC(AC)TGGCTCAG-30) and 1492R primer (50-CGG(CT)TACCTTGTTACGACTT-30) were used for PCR amplification of 16S rDNA and phylogenetic relationship of KE2 indicated the bacterial name was *Bacillus methylotrophicus* KE2 (Radhakrishnan & Lee, 2016).

***In vitro* antagonistic activity:** *B. methylotropicus* KE2 and two un-identified bacterial (KE1 and KE3) cultures were co-inoculated with *M. phaseolina* NICS01 on PDA plates and incubated for 5 days. The antagonistic activity of *B. methylotropicus* KE2 against *M. phaseolina* NICS01 was noticed by clear zone formation.

Results and Discussion

The sesame plants cultivated at agricultural area showed significant disease infected stems and roots as brown, dark brown and black colour. The identification of disease causing agent would help to control the infection in sesame plants, so we isolated (Fig. 1) and identified the charcoal rot disease causing organism, *Macrophomina phaseolina* NICS01 by molecular analysis. The sequence of NICS01 fungal isolate was compared with other closely related fungal isolates such as *Macrophomina phaseolina* MPKS138, MPKS175, MPKS203, MPKS271, MPKS283, MPAZ134, MPKS327 and MPKS313, and while *Fusarium sp.* OTU011 was used as out group of those isolates to confirm the name of the isolates. The sequence of our isolate, NICS01 was 97 % similar with other isolates of *M. phaseolina* (Fig. 2) and submitted to NCBI GenBank and also assigned Accession No. JX945162. Charcoal root rot disease caused by *M. phaseolina* on sesame was previously described by Maheshwari *et al*. (2012) and Chowdhury *et al*. (2014).

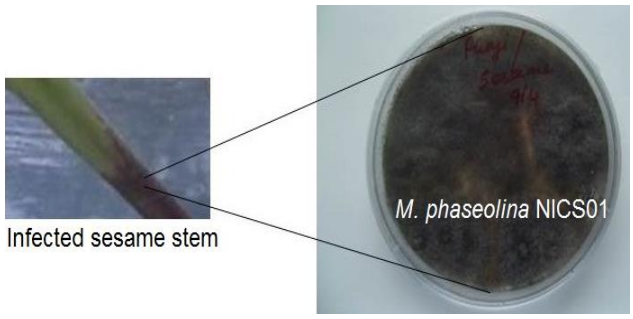

Fig. 1. *M. phaseolina* NICS01 growth over potato dextrose agar medium.

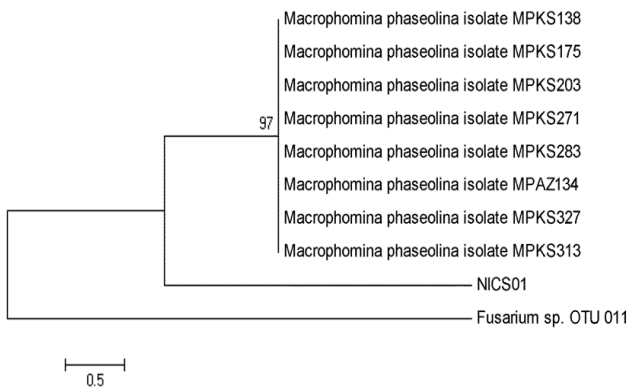

Fig. 2. Phylogenetic relationship of *M. phaseolina* NICS01 with other isolates of *M. phaseolina*.

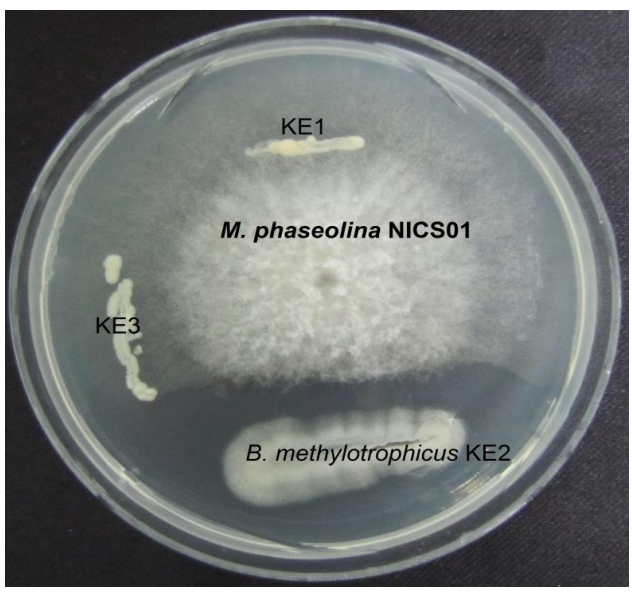

Fig. 3. Antagonistic activity of *B. methylotrophicus* KE2 against *M. phaseolina* NICS01.

A bacterial agent, *B. methylotrophicus* KE2 (NCBI GenBank Accession No. *KM875559.1*) was isolated from Kimchi food showed that the plant growth promoting activity on several plants in a preliminary study (Radhakrishnan & Lee, 2016) and it suggested that the production of gibberellins (GA_1, GA_3, GA_7, GA_8, GA_9, GA_{12}, GA_{19}, GA_{20}, GA_{24}, GA_{34} and GA_{53}) and indole-acetic acid from the bacterial cells helped to increase the nutrients and plant growth. In addition, the treatment of *B. methylotrophicus* KE2 was favor to improve the health of sesame plants by regulating their functional metabolites

(Radhakrishnan & Lee, 2017). In current study, the inoculation of *B. methylotrophicus* KE2 exhibited an anti-fungal activity against *M. phaseolina* NICS01 in fungal medium by the formation of clear zone between bacterial colonies and pathogen (Fig. 3), and while other bacterial isolates (KE1 and KE3) could not prevent the pathogen growth. The *In vitro* antagonistic activity of bio-control agent against pathogen is used to describe the actions of plant-pathogen communication at early infection period (Bressano *et al*., 2010). The degradation of mycelial growth of pathogenic fungi might be due to the action of lytic enzymes and toxic metabolites secreted from the bio-control agents. For example, *Azotobacter chroococcum* TRA2 secretes chitinase and β-1,3-glucanase to inhibit the fungal cell wall of *M. phaseolina* (Maheshwari *et al.,* 2012). However, other substances like siderophores, hydrogen cyanides and antibiotics production from bacteria prevent the pathogen growth by degrading the cell wall components which resulted in hyphal perforations, empty cell formation, shrinking and lysis of fungal mycelia and conidia (Maheshwari *et al*., 2012). The obtained results revealed that *B. methylotrophicus* prevented *M. phaseolina* growth it was assumed that it would reduce the disease index of *M. phaseolina* infection in sesame plants.

Conclusions

The sesame plant disease infection caused by *M. phaseolina* was identified by molecular analysis and the suitable bacterial antagonist; *B. methylotrophicus* was detected and confirmed by their bio-control activity due to presence of clear zone. The results of present study suggest that *B. methylotrophicus* can be a useful bio-control agent to prevent the *M. phaseolina* induced charcoal root rot disease in crop plants.

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