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CROP PROTECTION

Endophytic *Enterobacter cloacae* exhibits antagonistic activity against *Pythium* damping-off of cucumber

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ABSTRACT: *The study was performed to examine the potential presence of biological control agents against Pythium damping-off disease of cucumber.Examining eleven bacterial strains isolated from acid lime roots and rhizosphere soil showed that the bacterial strain RB1 was the most efficient in suppressing mycelial growth of P. aphanidermatum, producing an inhibition zone of 5mm. Scanning electron microscopy study of the mycelia at the interaction zone showed that the pathogen hyphae were deformed and shriveled by the bacterial strain.In pot experiments, pretreatment with the RB1 bacterial strain reduced disease incidence significantly by 63%.The bacterial strain did not exhibit any negative significant effects on cucumber growth (plant height and root dry weight) in comparison with untreated control under growth chamber conditions. Molecular identification of strain RB1 based on the 16S rRNA gene revealed that it is Enterobacter cloacae. Findings from this study suggested that E. cloacae has a potential to be used as a biocontrol agent for suppressingcucumber damping-off disease caused by P. aphanidermatum. This is the first report of the antagonistic activity of E. cloacae against P. aphanidermatum-induced damping-off of cucumber.*
Key words: *oomycetes, antagonism, biocontrol agent, endophytic bacteria.*

Enterobacter cloacae endofítica exibe atividade antagonista contra a podridão de pepino causada por *Pythium*

RESUMO: *O estudo foi realizado para examinar a presença potencial de agentes no controle biológico da doença do apodrecimento do pepino causado por Pythium. Examinando onze cepas bacterianas isoladas de raízes de cal ácida e solo da rizosfera mostraram que a cepa bacteriana RB1 foi a mais eficiente na supressão do crescimento micelial de P. aphanidermatum, produzindo uma zona de inibição de 5 mm. O estudo de microscopia eletrônica de varredura dos micélios na zona de interação mostrou que as hifas do patógeno foram deformadas e enrugadas pela cepa bacteriana. Em experimentos com vasos, o pré-tratamento com a cepa bacteriana RB1 reduziu significativamente a incidência da doença em 63%. A cepa bacteriana não exibiu nenhum efeito negativo. Efeitos significativos no crescimento do pepino (altura da planta e peso seco da raiz), em comparação com o controle não tratado sob condições da câmara de crescimento. A identificação molecular da cepa RB1 com base no gene 16S rRNA revelou que é a Enterobacter cloacae. Os resultados deste estudo sugerem que E. cloacae tem potencial para ser usado como agente de biocontrole para suprimir a doença da podridão de pepino causada por P. aphanidermatum. Este é o primeiro relato da atividade antagônica de E. cloacae contra o amortecimento induzido por P. aphanidermatum de pepino.*
Palavras-chave: *oomycetes, antagonismo, agente de biocontrole, bactérias endofíticas.*

Cucumber (*Cucumis sativus* L.) is a widely cultivated and economically important vegetable crop. High value cucumber cultivars are cultivated under controlled environments. In Oman, the majority of greenhouses (≥ 90%) are used for cucumber cultivation. Cucumber cultivation is usually in soil, with some farmers moving towards the use of hydroponics system (AL-SADI et al., 2011).

Damping-off disease is as a serious and worldwide problem on numerous agricultural and horticultural crops in fields, greenhouses, gardens, nurseries and forests. Various pathogenic oomycetes (*Pythium* and *Phytophthora*) and fungi (*Fusarium* and *Rhizoctonia*) have been reported as causal agents of damping-off (LOPEZ et al., 2018; ZHAO et al., 2019). *Pythium aphanidermatum* is one of the most common causes of damping-off disease, causing 25-75% cucumber seedling losses in Oman (AL-SADI et al., 2012).Management strategies in Oman rely on the use of mefenoxam, hymexazole, and propamocarb

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(AL-BALUSHI et al., 2018). However, application of these fungicides in large amounts can lead to reduction of their efficacy and building up of resistance in the fungal pathogens (MATIĆ et al., 2019).

Endophytic bacteria are plant associated microorganisms that reside in plant internal tissues without causing negative effects in their host (RYAN et al., 2008). Bacterial microorganisms including *Streptomyces* spp., *Serratia* spp., and *Pseudomonas* spp. and *Enterobacter* spp. have been efficiently used as potential biocontrol agents against Pythium damping-off (LI et al., 2007; AL-HINAI et al., 2010; ROBERTS et al., 2011). The origin of most of the bacterial strains were either soil or vegetable crops.

In Oman, Citrus has been grown for centuries. Studies have shown that citrus roots have high endophytic bacterial diversity (TORRES et al., 2008), some of which can be used as antagonists (KALAI-GRAMI et al., 2014). However, no reports exist on the isolation of antagonistic endophytic bacteria citrus against Pythium damping-off of cucumber. The present study evaluated the antagonistic activity of endophytic and rhizospheric bacteria associated with acid lime (*Citrus aurantifolia* L.) against *P. aphanidermatum*, the cause of damping-off disease of cucumber.

Intact root systems (secondary roots) were taken from the rhizosphere of acid lime by excavating them to a depth of 15-30 cm. In addition, rhizosphere soil samples within 5 mm of the secondary roots were taken from three plots around each tree and homogenized thoroughly. The soil samples were sandy, with 8.1 pH, 2.9 m S electrical conductivity, 5.8% inorganic carbon, 2.3% organic carbon, 0.08% nitrogen, 0.09 mg kg^{-1} phosphorus and 26.8 mg kg^{-1} potassium. All samples were wrapped in sterile plastic bags and kept at 10 °C in anincubator.

Soil bacteria were isolated based on the serial dilution method. The isolation petri plates were sealed with parafilm before incubation at 28 °C (AL-SADI et al., 2016). Morphologically well-defined bacterial colonies on the surface of plate were selected and transferred to new plates. Isolations were also conducted from acid lime roots. After surface disinfection, the root samples were dried under a laminar flow, followed by crushing them aseptically (maceration method) in sterile PBS with sterile mortar and pestle. The root content was diluted in PBS to 10^{-3} to form a homogenous suspension and the suspension was streaked onto generalized nutrient medium.

A pathogenic isolate, *Pythium aphanidermatum* isolated from naturally infected cucumber was attained from the fungal culture collection located in the Sultan Qaboos University (AL-SADI, 2012). An initial screening assay was conducted for 11 bacterial strains to evaluate their antagonistic activity against *Pythium aphanidermatum*. The assay was carried out as explained by KAZEROONI et al. (2019) except for using afilter paper disc containing bacterial suspension (about 5 mm in diameter) that was placed at the other end of the petri dish. Then 10 µl of the bacterial strain suspension (0.4 McF)was placed on filter paper disks. Plates without antagonistic bacteria were considered as controls. The petri dishes were incubated at 28 ºC in upright position to avoid losing or movement of the bacterial suspension. The size of the inhibition zone around each filter paper disk (up to the growth of the *Pythium*) was recorded in millimeters (mm). The experiment was carried out three times with three replications to confirm the activity of the bacterial strains.

The most effective strain from the previous test was used in further studies. Morphological and ultrastructural changes of *P. aphanidermatum* under the effect of RB1 were screened and studied using a scanning electron microscope (SEM, INSTUMENT JSE- 5600). Pathogen hyphal tips were observed to identify the morphological differences between *P. aphanidermatum* hypha near the inhibition zone with RB1 and untreated *P. aphanidermatum* grown in PDA as control. Preparation of samples for the electron microscope was done as described by GOLDSTEIN et al. (2018).

Potting mix was autoclaved and sterilized two times at 24 h intervals, at 121 °C (15 psi, 20 min). The experiment was conducted in 10-cm diameter pots using three replicates per treatment/control. A one-day old bacterial suspension (20 ml) was mixed with sterilized peat moss. Two control pots were prepared for the experiment: one without bacterial strains, and the other one with nutrient broth (without bacteria). Six disinfected cucumber seeds were sown in sufficient amount of sterilized potting mix in each pot. Incubation was at 27 °C and 70% RH for 7 days. The experiment was repeated two times. The length of roots and shoots were recorded. In order to determine dry weight, cucumber seedlings were dried at 60-65 °C for 24 hours. Then the weight of each seedling was recorded.

The experiment was conducted under growth chamber conditions of 27 °C and 70% RH for 7 days. Bacterial treatment suspension (20 ml) was mixed with 600 g of sterilized potting mix. Then, a 90-mm PDA plate culture of *P. aphanidermatum* grown for 3 days was placed on the top of the

sterilized potting mix. The remaining 200 g of the sterilized potting-antagonist mix was added on top of the pathogen culture. Pots inoculated with *Pythium* alone and pots mixed with nutrient broth alone served as controls. Untreated control pots (sterile potting mix alone) were also maintained. Survival percentage of the cucumber seedlings was determined in each pot. *P. aphanidermatum* was re-isolated from seedlings developing damping-off symptoms. Differences among treatments were analyzed using Tukey's Studentized range test (SAS v.8.)

The antagonistic bacterial strain (RB1) was identified using sequences the 16S rRNA gene. DNA was extracted in 1000 µL extraction buffer as explained by AL-SADI et al. (2016). Polymerase chain reaction (PCR) was done using the 27F and 1492R primers, with the PCR mixture been according to AL-SADI et al. (2016) while PCR conditions were according to FRANK et al. (2008). Sequencing was done at Macrogen Inc., Korea. A maximum likelihood analysis method was carried out using raxml GUI v.1.3 (SILVESTRO & MICHALAK, 2012).

In vitro screening of the bacterial strains revealed that one strain was efficient at inhibiting the growth of *P. aphanidermatum*. Strain RB1 suppressed the mycelial growth of *Pythium*, producing an inhibition zone of 5 mm. Scanning electron microscope (SEM) studies of the interaction zone of RB1 bacterial strain with *Pythium aphanidermatum* showed deformed pathogen hyphae including wrinkle, distortion, shrinkage and degeneration (Figure 1). On the contrary, pathogen hyphae from the control plate looked intact (Figure 1).

Inoculation of cucumber seedlings with the antagonistic bacterium RB1 did not produce any negative effects on the root length, shoot length or dry weight of cucumber (Figure 2). Inoculation of cucumber with *P. aphanidermatum* reduced the survival of cucumber to 27% within 7 days of inoculation. However, treatment of *Pythium*-infested pots with RB1 significantly increased the survival of cucumber seedlings to 73% when compared with the control (90%) or to pots receiving media (80%) (Figure 3; $P \leq 0.05$).Thus, RB1 significantly increased the survival rate of cucumber seedlings from 27% to

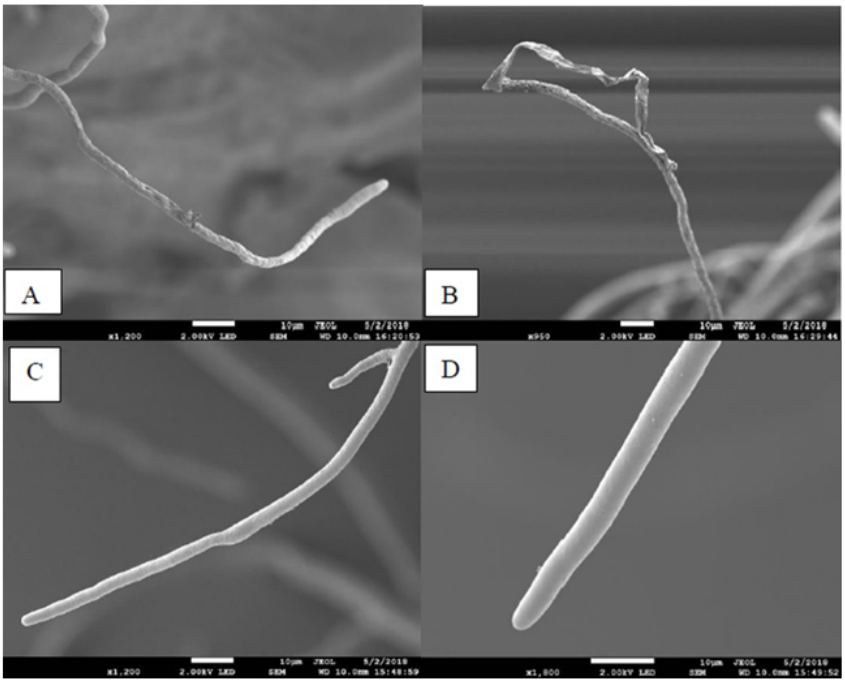

Figure 1 - Scanning electron microscope (SEM)showing the effect of RB1 strain on *Pythium aphanidermatum*hypha (A and B). Control hypha are shown in C and D.

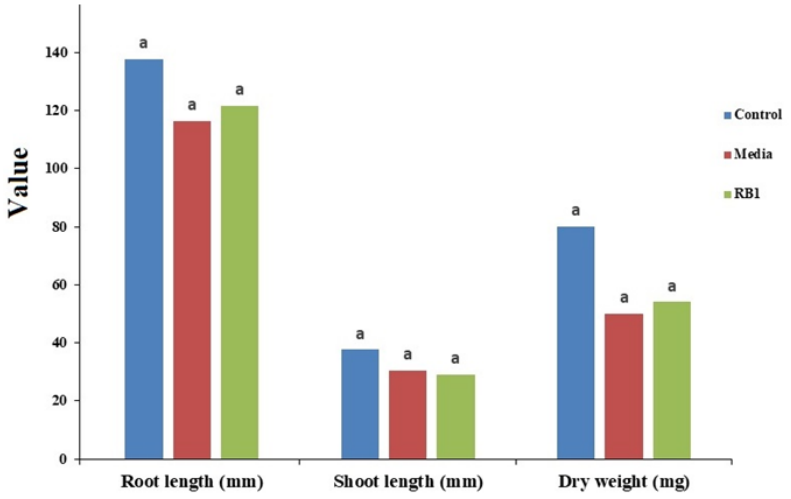

Figure 2 - Effect of RB1 bacterial strain on the root length, shoot length and dry weight of cucumber seedlings. Bars with different letters are significantly different from each other at P<0.05 (Tukeys' Studentized range test, SAS, v.8).

73% (Figure 3). *P. aphanidermatum* was re-isolated from the diseased cucumber seedlings. Based on sequence analysis, the antagonistic bacterial strain RB1 was identified as *Enterobacter cloacae*. The sequence of the strain was deposited in GenBank under the accession no. MK256309 (Figure 4).

Our scanning electron microscope results disclosed structural deformation of pathogen hyphae

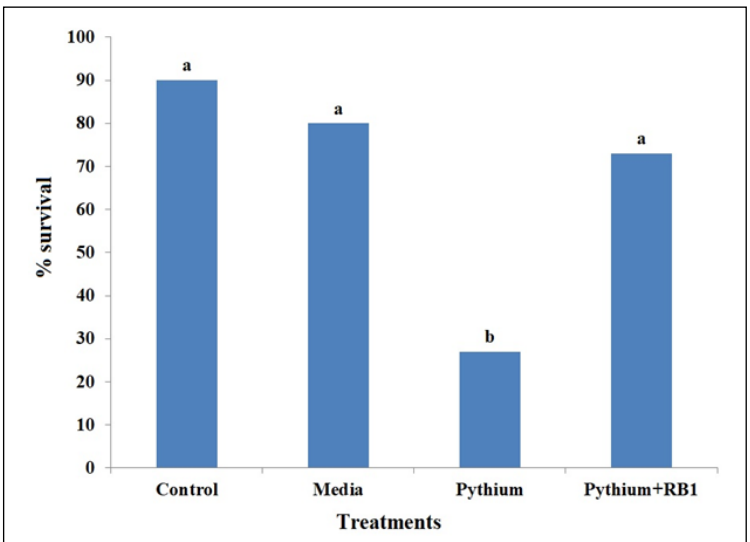

Figure 3 - Effect of RB1 strain on the percent survival of cucumber seedlings inoculated by *Pythium aphanidermatum*. Bars with different letters are significantly different from each other at P<0.05 (Tukeys' Studentized range test, SAS, v.8).

at the inhibition zone. *P. aphanidermatum* hyphae showed deformations, evident of the effect of the bacterial strain on its structure. Endophytic bacteria are capable of suppressing plant pathogenic fungi by producing antimicrobial compounds. These compounds can cause deformation and lysis of mycelia (YUAN et al., 2012). Our bacterial strain was identified as *E. cloacae*. Future studies may be required to assess the mode of action of this bacterial strain against *P. aphanidermatum*.

Growth chamber results demonstrated that *E. cloacae*showed considerable disease reduction compared to untreated control. *E. cloacae* appears to possess biocontrol potential against damping-off caused by *P. aphanidermatum*. The biocontrol potential of *E. cloacae* has been reported in other crops and pathogens, including its use against cucumber wilt (*Fusariumoxysporum* f. sp. *cucumerinum*) (SNEH et al., 1984), turfgrass blight (*Pythium aphanidermatum*) (NELSON & CRAFT, 1992), and dollar spot of turf (*Sclerotinia homoeocarpa*) (NELSON & CRAFT, 1991). Our study appears to be the first report of the effective use of *E. cloacae* in suppressing *P. aphanidermatum* and *Pythium*-induced damping-off in cucumber. Future studies should investigate the mechanisms associated with this suppression.

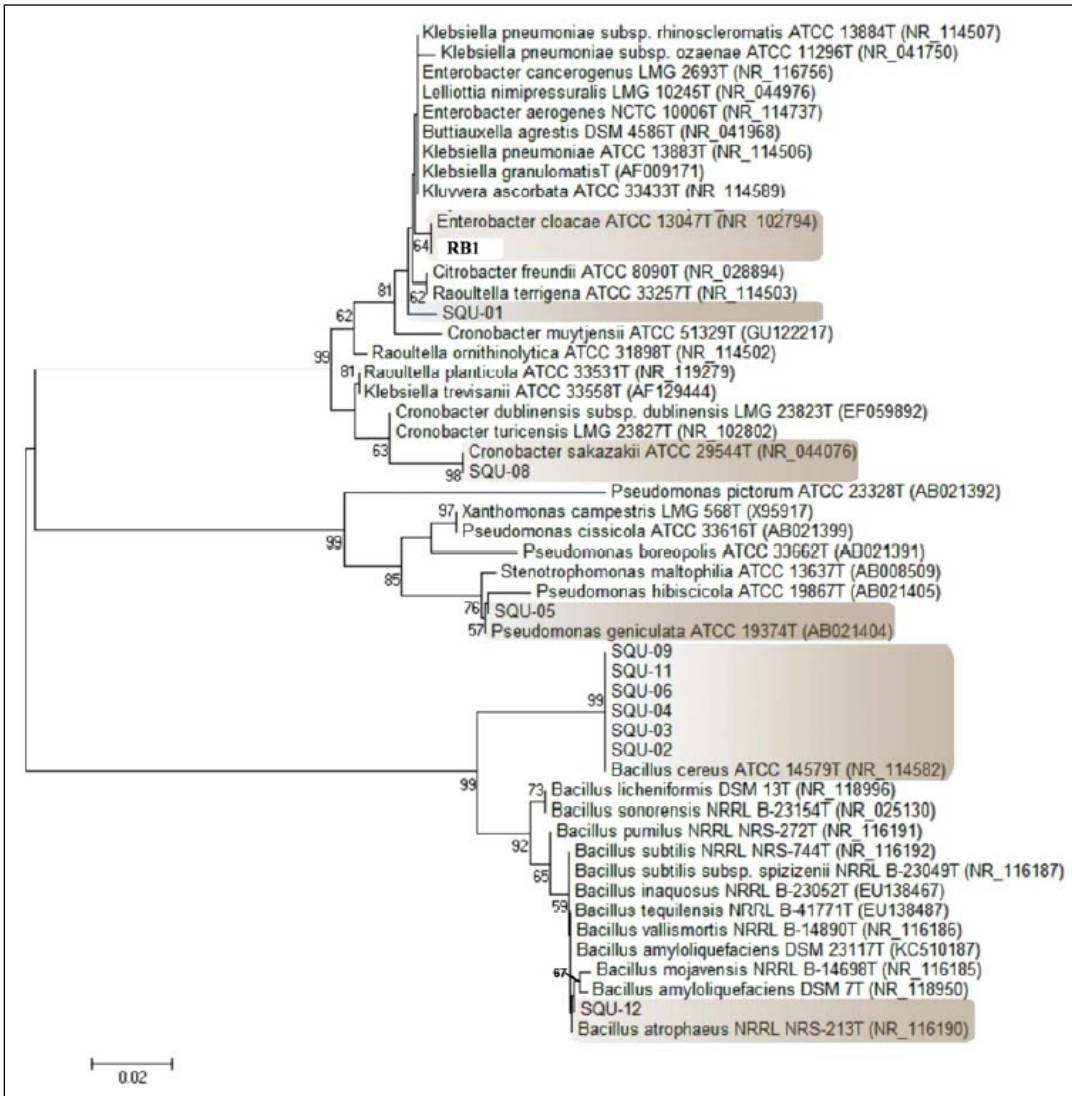

Figure 4 - Phylogram generated from Maximum Parsimony analysis based on sequence data of RB1 and related species.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

EA and HA conducted the experiments, and EA, HA, AN and AMA analyzed results and wrote the manuscript.

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