A simple method for assessing antagonistic activity of *Bacillus atrophaeus* against common root rot of barley

[*]Mohannad Harba, Mohammed Jawhar and Mohammed Imad Eddin Arabi

Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091, Damascus, Syria
[*]Corresponding author's email: ascientific@aec.org.sy

Abstract

Common root rot (CRR) caused by *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur is a serious disease of barley causing significant economic losses worldwide. An *in vitro* technique was developed for assessing directly the antagonistic potential of *Bacillus atrophaeus* isolate *SY15b* against CRR on barley. Seeds of the susceptible barely cv. WI 2291 were inoculated by mixing them thoroughly with *C. sativus SY15b* suspension and placed onto 1.5% water agar in sterile Petri dishes for 10 days. Our results demonstrated that *B. atrophaeus* had significant antagonistic capacities against the CRR by decreasing the percentage of barley infected area of sub-crown internodes (SCIs) as compared to controls after 10 days post incubation. Highly significant correlation coefficients ($r = 0.85$, $P = 0.001$) were found between *in vitro* and pot methods, indicating that *in vitro* co-culture testing procedure is reliable. The developed method is both simple and precise and could be used directly for a rapid assessment of the antagonistic activity of *Bacillus* on barley plants under uniform conditions for selecting suitable biocontrol agent against fungal soil-borne pathogens.

Keywords: *Bacillus atrophaeus*, Barley, Biocontrol, *Cochliobolus sativus*, *In vitro* test.

Introduction

Cochliobolus sativus is a soil-borne pathogenic fungus that causes common root rot of barley (*Hordeum vulgare* L.), a disease responsible for considered reduction in this crop worldwide (Van Leur *et al.,* 1997; Kumar *et al.,* 2002). CRR disease produces a brown to black discoloration of the sub-crown internode (SCI), which is commonly used for determining barley reaction towards this disease (Kokko *et al.,* 1995; Mathre *et al.,* 2003).

The economic damage caused by CRR can be avoided by using large amounts of fungicides, which has become one of the major concerns in barley fields due to great dangerous to humans and environment (Bailey *et al.,* 1997). Therefore, biocontrol has been suggested as an alternative approach to chemical control because it is highly selective method with less time consuming (Sellitto*et al.,* 2021). Many biocontrol agents were isolated by screening a big number of soil microorganisms for antagonism against fungal pathogens *in vitro* or in planta (Köhl *et al.,* 2019). Among those, *Bacillus* species have the benefit of being able to form endospores, which makes them very stable such as biofungicides or biofertilizers (Schisler *et al.,* 2004), they could suppress various soil borne diseases in different environments (Cao *et al.,* 2018).

*Bacillus atrophaeus*is a Gram-positive bacterium used in various studies as a classical non-pathogenic surrogate of *Bacillus anthracis* (Nakamura, 1989). It is easy to culture, has been identified for its resistance characteristics, and is being used in various applications (Sella *et al.,* 2014). Such bacteria have been reported to control different crop pathogens (Rajaofera *et al.,* 2017, 2020). The search for an environmental-friendly disease management approach has led to search bacterial agents with antagonistic activities. Although a wide spectrum of *Bacillus* antagonists has been reported during the past decades (Sharf *et al.,* 2021), more efficient and new *Bacillus* species antagonists might be around waiting for detection. However, it should be clearly understood that not all *Bacillus* are considered effective for controlling pathogens and therefore it is crucial to find out the *Bacillus* species with biocontrol potential, which is possible through *in vitro* tests prior to field experiments. The common method for assessing the antagonistic effects of bacterial and fungal antagonists against fungal pathogens is based on measuring the radial colony growth of fungi in co-culture system without plants (Khan and Javaid, 2020, 2021; Khan *et al.,* 2021; Sharf *et al.,* 2021). However, this method does not take into account changes in hyphal density or specific growth rate (Bolivar-Anillo *et al.,* 2021), and cannot give any information regarding this antagonism directly on plant for implementation as a biocontrol agent. The aim of this study was to develop a reliable *in vitro* laboratory assay for measuring directly the biocontrol capacity of *B. atrophaeus* isolate *SY15b* on barley plants against strains of *C. sativus* as a simple and rapid method.

Materials and Methods

Bacterial isolate

Soil samples were isolated from different locations in Syria (Ammouneh *et al.,* 2011). The samples (15 g each) were added to 100 mL of sterile deionized water and mixed for 10 min. Serial dilutions were made and plated on nutrient agar (NA) and incubated at 37 °C for 2 days. Bacterial colonies were screened among 525 isolates on nutrient broth (NB) culture according to Wulff *et al.* (2002). The *B. atrophaeus* isolate *SY15b* was identified based on both physiological characteristics and on 16S rDNA analysis (Harba *et al.,* 2020). Homology blast search was achieved against the database and deposited in GenBank (Accession No. MT159352). The *B. atrophaeusis SY15b* solution was diluted to 2×10^8 cell for using in the experiments.

C. sativus inoculum preparation

The most virulent pathotype Pt4 of *C. sativus* (Arabi and Jawhar, 2002) was used in this work. The isolate was grown on Petri dishes containing potato dextrose agar (PDA) medium (DIFCO, Detroit, MI. USA) at 22 ±1 ºC for 10 days in the dark. The conidial suspension was adjusted to 5×10^5 conidia mL^{-1} and 40 mL of spore suspension was mixed in a plastic Petri dish with 50 g sterile neutralized peat (Bailey *et al.,* 1997).

Seed inoculation by *C. sativus* and bacteria

Barley seeds of susceptible cultivar WI 2291 from Australia were surface sterilized with 5% sodium hypochlorite solution for 5 min and then soaked three times in sterilized distilled water. They were inoculated by mixing thoroughly with beat-gum conidia inoculums and *B. atrophaeusis SY15b* (Arabi and Jawhar, 2002).

Pot experiment

One part of inoculated seeds was planted in pots filled with sterilized peat moss in a depth of 6 cm for elongating the SCIs (Kokko *et al.,* 1995). Each experimental unit consisted of 10 seedlings with three replicates. A full replicate consisted of 10 pots inoculated with Pt4 isolate. Where control pots inoculated with *C. sativus* without bacteria was maintained as control. They were placed in a growth chamber at temperatures 22±1 ºC (day) and 17±1 ºC (night) with a daylength of 12 h and a relative humidity of 80–90%.

In vitro experiment

Second part of inoculated seeds were placed on 1.5% water agar, and to elongate SCIs, the Petri dishes were incubated 21±1 °C for 5 days in the dark. Then they were kept under a daylength of 12 h at the same temperature. Whereas, Petri dishes containing *C. sativus* inoculated seeds without bacteria were maintained as control.

Evaluation of antagonism

Common root rot responses based on the measurement of individual SCI discoloration (%) were assessed 7 weeks for pot assay, and 10 days for *in vitro* method post inoculation. The 0–5 scale (Kokko *et al.,* 1995) was used for both methods. All experiments were repeated three times. Data were analyzed using analysis of variance (ANOVA) and means were separated by Tukey's test ($P = 0.05$).

Results and Discussion

In this study, the antagonistic potential of the *B. atrophaeusis SY15b* was concluded and validated by reducing directly common root rot severity on barley SCI as shown in photo-plate of PDA culture plate assay comparing with controls (Fig. 1). The disease symptoms on the susceptible cv. WI 2291 were easily detected *in vitro* after 48 h of inoculation (Fig. 1).

Our results demonstrated that *B. atrophaeus* had significant antagonistic potential against the CRR by decreasing the percentage of barley infected area of SCIs as compared to controls after 10 days post incubation (Fig. 1). Inoculation with the *C. sativus* virulent pathotype Pt4 increased discoloration of the SCIs on cv. WI 2291 in pot and *in vitro* experiments (Fig. 2). The results clearly indicate that antagonistic between *B. atrophaeusis SY15b* and *C. sativus* was similar in the two methods and that the *in vitro* assay was useful for bio-control agent against CRR in a short time (Fig. 2). Highly significant correlation coefficients ($r = 0.85$, $P = 0.001$) were found between *in vitro* and pots methods, indicating that *in vitro* co-culture testing procedure is reliable (Fig. 3).

Our data are in accordance with those obtained by Schisler *et al.* (2004) and Cao *et al.* (2018) who reported that the antagonistic effect against plant pathogen could be attributed to the antifungal compounds production belonging to the family of iturins and subtilins that operate on the fungi's cell wall. Lyon and Muir (2003) found that the hydrophilic enzymes production to break down polysaccharides, lipids and nucleic acids and may have been also engaged. Hence the most likely explanation for reducing CRR symptoms by *B. atrophaeusis SY15b* is that antifungal activity is increased by *in vitro* co-culturing system, since *B. atrophaeus* has been proved to produce a wide spectrum of antimicrobial compounds, secretion metabolites preventing occurrence of systemic diseases (Rajaofera *et al.,* 2020).

Several factors have been reported to affect susceptibility of barley to common root rot disease during field experiments such as inoculum level and climate conditions (Kumar *et al.,* 2002). In our *in vitro* co-culture experiments, the inoculums concentration was controlled. Therefore, precise evaluation could be made. Furthermore, the soil may contain other fungi

and bacteria that can cause similar SCI rotting, which can lead to misdiagnosis of disease symptoms with root rot.

Conclusion

Despite the successful and importance use of *Bacillus* species, information regarding *B. atrophaeus* application is rare. Here we developed an *in vitro* technique that can be considered as a rapid, simple and relatively inexpensive method for *B. atrophaeus SY15b* isolate antagonisms against *C. sativus* under uniform conditions. The technique can help to replicate infection assays, as well as to test large numbers of bacterial and fungal pathogen isolates in a short time. The *SY15b* isolate could be promising as a bio-control agent against this disease.

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Fig. 1: (A): Symptoms of common root rot on barley SCI without *Bacillus* treatment. (**B**): *Bacillus atrophaeus* SY 15b showing antagonistic activity against *C. sativus* isolate Pt4 in the *in vitro* co-culture after 120 h of incubation.

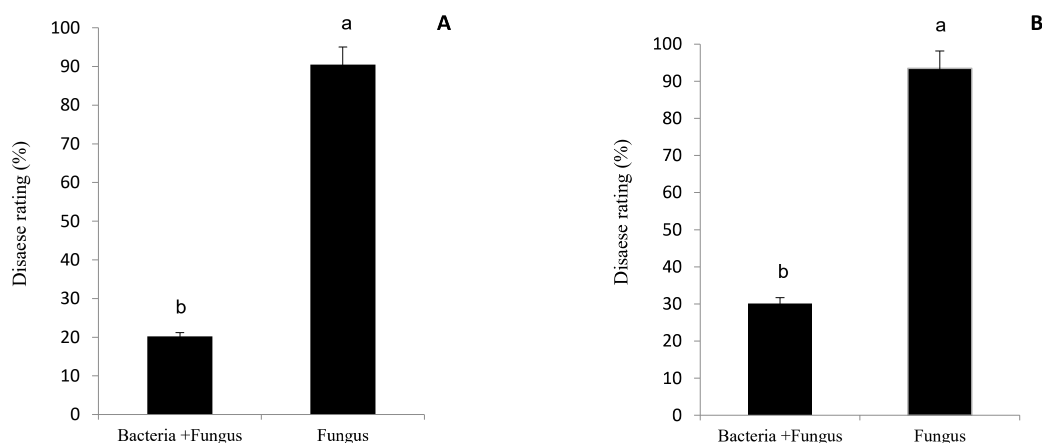

Fig. 2: *In vitro* (A) and pots (B) tests of barley reaction cv. WI2291 to Cochliobolus sativus isolate Pt4 after, 10 days of co-culture with Bacillus atrophaeus isolate SY15b. Error bars are representative of the standard deviation. Different letters on bar show significant difference at P = 0.001.

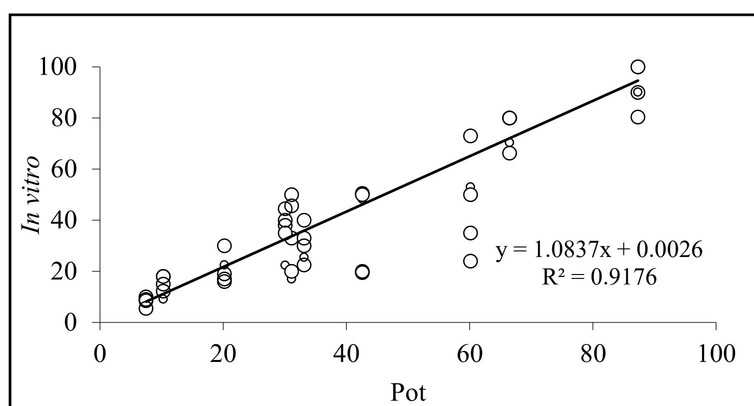

Fig. 3: Relationship between pot and *in vitro* co-culture for assessing the antagonistic activity of *Bacillus atrophaeus* against common root rot on barley susceptible cv. WI2291. Data points shown as (◦) represent many hidden observations.

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