Biological Control 71 (2014) 1–6

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

# Durable and broad-spectrum disease protection measure against airborne phytopathogenic fungi by using the detachment action of gelatinolytic bacteria

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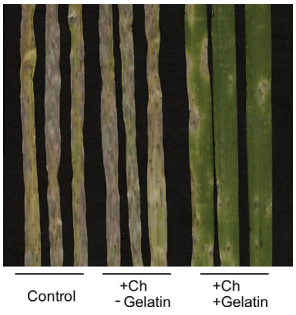
## HIGHLIGHTS

- The gelatinolytic *Chryseobacterium* sp. was most effective biocontrol agents as tested.
- Its protective effect was lost within 1 week because of incapable of settlement.
- The gelatin supplementation dramatically improved durability of disease protection.
- This disease protection measure was extended against other airborne pathogens.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 26 March 2013
Accepted 30 December 2013
Available online 8 January 2014

*Keywords:*
Adhesion
Extracellular matrix
Gelatin
*Magnaporthe oryzae*
*Alternaria alternata*
*Colletotrichum orbiculare*

## ABSTRACT

We had previously obtained collagenolytic/gelatinolytic bacteria, which degrade the fungal extracellular matrix, to establish a novel biological control measure that inhibits germling adhesion of airborne phytopathogenic fungi on the host plant surface. By using barley-*Magnaporthe oryzae* pathosystem, *Chryseobacterium* sp. was most effective biocontrol agents as tested. The selected bacteria were evaluated for durable disease protection against *M. oryzae* on barley leaves by using chloramphenicol-resistant mutants. *Chryseobacterium* sp. from the soil was less likely to settle on leaf surfaces. Therefore, we tried to manipulate *Chryseobacterium* sp. to inhabit the leaf's surface. The gelatin supplementation dramatically improved the settlement of gelatinolytic bacteria *Chryseobacterium* sp. from the soil, and the disease protection effect lasted for more than 2 weeks on barley. Moreover, exploitation of *Chryseobacterium* sp. for disease protection was extended against other airborne pathogens, *Alternaria alternata* Japanese pear pathotype on Japanese pear and *Colletotrichum orbiculare* on cucumber.



## 1. Introduction

Biological control of plant diseases has been investigated in numerous laboratories. Most approaches for biological control have focused primarily on soil-borne diseases (Sanford and Broadfoot, 1931; Kloepper and Schroth, 1979). Moreover, several successful studies that used biological control for protection against airborne diseases have been reported, e.g., chitinolytic activity of *Serratia marcescens* for rice blast disease (Someya et al., 2002) and antimicrobial compounds produced by *Bacillus licheniformis* for tomato gray mold disease (Lee et al., 2006). Most biological control agents protect against diseases by using antagonistic (i.e., killing) effects against pathogens. However, it is generally thought that biological control has some disadvantages (i.e., instability of the protection effect and difficulty in the establishment of the bio-control agents in the field) (Enkerli et al., 2004; Barratt et al., 2010). Numbers of successful reports were concurrent treatment with the pathogen and bio-control agents

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http://dx.doi.org/10.1016/j.biocontrol.2013.12.008

(Essghaier et al., 2009; Fukui et al., 1999; Inbar et al., 1996; Jones and Prusky, 2002; Ross et al., 2000; Shimoi et al., 2010).

We have been investigating a novel biological control measure that has a different protective mechanism against airborne diseases. A promising target for biological control is the inhibition of the attachment of the pathogen to the host plant surface. Adhesion of fungal spores to the host surface is considered a critical first step towards achieving infection (Deising et al., 1992; Braun and Howard, 1994). The spores of fungi secrete an extracellular matrix (ECM) as a factor in the adhesion. The ECM also offers a solid base for spores to penetrate the host cuticle. Consequently, inadequate adhesion of spores results in the failure of appressorium formation (Bae et al., 2007). The ECM of *Magnaporthe oryzae* is composed of animal cell adhesion factor-like compounds such as collagen, laminin, and fibronectin (Bae et al., 2007; Inoue et al., 2007). Inoue et al. (2007) have demonstrated that collagenase and gelatinase (glycoprotein-degrading enzymes) had an inhibitory effect on the spore adhesion of *M. oryzae* and suppressed disease occurrence. Therefore, we successfully screened collagenolytic/gelatinolytic bacteria from rice leaves and soil, and these selected bacteria suppressed disease occurrence in the concurrent treatment with the pathogen and selected bacteria (Shimoi et al., 2010).

In this study, we evaluated the durability and broad-spectrum of the disease protection effects of the selected bacteria. We used the barley-*M. oryzae* pathosystem. When compared with the growth of rice, the growth of barley is fast and insusceptible to light conditions. It was difficult to evaluate durability using rice-*M. oryzae* pathosystem because disease indices showed unstable between individual trials and were unable to compare them. The barley-*M. oryzae* pathosystem is promising to obtain reliable and comparable results.

We found that the disease protection effects of the selected bacteria decreased with time after bacterial treatment because of the selected soil bacterium species, *Chryseobacterium* sp., was quickly lost after 1 week of pretreatment. Therefore, we tried to modify the bacterial treatment of the plant's surface by using *Chryseobacterium* sp. (the most effective bacteria in our study, even though its survival on leaves was quite weak). Supplementation with spreading agents resulted in a suitable environment to establish the colonization of bio-control bacteria. We also evaluated the hypothesis that the disease protection effect could have applications for other pathosystems, such as black spot disease (*Alternaria alternata* Japanese pear pathotype) and anthracnose disease (*Colletotrichum orbiculare*). These pathosystems use different infection strategies (i.e., while *M. oryzae* and *C. orbiculare* are hemibiotrophic pathogens, *A. alternata* is a facultative saprophyte pathogen). On the basis of the results of this study, we established a durable and broad-spectrum disease protection measure against fungi dispersed via spores by using the detachment action of gelatinolytic bacteria.

## 2. Materials and methods

### 2.1. Gelatinolytic bacteria

The gelatinolytic bacteria *Chryseobacterium* sp., *Pseudomonas geniculata*, *Sphingomonas* sp., and *Acidovorax* sp. were described in our previous study (Shimoi et al., 2010). The bacteria were stored in skim milk medium at −80 °C. In the case of bacterial treatment, the stored bacteria were propagated in 3 ml of nutrient broth (NB) (Becton Dickinson and Company, NJ) at 26 °C for 1 day. The number of bacterial cells was counted using a spectrophotometer and adjusted to $5.6 \times 10^8$ cfu/ml.

### 2.2. Inoculation test on barley leaves

Blast fungus, *M. oryzae* isolate Guy11, was grown on oatmeal agar medium (i.e., 20 g of oatmeal [The Quaker Oats Company, Chicago, Ill. USA], 7.5 g of agar, 2.5 g of sucrose, and 500 ml of distilled water) and incubated at 25 °C for 7 days. The surfaces of aerial mycelia were rubbed with a spatula. The rubbed mycelia were exposed to near ultraviolet light (360 nm, 40 W) at 25 °C for 4 days to promote sporulation. Sterile, distilled water was then added to the spore-containing medium, and the fungal surfaces were rubbed with a spatula again. Subsequently, the spore suspension was filtered through tissue paper (Kimwipe S-200, Cresia Corp., Tokyo, Japan). Barley (*Hordeum vulgare* 'Nigrate', which is susceptible to *M. oryzae* Guy11, was used as the host plant. Seven barley seeds were sown per pot (7 × 15 cm) and incubated at 21 °C for 9 days. After that, the barley seedlings were inoculated with 20 ml of the spore suspension ($10^5$ spores/ml) and 1 ml of the bacterial culture. In the case of pretreatment with bacteria, 20 ml of distilled water, 1 ml of bacterial culture, and 0.001% (w/v) of Tween 20 were sprayed on the barley seedlings. After incubation at various time intervals (i.e., 0, 12, 24, and 48 h; 1, 3, 7, and 14 days), the seedlings were inoculated with 20 ml of the spore suspension ($10^5$ spores/ml) and 0.001% (w/v) of Tween 20. The inoculated seedlings were maintained at 23 °C under high moisture conditions overnight and subsequently transferred to a climate chamber. Finally, the disease index was calculated 5 days after the inoculation. The disease index was defined as follows: 0: no lesion; 1: small brown spots; 2: lesion area < 10%; 3: 10% ⩽ lesion area < 30%; 4: 30% ⩽ lesion area < 50%; 5: 50% ⩽ lesion area < 70%; 6: lesion area ⩾ 70%. The experiment was conducted in triplicate.

### 2.3. Transformation with chloramphenicol resistant gene

To detect the treated bacteria on the barley leaves, we introduced an antibiotic resistance gene into each bacterial strain (i.e., *Chryseobacterium* sp. [from soil], *Sphingomonas* sp. [from leaves], and *Escherichia coli* [non-adaptive bacteria]). We examined several antibiotics to inhibit bacterial growth. All the bacterial strains were sensitive to chloramphenicol. We used the plasmid pBT-LGF2 (Agilent Technologies, LaJolla, CA, USA) to introduce the chloramphenicol resistance gene. Each bacterium was propagated in NB medium to make competent cells for electroporation. The centrifuged bacterial cells were suspended in 10% (v/v) glycerol. The competent cell was added to 200 ng of pBT-LGF2 and transferred to an ice-cooled cuvette (BEX Co., Ltd., Tokyo, Japan). Electroporation was performed under the following conditions: 2.5 kV, 300 Ω, and 25 μF. After the reaction, the bacterial cells were transferred to 1 ml of SOC medium (20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 0.2 ml of 5 N NaOH, 20 ml of 1 M glucose, 10 ml of 1 M $MgCl_2$, 10 ml of 1 M $MgSO_4$/l) at 30 °C for 1 h and spread onto a Luria broth agar (LBA) plate (1% tryptone, 0.5% yeast extract, and 1% NaCl [w/v]) containing 50 μg/ml of chloramphenicol.

### 2.4. Genomic DNA extraction from the bacterial isolates and DNA manipulations

Bacterial genomic DNA extraction and ribosomal DNA (rDNA) sequencing were performed to identify bacterial species as previously described in Shimoi et al. (2010). To detect rDNA polymorphisms, rDNA fragments amplified using PCR with a forward (Fw) and reverse (Rv) primer (i.e., Fw: 5′-CAGGCCTAACACATGCAAGTC-3′, Rv: 5′-GGGCGGWGTGTACAAGGC-3′, where W is A or T) were digested with *Msp*I (Takara, Ohtsu, Japan) and electrophoresed in 1.5% agarose gel in TAE buffer (40 mM Tris–acetate pH 8.0, 1 mM EDTA). To detect chloramphenicol resistant mutants, the multi-cloning site of pBT-LGF2 was amplified