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Survival of the biocontrol agents *Brevibacillus brevis* ZJY-1 and *Bacillus subtilis* ZJY-116 on the spikes of barley in the field[*]

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Abstract: *Fusarium* head blight (FHB) caused by *Fusarium graminearum* is a devastating disease that results in extensive yield losses to wheat and barley. A green fluorescent protein (GFP) expressing plasmid pRP22-GFP was constructed for monitoring the colonization of two biocontrol agents, *Brevibacillus brevis* ZJY-1 and *Bacillus subtilis* ZJY-116, on the spikes of barley and their effect on suppression of FHB. Survival and colonization of the *Brevibacillus brevis* ZJY-1 and *Bacillus subtilis* ZJY-116 strains on spikes of barley were observed by tracking the bacterial transformants with GFP expression. Our field study revealed that plasmid pRP22-GFP was stably maintained in the bacterial strains without selective pressure. The retrieved GFP-tagged strains showed that the bacterial population fluctuation accorded with that of the rain events. Furthermore, both biocontrol strains gave significant protection against FHB on spikes of barley in fields. The greater suppression of barley FHB disease was resulted from the treatment of barley spikes with biocontrol agents before inoculation with *F. graminearum*.

Key words: GFP, Survival, *Brevibacillus brevis* and *Bacillus subtilis*, Spikes, Barley, Biocontrol
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INTRODUCTION

Soil-borne pathogens, including *Pythium* spp. and *Fusarium* spp., cause significant yield losses in horticulture and agriculture crops (Mao *et al*., 1997). Current practices for controlling plant diseases are based largely on disease resistant crops, cultivation management in fields and application of synthetic pesticides (Elizabeth and Emmert, 1999). Biological control using antagonistic microbes to reduce the use of chemical pesticides in a system of integrated plant disease management, offers a powerful alternative to control plant diseases.

Biocontrol of soil-borne pathogens depends on the successful establishment and survival of microbial inocula. While biocontrol agents are often effective in the laboratory, the level of control achieved in the field is sometimes unpredictable. Some of these failures can be attributed to inadequate establishment and survival of microbial inocula (O'Callaghan *et al*., 2001). To assess survival of biocontrol agents in fields, methods to detect the released strains and distinguish them from indigenous bacteria are needed. Classical studies of population dynamics of beneficial bacteria introduced into the environment have depended upon detection of culturable cells (Araujo *et*

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al., 1994; Boelens *et al*., 1994). To distinguish the introduced cells from the indigenous population, the former has generally been tagged with a traceable marker (Kragelund *et al*., 1995). Antibiotic resistance markers have so far almost always been relied on (Chao *et al*., 1986; Wilson, 1995; Nautiyal *et al*., 2002), but the value of this approach is limited by the fact that selective plating provides little information on the localization and distribution of the marked strains in the environmental samples (Scott *et al*., 1998). The green fluorescent protein (GFP) reporter has recently been found to be a potentially valuable tool for tracking the survival (Ramos *et al*., 2002) and localization of microbial cells (Xi *et al*., 1999) and for monitoring gene expression in micro-niches (Lewis and Marston, 1999). The *gfp* gene can be identified by noninvasive methods. Neither substrates, other enzymes nor co-factors are required for detection of GFP (Douka *et al*., 2001). In addition, mature GFP is extremely pH (pH 6~10) and temperature tolerant (up to 60 °C).

Fusarum head blight (FHB), primarily caused by *F. graminearum*, is a devastating disease that results in extensive yield and quality losses to wheat and barley throughout the world (Urrea *et al*., 2002; Capettini *et al*., 2003). After infection, *Fusarium* mycotoxins are accumulated and the products of these crops are harmful to both people and animal stock (Ingalls, 1996; Onji *et al*., 1998; Placinta *et al*., 1999). Among the pathogens, *F. graminearum* is the most common *Fusarium* species producing deoxynivalenol (DON) (Olsson *et al*., 2002). Two biocontrol agents (BCAs) which were isolated in our laboratory inhibited *F. graminearum* growth significantly in laboratory assays. The two strains were identified as *Bre vibacillus brevis* and *Bacillus subtilis* respectively by the methods of Klement *et al*.(1990), combined with 16 srDNA sequence analysis (GenBank accession number is AY897210 and AY897211, respectively). In order to track the biocontrol strains on the spikes of barley, a plasmid pRP22-GFP based on the pGFPuv replicon constitutively expressing the *gfp* gene was constructed. This paper described the construction of a broad-host-range plasmid expressing the *gfp* gene. Its use in survival and colonization studies of two biocontrol agents, *B. brevis* ZJY-1 and *B. subtilis* ZJY-116 on spikes of barley was demonstrated. In addition, the effect of disease suppression of two FHB biocontrol agents of barley in fields was also investigated.

MATERIALS AND METHODS

Plasmid construction

The vector pRP22, an *E. coli-B. subtilis* shuttle plasmid was kindly provided by Professor Chen, Jiangsu Academy of Agriculture Science. The plasmid (pRP22-GFP) carrying the *gfp* gene was constructed by the following strategy. The *xylR* gene and the *xylA* promoter were amplified as a single product from *B. subtilis* 168 chromosomal DNA using the primers Pr1 and Pr2. Gene *GFPuv* was amplified from the plasmid pGFPuv using the primers Pr3 and Pr4. The above PCR products were digested with *Hin*d III and ligated together. The resulting ligation product was reamplified with primers Pr1 and Pr4 and inserted into the *Sph*I-*Kpn*I site of plasmid pRP22 (Table 1).

Table 1 Strains, plasmids and DNA primers used in the study

Strains	Relevant characteristics	Antibiotic resistance	Reference/source
B. subtilis 168	Contains *xy1R* gene and *xy1A* promoter		This study
E. coli TG1			This study
Vectors			
pGFPuv	*gfp* gene	Amp^r	Clontech
pRP22	Shuttle vector	Cm^r Amp^r	Zhi-Yi Cheng
Oligo-nucleotide	Sequence		Restriction site(s)
Pr1	5′-CACATGCATGCCATGTCACTGTTGCTTCAG-3′		*Sph*I
Pr2	5′-CGGTACCCATAAGCTTGTGATTTCCCCCTTA-3′		*Kpn*I/ *Hin*d III
Pr3	5′-GCGCAAGCTTATGAGTAAAGGAGAAGAA-3′		*Hin*d III
Pr4	5′-GGGGTACCATTATTTTTGACACCAGA-3′		*Kpn*I

Underlining indicates *Sph*I (GCATGC), *Kpn*I (GGTACC), *Hin*d III (AAGCTT) restriction sites

Plasmid transformation

The plasmid pRP22-GFP was transformed into the two biocontrol agents, *B. brevis* ZJY-1 and *B. subtilis* ZJY-116, by the methods described in Chang and Cohen (1979) with slight modifications. Media and solutions compositions were as follows: SMM buffer consisting of 0.5 mol/L sucrose, 0.02 mol/L maleate and 0.02 mol/L $MgCl_2$, pH 6.5 adjusted with NaOH. SMMP medium was prepared by mixing equal volumes of fourfold strength Penassay broth (Difco) and double strength SMM. PEG solution (40%, *w*/*V*) containing 40 g PEG (Sigma, approx. MW 6000) and 50 ml double strength SMM buffer in 100 ml. Luria-Bertain (LB) broth containing Tryptone 10 g, yeast extract 5 g, NaCl 10 g in 1 L, adjusted pH 7.5 with NaOH. The DM3 regeneration medium consisted of the following sterile solutions (per liter: 200 ml 4% agar, 500 ml 1 mol/L sodium succinate (pH 7.3), 100 ml 5% Difco Casamino acids, 50 ml 10% Difco yeast extract, 100 ml 3.5% K_2HPO_4 and 1.5% KH_2PO_4, 25 ml 20% glucose, 20 ml 1 mol/L $MgCl_2$, and 5 ml filter-sterilized 2% bovine serum albumin (added to the mixture when the temperature was ~55 °C).

Mid-log phase cell cultures, freshly grown in LB at 37 °C to $A_{600\ nm}$ 0.6, were harvested by centrifugation (2000 r/min) for 10 min with a 10 ml Eppendorf tube and re-suspended in a 500 µl volume of SMMP solution. Four µl lysozyme (50 mg/ml) was added into the suspension and the suspension was incubated at 37 °C with gentle shaking (100 r/min) for about 2 h. Cells were then harvested by centrifugating the suspension at 2000 r/min for 10 min, washed once by resuspending them gently in SMMP and pelleted a second time. The washed protoplast was brought to 500 µl volume by adding SMMP. After mixing completely, 1×10^{-6}~5 µg plasmid DNA in 50 µl TE buffer (10 mmol/L Tris-HCl, 1 mmol/l EDTA, pH 8.0) was mixed immediately with an equal volume of double strength SMM, followed by 1.5 ml 40% (*w*/*V*) PEG. After 2 min, 5 ml SMMP medium was added to the mixture to dilute the PEG, and protoplasts were recovered by centrifugation for 10 min at 2000 r/min. The treated protoplasts were re-suspended in 1 ml SMMP and incubated for 2 h at 30 °C on shaker (100 r/min) to enable phenotypic expression of genetic determinants carried by the plasmid before plating them onto chloramphenicol (C_m) amended (3×10^{-6} g/L) DM3 media for direct selection of transformants. The final fluorescent colonies were obtained by incubation of protoplasts on the DM3 regeneration plates at 37 °C for 2 d.

Plasmid stability

To determine the stability of the pRP22-GFP, transconjugants of two BCA were grown in liquid LB medium for several generations. Plasmid loss under these conditions was determined by plating onto solid LB medium with and without selective pressure (C_m) and assaying for the GFP phenotypes. The number of colony forming units (CFU) was determined by the Miles and Misra drop count method on solid agar medium (Somasegaran and Hoben, 1994). The number of generations was determined by using the equation (Miller, 1972): $n=[\ln(N_t)-\ln(N_0)]/(\ln2)$ where n is the number of generations, N_t is CFU of the culture at final time (before each successive dilution) and N_0 is the CFU at zero time of the culture (after each successive dilution).

Pathogen culture and inoculation

The pathogen *F. graminearum* was isolated from diseased spikes of barley on the farm of Zhejiang University, Hangzhou, China. For experiment use, the pathogen was grown on potato dextrose agar (PDA) at 28 °C for 5 d and the resulting mycelial plugs were inoculated into 500 ml flasks containing 250 ml of sterilized Charles culture medium with the following compositions (per liter: 2 g KNO_3, 0.5 g KCl, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.1 g $FeSO_4$, 30 g Sucrose). *F. graminearum* spores were formed by incubation in flasks at 25 °C on a rotary shaker (220 r/min) for 7 d. The spore filtrate was obtained by filtration of the culture medium with double layer sterile gauzes. The spores were counted using hemacytometer under a light microscope and then diluted them to an ultimate sporule of 1×10^6 CFU/ml. The resulting suspension was ready for spraying on spikes in the field during mid-anthesis (50% of the flowering barley).

Population dynamics of the biocontrol agents in the barley spikes

The two transformed strains, of *B. brevis* ZJY-1 and *B. subtilis* ZJY-116, were streaked onto LB plate containing 3×10^{-6} g/L C_m(LB+C_m). After one day incubation at 37 °C, a single colony was used to in-

oculate sterile LB (250 ml) into a 500 ml flask which was then incubated overnight on a shaker (200 r/min) at 30 °C. Samples of cultures were centrifuged at 3000 r/min for 15 min at 4 °C, the cells were then resuspended in sterile distilled water (SDW). Bacterial suspensions of *B. brevis* ZJY-1 and *B. subtilis* ZJY-116 were adjusted to 6.8×10^7 CFU/ml and 7.4×10^7 CFU/ml respectively before inoculation onto barley spikes.

The experiments were conducted at the farm of Zhejiang University in Hangzhou, China. In order to study whether the existence of the pathogen affects the population dynamics of *B. brevis* ZJY-1 and *B. subtilis* ZJY-116 in barley spikes, during the mid-anthesis of barley, the suspension of the two GFP-tagged biocontrol agents was sprayed onto spikes that has been inoculated with *F. graminearum* or SDW the day before application of these biocontrol agents. Two groups of treatments (barley spikes inoculated with either *F. graminearum* or SDW), three replicates with a total of 6 plots (1 m^2 per plot) for each bacteria.

Sampling and statistical analysis

After inoculation, sampling was carried out at intervals of 1, 4, 7, 10 d… until the barley could be harvested. Ten grains were harvested each time at random from the spikes of each replicate, a total of 30 grains were removed from each treatment aseptically and placed in SDW (30 ml) in a 150 ml flask, shaken by hand for 5 min and left to stand for 10 min. An SDW dilution series was set up (0.1 ml to 0.9 ml), and 0.1 ml serial dilutions were plated onto LB+C_m plates incubated at 37 °C for about 24 h. Then the colony forming units (CFU) with obvious fluorescence were counted under ultraviolet (UV) light. To understand the final distribution of GFP-tagged strains on spikes when the spikes matured, the husks, awns and kernels from 30 harvested grains of each treatment were aseptically detached with sterile nipper and placed in SDW (30 ml) respectively. The introduced strains from every part were obtained and counted using the previously described methods.

Biocontrol effects of two biocontrol agents

The experiment was conducted in 2 m×1 m plots at the same farm. Survival test was conducted in November 2003. The barley seeds (variety: MOREX) were planted in the field with conventional patten. During the mid-anthesis (April, 2004), 5 L spore concentration of *F. graminearum* was sprayed onto the spikes until runoff. To study the optimal time to apply *B. brevis* ZJY-1 and *B. subtilis* ZJY-116 in fields, 1.5 L biocontrol agents of the same concentration as that used in the survival test were inoculated the day before onto the pathogen-sprayed spikes, and after being inoculated respectively. There were three controls designed for this study: (1) healthy control−the spikes treated with SDW only; (2) infected control−the spikes sprayed with pathogen *F. graminearum*; and (3) chemical control−the spikes previously sprayed with *F. graminearum* and treated with 0.75 L carbendazim (1 mg/ml) a day later. The experiments were randomly designed with two treatment groups (the biocontrol agents inoculated before and after the spikes were sprayed with *F. graminearum*), three replicates of each group, three replicates of each control, resulting in a total of 21 plots.

Sampling and statistical analysis

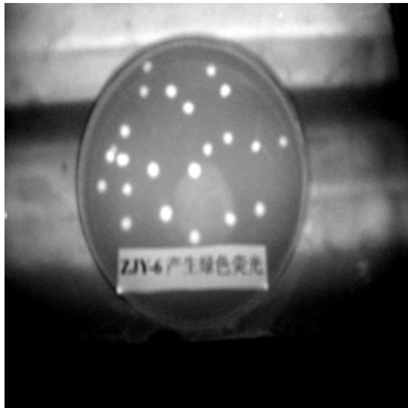
When the spikes ripened, 20 spikes from each plot were randomly collected. Infection percentage of the spikes infected by fungi was calculated as follows: Infection (%)=(number of grains infected by a fungus /total number of grains)×100%. The mean infection percentage and disease suppression were respectively calculated. Statistical analyses were conducted using the general linear models procedures of the Statistical Analysis System version 6.08 (SAS Institute, Inc., Cary, NC). Experiments results were analyzed using standard analysis of variance (ANOVA) and results were separated by the LSD test at P=0.05.

RESULTS

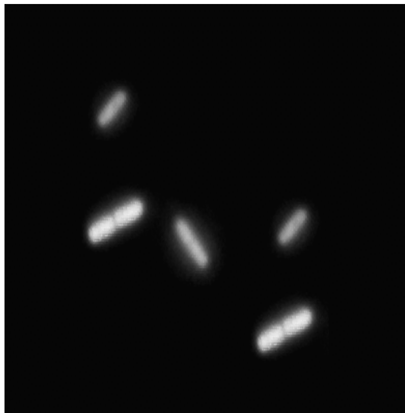
Plasmid construction and strains transformation

The vector pRP22-GFP constructed in this work was an *E. coli-B. subtilis* shuttle plasmid carrying *gfp*, chloramphenicol and ampicillin resistance genes. The transformants of *B. brevis* ZJY-1 and *B. subtilis* ZJY-116, designated as ZJY-1G and ZJY-116G, were successfully obtained using bacterial protoplasts as transformants. Sixty percent of the transformants of each strains regenerated cell walls. The GFP fluo-

rescence from all transformants was easily observed under UV radiation by fluorescence microscopy (Fig.1a and Fig.1b). The high levels of expression of fluorescence in the present constructs make them suitable for ecological studies even under normal field environments.



(a)



(b)

Fig.1 (a) Fluorescent colonies of ZJY-116G observed on LB agar plates under UV irradiation; (b) Fluorescence microscopy showing ZJY-116G cells harboring the plasmid pRP22-GFP

Stability of pRP22-GFP plasmid in BCAs

Studies showed that after 50 generations in the absence of antibiotic pressure, 55.3% strain ZJY-1G cells and 47.6% strain ZJY-116G cells were C_m resistant and showed GFP fluorescence.

Survival of GFP-tagged strains on spikes

The behaviour of the marked strain on both infected and healthy spikes was broadly similar. Although the number of ZJY-1G cells on healthy spikes showed a small increase a day after inoculation, the number steadily decreased in the first 13 d in both infected and healthy spikes, from 6.8×10^7 CFU/ml initially to 4×10^5 CFU/grain on infected spikes and 1×10^5 CFU/grain on healthy spikes, then the population began to recover gradually and the dynamics of introduced strains was closely related to the rain events in the field. Fig.2 showed that each period of rain inevitably reduced the number of marked agents and that heavier rain led to fewer surviving strains. For example, a heaviest rainfall (25 mm) corresponded with a lowest population on both infected and healthy spikes 25 d after inoculation.

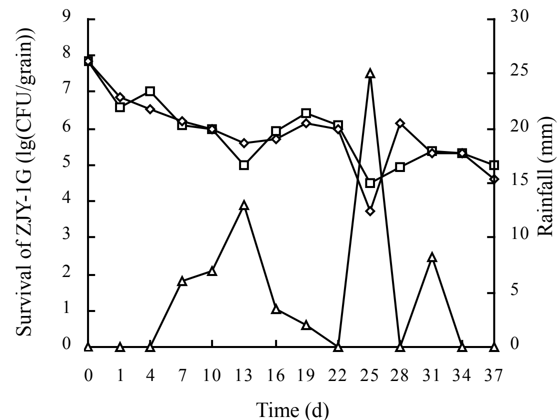


Fig.2 Survival of ZJY-1G on both infected (◇) and healthy (□) barley spikes in the field. Rainfall was computed based on the two-day accumulation of rain before each sampling (△)

Compared with ZJY-1G, smaller population fluctuation was observed in ZJY-116G on both infected and healthy spikes. In addition, a higher number of ZJY-116G cells was observed on healthy spikes than on *Fusarium* infected spikes throughout the experiments (Fig.3). Applied at suspension of 7.4×10^7 CFU/ml, strain ZJY-116G decreased steadily to 1×10^5 CFU/grain on *Fusarium* infected spikes and 2.9×10^5 CFU/grain on healthy spikes 13 d later, followed by a gradual increase, then the trend resembled the survival seen in the strain ZJY-1G.

Comparison of the distribution of these two GFP-tagged strains on the barley spikes showed that the inoculated strains were mostly distributed on the husks with the least amount distributed on the kernels (Table 2).

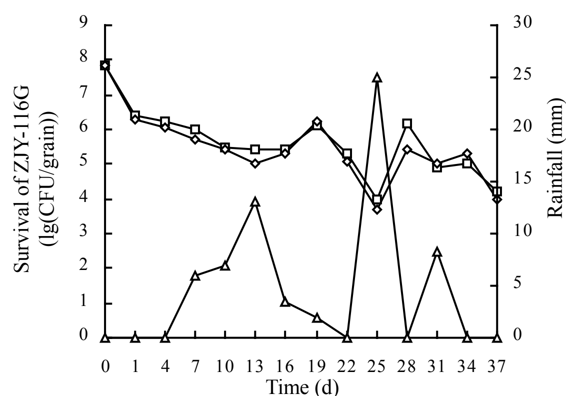


Fig.3 Survival of ZJY-116G on both infected (◇) and healthy (□) barley spikes in the field. Rainfall was computed based on the two-day accumulation of rain before each sampling (△)

The biocontrol effects of two biocontrol agents

Typical symptom of FHB developed about 10 d after inoculation with the suspension of *F. graminearum*. Some spikes took on a light brown colour in initial stages and brown grains resulted when the spikes matured, however, no visual infection was observed on the untreated control, the spikes treated with SDW only. All biocontrol agents treatments showed effective suppression of FHB as compared with the no-bacteria control treatment (Table 3). Greater disease suppression resulted from treatments when the biocontrol agents were sprayed before *F. graminearum* was inoculated.

DISCUSSION

A constitutively expressing *gfp* plasmid was constructed to monitor the colonization of two BCAs on barley spikes. The constructed plasmid was successfully transformed into strains *B. brevis* ZJY-1 and *B. subtilis* ZJY-116. GFP fluorescence from bacterial transformants was easily observed under UV radiation by fluorescence microscopy. Although the pRP22-GFP replicon was not very stably maintained in the two BCAs, and only 55.3% of strain ZJY-1G cells and 47.6% of strain ZJY-116G cells were C_m resistant and showed GFP fluorescence under laboratory condition, significant stability of the plasmid was achieved in the field. The abundance of GFP-tagged bacteria colonizing the spikes after 37 d of inoculation confirmed this. The reason for this may be that the nutrition in medium is more abundant than that in spikes for strains to grow, so the generation time in the medium is shorter than that in spikes in the field and the pRP22-GFP plasmid can be maintained a long time in the BCAs.

Tracking the bacterial strains introduced on the barley spikes revealed that the two strains showed good survival on both infected and healthy spikes in the field. When the biocontrol agents were inoculated during mid-anthesis, these bacterial strains still maintained a numerical level ($\times10^5$ CFU/grain of barley)

Table 2 The distribution of GFP-tagged bacterial strains on the spikes during harvesting in fields

Strains	Part of spike					
	Kernel		Crust		Awn	
	G[a]	T[b]	G	T	G	T
ZJY-1	1.01×10^4	1.00×10^8	2.16×10^5	6.72×10^7	5.60×10^4	3.80×10^7
ZJY-116	1.21×10^4	7.98×10^7	4.32×10^5	1.33×10^8	5.40×10^4	3.80×10^7

[a]G: The number of GFP-tagged bacterial strains (CFU/grain); [b]T: The number of total bacteria (CFU/grain)

Table 3 Disease protection induced by two biocontrol agents in the barley field

Treatment	Mean percentage of disease (%)	Mean percentage of disease suppression (%)
^{Pre}ZJY-1	16.01	58.60 A
^{Post} ZJY-1	17.50	54.72 B
^{Pre} ZJY-116	18.46	52.23 B
^{post} ZJY-116	21.20	45.15 C
Infected control	38.65	0.00 D
Chemical control	18.00	53.40 B
Healthy control	0.00	/

^{pre}: Biocontrol agents were sprayed a day before the spikes were inoculated with *F. graminearum*; ^{post}: Biocontrol agents were sprayed a day after the spikes were inoculated by *F. graminearum*; Treatments with different letters are significantly different according to the protected least significant difference (LSD) test at *P*=0.05 using the models procedure in SAS. Healthy control: The spikes were treated with sterilized water only; Infected control: The spikes were sprayed with pathogen *F. graminearum*; Chemical control: The spikes were previously sprayed with *F. graminearum* and treated with carbendazim a day later

that could be detected until the spikes matured. Although the cells applied on the spikes were initially vegetative, Bennett *et al*.(2003)'s study showed that introduced organism might survive as spores in the environment afterwards. Presumably, the strains' extensive adaptability to the environment is due to their ability of producing heat- and desiccation-resistant spores. Results from the field tests also suggested that on *Fusarium* infected spikes, the pathogen may compete with BCAs for micro-niches and nutrition. To obtain a higher population of the biocontrol bacteria on barley spikes, *B. subtilis*, and *B. brevi*s should be sprayed on the barley spikes before *Fusarium* infection. Fig.2 and Fig.3 showed that the number of the two bacterial strains can be dramatically affected by rain events. Each period of rain caused a decrease in the population of the introduced strains, although the cell number recovered several days later.

This study on the distribution of GFP-tagged strains on barley spikes showed that the inoculated strains were mostly distributed on the husks, least on the kernel, even though the number of total indigenous microorganisms was highest on the kernels and lowest on the awns, probably because the dominant indigenous bacteria's occupation of space and assimilation of nutrition inhibit the biocontrol agents from colonizing the kernel.

A better biocontrol effect was obtained when barley spikes were sprayed with biocontrol agents before *F. graminearum* inoculation. Our results indicated that these biocontrol strains might compete for space and resources with the fugal pathogen, which inhibit fungal establishment on barley spikes.

Both biocontrol agents strongly inhibited colonial growth of *F. graminearum* and produced clear inhibition zones in vitro. In the field, both strains induced significant disease suppression. One feature involved in suppressing disease might be that the biocontrol agents released secondary metabolites that inhibited the growth of *F. graminearum*. Although production of secondary metabolites is most often associated with fungal suppression by fluorescent *Pseudomonas* (Handelsman and Stabb, 1996; Mavrodi *et al*., 2000; Thomashow and Weller, 1988), the reports on the sporulating gram-positive bacteria producing antifungal compounds are not rare (Yu *et al*., 2002; Cho *et al*., 2003; Kim and Chung, 2004). *B. brevis* is now well established as a biocontrol agent in Europe showing efficacy against Botrytis and powdery mildew disease (Edwards and Seddon, 1992). By comparing the activity of *B. brevis* Nagano against *Botrytis cinerea* with that of pure gramicidin S and the antibiotic-negative mutant *B. brevis* E-1, Edwards and Seddon (2001) showed that the mode of antagonism exhibited was antibiosis due to the presence of gramicidin S. There are some other antibiotics (for example tyrocidins and gramicidin D) reported be produced by *B. brevis* (Saito *et al*., 1970), so further studies are required to identify the metabolites produced by the two bacteria strains and their effects on biocontrol. Under natural conditions, climatic conditions fluctuate naturally, Studies are also needed to explore how these factors influence the survival and the biocontrol effects of these two bacterial strains.

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