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# Biological Control of Tea Anthracnose Using an Antagonistic Bacterium of *Bacillus subtilis* Isolated from Tea Leaves

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**An antagonistic bacterium of *Bacillus subtilis* BD0310 against *Colletotrichum theae-sinensis* was isolated from the phylloplane of tea trees at a tea plantation in Korea. SC (suspension concentrate)-type biofungicide was formulated with the antagonist. Cell viability and antifungal activity of *B. subtilis* were maintained in the formulation more than 12 months at room temperature. The antagonist was sensitive only to copper sulfate among the chemical pesticides currently registered for tea trees in Korea. Greenhouse application demonstrated that the biofungicide controlled more effectively the disease in a protective mode than in a curative mode. Field trial showed that alternate applications of the biofungicide and chemical fungicide were more effective in controlling tea anthracnose than single application of the biofungicide or chemical fungicide with less use of chemicals. This study suggests that the biofungicide of *B. subtilis* BD0310 is an effective method for biological control of anthracnose in tea plantations.**

***Keywords*** : anthracnose, *Bacillus subtilis*, biocontrol, *Colletotrichum theae-sinensis*, tea

Tea is the most popular healthy beverage across the world only next to water. Due to a functional beverage, tea consumption remarkably increased in Korea during the last few years and tea plantations significantly enlarged across the southern coastal area of Korea (Shin et al., 2000). Because tea is manufactured from young shoots of tea trees, foliar diseases are of great concern in tea production. The bush of tea trees facilitates annual outbreak of the foliar diseases and their development during the growing season. Among them, anthracnose caused by *Colletotrichum theae-sinensis* and gray blight caused by *Pestalotiopsis longiseta* and *P. theae* are the economically important diseases in tea production (Koh et al., 2001; Park, 1995; Park et al., 1996; Shin et al., 1999). Fungicide protection is the primary strategy in the control of tea diseases in Korea (Shin et al., 2000). However, extensive use of fungicides has led serious side effects such as development of resistance in pathogen populations and negative consequences for human health and the environment (Horikawa, 1986, 1987; Oniki et al., 1985, 1986). Moreover, food safety of tea beverage became a great concern of consumers due to pesticide residuals on tea leaves. Biological control has been developed as an alternative to synthetic pesticide treatment (Janisiewicz and Korsten, 2002). A variety of microbial antagonists and their metabolites have been reported to control several different pathogens on various fruit and vegetables (Fravel, 2005), but only little information is available on the biological control of tea foliar diseases (Sanjay et al., 2008; Saravanakumar et al., 2007). In our previous study, an antagonistic bacterium of *Bacillus subtilis* BD0310 isolated from tea leaves effectively inhibited fungal growth of anthracnose pathogens and a novel biofungicide was successfully formulated with use of the antagonist (Oh et al., 2005; Kim et al., 2006). The present study was attempted to evaluate the biofungicide performance against tea anthracnose in the field. Alternate use of the biofungicide and chemical fungicide was also discussed to develop environmental-friendly control measures for tea anthracnose in tea plantations.

The antagonistic bacterium of *B. subtilis* BD0310 against *C. theae-sinensis* was also isolated from tea leaves (Oh et al., 2005) and deposited to KCCM (Korean Culture Center of Microorganisms) (No. KCCM 10640). Sensitivity of the antagonist was evaluated to 12 different fungicides and insecticides, which are currently registered for pest control of tea trees in Korea (Korea Crop Protection Association, 2007). The pesticides were diluted according to manufacture's instruction and dripped on paper discs (100 μl on 6 mm in diam.). The discs were placed on nutrient agar plates where bacterial suspension of *B. subtilis* BD0310 (100 μl) was smeared. The plates were incubated at 25°C incubator and clear zones were determined 3 days after inoculation. The biofungicide was prepared by making a suspension of

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*B. subtilis* ($1.0-1.6\times10^{10}$ CFU/ml) in sterilized distilled water and adding starch (4 g/L), skim milk powder (5 g/L), dextrose (20 g/L), 0.9% (w/v) NaCl, 1% (v/v) Tween 20 and 0.01% (w/v) ascorbic acid to the bacterial suspension (Kim et al., 2008). The mixture was homogenized by shaking at 150 rpm for 10 min. Cell viability of the antagonistic bacterium was determined by recovering *B. subtilis* BD0310 from the biofungicide every 2 month during 1 year storage at room temperature. The recovered cells were tested for antifungal activity against mycelial growth of *C. theae-sinensis* by dual-culture on the combined medium of PDA and NA (1:1 v/v) at 25°C.

Pot experiments were carried out to evaluate the control efficacy of the biofungicide against tea anthracnose in a glasshouse. The experiment was established in a randomized complete block design with three replicates. Each treatment has five pots in each replicate block. A seedling of 3 year-old tea plant of 'Yabukita', Japanese variety, was planted in each pot and acclimated in the same glasshouse until the experiment. Two or three leaves of young shoots were wounded by a sterilized scissor and then inoculated with the fungal pathogen by spraying the conidial suspension ($3.6\times10^5$ conidia/ml) on tea leaves until runoff 2 days before or after chemical or the biofungicide treatment. The inoculated plants were maintained in a controlled room (25±1°C, 90% RH) for 2 days and then transferred into the glasshouse. Disease incidence and control efficacy were evaluated 7 days later. The biofungicide was diluted 100-fold concentration and chemical fungicide of triflumizole wp was diluted 2,000-fold concentration before use. Only inoculated plants and non-treated plants were employed as negative and positive control, respectively. A field trial was conducted with use of 'Yabukita' variety of tea plants from June 26, 2006 in a tea plantation at Jangheung, Jeonnam Province, Korea. Single or alternate application of the biofungicide and chemical fungicide of triflumizole wp was evaluated to determine optimum spray programs for the control of tea anthracnose with less use of chemicals. The experiment was established in a randomized complete block design with three replicates. Each treatment had tea plants covering $1\times1.5$ m$^2$ area in each replicate block. There were 5 treatments; 1) 4-time foliar spray of the biofungicide with 1 week intervals, 2) 6-time foliar spray of the biofungicide with 1 week intervals, 3) 4-time foliar alternate spray of the biofungicide and the chemical fungicide with 1 week intervals, 4) 4-time foliar spray of the chemical fungicide with 1 week intervals and 5) no spray as a control. The biofungicide was diluted 100-fold concentration ($10^8$ CFU/ml) and chemical fungicide of triflumizole wp was diluted 2,000-fold concentration before use. Disease incidence and control efficacy were evaluated 44 days after the first spray on August 9, 2006. Disease occurrence was calculated by the number of diseased leaves per total leaves in $20\times20$ cm$^2$ quadrate. Three quadrates were randomly placed in each replicate and total 9 quadrates were employed for each treatment. Data from the pot experiments and the field experiments were analyzed using analysis of variance (ANOVA) in a complete randomized design. Duncan's multiple range test was used to compare the means of the treatments in each experiment. All statistical analyses were conducted using SPSS software.

Among the tested 12 pesticides registered for tea trees in Korea, only copper sulfate showed inhibitory effect on the bacterial growth of *B. subtilis* BD0310 on nutrient agar. No clear zones were detected in other pesticides plate (Table 1). Application of chemicals and biofungicides together may

**Table 1.** Sensitivity of antagonistic bacterium *Bacillus subtilis* BD0310 against various pesticides registered for diseases and insect pests on tea plants

| Pesticides | Target pests | Formulation (%)[a] | Applied Conc. (ppm) | Sensitivity |
|---|---|---|---|---|
| Azoxystrobin | Gray blight | SC 20 | 100 | – |
| Copper sulfate | Anthracnose | WP 58 | 20 | + |
| Thiophanate-methyl | Anthracnose | WP 70 | 150 | – |
| Triflumizole | Anthracnose | WP 30 | 750 | – |
| *Basillus thuringiensis* subsp. *aizawai* | Smaller tea tortrix | WG 35,000 DBMU/mg | – | – |
| Chlorfenapyr | Tea red spider mite | SC 10 | 33.5 | – |
| Chlorfluazuron | Smaller tea tortrix | EC 5 | 25 | – |
| Fenpyroximate | Tea red spider mite | SC 5 | 25 | – |
| Flufenoxuron | Spider mite | DC 5 | 50 | – |
| Lambda cyhalothrin | Leaf miner | EC 1 | 10 | – |
| Methidathion | Wax scale | EC 50 | 400 | – |
| Tebufenpyrad | Tea red spider mite | WP 10 | 50 | – |

[a]SC, Suspension concentrate; WP, Wettable powder; EC, Emulsifiable concentrate; DC, Dispersible concentrate; WG, Water dispersible granule.
–, Insensitive; +, Sensitive.

**Table 2.** Recovery concentrations of antagonistic bacterium *Bacillus subtilis* BD0310 from the suspension concentrate formulation during 12 month storage at room temperature

| Storage duration | *Bacillus subtilis* BD0310 (cfu/ml) |
|---|---|
| 0 | $1.6 \times 10^{10}$ |
| 2 | $1.1 \times 10^{10}$ |
| 4 | $9.3 \times 10^{9}$ |
| 6 | $7.4 \times 10^{9}$ |
| 8 | $4.5 \times 10^{9}$ |
| 10 | $2.1 \times 10^{9}$ |
| 12 | $1.3 \times 10^{9}$ |

be a practical method for plant diseases control with less use of chemicals in the field. Simultaneous use of chemicals and biofungicides has never been attempted for disease control of tea plants. Thus, the information will be very useful to develop more effective spray programs with less use of chemicals during the growing period of tea plants.

SC (suspension concentrate)-type formulation of the antagonist was developed in this study. Because the biofungicide should be applied on tea leaves, SC-type formulation can avoid the residual problems of WP (Wettable powder)-type formation which is a common type formulation for many biofungicides, mainly against soil-borne pathogens (Hynes and Boyetchko, 2006). Recovered cells from the formulation showed that the biofungicide maintained relatively high concentrations of antagonistic bacterium even after 12 month storage at room temperature. Compared to initial concentration of $1.6\times10^{10}$ cfu/ml, about 10% of cells ($1.3\times10^{9}$ cfu/ml) were still viable at 12 month storage (Table 2). The recovered cells also maintained strong antifungal activity against *C. theae-sinensis in vitro* (data not shown). The biofungicide of SC-type formulation was proved to be very stable for the antagonist and to maintain strong antifungal activity for long storage period at room temperature.

The biofungicide and chemical fungicide effectively controlled tea anthracnose in glasshouse experiments (Table 3). Wounding inoculation of *C. theae-sinensis* caused severe disease incidence at the rate of 81.5% in control treatment. Chemical fungicide was more effective in controlling the disease than biofungicide. Two-day protective application of the biofungicide showed lower anthracnose incidence on tea leaves than its 2-day curative application. This was also true for chemical fungicide treatments. Control efficacy was calculated in the order of protective application of chemical fungicide (90.9%), protective application of biofungicide (77.3%), curative application of chemical fungicide (72.8%) and curative application of biofungicide (59.1%). This indicated that the biofungicide acted more likely in a protective mode than in a curative mode. Although control efficacy of the biofungicide was still lower than that of chemical fungicide of triflumizole wp, the biofungicide effectively controlled tea anthracnose with much higher than 60% of control efficacy, which level is defined to be the minimum requirement for biofungicide registration in Korea. Thus, the biofungicide can be applied

**Table 3.** Comparison of the control efficacy of the biofungicide (suspension concentrate formulation of *Bacillus subtilis* BD0310) and chemical fungicide (triflumizole wp) against anthracnose of tea plants in a glasshouse

| Treatments | Diseased leaves (%) | Control value (%) |
|---|---|---|
| Non-treatment (+ control) | 0.0 a[a] | – |
| Postspray of the biofungicide 2 days after inoculation | 33.3 c | 59.1 |
| Prespray of the biofungicide 2 days before inoculation | 18.5 b | 77.3 |
| Postspray of the chemical fungicide 2 days after inoculation | 22.2 b | 72.8 |
| Prespray of the chemical fungicide 2 days before inoculation | 7.4 a | 90.9 |
| Only inoculation (– control) | 81.5 d | – |

[a] Means with the same letter are not significantly different at $P=0.05$.

**Table 4.** Control efficacy of the biofungicide (suspension concentrate formulation of *Bacillus subtilis* BD0310) and chemical fungicide (triflumizole wp) against anthracnose of tea plants in the field

| Treatments[a] | Diseased leaves (%) | Control value (%) |
|---|---|---|
| Four times spray of the biofungicide | 3.0 c[b] | 52.4 |
| Six times spray of the biofungicide | 2.1 bc | 66.7 |
| Four times alternate spray of the biofungicide and chemical fungicide | 1.8 ab | 71.4 |
| Four times spray of the chemical fungicide | 0.9 a | 85.7 |
| Untreated control | 6.3 d | – |

[a] The fungicides were treated with 1 week interval.
[b] Means with the same letter are not significantly different at $P=0.05$.

to tea plantations as an alternative of chemical fungicides for the protective purpose.

In the field trial, the disease was alleviated by the biofungicide or chemical fungicide treatments. Although disease incidence of control plots was estimated to be 6.3%, 3.0% and 2.1% of disease incidence were recorded in the treatment of 4-time foliar spray and 6-time foliar spray of the biofungicide with 1 week intervals, respectively. Four-times foliar spray of chemical fungicide with 1 week intervals resulted in 0.9% of disease incidence. Four-time alternate foliar spray of the biofungicide and chemical fungicide showed 1.8% of disease incidence (Table 4). Control efficacy was calculated in the order of the chemical spray (85.7%), the alternate spray (71.4%), 6-time spray of the biofungicide (66.7%) and 4-time spray of the biofungicide (52.4%). Single use of the biofungicide with 4-or 6-time foliar spray showed lower control efficacy than that of chemical fungicide of triflumizole wp with 4-time foliar spray in the field. However, simultaneous use of the biofungicide and chemical fungicide can overcome the disadvantages of single use of the biofungicide or chemicals in the point of control efficacy and environmental harmfulness. In fact, alternate sprays of the biofungicide and chemical fungicide resulted in improved control efficacy, compared with single use of the biofungicide in the field. At the same time, the applications also reduced number of chemical sprays with a similar control efficacy, compared with single use of chemical fungicide. It was previously reported in Japan and Korea that thiophanate methyl has been intensively used to control tea gray blight and anthracnose for many years and, as a result, resistant stains to the chemicals seriously emerged in tea plantations (Horikawa, 1986, 1987; Oniki et al., 1985, 1986; Shin et al., 2000). Therefore, alternate use of biofunicides and chemicals will also retard the development of resistance in pathogen populations.

The results demonstrated that the biofungicide of *B. subtilis* BD0310 could be used for the control of tea anthracnose and simultaneous use of the biofungicide and chemicals could improve the controlling strategy with less use of chemicals. Our previous study also showed that the biofungicide effectively controlled tea gray blight disease in the field (Kim et al., 2008). Therefore, the biofungicide can be applied to control two economically important diseases on tea leaves in the field.

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