World J Microbiol Biotechnol (2015) 31:1517–1527
DOI 10.1007/s11274-015-1896-0

ORIGINAL PAPER

Bacillus amyloliquefaciens subsp. *plantarum* GR53, a potent biocontrol agent resists *Rhizoctonia* disease on Chinese cabbage through hormonal and antioxidants regulation

Sang-Mo Kang[1] · Ramalingam Radhakrishnan[1] · In-Jung Lee[1]

Received: 24 September 2014 / Accepted: 29 June 2015 / Published online: 10 July 2015

Abstract The fungus *Rhizoctonia solani* is one of the causal agents of numerous diseases that affect crop growth and yield. The aim of this present investigation was to identify a biocontrol agent that acts against *R. solani* and to determine the agent's protective effect through phytohormones and antioxidant regulation in experimentally infected Chinese cabbage plants. Four rhizospheric soil bacterial isolates GR53, GR169, GR786, and GR320 were tested for their antagonistic activity against *R. solani*. Among these isolates, GR53 significantly suppressed fungal growth. GR53 was identified as *Bacillus amyloliquefaciens* subsp. *plantarum* by phylogenetic analysis of the 16S rDNA sequence. The biocontrol activity of *B. amyloliquefaciens* subsp. *plantarum* GR53 was tested in Chinese cabbage plants under controlled conditions. Results showed that *R. solani* inhibited plant growth (length, width, fresh and dry weight of leaves) by reducing chlorophyll and total phenolic content, as well as by increasing the levels of salicylic acid, jasmonic acid, abscisic acid, and DPPH scavenging activity. By regulating the levels of these compounds, the co-inoculation of *B. amyloliquefaciens* subsp. *plantarum* GR53 heightened induced systemic resistance in infected Chinese cabbage, effectively mitigating *R. solani*-induced damaging effects and improving plant growth. The results obtained from this study suggest that *B. amyloliquefaciens* subsp. *plantarum* GR53 is an effective biocontrol agent to prevent the damage caused by *R. solani* in Chinese cabbage plants.

Keywords *Bacillus amyloliquefaciens* subsp. *plantarum* GR53 · Biocontrol · Chinese cabbage · *Rhizoctonia solani*

Introduction

Chinese cabbage belongs to the Brassicaceae family and is a major ingredient in kimchi, which is a traditional fermented dish in the Republic of Korea. Over 30,000 ha of land are used to grow Chinese cabbage and 90 % of the domestic production is used for manufacturing kimchi (Lee et al. 2014). Hence, diseases that affect this crop severely hamper the profitable manufacture of cabbage. Of particular consequence is the damping-off in cabbage seedlings caused by the fungus *Rhizoctonia solani*, which is a major challenge to manage and control (Shiau et al. 1999). More generally, the fungus can persist as sclerotia for several years in the soil and causes diseases in a wide range of their host plants (Gonzalez-Garcia et al. 2006). Soil fumigation is the current strategy used to manage crop diseases, but it also negatively affects beneficial soil microorganisms that assist crop growth (Solanki et al. 2012). Additionally, the application of agrochemicals to plants or soil has been a risk to sustainable agriculture due to resulting contamination of soil, water, and air (Correa et al. 2009). The absence of targeted plant disease control approaches and the growing demand for naturally developed food has inspired the study on biological control (Chowdhury et al. 2013), or the use of microorganisms to eradicate plant pathogens. This method is more environmentally friendly and could be an alternative to chemical fungicides, bactericides, and pesticides (Choudhary 2011; Compant et al. 2005;

Electronic supplementary material The online version of this article (doi:10.1007/s11274-015-1896-0) contains supplementary material, which is available to authorized users.

✉ In-Jung Lee
ijlee@knu.ac.kr

[1] School of Applied Biosciences, Kyungpook National University, Daegu 702-701, Republic of Korea

Radhakrishnan et al. 2013). In the specific case of *R. solani*, the fungus *Trichoderma atroviride* and the rhizobacteria *Trichoderma harzianum*, *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Bacillus pumilus* have been identified as active biocontrol microbes that can contain the spread of the fungal pathogen (Asaka and Shoda 1996; Hadar et al. 1979; Huang et al. 2012; Nagarajkumar et al. 2004; Reithner et al. 2007).

Several reports have revealed the possible use of rhizobacteria such as *Bacillus*, *Pseudomonas* and *Burkholderia* species can able to prevent the phytopathogen effects in crop plants. Specifically, *Bacillus* species occur ubiquitously in soil and produce a vast array of biologically active metabolites, such as indole acetic acid and gibberellin, in addition to solubilizing the organic phosphate that helps to increase plant growth (Kang et al. 2014). Pavlo et al. (2011) studied the role of microorganisms on plant growth and inhibition of disease causing pathogens; they demonstrated that the stimulation of rapid plant growth results in a failure to develop disease resistance. Previous studies suggest that *Bacillus* spp. exhibit their antagonistic activity against pathogens through synthesizing antimicrobial peptides, secretion of lytic enzymes, competition for nutrient and space, and importantly, induced systemic resistance, including the increase of pathogenesis-related proteins in plants (Ongena and Jacques 2008; Osman et al. 2011; Sharma et al. 2009). *Bacillus amyloliquefaciens* secretes antibiotics such as zwittermicin-A, kanosamine, iturin, fengycin, and lipopeptides from surfactins, which are effective against an extensive range of pathogens (He et al. 1994; Stabb et al. 1994). Due to its antibacterial and antifungal activities, *B. amyloliquefaciens* became commercially developed as an agricultural biomaterial. In fact, *B. amyloliquefaciens* mediated suppression rates of *Phytophthora*-blight of peppers and *Fusarium*-wilt of tomatoes were found to be higher than those of popular chemical fungicides (Chung and Kim 2005). Ji et al. (2013) demonstrated that *B. amyloliquefaciens* CNU114001 exhibited broad spectrum inhibitory activity against 12 phytopathogenic fungi such as *Alternaria panax, Botrytis cinerea, Colletotrichum acutatum, Colletotrichum orbiculare, Corynespora cassiicola, Fusarium oxysporum, Phytophthora capsici, Penicillium digitatum, R. solani, Stemphylium lycopersici, Pyricularia grisea*, and *Sclerotinia sclerotiorum*. Bacterial antibiotics control fungal growth by inhibiting the synthesis of fungal sterols and nucleic acids, as well as changing cell membrane permeability and destroying the fungal cell wall (Michael and Nannette 2003).

Despite this, while the usage of *Bacillus* strains to improve plant growth has been documented widely, only a limited number of studies were conducted to understand their role in suppressing *R. solani*-induced damage in plants (Solanki et al. 2012). Moreover, in spite of these few reports regarding the improved suppression of *R. solani* by *B. amyloliquefaciens*, no data are available on the hormonal and antioxidant changes in the host plant. An increase of endogenous salicylic acid in plants is involved in signal transfer during pathogen infection and activates the pathogenesis-associated gene expression (Buonaurio et al. 2002). The purposes of the current study were twofold: first, to identify a potent biocontrol rhizobacteria against *R. solani* from available strains collected in the field, and second, to evaluate their mitigation effects by analyzing salicylic acid, jasmonic acid, abscisic acid, and antioxidant activity in experimentally infected Chinese cabbage.

Materials and methods

Isolation of bacterial strains from rhizosphere soil

Rhizosphere soil samples were collected from various agricultural field of Chungcheongbuk-do, Danyang, Republic of Korea. One gram of soil sample was added to 50 mL of sterilized saline solution. Resultant suspensions were successively diluted (10^{-4}) and 0.1 mL aliquots were spread on plates containing tryptic soy/agar (TSA; Merck Co., Germany) medium. The plates were incubated for 48 h at 30 °C. Bacterial colonies were separated by their morphology, pigmentation, and growth rate.

In vitro antifungal activity of bacterial strains against *R. solani*

The effects of bacterial strains GR53, GR169, GR786, and GR320 against *R. solani* were assayed by dual culture method, following Radhakrishnan et al. (2013). *Rhizoctonia solani* culture was obtained from the National Institute of Agricultural Science and Technology (Suwon, Korea). GR53, GR169, GR786, and GR320 bacterial strains were applied to *R. solani* grown on plates containing potato dextrose agar. Plates were then incubated for 10 days at 28 ± 2 °C. Subsequently, *R. solani* mycelial growth was measured, and the inhibition of mycelial growth was calculated by measuring the clear zone between the fungus and bacterial isolates.

Identification and phylogenetic analysis of bacterial isolate GR53

An isolated antagonistic bacterial strain GR53 was identified by a partial 16S ribosomal DNA (rDNA) sequence. Chromosomal DNA was isolated and extracted by following the methods (Adachi et al. 1996). Primers 27F

(5′-AGAGTTTGATC(AC)TGGCTCAG-3′) and 1492R (5′-CGG (CT) TACCTTGTTACGACTT-3′) were used for PCR amplification of the 16S rDNA. We then used BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the nucleotide sequence homology of this bacterial isolate. Closely related nucleotide sequences were aligned using ClustalW and MEGA (version 5.0), and the neighbor-joining tree was generated using same software. Bootstrap analysis (1000 replications) was performed to assess the stability of tree nodes and branches.

Bacterial and fungal treatments and plant growth condition

Chinese cabbage seeds were surface sterilized with 1 % Tween 80 solution and 2 % perchloric acid, then washed thoroughly with sterile distilled water: first, for 5 min in a shaking incubator (120 rpm), followed by rinsing three times. The seeds were aseptically cultured in magenta boxes (10.0 × 7.0 × 7.0 cm) containing 50 mL of MS basal medium (Murashige and Skoog 1962) supplemented with 0.8 % agar at 25 °C; untreated seed cultures served as controls. The bacterial strain GR53 was cultured in LB media for 3 days at 30 °C in a shaking incubator (200 rpm). The pathogenic fungus *R. solani* was inoculated in potato dextrose broth media and cultivated in a shaking incubator with 200 rpm at 25 °C for 5 days. One hundred micro-litter of bacterial culture GR53 was applied on the surface of MS agar media after the emergence of the first leaf from Chinese cabbage seeds. 3 days later, 200 micro-litter of *R. solani* culture was inoculated to GR53-treated and untreated plants. The length, width, fresh and dry weight of leaf, chlorophyll, salicylic acid, jasmonic acid, abscisic acid, total polyphenol, and DPPH scavenging activity were recorded in Chinese cabbage plants at 10 days post *R. solani* treatment.

Quantification of salicylic acid (SA)

SA was extracted and quantified using the methods of Enyedi et al. (1992) and Seskar et al. (1998). One hundred milligrams of leaf samples were extracted with 90 and 100 % methanol by centrifuging at 10,000×*g*. The combined methanol extracts were vacuum-dried and re-suspended in 2.5 mL of 5 % trichloroacetic acid, and the supernatant was partitioned with ethyl acetate/cyclopentane/isopropanol (49.5:49.5:1, v/v). The top organic layer was transferred to a 4 mL vial for drying with nitrogen gas. After drying, it was again suspended in 1 mL of 70 % methanol. HPLC analyses were carried out on a Shimadzu fluorescence detector (Shimadzu RF-10AXL, excitation and emission detected at 305 and 365 nm, respectively), fitted with a C18 reverse-phase HPLC column (HP hypersil ODS). The flow rate was 1.0 mL/min. Salicylic acid content in samples was calculated by authentic standard peak values.

Quantification of abscisic acid (ABA)

ABA contents were extracted from the leaf samples following the procedures of Qi et al. (1998). An extraction solution containing 95 % of isopropanol and 5 % glacial acetic acid was first filtered through filter paper, and then 10 ng ABA was added as internal standard. The filtrate was concentrated by a rotary evaporator. The concentrated residue was dissolved in 1N NaOH solution and then washed with methylene chloride to remove lipophilic materials. Next, the aqueous phase was adjusted to pH 3.5 with the addition of 6N HCl and partitioned with ethyl acetate (EtOAc). EtOAc extract was then evaporated; the residue was dissolved in phosphate buffer (pH 8.0) and run through a polyvinylpolypyrrolidone (PVPP) column. The phosphate buffer mixture was adjusted to pH 3.5 with the addition of 6 N HCl and partitioned with EtOAc. The EtOAc extract was again evaporated; the residue was dissolved in dichloromethane and passed through a silica cartridge (Sep-Pak; Waters Associates, Milford, MA, USA), which was pre-washed with diethyl ether:methanol (3:2, v/v) and dichloromethane. ABA was recovered from the cartridge by elution with diethyl ether:methanol (3:2, v/v). The extracts were dried and methylated by adding diazomethane for GC–MS SIM (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, Palo Alto, CA, USA) analysis. For quantification, the Lab-Base (ThermoQuset, Manchester, UK) data system software was used to monitor the responses to ions of m/e 162 and 190 for Me-ABA and 166 and 194 for Me-[2H6]-ABA.

Quantification of jasmonic acid (JA)

JA from plant leaf samples was extracted according to the method of McCloud and Baldwin (1997). The Me esters of extracts were analyzed by GC–MS SIM (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, Palo Alto, CA, USA). For JA determination, the fragment ion at $m/z = 83$ amu, corresponding to the base peaks of JA and (9,10-2H2)-9,10-dihydro-JA, was monitored. The amount of endogenous JA was calculated from the peak areas of endogenous JA in comparison to the corresponding standards.

Total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu colorimetric method (Amerine and Ough 1980).

Leaf tissues were extracted with 80 % MeOH. Fifty microliters of extract was mixed with 1 mL of 2 % Na_2CO_3 and 50 µL of 1N Folin–Ciocalteu reagent at room temperature for 30 min. The absorbance of total phenol content was measured at 750 nm. Gallic acid was used as the standard to quantify total phenol levels.

DPPH scavenging activity

The free radical scavenging activity of leaf samples was determined using a DPPH assay following an established method (Blois 1958). Leaf samples were extracted with MeOH; the reaction mixture contained 5 mg diphenyl-1-picrylhydrazyl (DPPH) dissolved in 50 mL MeOH. The reaction mixture and MeOH extract (1:1) were incubated at room temperature in the dark for 30 min. The absorbance of samples was detected at 517 nm. The radical scavenging activity was calculated and expressed as percentage by following equation.

$$\text{DPPH scavenged}\,(\%) = (A_{con} - A_{test})/A_{con} \times 100$$

A_{con}—the absorbance of the control reaction; A_{test}—the absorbance in the presence of the sample of the extracts.

Statistical analysis

The experiments were repeated three times. Sigma Plot Software (10.0) was used to calculate the standard errors and compare the statistical difference of treatments based on Duncan's multiple range test (DMRT), at a significance level of $p \leq 0.05$.

Results

Identification of bacterial strains antagonistic against *R. solani*

In this study, the four bacterial isolates, GR53, GR169, GR786, and GR320, were tested to identify the strain that effectively prevented the spread of *R. solani*. Among these, the bacterial strain GR53 (*Bacillus amyloliquefaciens*) alone inhibited the mycelial growth of *R. solani* (Supplementary material Fig. 1). The clear zone formation between bacterial and fungal mycelial growth was observed in petri-plates, revealing that bacterial isolate GR53 effectively prevented the spread of *R. solani* (Fig. 1). The rate of mycelial growth inhibition was checked at different time intervals, and within seven days, *R. solani* growth was completely halted by the effect of co-inoculation of GR53 (Fig. 2).

Phylogenetic analysis was carried out on GR53 after DNA extraction and subsequent sequencing of the 16S rDNA. Sequence of the selected strains was analyzed by BLAST, presenting the highest sequence homology proportion, query coverage, and the lowest E values. Sequences of other genera were also used to determine the actual relationship among participating candidates in the group. BLAST search results revealed that GR53 shares a 100 % sequence homology with *B. amyloliquefaciens* (Fig. 3). The sequence was submitted to NCBI GenBank (accession no. KJ937782). Based on sequence similarity and phylogenetic analysis, we identified bacterial isolate GR53 as *B. amyloliquefaciens*.

Biocontrol activity of *B. amyloliquefaciens* subsp. *plantarum* GR53 on *R. solani* infection in Chinese cabbage

The treatment of *B. amyloliquefaciens* subsp. *plantarum* GR53 did not cause any significant change in Chinese cabbage growth under normal conditions (Table 1). The disease causing agent *R. solani* significantly inhibited the length and width of experimentally infected cabbage leaves when compared to uninfected control plants. The co-treatment of *B. amyloliquefaciens* subsp. *plantarum* GR53 to infected plants mitigated the damaging effect on leaf growth by enhancing the length and width of leaves. Similarly, Chinese cabbage infected by *R. solani* had lower fresh and dry leaf weight in comparison to controls. GR53 treatment effectively prevented this damage; Chinese cabbage co-treated with *B. amyloliquefaciens* experienced an increase in their fresh weight (3.08–3.20 g) and dry weight (0.21–0.26 g). Indeed, photosynthetic pigment chlorophyll content declined in *R. solani* infected plants, while *B. amyloliquefaciens* subsp. *plantarum* GR53 co-treatment enhanced the chlorophyll content in diseased plants. To confirm the mitigation effect of GR53 against *R. solani* infection, same experiment was conducted on pots containing autoclaved horticultural soil. Fifteen-days-old plants were treated with *B. amyloliquefaciens* subsp. *plantarum* GR53 and subsequently *R. solani* culture was applied after 7 days. The significant increase of plant growth by biocontrol agent on diseased plants was found in 1 month old plants (Supplementary table 1), and the *R. solani* and *B. amyloliquefaciens* subsp. *plantarum* GR53 colonies in roots of Chinese cabbage were observed by SEM analysis (supplementary figure 2).

Hormonal and antioxidants changes in diseased plants during *B. amyloliquefaciens* subsp. *plantarum* GR53 interaction

The mitigation effect of *B. amyloliquefaciens* subsp. *plantarum* GR53-inoculated Chinese cabbage was assessed by measuring levels of stress hormones (SA, JA, and ABA)

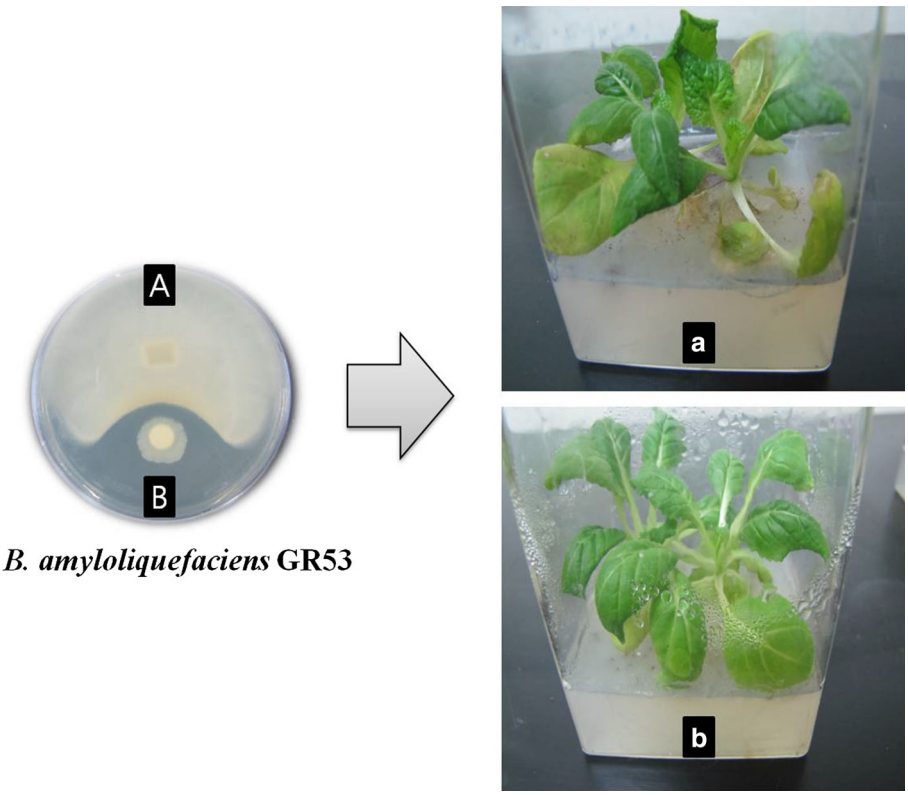

Fig. 1 In vitro antagonistic activity of *B. amyloliquefaciens* subsp. *plantarum* GR53 against *R. solani* in Chinese cabbage. (*A*) *R. solani*, (*B*) *B. amyloliquefaciens* subsp. *plantarum* GR53, **a** *R. solani*-infected plants, and **b** *B. amyloliquefaciens* subsp. *plantarum* GR53 treatment on *R. solani*-infected plants

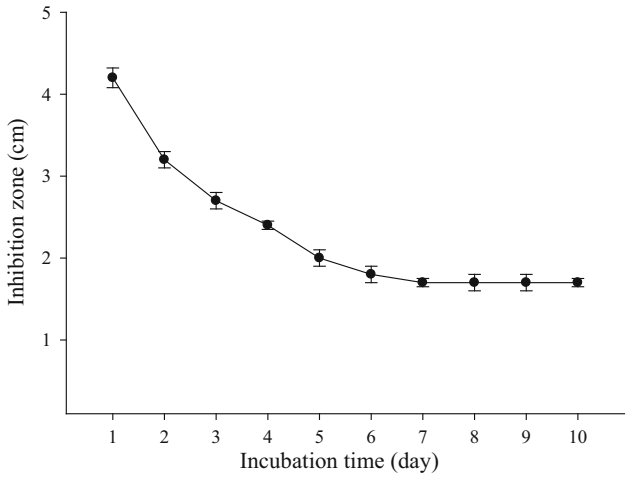

Fig. 2 Inhibition of *R. solani* growth with co-cultivation of *B. amyloliquefaciens* subsp. *plantarum* GR53. *Bars* represent means plus standard error (n = 3)

and antioxidants (total phenolic content and DPPH scavenging activity). In comparison to controls, SA content increased in both plants treated with *R. solani* only and co-treated with *B. amyloliquefaciens* subsp. *plantarum* GR53 (Fig. 4). The synergistic interaction of disease causing fungi *R. solani* and biocontrol agent GR53 on cabbage plants resulted in a drastic elevation of SA synthesis. Moreover, our results from measuring JA levels demonstrated the alleviating effects of *B. amyloliquefaciens* subsp. *plantarum* GR53 on diseased plants. First, we found that, as expected, *R. solani*-infected plants had higher levels of JA than healthy control plants. However, although JA synthesis was also enhanced in plants treated only with GR53, co-inoculation of *B. amyloliquefaciens* subsp. *plantarum* GR53 with *R. solani* significantly reduced JA levels in disease-infected plants. In addition, differential expression of ABA synthesis was observed in plants during pathogen-only treatment and bacterial + pathogen co-treatment. The accumulation of ABA was two times higher in *R. solani*-infected Chinese cabbage plants than in untreated control plants. Bacterial co-treatment using GR53 significantly blocked ABA accumulation in plants and mitigated disease-induced stress.

Finally, the microbial interaction also alters the antioxidant levels in affected plants. Chinese cabbage infected by *R. solani* failed to synthesize phenolic compounds (Fig. 5), while no significant changes to total phenolic levels were observed between control plants and those given a *B. amyloliquefaciens* subsp. *plantarum* GR53 treatment. The high production of phenolic compounds in diseased plants during the effect of *B. amyloliquefaciens* subsp. *plantarum* GR53 determined their biocontrol effect. In contrast, when compared to healthy controls, plants infected by *R. solani* had higher DPPH scavenging activity, while plants treated with *B. amyloliquefaciens* subsp. *plantarum* GR53 alone

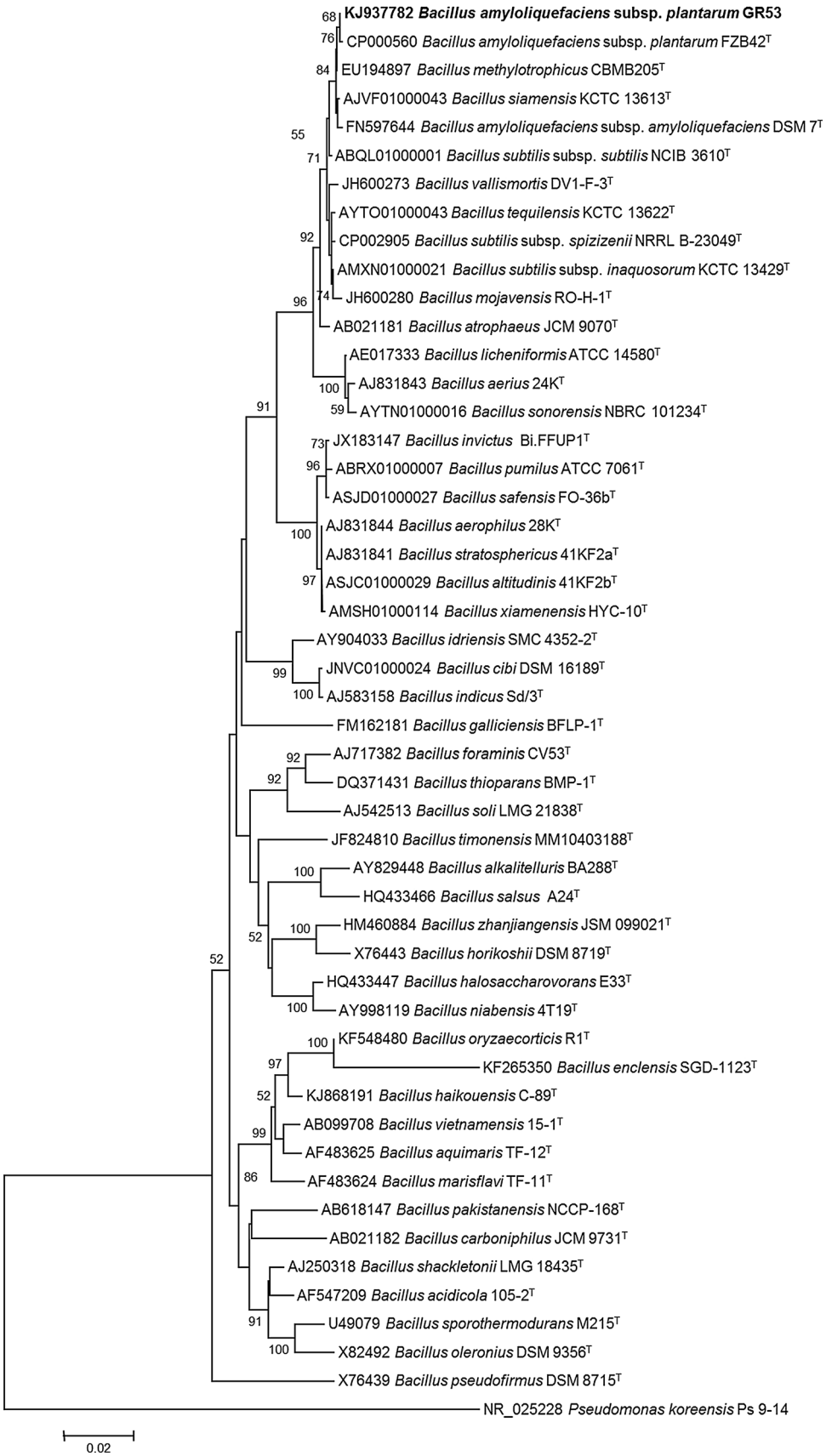

Fig. 3 Neighbor-joining phylogenetic tree based on the sequence obtained from 16S rDNA of GR53 and related bacteria. Percentage of confidence levels, generated from 1000 bootstrap trees, is indicated in each node

Table 1 Effect of *B. amyloliquefaciens* subsp. *plantarum* GR53 on Chinese cabbage plant growth and chlorophyll content in *R. solani*-infected plants

Treatments	Leaf length (cm/plant)	Leaf width (cm/plant)	Fresh weight (g/plant)	Dry weight (g/plant)	Chlorophyll content (SPAD)
Control	8.0 ± 0.19 a	1.32 ± 0.03 a	3.35 ± 0.10 a	0.28 ± 0.08 b	28.34 ± 0.26 a
B. amyloliquefaciens GR53	8.08 ± 0.14 a	1.32 ± 0.04 a	3.49 ± 0.13 a	0.31 ± 0.05 a	28.80 ± 0.28 a
R. solani (disease)	6.84 ± 0.17 c	1.16 ± 0.05 b	3.08 ± 0.04 b	0.21 ± 0.04 c	26.26 ± 0.27 c
B. amyloliquefaciens GR53 + *R. solani* (disease)	7.38 ± 0.01 b	1.30 ± 0.05 a	3.20 ± 0.06 c	0.26 ± 0.05 b	27.14 ± 0.15 b

Bars represent means plus standard error (n = 3). Means followed by the same letter are not significantly different ($p < 0.05$), as determined by Duncan's multiple range test

did not exhibit any changes. However, infected plants co-treated with *B. amyloliquefaciens* subsp. *plantarum* GR53 had lower levels of DPPH scavenging activity than those untreated with the bacteria.

Discussion

The biological control of plant diseases is a promising alternative approach to maintaining plant health and promoting crop yield. In the current study, we showed that GR53, a bacterial isolate from soil, was effective against the fungal pathogen *R. solani.* GR53 was identified as *B. amyloliquefaciens* through phylogenetic analysis. Previous studies have also documented the ability of *B. amyloliquefaciens* to inhibit *R. solani* fungal growth (Chowdhury et al. 2013; Huang et al. 2012; Ji et al. 2013; Yu et al. 2002) via the production of antifungal substance iturin. Overall, *B. amyloliquefaciens* has been studied extensively as a producer of α-amylase, subtilisin, barnase, and iturins, all of which can control pathogen growth (Yu et al. 2002). Although seed and root colonization of *Bacillus* species has been reported in the literature, less consideration has been given to the colonization of diverse plant cultivars. Correa et al. (2009) suggested that *B. amyloliquefaciens* BNM122 is a probable microbial biocontrol mediator able to restrict the damping-off disease caused by *R. solani* when inoculated in soybean seeds. In the present study, *R. solani* significantly reduced the leaf length, leaf width, fresh weight, dry weight, and chlorophyll content in Chinese cabbage plants, but *B. amyloliquefaciens* subsp. *plantarum* GR53 effectively mitigated all of these effects. Similar to our results, Solanki et al. (2012) found that damping off disease caused by *R. solani* inhibited the growth and yield of tomato plants, while *B. amyloliquefaciens* MB101 application on infected plants caused a severe drop in disease index and significant improvement of plant growth and yield. Additionally, co-treatment of *B. amyloliquefaciens* 7079 not only significantly suppressed *Fusarium*-wilt disease in tomatoes and *Phytophthora*-blight disease in peppers, but the bacteria's biocontrol effect was actually higher than chemical fungicides (Chung and Kim 2005). The greater efficacy of biological control agents compared to traditional pest control is encouraging, because application of fungicides thiram and carbendazim negatively disturbs both phytopathogenic and non-pathogenic fungi and bacteria (Johnsen et al. 2001; Niewiadomska 2004). Biocontrol approaches thus appear to be extremely favorable alternatives to reduce crop yield loss as limitations on the usage of chemical pesticides increase (Naegely 1997). With biocontrol methods, long-term solutions for pest control potentially exist; Chowdhury et al. (2013) suggested that the inoculating plants with *B. amyloliquefaciens* FZB42 could allow the bacteria to establish itself effectively in lettuce rhizospheres, reducing the effects of bottom rot without displaying any strong effect on the rhizosphere bacterial community itself.

Stress responsive hormones and antioxidants in plants are major defense factors against diseases. It can accrue through the regulation of plant hormones such as salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA), as well as antioxidants. SA is a phenolic compound that mediates the phenylpropanoid pathway in the cytoplasm initiates from phenylalanine, and the isochorismate pathway takes place in the chloroplast (Mercado-Blanco et al. 2001; Vicente and Plasencia 2011), and it has been identified as a key signal for the expression of pathogen resistance proteins during resistance (Loake and Grant 2007). In the current study, SA levels increased in plants co-treated with *B. amyloliquefaciens* subsp. *plantarum* GR53 and *R. solani*. The combined effect of both bacterial and fungal application caused a significant increase of SA levels in plants when compared to their controls. Our results are in agreement with a previous report, which documented that pathogen treatment of *Arabidopsis* elevated SA accumulation and increased the expression of pathogenesis-related genes, thereby enhancing plant disease resistance (Lee et al. 2006). At the time of infection, resistant plants increase the production of reactive oxygen species (ROS), which may play a role in killing the entering pathogens. Particularly, enhancement of hydrogen peroxide (H_2O_2) accumulation induces an increase of the

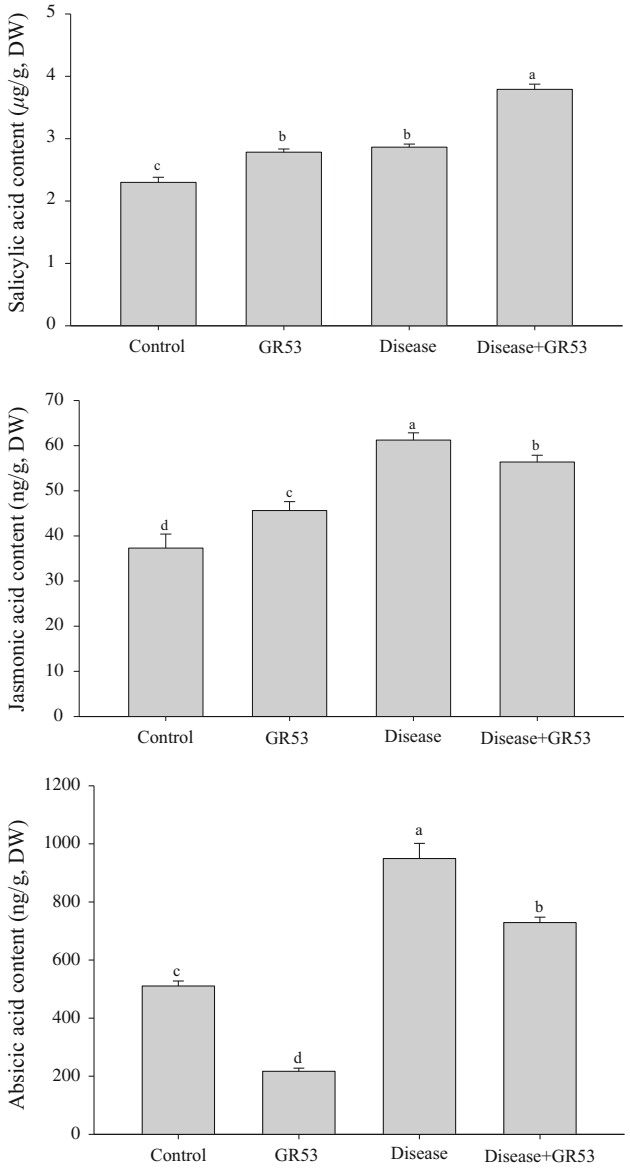

Fig. 4 Influence of *B. amyloliquefaciens* subsp. *plantarum* GR53 on salicylic acid, jasmonic acid, and abscisic acid levels in *R. solani*-infected plants. *Bars* represent means plus standard error (n = 3). Means followed by the *same letter* are not significantly different ($p < 0.05$), as determined by Duncan's multiple range test

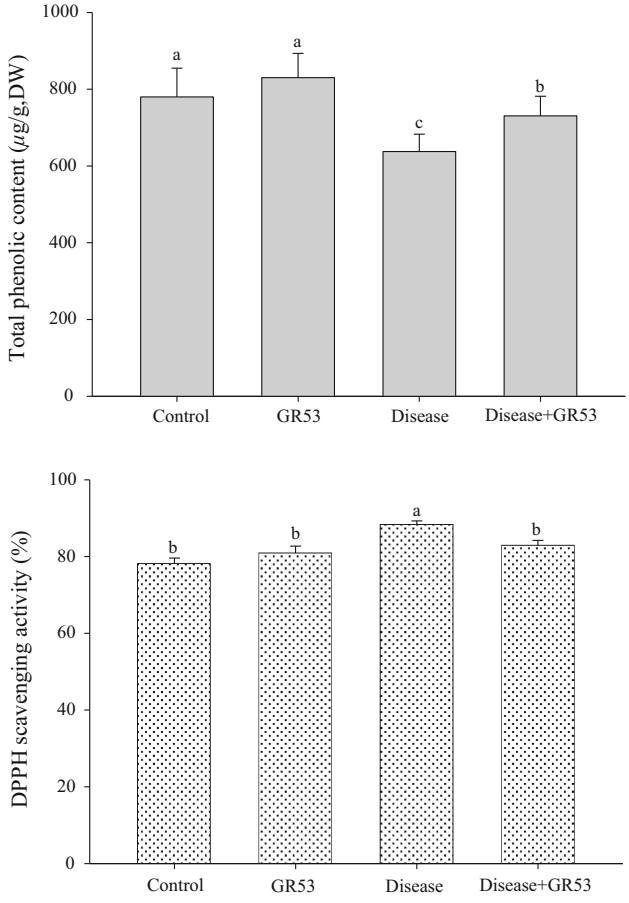

Fig. 5 Effect of *B. amyloliquefaciens* subsp. *plantarum* GR53 on antioxidant activity in *R. solani*-infected plants. *Bars* represent means plus standard error (n = 3). Means followed by the *same letter* are not significantly different ($p < 0.05$), as determined by Duncan's multiple range test

peroxidase-catalyzed synthesis of lignin, thus generating a physical barrier against pathogens (Dixon and Harrison 1992). SA attaches to catalase and prevents breakdown of H_2O_2, promoting an increase in H_2O_2 cellular concentration, which in turn, might serve as a second messenger for the stimulation of a defense reaction (Chen et al. 1993). Thus, the overproduction of SA stimulated by *B. amyloliquefaciens* subsp. *plantarum* GR53 in diseased plants may be one of the important factors to prevent plant damage.

Our quantification results of stress hormone JA in Chinese cabbage revealed that *R. solani*-infected samples had higher levels of JA when compared to controls, but *B. amyloliquefaciens* subsp. *plantarum* GR53 treatment effectively reduced JA concentration in diseased plants. JA and SA can act either antagonistically or synergistically in adjusting stress reactions (Rao et al. 2000). Unfortunately, to date, we have only a limited understanding of complex regulatory systems where several hormonal pathways interrelate and affect plant defense responses (Bari and Jones 2009). Elevated levels of JA in plants responding to infection have been demonstrated by several studies (Bari and Jones 2009; Lorenzo and Solano 2005; Wassternack 2007). Thus, since *B. amyloliquefaciens* subsp. *plantarum* GR53 modulates the relative abundance of JA, its presence may reprogram the communication of defense-related genes and organize multifaceted interactions between defense signaling pathways to trigger an active defense response against *R. solani* infection.

ABA is another stress hormone involved in numerous aspects of plant development, including the regulation of

stomatal aperture and adaptive responses to environmental conditions, but the role of ABA in plant disease resistance is not well defined (Mauch-Mani and Mauch 2005). In this study, ABA accumulation was significantly higher in *R. solani*-infected plants than in controls, and co-treatment with *B. amyloliquefaciens* subsp. *plantarum* GR53 reduces ABA levels. Previous research studies have shown that high accumulation of ABA in plants under disease conditions correlated with increased susceptibility (Mohr and Cahill 2003; Thaler and Bostock 2004). ABA inhibits the transcription of a basic β-1,3-glucanase that can destroy β-1,3-glucan callose, which regulates plasmodesmata to establish physical blocks against invading pathogens (Rezzonico et al. 1998). ROS production, Ca^{2+} signaling, and mitogen-activated protein kinase are inter-related with ABA signaling for disease resistance. Therefore, the alteration of these components and a high concentration of ABA all affect the plant's response to disease causing agents, controlled by the signaling pathway of SA (Mauch-Mani and Mauch 2005). This result suggests that the mechanism behind the ability of *B. amyloliquefaciens* subsp. *plantarum* GR53 to heighten plant resistance against *R. solani* might be a reduction of ABA and an increase of SA content.

Another, non-mutually exclusive possibility is that *B. amyloliquefaciens* subsp. *plantarum* GR53 affects DPPH scavenging levels in diseased plants. Plants under stress conditions, such as fungal infection, produce antioxidants to scavenge ROS. In this experiment, we observed that DPPH scavenging activity of diseased plants was higher than healthy controls, but DPPH scavenging capacity was effectively normalized by the activity of *B. amyloliquefaciens* subsp. *plantarum* GR53. The alteration in ROS-scavenging enzymes could be a vital process in phytopathogen defense (Gara et al. 2003). Non-pathogenic microbial inoculation can heighten the resistance of host plants against their pathogens by an increase of phenolic compounds (Gasoni and Gurfinkel 2009; Sneh et al. 1989). In support of this, we found that the phenolic content of *R. solani*-infected plants was less than that of the controls, but again, *B. amyloliquefaciens* subsp. *plantarum* GR53 treatment attenuated that consequence. Thimmaiah (1999) and Chatterjee and Ghosh (2008) suggested that the inhibition of phenolic compounds synthesis in plants during pathogen infection indicates the susceptibility of plants. The lower level of phenolic content in *R. solani*-infected Chinese cabbage plants revealed that this cultivar was susceptible to *R. solani* induced disease. A different rhizobacteria strain, *B. pumilus* SE34, had a similar effect in bean plants against *F. oxysporum* by triggering the release of callose and phenolic substances for inhibiting pathogenic symptoms (Benhamou et al. 1996).

In conclusion, although reports exist on the biocontrol effects of *B. amyloliquefaciens* against *R. solani* infection, to our knowledge, this is the first study to examine the mechanism behind protective qualities of *B. amyloliquefaciens* with plant hormones and antioxidants analysis. The current findings reveal that *B. amyloliquefaciens* regulates levels of plant stress hormones and antioxidant activity by increasing total phenolic content. In this way, *B. amyloliquefaciens* mitigated the biotic stress effects in *R. solani* infected Chinese cabbage. Thus, *B. amyloliquefaciens* application to agricultural fields can be a sustainable alternative to chemical fungicides, acting as an effective biocontrol agent to prevent diseases caused by *R. solani*.

Acknowledgments This work was financially supported by National Research Foundation of Korea (NRF), Ministry of Science, ICT and Future Planning through Basic Science Research Program (2014R1A1A1004918).

References

Adachi M, Sako Y, Ishida Y (1996) Analysis of Alexandrium (Dinophyceae) species using sequences of the 5.8 S ribosomal DNA and internal transcribed spacer regions. J Phycol 32:424–432

Amerine MA, Ough CS (1980) Methods for analysis of musts and wine. Wiley, New York, pp 205–206

Asaka O, Shoda M (1996) Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Appl Environ Microbiol 62:4081–4085

Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. Plant Mol Biol 69:473–488

Benhamou N, Kloepper JW, Quadt-Hallman A, Tuzon S (1996) Induction of defense-related ultrastructural modification in pea root tissues inoculated with endophytic bacteria. Plant Physiol 112:919–929

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. Nature 181:1199–1200

Buonaurio R, Scarponi L, Ferrara L, Sidoti P, Bertona A (2002) Induction of systemic acquired resistance in pepper by acibenzolar-S-methyl against bacterial spot disease. Eur J Plant Pathol 108:41–49

Chatterjee A, Ghosh SK (2008) Alterations in biochemical components in mesta plants infected with yellow vein mosaic disease. Braz J Plant Physiol 20:267–275

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262:1883–1886

Choudhary DK (2011) Plant growth promotion (PGP) activities and molecular characterization of rhizobacterial strains isolated from soybean (*Glycine max* L. Merril) plants against charcoal rot pathogen, *Macrophomina phaseolina*. Biotechnol Lett 33:2287–2295

Chowdhury SP, Dietel K, Randler M, Schmid M, Junge H, Borriss R, Hartmann A, Grosch R (2013) Effects of *Bacillus amyloliquefaciens* FZB42 on lettuce growth and health under pathogen pressure and its impact on the rhizosphere bacterial community. PLoS ONE 8:e68818

Chung SH, Kim SD (2005) Biological control of phytopathogenic fungi by *Bacillus amyloliquefaciens* 7079; suppression rates are better than popular chemical fungicides. J Microbiol Biotechnol 15:1011–1021

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71:49–51

Correa OS, Montecchia MS, Berti MF, Ferrari MCF, Pucheu NL, Kerber NL, Garcia AF (2009) *Bacillus amyloliquefaciens* BNM122, a potential microbial biocontrol agent applied on soybean seeds, causes a minor impact on rhizosphere and soil microbial communities. Appl Soil Ecol 41:185–194

Dixon RA, Harrison MG (1992) Activation, structure and organization of genes involved in microbial defense in plants. Adv Genet 28:165–234

Enyedi AJ, Yalpani N, Silverman P, Raskin I (1992) Localization, conjugation and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc Natl Acad Sci USA 89:2480–2484

Gara LD, de-Pinto MC, Tommasi F (2003) The antioxidant systems vis-a-vis reactive oxygen species during plant–pathogen interaction. Plant Physiol Biochem 41:863–870

Gasoni L, Gurfinkel BSD (2009) Biocontrol of *Rhizoctonia solani* by the endophytic fungus *Cladorrhinum foecundissimum* in cotton plants. Australas J Plant Pathol 38:389–391

Gonzalez-Garcia V, Portal-Onco MA, Rubio-Susan V (2006) Review: biology and systematics of the form genus *Rhizoctonia*. Span J Agric Res 4:55–79

Hadar Y, Chet I, Henis Y (1979) Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderma harzianum*. Phytopathology 69:64–68

He H, Silo-Suh LA, Handelsman J, Clardy J (1994) Zwittermicin A, an antifungal and plant protection agent from *Bacillus cereus*. Tetrahedron Lett 35:2499–2502

Huang X, Zhang N, Yong X, Yang X, Shen Q (2012) Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43. Microbiol Res 167:135–143

Ji SH, Paul NC, Deng JX, Kim YS, Yun BS, Yu SH (2013) Biocontrol activity of *Bacillus amyloliquefaciens* CNU114001 against fungal plant diseases. Mycobiology 41:234–242

Johnsen K, Jacobsen C, Torsvik V, Sorensen J (2001) Pesticide effects on bacterial diversity in agricultural soils—a review. Biol Fertil Soils 33:443–453

Kang SM, Radhakrishnan R, You YH, Joo GJ, Lee IJ (2014) Phosphate solubilizing *Bacillus megaterium* mj1212 regulates endogenous plant carbohydrates and amino acids contents to promote mustard plant growth. Indian J Microbiol 54:427–433

Lee J, Nam J, Park HC, Na G, Miura K, Jin JB, Yoo CY, Baek D, Kim DH, Jeong JC, Kim D, Lee SY, Salt DE, Mengiste T, Gong Q, Ma S, Bohnert HJ, Kwak SS, Bressan RA, Hasegawa PM, Yun DJ (2006) Salicylic acid-mediated innate immunity in *Arabidopsis* is regulated by SIZ1 SUMO E3 ligase. Plant J 49:79–90

Lee MK, Chun JH, Byeon DH, Chung SO, Park SU, Park S, Arasu MV, Al-Dhabif NA, Limg YP, Kim SJ (2014) Variation of glucosinolates in 62 varieties of Chinese cabbage (*Brassica rapa* L. ssp. pekinensis) and their antioxidant activity. LWT Food Sci Technol 58:93–101

Loake G, Grant M (2007) Salicylic acid in plant defence—the players and protagonists. Curr Opin Plant Biol 10:466–472

Lorenzo O, Solano R (2005) Molecular players regulating the jasmonate signaling network. Curr Opin Plant Biol 8:532–540

Mauch-Mani B, Mauch F (2005) The role of abscisic acid in plant–pathogen interactions. Curr Opin Plant Biol 8:409–414

McCloud ES, Baldwin IT (1997) Herbivory and caterpillar regurgitants amplify the wound induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*. Planta 203:430–435

Mercado-Blanco J, van-der-Drift KM, Olsson PE, Thomas-Oates JE, van-Loon LC, Bakker PA (2001) Analysis of the pmsCEAB gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. J Bacteriol 183:1909–1920

Michael RY, Nannette YY (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 55:27–55

Mohr PG, Cahill DM (2003) Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. tomato and *Peronospora parasitica*. Funct Plant Biol 30:461–469

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Naegely SK (1997) Greenhouse vegetables: business is booming. Greenh Grow 15:14–18

Nagarajkumar M, Bhaskaran R, Velazhahan R (2004) Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. Microbiol Res 159:73–81

Niewiadomska A (2004) Effect of carbendazim, imazetapir and thiram on nitrogenase activity, the number of microorganisms in soil and yield of red clover (*Trifolium pratense* L.). Pol J Environ Stud 13:403–410

Ongena M, Jacques P (2008) *Bacillus lipopeptides*: versatile weapons for plant disease biocontrol. Trends Microbiol 16:115–125

Osman MS, Sivakumar D, Korsten L (2011) Effect of biocontrol agent *Bacillus amyloliquefaciens* and 1-methylcyclopropene on the control of postharvest diseases and maintenance of fruit quality. Crop Prot 30:173–178

Pavlo A, Leonid O, Iryna Z, Natalia K, Maria PA (2011) Endophytic bacteria enhancing growth and disease resistance of potato (*Solanum tuberosum* L.). Biol Control 56:43–49

Qi QG, Rose PA, Abrams GD, Taylor DC, Abrams SR, Cutler AJ (1998) (+)-Abscisic acid metabolism, 3-ketoacyl-coenzyme A synthase gene expression, and very long chain monounsaturated fatty acid biosynthesis in *Brassica napus* embryos. Plant Physiol 117:979–987

Radhakrishnan R, Shim KB, Lee BW, Hwang CD, Pae SB, Park CH, Kim SU, Lee CK, Baek IY (2013) IAA-producing *Penicillium* sp. NICS01 triggers plant growth and suppresses *Fusarium* sp.-induced oxidative stress in sesame (*Sesamum indicum* L.). J Microbiol Biotechnol 23:856–863

Rao MV, Lee H, Creelman RA, Mullet JE, Davis KR (2000) Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. Plant Cell 12:1633–1646

Reithner B, Schuhmacher R, Stoppacher N, Pucher M, Brunner K, Zeilinger S (2007) Signaling via the *Trichoderma atroviride* mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection. Fungal Genet Biol 44:1123–1133

Rezzonico E, Flury N, Meins FJ, Beffa R (1998) Transcriptional downregulation by abscisic acid of pathogenesis-related beta-1,3-glucanase genes in tobacco cell cultures. Plant Physiol 117:585–592

Seskar M, Shulaev V, Raskin I (1998) Endogenous methyl salicylate in pathogen-inoculated tobacco plants. Plant Physiol 116:387–392

Sharma RR, Singh D, Singh R (2009) Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: a review. Biol Control 50:205–221

Shiau FL, Chung WC, Huang JW, Huang HC (1999) Organic amendment of commercial culture media for improving control

of *Rhizoctonia* damping-off of cabbage. Can J Plant Pathol 21:368–374

Sneh B, Ichielevich-Auster M, Plaut Z (1989) Mechanisms of seedling protection by a hypovirulent isolate of *Rhizoctonia solani*. Can J Bot 67:2135–2141

Solanki MK, Kumar S, Pandey AK, Srivastava S, Singh RK, Kashyap PL, Srivastava AK, Arora DK (2012) Diversity and antagonistic potential of *Bacillus* spp. associated to the rhizosphere of tomato for the management of *Rhizoctonia solani*. Biocont Sci Technol 22:203–217

Stabb EV, Jacobson LM, Handelsman J (1994) Zwittermicin A producing strains of *Bacillus cereus* from diverse soils. Appl Environ Microbiol 60:4404–4412

Thaler SJ, Bostock RM (2004) Interactions between abscisic-acid mediated responses and plant resistance to pathogens and insects. Ecology 85:48–58

Thimmaiah SR (1999) Standard method of biochemical analysis. Kalyani Publishers, New Delhi, pp 230–231

Vicente MR, Plasencia J (2011) Salicylic acid beyond defence: its role in plant growth and development. J Exp Bot 62:3321–3338

Wassternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot 100:681–697

Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biol Biochem 34:955–963