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REGULAR ARTICLE

Isolation and characterization of *Bacillus altitudinis* JSCX-1 as a new potential biocontrol agent against *Phytophthora sojae* in soybean [*Glycine max* (L.) Merr.]

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Abstract
Aims Phytophthora root and stem rot caused by the oomycete plant pathogen *Phytophthora sojae* (Kaufmann & Gerdemann), is a destructive disease of soybean [*Glycine max* (L.) Merr.]. There is no straightforward available method to control this disease. The present study aimed to isolate a biocontrol agent (BCA) to control *Phytophthora* rot and gain insights into the mechanisms of biocontrol activity.
Methods Antagonistic bacteria screening, inoculation assays, histochemical and fluorometric staining and real-time polymerase chain reaction (RT-PCR) were used to achieve the goals of the present study.
Results The results indicated that the isolated BCA strain JSCX-1 was characterized as *Bacillus altitudinis*. Further studies showed that JSCX-1 bacterial filtrate inhibited the mycelial growth and zoospore germination of *P. sojae.* Greenhouse experiments showed that biocontrol efficiency of JSCX-1 against *P. sojae* was 49.28 ± 3.42%. Our results revealed that JSCX-1 increased the reactive oxygen species (ROS) production and callose deposition of soybean leaves. Moreover, JSCX-1 up-regulated the transcriptional level of the *G. max PR1a* gene but not that of the *LOX* and *ERF* genes.
Conclusions B. altitudinis JSCX-1 can effectively reduce the infectivity of *P. sojae* via increasing the ROS production and callose deposition on soybean, and up-regulating the expression of salicylate-responsive gene *GmPR1a*.

Keywords *Phytophthora sojae* · *Bacillus altitudinis* · Biocontrol · Induced resistance · Salicylic acid signaling pathway

Abbreviations

BCA	Biocontrol agent
RT-PCR	real-time polymerase chain reaction
ROS	Reactive oxygen species

Xiaoxue Lu and Dongmei Zhou contributed equally in this study.

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ISR induced systemic resistance
SAR Systemic acquired resistance

Introduction

Phytophthora root and stem rot caused by the oomycete plant pathogen *Phytophthora sojae* (Kaufmann & Gerdemann), is a destructive disease of soybean [*Glycine max* (L.) Merr.], resulting in great yield reductions and capital losses each year worldwide (Qiao et al. 2013). This disease can be controlled predominately through the incorporation of resistance genes (Anderson and Buzzell 1992; Shan et al. 2004; Song et al. 2013), applying fungicides, and using cultural practice to improve field drainage and soil tillage. Surveys on *P. sojae* indicated that it has a diversity of races and the races are apparently becoming more diverse and difficult to manage. The most effective way to combat the disease is by restricting the spread of the fungus and limiting its impact, however, unfortunately, there is no straightforward available method to achieve this.

Rhizospheres contain a plethora of microflora, and thus, it is probable that there are potentially effective microorganisms that act as biocontrol agents against both bacterial and fungal plant pathogens. These microbes are able to trigger induced systemic resistance (ISR) in plants against a broad range of pathogens (Van Loon and Bakker 2005; Van Wees et al. 2008) by activating a series of plant responses, including cell-wall reinforcement (Benhamou et al. 1996), accumulation of defense-related enzymes (Benhamou and Belanger 1998), oxidative burst (Iriti et al. 2003), and secretion of secondary metabolites (Yedidia et al. 2003). Given the broad spectra and resistance levels that beneficial microbes induce, the application of a biocontrol agent (BCA) is considered a promising alternative way to control *Phytophthora* rot.

Among the various antagonists applied for the management of *Phytophthora* root and stem rot of soybean, the biocontrol agent *Trichoderma brevicompactum* and its volatile metabolites recently had been shown that can suppress the mycelial growth of *P. sojae* in vitro. Application of *T. brevicompactum* obviously reduced the disease severity of soybean caused by *P. sojae* in the greenhouse experiment (Ayoubi et al. 2012). Rhizobacteria such as *Bacillus* is one of the most common soil inhabitant, and widely used as antagonistic bacteria (Chen et al. 2007), studies showed that *Bacillus pumilus* had an excellent potential to be developed as BCA against *P. sojae* on soybean plant (Fu et al. 2011).

Bacillus is one of the most studied genera and has been shown to enhance plant growth, induce plant resistance, and confer abiotic stress (Thordal-Christensen et al. 1997; Pertot et al. 2013). *Bacillus* sp. can produce numerous antifungal compounds, such as lipopeptides (Ongena and Jacques 2008), bacillomycin (Chen et al. 2007), fengycin (Vanittanakom et al. 1986), surfactin (Thimon et al. 1992), and bacillibactin (Dertz et al. 2006). Thus, *Bacillus* has a broad spectrum of activity against multiple fungal pathogens. Lipopeptides extracted from *Bacillus amyloliquefaciens* CNU114001 inhibited the mycelial growth of six pathogenic fungi and the elongation of spore germ tubes (Ji et al. 2013). In addition, *Bacillus* releases volatile compounds, belonging to alkyls, alcohols, esters, ketones, phenols and heterocyclics, to suppress the mycelial growth and the spore germination of fungal pathogens such as *Fusarium oxysporum*, *Fusarium solani*, *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Fiddaman and Rossall 1994; Yuan et al. 2012; Li et al. 2014).

The elicitation of induced resistance by *Bacillus* and its metabolites has been demonstrated on a variety of crops to defend against pathogen attack in both greenhouse and field trails (Kloepper et al. 2004). Niu et al. (2011) found that *Bacillus cereus* AR156 induced hydrogen peroxide accumulation and callose deposition; moreover, it activated ISR in *Arabidopsis* through salicylic acid (SA)-and jasmonic acid (JA)/ethylene (ET)-dependent signaling pathways. Desoignies et al. (2013) investigated the impact of lipopeptides produced by *Bacillus amylolequifaciens* on the biocontrol of rhizomania disease caused by the fungus *Polymyxa betae*. Their results showed an effective ISR in sugar beet resulting in a significant reduction in *P. betae* infection. Additionally, *Bacillus* isolated from rainforest soil promoted plant growth and triggered ISR against the pathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis* (Huang et al. 2015).

In the present study, we aimed to isolate BCA(s) against *P. sojae* from the rhizosphere soil of healthy soybean plants present in diseased field, and gain insights into the mechanisms of the disease control. The study included: (1) the isolation and screening of bacteria against *P. sojae*, (2) the evaluation of the biocontrol effects of selected BCA in the greenhouse, (3) the

identification of the isolated BCA by 16S rRNA and *gyrB* sequencing, physiological and morphological characteristics analyses, and (4) the investigation of the biocontrol mechanisms of the BCA strain by determining the inhibition of mycelial growth and zoospore germination in *P. sojae*, reactive oxygen species (ROS) production, callose deposition, and defense-related gene induction. In our study, we isolated a BCA strain *B. altitudinis* JSCX-1 and investigated the biocontrol efficiency of *B. altitudinis* JSCX-1 against *Phytophthora* root and stem rot of soybean caused by *P. sojae*, and gained insights into the mechanisms of biocontrol activity. Our study indicated that application of this BCA could be an available and promising method to control this disease.

Materials and methods

Tested fungi, plant lines and growth conditions

The *P. sojae* isolate P6497 (race 2) was provided by Professor Yuanchao Wang (Nanjing Agricultural University) (Song et al. 2013), and routinely grown and maintained on 10% V8 agar at 25 °C in the dark (McLeod et al. 2008). *P. sojae* zoospores were obtained as described previously (Zhang et al. 2012). Soybean plants ('He Feng 35') were grown in a greenhouse at 20–23 °C (day) and 18–20 °C (night) with 16 h of light, and leaves used for biocontrol experiments were obtained from 14-day-old plants. Soybean that was grown in the darkness at 20–23 °C for 3 days was used for etiolated seedlings. R_2A (Reasoner and Geldreich 1985) and Luria-Bertani (LB) (Bertani 1951) media were used to grow bacterial strains in this study.

Screening of antagonistic bacterial strains to *P. sojae*

Antagonistic bacteria were isolated from rhizosphere soils of soybean roots collected from soybean fields in Jiangsu province (N34°12′33.24″; E119°03′31.45″) in China. Suspensions with 10 g of soil samples and 90 mL of sodium chloride (0.9%) were mixed in a shaker incubator at 30 °C for 15 min, and diluted to 10^{-4}. Then, 100 μL of the dilution sample was placed on R_2A medium. After 24 h, bacteria of different sizes and morphologies appeared on plates, and were individually isolated as single colonies and preserved in LB medium.

The screening of the antagonist activities of these isolated strains was carried out as follows: a small piece of *P. sojae* mycelial disk from the edge of a 5-day-old colony was transferred onto the center of a plate with V8 at 25 °C in the dark for 24 h, and each isolated bacterium for triplicate in one plate was sown at a distance of 3 cm from *P. sojae*. Petri dishes were grown for an additional 7 days. The antagonism efficiency was equal to: [(A–B)/A × 100], where 'A' was the mycelial diameter of control, and 'B' was the mycelial diameter of the fungus with the bacterial inoculation. Each treatment contained 3 plates and the experiment was repeated three times.

Characterization and identification of antagonistic bacteria

Physiological and morphological characteristics of the JSCX-1 strain were identified according to Bergey's Manual of Determinative Bacteriology. The tests included spore formation, gram staining, oxidase activity, catalase activity, gelatin liquefaction, starch hydrolysis and the citrate test. The genomic DNA of the strain JSCX-1 was extracted with Bacteria Genomic DNA Kit (CW Biotech, China). The 16S rRNA and *gyrB* gene sequences were amplified from the integrated chromosomal DNA of the isolate JSCX-1 using the universal primers 27F/1492R and UP-1S/UP-2Sr (Yamamoto and Harayama 1995; Galkiewicz and Kellogg 2008) (Table 1). The amplified fragments were sequenced by GenScript Co., Ltd. (Nanjing, China). The 16S rRNA and *gyrB* gene sequences were analyzed using BLAST network services at NCBI, and the genes with high similarities were selected as the references. Phylogenetic trees were constructed using MEGA version 5.1 (Tamura et al. 2011).

Antagonistic effect experiments

To test the antagonistic effects of JSCX-1 on the mycelia and zoospores of *P. sojae* in vitro, JSCX-1 was inoculated in 5 mL of LB in a shaker incubator at 30 °C for 24 h. Then, the culture filtrate was harvest and filtered using acrodisc syringe filter of 0.2 μm Supor Membrane. Five mycelial disks of 12 mm diameter (from the edge of a 5-day-old colony) of *P. sojae* were placed in 10 mL of sterile cultural filtration or water in a plate at 25 °C for 24 h. Zoospores (1×10^4/mL) of *P. sojae* were mixed with the sterile cultural filtration

Table 1 Primers used in this study

Gene	Primer name	Primer sequences	Source
16S rRNA	27F 1492R	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-TACCTTGTTACGACTT-3′	Galkiewicz and Kellogg 2008
gyrB	UP-1S UP-2Sr	5′-GAAGTCATCATGACCGTTCTGCA-3′ 5′-AGCAGGGTACGGATGTGCGAGCC-3’	Yamamoto and Harayama 1995
GmPR1a	PR1a-RT-F PR1a-RT-R	5′-GGGTGATGTTGCCTACGCTCAA-3’ 5′-CAGCAACCGTATCATCCCAAGC-3’	This study
GmLOX	LOX-RT-F LOX-RT-R	5′-TGGAGGTTTTAAGAGGAGATGG-3’ 5′-CCTGCGAGGGTAAGGATAGTTG-3’	This study
GmEREBP	EREBP-RT-F EREBP-RT-R	5′-GATTACTCCCACATCGCTACCC-3’ 5′-AGATTCTTCCTCTGCCTCTTCA-3’	This study
ELF1B	ELF1B-F ELF1B-R	5′-CCACTGCTGAAGAAGATGATGATG-3’ 5′-AAGGACAGAAGACTTGCCACTC-3’	This study

or water at the volume ratio of 1:1 in a plate at 25 °C for 24 h. The mycelial growth and zoospore germination were observed under a light microscope by checking five randomly selected fields in each treatment.

To test the antagonistic effects of JSCX-1 in vivo, the experiments were performed on mature soybean leaves and etiolated seedlings (Dong et al. 2009). JSCX-1 was inoculated as above, soybean leaves and etiolated seedlings were fully submerged in cell suspensions of JSCX-1 (OD_{600} = 1) or sterile water for 1 min, then the center of a *G. max* leaf or etiolated seedling was infected with a small piece of *P. sojae* mycelial disk from the edge of a 5-day-old colony, mycelial-side down. The antagonistic effects of JSCX-1 were quantified by measuring the lesion lengths at 48 h post inoculation (hpi). Each treatment had 6 mature leaves or etiolated seedlings. The experiments were repeated three times.

Greenhouse pot bioassay

The biocontrol effects of JSCX-1 were evaluated by hypocotyl inoculation (Tyler et al. 1995) in a greenhouse. Soybean seeds were cultivated in vermiculite pots (vermiculite was sterilized 3 times by autoclaves for 121 °C, 20 min) and on the 10th day of development, each plant was suspended in 3 mL of JSCX-1 (OD_{600} = 1) or sterile water. Three days later, the soybean hypocotyl was incised with a small wound and the wound was infected with a small piece of *P. sojae* mycelial disks from the edge of a 5-day-old colony. After 2 days, the disease incidence and biocontrol effects of JSCX-1 were evaluated. The disease incidence was equal to A/B × 100%; where ‘A’ was the dead plants and ‘B’ was the total number of infected plants. The biocontrol effect was equal to: (disease incidence $_{control}$–disease incidence $_{treatment}$)/disease incidence $_{control}$ × 100%. Each treatment had 15 soybean plants and the experiment was repeated three times.

Histochemical and fluorometric staining assays

Soybean leaves selected for the investigation of biocontrol mechanisms of JSCX-1 were treated as described above for the antagonistic effects assays. At 16 and 24 hpi with *P. sojae* mycelia, 6 mature leaves were collected for 3, 3′-diaminobenzidine (DAB) staining for ROS detection, and 6 mature leaves for aniline blue staining for callose detection, respectively. The methods of DAB and aniline blue staining were described previously (Thordal-Christensen et al. 1997; García-Andrade et al. 2011). ROS production was visualized as a reddish-brown precipitate in soybean leaves (Karimi et al. 2002), and callose deposition was observed and photographed under a ZEISS LSM 710 confocal microscope (ZEISS Microsystems).

Transcriptional profiling analysis

To test whether JSCX-1 could activate soybean plant defenses, we characterized *GmPR1a*, *GmLOX* and *GmEREBP* defense gene expression during *P. sojae* infection in the presence or absence of JSCX-1 by quantitative real-time PCR. Soybean leaves were treated as in the antagonistic effects assays, and collected at 0 (uninfected by *P. sojae*), 6, 12, 24, and 48 hpi (Niu et al. 2011). The total RNA of leaves was extracted using

TRIzol reagent (Invitrogen/Life Technologies, Paisley, UK). cDNA was synthesized using a PrimeScript RT reagent Kit with gDNA Eraser (Takara RR047A) following the manufacturer's instructions. SsoFast EvaGreen Supermix (Bio-Rad Corporation, USA) was used for these genes transcriptional analyses, the quantitative RT-PCR amplification was performed in the presence of EvaGreen Dye (Bio-Rad) with an iQ5 iCycler (Bio-Rad), and the thermal cycler conditions and reaction mixtures were carried out following the manufacturer's instructions.

The expression levels of the three genes were normalized to the *ELF1B* reference gene of soybean (Genbank accession no. NM_001249608.1). Primers were designed using Primer premier 5 (Premier corporation, Canada) and listed in Table 1. The Bio-Rad iQ5 Optical System Software (version 2.1) was used to analyze the threshold cycle (Ct) value. The transcriptional levels of the three genes were determined in accordance with the function $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$, to compare untreated and treated expression levels, and $\Delta\Delta Ct = \Delta Ct_{\text{treatment}} - \Delta Ct_{\text{control}}$, where the control was the H_2O-treated leaves. The induction ratio of treatment to control was calculated according to $2^{(-\Delta\Delta Ct)}$ (Livak and Schmittgen 2001). This experiment was repeated three times.

Statistical analyses

A one-way analysis of variance (ANOVA) was carried out followed with Duncan's new multiple range test ($P < 0.05$, DPS 7.05) to compare the difference in mycelium diameter of control and the treatments and *GmLOX* expression of different time intervals of each treatment. The *t*-test was conducted to compare the difference in zoospore germination, lesion length, disease incidence and genes expression of treatments and controls ($P < 0.05$, SPSS 19.0).

Results

Screening and characterization of antagonistic bacteria

A total of 10 bacterial isolates isolated from soybean rhizosphere soil showed antagonistic activity to *P. sojae*. Among these isolates, strain JSCX-1 had the strongest antifungal activity towards the growth of *P. sojae* in in vitro assays compared with the other strains (Fig. S1). The growth inhibition ratio of JSCX-1 to *P. sojae* reached 63.94 ± 3.94% at 7 days post confrontation (Fig. 1).

The physiological and morphological characteristics of the strain JSCX-1 were analyzed (Table S1). JSCX-1 exhibited positive results in most of the tests, such as spore formation, gram staining, oxidase activity, catalase activity, gelatin liquefaction, starch hydrolysis and the citrate test, indicating that JSCX-1 belonged to the genus *Bacillus*. Further studies based on 16S rRNA (GenBank accession no. KU955326) and *gyrB* gene (GenBank accession no. KU955327) sequencing and the phylogenetic analysis identified JSCX-1 as *Bacillus altitudinis* (Fig. S2; Fig. 2).

Inhibition effects of bacterial filtration on the mycelial growth and zoospore germination of *P. sojae*

The suppression of bacterial filtration on *P. sojae* mycelial growth and zoospore germination was evaluated under a light microscope (Fig. 3). *P. sojae* mycelia had a disorganized development, highly vesiculated protoplasm and fewer branches in the cell free culture filtrate of *B. altitudinis* JSCX-1 (Fig. 3a). In contrast, the H_2O-treated *P. sojae* mycelia exhibited regular development, typical protoplasm, and rectangular branching (Fig. 3a). Furthermore, the zoospore germination of *P. sojae* was strongly inhibited by the culture filtrate of strain JSCX-1, with an inhibition of 42.50 ± 1.56%, in comparison with H_2O-treated control (Fig. 3b and c).

BCA effects of JSCX-1 on *P. sojae* in soybean leaves and etiolated seedlings

The antagonistic effects of JSCX-1 against *P. sojae* in soybean leaves and etiolated seedlings were investigated. Two days after *P. sojae* infection, H_2O-treated leaves showed extensive lesion (Fig. 4a). However, applications of JSCX-1-treated leaves exhibited a significant reduction in lesion length compared with the control at 48 hpi (Fig. 4a), with the control effect being 61.11 ± 3.29% (Fig. 4c). Similarly, JSCX-1 dramatically protected against the infection of *P. sojae* in soybean etiolated seedlings at 48 hpi (Fig. 4b), with an efficiency of 75.94 ± 4.55% (Fig. 4d).

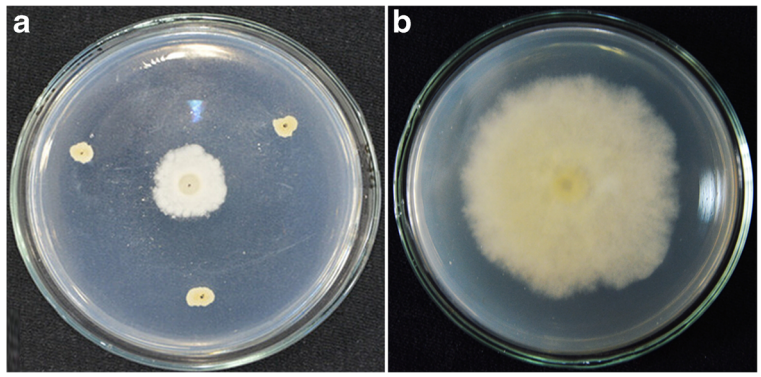

Fig. 1 Inhibition effect of JSCX-1 on the mycelial growth of *P. sojae* at 7 days post confrontation. *P. sojae* in the presence of strain JSCX-1 (**a**), and *P. sojae* alone (**b**). The experiment was repeated three times with similar results

Biological control of JSCX-1 against *P. sojae* in soybean plants

Hypocotyl inoculation assay was performed to evaluate the biocontrol activities of JSCX-1 against *P. sojae* in soybean. Two day after *P. sojae* inoculation, the H_2O-treated plant showed representative phenotypes with leaves drooping and wilting and had reached the disease incidence up to 80%. Soybean plants pretreated with a cell suspension of *B. altitudinis* had an obvious decrease in disease incidence when compared with

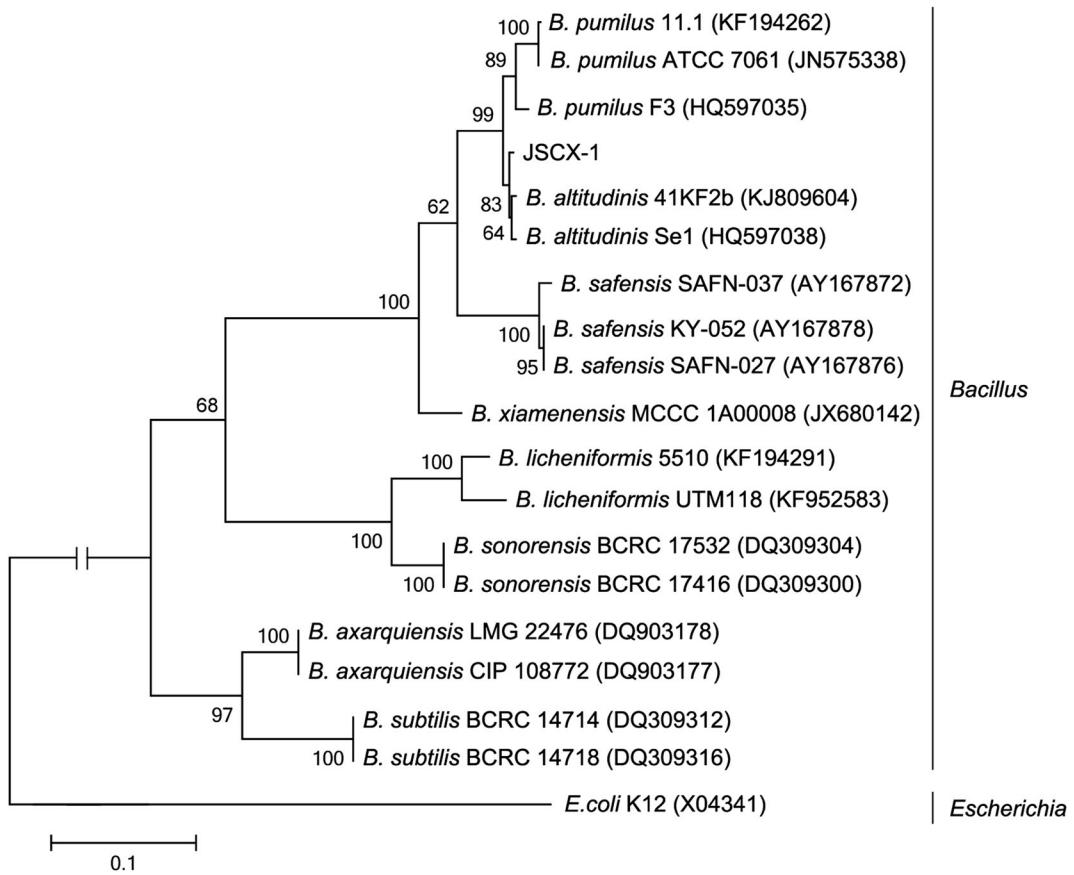

Fig. 2 Neighbor-joining phylogenetic trees based on *gyrB* gene sequence, showing the position of strain JSCX-1 among other members of the genus. Numbers at nodes are percentages that indicate the bootstrap values (expressed as 1000 replications). The scale bar =0.1 nt substitutions per site

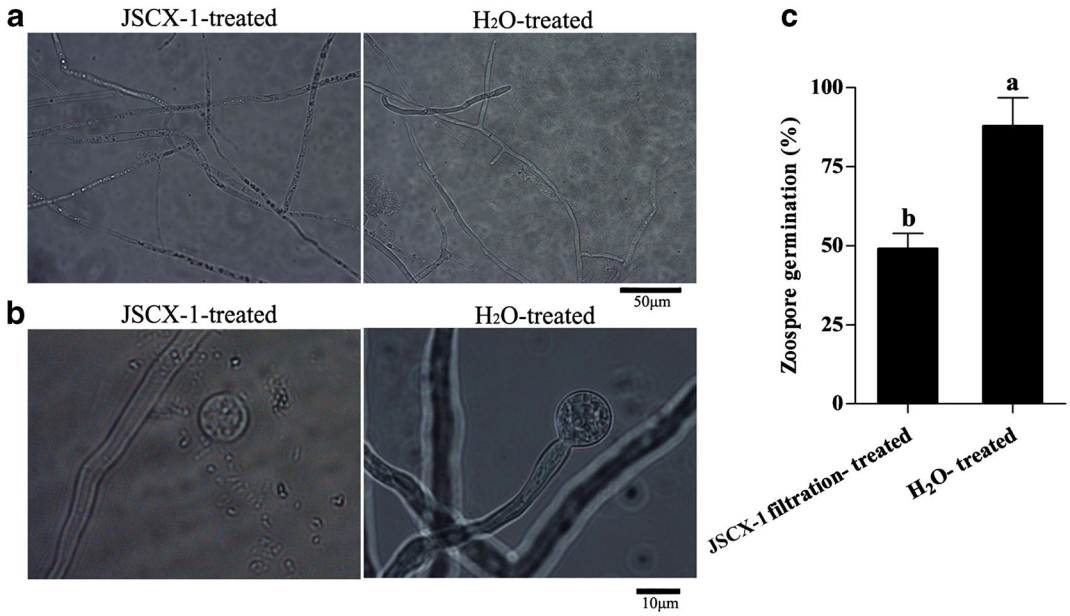

Fig. 3 The strain JSCX-1 suppressed *P. sojae* mycelial growth and zoospore germination. **a** Abnormal changes in *P. sojae* mycelia were observed following treatments with JSCX-1 for 24 h compared to H_2O-treated control, as observed under a light microscopy. Bar =50 μm. **b** Fewer *P. sojae* zoospores germinated in the presence of the JSCX-1 filtrate after 24 h compared to H_2O-treated control, as observed under light microscopy. Bar =10 μm. **c** Inhibitory effects of the JSCX-1 filtrate on *P. sojae* zoospore germination compared with the control. Data are means ± SE, where SE = SD/sqrt (n) and $n = 5$. Different lowercase letters above the bars indicate significant differences among the treatments at the $P < 0.05$ level as indicated by *t*-test

the H_2O-treated control (Fig. 5a) at an efficiency of 49.28 ± 3.42% (Fig. 5b).

The antagonistic bacteria JSCX-1 activates soybean plant defense responses

To assess whether JSCX-1 could activate soybean defense responses, ROS production and callose deposition in soybean were evaluated (Fig. 6). Treatment of leaves with cell suspensions of JSCX-1 led to an increase in ROS production (Fig. 6a) and callose deposition (Fig. 6b) in soybean leaves at 16 hpi and 24 hpi when compared with the H_2O-treated control.

The relative expression levels of the SA-responsive gene *GmPR1a* (Van Loon and Van Strien 1999), JA-responsive gene *GmLOX* (Wang et al. 2000), and ET-responsive gene *GmEREBP* (Lorenzo et al. 2003) were further measured at various time points of infection to validate that JSCX-1 could induce soybean systemic resistance possibly through a single defense pathway. Transcriptional levels of the three genes were evaluated after *P. sojae* infection at 0, 6, 12, 24, and 48 hpi. The transcriptional level of *GmPR1a* in JSCX-1-pretreated leaves appeared significantly faster and stronger at 0, 6, and 12 hpi than those of the H_2O-treated control leaves. In contrast, the transcriptional level of *GmLOX* was reduced at 6 and 12 hpi by *P. sojae* in H_2O- and JSCX-1-treatments when comparing to those at 0 hpi. However, the ET-responsive gene *GmEREBP* was constantly expressed at a steady level and did not show any differences in the H_2O-treated and JSCX-1-treated leaves (Fig. 6c).

Discussion

Phytophthora rot occurs in soybean worldwide and is difficult to manage. Studies on the use of BCA to control this disease are limited. In the current study, we isolated a bacterial strain, JSCX-1, from the rhizosphere soil of healthy soybean from disease fields with biocontrol abilities to *P. sojae* infection. This bacterial strain JSCX-1 was identified as *B. altitudinis* and found to induce resistance to *P. sojae* in soybean by significantly reducing *P. sojae* infections.

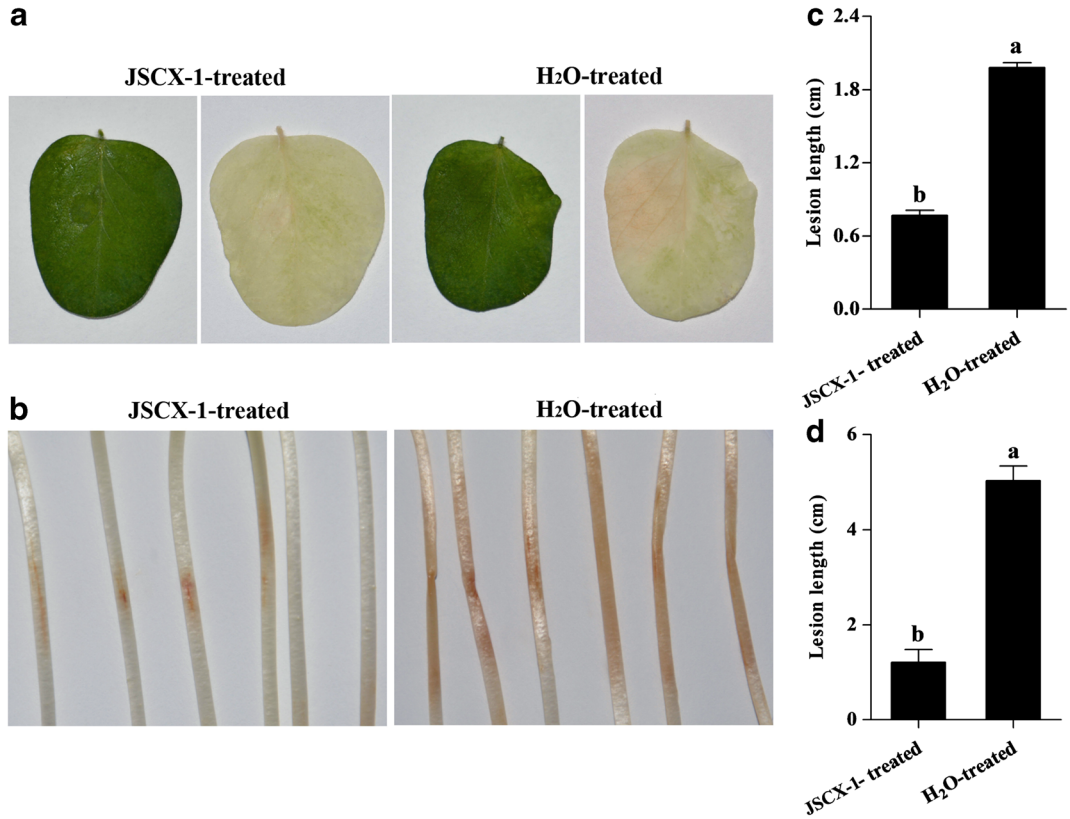

Fig. 4 Pretreatment with JSCX-1 enhanced the resistance of soybean to *P. sojae*. (a and b) Soybean leaves and etiolated seedlings upon *P. sojae* infection. The lesion lengths were measured at 48 hpi. (c and d) Biocontrol efficiency of JSCX-1 against *P. sojae* in soybean leaves and etiolated seedlings. The experiments were repeated three times with similar results, and the figures represented one of the results. Data are means ± SE where SE = SD/sqrt (n) and $n = 6$. Different lowercase letters above the bars indicate significant differences among the treatments at the $P < 0.05$ level as indicated by *t*-test

Fig. 5 The pretreatment of JSCX-1 enhanced the resistance of soybean to *P. sojae* in greenhouse pot bioassays. **a** Phenotypes in hypocotyl inoculation assay of *P. sojae* on soybean, and photographs were taken at 48 hpi. **b** Biocontrol efficiency of JSCX-1 to *P. sojae* in hypocotyl inoculation assay. The experiment was repeated three times with similar results. Data are mean ± SE from 15 plants each from three independent experiments, SE = SD/sqrt (n) and $n = 3$. Different lowercase letters above the bars indicate significant difference between the treatments at $P < 0.05$ level by *t*-test

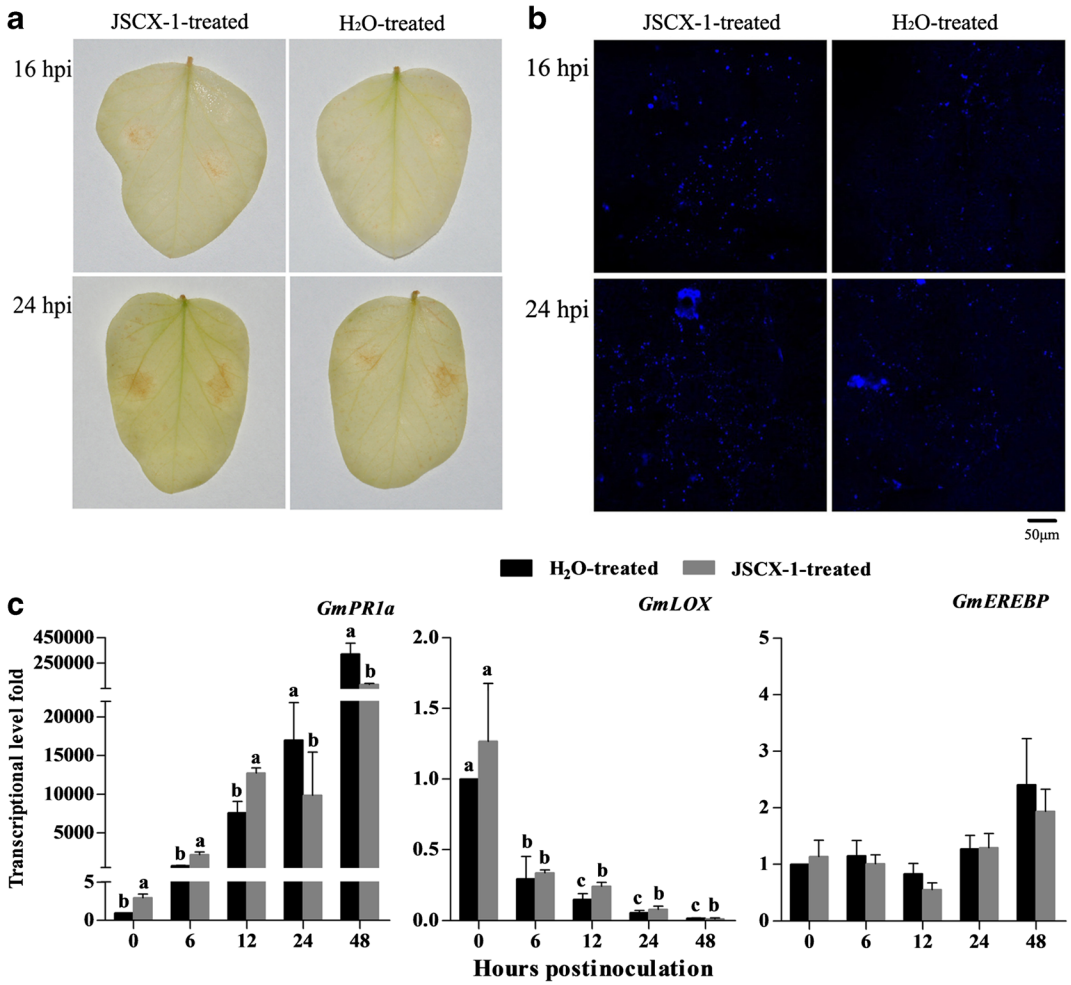

Fig. 6 JSCX-1 increased ROS production, callose deposition and expression of *GmPR1a* in soybean leaves. **a** ROS production and **b** callose deposition after *P. sojae* infection of soybean leaves. DAB (for ROS production) and aniline *blue* staining (for callose deposition) were performed at 16 hpi and 24 hpi, respectively, in JSCX-1- and H_2O-treated soybean leaves. Bar =50 μm. **c** JSCX-1 up-regulated the transcriptional level of *G. max PR1a*. Transcript levels of the *GmPR1a*, *GmLOX* and *GmEREBP* genes in JSCX-1-treated soybean leaves compared with the H_2O-treated control after *P. sojae* infection at different time intervals, measured by qRT-PCR. The experiment was repeated three times with similar results. Error bars represent standard errors from three independent RNA isolations and qRT–PCR replicates. Lowercase letters above the bars indicate significant differences between the H_2O-treated control and JSCX-1-treated soybean leaves at $P < 0.05$ level by *t*-test (left of Fig. 6c). Different lowercase letters above the bars indicate significant difference of different time intervals of each treatment at $P < 0.05$ level by Duncan's new multiple range test (middle of Fig. 6c). No significant differences of *GmEREBP* expression were found between the H_2O-treated control and JSCX-1-treated soybean leaves (right of Fig. 6c)

Many studies reported that the application of *Bacillus* spp., such as *B. subtilis*, *B. cereus*, *Bacillus licheniformis*, could manage diseases caused by *Phytophthora* species, such as *Phytophthora capsici*, *P. sojae*, *Phytophthora fragariae* var. *fragariae*, *Phytophthora infestans*, and *Phytophthora cactorum* on pepper, soybean, potato, cucumber, alfalfa, strawberry and apple (Utkhede 1984; Handelsman et al. 1990; Osburn et al. 1995; Anandhakumar and Zeller 2008; Özyilmaz and Benlioglu 2013; Maksimov et al. 2014; Khabbaz et al. 2015).

Biocontrol agents against *P. sojae* that have shown effectiveness in disease reduction include several *Bacillus* (Osburn et al. 1995), *Trichoderma* (Ayoubi et al. 2012), and *Actinomycetes* (Filonow and Lockwood 1985). Fu et al. (2011) found that *B. pumilus* B048 could suppress the occurrence of *P. sojae* on soybean plant. Application of

T. brevicompactum to soybean seeds was an effective strategy for management of damping-off, disease seveirity as well as increasing growth of treated seeds (Ayoubi et al. 2012). *Streptomyces* isolate GS93–96 reduced the percentage of dead plant caused by *P. sojae* under low pathogen inoculum, with the biocontrol efficiency of 80% (Xiao et al. 2002). However, under high pathogen inoculum, the same pathogen inoculum with in this study, application of *Streptomyces* GS93–96 could not reduce the percentage of dead plant (Xiao et al. 2002).

In the present study, the bacterial strain JSCX-1 belonging to *B. altitudinis* showed significant antagonistic activity to *P. sojae* with an inhibition ratio of 63.94 ± 3.94% (Fig. 1). Recently, reports of *B. altitudinis* having the ability to promote growth and suppress root fungal pathogens, such as *Macrophomina phaseolina* and *Thanatephorus cucumeris*, were described (Gopalakrishnan et al. 2011; Sunar et al. 2015), indicating that *B. altitudinis* was a promising BCA for controlling fungal diseases. In our study, *B. altitudinis* JSCX-1 showed significant biocontrol activity in in vivo and in vitro assays (Figs. 4 and 5), suggesting that *B. altitudinis* JSCX-1 is a new BCA against *P. sojae.* Notably, this is the first report that *B. altitudinis* is able to control *Phytophthora* rot disease.

B. altitudinis has been recently characterized as a new causative agent of bacterial soft rot on apple and pear fruit (Elbanna et al. 2014). Accordingly, we tested whether the *B. altitudinis* JSCX-1 isolated in this study could cause soft rot on these fruit. We did not observe the soft rot disease symptoms as reported on apple and pear fruit (data not shown). This illustrated that *B. altitudinis* JSCX-1 is not a pathogenic agent of the bacterial soft rot.

The production of antifungal compounds is an important way in which BCAs from *Bacillus* spp. defend against pathogens. Antifungal compounds can directly function against pathogens. For instance, antifungal metabolites produced by *B. pumilus* inhibited the mycelial growth of many species of *Aspergillus, Penicillium* and *Fusarium*, as well as the production of their respective toxic compounds. The active antifungal compounds were further characterized as either cyclic polypeptides or non-peptidic compounds (Munimbazi and Bullerman 1998). *Bacillus* sp. IBA 33 showed an inhibitory effect against the growth of *Geotrichum candidum*, the sour rot disease agent in lemon, due to the secretion of two thermo-resistant proteins (Maldonado et al. 2009). Accordingly, we tested the effects of the JSCX-1 filtrate on the growth and zoospore germination of *P. sojae* and found that JSCX-1 had negative effects on both parameters (Fig. 3). An antifungal molecule produced by *B. licheniformis* BC98 had a negative effect on *Magnaporthe grisea* and exhibited bulbous hyphae, showing a patchy and vacuolated cytoplasm (Tendulkar et al. 2007). Microscopic observations of the effects of the antagonist on *P. sojae* revealed that JSCX-1 induced morphological changes in *P. sojae* (Fig. 3). Thus, the presence of antifungal compounds may be part of the mechanism by which JSCX-1 is able to control *Phytophthora* rot. Further experimentation is needed to identify what kind of antifungal compounds play this role in JSCX-1.

Antifungal compounds could also indirectly affect the pathogen by inducing plant resistance to defend against pathogen infections (Choudhary and Johri 2009). *B. subtilis* UMAF6639 secretes lipopeptides and enables plants to prepare against powdery mildew by activating JA- and SA-dependent defense responses (García-Gutiérrez et al. 2013). In the current study, we did not measure whether the JSCX-1 filtrate possesses this ability. However, there is a great possibility that JSCX-1 has this potential. Further experiments should be performed to validity this speculation.

An array of defense responses at both the cellular and molecular levels can be triggered by pathogenic fungi in plants (Jones and Dangl 2006; Boller and He 2009). The oxidative burst and callose deposition are ubiquitous early steps in response to microbial pathogenic attacks (Bolwell et al. 2002; Underwood 2012; Ellinger et al. 2013). The accumulation of hydrogen peroxide is a characteristic early feature of the hypersensitive response (Lamb and Dixon 1997) and callose acts as a physical barrier to slow pathogen invasion (Beffa et al. 1996).

In addition, an accelerated and enhanced accumulation of hydrogen peroxide, and callose deposition, could be conducted using rhizosphere bacteria, especially plant growth promoting rhizobacteria (Conrath et al. 2002; Van Wees et al. 2008), which occur in different plants (Silva et al. 2004; Niu et al. 2011). In the present study, pre-inoculation with JSCX-1 stimulated hydrogen peroxide activity and callose deposition (Fig. 6), inferring that JSCX-1 successfully activated the cellular defenses of plant cells, conferring at least a partially resistance to *P. sojae.*

The accumulation of PR proteins is an important part of plant defense responses (Van Loon and Van Strien 1999; Van Loon et al. 2006) that has been useful for protecting against fungal pathogens in different plants (Epple et al. 1997; Van Loon and Van Strien 1999). In addition, non-pathogenic bacteria and fungi have the ability to trigger plant basal defenses. *Bacillus* spp. and their secondary metabolites can act as elicitors to induce or stimulate plant resistance, including ISR (Choudhary and Johri 2009). *Bacillus* spp. protect plants through ISR and successfully control the disease severity in many plants, such as tobacco, maize, melon and *Arabidopsis* (Choudhary and Johri 2009; García-Gutiérrez et al. 2013; Gond et al. 2015; Huang et al. 2015; Kim et al. 2015). Therefore, it is possible that the BCA isolated in the present study might act as an ISR inducer. The expression level of *GmPR1a* gene was up-regulated in our results (Fig. 6), demonstrating that the BCA was able to activate plant basal defense responses, thereby attenuating the *Phytophthora* infection. These results are similar with studies on the strains of *B. cereus* against DC3000 in *Arabidopsis* (Niu et al. 2011) and *B. amyloliquefaciens* against *Ralstonia solanacearum* in tomato (Tan et al. 2013).

ISR is typically independent of SA and is mostly dependent on the JA- and/or ET-signaling pathways (Verhagen et al. 2004; Pieterse et al. 2009). However, some ISR inducers also appear to activate an SA-dependent pathway, indicating that different signaling pathways may operate when ISR is elicited (Ryu et al. 2003; Niu et al. 2011). In the present study, we tested three genes related to the typical signaling pathways and found that the SA-regulated defense-related gene *GmPR1a* had been activated by JSCX-1, the expression of JA-regulated defense-related gene *GmLOX* was impaired in the control and JSCX-1 treatments (Fig. 6c). However, the ET-regulated defense-related gene *GmEREBP* did not show any significant difference in leaves treated with H_2O and JSCX-1 combination with *P. sojae* infection (Fig. 6c). The *PR1* gene is mostly used an indicator of systemic acquired resistance (SAR) (Van Loon and Van Strien 1999). This indicates that the disease resistance induced by JSCX-1 may be controlled by an SA-dependent signaling pathway. In many characterized examples, the SA- and JA/ET-signaling pathways mutually interact antagonistically (Koornneef and Pieterse 2008). The bacterial pathogen *Erwinia carotovora* activated genes expression through JA-dependent signaling pathway in *Arabidopsis* (Norman-Setterblad et al. 2000). However, by exogenous application of SA, *E. carotovora* induced gene expression was antagonized (Norman-Setterblad et al. 2000). The results in this study suggest that *P. sojae*-mediated SAR in soybean indeed involves this type of negative cross-talk (Fig. 6c). *P. sojae* stimulated the expression of SA-responsive gene *GmPR1a*, which lead to the suppression of JA-responsive gene *GmLOX* (Fig. 6c).

In conclusion, *B. altitudinis* JSCX-1 isolated from a healthy soybean rhizosphere triggered plant resistance against *P. sojae* by an SA-dependent signaling pathway. This indicates that JSCX is a potential biological agent and has promise in enhancing plant disease resistance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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