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Decreased incidence of disease caused by *Sclerotinia sclerotiorum* and improved plant vigor of oilseed rape with *Bacillus subtilis* Tu-100

Received: 23 November 2004 / Revised: 27 January 2005 / Accepted: 8 February 2005 / Published online: 3 March 2005
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Abstract *Sclerotinia sclerotiorum* causes serious yield losses in oilseed crops worldwide. *Bacillus subtilis* Tu-100 significantly reduced ($P \leq 0.05$) the incidence of disease caused by *S. sclerotiorum* on oilseed rape at harvest in two trials conducted in fields artificially infested with this pathogen. Mean plant dry weight was significantly greater ($P \leq 0.05$) and mean plant length was significantly greater ($P \leq 0.07$) at the seven-true-leaf stage with the Tu-100 treatment than with the control. Mean seed yield per 120 plants at harvest was significantly greater ($P \leq 0.05$) in the second field trial with treatments containing isolate Tu-100. *B. subtilis* Tu-100 also promoted the growth of hydroponically grown oilseed rape. Plants were approximately 15% greater in dry weight ($P \leq 0.0001$) and 6% greater in length ($P \leq 0.0025$) when grown in the presence of isolate Tu-100 in Hoagland's solution, compared with the noninoculated control. In gnotobiotic studies, the *lacZ*-tagged strain *B. subtilis* Tu-100(pUC18) was detected within all roots of oilseed rape. Isolate Tu-100 did not persist in the ectorhizosphere of oilseed rape. Populations of this isolate decreased from 8.5×10^8 colony-forming units (CFU) per seed to approximately 10^2 CFU in the plant ectorhizosphere within 30 days of sowing in autoclaved soil.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is distributed worldwide and causes disease on oilseed rape and many other important crops (Boland and Hall 1994; Purdy 1979). Serious yield losses can result with annual losses to *S. sclerotiorum* on oilseed rape (*Brassica napus* L.), sunflower (*Helianthus annuus* L.), and soybean [*Glycine max* (L.) Merr.] estimated at more than U.S. \$ 60×10^6 (Lu 2003). This pathogen overwinters as sclerotia in soil and generally infects plants as mycelia originating from eruptive germination of sclerotia in soil or as airborne ascospores that directly penetrate host tissue (Lu 2003). Diseases caused by this pathogen are typically initiated on above-ground plant parts (Abawi and Grogan 1979). On oilseed rape, stem lesions develop from the soil line or axils of branches or leaves. Lesions can eventually expand to girdle the stem and kill the plant (Nyvall 1979).

Oilseed rape is the major oilseed crop in the People's Republic of China, with approximately 70×10^6 ha in production (Zhao and Meng 2003). Crop rotation to nonsusceptible hosts and fungicide application are the prominent methods for control of diseases caused by *S. sclerotiorum* (Lu 2003; Yu and Zhou 1994). Although several fungicides are available, these chemicals can be expensive, ineffective, and hazardous (Lu 2003). There are also concerns regarding fungicide resistance in populations of *S. sclerotiorum* (Gossen et al. 2001).

Traditional oilseed rape breeding programs for disease resistance have been hampered by a limited gene pool (Lu 2003). Furthermore, selection of oilseed rape cultivars with low seed glucosinolate content has become a priority for plant breeders. Glucosinolates are secondary metabolites present in crops of the Brassicaceae family, some of which produce toxic compounds when degraded (Zhao and Meng 2003). It is suspected that low glucosinolate levels in seeds lead to reduced resistance to *S. sclerotiorum* and other pathogens (Liu 2000). Certain degradation products of glucosinolates, especially hydrolysates of indoyl glucosinolate, have been reported to defend plants from insects and fungal pathogens on leaves (Brown and Morra 1997; Mithen

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1992). Because of these health and environmental concerns, and the current breeding trends, alternative control measures are needed for disease caused by *S. sclerotiorum* on oilseed rape in the People's Republic of China.

Biological control of diseases caused by *S. sclerotiorum* receives considerable attention. *Coniothyrium minutans* (Campbell) and several other biological control agents suppress various diseases caused by *S. sclerotiorum* on a number of crops (Adams and Fravel 1990; Escande et al. 2002; Huang et al. 1993; Li et al. 2003; McQuilken et al. 1997). Commercial products based on *C. minutans*, developed for the control of *S. sclerotiorum* on oilseed rape and other crops, are marketed in Europe (de Vrije et al. 2001). These products are not used in the People's Republic of China, possibly due to high cost at current exchange rates. Consequently, there is a need for biological control agents for the suppression of *S. sclerotiorum* on oilseed rape that can be marketed for use in China. We report here decreased incidence of disease caused by *S. sclerotiorum* and improved plant vigor of oilseed rape in field trials in China with treatments containing *Bacillus subtilis* (Ehrenberg) isolate Tu-100.

Materials and methods

Bacterial and fungal isolates

B. subtilis Tu-100 was isolated from an oilseed rape rhizosphere (Hu et al. 1992) and shown to inhibit a number of bacterial and fungal plant pathogens on agar media (Hu, unpublished data). Isolate Tu-100 was cultured in Luria-Bertani (LB) broth or agar with or without 30 $\mu\text{g ml}^{-1}$ kanamycin. *B. subtilis* Tu-100 is naturally resistant to 30 $\mu\text{g ml}^{-1}$ kanamycin. Strain Tu-100(pUC18) was constructed by introduction of plasmid pUC18 (Promega Corp., Wuhan, People's Republic of China), containing *lacZ*, into isolate Tu-100 by electroporation (Chassy et al. 1988). Strain Tu-100(pUC18) was similar to strain Tu-100 in growth rate, colony morphology, in vitro inhibition of *S. sclerotiorum* Ss-1, and inhibition of isolate Ss-1 in detached-leaf assays (data not shown). Strain Tu-100(pUC18) was cultured on LB broth or agar plus 50 $\mu\text{g ml}^{-1}$ ampicillin. Bacterial broth cultures were incubated at 150 rpm and 28°C. *S. sclerotiorum* Ss-1 was isolated from a sclerotium formed on oilseed rape. Isolate Ss-1 was maintained on potato dextrose agar (PDA) at 4°C. All microorganisms were obtained from the culture collection of the Plant Protection Laboratory, Oil Crops Research Institute (Chinese Academy of Agricultural Sciences, Wuhan, People's Republic of China).

In vitro inhibition assays

For in vitro inhibition assays, one disk (9 mm diam.) of isolate Ss-1 (from a PDA plate) was added to the center and four disks (9 mm diam.) of Tu-100 (from a PDA plate) were added to the periphery of each fresh PDA plate. These PDA plates were incubated at 22°C for 5 days prior

to measuring the zone of inhibition of hyphal growth. For this, the distance between the bacterial colony and the edge of the Ss-1 mycelium was determined. The experiment was performed three times with five replicate PDA plates.

Detached-leaf assays

Leaves from healthy, greenhouse-grown oilseed rape (*B. napus* cv Zhong You 119) plants were detached, washed with sterile distilled water (SDW), blotted with sterile filter paper to remove excess water, and air-dried. A single leaf was detached from each plant. Leaf age, growing position, and leaf size were similar for all treatments in these experiments. Leaves were spotted with a 0.1-ml suspension of isolate Tu-100 in SDW [8.6×10^9 colony-forming units (CFU) ml^{-1}] or with 0.1 ml of SDW and incubated for 1 min. A single, 9-mm-diam. disk of Ss-1 on PDA was subsequently applied to detached leaves. Treated leaves were placed on glass rods that were positioned on moist gauze, covered with plastic wrap, and incubated at 23°C in a growth chamber. Area of hyphal growth on each detached leaf was recorded after 4 days of incubation. For this, the lengths of the long axis and short axis were averaged and the radius was determined and used in the formula: $\text{Area} = \pi(\text{radius})^2$. The experiment was performed two times with seven replicates per treatment.

Plant growth promotion assays

Oilseed rape seeds were surface-sterilized with 0.1% HgCl_2 , rinsed thoroughly with SDW, and germinated. Seedlings with 3- to 5-cm tap roots were secured with sterile cotton in holes (1.2 cm diam.) in covers of sterile containers (18.5 cm deep, 13.5 cm diam.). A suspension (0.5 ml) containing 5×10^9 CFU ml^{-1} isolate Tu-100 in LB broth, or 0.5 ml of sterile LB broth for the control, was added to 1,700 ml of sterile Hoagland's solution (Hoagland and Arnon 1938) in the containers. Containers were incubated in the greenhouse at approximately 16°C (day) and 10°C (night). An additional 300 ml of sterile Hoagland's solution was added as needed over the course of the 90-day experiment. Containers were tested periodically for contamination by plating on PDA. Mean dry weight per plant and mean plant length were determined and compared using SAS Least squares means (SAS, Cary, N.C.). The experiment was performed three times with three replicates per treatment. Data from experiments were combined prior to analysis.

Root colonization assays

Experiments to determine the colonization of internal root tissue by *B. subtilis* Tu-100(pUC18) were performed essentially as described by O'Callaghan et al. (2000). Seeds were surface-sterilized with 0.1% HgCl_2 , rinsed thoroughly with SDW, and blotted dry with sterile filter paper.

Seed (1 g) was incubated with 0.2 ml of a 24-h culture of Tu-100 (pUC18) in LB broth (9×10^{10} CFU ml⁻¹), or sterile LB broth for the control, for 20 min at 25°C. Treated seeds were sown in 1% agar plus sterile Hoagland's solution in sterile tubes (18 cm long, 1.8 cm diam.) and incubated for 21 days at 25°C (day) and 20°C (night) in the growth chamber. At sampling time, shoots were excised and roots separated from the agar. Root systems were fixed in glutaraldehyde and incubated in a solution containing 5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside (X-gal) as described by Boivin et al. (1990). Regions of roots with dark blue precipitate, due to the degradation of X-gal, were processed for light microscopy as described by Davey et al. (1993). Plant cell walls were stained with a phenolic-magenta stain (Davey et al. 1993). The experiment was performed twice with ten replicates per experiment.

For ectorhizosphere colonization experiments, oilseed rape seeds were surface-sterilized and treated with isolate Tu-100, or sterile LB broth for the control, as above. Individual oilseed rape seeds, treated with Tu-100 or sterile LB broth, were placed on top of 700 g of an autoclaved silty clay loam soil (60.4 mg kg⁻¹ P, 22.8 mg kg⁻¹ N, 147.9 mg kg⁻¹ K, 1.38% organic matter) in sterile glass bottles (18 cm deep, 7 cm diam.), covered with a 0.5-cm layer of autoclaved soil, and the bottle openings covered with sterile paper. Soil was autoclaved for 1 h at 121°C prior to use. Bottles were incubated at 25°C (day) and 20°C (night) in the growth chamber. When seedlings emerged, the paper cover was slit and the bottles were moved to the greenhouse and incubated at approximately 25°C (day) and 20°C (night). Plants were watered with sterile water as needed, through the slit in the bottle cover. At sampling time, the bottles were broken, shoots were excised at the soil line, and root systems were removed from the soil and aseptically cut into 2-cm sections. CFU of isolate Tu-100 per root segment was determined by dilution-plating individual root segments onto LB agar plus 30 µg ml⁻¹ kanamycin. The experiment was performed twice with three replicates per treatment. Data from experiments were combined for analysis.

Field trials

Field experiments were performed twice over consecutive years at the Oil Crops Research Institute. The January prior to seeding, the field (soil characteristics as in root colonization assays) was artificially infested with sclerotia of *S. sclerotiorum* Ss-1 by imbedding sclerotia in the field at a depth of 2–3 cm and a rate of ten sclerotia per replicate block. Three replicate blocks per treatment were arranged in a random complete block design surrounded by a 1-m protective belt of oilseed rape plants. Each replicate block was 3.3 × 2.0 m with ten rows planted at a density of 10 seeds m⁻¹ and rows spaced 33 cm apart. Treatments used were oilseed rape seed incubated with overnight cultures of isolate Tu-100 in LB broth or oilseed rape seed incubated

with sterile LB broth. Seeds treated with isolate Tu-100 contained approximately 8.1×10^8 CFU of isolate Tu-100 per seed. Treatments were prepared as in the ectorhizosphere colonization experiment, except the oilseed rape seed was not surface-sterilized. Field trials were conducted from October through May of each year.

When plants had seven true leaves, five plants were collected at random from each replicate block and the plant length and dry weight were determined. After this sampling, rows were thinned to 12 plants per row. Five days prior to harvest, 120 plants from each replicate block were rated for disease incidence. An oilseed rape plant was considered diseased if one-third of the branches on the plant contained one or more lesions resulting from infection by *S. sclerotiorum* or if the plant contained a lesion on the caulis (Zhou 1994). At harvest, 120 plants from each replicate block were sampled for seed fresh weight. Mean plant length, mean dry weight, mean disease incidence, and mean seed weight per 120 plants were determined and compared using SAS Least squares means. Experiments were analyzed independently.

Results

Inhibition of *S. sclerotiorum* in vitro and in detached leaves

Cultures of *B. subtilis* Tu-100 grown on PDA produced a metabolite(s) that was released into the agar and inhibited the hyphal growth of *S. sclerotiorum* Ss-1. The zones of inhibition in three separate experiments were 0.92 ± 0.12 , 0.90 ± 0.14 , and 0.96 ± 0.14 cm. Detached leaves from oilseed rape spotted with a suspension of isolate Tu-100 also had substantially less *S. sclerotiorum* Ss-1 hyphal growth than leaves spotted with SDW. Areas of *S. sclerotiorum* Ss-1 hyphal growth on leaves spotted with Tu-100 and SDW were 2.86 ± 0.49 cm² and 8.03 ± 0.61 cm², respectively, in one experiment and 3.04 ± 0.23 cm² and 8.53 ± 0.85 cm², respectively, in a second experiment.

Plant growth promotion

B. subtilis Tu-100 promoted growth by oilseed rape plants when grown hydroponically for 90 days in Hoagland's solution. Mean dry weight of oilseed rape plants grown in the presence and absence of isolate Tu-100 was 2.10 g and 1.82 g, respectively, after 90 days. Plants grown in the presence of Tu-100 were approximately 15% greater in dry weight than plants grown without this bacterium ($P \leq 0.0001$). Plants grown in the presence of isolate Tu-100 were also slightly greater in length (5%) than plants grown in the absence of this bacterium ($P \leq 0.0025$) after 90 days. Mean plant length was 23.74 cm and 22.46 cm, respectively, for oilseed rape plants grown in the presence and absence of isolate Tu-100.

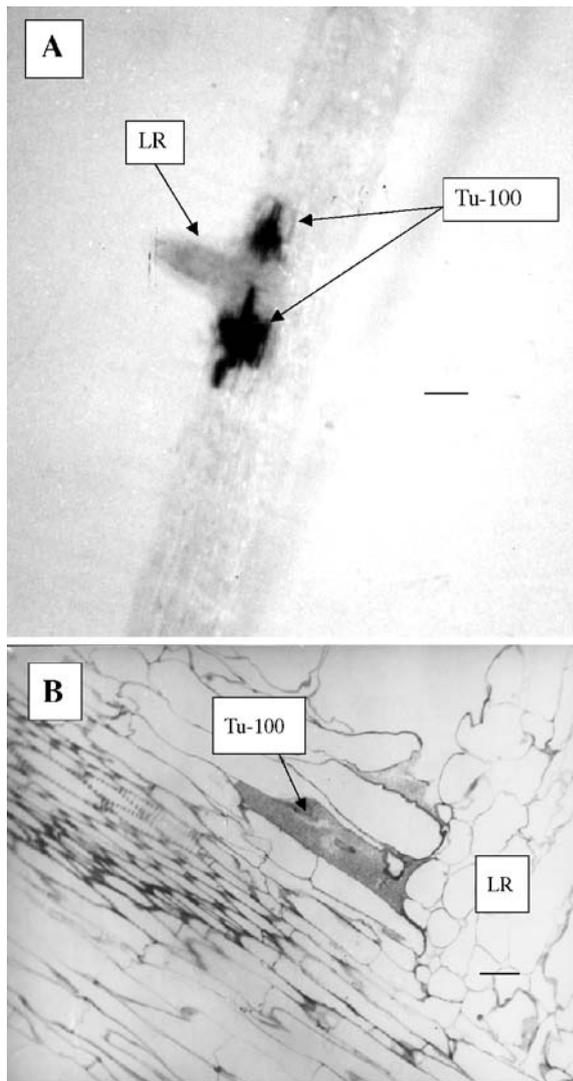


Fig. 1 Light micrographs showing colonization of internal regions of oilseed rape roots by *B. subtilis* Tu-100(pUC18). The location of *B. subtilis* Tu-100(pUC18) was determined after staining root tissue with X-gal. Degradation of X-gal by β -galactosidase, encoded by *lacZ* on pUC18, resulted in the production of a dark pigment. Root cells were stained with a phenolic-magenta stain. The size bar represents 500 μ m in **a** and 35 μ m in **b**. LR Lateral root, Tu-100*B. subtilis* Tu-100(pUC18)

Root colonization

The *lacZ*-tagged strain *B. subtilis* Tu-100(pUC18) was detected within roots of oilseed rape in gnotobiotic studies in all ten replicate plants in both experiments. The location of strain Tu-100(pUC18) was evident, due to the dark pigment formed from the degradation of X-gal by the activity of β -galactosidase encoded by *lacZ* on pUC18 (Fig. 1a). Sections of root regions under higher magnification confirmed that Tu-100(pUC18) was detected within root tissue (Fig. 1b). There was no similar dark pigment present in root sections from non-inoculated controls (data not shown). Colonization of internal regions was always in the region of lateral root formation. *B. subtilis* Tu-100 did not effectively colonize the ectorrhizosphere of oilseed rape plants in experiments conducted in autoclaved soil (Table 1). Populations of this strain decreased rapidly over 30 days, from 8.5×10^8 CFU per seed to 10^2 CFU in the entire ectorrhizosphere.

Suppression of *S. sclerotiorum* in the field

B. subtilis Tu-100 effectively suppressed *S. sclerotiorum* in field trials conducted over two consecutive years at the same location with soil artificially infested with this pathogen (Table 2). Disease incidence at harvest was significantly lower ($P \leq 0.05$) in plants treated with isolate Tu-100 than with the control. Plants were harvested approximately 7.5 months after sowing. In addition, plant vigor (determined 85 days after sowing) was improved in treatments containing isolate Tu-100. Mean dry weight per plant was 27% and 11% greater with the Tu-100 treatment in experiments 1 and 2, respectively, than with the control treatment. Mean plant length was slightly greater (significant at $P \leq 0.07$) in both experiments. Finally, mean seed yield per 120 plants at harvest was increased 6% in experiment 1 and 18% in experiment 2 with treatments containing Tu-100, as compared with the control. There was a significant increase in mean seed yield per 120 plants ($P \leq 0.05$) in the second experiment in the treatment containing isolate Tu-100, relative to the control (Table 2).

Table 1 Distribution of populations of *B. subtilis* Tu-100 in the ectorrhizosphere of oilseed rape. *B. subtilis* Tu-100 was applied as a seed treatment and seeds were sown in autoclaved soil. Seeds treated

with isolate Tu-100 contained 8.5×10^8 CFU of isolate Tu-100 per seed. ND No CFU of isolate Tu-100 detected, No root root tissue absent at this distance from the soil line

Root segment ^a (cm)	Days after planting ^b						
	3	6	9	12	15	18	30
0–2	$4.5 \pm 0 \times 10^5$	$3.9 \pm 0.3 \times 10^4$	$5.7 \pm 0.7 \times 10^3$	$2.4 \pm 0.4 \times 10^3$	$4.9 \pm 1.3 \times 10^2$	$3.2 \pm 0.4 \times 10^2$	$2.3 \pm 0.4 \times 10^2$
2–4	$8.1 \pm 0.2 \times 10^5$	$3.9 \pm 1.2 \times 10^4$	$3.5 \pm 0.2 \times 10^2$	$1.8 \pm 0.8 \times 10^2$	$2.6 \pm 0.8 \times 10^1$	ND	ND
4–6	No root	$5.2 \pm 0.5 \times 10^2$	$1.6 \pm 0.2 \times 10^2$	$3.5 \pm 0.9 \times 10^1$	ND	ND	ND
6–8	No root	No root	$3.9 \pm 0.6 \times 10^1$	ND	ND	ND	ND
8–12	No root	No root	ND	ND	ND	ND	ND

^aDistance from point of excision from the plant. Roots were excised at the soil line

^bColony-forming units of isolate Tu-100 were determined by dilution-plating root segments

Table 2 Biological control of *S. sclerotiorum* on oilseed rape with *B. subtilis* Tu-100 in field trials conducted over two consecutive years at the Oil Crops Research Institute. Numbers in columns followed by the same letter are not significantly different ($P>0.05$) in that particular experiment (SAS Least squares means). Seeds were treated either with overnight cultures of isolate Tu-100 grown in LB broth or with sterile LB broth. Seeds treated with isolate Tu-100

contained approximately 8.1×10^8 CFU of isolate Tu-100 per seed. Mean plant dry weight and mean plant length were determined approximately 85 days after planting. Five plants per replicate block were randomly sampled and used for these determinations. Mean disease incidence was determined 5 days prior to harvest. Mean seed yield per 120 plants was determined at harvest

Experiment	Treatment	Mean plant dry weight (g)	Mean plant length (cm)	Mean disease incidence	Mean seed yield per 120 plants (kg)
1	Tu-100	1.61a	19.0a	9.3a	1.60a
	Control	1.26b	16.9a	25.7b	1.51a
2	Tu-100	2.22a	28.0a	6.7a	1.91a
	Control	2.00b	25.2a	19.0b	1.61b

Discussion

B. subtilis Tu-100 has beneficial traits that show promise for use in the production of oilseed rape in China. Isolate Tu-100 significantly reduced the incidence of disease in field trials conducted over two consecutive years in fields artificially infested with *S. sclerotiorum*. Oilseed rape plants treated with isolate Tu-100 were also significantly improved in plant vigor in both trials; and seed yield was significantly greater in the second field trial.

The mechanisms by which *B. subtilis* Tu-100 suppressed the incidence of disease caused by *S. sclerotiorum* on oilseed rape and improved the plant vigor are unknown. However, cultures of isolate Tu-100 grown on PDA produced a metabolite(s) that was released into the medium and inhibited hyphal growth by this pathogen. Other isolates of *B. subtilis* are reported to inhibit bacterial and fungal pathogens in plant and animal systems (Logan 1988; Phae and Shoda 1990; Walker et al. 1998). This inhibitory activity is attributed in part to the production of antibiotic compounds, including peptides (Banerjee and Hansen 1988; Paik et al. 1998), lipopeptides (Arima et al. 1968), phenylpropanol derivatives (Pinchuk et al. 2002), and a novel phospholipid compound (Tamechiro et al. 2002). It is also possible that plant growth promotion resulted in the improved plant vigor observed in the field trials. *B. subtilis* Tu-100 promoted the growth of oilseed rape when grown hydroponically in Hoagland's solution in the absence of pathogens.

Strains of *Bacillus* are among the bacteria commonly found to colonize internal plant parts (Lilley et al. 1996; Mahaffee and Kloepper 1997). As with other isolates of *Bacillus*, *B. subtilis* Tu-100 colonized roots of oilseed rape endophytically (Fig. 1). This bacterium was detected in the internal portions of roots of every oilseed rape plant sampled in tissues associated with lateral root formation. The main entry for bacterial endophytes appears to be through wounds resulting from plant growth, such as in areas of lateral root formation (Huang 1986; Hallman et al. 1997). In contrast, isolate Tu-100 did not effectively colonize the ectorhizosphere of oilseed rape plants (Table 1). Poor colonization of the ectorhizosphere occurred in autoclaved soil, where competition with the indigenous microflora was reduced. Bacterial endophytes have been shown to be an-

tagonists of plant pathogens in vitro and to suppress plant disease. However, this report concerning *B. subtilis* Tu-100 is one of the few where disease suppression by a bacterial endophyte is confirmed with field trials (Wei et al. 1996; Hallman et al. 1997).

Future work with *B. subtilis* Tu-100 will focus on experiments needed to demonstrate the commercial viability of this bacterium for the suppression of disease caused by *S. sclerotiorum* on oilseed rape. Large-scale field trials will be conducted in China under varying environmental conditions, using a number of cultivars of this crop and a genetically diverse collection of *S. sclerotiorum* isolates. Genetic and biochemical studies will also be conducted to determine the mechanisms by which isolate Tu-100 suppresses disease caused by *S. sclerotiorum* and promotes growth by oilseed rape.

Acknowledgement This project was supported by the National Natural Science Foundation of China (Project number 39870043).

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