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The Use of *Pseudomonas fluorescens* P13 to Control Sclerotinia Stem Rot (*Sclerotinia sclerotiorum*) of Oilseed Rape

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Sclerotinia stem rot (SSR) caused by the fungus *Sclerotinia sclerotiorum* has been an increasing threat to oilseed rape (*Brassica napus* L.) cultivation. Efficient and environment-friendly treatments are much needed. Here we focus on microbial control. The *Pseudomonas fluorescens* P13 that was isolated from oilseed rape cultivation soil, proved to be a useful biocontrol strain for application. Morphology, physiological and biochemical tests and 16S rDNA analysis demonstrated that it was *P. fluorescens* P13 and that it had a broad antagonistic spectrum, significantly lessening the mycelial growth of *S. sclerotiorum* by 84.4% and suppressing sclerotial formation by 95-100%. Scanning electron microscopy studies attested that P13 deformed *S. sclerotiorum* mycelia when they were cultured together. P13 did not produce chitinase but did produce hydrogen cyanide (HCN) which was likely one of the antagonistic mechanisms. The density of P13 remained at a high level (≥10^6 CFU/ml) during 5 weeks in the rhizosphere soil and roots. P13 reduced SSR severity at least by 59% in field studies and also promoted seedling growth (p<0.05) at the seedling stage. From these data, our work provided evidence that P13 could be a good alternative biological resource for biocontrol of *S. sclerotiorum*.

***Keywords*:** *Pseudomonas fluorescens*, biocontrol, sclerotinia stem rot, mechanisms, growth promotion

Sclerotinia stem rot is caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, which has a broad ecological distribution and infects a wide variety of plant species including economically important crops (Abawi and Grogan, 1979; Adams and Ayers, 1979; Purdy, 1979; Nelson, 1984). Sclerotinia stem rot leads to huge economic losses each year. Just in the growing regions of the Yangtze River in China, the annual incidence rate of SSR reaches up to 15-30%, which accounts for nearly 60% of the total losses of oilseed rape. (http://www.farmer.com.cn/agri/zy/lzlf/201103/t20110331_622430.htm). Sclerotinia stem rot has already become one of the major agents contributing to yield loss in rape cultivation (Purdy, 1979; Gao *et al*., 2009).

S. sclerotiorum, which primarily disperses by spores and usually forms sclerotia, can infect stems, leaves, flowers and siliques and easily spread to adjacent individuals. Warm and moist environmental conditions are favorable for epidemics of this notorious fungus (Abawi and Grogan, 1979). Due to its wide host-range, effective reproduction and sclerotia, it is very difficult to control *S. sclerotiorum*. Cultivation management practices in fields, such as crop rotation, using healthy seeds, and avoiding water logging in the field, contribute to a reduction of the disease, but the effects are usually limited (Abd-Elgawad *et al*., 2010). Although the application of chemical synthetic pesticides is much more effective than cultivation management, it has negative environmental impacts and its efficacy decreases over time (Steadman, 1979; Yu *et al*., 2002).

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Biocontrol is a sustainable method for long-term application. It has been reported that some microbes such as *Coniothyrium minitans* CCTCC M203020 and *Bacillus* spp. can antagonize *S. sclerotiorum* (Shi *et al*., 2004; Li *et al*., 2006). Endophytic bacterium yc8 and endobacterium BY-2 can inhibit mycelial growth and sclerotia formation (Jiang *et al*., 2007; Ren *et al*., 2007). However, not enough information has been reported on biocontrol of SSR in oilseed rape and the effects of biocontrol agents on plant growth. Many *Pseudomonas fluorescens* strains, which are important plant growth promoting rhizobacteria (PGPR), have the ability to suppress fungal plant pathogens (Walsh *et al*., 2001; Dutta *et al*., 2008). These studies have offered the prospect of exploiting *P. fluorescens* to prevent and treat SSR, but this possibility has not yet been documented.

The aims of this research were to: (a) identify the *P. fluorescens* P13 strain and evaluate its inhibiting ability *in vitro*; (b) elucidate the probable biocontrol mechanisms of P13; (c) evaluate *in vitro* the performance of P13 and its potential for practical application.

Materials and Methods

Identification of *P. fluorescens* P13

P. fluorescens strains were isolated from the rhizosphere of oilseed rape in long-term rotation farmland in Shanghai, China, by using King's B medium (KB; peptone 20 g, glycerol 10 ml, $K_2HPO_4 \cdot 3H_2O$ 1.5 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, agar 20 g, distilled water 1,000 ml, pH 7). Plant fungal pathogens were provided by the Shanghai Research and Development Centre for Pesticides, and maintained on potato

dextrose agar (PDA; potato 200 g, glucose 20 g, agar 20 g, distilled water 1,000 ml, pH 7. They were *Bipolaris maydis* (CCAM 041043), *Botrytis cinerea* (CCAM 041128), *Colletotrichum orbiculare* (SCTCC 400538), *Fusarium graminearum* (CCTCC AF 97003), *Pyricularia oryzae* (SCTCC 400536), *Rhizoctonia solani* (ATCC 90101), *Sclerotinia sclerotiorum* (ACCC 30096). The inhibition zone test was used to detect the antagonistic spectrum of *P. fluorescens* at 25°C for 7-10 days (Li *et al*., 2005). The growth of fungal mycelia was recorded by measuring the diameter of clear zones between each fungus and *P. fluorescens*. *P. fluorescens* P13 was selected for further study because it showed the highest antagonistic capability.

The identification of *P. fluorescens* P13 was based on morphology, Gram staining, physiological and biochemical tests according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). The microscopic structure of P13 was observed by transmission electron microscope (H-600; Hitachi, Japan). Further, the bacterial identification was confirmed by the 16S rDNA sequence, which was amplified from the purified genomic DNA by polymerase chain reaction (PCR). The forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer (5′-ACGGCTACCTTGTTACGACTT-3′) were used. The DNA fragment was amplified under the following conditions: 95°C for 5 min, 40 cycles of 93°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension at 72°C for 10 min. The amplified DNA fragment was sequenced (Invitrogen, China) and submitted to GenBank (accession number: EF487999). This *P. fluorescens* P13 strain has been deposited in the China Center for Type Culture Collection (CCTCC) and its accession number is CCTCC AB 209102.

Antagonistic mechanisms of *P. fluorescens* P13

The influence of P13 on *S. sclerotiorum* growth was analyzed by scanning electron microscope (JSM-840; JEOL Ltd., Japan). The mycelial disc (5 mm) at the edge of the inhibition zone was picked out to prepare the scanning electron microscope samples as previously described (Kang, 1996). Healthy mycelia of *S. sclerotiorum* were used as a control.

The qualitative assay for chitinase activity of P13 was performed according to the chitin clear zone method as described by Xu and Gross (1986). Clearance halos around and beneath the culture were recorded at 28°C after 2-14 days.

The test for hydrogen cyanide (HCN) production was carried out as follows: 1 μl of the P13 cell suspension was spread on the fresh KB culture medium in one plate. Two drops of 20% ferrous sulfate ($FeSO_4$) solution and 10% sodium hydroxide (NaOH) solution were successively dropped onto a sterile piece of the Whatman filter paper in the other plate. Then one plate was inverted over the other and the joined plates were made airtight by pasting them with adhesive cellophane tape, and then they were cultured in 28°C. The plates without P13 served as a control. Three days later the sterile filter paper in the plate was dipped in 6 N hydrochloric acid (HCl) and rinsed with distilled water. If there were Prussian blue dots on the filter paper, it indicated that P13 could secrete HCN. All the chemical reagents were provided by Shanghai Mountain and Sea Engineering Experimental Company. The effect of HCN produced by P13 on *S. sclerotiorum* was estimated as previously described (Gupta *et al*., 2006). The growth of fungal mycelia was recorded for 14 days.

Applied potential of *P. fluorescens* P13

To study colonization, P13 with a marker was needed. P13 was found to be resistant to streptomycin (25 μg/ml) but sensitive to rifampicin, thus a rifampicin-resistant P13 strain was selected as described by Shen *et al.* (2002).

Susceptible rape seeds (*B. napus*, cv. You Yan NO.7) were obtained from Hefei Fengle Seed Industry Co., Ltd. in China. The seeds were surface sterilized with 0.1 % potassium permanganate ($KMnO_4$) for 10 min and immediately washed with sterile distilled water 3-4 times. Four seeds were planted in one black plastic pot (9.5 cm × 8.5 cm × 6.5 cm) filled with 200 g cultivation soil in the greenhouse. Each pot was inoculated with 10 ml of the rifampicin-resistant P13 suspension (10^8 CFU/ml) 1 day after planting. Sterile distilled water treatment served as a control. Each treatment contained five pots and all treatments were replicated three times. Seedlings were carefully pulled out weekly for 5 weeks and the soil adhering to the roots shaken off gently and collected. Soil dilution was prepared and the colony forming units (CFU) of the rifampicin-resistant P13 on the KB plate containing 100 μg/ml rifampicin were recorded according to Shen *et al*. (2002). Roots were washed and the last eluant was collected. The clean roots were ground with 1 ml sterile distilled water under aseptic conditions. The slurry was prepared for different dilutions. One hundred microliter of the dilution was poured onto a KB plate containing 100 μg/ml rifampicin and incubated at 28°C. Then the number of colony forming units was recorded.

Performance of *P. fluorescens* P13 in the experimental plots

Oilseed rape was planted in an experimental plot (2 m × 2 m) at Shanghai Normal University. At florescence, the P13 culture (10^8 CFU/ml) was sprayed onto the petioles and the main stems. After 3 days, 8 mm discs of *S. sclerotiorum* on the PDA plate were placed on the petioles with one disc per petiole and two petioles per seedling. The inoculated areas were wrapped with wet cotton and were sprayed with distilled water three times per day to keep the humidity. A control group was treated with identical agar plates without *S. sclerotiorum*. Disease symptoms were recorded every day. Disease severity was scored on a seven-point scale, where 0=no disease; 1=main stem dry or brown lesion area <20 mm^2; 2=main stem wilting or brown lesion area of 20 to 50 mm^2; 3=main stem splitting or brown lesion area of 50 to 100 mm^2; 4=main stem broken or brown lesion area $\geq$100 mm^2; 5=petiole rotting or water-soaked lesion on leaf $<50\%$ of a whole leaf; 6= water-soaked lesion on leaf $\geq 50\%$ or the whole plant rotting. Disease incidence was calculated as

$$\text{Disease incidence} = N_1/N_2 \times 100\%$$

N_1 means the number of diseased plants and N_2 means the number of all investigated plants. Disease index is calculated as

$$\text{Disease index} = \frac{\sum(g \times n)}{N \times 7}$$

where g is the grade of disease severity, n is the number of plants at that grade, N is the number of all investigated plants.

The P13 culture (10^8 CFU/ml) was prepared into 4, 6, 8, 10, 30, 50, 70, 90, and 100-fold dilutions using sterilized distilled water. Sterilized rape seeds were put into 9 cm Petri dishes with sterilized filter paper and immersed into the various P13 culture dilutions. Each treatment contained 30 seeds and all treatments were replicated six times. Sterile distilled water treatment served as a control. Seed germination rate was recorded after 10 days.

The seedling growth-promotion test was carried out in a 3 m×1.5 m plot. Ten mililiter of the P13 culture (10^8 CFU/ml) were poured

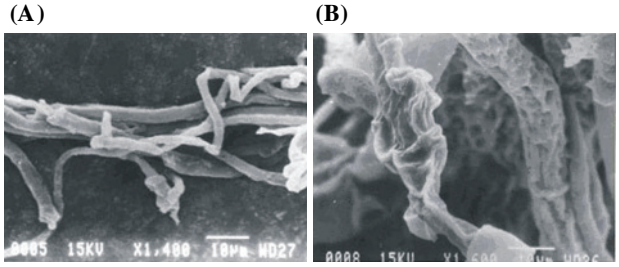

Fig. 1. Changes of *S. sclerotiorum* cultured with P13. (A) Normal mycelia without P13 (control), (B) Injured mycelia cultured with P13.

into the soil around each seedling three times before inoculating *S. sclerotiorum*. Irrigation with distilled water served as a control. Each treatment contained 10 seedlings and all treatments were replicated six times. Root length, seedling height, leaf number and dry biomass were measured after two months growth.

Statistical analysis

Analysis of variance (ANOVA) and t-test were used to test for correlation and significant differences ($P < 0.05$). Statistical analysis was performed in R (http://www.R-project.org/) with R version 2.12.2 (R Development Core Team, 2011).

Results

Identification of *P. fluorescens* P13

P. fluorescens P13 was selected from 254 strains based on its high antagonistic capability against *S. sclerotiorum in vitro*. P13 suppressed mycelial growth of *S. sclerotiorum* by 84.4% and impeded sclerotial formation by 95 to 100%, when compared with the 90 mm colony of *S. sclerotiorum* and an average of 20 sclerotia in the control. The P13 strain also restrained the growth of *Rhizoctonia solani*, *Fusarium graminearum*, *Botrytis cinerea*, *Bipolaris maydis*, and *Colletotrichum orbiculare*. Repeated experiments indicated that the antagonistic capability of the P13 strain was stable and persistent (data not shown).

P13 formed a slightly raised, light yellow, easily-picked colony with a smooth margin on KB medium. It produced yellow-green, water-soluble pigment on KB medium and gave off strong yellow-green fluorescence, which could be seen under 254 nm ultraviolet radiation. It also secreted light blue-green pigment on PDA medium. P13 lived over a wide temperature range (4-40°C) without forming spores and the optimal temperature was 26-28°C. The strain was Gram negative, aerobic and rod-shape (0.5-0.8 μm×1.4-1.7 μm) with many polar flagella about 5 μm long. P13 could liquefy gelatin and ferment glucose, sucrose, lactose and citrate but not starch. It also was catalase positive, denitrification positive and lipase negative. In addition, P13 was resistant to penicillin and did not excrete pyocyanine. The 16S rDNA sequences were obtained by PCR with the established primers. The alignment analysis showed that the 16S rDNA sequence of P13 was nearly identical to that of *P. fluorescens*. Based on these observations, the P13 strain was provisionally classified as *P. fluorescens*.

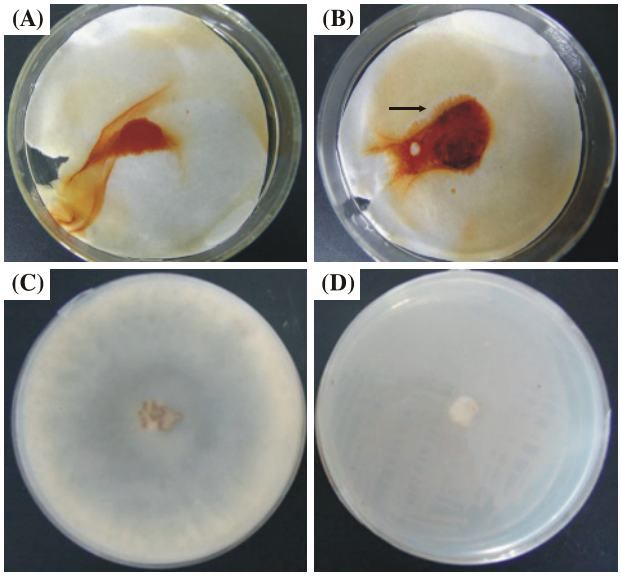

Fig. 2. Inhibiting mechanism of P13 on *S. sclerotiorum*. (A) No Prussian blue dots appear (control). (B) The arrow shows the Prussian blue dots on the filter paper. (C) *S. sclerotiorum* without P13 (control). (D) *S. sclerotiorum* stopped growing when cultured with P13.

Antagonistic mechanisms of *P. fluorescens* P13

Antagonistic interaction between P13 and *S. sclerotiorum* was investigated by scanning electron microscopy. The antagonistic effect of P13 on *S. sclerotiorum* was pronounced (Fig. 1). The healthy germinating mycelia were intact and plump (Fig. 1A), whereas the mycelia adjacent to P13 were irregularly distorted and mycelia were perforated and fractured (Fig. 1B).

A chitin clear zone did not appear in the qualitative assay for chitinase activity. Prussian blue dots were clearly detected (Fig. 2B), so it appeared likely that HCN was one of the metabolites of P13. After the culture system was improved, *S. sclerotiorum* yielded thick mycelia in the control group (Fig.

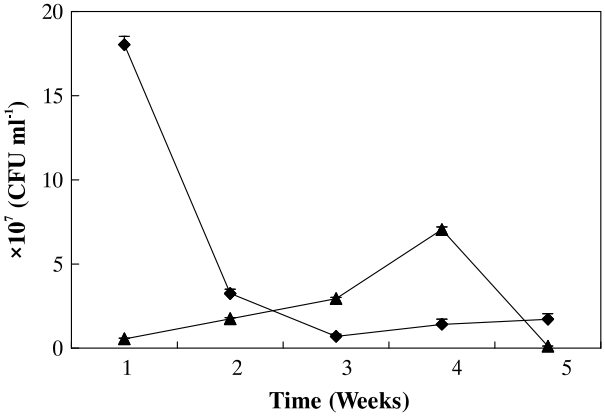

Fig. 3. Density dynamics of P13 in the rhizosphere soil and roots. Colony forming units (CFU) of the rifampicin-resistant P13 strain in the rhizosphere soil (◆) and roots (▲) were measured at the indicated times.

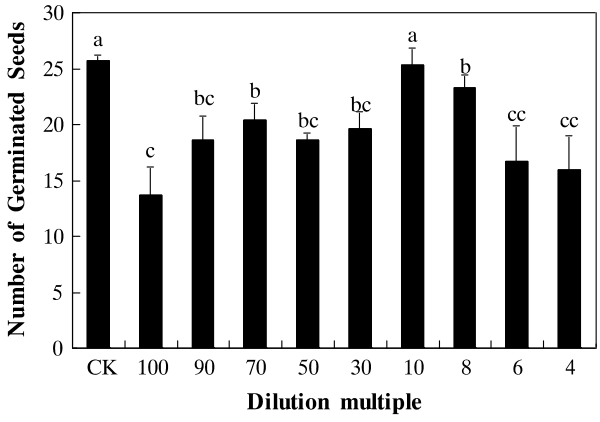

Fig. 4. The effect of P13 on seed germination. CK, control group treated without P13; 4, 6, 8, 10, 30, 50, 70, 90, and 100, different dilutions of P13 suspension. Different letters represent significant differences ($P<0.05$).

2C), whereas the mycelia stopped germinating in the treatment group during the two-week testing period (Fig. 2D). Hence, producing HCN was likely one of the biocontrol mechanisms.

Applied potential of *P. fluorescens* P13

To investigate the colonization of P13 in the rhizosphere soil and roots of oilseed rape, a rifampicin-resistant derivative was obtained. The characteristics of the rifampicin-resistant P13 were the same as that of the original strain, thus the rifampicin-resistant P13 was introduced into the rhizosphere. Colony forming units of the rifampicin-resistant P13 that survived in the rhizosphere soil and root tissues were measured at the indicated times. The population number of P13 fluctuated in the soil and roots during the 5 weeks after inoculation (Fig. 3). In the soil, the P13 density declined rapidly during the first three weeks and reached the lowest point (9.2×10^6 CFU/ml) on the third week, then rose slowly and reached 2.0×10^6 CFU/ml at the fifth week. The P13 density in the roots increased during the first four weeks with the maximum number 7.4×10^7 CFU/ml. Although it began to drop after the fourth week, the density did not go below 10^6 CFU/ml.

Performance of *P. fluorescens* P13 in the experimental plots

Disease incidence in the control group was 89%, showing that the method of inoculating *S. sclerotiorum* was effective. P13 showed fairly good biocontrol ability on sclerotinia stem rot during the flowering stage. The disease index of the treatment group and control were 19.3 and 47.1, respectively. The control effectiveness was as high as 59%.

All the concentrations, except the 10-fold dilution, clearly inhibited germination compared to the control group ($P<0.05$; Fig. 4). P13 significantly increased seedling height by 16.3% in a very short time after irrigating the soil ($P<0.05$). P13 also appeared to promote root length, leaf number and dry biomass, but the difference was not significant during the first two months ($P>0.05$).

Discussion

In this paper, we selected the P13 strain from the rhizosphere of oilseed rape, and identified it as *P. fluorescens.* It had an antagonistic effect against several familiar plant fungal pathogens. P13 showed the highest growth inhibition (14 mm) against *S. sclerotiorum*, suppressed sclerotinia formation by 95-100% *in vitro* and efficaciously decreased SSR (≥59%) *in situ* at florescence. To our knowledge, there is little information on application of *P. fluorescens* strains for prevention of SSR. Therefore, our application of P13 will add to the knowledge in this field.

P. fluorescens strains have been reported to succeed in reducing plant diseases and promoting plant growth by inducing systemic resistance (Maurhofer *et al*., 1994; Lee *et al*., 1995; Liu *et al*., 1995a, 1995b). These mechanisms include competing for nutrients and site and producing chitinase, lytic enzyme, siderophores as well as many antibiotics viz, phenazin-e-1-carboxylic acid, pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, pyocyanin, and oomycin A (Ganeshan and Kumar, 2005; Zhang *et al*., 2007; Fgaier and Eberl, 2011). The scanning electron microscope results showed that the fungal mycelia cultured with P13 were badly deformed and appeared to be degraded. Because the main component in the fungal cell walls is chitin, we investigated whether P13 could secret chitinase. The experimental results demonstrated P13 did not secrete chitinase. It was reported that there was no correlation between chitinase production by *P. putida* or *P. fluorescens* and their ability to suppress rice sheath blight (Thara and Gnanamanickam, 1994). Biocontrol bacteria may stimulate plants to secrete more chitinase when colonizing the rhizosphere, and the plant chitinase may play a more effective role in plant defense (Thara and Gnanamanickam, 1994). Whether P13 could induce plants to secrete chitinase remains to be tested. In our study, P13 produced volatile hydrogen cyanide, which stopped the growth of *S. sclerotiorum* for a long period (Fig. 2). Berry *et al*. (2010) reported that *Pseudomonas* sp. DF41 produced several compounds which might be helpful for biological control including HCN and protease, but the definite role of HCN has not been confirmed. Whether HCN is directly responsible for inhibiting SSR, and the exact mechanism(s) should be tested in controlled experiments. In addition, Wang *et al.* (2010) have discovered that P13 could excrete siderophores that might inhibit *S. sclerotiorum*. More studies are still required if we are to deeply understand the mechanisms by which P13 defeats *S. sclerotiorum*.

In large-scale application, biocontrol bacteria must colonize their targets continuously and the range of colonization should also be considered (Weller and Cook, 1983). Moreover, antagonists should not be harmful to target plants (Zachow *et al*., 2010). It has been reported that the best applied concentration of biocontrol bacteria is at least 10^8 CFU/ml (Chao *et al*., 1986). In the present study, we inoculated plants with P13 at 10^8 CFU/ml and the bacteria formed a relatively constant population (106 CFU/ml) in the rhizosphere soil and roots during 5 weeks. When repeatedly irrigating with P13 in advance of pathogen application, the plants in the treatment group had a lower incidence of disease and reduced disease symptoms, such as smaller lesions, compared to the plants in the control. This indicates that multiple inoculation

with P13 at an early stage enhances the colonization by the antagonist, which makes the antagonists more effective. Many experiments also showed that the most effective inoculation concentration for various biocontrol bacteria was 10^8 CFU/ml (Zhang *et al*., 2010). An important point we have to face is that the density of biocontrol agents will decrease over time, which is related to biotic and abiotic factors. Plant-bacteria interactions may facilitate the colonization of antagonists. Allelopathic compounds also play an important role in determining the aggregation and dispersion of the microorganisms in the rhizosphere environment (Wang *et al*., 1996). Hence, we need to do more quantitative experiments to ensure their validity before we apply biocontrol agents or products in practice. In the plot, the plants treated with P13 accumulated more above-ground biomass than the untreated plants (Fig. 4B) in the very early growing phase. This points out that P13 will have advantage as a biocontrol agent not only for preventing SSR, but also for promoting plant growth.

In this paper we reported the initial findings on using *P. fluorescens* P13 as a biocontrol agent for preventing SSR, but more studies are needed. For instance, before popularizing P13 as an alternative agent, it is better to compare the biocontrol effectiveness of P13 with other microbes or existing chemical pesticides. In addition, our team has found the materials suitable for developing the P13 biocontrol agents that will be appropriate for large-scale practical application (Li *et al*., 2009). So, considering its good performance in controlling SSR and promoting seedling growth during these trials it appears feasible to develop P13 into a biocontrol product. In conclusion, we predict that the *P. fluorescens* P13 strain is very likely to have a good future as a biocontrol agent for managing SSR.

Acknowledgements

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