

## *In Vitro* Screening for Effective Antagonists of *Sclerotium rolfsii* Sacc., A Causal Agent of Tomato Stem Rot

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### ABSTRACT

Ninety-seven soil samples were collected from five locations in Kamphaeng Saen; Nakhon Pathom. Each sample consisted of three subsamples including soils around *Sclerotium* infected, and healthy tomato plants as well as sclerotia-adhering soil. Fungi (936 isolates) and bacteria (115 isolates) were isolated through the use of serial dilution technique by placing soil suspension on the agar surface of Martin's medium, and Thornton's medium and KMB, respectively. Attained fungi could be divided in seven groups including, *Aspergillus flavus* group (80 isolates), *A. niger* group (129 isolates), *Aspergillus* group "A" (66 isolates), *Aspergillus* group "B" (62 isolates), Phycomycetous group (30 isolates), *Trichoderma-Gliocladium* group (147 isolates) and unidentified group (422 isolates). For bacteria, *Bacillus* spp. (50 isolates) and *Pseudomonas* spp. (fluorescent group) (65 isolates) were identified. Evaluation of bacteria for the inhibition of mycelial growth of *Sclerotium rolfsii* revealed that 18 isolates of *Bacillus* species and one isolate of *P. fluorescens* produced inhibition (clear) zone (2-5 mm. width). *In vitro* evaluation for the *Sclerotium* inhibitive isolates of *Trichoderma-Gliocladium* group indicated 123 isolates showing inhibitive reaction.

### INTRODUCTION

Tomato stem and root rot caused by *Sclerotium rolfsii* Sacc. is one of the most serious diseases occurring on commercial tomato varieties in Thailand. Severe losses have been found during the flowering and fruiting stage up to the harvesting period. Basal stem as well as upper roots of tomato plant are usually attacked by the fungus causing pale to dark brown lesion around the stem. Sunken cortical tissues covered with white rough mycelium or frequently with white, brown or black sclerotia are obviously observed. Infected plants will show sudden wilting while all leaves are still green or without yellowing of the lower leaves. Chemical control is effective but uneconomically accepted by most growers. Crop rotation,

sanitation, and reduced irrigation can not provide consistent control of root and stem rot caused by *S. rolfsii*. The potential means to suppress this disease is the biological control by adding antagonistic microorganisms to the soil. Several species of *Trichoderma* have been reported to be efficient antagonists of *S. rolfsii*. *T. lignorum* (Weindling, 1932), *T. viride* (Inwang and Chamswarn, 1986) and *T. harzianum* (Elad *et. al.*, 1980 ; Papavizas, 1985 ; Well *et. al.*, 1972) have been proved to be parasites of *S. rolfsii*. For bacterial antagonists, *Bacillus subtilis*, *Pseudomonas aeruginosa* (Brathwaite, 1978), *P. fluorescens* and *Streptomyces* sp. (Inwang and Chamswarn, 1986) show antagonistic ability against *S. rolfsii* by the production of inhibitive (clear) zones on the test medium.

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This paper is concerned with the isolation and grouping of fungi and bacteria from soils surrounding the tomato plants and their efficacy for inhibition of mycelial growth of *S. rolf sii* on potato dextrose agar (PDA) medium.

## MATERIALS AND METHODS

### Isolation of *Sclerotium rolf sii* Sacc.

Tomato plants showing stem rot with sudden wilting symptoms were collected from natural fields, cut into pieces of 0.5 cm. in length which were then used for isolation of *S. rolf sii*. Tissue transplanting method was performed by placing infected tissues in 10% clorox for 3 min. before transferring to PDA medium. Several days after the incubation at room temperature (25-30°C), small pieces of PDA containing mycelium of *S. rolf sii* were transferred to another PDA plates or slants for securing the pure culture.

### Isolation of Fungi and Bacteria

Ninety-seven soil samples were collected from five natural fields in Nakhon Pathom. Each soil sample consisted of three sub-soil samples including soil around *Sclerotium* - infected plants, healthy plants and sclerotia-adhering soil. All samples were ground and sieved through the screen with 1 × 1 mm<sup>2</sup> opening. Soil suspension was made by mixing 5 g-soil or 1 g-sclerotia-adhering-soil in 10 ml. of sterile distilled water (SDW), suspension was then shaken on automatic shaker for 30 min. prior diluted with SDW. Isolation of both fungi and bacteria was accomplished through the use of surface soil dilution plate (SSPP) method. To isolate fungi, 0.1 ml. -diluted soil suspension was spread over the surface of Martin's medium (Johnson and Curl, 1972). Three replicate plates were used for each dilution. After 3-4 days of incubation, fungal colonies developed on agar surface were transferred to PDA slant for further study. Thornton's medium (Johnson and Curl, 1972) was used for isolation of a bacterium, *Bacillus* spp. by following the SSDP procedure. Gram reaction of isolated bacteria

was assessed through the use of KOH solubility test (Suslow and Schroth, 1982). All bacterial isolates were cultured and maintained on Nutrient Agar (NA) slant (Schaad, 1980). In order to isolate *Pseudomonas fluorescens*, King's Medium B agar (KB) was used (Schaad, 1980).

### Evaluation of Antagonistic Activity in Laboratory

All bacterial isolates and *S. rolf sii* were cultured on PDA at room temperature for 24 and 40 hrs, respectively. Six millimeter-diameter disc of *S. rolf sii* was placed at the center of plate while four tested bacteria were spotted at 3.0 to 3.5 cm. apart from a disc.

Five millimeter-diameter discs of fungal isolates were removed from the edge of colonies of 2-day-old PAD cultures and placed on one side of a 100 mm.-plate containing PDA medium, one disc per plate. A disc of *S. rolf sii* isolate grown in the similar manner was placed on the opposite side of plate, 6 cm. apart from fungal disc.

Each treatment was replicated three times. Cultures were observed daily up to 10 days and recorded for mycelial growth and interaction.

## RESULTS

### Isolation and Grouping of Fungi and Bacteria

Isolation of fungi and bacteria from 97 soil samples (297 sub-soil samples) revealed 115 and 936 isolates of bacteria and fungi, respectively. As shown in Table 1, attained fungi could be divided in seven groups including *Aspergillus flavus* group (80 isolates), *A. niger* group (129 isolates), *Aspergillus* Group "A" (66 isolates), *Aspergillus* group "B" (62 isolates), Phycomycetous group (30 isolates), *Trichoderma-Gliocladium* group (147 isolates) and unidentified group (422 isolates). For bacteria, *Bacillus* spp. (50 isolates) and *Pseudomonas* spp. (fluorescent group)(65 isolates) were identified (Table 1). Numbers of fungal isolates achieved from the infected plant-soil were similar to those from the healthy plant-soil but markedly higher as com-

**Table 1** Numbers of bacterial and fungal isolates obtained from soils around *Sclerotium*-infected and healthy tomato plants and sclerotium-adhering soil.

| Bacteria/Fungi                       | Number of isolate <sup>1</sup> |                    |                          | Total |
|--------------------------------------|--------------------------------|--------------------|--------------------------|-------|
|                                      | Infected plant-soil            | Healthy plant-soil | Sclerotium-adhering-soil |       |
| <i>Bacteria</i> <sup>2</sup>         |                                |                    |                          |       |
| <i>Bacillus</i> spp.                 | 22                             | 23                 | 5                        | 50    |
| <i>Pseudomonas</i> spp. <sup>3</sup> | 22                             | 39                 | 4                        | 65    |
| <i>Fungi</i> <sup>4</sup>            |                                |                    |                          |       |
| <i>Aspergillus flavus</i> group      | 27                             | 34                 | 19                       | 80    |
| <i>A. niger</i> group                | 44                             | 67                 | 18                       | 129   |
| <i>Aspergillus</i> group "A"         | 26                             | 30                 | 10                       | 66    |
| <i>Aspergillus</i> group "B"         | 21                             | 39                 | 2                        | 62    |
| Phycomycetes                         | 12                             | 15                 | 3                        | 30    |
| <i>Trichoderma-Gliocladium</i> group | 75                             | 68                 | 4                        | 147   |
| Unidentified                         | 170                            | 184                | 68                       | 422   |

<sup>1</sup> All isolates were obtained from 97 soil samples (297 sub-samples).

<sup>2</sup> Thornton's Medium (Johnson and Curl, 1972) and King's Medium "B" (Schaad, 1980) were used for qualitative isolation of general bacteria and fluorescent pseudomonads, respectively.

<sup>3</sup> Consisting of fluorescent pseudomonads.

<sup>4</sup> Martin's medium (Johnson and Curl, 1972) was used for isolation of fungi.

pared with the numbers obtained from sclerotium-adhering soil (Table 1).

Evaluation of 115 bacterial isolates for the inhibition of mycelial growth of *S. rolf sii* on PDA indicated that 18 out of 50 isolates of *Bacillus* species produced inhibition (clear) zone by 1 to 7 mm. in width, whereas only one isolate of *P. fluorescens* out of 65 isolates of *Pseudomonas* spp. (fluorescent group) showed similar efficacy. Both bacteria obtained from soil samples collected from the vicinity around the infected or healthy plants provided higher numbers of inhibitive isolates as compared to those obtained from the sclerotium-adhering soil (Table 2).

*In vitro* evaluation of 147 isolates of *Trichoderma-Gliocladium* group on the inhibition

of *S. rolf sii* on PDA indicated that 24 isolates not only failed to inhibit but were also overgrown completely by mycelium of *S. rolf sii* (Figure 1. 1 A-C). Only three isolates strongly inhibited mycelial growth of *S. rolf sii* and also overgrew on the pathogen's colony (Figure 1. 5 A-C) whereas 8 isolates could inhibit mycelial growth without overgrowing of the pathogen's colony (Figure 1. 4 A-C). Seventy-four isolates of *Trichoderma-Gliocladium* group provided inhibitive reaction at the early meeting with *S. rolf sii*, however several days later, they were partially overgrown by mycelium of pathogen (Figure 1. 2 A-C). Similar reaction could be observed on eighty testing isolates of which their colonies were 40 to 80% overgrown by mycelia of *S. rolf sii* (Figure 1. 3 A-C) (Table 3).

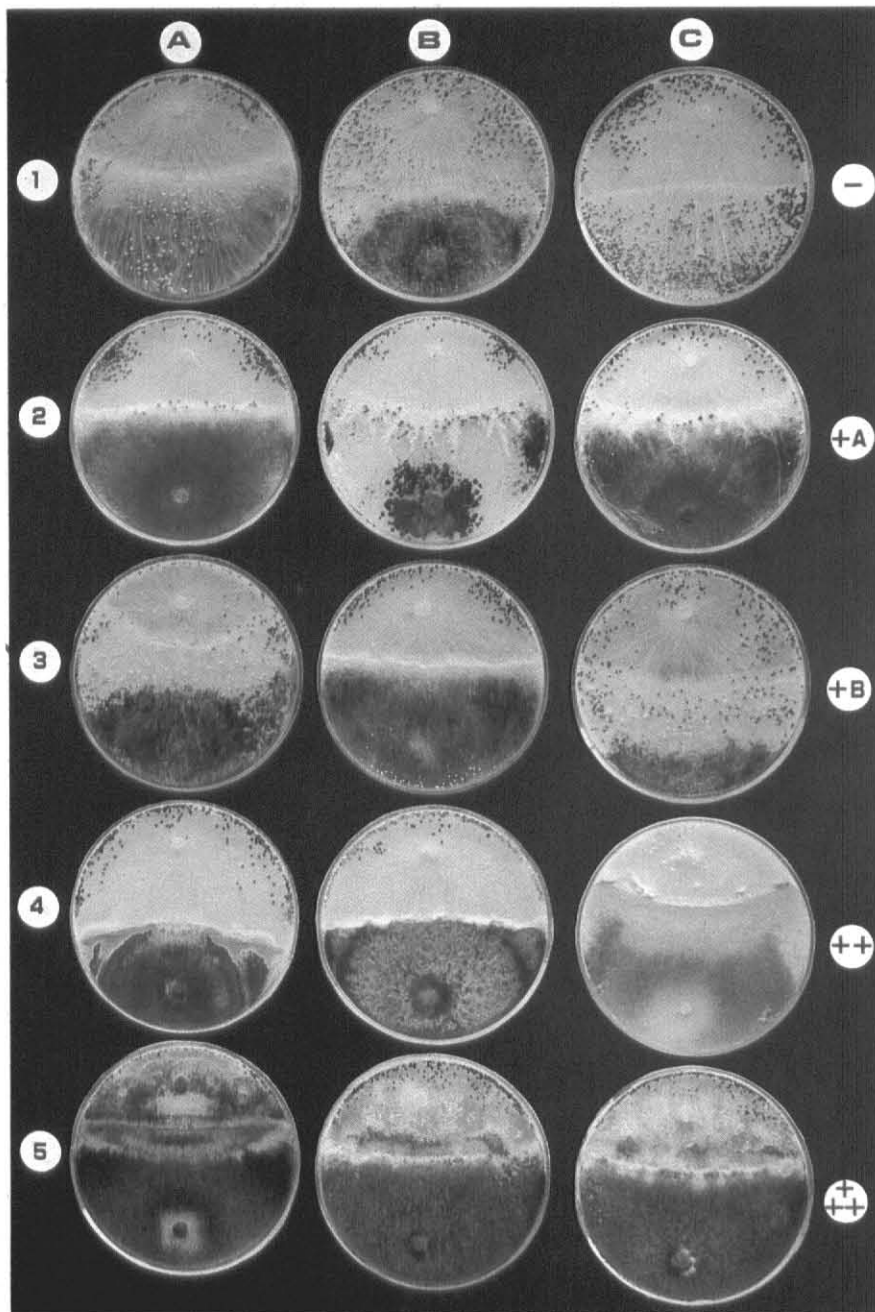


Figure 1 Types of reactions between *Sclerotium rolfsii* (upper disc) and testing isolates of *Trichoderma-Gliocladium* group (lower disc) 7 days after placing on PDA as dual culture.

1A = CF-30  
 2A = CF-117  
 3A = CF-133  
 4A = CF-22  
 5A = CF-6

1B = CF-3  
 2B = CF-106  
 3B = CF-12  
 4B = CF-21  
 5B = CF-28

1C = CF-119  
 2C = CF-5  
 3C = CF-1  
 4C = CF-121  
 5C = CF-31

**Table 2 Reactions of bacteria on mycelial growth of *Sclerotium rolfsii* when assayed on potato dextrose agar (PDA) and incubated for 3 days.**

| Source of<br>bacterium   | Number of isolated/Reaction <sup>1</sup> |   |    |     |                  |   |    |     |
|--------------------------|--|---|----|-----|------------------|---|----|-----|
|                          | Bacillus spp.                            |   |    |     | Pseudomonas spp. |   |    |     |
|                          | —  | + | ++ | +++ | —                | + | ++ | +++ |
| Sclerotium-adhering-soil | 4  | 0 | 1  | 0   | 4                | 0 | 0  | 0   |
| Infected plant-soil      | 14                                       | 4 | 2  | 4   | 22               | 0 | 0  | 0   |
| Healthy plant-soil       | 14                                       | 3 | 3  | 1   | 38               | 0 | 0  | 1   |
| Total                    | 32                                       | 7 | 6  | 5   | 64               | 0 | 0  | 1   |

<sup>1</sup> Reactions were identified by measuring the width of inhibition (clear) zone occurred between the edges of colonies of the pathogen and testing bacteria.

- No inhibition zone, *S. rolfsii* overgrows bacterial colony
- + Inhibition zone 1-3 mm.
- ++ Inhibition zone 3-5 mm.
- +++ Inhibition zone > 5 mm.

**Table 3 Reactions<sup>1</sup> of *Trichoderma-Gliocladium* group on mycelial growth of *Sclerotium rolfsii* when assayed on potato dextrose agar (PDA), 7 days after incubation.**

| Source of<br>fungus          | Number of isolate |    |    |    |     | Total |
|------------------------------|-------------------|----|----|----|-----|-------|
|                              | —                 | +a | +b | ++ | +++ |       |
| Sclerotium-<br>adhering-soil | 1                 | 3  | 0  | 0  | 0   | 4     |
| Infected plant-soil          | 12                | 39 | 3  | 19 | 3   | 76    |
| Healthy plant-soil           | 11                | 32 | 5  | 19 | 0   | 67    |
| Total                        | 24                | 74 | 8  | 38 | 3   |       |

<sup>1</sup> Reactions were classified by observing mycelial growth patterns when colony of testing fungus met with colony of *S. rolfsii*

- Testing fungus is overgrown by *S. rolfsii* mycelium.
- +a Mycelium of *S. rolfsii* partially overgrows the colony of testing fungus.
- +b Mycelium of *S. rolfsii* overgrows approximately 40 to 80% of the colony of testing fungus.
- ++ Mycelial growth of both testing fungus and *S. rolfsii* is ceased at their meeting lines.
- +++ Testing fungus inhibits mycelial growth and overgrows colony of *S. rolfsii*.

## DISCUSSION

Generally, there is a suggestion that antagonists should be sought in areas where the disease can not develop, despite the presence of a susceptible host, rather than where the disease occurs (Cook and Baker, 1983 ; Baker and Cook, 1974). However, in this study, higher numbers of promising antagonistic bacteria (*Bacillus* spp., *Pseudomonas* spp.) and fungi (*Trichoderma* pp. and *Gliocladium* spp.) were achieved from soil samples collected from the vicinities of infected plants rather than from the healthy plant soils. Mycelium of *S. rolfsii* in soil or on infected roots or stems as well as sclerotial exudates served as the baits or food bases for rapid development and multiplication of antagonists may explain this expression. For an exceptional case, only one isolate of effective antagonistic pseudomonad was obtained from soil around the healthy plants. Less numbers of fungi and bacteria accomplished from sclerotia-adhering-soil was probably due to less amount of sclerotia and their adhering soil used in soil dilution preparation (1 g/10 ml.). Moreover, sclerotia collected from soil or on lesions of infected plants were not appropriately deteriorated (Smith, 1972).

For isolation of soil fungi and bacteria, the SSDP method yielded so many distinct colonies on the agar surface of each medium. Thornton's medium was an efficient medium for bacterial isolation especially *Bacillus* spp. while Martin's medium was suitable for isolation of *Trichoderma-Gliocladium* group as previously described by Inwang and Chamswarn (1986).

At least five types of reaction were produced after challenging the testing fungi with *S. rolfsii* on PDA agar surface. Overgrowing of mycelium on the opposed fungal colony was a major criterion for determination of antagonistic effectiveness. By following this method, several isolates of *Trichoderma-Gliocladium* group were found to be highly antagonistic (+ + +) against *S. rolfsii* (Figure 1. 5 A-C). However, some testing fungal

isolates with moderate antagonistic reaction (+ +) could markedly reduce the number of sclerotia produced on the agar surface (Figure 1. 4C). Integrated use of those isolates of *Trichoderma-Gliocladium* group which possess either overgrowing capability and ability to inhibit or reduce sclerotial production may provide the promising mean for the control of tomato root and stem rot.

Expression of inhibition (clear) zone between the colony margins of testing bacteria and *S. rolfsii* indicated the production of some antibiotic substances by the bacteria. It is of interest to note that higher numbers of isolates with strong antagonistic reaction (+ + +) are *Bacillus* spp. rather than *Pseudomonas* spp.

Further experiments should be performed especially on the evaluation of all antagonists against *Sclerotium* stem and root rot under greenhouse as well as field conditions. *In vitro* testing of some *Aspergillus* spp. and some fast growing isolates in unidentified group for the inhibition of mycelial growth of *S. rolfsii* and species identification of *Trichoderma* and *Gliocladium* are also needed.

## CONCLUSION

By using serial dilution technique, fungi (936 isolates) and bacteria (115 isolates) were isolated from soils samples collected from five tomato fields. *In vitro* screening of those isolates against mycelial growth of *Sclerotium rolfsii* revealed that *Bacillus* spp. (18 isolates) and *Pseudomonas fluorescens* (1 isolate) produced inhibition (clear) zone of 1-7 mm. in width. *Trichoderma-Gliocladium* group (123 isolates) could inhibit mycelial growth of *S. rolfsii*. Among these, only three isolates showed strong antagonistic reaction.

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