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Production of Thaxtomin A by Korean Isolates of *Streptomyces turgidiscabies* and Their Involvement in Pathogenicity

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Crude extracts of pathogenic *Streptomyces turgidiscabies* isolates produced necrotic reaction on potato slices. Necrosis was first visible 24 hr after application and increased in severity over several days. However, necrosis was not observed when crude extracts of nonpathogenic strains were applied onto tuber slices. Presence of thaxtomin A from crude extracts of pathogenic *S. turgidiscabies* was identified by thin layer chromatography and high performance liquid chromatography. Nonpathgenic strains did not produce thaxtomin A in oatmeal broth. Inoculation of potato tuber slices with thaxtomin A partially purified from crude extract of a pathogenic *S. turgidiscabies* ST5 reproduced necrotic reaction suggesting that thaxtomin A is a pathogenicity determinant in *S. turgidiscabies*.

Keywords : potato scab, *Streptomyces turgidiscabies*, thaxtomin A.

Potato scab disease represents a main economic problem in potato production (Faucher et al., 1993; Faucher et al., 1995; Goyer et al., 1996; Healy et al., 1991; Kim et al., 1996). The disease is characterized by shallow, raised or deep-pitted corky lesions on potato tubers (Loria et al., 1997). *Streptomyces scabies* is the predominant causal agent (Lambert et al., 1989) and other *Streptomyces* species (Doering-Saad et al., 1992; Goyer et al., 1996) such as *S. acdiscabies* (Lambert et al., 1989) and *S. caviscabies* (Goyer et al., 1996) are also known to cause the same symptoms on potato tubers.

Thaxtomins, 4-nitroindol-3-yl-containing 2,5-dioxopiperazines, are known to be associated with potato common scab disease caused by *Streptomyces* species (King et al., 1989a). Host interaction studies involving *S. scabies* and *S. acidiscabies* determined that thaxtomins play an important role in the pathogenicity of these organisms (King et al., 1991). Of these, thaxtomin A is the predominant phytotoxin produced by both *S. scabies* and *S. acidiscabies* along with about nine minor thaxtomins (King et al., 1996; Laszlo et al., 1997; Leiner et al., 1996). A number of studies have demonstrated a positive correlation between thaxtomin A production and pathogenicity (King et al., 1989a; King et al., 1989b; Loria et al., 1995). Recently *Sreptomyces turgidiscabies* that was first reported in Japan (Miyajima et al., 1998) was also observed in Korea to cause the common scabs on potato tubers (Kim et al., 1999). This study describes thaxtomin A production by Korean *S. turgidiscabies* strains and its involvement of pathogenicity in potato scab disease.

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Materials and Methods

Bacterial strains and media. The pathogenic strains used in this study were *S. trugidiscabies* ST1, ST3, ST4, ST5, ST6 and ATCC 700248. The nonpathogenic strains were *S. turgidiscabies* ST2 and ST7 (Table 1). All strains were isolated from potato scab lesions and pathogenicity was confirmed in greenhouse studies (Table 2) using the methods described by Loria and Kempter (1986) and potato tuber slice assay (Table 2). Bacterial strains were grown in oatmeal broth (OMB) or oatmeal agar (OMA). OMB was prepared by boiling rolled oats (40 g/ 600 ml) in distilled water for 20 min and straining through cheesecloth. ZnSO4 · $7H_2O$ stock solution (1 g/ 100 ml) was added, the volume was brought up to 1 liter and the pH was adjust to 7.2 before autoclaving. For OMA, agar (1.5%) was added to OMB.

Table 1. *Streptomyces turgidiscabies* strains used in this study

Bacterial strains	Origin of strains	Host
ST1	Suwon, Kyungki-Do	potato
ST2	Suwon, Kyungki-Do	potato
ST3	Suwon, Kyungki-Do	potato
ST4	Jinbu, Kangwon-Do	potato
ST5	Hoengke, Kangwon-Do	potato
ST6	Daechung, Cheju Island	potato
ST7	Iksan, Chunbuk	potato
ATCC 700248[a]	Hokkaido, Japan	potato

[a] American Type Culture Collection of *Streptomyces turgidiscabies*

Table 2. The pathogenicity of *Streptomyces turgidiscabies* strains on potato tuber

Bacterial strains	Pathogenicity	
	Greenhouse test	Potato tuber slices
ST1	+[a]	++
ST2	–	–
ST3	++	+
ST4	+	+
ST5	++	++
ST6	+	+
ST7	–	–
ATCC700248	++	++

[a] ++, strongly pathogenic; +, moderately pathogenic; –, non-pathogenic

Extraction and thin layer chromatography of thaxtomin A. Authentic thaxtomin A was provided by Akira Ogoshi at Hokkaido University, Japan. Methods for extraction, purification and identification of thaxtomin A were modification of those described by King et al. (1989b) and Fumio Tanaka et al. (1997). OMB (100 ml) was inoculated with bacterial strains and incubated at 28°C on a rotary shaker (150-180 rpm) for 5 days. Cells were removed from the liquid cultures by centrifugation and were filtered through Whatman NO. 1 filter paper. The filtered liquid cultures were extracted twice with equal volumes of chloroform. The chloroform extracts that remained when the chloroform was evaporated to dryness was stored in the dark at 4°C until used for assays. Crude extracts were made by dissolving dried chloroform extracts in 1 ml of chloroform again and loaded onto precoated 0.25 mm Merk silica-gel 60 F_{254} thin layer chromatography (TLC). Yellow bands comigrating with authentic thaxtomin A standard were recorded.

Purification and high performance liquid chromatography of thaxtomin A. For purification of thaxtomin A from strain ST5, the yellow band on comigrating with authentic thaxtomin A onto precoated 0.25 mm Merk silica-gel 60 F_{254} TLC was scraped off and eluted with methanol and chloroform (1 : 9). The yellow band eluted was concentrated to dryness, dissolved in 1 ml of methanol and used in potato tuber slice assay to assess its necrotic ability. Presence of thaxtomin A in crude extracts from pathogenic strains was identified by high performance liquid chromatography (HPLC) using a Shimadzu 10A system with a symmetry C18 column (Waters®, 3.9×150 mm). Thaxtomin A was eluted with a 25% acetonitrile over 20 min and monitored at 380 nm.

Evaluation of pathogenicity in greenhouse. Pathogenicity tests were carried out according to Loria and Kempter with some modifications (1986). Inoculum was prepared by adding 5 ml of sterile water to sporulating cultures growing on OMA plates. Two milliliters of the suspension were added to Erlenmeyer flasks (500 ml) containing 50 ml of potato-dextrose broth (PDB, Difco Laboratories, Detroit, MI, USA); cultures were then incubated for 2 weeks at 30°C. Sterile distilled water (250 ml) was added to each flask to form a spore suspension. Potato tubers were cut from grown in scab-susceptible Daejima, planted in the 19-cm-diameter pots containing a peat/vermiculite based growing medium and grown in greenhouse. Nutrients in the medium were supplemented with weekly applications of a soluble fertilizer. Greenhouse temperatures were about 24±5°C. Three potato tubers were placed in each pot, which as considered an experimental unit. Each pot was throughly drenched with 40 ml of the spore suspension, harvested 21 days after inoculation, and evaluated for scab infection.

Potato tuber slice assay. The potato tuber slice assay was used to assess the necrotic abilities of *S. turgidiscabies,* crude extracts and the partially purified thaxtomin A. Cultures of test strains were grown on OMA for 5-7 days at 28°C and agar plugs from the sporulating colonies were inverted onto the tuber slices from scab-susceptible cultivar Dejima tubers, which were surface sterilized in 0.5% NaOCl. Tuber slices were incubated in a moist chamber at 22-24°C in the dark. Potato tuber assays were repeated three to five times and necrosis and collapse of tuber cells under and surrounding agar plugs were observed. Potato slice assays were also used to test necrotic abilities of crude extracts and thaxtomin A; 10 μl of crude extracts and 10 pico M of partially purified thaxtomin A were separately inoculated on the tuber slices and collapse of tuber cells was observed.

Results and Discussion

At least three species of *Streptomyces* have been reported to cause scab disease on potato tuber in Korea (Kim et al., 1996; Kim et al., 1999). Two of these, *S. scabies* and *S. acidiscabies*, are well-known as potato pathogens. A third species, *S. turgidiscabies,* was first described in Japan (Miyajima et al., 1998) and has been recently observed in Korea as well (Kim et al., 1999). This species is distinct from *S. scabies* and *S. acidiscabies* based on physiological, morphological, DNA-DNA hybridization and ribosomal analyses (Miyajima et al., 1998). However, similarities in host range and disease symptomology for three *Streptomyces* species suggest a possible common mechanism of pathogenicity.

Intensive studies have showed that a phytotoxic secondary metabolite, thaxtomin A, originally purified from *S. scabies*-infected plant tissue, was also produced by *S. ascidiscabies* (Loria and Kempter, 1986; Loria et al., 1995). Thaxtomin A production has been positively correlated with plant pathogenicity of *S. scabies* and *S. acidiscabies.* Meanwhile pathogenicity of *S. turgidiscabies* is obscure because of its recent observation only in Japan and Korea. In this study, a total of 7 strains of *S. turgidiscabies* were screened for pathogenicity by the greenhouse and tuber slice assays (Table 2). The isolates pathogenic on potato tubers in greenhouse assay also produced necrotic reactions on tuber slices with a positive correlation between the two pathogenicity assays. The tuber slice assay appears to be a convenient and efficient technique for assessing pathogenicity of *Streptomyces* strains.

Crude extract of pathogenic *S. turgidiscabies* grown in OMB produced necrotic reaction on potato slices. Necrosis

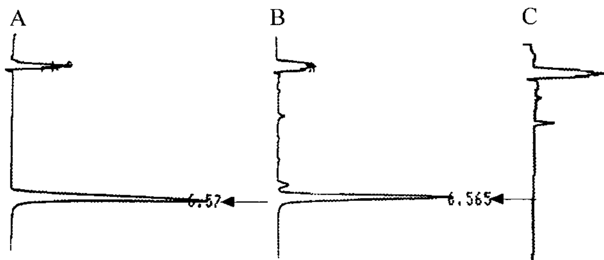

Fig. 1. Identification of thaxtomin A from pathogenic strain ST5 of *S. turgidiscabies* by HPLC analysis. A: an authentic thaxtomin A, B: a pathogenic strain, C: a non-pathogenic strain. Arrows indicate thaxtomin A peaks.

was first visible 24 hr after application and increased in severity over several days. Necrosis was not observed when crude extracts of OMB cultures of non-pathogenic strains were applied to tuber slices. The tuber slice assay results clearly indicated that cell free extracts of pathogenic strains contain phytotoxic compounds for necrosis on tubers. Consequently, attempts were made to verify the role of these compounds as incitants of the common scab disease by TLC and HPLC assays. All pathogenic strains of *S. turgidiscabies* produced a yellow component comigrating with authentic thaxtomin A and other compounds on TLC. Two non pathogenic strains, ST2 and ST7 did not produce any compounds comigrating with a authentic thaxtomin A, suggesting a positive correlation between pathogenicity and production of thaxtomin A. Consistent with our expectations, thaxtomin A was detected by HPLC analysis from pathogenic stains positive for production of thaxtomin A on TLC (Fig. 1). Accordingly, two non-pathogenic strains of ST2 and ST7 were negative for production of thaxtomin A by HPLC analysis. On the other hand, intensity of a yellow component comigrating with authentic thaxtomin A on TLC was not correlated with the quantitiy of thaxtomin A on HPLC, however. The result clearly displays HPLC analysis is more efficient and sensitive than TLC method for identifying and quantifying thaxtomin A.

A tuber slice assay was also used to confirm the phytotoxicity of the partially purified thaxtomin A. Partially purified thaxtomin A from ST5 strain produced a necrotic reaction on tuber slices. The pattern and type of necrosis was identical to that produced by inoculation of crude extracts from pathogenic strains. Thaxtomin A was again the phytotoxic compound involved in pathogenicity of scab disease and other data combined with this study proved that all three species of *S. scabies, S. acidiscabies* and *S. turgidiscabies* produce thaxtomin A therein. These three species are morphologically, physiologically and genetically diverse (Takeuchi et al., 1996; Miyajima et al., 1998), suggesting that pathogenicity genes required for thaxtomin A production have been transferred among species. Gene exchange among *Streptomyces* strains has been reported (Charter and Hopwood, 1989).

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