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Genetic Study of Soybean Sudden Death Syndrome Pathogen (*Fusarium solani* f. sp. *glycines*) isolated from Geographically Different Fields based on RFLPs of Mitochondrial DNA

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ASTRACT: From the soils of soybean fields in Cotton Branch Station (CBS) and Pine Tree Station (PTS), Arkansas, USA, various single spore isloates of sudden death syndrome (SDS) pathogen were obtained on modified Nash & Snyder's medium (MNSM) with dilution plating technique and transferred to potato dextrose agar (PDA) medium to identify the cultural colony shape. The colony shapes of these isolates resembled *F. solani* isolate 171 which was white and chalky shaped on MNSM and most of them had unique form of morphology which produced white margin and blue center colony on PDA. Although, some of these isolates had more dark blue or showed slightly different color, all isolates that were selected randomly for greenhouse inoculation assay produced typical foliar symptoms on leaves of soybean, Hartz 6686. To determine the genetic differences among the isolates, mitochondrial DNA restriction fragment length polymorphism (RFLP) was conducted with fourty isolates from both fields, using mtDNA probes, 2U18 and 4U40, derived from *Colletotrichum orbiculare*. We obtained distinctive RFLPs in each treatment of restriction enzyme, *Eco*RI and *Hae*III. Isolates, 11-2-5 and 14-3-1-1, from CBS and isolates, 104-3-1-2 and 701-1-5-1, from PTS showed different band patterns from 171 in both or in either treatment of restriction enzymes. Even if some of these isolates showed heterogeneous, they were more closer to 171 than PN603. And, also, rest of the thirty-six isolates had exactly same polymorphisms as 171 in each treatment of restriction enzyme. Although, some of the isolates showed the different morphological shape on PDA and slightly different band patterns on RFLPs, all of the isolates selected on MNSM due to their distinctive colony shape from other fungi produced the typical foliar symptoms on soybean leaves in greenhouse inoculation assay. It might be suggested that these isolates were not genetically different from check isolate 171 and they were unique strain of *F. solani*.

Keywords : mitochondria DNA probes, RFLPs, enhanced chemiluminescent gene detection (ECL), Southern blotting, soybean, sudden death syndrome, *Fusarium solani*.

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Sudden death syndrome (SDS), a soil born disease of soybean (*Glycine max* (L.) Merr.) caused by the *Fusarium solani* f. sp. *glycines*, was first observed in Arkansas, USA, in 1971 and was named by Hirrel in 1983 in response to perceived rapidity with which above ground leaf symptom developed. In the United States, SDS has been reported in other states including many important soybean fields of southern and northern areas (Doupnik, 1993; Wrather *et al.*, 1995). SDS also reported to occur in other countries, Argentina, Brazil, and Canada(Anderson & Tenuta, 1998; Ivancovich *et al.*, 1996; Nakajima *et al.*, 1994).

In symptomatic plants, SDS produces necrotic streaks of interveinal leaves and, eventually, result in defoliation with the petioles remaining attached to the stem. The unique foliar symptoms of SDS, appear to be produced by a 17 kda polypeptide toxin since the fungus is confined to the root system (Jin *et al.*, 1993). Cultivar reactions to the toxin are similar to the reactions to SDS in greenhouse inoculation tests.

The typical root symptoms of SDS include root rot, crown necrosis, vascular discoloration of roots and stems. In greenhouse inoculation assay, root rot symptom was observed before leaf symptom appeared and there were no significant differences among cultivars in average proportions of root rot caused by this pathogen (Cho *et al.*, 1999). External symptoms of SDS are similar to those of brown stem rot caused by *Phialophora gregata*, fusarium wilt by *F. oxysporum*, stem canker by *Diapothe phaseolorum*, and red crown rot by *Cylindrocladium*. However, pith of plants infected with *F. solani* f. sp. *glycines* remains white (Hirrel, 1983).

The SDS pathogen appears to be a unique strain of the fungus. Based on pathogenicity tests, Roy *et al.* (1989) designated the SDS pathogen as *F. solani* f. sp. *glycines*. Rupe *et al.* (1996) also reported that causal fungus as blue pigment strain of *F. solani* is considered to be the same as *F. solani* f. sp. *glycines*. Various isolates of *F. solani* produced root rot and leaf symptoms similar to SDS in greenhouse and field but the symptoms varied greatly in intensity. *F. solani* recov-

ered from the roots and crown roots of inoculated plants and infested plants in the field but not from stem tissue above the soil line.

On PDA, *F. solani* f. sp. *glycines* grows slowly producing an appressed colony with a blue center and a white margin. Numerous three to five celled macroconidia are produced, but microconidia are rare. Macroconidia measure 34 to 66 μm 3.9 to 5.0 μm. Chlamydospores are produced in hyphae and in macroconidia (Lowlence *et al.*, 1988; Rupe, 1989).

Many studies have been conducted to identify the soil borne disease and to determine the distribution of the pathogen in soils and plants with selective media. In many results, *F. solani* f. sp. *glycines* appeares to be closely related to another soil borne pathogen *F. solani* f. sp. *phaseoli*, for which a selective medium has been developed. The two pathogens, *F. solani* f. sp. *glycines* and *F. solani* f. sp. *phaseoli* do share many cultural characteristics and produce a colony distinctive from any other soil fungi on selective medium developed by Nash and Snyder (1962). However, *F. solani* f. sp. *glycines* caused typical SDS foliar symptomes on soybean in greenhouse and field inoculation assay; but *F. solani* f. sp. *phaseoli* did not (Roy, 1997). Using dilution plating technique and MNSM including antibiotics and pentachloronitrobenzene (PCNB), soil distributions of *F. solani* with this colony type have been determined in soils from fields with SDS (Rupe *et al.*, 1993; 1999).

Analysis of mitochondrial, ribosomal, and genomic DNA of *F. solani* f. sp. *glycines* have found this fungus to be in an extremely homologous group of fungi and distinct from other, non-SDS *F. solani* strains, although it may be closely related to *F. solani* f. sp. *phaseoli* (Achenbach *et al.*, 1996; Li *et al.* 1998; O'Donnell and Gray, 1995; Rupe *et al.*, 1993). Using RFLP technique, other species of *Fusarium* was studied to determine genetic relationships among isolates collected from different areas (Marlatt *et al.*, 1996). In this study, restricted DNA fragments of *F. oxysporum* f. sp. *licopersici* were hybridized with two large, non overlapping, mitochondrial DNA clones, 2U18 and 4U40, and detected by an enhanced chemiluminescent gene detection technique. These mtDNA probes, 2U18 and 4U40, were derived from mtDNA clones (38.6 kbp) of *Colletotrichum orbiculare* and their size were 10.1 kbp and 13.7 kbp, respectively (Correll *et al.*, 1993).

The objects of this study were : 1) using MNSM, to identify the SDS pathogen from the soils collected in geographically different areas, 2) to determine virulences of the isolates in greenhouse inoculation assay, and 3) using RFLPs with mtDNA probes, to determine the genetic differences among these isolates selected on this medium.

MATERIALS AND METHODS

Isolation of SDS pathogen

The colonies that resembled *F. solani* f. sp. *glycines*, on MNSM were isolated from the soil samples in each of two soybean fields with a history of SDS on 27 July 1997. The fields were located in PTS, Colt and CBS, Marianna, Arkansas, USA. The soil at PTS was a Crowley silt loam and the soil at CBS was a Loring silt loam. MNSM was consisted of 2% (w/v) agar, 1.5% (w/v) peptone, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$ with deionized water. After autoclaving and cooling to 45°C, 0.01% (w/v) streptomycin, 0.024% (w/v) pentachloronitrobenzene (PCNB, 75% WP), 0.01% (w/v) chlorotetracycline, 0.005% (w/v) rifampicin were added and mixed throughly.

Dilution plating technique was used to isolate SDS pathogen form the soil (Rupe *et al.*, 1999). After spreading diluted soil suspensions on MNSM, single spore colonies were grown for 6 days at room temperature. Colonies that resembled to check siolate 171 of *F. solani* f. sp. *glycines* were selected randomly, transferred to PDA medium (Difco Laboratories, Detroit) in 9 cm plate petri-dishes, and then grown for 18 days at room temperature.

Greenhouse inoculation assay

From the isolates identified on MNSM, fifty-seven were randomly selected from PTS and fifty-five from CBS for pathogenicity tests in the greenhouse. Preparation of sorghum seed inoculum and greenhouse inoculation assay were conducted as our previous research (Cho, 1999; Cho *et al.*, 1999).

Six equally spaced furrows were dug across the width of the plate (30 cm × 60 cm × 6 cm : W × L × D) to a depth of 5 cm, and 20 ml of inoculum was evenly spread along the bottom of the furrow. Inoculum were covered with 2 cm of soil. Ten seeds per furrow of the SDS-susceptible cultivar, Hartz 6686, were placed onto the covered soil in the furrow and then covered with 2 cm of soil. Plants were grown in greenhouse for 3~4 weeks from March to July, 1998. Each row represented one plot of a single isolate. Arrangement of each isolate was followed by the plot plan of randomized complete block design with three replications and tested twice. To compare the SDS foliar symptoms severities, check isolate 171 (SDS-pathogen) and uninoculated plot were includes as controls.

SDS foliar symptom severities were rated by Horsfall-Barratt Scale as our previous study (Cho, 1999; Cho *et al.*, 1999). Horsfall-Barratt Scale was converted to mid point percentage before analysis. The fresh weight of the plant in

each row, also, was measured to determine the responses of the cultivar to each isolate. Since the level of the symptom severities and the fresh weights of the plants varied from one test to another, level of the scores in each isolate in each run of the test were adjusted to controls and expressed as the ratio to the proportion of disease of the check isolate 171. The resulting symptom severities and fresh weights of the plants were than analyzed by ANOVA and significant differences of the means among isolates were determined using the Least Significant Difference Test. The analysis was conducted using Proc GLM of SAS (SAS Institute, Cary, NC).

Mycelium growing and DNA extraction

A mini-prep procedure was used to recover total DNA from all isolates. Mycelium from each isolate of *Fusarium solani* was grown at room temperature for 5 days on an orbital shaker in a complete broth media, collected by filteration, lyophilized and ground to fine powder in liquid nitrogen. Complete broth media was prepared with 30g of sucrose, 2 g of $NaNO_3$, 1 g of KH_2PO_4 (monobasic), 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 10 mg of $FeSO_4 \cdot 7H_2O$, 2 g of casein acid hydrolysate, 1 g of yeast extract, 0.2 ml of trace elements solution and 10 ml of zambino vitamin solution in 1000 ml of dH_2O, then autoclaved for 20 minutes. Trace element solution was prepared with 5 g of citric acid, 5 g of $ZnSO_4 \cdot 7H_2O$, 0.25 g of $CuSO_4 \cdot 5H_2O$, 50 mg of $MnSO_4 \cdot H_2O$, 50 mg of H_3BO_3, and 50 mg of $NaMoO_4 \cdot 2H_2O$ in 95 ml of dH_2O. Zambino vitamin solution was prepared with 100 ml of 100% ethanol, 100 ml of distilled water, 20 mg of thiamine · HCl, 15 mg of pyridoxine · HCl, and 1 mg of Biotin. 200 ml of complete broth media was used to each isolate.

Approximately 100 mg of mycelium was mixed with 1.3 ml of extraction buffer (0.5 M Tris, pH 8.0, 0.25 M NaCl, 0.025 M EDTA, 0.5% (w/v) Sodium dodecyl sulfate) in a 2 ml micro centrifuge tube and incubated for 30 minutes at room temperature. Cell debris was pelleted by centrifugation at 14 krpm for 5 minutes at room temperature and 1 ml of supernatant was transferred to a new 2.0 ml tube, followed by a phenol : chloroform : isoamyl-alcohol (25:24:1) extraction. The aqueous phase was treated with 100 ug/ml RNase A and incubated for 30 minutes at 37°C, followed by a chloroform : isoamyl-alcohol (24:1) extraction. The aqueous phase was removed, treated with 1/10 vol (100 ul) of 3.0 M NaOAc, precipitated with 1 vol (1 ml) of 100% iso-propanol followed by cetrifugation at 14 krpm for 1 minute. The resultant pellet was resuspended in 360 ul of TE (10 mM Tris, 1 mM EDTA, pH 7.4) followed by addition of 180 ul of 7.5 M NH_4OAc. After chilling on ice for 10 minutes, proteins were pelleted by centrifugation for 20 minutes at 14 krpm at 4°C. DNA was precipitated by 95% ethanol from the resultant supernatant and resuspended in 50~200 ul of Te (10 mM Tris, 0.1 mM EDTA) depending on the size of the pellet. Quantification of DNA concentration was performed with TKO 102 fluorometer standard kit (Hoffer Sci. Co.) by using Hoechst dye and 1 mg/ml Calf-thymus DNA.

RFLP analysis

Forty of one hundred twelve isolates of *F. solani* from CBS and PTS were selected for RFLP analysis based on the symptom severity of greenhouse tests. Check isolates 171 (SDS-pathogen) and PN603 (non-SDS pathogen) were used to compare the RFLP data of fourty isolates. Overall procedures of this experiment was conducted as Marlatt's (Marlatt *et al.*, 1996)

Total DNA was digested with restriction enzymes, *Hae*III and *Eco*RI, for 6~10 hours according to the manufacturers instructions and electrophoresed in a 0.8% agarose/0.5× TBE at 25 V for 15 hours using 12×15 cm gel electrophoresis kit. Capillary transfer of DNA to nylon membrane (Hybond-N+, Amersham, Arlington, Heights, IL) was conducted overnight. An enhanced chemiluminescent (ECL) direct nucleic acid labeling and detection kit (Amersham, Arlington Heights, IL) was used to label and to detect mtDNA probes, 2U18 and 4U40 derived from mtDNA of *Colletotrichum orbiculare* (Correll *et al.*; 1993). Prehybridization and hybridization were performed according to the manufacturer's instructions on a shaker at 42°C in a sealed plastic bag. These two mtDNA probes were covalently labeled with horseradish peroxidase with glutaraldehyde. Hybridization reaction were allowed to proceed for 12~16 hours on a shaker at 42°C.

Membrane blots were washed twice at 42°C in primary wash buffer (0.5×SSC [1×SSC is consisted with 0.05 M NaCl and 0.015 M Sodium citrate], 36% Urea, and 0.4% Sodium dodecyl sulfsate) for 20 minutes each time, followed by washing twice at room temperature in 2×SSC for 5 minutes each time. Light sensitive Film (Hyperfilm-ECL, Amersham) was placed on the membrane and exposed for 10~120 minutes in X-ray film casset depending on the strength of the enzyme reaction signal.

RESULTS AND DISCUSSIONS

Isolation and identification of SDS pathogen

The colony shape of the SDS pathogen was determined on MNSM using dilution plating technique. *F. solani* check isolate 171, SDS pathogen, produced the white and chalky shaped colonies on this selective media, MNSM, when it

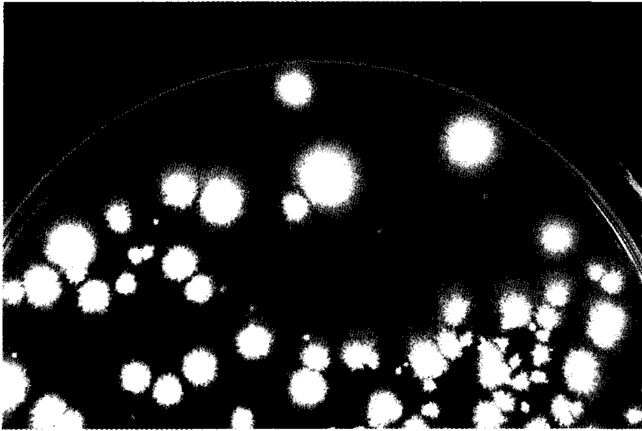

Fig. 1. Six days old colony shape of *F. solani* isolate 171, SDS pathogen, on modified Nash and Snyder's medium.

grew for 6 days at room temperature (Fig. 1.). From the soil suspensions of CBS and PTS, *Paecilomyces* spp., *Phytium* spp., and *F. merisoides* were also identified on MNSM, but they produced different colony types and were easily distinguished from SDS pathogen of *Fusarium solani* (Cho *et al.*, In press, Dr. Rupe's lab. Department of Plant Pathology, University of Arkansas).

In 1962, Nash and Snyder identified the colony shape of *Fusarium* sp. with the medium including PCNB and streptomycin, however, other type of fungi such as *Penicillium, Gliocladium,* and *Aspergillus* produced a colny on this medium and these were usually very tiny and sporulation was sparse and distinct from *Fusarium* sp. (Nsah and Snyder, 1962).

Cultural characteristics on PDA

We already represented the cultural colony shape of 171 on PDA in our previous study (Cho *et al.*, 1999). This isolate showed the blue centered pigment on PDA and its diameter reached approximately 3 cm, when it grew for 7 days at room temperature. As this colony grew older, blue colored center became more dark. All isolates that resembled 171 on MNSM produced similar colonies on PDA (Fig. 2. B, C) : slow growing, appressed with a blue center and white margin, abundant microconidia and macroconidia. When these isolates grew on PDA at room temperature under the light for 18 days, they had the unique form of morphological shape of colonies and were distinctive to other type of colonies, especially, green bean pathogen, PN603 (Fig. 2 : A-1). However, some of the isolates we obtained on MNSM showed different color from that of 171 (Fig. 2. : A-3). As shown as other results, some of the isolates of *F. solani* selected for this study showed slightly different color from the typical shape of 171. The isolates seldom had other kinds of color such as dark blue, purple, and violet in center of the colony (Roy *et al.*, 1997).

mtDNA RFLP

To compare genetic differences between isolates originated from two different fields, RFLPs were conducted with twenty isolates from each location. With ECL methods, we obtained two distinctive RFLPs in each treatment of restriction enzymes, *Eco*RI and *Hae*III (Fig. 3., Fig. 4.). Most of the isolates analyzed had unique form of polymorphisms and they were genetically homologous. So, we represent here several of them because of their genetic similarity among the isolates. The results obtained here were same as those of other's (Rupe *et al.*, 1993). They reported that SDS pathogen collected from SDS symptomatic plants of soybean were homogeneous with respect to mtDNA RFLPs and distinct from the non SDS isolates from other hosts.

More bands between two restriction enzymes were produced in *Hae*III than *Eco*RI. Most of the isolates from both locations of fields showed the same band patterns as check isolate 171 but not non SDS pathogen, PN603. However, some of them were slightly different from 171.

Isolate 14-3-1-1 from CBS and isolate 104-3-1-2 from PTS showed different band pattern from 171 in both treatment of restriction enzymes, *Eco*RI and *Hae*III. Isolate 11-2-5-2 from CBS showed different band pattern from 171 in *Eco*RI but same as 171 in *Hae*III. Furthermore, isolate 701-1-5-1 from PTS showed opposite result with these restriction enzyme : same as 171 in *Eco*RI but not in *Hae*III.

Even if these four isolates out of fourty isolates showed different RFLP polymorphism from 171, they are slightly different and their RFLPs had just one or two more band comparing to other lane and, also, these were more similar to 171 than to PN603. Rest of the thirty-six isolates had exactly same RFLPs as 171 and extremely homologous.

Greenhouse inoculation assay

Table 1. represents the foliar symptoms severities and plant top weight of Hartz 6686 inoculated with the isolates that used the RFLP analysis. Scores represented were the ratio of symptom severities and plant top fresh weight of the plants inoculated with each isolate to those of check isolate 171. The severity proportion and the plant top fresh weight in check isolate 171 were 67% and 1.03 g, respectively. So far as those of uninoculated control, no symptoms were observed and plant top fresh weight was recorded as 3.45 g.

Comparing to 171, isolate 14-3-1-1 from CBS produced significantly more severe foliar symptoms and isolate 804-3-1-1 from PTS was included in the same group as 171, respectively. The rest of the isolates showed significantly less severe symptoms than 171. Plants weight of soybean infected by the isolates beside 11-2-5-2, 12-2-4-1, and 703-

Table 1. SDS foliar symptoms and plant top weight of Hartz 6686 treated with various isolates collected from different soybean fields.

Locations	Isolates	SDS foliar symptom		Top plant weight	
		Ratio to check isolate	Comparison to check isolate	Ratio to check isolate	Comparison to check isolate
CBS	12-2-4-1	0.51	< *F. solani* 171	1.75	= *F. solani* 171
	12-3-1-2	0.48	< *F. solani* 171	3.29	> *F. solani* 171
	12-3-5-1	0.45	< *F. solani* 171	2.41	> *F. solani* 171
	14-1-1-2	0.61	< *F. solani* 171	2.05	> *F. solani* 171
	14-2-1-2	0.08	< *F. solani* 171	3.07	> *F. solani* 171
	11-2-5-2	0.64	< *F. solani* 171	1.56	= *F. solani* 171
	14-3-1-1	1.32	> *F. solani* 171	1.00	< *F. solani* 171
PTS	703-2-4-1	0.54	< *F. solani* 171	1.83	= *F. solani* 171
	703-3-5-1	0.44	< *F. solani* 171	2.30	> *F. solani* 171
	804-1-3-1	0.12	< *F. solani* 171	4.64	> *F. solani* 171
	804-3-1-1	0.72	= *F. solani* 171	2.14	> *F. solani* 171
	805-2-5-1	0.16	< *F. solani* 171	3.85	> *F. solani* 171
	701-1-5-1	0.57	< *F. solani* 171	2.17	> *F. solani* 171
	104-3-1-2	0.21	< *F. solani* 171	4.85	> *F. solani* 171

†Ratio (%) of SDS symptom severity proportion=tested isolates from CBS and PTS/check isolate
‡Ratio (%) of plant top fresh weight=tested isolates from CBS and PTS/check isolate
§171 is a check isolate of *F. solani*, SDS pathogen, from PTS, Arkansas, USA.
< and> are less and more significant differences comparing to 171 in each catagories at P<0.05 in LSD.
=There are no significant differences between isolates tested and 171 in each catagories at P<0.05 in LSD.
Severity proportion of SDS symptom and plant top fresh weight in check isolate 171 were 67% and 1.03 g, respectively.

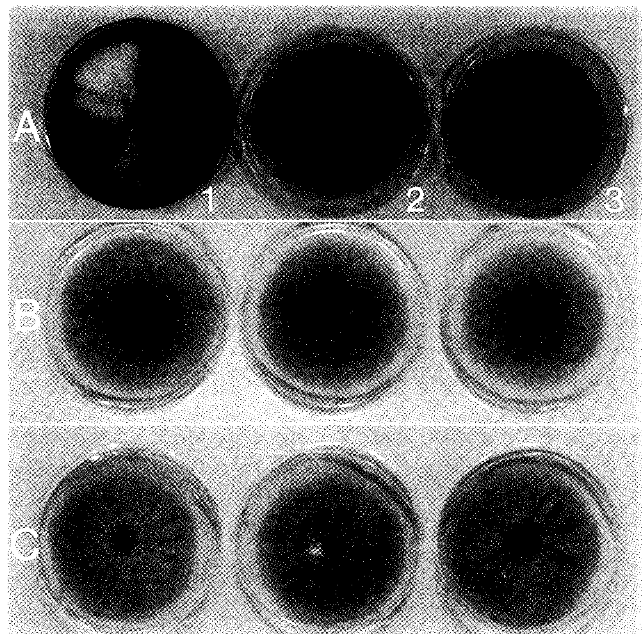

Fig. 2. Cultural morphology of various isolates of *Fusarium solani* on PDA. These isolates were selected on MNSM due to their resemblance to check isolate 171, *F. solani* f. sp. *glycines*, SDS pathogen, and then cultured on PDA for 18 days at room temperature. A-1 : PN603, green bean pathogen (non-SDS pathogen). A-2 : Check isolate 171, *F. solani* f. sp. *glycines*, SDS pathogen. A-3 : 1004-3-5-2, isolate from PTS. B : Isolates from CBS. C : Isolates from PTS

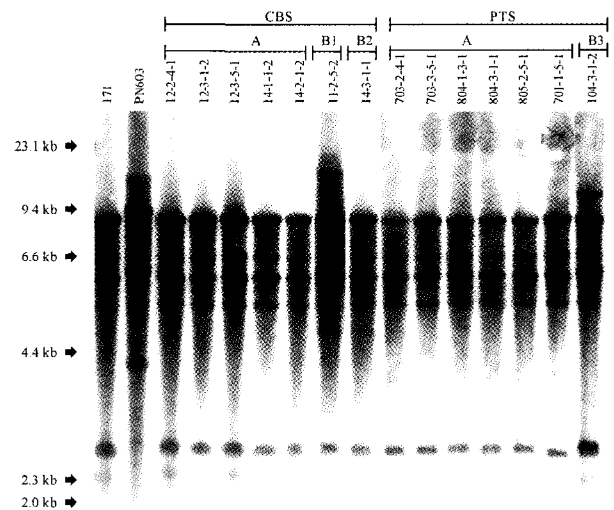

Fig. 3. Mitochondrial DNA restriction fragment length polymorphisms with *Eco*RI among isolates of *F. solani* f. sp. *glycines* from geographically different soybean fields. Total DNA of each isolate was digested with *Eco*RI and detected with mtDNA probes, 2U18 and 4U40, derived from *Colletotrichum orbiculare* using ECL southern blooting. λ/*Hind*III was used as a size marker. Check isolate 171 of *F. solani*, SDS pathogen, and PN603, green bean pathogen, were included in first two lanes for comparing the polymorphisms. Each lane has the same character such as A, B1, B2 or B3 represents the same group in RFLPs. CBS: Cotton Branch Station, Arkansas, PTS : Pine Tree Station, Arkansas.

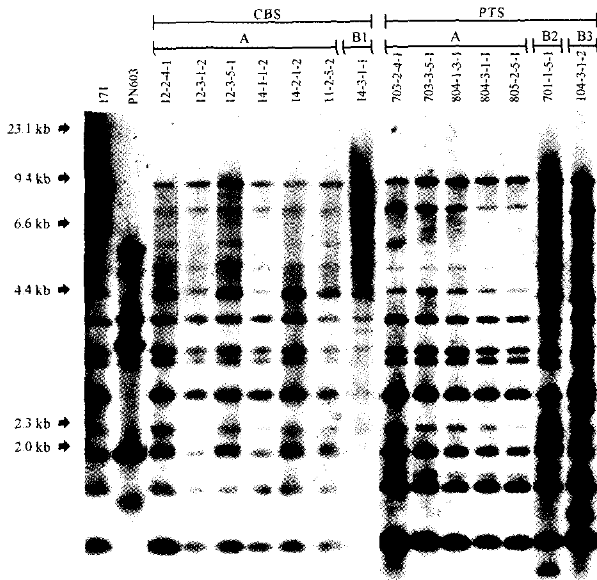

Fig. 4. Mitochondrial DNA restriction fragment polymorphisms with *Hae*III among isolates of *F. solani* f. sp. *glycines* from geographically different soybean fields. Total DNA of each isolate was digested with *Hae*III and detected with mtDNA probes, 2U18 and 4U40, derived from *Colletotrichum orbiculare* using ECL southern blooting. λ/*Hind*III was used as a size marker. Check isolate 171 of *F. solani*, SDS pathogen, and PN603, green bean pathogen, were included in first two lanes for comparing the polymorphisms. Each lane has the same character such as A, B1, B2 or B3 represents the same group in RFLPs. CBS: Cotton Branch Station, Arkansas, PTS : Pine Tree Station, Arkansas.

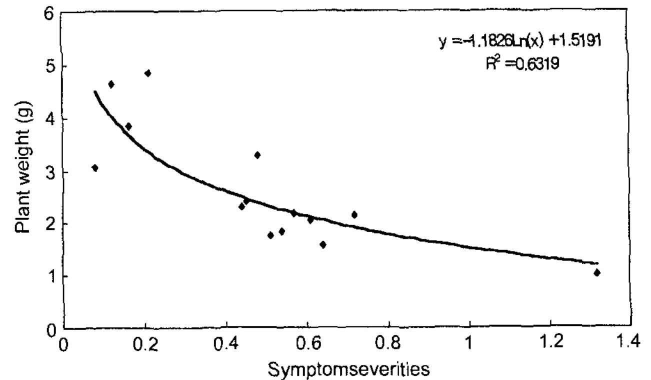

Fig. 5. Correlations between severity proportions and plant top fresh weight of soybean (Hartz 6686) inoculated with various isolates of *F. solani*.

2-4-1 were higher than by 171, Especially, in results of LSD(0.05), plants weight of soybean inoculated with 14-3-1-1 which showed more severe virulence than 171 was significantly less than that with 171. Correlations between symptom severities and plant weight were analyzed to identify how virulence of the isolates affect plant growth. There was negative correlation between these two factors and these results had the similar tendency to our previous the results (Cho, 1999; Cho *et al.*, 1999).

In this study, dilution plating technique with MNSM (Rupe *et al.*, 1999) was useful and satisfactory to identify the soil born pathogen. Although some of the isolates had slightly different shape from 171 on PDA (Fig. 2., A-3) and heterogeneous in RFLP (Fig. 3., Fig. 4. : B1, B2), all isolates selected due to their resemblance to 171 on MNSM produced SDS foliar symptoms in greenhouse inoculation assay and there were significant differences among the isolates in foliar symptom severity and plant fresh weight at $P<0.05$. However, in other experiments, uninoculated plants and the plants inoculated with PN603, green bean pathogen, did not show SDS symptoms.

As results, most of isolates selected on MNSM selective media from which geographically distinctive fields were not genetically different from check isolate 171 and they were unique strain of *F. solani*. However, further studies were needed with the isolates that showed the different colony shape from 171 on PDA or different band patterns in RFLPs with mtDNA probes. And, also, with these isolates virulence in relationship with phytotoxin produced by the fungus, may be needed.

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