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Phytotoxin Production of *Nigrospora sphaerica* Pathogenic on Turfgrasses

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A causal fungus of turfgrass blight was isolated from the infected leaves of zoysiagrass (*Zoysia japonica* Steud.) and identified as *Nigrospora sphaerica* (Sacc.) Mason by using a light microscope. Its conidia are large (14-20 μm diameter), shiny, black, aseptate, and smooth-walled spheres. The fungus caused typical blighting symptoms on the two turfgrass plants of bermudagrass (*Cynodon dactylon* (L.) Pers.) and bentgrass (*Agrostis palustris* Huds.). The fungus was found to produce a phytotoxic substance to be associated with the pathogenic mechanism. A phytotoxin was isolated from the liquid cultures of *N. sphaerica* by repeated silica gel column chromatography and its structure was determined to be 5,6-dihydro-5-hydroxy-6-propenyl-2*H*-pyr-2-one (T-3 compound). It was not a host-specific toxin showing phytotoxic effects to various plants including turfgrasses in the leaf-wounding assay, the whole plant test, and the cellular leakage test. The compound caused leaf tip dieback symptoms in turfgrass plants similar to those caused by the pathogen. Thus, T-3 compound is thought to be involved in the development of Nigrospora blight.

Keywords **:** turfgrass, Nigrospora blight, phytotoxin, *Nigrospora sphaerica.*

Zoysiagrass (*Zoysia japonica* Steud.) is a warm-season turfgrass. It is predominantly grown at home lawns, lawn grounds, and golf course fairways in Korea. Although it is subjected to injury by various plant pathogens, few diseases caused by *Rhizoctonia* spp., *Pythium* spp., *Colletotrichum* sp., and *Curvularia* sp. have been reported (Beard, 1973; Chung et al., 1991; Kim et al., 1991a; Kim et al., 1991b; Kim et al., 1992; Kim et al., 1993; Kim et al., 2000; Kim and Park, 1997; Sung et al., 1992).

Nigrospora blight caused by *Nigrospora sphaerica* has been observed in North America during the midsummer months on perennial ryegrass (*Lolium perenne* L.), chewing fescue (*Festuca rubra* L.), and Kentucky bluegrass (*Poa pratensis* L.) and in the spring and early summer on St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kuntze) (Shurtleff et al., 1987; Smiley et al., 1992). A small patch-type disease occurred in lawn grounds of zoysiagrass from June, 1998 because of unusual hot, very humid weather. Since the etiology of the patch disease in hot weather has hardly been defined in zoysiagrass, we tried to identify the causal agent of the disease. In the course of the study, we found that *N. sphaerica* (Sacc.) Mason could cause the blight symptom on turfgrass.

Plant pathogens have long been considered to produce toxic substances that play a role in pathogenesis. For example, pyrenocines A and B are thought to be involved in the development of turfgrass Curvularia blight caused by *Curvularia inaequalis* (Kim et al., 2000). The toxic metabolite induced phytotoxic effects to various plants including turfgrasses in leaf-wounding bioassay and whole plant test. However, a phytotoxin involved in development of Nigrospora blight caused by *N. sphaerica* has not been reported yet.

The isolate of *N. sphaerica* isolated from infected leaves of zoysiagrass by us could cause blight symptom on juvenile leaves of turfgrasses such as bentgrass (*Agrostis palustris* Huds) and bermudagrass (*Cynodon dactylon* (L.) Pers). The isolate was also found to produce a phytotoxic substance which may be associated with its pathogenicity. Therefore, the purpose of this study was to isolate and identify the isolate of *Nigrospora* species, to determine the pathogenicity of the isolate on turfgrasses, to isolate and identify a phytotoxic substance, and to examine phytotoxicity to various plants including turfgrasses.

Materials and Methods

Fungal isolation and identification. Fungal isolation was attempted from the leaves of diseased zoysiagrass collected from lawn grounds in Taejon, in June, 1998. Samples were washed in tap water to remove soil and then 1- to 2-cm sections of leaves were removed with a scalpel. The pieces were disinfested in 1% NaOCl for 1 min, rinsed in sterile water, and placed on potato dextrose agar (PDA) amended with streptomycin (200 μg/ml). *Nigrospora* sp. T-3 isolate was obtained and transferred to PDA slant tubes, which were stored at 4°C.

The isolate was incubated on PDA plates at 25°C for 2 weeks with lighting for 12 hr daily. The fungus was employed for taxo-

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nomic identification and identified according to Ellis (Ellis, 1971).

Temperature effect on radial growth of T-3 isolate was examined. Mycelial agar discs, 6 mm in diameter were cut from vegetative areas of 4-day-old PDA cultures grown at 28°C. The discs were placed onto PDA plates and three plates were incubated at each of six temperatures between 15°C and 33°C for 4 days. Colony diameters were then measured.

Pathogenicity of *N. sphaerica*. Pathogenicity experiment was conducted on bentgrass and bermudagrass. Seeds of bentgrass (*A. palustris* Huds.) and bermudagrass (*C. dactylon* (L.) Pers) were sown in 4.8-cm-diameter × 5-cm-deep plastic pots containing in a steamed clay loam soil:peat:perlite mixture (2:2:1, v/v/v) at 25 ± 7 °C in a greenhouse. The pots were incubated for 3 weeks in the greenhouse.

T-3 isolate was inoculated in potato dextrose broth (PDB) and then incubated with agitation (150 oscillations/min) at 25°C for 3 days in the dark. The mycelia were macerated using a Waring blender. The potted turfgrass plants were inoculated by spaying mycelial suspension on the foliage and crowns of the plants, and incubated in a moist chamber at 30°C for 2 days. The plants were then transferred to the greenhouse and incubated for 10 days. Control pots were sprayed with distilled water alone. The disease severity on the each turfgrass was rated on a 0-5 scale by a slight modification of a previously published method (Falloon, 1975); 0: No symptoms, 1: Leaf tip dieback 0-5 mm, 2: Leaf tip dieback > 5 mm, 3: Chlorotic leaf lesions plus leaf tip dieback, 4: 50% tillers with leaf sheath lesions, 5: 50% tillers with dead leaves. Pots were arranged in a randomized complete block with five replications per treatment. The experiment was repeated once and results shown are the averages of two runs with five replicates each.

Isolation of T-3 compound. Erlenmeyer flasks (250 ml), each containing 100 ml of PDB medium, were inoculated with a mycelial agar disc from 5-day-old PDA plates of *N. sphaerica* T-3. The flasks were incubated for 10 days at 25°C with a agitation of 150 rpm, and then 8.3 L of PDB cultures was centrifuged at 5,000 rpm for 15 min. The supernatant and the acetone extract of fungal mycelia were extracted two times with an equal volume of ethyl acetate and then concentrated to dryness. The residue (4.9 g) was bioassayed in a leaf-wounding test with zoysiagrass; a portion of the extract was dissolved in 2% methanol in distilled water at a concentration of 1,000 μg/ml and 5-μl aliquot was applied to each pin hole of a zoysiagrass leaf. It caused a dark necrotic lesion within 24 hr.

The ethyl acetate extract was dissolved in chloroform-methanol (99:1, v/v) and loaded onto a silica gel column (3.6 cm [inner diameter] by 60 cm) containing 260 g of silica gel (Kiesel gel 60, 70/230 mesh; E. Merck, Darmstadt, Germany). The column was eluted with chloroform-methanol (99:1, v/v) and collected in 15-ml fractions with a fraction collector. The fractions were monitored by TLC and reduced to six fractions called F1, F2, F3, F4, F5, and F6, which were bioassayed in a leaf-wounding test. Since F4 caused necrotic brown lesions, it was purified further. The active F4 fraction (1.6 g) was suspended in chloroform-methanol (95:5, v/v) and loaded onto a silica gel column (3.2 cm [inner diameter] by 60 cm) containing 150 g of silica gel (Kiesel gel 60, 70/230 mesh; E. Merck), which was eluted with the same solvent system to give two fractions called F41 and F42. The active F42 (1.4 g) was suspended in chloroform-methanol (97:3, v/v) and loaded onto a silica gel column (3.2 cm [inner diameter] by 60 cm) containing 150 g of silica gel (Kiesel gel 60, 230/400 mesh; E. Merck). The column chromatography yielded ca. 1,3 g of T-3 compound as a white powder.

Spectral measurements. Low-resolution (LR) mass spectra were recorded on a double-focusing high-resolution (HR) mass spectrometer (JEOL JMS-DX303; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker AMX-500 (500 MHz) NMR spectrometer (Bruker Analytische Messtechnik Gmbh, Rheinstetten, Germany). Spectra were referenced to TMS (^{1}H) or to solvent signals (^{13}C).

Phytotoxicity tests of T-3 compound. The phytotoxic activities of T-3 compound to various plants were tested by using a leaf-wounding assay, a whole plant test, and a cellular leakage test. For the leaf-wounding assay, cocklebur (*Xanthium strumarium* L.), pea (*Pisum sativum* L.), corn (*Zea mays* L.), barley (*Hordem sativum* Jessen), shattercane (*Sorghum bicolar* Moench), soybean (*Glycine max* L.) were used. The plants (2-weeks-old) were grown in a greenhouse and centers of detached plant leaves were nicked with a pin. The damaged leaf surfaces were immediately covered with 5 μl of each toxin solution. T-3 compound was dissolved in methanol and diluted with distilled water to give final toxin concentration of 0.5, 1.0, and 2.0 mM. The control used was a 2% methanol in distilled water. The leaf blades treated were subsequently incubated in a sealed Petri dish containing moistened filter paper. After a 72-hr incubation at room temperature, phytotoxicity was assessed.

Whole plant tests were done on large crabgrass (*Digitaria sanguinalis* (L.) Scop.), black nightshade (*Solanum nigrum*), bentgrass, bermudagrass, indian joint-vetch (*Aeschynomene indica* L.), barnyard grass (*Echinochloa crus-galli* P. Beau.), grain sorghum, cocklebur, quack grass (*Agropyron repens* (L.) P. Beauv.), and fall panicum (*Panicum dichotomiflorum* Michx.). The 10 plant species consisting of 5-10 plants per each species were grown in a pot (15.6 × 21 cm, 4-cm depth) containing sandy loam soil for 2 weeks in a greenhouse. These plants were treated by spraying each pot with 14 ml of toxin solution at concentrations of 500 and 1,000 μg/ml. The toxin solution was dissolved in 60% acetone containing 0.1% Tween 20 and the control plants were treated with 60% acetone containing 0.1% Tween 20. After treatment, the plants were placed in a ventilated hood to dry solvent and then transferred to a greenhouse. The phytotoxicity was assessed 4 days after the treatment based on visual observations of symptoms using the 0 to 100 rating system (Fans et al., 1986).

The cellular leakage from plant tissues was determined periodically by the detection of electrolyte leakage into the incubation medium, using a conductivity meter (Denki Kagaku Keiki Co., Ltd., Japan) (Lee et al., 1995). The 5-mm leaf sections of leaves were cut with a razor from bermudagrass plants grown for 1 month in a greenhouse and washed in 5 ml of incubation medium, containing 1% sucrose and 10 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5). They were then placed in 6-cm diameter

Petri dishes (approximately 0.15 g fresh weight of leaf pieces per dish) with 7 ml of incubation medium with T-3 compound at concentrations of 0.1 mM and 1 mM. The T-3 compound was dissolved in acetone. Control contained the same amount of the solvent without the compound and the final concentration of the solvent in the dishes was 1% (v/v). The Petri dishes were incubated at 25°C in the dark and conductivity was measured periodically. Because of differences in background conductivity of the different treatment solutions, results are expressed as changes in conductivity from the initial measurement. The experiments were repeated and results shown are the average of two run with three replicates each.

Results

Symptoms. Nigrospora blight of zoysiagrass began to occur from June due to early high temperature and large amount of rainfall in 1998. On zoysiagrass, large areas of turfgrass became irregular patches, 20 to 30 cm in diameter. Individual leaves predominantly started dying back from the tip and had uniform lesions extending all the way down to the leaf sheath. The diseased leaves turned yellow, and died resulting in a general thinning or patchiness of diseased turfgrass.

Isolation and Identification of T-3 isolate. *Nigrospora* sp. isolates were found from blighted leaves of zoysiagrass samples collected from patches. Among a number of *Nigrospora* sp. isolates detected on PDA plates, one isolate of T-3 was selected due to the their same morphological appearances and colony color and used for further study.

The T-3 isolate was identified as *N. sphaerica* according to Ellis (1971). Mean conidial dimensions in culture were 17 μm long (mostly 15-18 μm). Conidia were solitary, acrogenous, spherical or ellipsoidal, compressed dorsiventrally, black, shining, smooth, and aseptate (Fig. 1). Conidiophores were branched, flexuous, colorless to brown, and smooth, and conidiogenous cells were monoblastic, discrete, solitary, ampulliform or subspherical, and colorless.

The optimum temperature for radial growth of the isolate

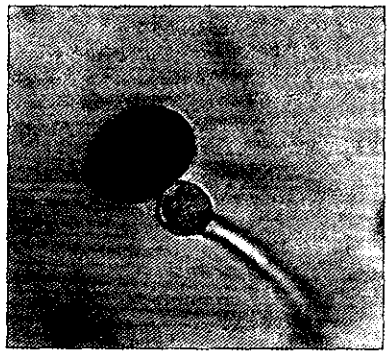

Fig. 1. Conidia of *Nigrospora sphaerica* isolated from zoysiagrass.

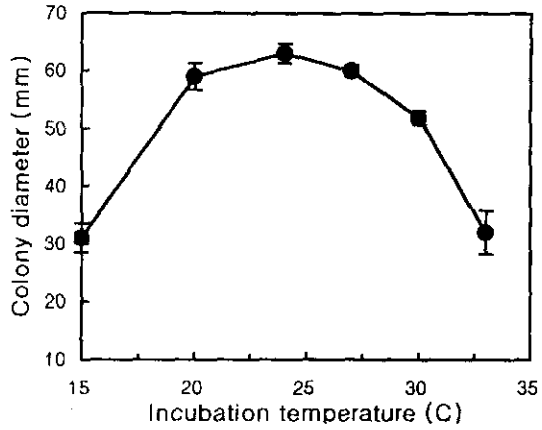

Fig. 2. Effect of temperature on mycelial growth of *Nigrospora sphaerica* T-3 on PDA for 4 days.

on PDA was 24°C. But the isolate showed good growth at 20 to 30°C except for 15 and 33°C (Fig. 2).

Pathogenicity. *N. sphaerica* T-3 isolate caused typical yellow lesions, extending downward from the leaf tip, on the two turfgrass plants of bermudagrass and bentgrass. After several days, there was a tip dieback and extensive blighting of the turfgrasses. At the beginning, bermudagrass appeared more susceptible to *N. sphaerica*, but both of bermudagrass and bentgrass was finally diseased (Table 1). The pathogen was reisolated from the diseased leaves sampled from each turfgrass species. No symptoms were observed on any uninoculated contol plants.

Characterization of the phytotoxin. The LR-electro impact MS data indicated that T-3 compound did not give molecular ion peak (M^+) but showed fragment ions at m/z 84, 71, and 55. The LR-chemical ionization spectrum showed the molecular ion $[M+1]^+$ at m/z 155. ^{13}C-NMR spectra showed that the compound has 8 carbon signals. ^{1}H-NMR spectra showed the presence of one secondary methyl in the compound. By copilation of all data, the active substance was identified as 5,6-dihydro-5-hydroxy-

Table 1. Effect of *Nigrospora sphaerica* T-3 from zoysiagrass on Nigrospora blight disease severity of bentgrass and bermudagrass[a]

Turfgrass	Disease severity[b]
Bentgrass	4.8 a[c]
Bermudagrass	5.0 a

[a]Turfgrass plants were inoculated with a mycelial suspension, incubated for 48 hr in a humidity chamber, then maintained in a greenhouse through the test.

[b]Disease severity was rated 10 days after inoculation on a 0-5 scale as follows; 0: No symptoms, 1: Leaf tip dieback 0-5 mm, 2: Leaf tip dieback > 5 mm, 3: Chlorotic leaf lesions plus leaf tip dieback, 4: 50% tillers with leaf sheath lesions, 5: 50% tillers with dead leaves.

[c]Means followed by the same letter are not significantly different (P=0.05) according to the least significant difference test.

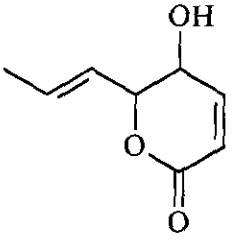

Fig. 3. Chemical structure of T-3 compound produced by *Nigrospora sphaerica*.

6-propenyl-2*H*-pyr-2-one, which were previously reported as a metabolite from unknown *Nigrospora* species (Fig. 3) (Evans et al., 1969).

Phytotoxicity of T-3 compound. In the leaf-wounding assay, T-3 compound exhibited phytotoxic activities to most of the plants tested except for cocklebur (Table 2). It caused chlorotic lesions, sunken lesions, or dark necrotic lesions. The compound induced phytotoxicity on the test plants in a concentration-dependent manner. Among them, barley was most susceptible to T-3 compound, and pea was relatively resistant.

T-3 compound was also tested on whole plants. When treated at concentrations of 500 and 1,000 μg/ml, It caused a severe growth reduction of large crabgrass and black nightshade (Table 3). Of the plants tested, bentgrass, bermudagrass, indian joint-vetch, barnyard grass, and grain sorghum were moderately susceptible to the compound. Cocklebur, quack grass, and fall panicum were relatively tolerant to the phytotoxin. The symptoms of T-3 compound on all the plants were chlorosis, which became necrosis; on the two turfgrass plants, the phytotoxin caused leaf tip dieback symptoms in the turfgrass plants similar to those caused by the pathogen.

Significant and differential cellular leakage, as measured by the detection of electrolyte leakage, did not occur until 44 hr incubation for bermudagrass treated with T-3 compound at concentrations of 0.1 and 1 mM (Fig. 3). Cellular leakage from the plants began to increase after 44 hr incubation, depending on the concentration of the compound and the duration of incubation time. They reached up to 380 mho/cm for I mM at 98 hr after incubation.

Table 2. Phytotoxicity of T-3 compound from *Nigrospora sphaerica* T-3 by the leaf-wounding assay[a]

Plant	Phytotoxicity		
	0.5 mM[b]	1.0 mM	2.0 mM
Cockle bur	–[c]	–	–
Pea	+[d]	++	++
Corn	+[e]	++	+++
Barley	+[f]	+++	++++
Shattercane	+[e]	++	+++
Soybean	++[d]	++	+++

[a]The leaves on filter papers moistened in petri dishes were incubated at room temperature for 72 hr.
[b]The compound was dissolved in 2% methanol in distilled water and 5 μl of each toxin solution was applied onto each pin hole.
[c]Lesion size : ++++, > 4.5 mm; +++, 3.0 mm to 4.5 mm; ++, 1.5 mm to 3.0 mm; +, 0.5 mm to 1.5 mm; –, < 0.5 mm.
[d]Sunken lesion; [e]Dark necrotic lesion; [f]Chlorotic lesion.

Table 3. Phytotoxicity of T-3 compound from *Nigrospora sphaerica* T-3 in the whole plant test

Test plant[a]	Growth inhibition (%)	
	500 μg/ml[b]	1,000 μg/ml
Large crabgrass	60	80
Black nightshade	30	100
Bentgrass	30	60
Bermudagrass	30	45
Indian joint-vetch	40	40
Barnyard grass	35	40
Grain sorghum	20	40
Cocklebur	0	40
Quack grass	0	35
Fall panicum	0	30

[a]The plants grown for 14 days after sowing were used and incubated for 4 days in a greenhouse after treatment.
[b]T-3 compound was dissolved in 60% acetone containing 0.1% Tween 20.

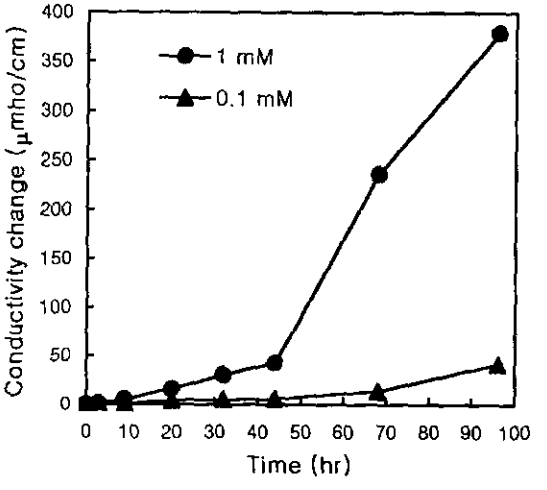

Fig. 4. Effects of T-3 compound on cellular leakage from the leaf tissues of bermudagrass. Values are differences between the treated and control tissues.

Discussion

Nigrospora blight, caused by the common fungus *N. sphaerica*, has been observed in the northern United States during midsummer on Kentucky bluegrass, perennial ryegrass, and creeping red and chewing fescues. In the

south it occurs on St. Augustinegrass in spring and early summer (Shurtleff et al., 1987; Smiley et al., 1992). In Korea, it occurred in lawn grounds of zoysiagrass during early summer because of high temperature and humidity.

As for symptoms, individual leaves on Kentucky bluegrass generally start dying back from the tip and may have uniform lesions extending all the way down to the leaf sheath (Shurtleff et al., 1987; Smiley et al., 1992). The symptoms were similar to those occurred on zoysiagrass. On the other hand, the dying leaf of some Kentucky bluegrass cultivars turns deep purple (Smiley et al., 1992), but zoysiagrass turned uniformly yellow without the color. Deep purple leaves were not observed on zoysiagrass.

Tompson reported that Nigrospora blight is caused by a *Nigrospora* sp. tentatively identified as *N. sphaerica* (Sacc.) Mason (Tompson et al., 1982). The T-3 isolate collected from infected leaves of zoysiagrass by us was also identified as *N. sphaerica*. The conidia of *Nigrospora* species were identical in morphology and different to conidia diameter (Domsch et al., 1980; Ellis, 1971; Hudson, 1963). Compared with the conidia of *N. sphaerica*, those of *N. oryzae* are smaller (10-16 [mostly 12-14] μm) and *N. sacchari* are larger (17-24 [mostly 20-22] μm). Conidia of *N. sphaerica* T-3 were almost identical in conidial size to those described by Ellis (1971).

N. sphaerica from the diseased leaves of zoysiagrass (warm-season turfgrass) exhibited a strong pathogenicity to bentgrass (cool-season turfgrass) and bermudagrass (warm-season turfgrass) and a lack of host specificity. Thompson also reported that isolates of *Nigrospora* obtained from ryegrass and bluegrass are not host specific in greenhouse pathogenicity studies (Thompson et al., 1982).

N. sphaerica T-3 produced a pyrone metabolite, 5,6-dihydro-5-hydroxy-6-propenyl-2*H*-pyr-2-one (T-3 compound), showing phytotoxicity by leaf-wounding assay, whole plant test, and cellular leakage test. It was originally reported from the liquid cultures of an unidentified *Nigrospora* species without biological activity (Evans et al., 1969). In the leaf-wounding assay and the whole plant test, T-3 compound was phytotoxic to various plants including turfgrasses. This indicates that it is not a host-specific toxin. On bentgrass and bermudagrass, the phytotoxin caused leaf tip dieback symptoms in the turfgrass plants similar to those caused by the pathogen. These results suggest that T-3 compound may be involved in the disease development by *N. sphaerica*.

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