Research Article

Triazole Fungicides Sensitivity of *Sclerotinia homoeocarpa* in Korean Golf Courses

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Chemical management of dollar spot in turf may lead to the development of *Sclerotinia homoeocarpa* populations with reduced fungicide sensitivity. The objective of this study was to investigate resistance of *S. homoeocarpa* isolates to triazole fungicides and to test cross-resistance among three triazole fungicides. A total of 66 isolates of *S. homoeocarpa* were collected from 15 golf courses across Korea, and tested via *in vitro* sensitivity assay against hexaconazole, propiconazole and tebuconazole. EC$_{50}$ values of the isolates to these fungicides were distributed in the range of 0.001-1.1 a. i. μg ml$^{-1}$. Based on the EC$_{50}$ values, twelve representative strains were selected as sensitive isolates including control and insensitive isolates with respect to each fungicide. At a concentration of 0.1 a. i. μg ml$^{-1}$ for all fungicides, the selected strains were distinguished as sensitive or resistant isolates with the mycelial growth inhibition rate of 50% as the criterion. The EC$_{50}$ values of resistant strains exposed to hexaconazole, propiconazole and tebuconazole were 20-50 times, 50-70 times, and 77 times greater, respectively, than that of the control strains. Two isolates of *S. homoeocarpa* S0-41 and Sh14-2-1 showed sensitivity toward all the fungicides used, while two other isolates Sh7-5-1 and Sh2-1-1 showed resistance to all fungicides. Each isolate showed similar resistance to the three types of triazole fungicides, whereby cross-resistance of isolates was confirmed in the present study; all three triazole fungicide combinations displayed significant correlation coefficients equivalent to or greater than 0.8.

**Keywords**: dollar spot, fungicides sensitivity, golf courses, triazole, *Sclerotinia homoeocarpa*

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Dollar spot, caused by *Sclerotinia homoeocarpa*, is a common and persistent disease of cool-and warm-season turfgrass species occurring in golf courses in South Korea, North America, Europe, Japan and elsewhere. Chemical preventive measures employing fungicides are in use for the prevention of dollar spot. Nevertheless, due to repetitive usage of these fungicides, the manifestation of fungicide resistant strains is increasingly reported. Appearance of resistant isolates to the fungicides containing heavy metal additives was reported as early as in the late 1960s (Cole et al., 1968; Massie et al., 1968). Benzimidazole fungicides experienced numerous reports of occurrences of resistant isolates in the early 1970s soon after its registration (Burpee, 1997; Detweiler et al., 1983; Warren et al., 1974). Among the translocating fungicide, a dicarboxamide fungicide was reported in 1980s (Detweiler et al., 1983), and a demethylation inhibitor (DMI) fungicide was too highlighted in 1990s that the appearance of resistant isolates was occurred (Vargas et al., 1992). Since the first report of the resistant isolates of DMI fungicides on a dollar spot was filed by Vargas et al. (1992), a lot of research reports on the resistance of DMI fungicides in many countries such as the United States, Europe, and Canada (Burpee, 1997; Golembiewski et al., 1995; Hsiang et al., 2007; Jo et al., 2006; Miller et al., 2002; Mocioni et al., 2001; Ok et al., 2011; Popko et al., 2012) have been published. In South Korea, appearance of resistance to propiconazole has been reported (Shim et al., 2001).

Same class of fungicides usually show cross-resistance,
but cases of not exhibiting cross-resistance or showing weak correlations have been reported for DMI fungicides (Hsiang et al., 1997; Leroux, 1992; Miller et al., 2002; Robbertse et al., 2001). The maiden attempt to study the cross-resistance to DMI on grass dollar spot was by Doney and Vinceli (1993), and research reports on cross-resistance of triazole fungicides has been well documented (Golembiewski et al., 1995; Hsiang et al., 1997; Miller et al., 2002; Ok et al., 2011).

The aims of the present research were to (i) identify modified sensitivity of \textit{S. homoeocarpa} isolates in golf courses treated with triazole fungicides and (ii) investigate the cross-resistance of \textit{S. homoeocarpa} isolates towards three triazole fungicides i.e., hexaconazole, propiconazole and tebuconazole, which are typically used for the prevention of dollar spot in the fields.

**Materials and Methods**

**Collection and isolation of \textit{S. homoeocarpa}.** \textit{S. homoeocarpa} isolates were collected from golf courses green, tee and fairway exhibiting dollar spots across ten golf courses in South Korea. Isolation was made by collecting hole-cup sized cylindrical turfgrass patches taken from ten golf courses in Korea. Two hundred and sixty-five isolates of \textit{S. homoeocarpa} were collected from four different species of turfgrasses: 161 isolates from creeping bentgrass, 67 isolates from Kentucky bluegrass, 21 isolates from perennial ryegrass and 16 isolates from zoysiagrass (Table 1). The isolates were sampled in spring and fall of 2014 and 2015. Of the 265 isolates, sixty strains of \textit{S. homoeocarpa} were chosen for use in the study by selecting only one strain per diseased patch in the sample and additional six strains were obtained from the Korea Turfgrass Research Institute (KTRI) (Table 2).

Each isolate was picked up from the margins of the infection center on symptomatic leaf blades as described by Jo et al. (2006). Individual leaf blades infected within the dollar spot showing distinctive brown edge at the rim were surface disinfected in 75% EtOH and 1% sodium hypochlorite solution, and rinsed three times using sterilized water. The disinfected leaf blades were dried on a sterilized filter paper prior to inoculation of the fungi on water agar (WA) and stored at 25°C for 48 h under cycles of 12 h light/12 h darkness.

Mycelium formed from the section was isolated and transferred to potato dextrose agar (PDA; Difco, USA). After 7 days of inoculation on PDA, \textit{S. homoeocarpa} isolates were identified based on morphological features and by comparison to reference isolates. Pure cultures of \textit{S. homoeocarpa} were obtained by subculturing hyphae from the edge of an actively growing colony and subsequently transferred to PDA plates.

**In vitro sensitivity assay.** \textit{In vitro} sensitivity assays of \textit{S. homoeocarpa} isolates to triazole fungicides including hexaconazole SC (a. i. cont. 2%), propiconazole EC (a. i. cont. 25%), and tebuconazole SC (a. i. cont. 20%) were conducted. Each triazole fungicide was diluted to prepare five different concentrations containing 10.0, 1.0, 0.1, 0.01, and 0.001 a. i. μg ml⁻¹.

One milliliter of each diluted fungicide was added to 599...
ml of PDA cooled to 50-55°C and evenly mixed using a magnetic stirrer. The fungicide-amended PDA was poured into Petri-dishes (9 cm in diameter).

*S. homoeocarpa* isolates were inoculated at 25°C and incubated for 2-3 days under 12 h light/12 h dark cycles. Agar plugs (5 mm in diameter) from the margins of actively growing *S. homoeocarpa* cultures were transferred to the center of fungicide-amended PDA plates. For each fungicide treatment, three replicates of fungicide amended PDA plates and three additional PDA petri-dishes without fungicide (control) were used. All fungal isolates were incubated at 25°C. Four radial points located approximately 90° apart on the actively growing *S. homoeocarpa* mycelium colonies were marked, and the growth of mycelium in outer radial directions was measured within 48 h following transfer.

To determine the rate of mycelium growth inhibition (%), length of mycelium growth of *S. homoeocarpa* isolates were also measured.

Fifty percent effective concentration (EC<sub>50</sub>) values of *S. homoeocarpa* isolates was estimated based on their relative mycelium growth on the control PDA and fungicide amended PDA plates. The fungicide concentrations were log-transformed and the EC<sub>50</sub> values were calculated from the linear regression model generated. Linear regression was used to determine the relationship between mycelium growth and fungicide concentrations using the PROC PROBIT (SAS 9.4: SAS Institute, Cary, NC) software.

### Cross-resistance

Using the log EC<sub>50</sub> value of 66 *S. homoeocarpa* isolates against the registered fungicides, the relationship between the 3 triazole fungicides was analyzed via paired comparison. Through these inter relational results, cross-resistance was analyzed for hexaconazole-propiconazole, hexaconazole-tebuconazole, propiconazole-tebuconazole fungicide combinations by calculating the correlation coefficients of these three fungicides.

### Results

#### Isolate sensitivity to triazole fungicides

Sensitive and resistant isolates were not clearly distinguished and showed...
quantitatively random distribution (Fig. 1). EC_{50} result for each fungicide was as follows; EC_{50} for hexaconazole was in the range of 0.001 a. i. μg ml^{-1} to 0.17 a. i. μg ml^{-1}; propiconazole from 0.001 a. i. μg ml^{-1} to 0.47 a. i. μg ml^{-1}, and tebuconazole from 0.002 a. i. μg ml^{-1} to 1.1 a. i. μg ml^{-1}.

Three S. homoeocarpa isolates that recorded the lowest EC_{50} value were selected as the representative sensitive strains, while five S. homoeocarpa isolates showing EC_{50} values greater than the isolates from golf course without prior history of fungicide application [(Sh14-2-1) by 20 times in hexaconazole, 50 times in propiconazole, and 35 times in tebuconazole] were selected as insensitive strains, short listing a final selection of 12 strains in total (Table 3).

In the sensitive S. homoeocarpa isolates, the EC_{50} value was below 0.003 μg ml^{-1} for hexaconazole, 0.006 μg ml^{-1} for propiconazole, and 0.016 μg ml^{-1} for tebuconazole. EC_{50} value of insensitive strains was shown to be 50 times greater in hexaconazole, 50-70 times in propiconazole, and 77 times in tebuconazole (highest), compared to the sensitive isolates. On the concentration-reaction graph of the 12 selected isolates, hexaconazole, propiconazole, tebuconazole all showed distinguished inhibition rates in the concentration of 0.01 μg ml^{-1}, with 50% inhibition rate at concentrations of 0.1 μg ml^{-1} (Fig. 2). Strains showing mycelial growth rates greater than 50% were 11 in hexaconazole among 66 isolates, 42 in propiconazole amongst

Fig. 1. Differential responses of 66 isolates of Sclerotinia homoeocarpa to hexaconazole, propiconazole, and tebuconazole. The isolates were grown on PDA with various concentrations of hexaconazole, propiconazole, and tebuconazole for two days at 25°C in darkness.

Fig. 2. Differential responses of twelve isolates of Sclerotinia homoeocarpa to hexaconazole, propiconazole, and tebuconazole. The isolates were grown on PDA amended with various concentrations of hexaconazole, propiconazole, and tebuconazole for two days at 25°C in darkness. Relative mycelial growth in 0.1 a. i. μg ml^{-1} of each fungicide had a distinct gap between sensitive (S) and resistant (R) isolates.
Dollar Spot Resistance to Triazole Fungicides

593

66 isolates, and 51 in tebuconazole amongst 66 isolates (Fig. 2).

Fungicide resistance distribution for each strain group was analyzed by indicating the value of resistance factor (RF), range of EC$_{50}$ value, its mean with distinguished populations. Resistance Factor is a degree of increase in the fungicide resistance in comparison with isolate groups that were sensitive towards fungicides for there was no previous encounter reported early. Resistance factor was calculated by dividing the mean EC$_{50}$ value of strain groups with decreased sensitivity towards fungicides with the mean EC$_{50}$ value of strain groups showing selective sensitivity from golf courses that had no history of fungicide. Groups, with greater RF value from 2.7 to 13.3 times than that of Sh14 strain groups which had no history of fungicide, were separated (Table 4).

Cross-resistance between triazole fungicides. As a result of pairwise comparison for determination of cross-resistance of 3 triazole fungicides (hexaconazole, propiconazole, and tebuconazole), based on EC$_{50}$ values from 66 isolates (Fig. 3), significant ($P < 0.0001$) relation coef-

### Table 3. EC$_{50}$ values and the EC$_{50}$ ratio of resistant isolates (R) to sensitive isolates (S) of twelve selected isolates for each fungicide

| Isolates | Hexaconazole | Propiconazole | Tebuconazole |
|----------|--------------|---------------|--------------|
|          | EC$_{50}$    | R/S ratio$^a$ | EC$_{50}$    | R/S ratio$^a$ | EC$_{50}$    | R/S ratio$^a$ |
| S1 S0-41 | 0.002        | -             | 0.001        | -             | 0.004        | -             |
| S2 Sh14-2-1 | 0.003      | -             | 0.006        | -             | 0.014        | -             |
| S3 Sh11-2-1 | 0.003      | -             | 0.005        | -             | -            | -             |
| S4 Sh5-1-1 | -            | -             | -            | -             | 0.016        | -             |
| R1 Sh9-8-1 | 0.169        | 49.1          | -            | -             | 0.526        | 36.5          |
| R2 Sh7-5-1 | 0.091        | 26.5          | 0.341        | 53.5          | 1.111        | 77.1          |
| R3 Sh2-1-1 | 0.092        | 26.7          | 0.451        | 70.8          | 0.670        | 46.5          |
| R4 S0-7    | 0.069        | 20.1          | 0.401        | 63.0          | -            | -             |
| R5 Sh12-4-1 | -           | -             | 0.401        | 62.9          | 0.556        | 38.6          |
| R6 Sh10-3-1 | 0.130        | 37.7          | -            | -             | -            | -             |
| R7 Sh3-2-1 | -            | -             | 0.426        | 66.9          | -            | -             |
| R8 Sh13-3-1 | -            | -             | -            | -             | 0.717        | 49.7          |

$^a$R/S ratio was calculated as EC$_{50}$ value (a. i. μg ml$^{-1}$) of each resistant isolates divided by EC$_{50}$ value of Sh14-2-1 which isolated from golf courses with no history of fungicides.

### Table 4. Range, mean EC$_{50}$, and resistance factor (RF) for each fungicide in each population of Sclerotinia homoeocarpa from golf courses in Korea

| Population | Isolates | Hexaconazole | Propiconazole | Tebuconazole |
|------------|----------|--------------|---------------|--------------|
|            |          | Range        | mean | RF$^*$         | range | mean | RF$^*$         | range | mean | RF$^*$         |
| Sh1        | 5        | 0.003-0.126 | 0.054 | 9.4            | 0.025-0.263 | 0.121 | 5.6          | 0.027-0.231 | 0.123 | 3.0          |
| Sh2        | 4        | 0.011-0.092 | 0.053 | 9.3            | 0.068-0.451 | 0.263 | 12.1         | 0.089-0.653 | 0.313 | 7.6          |
| Sh5        | 5        | 0.005-0.050 | 0.027 | 4.7            | 0.046-0.210 | 0.119 | 5.5          | 0.016-0.172 | 0.128 | 3.1          |
| Sh6        | 3        | 0.027-0.049 | 0.035 | 6.1            | 0.023-0.177 | 0.093 | 4.3          | 0.054-0.344 | 0.162 | 3.9          |
| Sh7        | 5        | 0.009-0.091 | 0.035 | 6.1            | 0.015-0.341 | 0.102 | 4.7          | 0.051-1.111 | 0.352 | 8.6          |
| Sh8        | 4        | 0.020-0.037 | 0.030 | 5.2            | 0.033-0.112 | 0.071 | 3.3          | 0.053-0.194 | 0.109 | 2.7          |
| Sh9        | 8        | 0.045-0.169 | 0.070 | 12.3           | 0.067-0.257 | 0.150 | 6.9          | 0.061-0.526 | 0.272 | 6.6          |
| Sh10       | 5        | 0.029-0.130 | 0.076 | 13.3           | 0.070-0.225 | 0.131 | 6.0          | 0.150-0.345 | 0.266 | 6.5          |
| Sh12       | 6        | 0.007-0.087 | 0.042 | 7.4            | 0.036-0.401 | 0.154 | 7.1          | 0.067-0.556 | 0.181 | 4.4          |
| Sh13       | 5        | 0.020-0.076 | 0.042 | 7.4            | 0.099-0.344 | 0.249 | 11.5         | 0.126-0.717 | 0.400 | 9.7          |
| Sh14       | 6        | 0.003-0.010 | 0.006 | 1.0            | 0.006-0.052 | 0.022 | 1.0          | 0.014-0.111 | 0.041 | 1.0          |

$^*$Resistance factors were calculated as mean EC$_{50}$ (a. i. μg ml$^{-1}$) of each population divided by mean EC$_{50}$ of Sh14 population which isolated from golf courses with no history of fungicides.
Among the 10 golf courses from which *S. homoeocarpa* isolates were collected, 9 had a history of using more than one type of triazole fungicides, and Ecoland golf course had no history of using fungicides since its establishment, thus enabling baseline sensitivity of the fungi towards the fungicide. Baseline sensitivity is the standard sensitivity of the fungi towards the fungicide. Isolates with baseline sensitivity are useful in investigating and analyzing the degree of induction of resistance against the fungicide in certain strain groups, and differences in genetic composition of strains transited to resistance, and researching on genes related to fungicide resistance (Russell, 2004). Hsiang et al. (2007) researched on the distribution of baseline sensitivity in the *S. homoeocarpa* isolates before the registration and usage of triazole in all regions of Ontario, Canada, and all areas except for the ones along the borders of the United States, propiconazole showed EC$_{50}$ value lower than 0.009 μg ml$^{-1}$ while tebuconazole showed mean EC$_{50}$ value lower than 0.03 μg ml$^{-1}$ in all groups. EC$_{50}$ value of selected sensitive strains in this research lie within the EC$_{50}$ range of representative sensitive strains obtained from golf courses that had no history of fungicide in Canada, United States, and others, thus showing the baseline sensitivity towards these triazole fungicides.

When analyzing the concentration reaction graph of 12 selected isolates, inhibition rates were evident at concentrations of 0.01 μg ml$^{-1}$ for hexaconazole, propiconazole, and tebuconazole, and sensitive or resistant isolates could be distinguished (Fig. 2) based on the 50% inhibition rates at concentrations of 0.1 μg ml$^{-1}$. Concentration of 0.1 μg ml$^{-1}$ is thus used as threshold concentration to distinguish between sensitive and resistant strains in earlier reports by Miller et al. (2002), Jo et al. (2006, 2008), and Popko et al. (2012, 2013). In this current work sensitive and insensitive strains could be distinguished at concentrations of 0.1 μg ml$^{-1}$ based on the 50% inhibition rate. Popko et al. (2012) used fungicides in 4 golf courses whereby the effectiveness of fungal prevention decreased after spraying of propiconazole, and infection appeared after 7 days. Separated isolates showed mycelium growth rates greater than 50% in the determinant concentration (0.1 μg ml$^{-1}$). Based on this result, Popko et al. (2013) reported that by marking the 50% of mycelium growth rate 0.1 μg ml$^{-1}$ based on the 50% inhibition rate. Popko et al. (2012) used fungicides in 4 golf courses whereby the effectiveness of fungal prevention decreased after spraying of propiconazole, and infection appeared after 7 days. Separated isolates showed mycelium growth rates greater than 50% in the determinant concentration (0.1 μg ml$^{-1}$). Based on this result, Popko et al. (2013) reported that by marking the 50% of mycelium growth rate 0.1 μg ml$^{-1}$, strain groups showing higher mycelium growth rate can easily be distinguished as strains indicating practical field resistance (PFR). In this research, strains showing mycelium growth rate of greater than 50% in the concentration of 0.1 μg ml$^{-1}$ were 11 out of 66 strains in hexaconazole, 42 out of 66 strains in propiconazole, and 51 out of 66 in tebuconazole (Fig. 2), predicting greater occurrence of strains showing PFR in propiconazole and tebuconazole, also requiring further verification in field experiments.

As a result of pairwise comparisons between the 3 triazole fungicides, relation coefficient of greater than 0.8 was obtained, confirming cross-resistance. Relation to
Dollar Spot Resistance to Triazole Fungicides

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