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# Vegetative Compatibility Grouping of *Sclerotinia homoeocarpa* Isolates Infecting Turfgrass in South Korea

**Seog-Won, Chang[1]*, Tae-Hyun Chang[2], Jeum Kyu, Hong[3], Jong-Hyun Park[1], and Suk-Woo Jung[1]**

*[1]Department of Golf Course Management, Korea Golf University, Hoengseong, 225-811, Korea*
*[2]Plant Resources and Environment Major, College of Ecology & Enviromental Sciences, Kyungpook National University, Sangju-city, Gyeongsang Buk-Do, 742-711, South Korea*
*[3]Department of Horticultural Sciences, Gyeongnam of Science and Tecnology, Kyeongnam 660-758, Korea*

**ABSTRACT.** *Sclerotinia homoeocarpa*, the causal agent of dollar spot, is one of the most common pathogens of cool season turfgrasses in South Korea. The vegetative compatibility group (VCG) assay was carried out using nitrate-nonutilizing (*nit*) mutants recovered from 13 South Korean isolates with various geographical origins. The mutants were divided into four phenotypic classes based on mutation loci associated with nitrogen assimilation: *nit*1, *nit*2, *nit*3, and NitM. The recovered number of *nit* mutants greatly varied among the isolates, ranging from 0 to 15 mutants. Of the mutants isolated, *nit*1 and *nit*2 mutants were most common (80%) while NitM and *nit*3 were relatively rare. One dominant and four minor VCGs were determined from 18 mutant isolates tested. To study population structures of Korean *S. homoeocarpa* isolates and increase our understanding of its ecological and epidemiological aspects for dollar spot management on turfgrass, more generated mutants should be tested with more diverse isolate collections.

***Key words*:** *Sclerotinia homoeocarpa*, Vegetative Compatibility Group (VCG), *Nit*-mutant

## Introduction

Dollar spot caused by *Sclerotinia homoeocarpa* F. T. Bennett is one of the most common fungal diseases on cool season turfgrass worldwide (Mitkowski & Colucci, 2006; Vargas, 1994). The disease is most damaging and prevalent during the turfgrass growing season from spring to fall. Warm and humid conditions during a day coupled with cool nights promote an extended period of leaf wetness, particularly conducive to the disease development (Bennett, 1937). Golf course with low mowing heights increases the susceptibility of turfgasses to dollar spot infection, therefore, more money is spent on managing dollar spot than any other diseases in most countries (Smith et al., 1989).

*S. homoeocarpa* most likely spreads by vegetative growth of mycelium, because of conidia or the teleomorphic form of the fungus has not been observed in the field (Smith et al., 1989). Long distance dissemination may occur via infected grass clippings in wind, water, machinery such as mowers, or by human traffic. During the vegetative phase, hyphal fusion and heterokaryosis may occur between mycelia within the same compatibility group. Vegetative hyphal fusion plays a key role in intra- and inter-hyphal communication, translocation of water and nutrients, genetic exchange and general homeostasis within or between fungus populations (Glass et al., 2004; Smith et al., 1989). Vegetative compatibility refers to the ability of individual fungal isolates to undergo mutual hyphal anastomosis and subsequently form viable heterokaryons (Glass et al., 2000; Powell and Vargas, 2001). In fungi lacking sexual stages in their life cycle, vegetative compatibility may serve as an important means of genetic exchange and diversity (Leslie, 1993). Isolates that are vegetatively compatible with each other are members of the same vegetative compatibility group (VCG), whereas vegetatively incompatible isolates are incapable of anastomosis with each other or fail to establish stable heterokaryosis (Glass et al., 2000).

The VCG assay has been used to measure population diversity of plant pathogenic fungi, and to enable appropriate identification and characterization of individual isolates (Brooker et al., 1991; Joaquim & Rowe, 1991; Klittich & Leslie, 1988). The most effective and widely used VCG assay method in many plant pathogenic fungi is based on the complementation between nitrate-nonutilizing (*nit*) mutants (Jo et al., 2007; Leslie, 1993). A big advantage of using nit mutants for vegetative compatibility tests is that nit mutants can be readily recovered on selective, and complementation or no complementation were easily identified (Jo et al., 2007). In this study, we generated 65 *nit* mutants from 13 isolates and evaluated genetic diversity among isolates.

*Corresponding author; Tel: +82-70-7877-2106
E-mail : changsw802@hanmail.net
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## Materials and Methods

### Fungal isolates

All 13 isolates were taken from a variety of turfgrass species in different golf courses. These samples were obtained from golf courses in Anseong (isolates-13, 14 and 15), Paju (isolates 1 and 2), Pocheon (isolates 17 and 18), Gaeryung (isolates 3, 4 and 5), Muzu (isolates 6 and 7), and Sangju (isolates 8, 9, 10 and 11) from 2009 to 2010 (Table 1).

### Isolation of *nit* mutants

*Nit* mutants of each *S. homoeocarpa* isolate were attempted for isolation using the same procedure developed by Jo et al. (2007). Water agar medium amended with chlorate (WAC: 2% agar, 0.2% glucose and 4% potassium chlorate) was used for screening *nit* mutants.

One 5-mm diameter mycelial plug was taken from the colony edge of each *S. homoeocarpa* isolate grown on potato dextrose agar (PDA), and transferred to 10 ml potato dextrose broth (PDB) in a 9.0-cm Petri dish. After incubation for 10 days at 25°C, mycelia were harvested and dried on sterilized paper towels. A 5 g sample of the mycelia was homogenized with 5 ml of sterile, distilled water using a blender at 15,000 rpm for 30 seconds. A 200 μl aliquot of shredded mycelial suspension was spread on ten plates of WAC for the each isolate. After spreading, all plates were air-dried in a laminar flow hood for 5 min and incubated at 25°C. When colonies of *nit* mutants became distinct with an expansive and thin mycelial growth on WAC after 30 days of incubation, the hyphal tip of each colony was transferred to Czapek-Dox solution agar medium (CDA) containing nitrate as a single source of nitrogen. Each colony transferred was determined whether it was a *nit* mutant by examining the presence of typical *nit* mutant phenotypes producing a thin and expansive growth with no aerial mycelium on CDA. *Nit* mutants confirmed were sub-cultured on WAC and stored at 4°C.

### Characterization of *nit* mutant phenotypes

As described in Jo et al. (2007), phenotypes of *nit* mutants were determined by their utilization of different nitrogen substances: $NaNO_2$ and hypoxanthine. Agar plugs (5 mm in diameter) were taken from the edge of each *nit* mutant culture growing on the CDA, and transferred to three types of media: CDA, CDA amended with $NaNO_2$ (0.05% w/v),

**Table 1.** The 18 *nit* mutant isolates and its origin of *Sclerotinia homoeocarpa*, and the site of collection and mutant type used in this study.

| No. | *Nit* mutant isolate | Original isolate of mutant | Site of collection | Mutant type [Z] |
|---|---|---|---|---|
| 1 | BC-5F-2-1 | BC-5F-2 | Paju, Gyeonggi | *nit*1 |
| 2 | BC-Nur-2-1 | BC-Nur-2 | Paju, Gyeonggi | *nit*2 |
| 3 | KR-Nur-3-1 | KR-Nur-3 | Gyeryong, Chungnam | *nit*1 |
| 4 | KR-Nur-3-4 | KR-Nur-3 | Gyeryong, Chungnam | NitM |
| 5 | KR-Nur-7-1 | KR-Nur-7 | Gyeryong, Chungnam | *nit*3 |
| 6 | Mu-18G-2-2 | Mu-18G-2 | Muju, Jeonbuk | *nit*2 |
| 7 | Mu-18R-1-7 | Mu-18R-1 | Muju, Jeonbuk | *nit*1 |
| 8 | OR-10R-2-1 | OR-10R-2 | Sangju, Gyeongbuk | *nit*2 |
| 9 | OR-10R-2-2 | OR-10R-2 | Sangju, Gyeongbuk | *nit*1 |
| 10 | OR-13F-1-1 | OR-13F-1 | Sangju, Gyeongbuk | *nit*3 |
| 11 | OR-13F-1-5 | OR-13F-1 | Sangju, Gyeongbuk | *nit*1 |
| 12 | SH-8R-6 | SH-8R | Anseong, Gyeonggi | NitM |
| 13 | SH-S-2-2-6 | SH-S-2-2 | Anseong, Gyeonggi | *nit*2 |
| 14 | SH-3T-1-1 | SH-3T-1 | Anseong, Gyeonggi | *nit*2 |
| 15 | SH-2T(F)-2-5-1 | SH-2T(F)-2-5 | Anseong, Gyeonggi | *nit*1 |
| 16 | SH-2T(F)-2-5-3 | SH-2T(F)-2-5 | Anseong, Gyeonggi | NitM |
| 17 | WS-Nur-2-2 | WS-Nur-2 | Pocheon, Gyeonggi | *nit*1 |
| 18 | WS-Nur-2-3 | WS-Nur-2 | Pocheon, Gyeonggi | *nit*2 |

[Z] *Nit* mutant phenotypes were determined by following the previously published nomenclature system (Jo et al., 2007). Mutants unable to use nitrate but able to utilize both nitrite and hypoxanthine were designated as *nit*1. Mutants incapable of using nitrate, nitrite and hypoxanthine were referred to as *nit*2. Mutants that could not use nitrate and nitrite but could use hypoxanthine were designated as *nit*3. Mutants capable of utilizing nitrite but not nitrate and hypoxanthine were designated as NitM.

and CDA amended with hypoxanthine (0.02% w/v).

An agar plug of each original wild-type isolate was also transferred to CDA as a wild-type control for each test run. *Nit* mutant phenotypes were determined by following the previously published nomenclature system (Jo et al., 2007). Mutants unable to use nitrate but able to utilize both nitrite and hypoxanthine were designated as *nit*1. Mutants incapable of using nitrate, nitrite and hypoxanthine were referred to as *nit*2. Mutants that could not use nitrate and nitrite but could use hypoxanthine were designated as *nit*3. Mutants capable of utilizing nitrite but not nitrate and hypoxanthine were designated as NitM.

Subsequently, all *nit*3 mutants were further tested for nitrite excretion. Each *nit*3 isolate was grown on urea medium (CDA amended with 0.04% urea) in a 9-cm Petri dish at 25ºC for 3 days. The plate was then flooded with a 10 ml solution of 3M $NaNO_3$. After 24 h incubation at 25ºC, $NaNO_3$ was poured off. By adding 1 ml of a sulfanilamide solution and 1 ml of a color indicator to the plate, the presence of nitrite was indicated by bright purple color. *Nit* mutants recovered from four isolates (BC-5F-2-1, KR-Nur-7-1, OR-10R-2-1, and SH-8R-6) were paired in all possible combinations to determine the compatibility among phenotypes.

### Vegetative compatibility assay

Different types of *nit* mutants screened from each of the 18 isolates were paired in all possible pair wise combinations. Agar plugs (5 mm in diameter) were cut from the edge of each *nit* mutant colony actively growing on CDA. Agar plugs from two different isolates were placed 2 cm apart on a 9-cm diameter plate of CDA; one plug on the center (tester isolate) and the other on the side of the plate (tester isolate). A pairing with the same *nit* mutant isolate (self-fusion) was also included for each test run by placing an additional agar plug of the tester isolate on the other side of the same plate as a negative control, representing no complementation (Jo et al., 2009). The plates were incubated at 25ºC for a month. A complementary reaction was evident by the development of dense aerial mycelial growth (Table 2). The pairing experiment was conducted twice.

**Table 2.** Complementation reactions between phenotypes of nitrate nonutilizing (*nit*) mutants of *Sclerotinia homoeocarpa*.

| Nit mutant phenotype | *nit*1 | *nit*2 | *nit*3 | NitM |
|---|---|---|---|---|
| *nit*1 | -[z] | + | - | + |
| *nit*2 | | - | + | + or - |
| *nit*3 | | | - | + or - |
| NitM | | | | - |

[z] -: prototrophic growth absent or inconspicuous between *nit* mutants of isolates. +: *nit* mutants of strains yielded a dense prototrophic growth at the mycelial interface between nit mutants of isolates. *Nit* mutants recovered from four isolates (BC-5F-2-1, KR-Nur-7-1, OR-10R-2-1, and SH-8R-6) were paired in all possible combinations to determine the compatibility among phenotypes.

## Results

Twenty-two isolates of *S. homoeocarpa* were collected from different commercial golf courses in the northern and middle region of South Korea during 2009 and 2010. Thirteen out of the 22 *S. homoeocarpa* isolates used in this study spontaneously generated a total of 65 *nit* mutants on WAC. Of isolates mutant was recovered, the frequency of the mutants resistant to chlorate varied among the isolates.

Over two runs, isolates SH-S-2 and SH-2T(F)-2 produced the greatest number of mutants, but the lowest number of mutants was generated by isolates BC-5F, KR-Nur and SH-3T, except isolates with no nit mutant. Nine isolates did not recovered *nit* mutants, indicating the mutant frequency of *S. homoeocarpa* depends on conditions for generation of *nit* mutants. Of these mutants 80% were characterized as *nit*1/*nit*2 mutants and the 20% as NitM mutants (Table 1).

Vegetative compatibility grouping determined by pairings between *nit* mutants are shown in Table 3. Except isolates Mu-18G-2-2, MU-18R-1-7, OR-10R-2-1, and SH-2T(F)-2-5-3, all isolates were consistently grouped into one VCG, which is a dominant group. Minor groups were not positively complemented with each other.

## Discussion

*Nit* mutants in culture media containing chlorate have commonly been used to define vegetative compatibility groupings of important plant pathogens (Brooker et al., 1991; Klittich & Leslie, 1988). It is reported that the genetic variation of a population in ascomycete fungi was caused by spontaneous natural mutation and heterokaryosis between compatible isolates due to a lack of sexual recombination in nature (Glass et al., 2000; Leslie, 1993).

The mutants could be visually identified and isolated from non-mutated colonies. At least one *nit* mutant in 13 out of 22 isolates was generated on WAC medium (data not shown). Although nine isolates were repeatedly inoculated on chlorate media, we failed to obtain more mutants, probably suggesting new condition for generation of *nit* mutants in *S. homoeocarpa*. Klittich and Leslie (1988) reported that the isolation frequency of *nit* mutant could be altered by changing the source of nitrogen in the chlorate medium.

Four phenotypic classes (*nit*1, *nit*2, *nit*3, and NitM) were identified from 65 *nit* mutants. Similar phenotypic recoveries were found for plant-pathogenic fungi such as *S. homoeocarpa* (Jo et al., 2007), *F. oxysporum* (Correll et al., 1987), *V. dahliae* (Korolev and Katan, 1997) and *C.*

**Table 3.** Vegetative compatibility (VC) based on complementary reaction of *nit* mutants on Czapek solution agar and VC grouping.

| Isolate | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | VCG group |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | ++[Z] | - | ++ | - | - | - | - | - | - | - | ++ | ++ | ++ | - | - | - | ++ | A |
| 2 | | - | ++ | ++ | ++ | - | - | - | ++ | ++ | ++ | ++ | - | - | - | - | ++ | - | A |
| 3 | | | - | ++ | - | - | - | - | - | - | - | ++ | ++ | ++ | - | - | - | ++ | A |
| 4 | | | | - | ++ | - | - | - | ++ | ++ | ++ | - | ++ | - | - | - | ++ | ++ | A |
| 5 | | | | | - | - | - | - | - | - | - | ++ | ++ | ++ | - | - | - | + | A |
| 6 | | | | | | - | - | - | - | - | - | - | - | - | - | - | - | - | B |
| 7 | | | | | | | - | - | - | - | - | +/- | - | - | - | - | - | - | C |
| 8 | | | | | | | | - | - | - | - | +/- | - | ++ | - | - | - | - | D |
| 9 | | | | | | | | | - | - | - | ++ | ++ | ++ | - | - | - | ++ | A |
| 10 | | | | | | | | | | - | - | ++ | ++ | ++ | - | - | - | ++ | A |
| 11 | | | | | | | | | | | - | ++ | ++ | ++ | - | - | - | ++ | A |
| 12 | | | | | | | | | | | | - | ++ | ++ | ++ | +/- | + | ++ | A |
| 13 | | | | | | | | | | | | | - | - | ++ | - | + | - | A |
| 14 | | | | | | | | | | | | | | - | + | - | ++ | - | A |
| 15 | | | | | | | | | | | | | | | - | +/- | - | + | A |
| 16 | | | | | | | | | | | | | | | | - | - | - | E |
| 17 | | | | | | | | | | | | | | | | | - | ++ | A |
| 18 | | | | | | | | | | | | | | | | | | - | A |

[Z] Diagonal interpret compatibility based on a complementation reaction of nit mutants was described in a report by Jo et al.(2007): ++ = strong heterokaryotic reaction; + = heterokaryotic reaction; - = no reaction; and +/- = not determined.

*coccodes* (Nitzan et al., 2002) on WAC medium. It appears that the genetic control of nitrogen catabolism including nitrate assimilation of *S. homoeocarpa* is similar to those of other plant pathogenic fungi such as *Colletotrichum*, *Fusarium*, and *Verticillium* (Leslie, 1993).

The frequency of the mutants resistant to chlorate varied among the isolates. All *nit*1 and *nit*3 mutants from an individual isolate readily complemented the NitM or *nit*2 mutants derived from different isolates within a same VCG. Of isolates tested, the type and frequency of the mutants resistant to chlorate varied among the isolates, suggesting dynamic process of mutation to chlorate resistant and underlying genetic differences in *S. homoeocarpa*. For example, isolate KR-Nur-3, OR-13F-1, and SH-2T(F)-2-5 produced more than eight mutants with three types, whereas isolate SH-2-2-2 only generated fifteen mutants of *nit*2. Also, Jo et al. (2007) reported that the variation of isolates to generate *nit* mutants was found in *S. homoeocarpa* isolates.

According to a report of Klittich and Leslie (1988), the frequency of mutant type could be altered by changing the nutritional sources in the chlorate medium. In this study, the dominant recovery of nit1 in *S. homoeocarpa* isolates was also similar to the phenomenon revealed in the plant pathogenic fungi such *C. sublineolum*, *V. dahlia*, and *C. cocodes* (Cecilia De Lima Favaro, 2007; Korolev and Katan, 1997; Nitzan et al., 2002).

Except one isolate (Mu-18G-2-2, MU-18R-1-7, OR-10R-2-1, and SH-2T(F)-2-5-3), all isolates with various geographic origins shared the same VCG within one dominant VCG, indicating the isolates could be similar in nitrate metabolism, possibly in many ways. Regular exchanges of turfgrass sod between golf courses might be a possible explanation (Vergara et al., 2004.). Another possibility is that most isolates tested were collected in the northern and middle part of south Korea, indicating additional isolates over region should be tested for VCG evaluation. Interestingly, SH and OR groups respectively originated from Sangju and Anseong belonged to two VCGs.

In conclusion, the prevalence of the same genetic lineage among the many isolates of *S. homoeocarpa* supports that isolates originated from a clonal population could be involved in outbreaks of dollar spot epidemics in golf courses of South Korea. But, the negative complementation of four isolates indicate that the isolates could be biologically distinct from the dominant VCG. Isolates in different groups would help to determine if there are more VCG in South Korea. Therefore, authors expect that the more evidence from many different regions or sites in South

Korea would provide valuable information on VCG epidemiology of *S. homoeocarpa*.

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# 한국의 잔디에서 분리한 *Sclerotinia homoeocarpa* 균의 체세포화합성군 분류

장석원[1]* · 장태현[2] · 홍점규[3] · 박종현[1] · 정석우[1]

[1]한국골프대학교 골프코스매니지먼트과, [2]경북대학교 생태환경대학 식물자원환경전공, [3]경남과학기술대학교 원예학과

**요 약**: 동전마름병을 일으키는 *Sclerotinia homoeocarpa*는 한국에서 가장 일반적인 한지형 잔디 병원균이다. 경기도, 충청남도, 전라북도, 경상북도 등 다양한 지역에서 분리한 13개 균주로부터 유도된 *nit* 변이균주를 이용하여 의한 체세포화합성군(vegetative compatibility group, VCG) 평가가 이루어졌다. 돌연변이주는 질소동화작용에 근거하여 *nit*1, *nit*2, *nit*3, NitM 등 4가지 유형으로 분류되었다. 균주간에 돌연변이주 발생정도는 크게 차이가 있었으며 최대 15개의 변이주를 생산한 균주가 있는 반면에 전혀 형성하지 못한 균주도 존재하였다. 선택배지에서 4가지 돌연변이주 유형 중에 *nit*1과 NitM의 발생빈도(80%)가 높았으며, *nit*2와 *nit*3은 발생빈도가 매우 낮았다. 한 개의 큰 체세포화합성군 그룹과 4개의 작은 그룹이 18개 돌연변이주로부터 결정되었다. Nit 변이균주를 이용하여 의한 VCG 평가는 매우 효율적이었으며, 향후 한국의 *Sclerotinia homoeocarpa*균의 효율적 관리를 위한 생태학적 · 역학적인 면을 연구하기 위해서는 보다 많은 지역으로부터 분리된 균주의 평가가 필요하다.

**주요어**: 잔디동전바른병균, 체세포화합성군, Nit 돌연변이주