

IDENTIFYING MYCELIAL COMPATIBILITY GROUPS OF *SCLEROTINIA SCLEROTIORUM* USING POTATO DEXTROSE AGAR AMENDED WITH ACTIVATED CHARCOAL

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Abstract: In this study, mycelial compatibility grouping (MCG) of *Sclerotinia sclerotiorum* (Lib.) de Bary was determined in 15 infected potato fields in Bahar and Lelehjin, Hamadan, Iran. Among 193 isolates, 37 MCGs were identified and 67% were represented by single isolates observed at single locations. Within Bahar fields, 29 MCGs were identified and 15 MCGs were recognized in Lalehjin samples. MCGs 2, 6, 18 and 25 were collected at high frequency from multiple locations. The efficacy of Potato Dextrose Agar (PDA) amended with activated charcoal for identifying MCGs in this fungus was tested in doubtful reactions and proved to be an effective medium. The activated charcoal produced a black reaction area in incompatible interactions as well as a red reaction line in PDA amended with McCormick's red food coloring.

Key words: clone, Hamadan, MCG, medium, stem rot

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is an important ascomycetous plant pathogenic fungus which has a wide geographic distribution and a diverse host range including many agronomic crops (Hartman *et al.* 1999). This pathogen has recently caused stem rot (also called white mold) on potato plants (*Solanum tuberosum*) in a large number of sprinkler irrigated fields at Hamadan, a potato-producing province in the west of Iran (Ojaghian 2009). Although there is extensive literature on the biology and ecology of *S. sclerotiorum* (Adams and Ayers 1979; Willetts and Wong 1980; Mitchell and Wheeler 1990), new information about the population biology of this fungus is still needed.

The populations of *S. sclerotiorum* are naturally found as a mosaic of clones (Kohn *et al.* 1991; Anderson and Kohn 1995; Cubeta *et al.* 1997) and a clone is defined as a genotype maintained in a single mitotic lineage (Anderson and Kohn 1995; Kohn 1995). Clones of *S. sclerotiorum* can be recognized by deoxyribonucleic acid DNA fingerprinting using molecular markers and/or mycelial compatibility groups (MCGs; Kohn *et al.* 1990; Kohn *et al.* 1991; Kohli *et al.* 1992). Mycelial compatibility is the ability of two strains of filamentous fungi to anastomose and form one confluent colony. Although this is synonymous with vegetative or somatic compatibility in some fungi like *Fusarium* spp. (Puhalla 1985) and *Rhizoctonia* spp. (Ceresini *et al.* 2002), respectively, movement of nuclei (via sexual or somatic ways) has not been reported be-

tween MCGs (Leslie 1993). Mycelial compatibility-incompatibility grouping system is a useful method in studying population dynamics of pathogenic and nonpathogenic isolates of fungi. This system is used for ascertaining the source of new races to a particular geographical area. Mycelial compatibility-incompatibility grouping system is an easy, quick, inexpensive, and macroscopic assay of the self-nonsel self recognition method and is determined using a side by side pairing system (Kohn *et al.* 1990).

It has been reported that MCG systems are not necessarily clonal and may be associated with one or more DNA fingerprints (Hambleton *et al.* 2002; Cubeta *et al.* 1997). This is ascribed to parallel gain or loss of fingerprint fragments resulting from transposable element activity or rare episodes of genetic exchange (Carbone *et al.* 1999). Phylogenetic analysis of clones using DNA sequence data from four regions, suggests a predominantly clonal mode of evolution with no evidence of contemporary genetic exchange and recombination between individual genotypes (Carbone *et al.* 1999). Kohn and co-workers (1991) analyzed the MCGs of *S. sclerotiorum* in two neighboring fields of canola (*Brassica napus*). They found high diversity of MCGs. They demonstrated with molecular data that the MCGs represented genotypically different strains. Because genetic regulation is observed in some ascomycetous fungi such as *Podospora anserine* and *Neurospora crassa*, it is probable that *S. sclerotiorum* is multigenic (Glass *et al.* 2000). Factors for the designation of MCGs or clones were shown to be stable and un-

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changed through successive sexual generations and after serial culturing. As Kohli and coworkers reported (1992), the correlation between an MCG and a DNA fingerprint or fingerprints supports the synonymous relationship between MCGs and clones of *S. sclerotiorum*.

Patterson (1985) determined MCGs of a *Sclerotinia minor* population on a specific culture medium named Patterson's medium. In the first study of MCGs on *S. sclerotiorum* strains, Kohn and coworkers (1990) amended this medium with 6 drops of McCormick's red food coloring per liter of medium, modified Patterson's medium (MPM). Pairings were evaluated in the dark at room temperature 4, 7 and 14 days after inoculation. Compatible pairings formed one confluent colony. Incompatible pairings produced a visible reaction in the interaction zone, such as a red line visible on the colony reverse, or a line of fluffy, aerial mycelium or thin mycelium on the colony surface. Microscopically, challenging hyphae in compatible interactions did not necessarily anastomose but were able to overgrow each other. In incompatible interactions that resulted in macroscopic red reaction lines, deterioration of hyphae was observed within and adjacent to the interaction zone. This medium can be inhibitory to isolates from some samples, confounding scoring of compatibility. Recently, Potato Dextrose Agar (PDA) amended with 75 µl/l of McCormick's red food coloring has been reported as an optimal medium for isolates inhibited by MPM from a recombining and highly diverse population sample. This precisely amended PDA is also suitable for isolates from highly clonal populations that are not inhibited by MPM or by higher concentrations of red food coloring (Schafer and Kohn 2006). Although PDA amended with activated charcoal is a suitable medium for determining somatic compatibility groupings in *Rhizoctonia solani* (Butler and Bolkan 1973; Ceresini *et al.* 2002), no information is available on the efficiency of this medium for determining MCGs within populations of *S. sclerotiorum*.

The objective of this study was to determine whether populations of this fungus in potato fields are predominantly from different MCGs, and whether each potato field is infected by different MCGs. In addition, the growth ability of several *S. sclerotiorum* isolates on charcoal-amended PDA was assessed. Patterson's medium and PDA amended with red food coloring were compared. Efficacy of charcoal-amended PDA in identifying MCGs in *S. sclerotiorum* was also tested.

MATERIALS AND METHODS

Isolates

From 15 naturally infected fields at Bahar, Hamadan, and Lelehjin, Hamadan, 224 isolates of *S. sclerotiorum* were sampled from potato plants. These fields had been under potato cultivation for more than four years. As described by Schafer and Kohn (2006), slow growing isolates were eliminated, and therefore 193 isolates (designated 1 to 193) were eventually used in this study (Table 1). All fields (sites) were in commercial production and under center-pivot sprinkler irrigation. Sites 1–9 were sampled in September 2008, and sites 10–15 were

sampled in October 2008. Ten to 18 plants were sampled per field. To ensure that isolates used in this study were a result of the current year's infection cycle, sclerotia were collected from stem surfaces or stem pith.

Table 1. List of studied sites where isolates of *S. sclerotiorum* were sampled

Site (field)	Isolate No.	Number of strains	Location	Host cultivar
1	1–15	15	Hamadan, Bahar	Agria
2	16–26	11	Hamadan, Bahar	Agria
3	27–39	13	Hamadan, Bahar	Marfauna
4	40–51	12	Hamadan, Bahar	Marfauna
5	52–63	12	Hamadan, Bahar	Agria
6	64–81	18	Hamadan, Bahar	Agria
7	82–93	12	Hamadan, Bahar	Agria
8	94–103	10	Hamadan, Bahar	Agria
9	104–113	10	Hamadan, Bahar	Marfauna
10	114–129	16	Hamadan, Lalehjin	Marfauna
11	130–144	15	Hamadan, Lalehjin	Agria
12	145–157	13	Hamadan, Lalehjin	Marfauna
13	158–169	12	Hamadan, Lalehjin	Agria
14	170–183	14	Hamadan, Lalehjin	Marfauna
15	184–193	10	Hamadan, Lalehjin	Agria

For fields 1–4, 10 and 13–15, sclerotia were sampled at 20 m intervals along a 200 m transect. In other fields, isolates were collected along several transects to sample the whole field.

One isolate was derived from a single sclerotium per plant, after surface sterilization in 3% sodium hypochlorite solution in sterile distilled water (SDW) for 2–3 min. Two or three rinses in SDW for 5 min followed. Heavy surface contamination of some samples necessitated rehydration in SDW for 30 min to 1 h, followed by an initial sterilization in 70% ethanol for 30 s. A higher concentration of sodium hypochlorite solution and a longer water rinse were also used. Surface disinfected sclerotia were cut into halves, and plated on PDA (Merck, Darmstadt, Germany) with the cut surfaces partially embedded. Isolates were maintained in test tubes containing PDA at 4°C or by storing sclerotia in glass vials at –20°C (Hambleton *et al.* 2002).

Mycelial compatibility grouping

Isolates were paired in all possible combinations including self-self on PDA. According to Schafer and Kohn (2006), under the standard protocol, inoculum was cut from 1 cm behind the growing hyphal front. Cutting was done in this way because hyphal tips have been shown to provide less repeatable results. As a rule, self-self pairings are the basis for determining a compatible interaction in self-nonsel self pairings. Mycelial reactions were recorded as compatible when a continuous colony was observed in the interaction zone. Mycelial reactions were noted as incompatible when an apparent line of demarcation (a barrage zone) was observed between the confronting paired isolates. Barrage zone reactions were a zone of sparse or no mycelium, or a thin to wide band of aerial and uniform mycelia. In both compatible and incompatible pairings, sclerotia developed in the interaction zone of two isolates.

Therefore, sclerotia formation was not considered as a criterion for MCGs designation. As described by Kohn and coworkers (1991), to facilitate MCG determination of all isolates listed in table 1, a subset of 15 isolates from each region (Bahar and Lalehjin) were selected and paired in all possible combinations. MCGs were determined, and an isolate was selected to represent each MCG. These representative isolates were paired with all remaining untested isolates until all isolates were assigned to an MCG. If an incompatible reaction occurred with any MCG representative isolates, the newly tested isolate was established as a newly observed MCG and was used to represent the new MCG. All pairings were conducted at least twice and scored independently by at least two people after incubation in the dark at 23°C for 4, 7, and 14 days. Because a universal MCG numbering system for *S. sclerotiorum* has not been established, a numbering system was used for this experiment (Kull *et al.* 2004).

Deployment of charcoal-amended PDA in difficult-scoring reactions

In the instances where the interaction between colonies was difficult to interpret or the barrage zone was not wide, pairings were repeated on PDA (Merck, Darmstadt, Germany) amended with 1 g/l activated charcoal (carbon amended PDA: CAP) as well as PDA modified with 75 µl/l of McCormick's red food coloring (red food coloring amended PDA: RFAP) (McCormack Corp., Dallas, Texas) (Schafer and Kohn 2006) and Patterson's medium (PM) (0.68 g/l KH_2PO_4 , 0.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g/l KCl, 1.0 g/l NH_4NO_3 , 18.4 g/l D-glucose, 0.5 g/l yeast extract, 15.0 g/l agar, 1.0 l distilled H_2O , 200 µl M.P.F.Y.E. trace element solution containing: 95 ml distilled H_2O , 5.0 g citric acid monohydrate, 5.0 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.25 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.05 g H_3BO_3 , 0.05 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) (Patterson 1985, Vogel 1964). A ml of CHCl_3 was added to 1 liter of the medium PM as a preservative (Schafer and Kohn 2006) and was maintained at room temperature (21–24°C). For pairing, 2 mm discs of mycelial inoculum were removed from the inner colony (less than 1 cm from the growing margin) and placed 3.5 cm apart from the challenging isolate in a 9 cm Petri dish (one pairing per dish) and incubated in the dark at 20–24°C. The pairings were replicated ten times on these three media. All pairings were scored by more than one person after 6, 8 and 10 days.

In order to assess the effect of charcoal on the growth of *S. sclerotiorum*, 5 isolates of different MCGs (isolates no. 35, 40, 52, 64 and 153) were selected randomly and cultured on PDA (as the control), RFAP, CAP and PM. After 3 and 6 days of incubation at a temperature of 25°C, in the dark, the average radial growth of this fungus on these media was calculated. In this *in vitro* trial, each treatment was replicated five times and the effects of different treatments were determined by the analysis of variance (ANOVA) using SAS software (SAS 8.2, 1999–2001; SAS Institute Inc., Cary, NC) in a completely randomized design.

Moreover, 20 isolates from diverse and similar MCGs (isolates 1–8 and 20–31) were paired four times on CAP with different amounts of activated charcoal (0.5, 1 and 2 g/l) in different combinations including self-self. Inter-

actions were observed after three days at 25°C by more than two persons and compared with their confronting reactions on PDA, PM and RFAP.

RESULTS AND DISCUSSION

Among 193 *S. sclerotiorum* isolates, 37 MCGs (designated 1 to 37) were identified (Table 2). Most (67%) of the MCGs were unique, which describes an MCG represented by a single isolate observed at a single location. Although both unique and common MCGs were observed at both Bahar and Lalehjin, MCGs and MCG frequencies were not completely similar within these two sampled regions (Table 3). In this study, frequently recorded MCGs (no. 2, 6, 18 and 25) included isolates sampled from both cultivars and they were sampled at high frequency from multiple locations.

Table 2. Mycelial compatibility group (MCG) designation for investigated *S. sclerotiorum* isolates

MCG No.	Isolate No. (Number of isolates in each MCG)
1	1 (1)
2	2–5, 28–31, 101–114, 169–170 (24)
3	6 (1)
4	7, 46–50, 158–160 (9)
5	8 (1)
6	11, 15–26, 116–136, 161–167, 174–182 (50)
7	10 (1)
8	9 (1)
9	12, 65, 71, 91–94 (7)
10	13 (1)
11	14, 168, 171 (3)
12	27 (1)
13	32 (1)
14	33 (1)
15	34 (1)
16	35 (1)
17	36 (1)
18	37, 40–45, 138–152 (22)
19	96–98, 184 (4)
20	38 (1)
21	39 (1)
22	51 (1)
23	52 (1)
24	53 (1)
25	54–56, 74–85, 154–156 (18)
26	57–63, 99–100 (9)
27	64, 66–70, 72 (7)
28	73 (1)
29	86–90, 95 (6)
30	115 (1)
31	137 (1)
32	153 (1)
33	157, 186–193 (9)
34	172 (1)
35	173 (1)
36	183 (1)
37	185 (1)

Table 3. Number of isolates and mycelial compatibility groups (MCGs) observed in both sampled areas

Sampled region	No. of isolates	No. of MCGs	Identified MCGs
Hamadan, Bahar	113	29	1–29
Hamadan, Lalehjin	80	15	2, 4, 6, 11, 18, 19, 25, 30–37

Table 4. Radial growth of five isolates of *Sclerotinia sclerotiorum* on 4 culture media including PDA, CAP, PM and RFAP after 3 and 6 days at 25°C

Isolates	Colony diameter [mm]									
	3 days					6 days				
	35	40	52	64	153	35	40	52	64	153
Culture media										
PDA (control)	37.2a*	33.4a	22.4a	39.6a	24.8a	83.6a	87.2a	58.9a	88.2a	74.1a
CAP	34.4a	34.5a	23.4a	35.5a	24.2a	83.1a	86.2a	57.2a	89.1a	75.2a
PM	32.2a	32.3a	17.2b	37.3a	23.9a	64.2b	83.4a	41.2b	71.4b	62.1b
RFAP	35.5a	30.5a	21.8a	37.5a	25.2a	82.2a	84.3a	56.8a	85.1a	73.5a

*means within columns followed by the same letter are not significantly different ($p < 0.05$) using the least significant difference (LSD) test

Compatible pairings formed one confluent colony on all three media (CAP, PM and RFAP). The charcoal and red food coloring produced a black and red reaction line in incompatible interactions, respectively. In CAP tests, this reaction zone was quite visible on colony reverse in front of a light. The more mycelial incompatibility there was, the thicker the line. In PM, incompatible reactions were mainly a line of sparse or no mycelium. No difference was observed in compatibility/incompatibility results of the three tested media.

In growth assessment of five isolates of *S. sclerotiorum* on the four different media, activated charcoal inhibited aerial growth of mycelia to some extent. Compared with PDA and RFAP, however, no inhibitory effect was significantly observed ($p < 0.05$) on horizontal growth of the fungus after 3 and 6 days of incubation (Table 4). Compared with the control, PM was the only medium that inhibited radial growth of no. 52 isolates after 3 days, and isolates no. 35, 52, 153 and 64, after 6 days.

After pairing 20 isolates on CAP with different amounts of activated charcoal, no inhibitory effect of activated charcoal was observed in concentrations 0.5 and 1 g/l. But concentration 2 g/l significantly reduced radial growth of *S. sclerotiorum* in isolates no. 2, 4, 5, 7, 8 and 20–31 (Table 5). Compared with concentration 0.5 g/l, incompatible interaction lines were more visible and interpretable in CAP with 1 g/l of activated charcoal.

Based on our results, it became clear that populations of *S. sclerotiorum* from two potato-growing regions were a heterogeneous mix of MCGs, and different MCGs were detected in each field. This study confirms the former reports of *S. sclerotiorum* MCG structure on several crops other than potato in the Americas and Northern Europe (Kohli *et al.* 1992, 1995; Cubeta *et al.* 1997; Carpenter *et al.* 1999; Durman *et al.* 2001; Hambleton *et al.* 2002; Atallah *et al.* 2004). The population structure of *S. sclerotiorum* based on MCGs, appears similar irrespective of host crop and field location (Kull *et al.* 2004). In this study, no association of MCG frequency with potato cultivar was evident.

Table 5. The effect of activated charcoal in three different concentrations on radial growth of *S. sclerotiorum* after 4 days at 25°C compared with PDA (the control)

isolate No.	Colony diameter [mm]			
	control	0.5 g/l	1 g/l	2 g/l
1	27 a	26 a	27 a	26 a
2	34 a	36 a	32 a	22 b
3	31 a	32 a	32 a	31 a
4	29 a	28 a	27 a	20 b
5	30 a	31 a	31 a	21 b
6	27 a	25 a	25 a	25 a
7	30 a	31 a	30 a	23 b
8	25 a	26 a	24 a	12 b
20	33 a	31 a	32 a	24 b
21	30 a	30 a	28 a	24 b
22	28 a	28 a	27 a	21 b
23	25 a	25 a	24 a	12 b
24	32 a	34 a	34 a	25 b
25	25 a	24 a	26 a	13 b
26	25 a	26 a	24 a	14 b
27	36 a	35 a	36 a	21 b
28	36 a	35 a	36 a	14 b
29	37 a	35 a	35 a	21 b
30	37 a	35 a	36 a	24 b
31	29 a	28 a	28 a	13 b

*means within rows followed by the same letter are not significantly different ($p < 0.05$) using the least significant difference (LSD) test

It has been reported that more recombination is evident in subtropical populations compared with temperate ones (Carbone *et al.* 1999; Carbone and Kohn 2001). The distance between Bahar and Lalehjin is 5–6 km and a number of unique MCGs were observed at these two sampled regions. In New Zealand, a study was done on the comparison of DNA fingerprint patterns from *S. sclerotiorum* populations. The results showed considerable

local movement of isolates but provided little evidence for long-range dispersion (Carpenter *et al.* 1999). Center-pivot sprinkler irrigation (Atallah and Johnson 2004), high crop density, close row width, and excess nitrogen fertilization are factors linked to the increased incidence of *S. sclerotiorum* disease (Natti 1971; Grau and Radke 1984). All these factors have recently become customary in potato fields at Hamadan. MCG structure of *S. sclerotiorum* on cultivated hosts appears to be more complex, indicating that agricultural practices may influence MCG frequencies and patterns (Kull *et al.* 2004). In this study, both high and low frequency of MCGs were observed at potato fields and this finding is in accordance with previous MCG studies. *S. sclerotiorum* MCGs on soybean may be unique and exist locally at low frequencies, as indicated by 65% of the MCGs sampled being represented by one isolate. Localization of unique MCGs was observed in vegetable-growing regions in New Zealand (Carpenter *et al.* 1999) and in winter canola in Harrison, Ontario (Kohn *et al.* 1991). Unique and low-frequency MCGs in a sampling area may be recent MCG introduction events or random mutation events (Kull *et al.* 2004). The appearance of new clones or MCGs in single fields may be an indication of genotypes adapted to specific field microclimates or hosts. Such an appearance is less likely the result of genetic exchange and recombination (Ben-Yephet *et al.* 1985; Hambleton *et al.* 2002). It is necessary to identify the factors which help the pathogen spread. An understanding of the population biology of this pathogen should offer some suggestions on this subject. MCGs may exist locally at high frequency. Growers who utilize a portion of each crop for seed for the next growing season or continually plant the same soybean cultivar may tend to localize and increase the frequency of specific MCGs (Kull *et al.* 2004). Prevalent MCGs may be due to human-caused movement of sclerotia or infested seed through different distribution ways. Moreover, isolate fecundity factors may affect increment of certain genotypes. It is necessary to study the populations of *S. sclerotiorum* in Hamedan to determine the most repeated isolates in potato fields. In addition to potato, canola (*Brassica napus* L.) is an economically important and highly susceptible crop cultivated in Hamadan province. This pathogen is the most destructive disease of canola in northern Iran (Hemmati *et al.* 2009). The results of this research can be used in population studies of this pathogen in probable relationship between infections of canola and potato fields.

MCGs No. 2, 6, 18 and 25 were sampled at high frequency in this research. MCGs may be found at high frequencies with wide geographic distribution (Kohli *et al.* 1992). Prevalent MCGs may be due to movement of sclerotia or infested seed through different distribution ways.

In classification of isolates into mycelial compatibility groups, appropriate conditions for growth are important (Schafer and Kohn 2006). Diverse compatibility reactions are difficult to categorize and score (*e.g.* in samples from populations with high genetic diversity). In this study, CAP was investigated for its application for determining MCG in *S. sclerotiorum* populations and it was compared with other media. CAP showed good efficacy in scoring unconvinced reactions and it is possible to use this me-

dium for this purpose. To study heterogeneous clones of this fungus, it is necessary to determine frequent MCGs in infected potato and other crops fields during consecutive and alternate growing seasons at Hamadan province.

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