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Variation in oxalic acid production and mycelial compatibility within field populations of *Sclerotinia sclerotiorum*

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Abstract

This study was aimed at detecting mycelial compatibility groups and variations in oxalic acid production in *Sclerotinia sclerotiorum*. For this purpose, 121 isolates of this plant pathogen recovered from lettuce, soybean and sunflower field crops, and grouped in 46 MCGs were tested for their ability to release oxalic acid and other organic acids to the medium. Oxalic acid production on liquid media was measured spectrophotometrically and release of organic acids was estimated by isolate abilities to discolour solid media amended with bromophenol blue. There were significant differences among MCGs in both oxalic acid and organic acids releasing, ranging the mean production of oxalic acid between 18 and 110 μg oxalic acid mg^{-1} dry wt. When isolates were grouped by their hosts, those obtained from soybean presented the highest release of oxalic acid (71 μg oxalic acid mg^{-1} dry wt), while those from sunflower showed the highest release of other acids to the medium. Solid medium discoloration was not correlated with oxalic acid concentration in liquid medium (Spearman $R=-0.085$; $P=0.126$).

Keywords: *Sclerotinia sclerotiorum*; Oxalic acid; Organic acids; Mycelial compatibility groups

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a widespread fungal pathogen that causes disease on many economically important vegetables and field crops (Boland and Hall, 1994). Approximately 90% of its life cycle is spent in soil as sclerotia and their high persistence make of *S. sclerotiorum* a very successful pathogen (Adams and Ayers, 1979).

This fungus produces and secretes millimolar concentrations of oxalic acid into their surroundings (Cessna et al., 2000). The observation that mutants unable to synthesize oxalate were non-pathogenic, whereas revertant strains displayed normal virulence confirms oxalate as a pathogenicity determinant in *S. sclerotiorum* (Godoy et al., 1990). Besides oxalic acid, *S. sclerotiorum* may release other dicarboxylic organic acids such as succinic, malic, fumaric and glycolic (Vega et al., 1970).

Considerable research interest has been focused on oxalic acid production by this pathogen and the following mechanisms of action have been proposed to explain its involvement in pathogenesis: (1) lowering infected tissues pH, what enhances the activity of extracellular enzymes produced by the pathogen (Bateman and Beer, 1965), (2) chelation of cell wall Ca^{2+} by the oxalate anion, what softens plant cell wall and compromises the function of Ca^{2+}-dependent defence responses (Bateman and Beer, 1965), (3) direct toxicity to host plants, what weakens the plant and facilitates invasion (Noyes and Hancock, 1981), and (4) suppression of the host plant oxidative burst (Cessna et al., 2000).

It has been established that field crops populations of *S. sclerotiorum* are clonal and that several clones may infect each field (Kohn, 1994). One of the criterions for detecting clonality is the mycelial compatibility grouping. When paired in culture, all members of an MCG can anastomose to form one confluent colony with no reaction line. These members also share a unique complex DNA fingerprint (Kohn et al., 1991; Carbone et al., 1999).

Identifying an association between MCGs and oxalic acid, one of the main determinants of *Sclerotinia*

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pathogenicity, is important for developing strategies to combat its spread among agronomically important crops, particularly because some MCGs have shown to persist from year to year specially as resistant sclerotia in soil, covering a very wide geographical area being some of them responsible for a large proportion of infections (Hambleton et al., 2002; Durman et al., 2003).

Therefore, the determination of oxalic acid in *S. sclerotiorum* is of importance, and numerous methods have been used including $KMnO_4$ tritation (Bateman and Beer, 1965), enzyme-based colorimetric assay (Yriberri and Possen, 1980), spectrophotometry (Allan et al., 1986; Zhou and Boland, 1999), high pressure liquid chromatography (Jarosz-Wilkolazka and Gad, 2003), for this and other fungi.

A previous report from Durman et al. (2003) based on more than 140 *S. sclerotiorum* isolates collected from soybean, sunflower and lettuce crops in the Buenos Aires province, demonstrated that these populations presented a clonal structure and that each one was made up by several MCGs. The objectives of this study were: (1) to evaluate the ability to release oxalic acid by different *S. sclerotiorum* isolates from economically important field crops and (2) to establish the presence of an association between oxalic acid release and specific genotypes (or MCGs) or host.

2. Materials and methods

2.1. Isolates

The study was performed on 121 *S. sclerotiorum* isolates confined, from a previous study (Durman et al., 2003), to 46 different MCGs. Nineteen isolates were obtained from sunflower, 49 from lettuce and 53 from soybean field crops in the Buenos Aires province, Argentina (Fig. 1).

2.2. Oxalic acid determination

For oxalic acid determination isolates were grown in 60 ml flasks containing 15 ml of potato dextrose broth (PDB, 2% glucose and 0.4% fresh potato extract in distil water). Flasks were statically incubated for 3 days at room temperature. Cultures were vacuum filtered and the supernatant was used as oxalic acid sample. Mycelial fractions dry weights were determined after drying at 80 °C, 72 h. Oxalic acid was determinated in the PDB culture following Xu and Zhang (2000) with few modifications. Reaction mix contained 0.2 ml of sample (or standard oxalic acid solution), 0.11 ml of bromophenol blue (BPB, 1 mM), 0.198 ml of sulfuric acid (1 M), 0.176 ml of potassium dichromate (100 mM) and 4.8 ml of distilled water. The reaction mix was placed in a water bath at 60 °C and quenched by adding 0.5 ml sodium hydroxide solution (0.75 M) after 10 min. The absorbance was measured at 600 nm by means of a spectrophotometer (Spectronic 20D+, Spectronic Instruments, Inc. USA) and PDB was used as the blank control. Oxalic acid concentration was calculated comparing with a standard curve and was expressed as μg oxalic acid mg^{-1} dry wt mycelium. Assay was run in triplicate and repeated once.

2.3. Estimation of total acids production

Plugs from 3-days old *S. sclerotiorum* colonies were inoculated onto Petri plates filled with potato dextrose agar and BPB (50 mg ml^{-1}). Plates were incubated for 3 days in darkness and 25 °C. Since bromophenol blue is a pH-indicator that turns from blue to yellow when pH is 3–4.6, the presence of a yellow halo was considered an evidenced of acid releasing by the fungus. Radii of yellow halo and colony were both

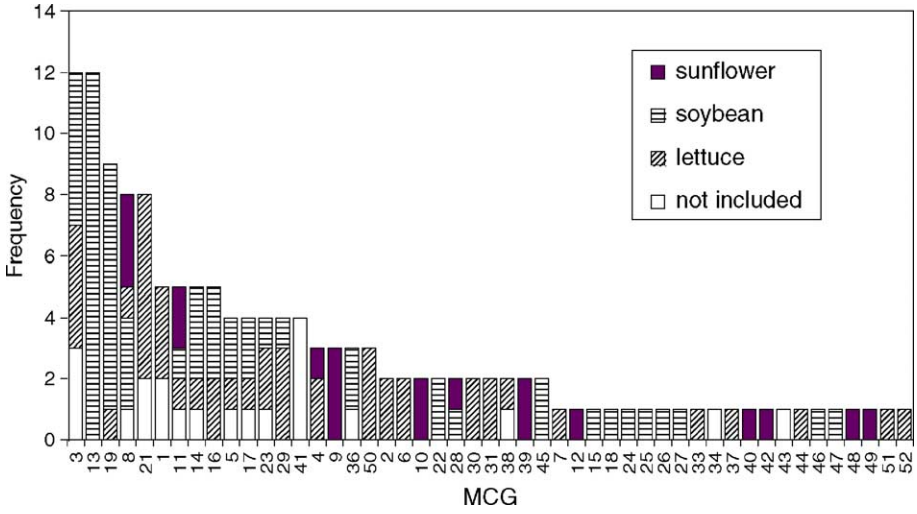

Fig. 1. *S. sclerotiorum* MCG frequencies. Shaded area in each column indicates number of isolates included in this study, discriminated by the host from which they were collected.

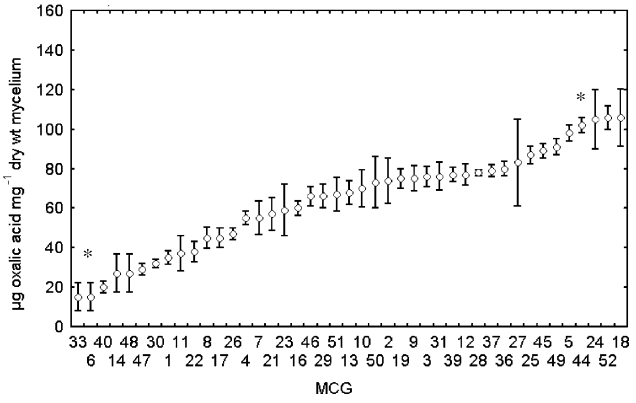

Fig. 2. Oxalic acid produced and released in PDB by the *S. sclerotiorum* isolates from the different MCGs. Oxalic acid was expressed as µg oxalic acid mg^{-1} of dry mycelium. Each point is the mean of all the isolates from the same MCG. Vertical bars represent the standard error of the mean. *, significantly different at $P \leq 0.05$.

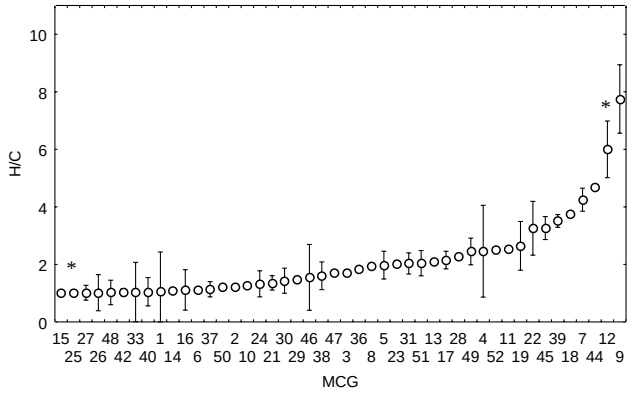

Fig. 3. Estimation of acid production and releasing on PDA amended with BPB by the *S. sclerotiorum* isolates from the different MCGs. Estimation of acid production was expressed as the ratio between radii of medium discoloration halo and colony (H/C). Each point is the mean of all the isolates belonging to the same MCG. Vertical bars represent the standard error of the mean. *, significantly different at $P \leq 0.05$.

measured from the centre of the inoculation plug and the ratio between them (H/C) was used as the parameter for the data analysis. Assay was run in quadruplicate and repeated once.

2.4. Data analysis

After grouping isolates by MCG or by isolate source host, oxalic acid concentration data were analysed using analysis of variance (ANOVA) and Tukey HSD (Honest Significant Difference) test for unequal sample sizes (Spjotvoll and Stoline, 1973) where appropriate. Data were checked for deviations from normality and homogeneity of variance prior to analysis. H/C data were analysed using Kruskal–Wallis tests (Kruskal and Wallis, 1952) and comparison of means ranks. Spearman rank correlation analysis was performed between H/C vs. oxalic acid concentration. Analyses were performed with the Statistix (Analytical Software, 1998) and Statistica packages (StatSoft, 1998).

3. Results

When oxalic acid productions were compared among MCGs, the mean production ranged between 18 and 110 µg oxalic acid mg^{-1} dry wt (Fig. 2). There was a high degree of variability in oxalic acid production among MCGs, however, significant differences among them could be detected (Table 1). Oxalic acid released by MCGs 44, 24, 52 and 18 was several folds higher than that released by MCGs 33 and 6. The remaining 37 MCGs produced intermediate levels of oxalic acid.

Ratio between yellow halo and colony radii ranged between 1 and 8 (Fig. 3). Ninety six percent of MCGs showed a H/C relation lower than 5. Despite the high variability within each MCG, weaker and stronger acid releasers (MCGs 15 and 25, and 12 and 9, respectively) could be distinguished (Table 1).

Grouping isolates by their host showed that those obtained from soybean and sunflower presented the highest release of oxalic acid in PDB and acids in the Petri plate assay, respectively (Table 2).

Table 1
Significance of ANOVA showing differences in (1) oxalic acid produced and released in PDB by the *S. sclerotiorum* isolates from the different MCGs and (2) estimation of acid production and releasing on PDA amended with BPB by the *S. sclerotiorum* isolates from the different MCGs

Summary of all effects						
	df effect	MS effect	df error	MS error	*F*	*P*-level
1	42	2939.03613	281	1476.88013	1.9900	0.0006
2	45	12.7602654	422	3.18112302	4.0112	0.0000

Table 2
Estimation of oxalic acid concentration in PDB and acids production and releasing on PDA amended with BPB by the *S. sclerotiorum* isolates sampled from the different hosts

Host	Oxalic acid[a] (µg oxalic acid mg^{-1} dry wt mycelium)	H/C[b]
Lettuce	54[c] ±40 a[d]	1.87±0.13 a
Soybean	71±50 b	1.88±0.12 a
Sunflower	60±30 ab	2.86±0.35 b

[a] Oxalic acid concentration was expressed as µg oxalic acid mg^{-1} dry wt mycelium.
[b] Estimation of acids production was expressed as the ratio between radii of medium discoloration halo and colony (H/C).
[c] Each point is the mean of all the isolates collected from the same host followed by the standard error of the mean.
[d] Points with the same letter do not significantly differ at $P \leq 0.05$.

Correlation analysis among variables showed that the ratio H/C in the Petri plate assay was not correlated with oxalic acid concentration in PDB (Spearman $R = -0.085$; $P = 0.126$).

4. Discussion

The present work constitutes the first attempt to detect differences in oxalic acid production among different MCGs of *S. sclerotiorum*. Results from oxalic acid determination showed levels above those reported by Zhou and Boland (1999) for the same pathogen.

The fact that the great majority of MCGs overlap in their ability to release oxalic acid, could explain the failures to correlate MCGs and aggressiveness in the works by Ekins (1999); Durman et al. (2003). This, in addition to the finding that those MCGs with the highest oxalic acid production do not correspond to those most frequently sampled, supports the idea that there are other traits determining aggressiveness and sclerotial resistance in soil.

In turn, the lower H/C ratio and oxalic acid release observed in isolates collected from lettuce is in accordance with a previous study in which these same isolates showed to produce lesions smaller in size than those produced by isolates collected from sunflower or soybean (Durman, 2004). These results contrast with Ekins (1999) who found that aggressiveness did not correlate with the host from which an isolate has been collected.

Previous studies (Price and Colhoun, 1975; Scott, 1984) considered that significant host specialization does not occur in *S. sclerotiorum*. However, differences found in this work in the ability to release acids in general and oxalic acid in particular, among isolates collected from different hosts, suggest that the host could exert some kind of selective pressure on *S. sclerotiorum* isolates, based on such an ability.

Since some organic acids could diffuse better in the PDA medium than others, differences found in the H/C ratio could be related not only to the amount of their production, but also to the variability in the proportions of dicarboxylic organic acids (besides oxalic acid) composition among MCGs. This variability could explain the absence of a correlation between the ratio H/C and oxalic acid concentration in the liquid medium and suggests that the Petri plate assays would not be useful for detecting isolates that are either strong or weak releasers of oxalic acid. However, null H/C rate producing isolates always showed very low levels of oxalic acid concentration in PDB (data not shown), suggesting that this method would be useful for the visual detection of isolates releasing extremely low amounts of oxalic acid.

Finally, qualitative differences in acid composition among MCGs would be of pathological relevance and deserve further research.

In conclusion, populations of *S. sclerotiorum* are highly diverse, irrespective of the source, and this could affect successful disease prediction and management. Therefore, it would be useful to take into account the differences in oxalic acid production among MCGs for screening purposes in breeding programs, since clonal propagation can yield a large amount of genetically uniform biomass for each clone and thus it is likely to quickly overcome the newly control measures.

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