Analysis of mycelial compatibility and pathogenicity of *Sclerotinia sclerotiorum* isolated from oilseed crops in Canada, China, USA and England

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Abstract

Objectives of this study were to understand the genetic and pathogenic variations of *Sclerotinia sclerotiorum* isolates originating from a worldwide collection. For this purpose, 160 isolates recovered from oilseed crops in China (71 isolates), Canada (39 isolates), the United States (20 isolates) and England (30 isolates) were evaluated for their mycelial compatibility and pathogenicity by comparing their differences on radial growth, aggressiveness, sclerotial yield and productions of oxalic acid and total acids. Mycelial compatibility group (MCG) testing identified 44 MCGs, of which, 56.8% were represented by single strains. Isolates from different countries were incompatible except those in MCG11 and MCG17, which were collected from Manitoba (MB), Canada and its neighboring North Dakota (ND), USA. There were significant differences in terms of radial growth, sclerotial yield, aggressiveness, production of oxalic acid and total acids within tested isolates and among MCGs ($P<0.0001$) regardless of their geographic origins. Correlation analysis indicated that mycelial growth was positively related to aggressiveness ($r=0.46$), oxalic acid production ($r=0.2$) and total acids secretion ($r=0.31$), but not to the yield of sclerotia produced on PDA ($r=0.09$). Aggressiveness was positively related to oxalic acid concentration ($r=0.74$) and negatively related to the production of total acids ($r=-0.75$). Additionally, there was high positive relationship between pH and oxalic acid production ($r=0.9$).

Key words: oilseed crops, *Sclerotinia sclerotiorum*, MCG, pathogenicity, aggressiveness, oxalic acid.

Introduction

Sclerotinia sclerotiorum is a necrotrophic pathogen that can infect over 400 species of plants, including dicotyledonous crops (sunflower, soybean, oilseed rape, edible dry bean, chickpea, peanut, dry pea, lentils and various other legumes) and a few monocotyledonous species such as onion and tulip (Boland and Hall, 1994). Sclerotia are the resting structures which allow the fungus to survive for a long time under adverse conditions. The secretions of cell-wall-degrading enzymes (CWDEs) and oxalic acid are the major virulence factors during infection (Bolton et al., 2006). Oxalic acid is proposed to facilitate the infection mainly: (a) by lowering pH of the host tissue and enhancing the activities of CWDEs (Bateman and Beer, 1965; Magro et al., 1984); (b) by weakening the plant due to the toxin of oxalate (Noyes and Hancock, 1981); (c) through chelation of cell wall Ca^{2+} by the oxalate anion, which softens plant cell wall and compromises the function of Ca^{2+}-dependent defense responses (Bateman and Beer, 1965); and (d) by suppressing the host plant oxidative burst (Cessna et al., 2000).

Previous studies have shown that *S. sclerotiorum* populations in canola in Canada and cabbage in the United States were clonal and isolates could be separated into distinct mycelial compatibility groups (MCGs) (Kohn et al., 1991; Cubeta et al., 1997). MCG testing is a phenotypic, macroscopic assay of the self/non-self recognition system controlled by multiple loci common in fungi (Carbone et al., 1999). In this study, by using 160 isolates of *S. sclerotiorum* isolated from oilseed crops from China, Canada, England and the United States, our aim were to (1) detect the genetic variability among isolates from long distance locations; (2) comprehensively compare the pathogenic differences among MCGs with regard to aggressiveness, radial growth, sclerotial production on medium and leaf of canola, production of oxalic acid and total acids; and (3) analyze the correlation among those factors related to pathogenicity.

Materials and Methods

Isolates 160 isolates of *S. sclerotiorum* from were grouped in four sets according to the country isolated. The China Set included 71 isolates; Canada Set, 39 isolates; England Set, 30 isolates; and the United States, 20 isolates. All isolates were inoculated onto potato dextrose agar (PDA) media and their mycelial tips were cut and re-cultured at least three times to obtain genetically identical cultures.

Radial growth and sclerotial yield on PDA Plates containing 20 ml of PDA amended with 50 mg/L of bromophenol blue (BPB) were made. A 7-mm plug removed from the actively growing colony margin was inoculated on PDA with 6 reps for each isolate.

Aggressiveness assessment and sclerotial yield on leaf The true and second leaves with a weight of 0.8-1.2g from

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30-day old seedling of *B. napus* cultivar Westar were used. In a 9-cm dish, a leaf was put on a layer of filter paper saturated with 2 ml water. A 7-mm PDA disc was inoculated on the center of leaf and 3 reps done for each strain. The diameter of necrotic lesions was measured at 48h after inoculation and the amount of sclerotia per leaf was recorded after incubation for 14 days. The sclerotial yield was expressed as sclerotia per gram of leaf.

Estimation of oxalic acid and total acid production Broth cultures of each isolate were vacuum filtered and the supernatant used for oxalic acid and pH tests. Oxalic acid was determined following Durman et al. (2005). Total acids production was estimated by testing pH value of supernatant using a pH meter. Assay was run in triplicate and repeated once.

MCG analysis 3 strains were co-cultured triangularly on PDA plates amended with 50mgL^{-1} bromophenol blue (BPB), which was added to enhance the visibility of the incompatible reaction. Each isolate was paired with itself as a control for compatibility. Assay was repeated at least once.

Results

1. Determination of MCGs For the 160 isolates tested, 44 MCGs were identified. Of the 44 MCGs, 25 groups (accounting for 56.8%) were unique, which described an MCG represented by single isolate, especially the Canada Set, which 39 isolates were grouped in 28 MCGs, contained 21 MCGs represented by a single isolate. There were 3, 6, 9 and 28 MCGs respectively identified for sets from China, England, USA and Canada. Isolates among sets of China, England, Canada/USA, were incompatible. In MCG11, isolate DF148 collected from Manitoba, Canada was compatible with 6 isolates from North Dakota, USA, and in MCG17, DF31 isolated from ND, USA was compatible with DF129 from MB, CA. Geographically, MB and ND are neighboring each other, and the MCG of isolates may be due to clonality in these regions.

2. Radial growth and aggressiveness All isolates of *S. sclerotiorum* grew rapidly and the color of medium changed quickly from blue to yellow due to the secretion of oxalic acid and other organic acids by mycelia. After incubation of 48 hours, colonies attained the diameters ranging from 3.67±0.15 cm (isolate DF10) to 8.93±0.24 cm (isolate DF112). Significant differences ($P<0.05$) were observed on the radial growth of isolates regardless of their geographic origin, hosts and MCGs (data not shown). When isolates were grouped by mycelial compatibility, the average growth rates varied between 5.06±0.09 cm/48h (MCG4) and 8.67±0.07 cm/48h (MCG16) (Fig. 1). The pathogenic variability estimated using detached leaf test yielded symptoms on leaf 12 h after inoculation. When performed the same analysis on MCGs, significant differences of pathogenic variability were observed among MCGs ($P<0.0001$), where lesions were between 1.65±0.2 cm/48h (MCG4, from England) and 5.02±0.25 cm/48h (MCG3, from China). These pathogenic differences of isolates and MCGs were not found to relate with their geographic origin.

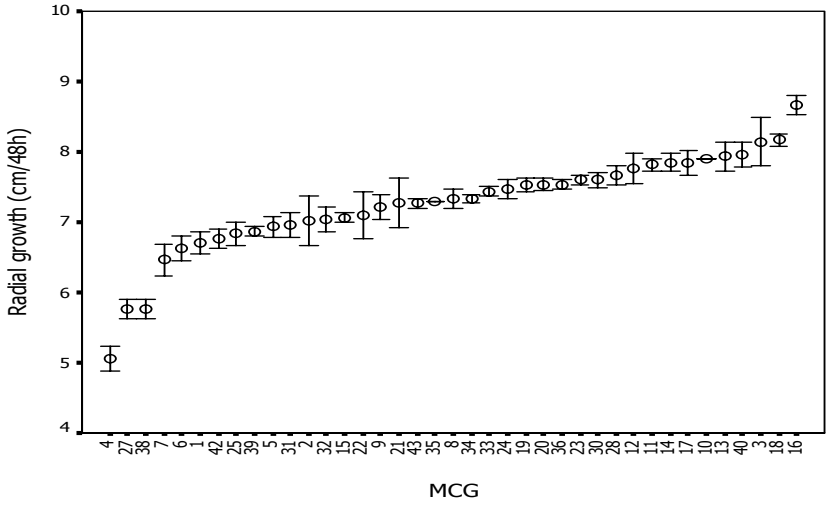

Fig. 1. Radial growth of MCGs of *S. sclerotiorum* from different worldwide locations. Each point was the mean of all the isolates from same MCG. Vertical bars represent the standard error of the mean. *, significantly different at P=0.05.

3. Determination of oxalic acid and estimation of total acids production When the productions were compared among MCGs, the mean production varied between 0.73±0.03 (MCG39) and 2.06±0.05 (MCG3) mg^{-1} ml. There was a high degree of variability in oxalic acid production among MCGs. These differences were observed both among MCGs from worldwide collections and also among the groups from same geographic set. All isolates secreted acids actively resulting pH values of culture medium decreasing from the original level of 5.12 to 3.97-2.00 (data not shown). As in oxalic acid production, MCG39 had the lowest production of total acids (pH=3.87), and the highest production by MCG32 (Fig. 2). Furthermore, there was high positive relationship between pH value and oxalic acid production; this suggested that oxalic acid contributed mostly to the production of total acids.

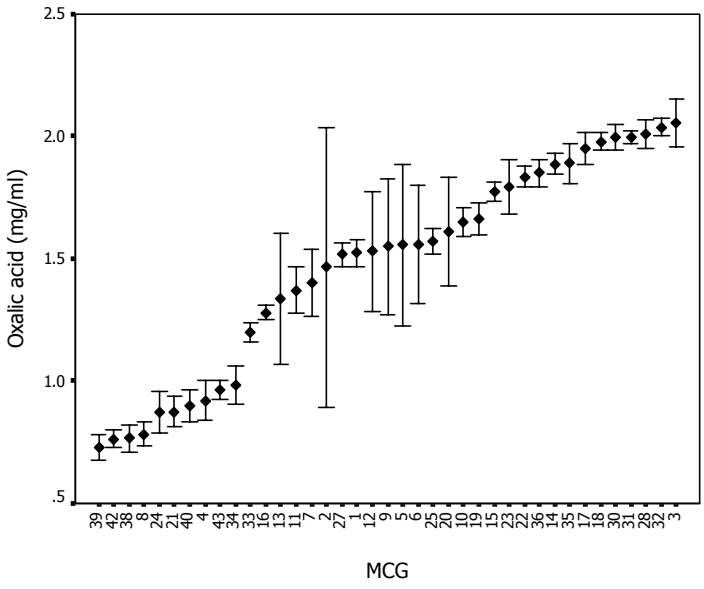

Fig. 2. Oxalic acid production of MCGs of *S. sclerotiorum*. Each point was the mean of all the isolates from same MCG. Vertical bars represent the standard error of the mean. *, significantly different at P=0.05.

Discussion

Populations of *S. sclerotiorum* using MCG analysis in this study suggested in a given location there were a heterogeneous mix of MCGs. This agreed with previous reports of *S. sclerotiorum* MCG population structure on different crops (Durman et al., 2003; Kull and Pedersen, 2004). A group of isolates sharing the same DNA fingerprint and MCG was interpreted as a clone (Hambleton et al., 2002). Population studies on *S. sclerotiorum* have suggested a predominantly clonal mode of reproduction (Cubeta et al., 1997; Kohli and Kohn, 1998) with some evidence of outcrossing contributing to the population structure in a few regions (Atallah et al., 2004; Sexton and Howlett, 2004). Ascospores of *S. sclerotiorum* was reported to deposit within the field where they are produced (Wegulo et al., 2000) although some can be carried several kilometers in air currents (Li et al., 1994). The isolates from different countries with long distances from each other, except those with close proximity (MB & ND), were incompatible. This could be resulted by geographic barrage and failure of sexual recombination.

Aggressiveness variation of *S. sclerotiorum* has been investigated previously. Our results indicated significant differences in aggressiveness presented not only within isolates but also among MCGs regardless of origin of isolates. Importantly, results suggested that to evaluate pathogenicity of *S. sclerotiorum* dispersed in a region, or to screen resistant cultivars, a more representative sample of isolates should be included.

Oxalic acid has been associated with pathogenesis. Marciano et al. (1989) found that highly aggressive and weakly aggressive isolates could equally utilize several components of host cell wall as nutrients for mycelial, but differed in their ability to utilize them for oxalate production. The poor ability to produce oxalic acid by weakly aggressive isolates seems to be due to a lower efficiency in the synthetic pathway. Godoy et al.(1990), showed mutants of *S. sclerotiorum* were deficient in the ability to synthesize oxalate were nonpathogenic, whereas revertant strains that regain their oxalate biosynthetic capacity exhibited normal virulence. It has been reported that there were significant differences among MCGs *S. sclerotiorum* in both oxalic acid and organic acids releasing (Durman et al., 2005). Our results confirmed this conclusion and indicated additionally that these differences not related to the geographic origins of MCGs.

Conclusion: our results supported the conclusion that there were high genetic diversity on *S. sclerotiorum* populations and high variations on pathogenicity of isolates and MCGs, irrespective of the source.

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