CHARACTERIZATION OF ISOLATES OF *Sclerotinia sclerotiorum* FROM CABBAGE IN AMBEWELA, SRI LANKA

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Introduction

***Sclerotinia sclerotiorum* is a destructive ubiquitous fungal plant pathogen that infects over 400 plant species including economically important crops such as potato, carrot, bean, lettuce and cabbage. Cabbage head rot has not been recognized as an economically important disease in Sri Lanka until our recent unexpected finding in Pattipola area in 2014. No disease free fields were found at the maturity during the wet season and the disease incidence was about 5% in September, 2014.**

Early pathogenicity events of *S. sclerotiorum* include oxalic acid production and secretion of cell wall degrading enzymes. However, whether frequently found isolates in Sri Lanka could produce acid or not is not known whereas variation in acid production has been reported in other countries. Many studies are available on genetic diversity in terms of mycelial compatibility groups (MCGs) and molecular markers on *S. sclerotiorum* worldwide. Mycelial compatibility is the ability of two strains of filamentous fungi to anastomose and form one continuous colony. As an easy test for self-nonself recognition, vegetative compatibility has been extremely useful in intraspecific strain comparisons. For instance, if isolates A, B and C represented one MCG, that means isolate A was compatible with isolate B, isolate B was compatible with isolate C, isolate A was compatible with isolate C. None of the studies have attempted to estimate the MCG diversity in Sri Lanka.

The pathogen produces brownish, cup-shaped apothecia arising from sclerotia, where apothecia are lined with asci, each filled with eight ascospores, which are forcibly ejected in puffs and carried by the wind current during carpogenic germination. In myceliogenic germination, soil borne sclerotia are produced from mycelia. Mycelial infections are usually initiated at the soil line. Airborne ascospores carried by wind can disperse the pathogen over wider areas. However, the source of inoculum for the cabbage head rot in Sri Lanka is not known. Small scale vegetable production system in Sri Lanka could heavily affect if the source of inoculum is ascospores. Crop rotation with host crops and the ability of sclerotia to survive in soil for up to eight years make the disease management very challenging.

Potato is an alternative host for *S. sclerotiorum*. In Ambewela and Patipola areas, where the pathogen was isolated, fungicide application is a routine practice to protect the crop from late blight infections (personal communications, farmers in the area). When the disease is severe during the wet season, farmers often apply fungicides every other day. Therefore, it is important to know if fungicide insensitivity has been developed among the isolates. Studying on this aspect is economically important on devising management strategies. Polyram M (Maneb 80% wp) is an extensively used fungicide by up-country potato farmers and therefore, it was used to test the hypothesis that fungicide

insensitivity has been developed among pathogen isolates. In addition, Neemtee®, a natural product based commercial fungicide was also included in the test to determine, natural product based fungicide, is effective against the pathogen.
Since the cabbage head rot is reported in Sri Lanka for the first time, no detailed study on characterization of isolates present in Sri Lanka has been carried out. The objectives of this study were to identify the source of inoculum, which is very important in devising disease management strategies, and to characterize randomly selected pathogen isolates in terms of acid producing ability, mycelial compatibility groups, sclerotial size, fungicide sensitivity and DNA sequence based methods.

Methodology
Sample collection was done from 15 randomly selected cabbage fields in Ambewela and Pattipola. Sclerotia were collected from infected cabbage heads at least 3-6 m apart from each other in early 2014. Pure cultures from thirty-five isolates were prepared from surface sterilized sclerotia in PDA plates.

Three medium sized sclerotia from two selected isolates were ground separately using sterilized mortar and pestle. Total genomic DNA was extracted using CEYGEN MycoSpin DTM (Ceygen Biotech (PVT) Ltd., Sri Lanka) fungal genomic DNA extraction kit following the manufacturer's instructions with some modifications. Polymerase chain reaction (PCR) was used to amplify fungal ITS 1, 5.8s and ITS 2 regions as described in Attanayake *et al.* (2014). PCR products from selected isolates were sent for sequencing to the genotyping facility at GenTech (Pvt) Ltd., Colombo, Sri Lanka. MCGs were determined by pairing selected 20 isolates in all possible combinations on PDA plates amended with red food coloring.

Ten randomly selected isolated were used to test fungicide insensitivity. The tested concentration for fungicide Maneb and Neemtee® were 5, 10, 25, 50, 75, 100, 250, 500 µg/mL. The variance was calculated for percentage inhibition to each concentration. The concentration which has the highest variance was selected as the discriminatory concentration and another 20 isolated were tested for the discriminatory concentration.

Semi selective medium for *S. sclerotiorum*, NEON was prepared according to by amending bromophenol blue (500 µg/mL) with PDA. Mycelial plugs (5 mm diameter) were added from each isolate into separate plates and incubated at 23 °C. The color change in the medium (blue-purple to yellow) was observed.

Soil was collected covering 1m x 1m x ~5 cm (depth) from a recently harvested commercial cabbage field and a non-harvested cabbage field. Two replicates were done to each field and were dried at room temperature for 3 days, weighed and passed through a 0.85 mm sieve. Number of sclerotia was counted per 1 kg of soil. Semi selective NEON plates were placed on the top of cabbage heads in commercial cabbage fields facing to the wind direction as explained in Atallah *et al. (*2004). Plates (spore traps) were kept covering 12 hours to collect wind borne spores of *S. sclerotiorum* and brought to the laboratory. Color change in the medium (blue to yellow) and the production of sclerotia were observed.

Results and Discussion

Sclerotia of all 35 isolates were black in color and had 2-10 mm in diameter, smooth and round. BLAST searches of sequences of ITS region were 99 % similar to *S. sclerotiorum* and ITS sequences of both isolates was identical. Twelve MCGs were identified out of 20 isolates of *S. sclerotiorum*. One MCG included four isolates, one MCGs included three isolates, three MCG's were formed by two isolates and another seven MCGs were formed by individual isolates.

***S. sclerotiorum* population from commercial cabbage fields in Pattipola is quite diverse giving 1.66 isolates per MCG. Among the ten isolates tested for fungicide Maneb, highest variance was obtained to 250 µg/mL. Four isolates achieved complete inhibition (100 % inhibition). From the 20 isolates tested to 250 µg/mL, five isolates had less than 50% inhibition, indicating Maneb insensitive isolates could be present in Sri Lanka. Rest of the isolates displayed 50-100% inhibition zone. Therefore, early detection and management of the disease is important.**

No tested isolates achieved 100% inhibition for tested concentrations to Neemtee®. Four isolates did not achieve at least 50% inhibition. One isolate achieved 60% inhibition at the 500 µg/mL, which was the highest showed inhibition. All tested isolates changed blue color of bromophenol blue in semi-selective media to yellow color indicating the production of an acid. No acid free cultures were observed. No sclerotia formation or color change was observed in the semi-selective media, which were exposed to the wind at cabbage fields. The average number of sclerotia found in both harvested and non-harvested fields was 4-5 per kilogram of soil.

Conclusions and Recommendations

All the collected *S. sclerotiorum* isolates were capable of producing acid in the semi-selective medium. Based on the MCG study, a high MCG diversity might present in Sri Lanka and further sampling and a thorough study is needed to understand the MCG diversity.

In Sri Lanka apparently the source of inoculum is soil borne sclerotia; however, more field testing is needed to capture correct ascospore release time, direction and to find the maximum ascospore release time of the year. Based on the fungicide insensitivity test, Maneb insensitivity could be developing in the population and therefore, one should exercise caution when applying fungicides in this area. Neem itself is not as effective as Maneb in controlling the pathogen *S. sclerotiorum in-vitro* and since it has some fungicidal activity, further development of the product can be used in organic agriculture.

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