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Conference Paper · February 2020

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INVESTIGATION OF MOLECULAR MECHANISMS ASSOCIATED WITH FUNGICIDE SENSITIVITY IN IRISH *PYRENOPEZIZA BRASSICAE* POPULATIONS

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Summary: Light leaf spot is amongst the most damaging diseases of oilseed rape and a significant threat to Irish crops. Unfortunately, the epidemiology of *Pyrenopeziza brassicae,* the agent causing this disease, remains poorly understood under Irish growing conditions and fungicides are relied upon to provide control. To investigate if the cropping strategies currently used are the best for the control of disease, we screened three populations of *P. brassicae* isolates from different regions of Ireland for alterations in the genes targeted by azole and Methyl benzamidazole carbamate fungicides. As molecular mechanisms associated with a decrease in fungicide sensitivity were observed in the populations, the results will be correlated with data from fungicide sensitivity tests using these classes of fungicides.

INTRODUCTION

Light leaf spot (LLS) disease of brassicas, caused by the hemibiotrophic fungal pathogen *Pyrenopeziza brassicae* (anamorph *Cylindrosporium concentricum*), is known as one of the most damaging diseases of winter oilseed rape (*Brassica napus* spp.) in Northern Europe (Boys *et al.*, 2007). In Ireland, *P. brassicae* was observed for the first time during the 1964-1965 season, causing light leaf spot disease on broccoli, cabbage and Brussels sprouts (Staunton, 1967). As the area sown to oilseed rape in Ireland has increased from 2,300 ha in 2003 to 17,000 ha in 2012 (Collins and Phelan, 2018) and the Irish Tillage Sector Development Plan (2012) highlighted the potential to increase its production even more, LLS is regularly observed in Irish oilseed rape (OSR) fields.

Although believed to be a significant threat to Irish oilseed rape crops, the epidemiology of *P. brassicae* under Irish growing conditions is poorly understood. Currently, LLS control involves the use of resistant cultivars and fungicide applications, typically one in late autumn and a second in early spring. However, as disease symptoms are frequently not visible until late winter (Gilles, 2000), the correct timing for the first fungicide application is difficult to determine (Fitt *et al.*, 1998). Equally, in early spring the second fungicide spray is often applied to crops with symptoms, which may be too late for the fungicides to be effective. In addition, the potential for the development of fungicide insensitivity in *P. brassicae* populations (Carter et al., 2014) to further compounds will affect our ability to control the disease. As limted data are available on the fungicide sensitivity of the Irish *P. brassicae* population we aimed to investigate the molecular mechanisms correlated with decreases in sensitivity to azole and MBC, two of the classes of fungicides currently/previously used in controlling LLS. As a decrease in sensitivity to azoles has been correlated with alterations in the regulatory and coding region of the sterol 14α-demethylase gene (*CYP51*), the gene targeted by these fungicides, we investigated the presence of alterations identified by Carter *et al.* (2014) in Irish *P. brassicae* populations. Similarly the presence of the mutations E198G and L240F in the β-

tubulin, previously confirmed by Carter *et al.* (2013) as conferring MBC resistance, was determined.

MATERIALS AND METHODS

Collection establishment

Leaves presenting characteristic symptoms of light leaf spot, such as the white acervular conidiomata pustules with a circular distribution (Ashby, 1997) were randomly sampled from three oilseed rape crops (Cv. Phoenix) located in three different regions of Ireland: Co. Carlow (East), Co. Cork (South) and Co. Louth (North). At the time of sampling, early March 2019, each crop had received a fungicide treatment (Proline) in late autumn. At sampling, diseased leaves collected from each field were further incubated separately in polyethylene bags for 2-4 days at 4°C in order to promote *P. brassicae* asexual sporulation. Following this, single conidiomata were identified using a dissection microscope and isolated from each leaf using a sterile needle. The colonies obtained were subcultured several times on potato glucose agar amended with ampicillin and streptomycin sulphate to obtain single spore colonies. A subsample of the collections established was used for further molecular analysis.

DNA extraction

Single spore colonies grown from glycerol stocks at 18°C for 19 days were used to inoculate 50 ml Falcon tubes containing 30 ml potato dextrose broth. After 5 weeks of growth at 20°C with 240 RPM, *P. brassicae* mycelium was separated by centrifugation, freeze-dried for 24 h and homogenised using a benchtop mixer mill (Retsch Mixer Mill). The DNA was extracted using a GenElute™ miniprep kit according to the manufacturer's protocol (Sigma-Aldrich, Missouri, United States).

Amplification of *CYP51* regulatory region

A 662 bp sequence representing the predicted regulatory region of *CYP51* was amplified using the primer pair CYP51upstreamF / CYP51upstreamR (Carter *et al.*, 2014). PCR was done using *Taq* DNA polymerase with ThermoPol Buffer (New England BioLabs Inc., Massachusetts, United States). The PCR conditions were 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 1 min, followed by a final extension of 5 min at 72°C. The PCR product obtained was migrated in electrophoresis on a 1% agarose gel and visualised under UV-light using an ENDURO™ GDS Gel Documentation System for Electrophoresis (Labnet International, Inc. New Jersey, United States).

Detection of codons 460 and 508 from *PbCYP51* coding region

The 1244 bp fraction of *PbCYP51* encompassing codons 460 and 508 was amplified using the primers pair CYP51expressionF1 / CYP51R (Carter *et al.*, 2014), using *Taq* DNA polymerase with ThermoPol Buffer (New England BioLabs Inc., Massachusetts, United States). The PCR reaction conditions used were as described above, with an annealing temperature of 56°C and an extension time of 1:30 min. Two units of *TspRI* (CASTG) (New England BioLabs Inc., Massachusetts, United States) were used to detect G460S, using approximately 60 ng of purified PCR product and 1 × NE buffer 4 in a total volume of 10 µl, and the mix was incubated for 2 h at 65°C. In order to detect the mutation S508T, approximately 60 ng of the purified PCR product were digested with 1 unit of *BssSI* (CACGAG) and 1 × NE buffer 3 in a total volume of 10 µl and the mix was incubated for 2 h at 37°C. The digested product obtained was separated on a 1% (w/v) agarose gel and exposed to UV-light to visualize DNA fragments.

PCR-RFLP detection of E198A and L240F substitutions in the *β-tubulin* gene

The primer pair PZtubF1 / PZtubR1 (Carter *et al.,* 2013) an 865 bp fragment from the *β-tubulin* gene, including codons 198 and 240 was amplified. The reaction mix used was similar to the one mentioned above, and the reaction conditions were 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 52°C for 1 min and 72°C for 1 min, with a final extension step of 5 min at 72°C. The PCR product obtained was purified and approximately 60 ng PCR product was digested with 1 unit of *BsmAI* enzyme (GTCTCN) (New England BioLabs Inc., Massachusetts, United States) and 1× NE buffer 4 in a total volume of 10 µl and incubated for 2 h at 55°C. The restriction fragments resulted were separated in a 3% (w/v) electrophoresis gel and visualised under UV-light.

RESULTS

A total of 47 *P. brassicae* isolates were used to detect the alterations in the genes targeted by azole and MBC fungicides (14 were sampled from Co. Carlow, 11 from Co. Louth and 22 from Co. Cork).

Detection of alterations in the regulatory and coding region of *PbCYP51* gene

The analysis of the regulatory region of *PbCYP51* showed the presence of the three inserts in all three populations screened. Wild type isolates without any type of insert were present in all three populations, representing 34% of the entire collection. Amongst the remaining isolates, the 151 bp insert was most frequent in the collection, followed by the 46 bp insert, however, differences existed between the collections in terms of the frequencies of the inserts in each (Figure 1).

Figure 1 Frequencies (%) of isolates presenting S508T and G460S alongside the different types of inserts observed for the regulatory region of *PbCYP51* in Irish populations of *P. brassicae* sampled from Carlow, Louth and Cork. Error bars represent SEM, df = 12.

The digestion of 1244 bp *PbCYP51* fragment with *BssSI* resulted in detection of S508T substitution in 4 out of the 47 isolates analysed, two of which came from the Louth population, one from Carlow and one from Cork (Figure 2).

Nine isolates presenting G460S were identified in the three populations screened following the digestion of the purified PCR product with *TspRI*, four of which came from the Louth population, two from Carlow and the remaining three from Cork (Figure 3).

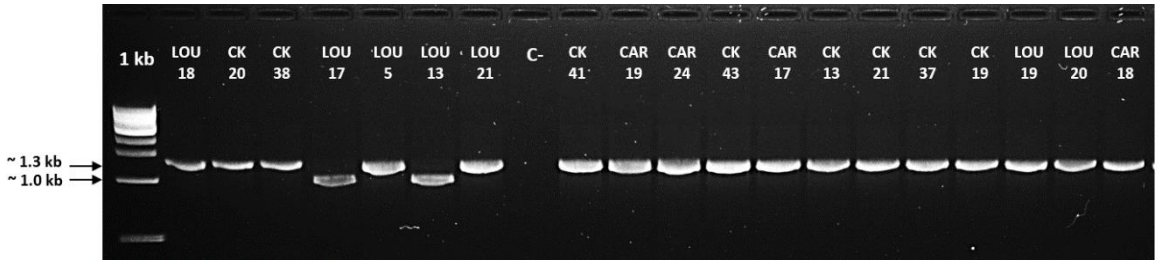

Figure 2 Restriction digest of 1244 bp fragment of *PbCYP51* using *BssSI to* detect the substitution S508T. Arrows indicate the size of bands obtained, 1 kb bands are specific for the isolates with the mutation. Sites from which *P. brassicae* isolates were obtained: LOU – Louth, CK – Cork, CAR – Carlow, C^- – Negative control.

Analysing the possible combinations of the two mutations in the coding region and the inserts from the regulatory region, six different combinations were observed in the *P. brassicae* populations: G460S with each of the different inserts, G460S + S508T, and G460S + S508T, with either the 151 bp or 232 bp insert.

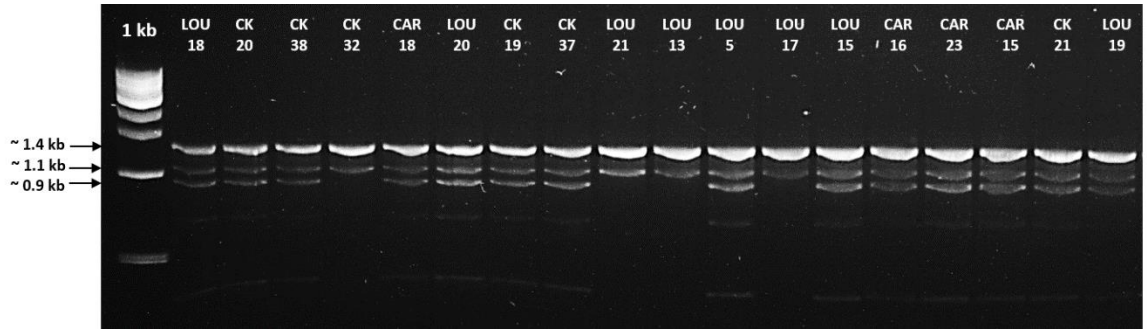

Figure 3 Restriction digest of 1244 bp fragment of *PbCYP51* using *TspRI to* detect the substitution G460S. Arrows indicate the size of bands obtained, the absence of 900 bp bands is characteristic for the isolates with the mutation. Sites from which *P. brassicae* isolates were obtained: LOU – Louth, CK – Cork, CAR – Carlow.

The average frequencies of all the possible alteration were not significantly different between the populations, and there were no significant differences observed between the three populations screened based on the frequencies of the two possible substitutions in the coding region (P-value = 0.125). This was also the case comparing the populations based on the different variants of the insert from the regulatory region, the P-value obtained being of 0.18225.

Population screening for detection of resistance to MBC fungicides

After the digestion with *BsmAI* of the 865 bp purified PCR product, the isolates were divided into three groups: Sensitive (S), Moderately Insensitive (MI) and insensitive (I), based on the size and number of bands obtained, as described by Carter *et al.* (2013): the isolates presenting five bands (461bp, 200 bp, 104 bp, 69 bp and 31bp) were grouped as sensitive, while the isolates with only 4 bands were classified as insensitive (565 bp, 200 bp, 69 bp and 31bp, the 69 bp) or moderately insensitive (461bp, 200 bp, 104bp and 100 bp) to methyl benzamidazole carbamates. According to Carter *et al.* (2013) the absence of 100/104 bp band is caused by the E198A substitution in the insensitive isolates, whereas the absence of 69 bp band is caused by L240F in the moderately insensitive isolates (Figure 4). The difference between the resistant isolates and the other two types was caused by the 565 bp band, while the difference between the sensitive and the moderately resistant isolates was caused by the presence of 69 bp and 31bp bands in the sensitive ones.

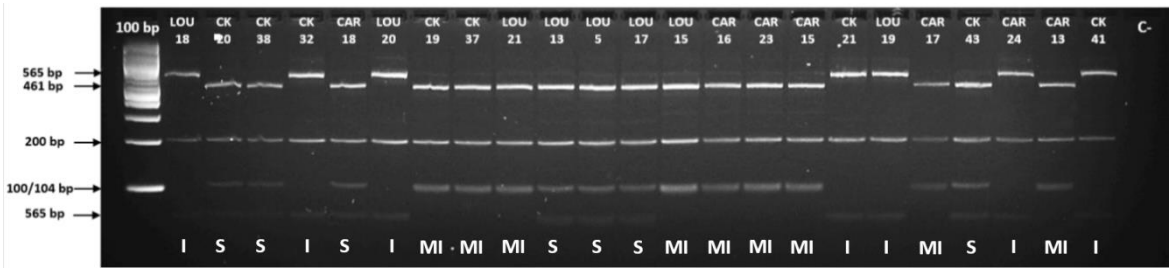

Figure 4 Restriction digest of 865 bp fragment *β-tubulin* gene detecting the substitutions E198A and L240F in order to differentiate between Insensitive (I), Moderately Insensitive (MI) and Sensitive (S) isolates. Arrows indicate the size of bands obtained. Sites from which *P. brassicae* isolates were obtained: LOU – Louth, CK – Cork, CAR – Carlow.

All types of isolates were present in all three populations screened. The isolates with sensitivity to MBC fungicides were predominant in the population sampled from Cork, while for Carlow the moderately insensitive isolates dominated in the population, whereas, in Louth, the same number of sensitive and insensitive isolates have been observed (Figure 5).

Figure 5 Frequencies (%) of isolates with different degree of insensitivity to MBC fungicides in Irish populations of *P. brassicae* sampled from Carlow, Louth and Cork. S – isolates without alterations in the β-tubulin gene, MI – isolates with L240F and I – isolates with E198A. Error bars as SEM, df = 4.

For all the locations screened, the frequency of isolates with E198A was the lowest (Figure 5). There were no significant differences observed between the three populations screened based on the frequencies of the three types of isolates monitored: presenting L240F, E198A or without any alterations, and the P-value obtained was of 0.4.

DISCUSSION

The results obtained from this preliminary molecular screening suggest that limited differences, if any exist between the main OSR producing regions in Ireland in terms of sensitivity of *P. brassicae* to either the azole or MBC fungicides. However, they do confirm the presence of alterations previously associated with decreased sensitivity to the azole and MBC fungicides in the Irish *P. brassicae* populations (Carter et al., 2014).

We observed that most of the population analysed had neither the substitution S508T or G460S, inserts in the regulatory region, potentially indicating a population largely sensitive to azoles. However, comparing the regulatory and coding regions of the gene confirmed the populations had a greater tendency to have inserts in the regulatory region than mutations in the coding region. Although Carter et al. (2014) detected the amino acid substitution G508T alone, in the Irish collection of *P. brassicae* isolates it was detected only in combination with

G460S alone, or with G460S and either the 151 bp or 232 bp insert. Interestingly neither G460S or S508T were detected in any of the isolates with the 46 bp insert in the regulatory region. Whilst most of isolates may not have G460S or S508T, complete sequencing of the *PbCYP51* is required to determine if additional mutations are present.

The frequencies of the two alterations in the *β-tubulin* gene, E198A and L240F, known to confer different levels of insensitivity, did not significantly vary between the three sites. However, as with the azoles, both mutations were detected in all three populations indicating the presence of insensitivity in the Irish populations. Again, isolates without either mutation dominated the populations. As the MBC fungicides have not been used in Irish OSR crops for a number of years, the low incidence of either mutation may reflect this.

Following the detection of the different inserts/mutations associated with azole and MBC insensitivity, the next steps are to determine the sensitivity of the Irish population to these two classes of fungicides in order to confirm the degree of sensitivity established in the *P. brassicae* population.

ACKNOWLEDGEMENTS

This work was funded by Teagasc - The Agriculture and Food Development Authority under its Walsh Fellow Scheme.

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