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SENSITIVITY OF *CERCOSPORA BETICOLA* ISOLATES TO AZOXYSTROBIN

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SUMMARY

Sugar beet leaf spot, caused by the air-borne fungus *Cercospora beticola* Sacc., leads to a decrease in sugar beet leaf mass and the consequent regrowth of leaves based on exploiting the sugar reserves stored in the plant's roots, thus ultimately resulting in lower yields and sugar contents of sugar beets. Azoxystrobin belongs to the group of QoI fungicides, which inhibit mitochondrial respiration by blocking cytochrome c reductase. The QoI fungicides are characterized by a very high risk of resistance interfering with their biological activity. For the purpose of testing the azoxystrobin sensitivity of the *Cercospora beticola* population found at the site of Rimski Šančevi, a collection of 84 isolates was assembled and tested for sensitivity to azoxystrobin by measuring the mycelial growth on fungicide-amended media with the addition of SHAM. The results obtained indicate that none of the isolates tested exhibited complete sensitivity to azoxystrobin, 4% were found to have reduced sensitivity, 26% were moderately resistant and 70% were highly resistant. A higher proportion of resistant isolates recorded is associated with the loss of azoxystrobin biological efficacy at the experimental site.

Key words: sugar beet, leaf spot, *Cercospora beticola*, azoxystrobin, fungicide, resistance, QoI

INTRODUCTION

Sugar beet leaf spot, caused by the phytopathogenic fungus *Cercospora beticola* Sacc., is the most serious and destructive sugar beet foliar disease (Holtshulte, 2000; Budakov, 2014). It poses an issue of growing complexity due to the abundance of potential hosts (namely sugar beets and various plants of different genera such as *Chenopodium*, *Atriplex*, *Amaranthus*, *Polygonum*, *Lactuca*, *Medicago*, *Glycine*, *Taraxacum*, *Malva*, *Spinacia*, *Limonium*, *Apium*, etc. (Ruppel, 1986; Weiland & Koch, 2004; Lartey et al., 2010)) and favourable environmental conditions such as warm and humid weather during the vegetative period (Budakov et al., 2014).

The symptoms of leaf spot disease first appear on older leaves as individual spots ranging from 3-5 mm in diameter (Jacobsen & Franc, 2009). The lesion tissue is tan to light brown with brown or reddish purple margins. Necrotic areas increase with the appearance of new lesions, which eventually lead to the complete leaf necrosis and regrowth of new leaves based on exploiting the sugar reserves stored in sugar beet roots (Weiland & Koch, 2004). Such depletion of root sugar reserves is reflected in lower yields and sugar contents of sugar beets, rendering the plant's roots more susceptible to rot during storage (Jacobsen & Franc, 2009). The control of *C. beticola*, as part of the integrated disease management, comprises cultural, biological and fungicide practices, which are carried out 2-5 times a year under the environmental conditions of Serbia (Budakov et al., 2014). As *C. beticola* is a polycyclic parasite, it produces a large number of spores annually, which are exposed to fungicide activity several times during the vegetation. The most effective fungicides against this disease are site-specific fungicides, which contain various

systemic active substances targeting one site in the fungal metabolism. According to FRAC (Fungicide Resistant Action Committee), these fungicides possess a very high risk of resistance interfering with their biological activity (Brent & Hollomon, 2007a, 2007b). The resistance to fungicides can be defined as a stable, inheritable adaptation of a pathogen through reducing its sensitivity to a fungicide, which allows individuals within the population to survive plant protection treatments which would, under normal conditions, lead to the suppression of harmful organisms (Gallian et al., 2001). Azoxystrobin is one of the Quinone outside inhibitors (QoIs) with a high risk of resistance which inhibits the mitochondrial respiration by blocking the transport of electrons between cytochrome b and cytochrome c1 and by inhibiting cytochrome c reductase. The first case of resistance to QoIs among the field population of *C. beticola* was recorded in 2011 in the USA (Kirk et al., 2012), after failing to control CLS in sugar beet fields treated with these fungicides over the period of several years. The occurrence and incidence of the QoI resistance in Serbia was argued by Trkulja et al. (2017). With regard to the *in vitro* testing of the *C. beticola* susceptibility to the QoI fungicides, the methods applied are mostly based on the measurements of spore germination inhibition (Secor et al., 2010) and/or mycelial growth reduction (Malandrakis et al., 2011). In the presence of the QoI fungicides, fungi have the ability to make use of alternative oxidase (AOX), which enables sensitive isolates to show mycelial growth on the medium amended with fungicide. The studies on the AOX importance in the QoI resistance have used salicylhydroxamic acid (SHAM) to inhibit the activity of AOX during the QoI resistance testing (Avila-Adame & Köller 2002, 2003). By inhibiting AOX, the resistance to the QoI fungicides becomes dependent on mutations in the cytochrome b gene, which as a consequence inhibits the mycelial growth of sensitive isolates, thus revealing only the resistance caused by mutations in the cytochrome b gene (Larson, 2004).

As the sensitivity of fungal populations to various fungicides is one of the most important aspects of the anti-resistant strategy (Brent & Hollomon, 2007a, 2007b), the purpose of this paper is to determine the level of resistance of a large number of *Cercospora beticola* isolates, collected from an experimental sugar beet field, to azoxystrobin.

MATERIAL AND METHODS

For the purpose of this research, sugar beet leaves exhibiting typical signs of leaf spot caused by *Cercospora beticola* were collected at the Rimski Šančevi trial site of the Institute of Field and Vegetable Crops, the Faculty of Agriculture in Novi Sad. Sampling was carried out in June 2017, prior to the first fungicide application in order to evaluate not only the level of resistance of individual isolates, but also the frequency of resistance to azoxystrobin at the experimental site. Sampling was carried out by randomly collecting 100 leaves along each of the two diagonals of a 0.5 ha sugar beet field.

The leaf samples with typical spots caused by *C. beticola* were analyzed at a phytopathological laboratory (Department of Environmental and Plant Protection, Faculty of Agriculture, University of Novi Sad). They were placed in a Petri dish with moist filter paper on the microscope slides and incubated at 25 °C for 24-36 hours in order to induce sporulation. Single lesion isolations of the parasite were performed using a sterile entomological needle by transferring one or more conidia from one lesion to the PDA (Potato Dextrose Agar).

The sensitivity level of the isolates tested was determined by employing the method described by Budakov (2014), which involves using the discriminative concentration of the fungicide tested (Karaoglanidis et al., 2003). The discriminative fungicide concentration is a fungicide concentration at which the tested isolates are clearly divided into two groups: inhibited and uninhibited isolates (Jo et al., 2006). A discriminative concentration of 0.1 µg azoxystrobin /ml was previously established (Budakov, 2014) by testing the reference isolates of *C. beticola* isolated from beetroots grown in the organic farming system, in which the pathogen population has a baseline sensitivity because the fungicide tested had never been used before at this site. Under sterile conditions and in a sterile cooled medium, a solution of technically pure azoxystrobin in methanol was added to obtain a concentration of 0.1 µg/ml. Salicylhydroxamic acid (SHAM), at a concentration of 0.5 mM, was added in order to inhibit the activity of alternative oxidase. The PDA was used as a negative control with the addition of SHAM (without fungicide). The fragments of mycelium (5 mm in diameter) were placed on the plates with agar surface with mycelia facing down in order to establish the contact with the fungicide.

The isolates tested were incubated at 25 °C for 6 days. Sensitivity evaluations were performed by calculating the average diameter of a colony based on measurements of two normal mycelium diameters. The data obtained were used to calculate the relative growth in the mycelium for each isolate using the following formula:

$$RP(\%) = \frac{A}{B} \times 100$$

Where:

RP is a relative growth of mycelium, A is the average mycelium growth on media with the addition of fungicides and SHAM, B is the average mycelium growth on media with SHAM, but without fungicide (negative control).

Based on the relative growth of the mycelium, all the isolates tested were divided into four groups:

1. Sensitive (RP <20%)
2. Less sensitive (RP = 20-39.9%)
3. Moderately resistant (RP = 40-69.9%)
4. Highly resistant (RP ≥ 70%).

RESULTS AND DISCUSSION

A total of 84 isolates of *Cercospora beticola*, collected at the site of Rimski Šančevi, were selected for sensitivity testing to azoxystrobin (0.1 µg/ml). The results obtained are shown in Table 1.

Table 1. Frequency of *Cercospora beticola* isolates with different sensitivity to azoxystrobin

Sensitivity level	Range of sensitivity level	Number of isolates	Percent of isolates	Isolate codes
Sensitive	/	/	/	/
Less sensitive	20.89-31.52	3	4%	15RŠ9, 19RŠ6, 15G2
Moderately resistant	40.00-69.69	22	26%	15G7, 15RŠ8, 14G3, 18RŠ1, 18G2, 18RŠ2, 15G4, 15RŠ5, 13RŠ5, 13RŠ6, 20RŠ2, 19RŠ5, 14RŠ5, 19RŠ12, 19RŠ9, 15RŠ4, 18G1, 15RŠ10, 14RŠ7, 13G3, 13RŠ2, 15RŠ3
Highly resistant	70.12-275.00	59	70%	20G1, 18G3, 15RŠ6, 18G4, 14G7, 13RŠ4, 19RŠ7, 14G4, 19G3, 13RŠ3, 18G5, 14G8, 15G8, 19RŠ4, 15RŠ13, 18RŠ4, 15RŠ12, 20G2, 18RŠ3, 20RŠ1, 19G2, 14RŠ2, 18G7, 19G1, 15G1, 15RŠ11, 18G8, 19G5, 15G6, 19RŠ3, 18RŠ5, 15RŠ, 13G8, 13G7, 19RŠ8, 19RŠ1, 18G6, 14G2, 15G5, 13G2, 19G6, 14RŠ3, 14RŠ6, 14G6, 14G1, 14RŠ4, 19RŠ10, 13RŠ1, 14G5, 14RŠ1, 19G4, 13G5, 15G3, 19RŠ2, 19RŠ11, 13G6, 13G1, 15RŠ7, 13G4

The results obtained indicate that none of the isolates tested was sensitive to azoxystrobin (RP < 20%), whereas 4% of the isolates showed reduced sensitivity. However, a total of 96% of the isolates tested exhibited resistance: 26% were moderately resistant (RP = 40-69.9%) and 70% were highly resistant (RP ≥ 70%). On balance, these results suggest a high percentage of resistance to azoxystrobin, which inevitably leads to additional challenges in sugar beet protection as this fungicide cannot successfully control sugar beet leaf spot.

Increased parasite resistance to fungicides leads to decreased efficacy of a given fungicide (Karaoglanidis & Ioannidis, 2010). Therefore, the predominance of isolates resistant to QoIs in the field population can result in significantly lower yields and sugar contents of sugar beets, i.e. major economic losses due to the application of fungicides with reduced biological efficacy. Moreover, the application of fungicides which have no effect on the target organism can be harmful to beneficial microorganisms and the environment (Budakov et al., 2014).

Determining the level of sensitivity of *C. beticola* populations to fungicides facilitates the control of fungicide efficacy in practice. It also aids establishing and implementing new strategies for the prevention of resistance emergence in order to successfully control CLS. Testing the sensitivity of pathogen populations to fungicides for disease control purposes is the most important aspect of the anti-resistance strategy (Karaoglanidis et al., 2003).

CONCLUSION

The purpose of this study is to emphasize the importance of monitoring changes in the sensitivity of *Cercospora beticola* populations to azoxystrobin, the predominant fungicide used in the sugar beet areas in Serbia.

The results obtained indicate that none of the isolates tested exhibited complete sensitivity to azoxystrobin, 4% were found to have reduced sensitivity, 26% were moderately resistant and 70% were highly resistant. A higher proportion of resistant isolates recorded is associated with the loss of azoxystrobin biological efficacy at the experimental site.

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