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Review on Agriculture and Rural Development 2014. vol. 3 (1) ISSN 2063-4803

SPATIAL DISTRIBUTION OF BENZIMIDAZOLE RESISTANCE OF *CERCOSPORA BETICOLA* SACC. IN SERBIA

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ABSTRACT
In order to check distribution of resistance of *C. beticola* isolates to benzimidazole fungicides we collected samples from four distinct geographical districts with different fungicides history in Serbia. From all localities about 40 - 50 isolates of *C. beticola* were collected and subjected to testing sensitivity to benzimidazoles in the laboratory. Due to RG of isolates on the discriminatory concentrations of carbendazim and thiophanate methyl we concluded level of resistance to benzimidazoles. Discriminatory concentration for carbendazim was 1 mg/l while for thiophanate methyl was 5 mg/l. Very high frequency of resistance of *C. beticola* isolates to benzimidazole fungicides on locality West Bačka (98 %), Srem (100%) and Moravica (97%), while on locality Rasina decrease frequency of benzimidazole resistance with 18% was established. Frequency of resistance of *C. beticola* populations to benzimidazoles was stable on locality where benzimidazoles were applied in the past, while on locality where they have never been applied it varies.

Keywords: Sugar beet, *C. beticola*, fungicides, benzimidazoles resistance, distribution

INTRODUCTION

Cercospora leaf spot caused by the fungus *Cercospora beticola* Sacc. is a destructive foliar disease of sugar beet (*Beta vulgaris* L.), present in all regions worldwide where sugar beet grows (HOLTSCHULTE, 2000). Severe epidemics of Cercospora leaf spot in production regions that prevails high temperature and humidity can cause substantial decreases in yield of sugar beet and extracted sucrose (SHANE AND TENG, 1992). In order to control Cercospora leaf spot disease in the sugar beet crop, farmers are applying an integrated concept that includes cultivation of resistant varieties of sugar beet to *C. beticola*, crop rotation and application of fungicides (MILLER ET AL., 1994). The benzimidazoles were first sistemic fungicides which used to control Cercospora leaf spot wherever the sugar beet grows. Benzimidazoles perform their fungicidal activity by binding to the β-tubulin protein, which is a subunit of the microtubule spindle, thus interfering with microtubule formation and mitosis (DAVIDSE, 1986). After several years of application of benzimidazole fungicides, resistant populations of *C. beticola* to benzimidazoles was recorded, first in Greece (GEORGEPULOS AND DOVAS, 1973) and then Italy (D`AMBRA ET AL. 1974), as well as in Serbia (MARIĆ ET AL., 1976). Benzimidazole resistance has developed independently in a number of fungal species and it has always been associated with point mutations in the β-tubulin gene (MA AND MICHAILIDES, 2005). In the case of *C. beticola*, resistance results from a single-step change in the β-tubulin gene, causing amino acid substitution of glutamic acid with alanine at position 198 (DAVIDSON ET AL., 2006) and tyrosine with phenylalanine at position 167 (TRKULJA ET AL., 2013). Due to fact that resistance relies on single mutations (qualitative resistance), pathogen such as *C. beticola* with abundant sporulations and fewest cycles in one growing season can quickly evolve

from mainly sensitive populations to mainly resistant. Recent studies conducted in Serbia revealed very high frequency of resistant populations of *C. beticola* to benzimidazoles in all localities where they were intensively used to control sugar beet, but also presence of benzimidazole resistance was recorded in beet root crop in the localities where benzimidazoles have never been used to control Cercospora leaf spot disease (TRKULJA ET AL., 2009; TRKULJA ET AL., 2012).

This study is carried out in order to determine spatial distributions of *C. beticola* resistant populations to benzimidazole fungicides in a different geographical districts in Serbia. Accurate decisions have to be made in the terms of implementation of the benzimidazole in the management for control Cercospora leaf spot disease, in accordance with the resistant strategies and environmental policy.

MATERIAL AND METHOD

Samples were collected during August and September of 2013. Four different geographical districts in Serbia with different history of fungicide application were chosen. Benzimidazole fungicides were applied at West Bačka and Srem districts while at Moravica and Rasina districts they have never been used to control Cercospora leaf spot disease *(Table 1.)*. Samples derived from districts of West Bačka and Srem originated from sugar beet crops while from districts of Moravica and Rasina originated from beet root crops.

Sampling and pathogen isolation

Sugar beet or beet root leaves with serious symptoms of Cercospora leaf spot and abundant sporulation of *C. beticola* were collected at 10 different sites within a district. The leaves are packed in paper bags, after that were stored in hand fridge and transferred to the laboratory. Conidia derived from one spot per a leaf were transferred to the water agar medium in order to establish monosporial culture of *C. beticola*. After two days single germinated conidia were transferred on a fresh potato-dextrose agar (PDA) and incubated ten days at 25 °C in the dark. List of isolates obtained per locality is shown in the *Table 1*.

Table 1. Locality and the number of tested isolates of *C. beticola*

Locality	Host	Number of isolates	History of benzimidazole applications
West Bačka	sugar beet	48	used
Srem	sugar beet	46	used
Moravica	beet root	44	not used
Rasina	beet root	50	not used

Quantitative sensitivity testing

In order to determine frequency of resistant isolates per locality we used appropriate discriminatory concentration for carbendazim 1 µg/ml (KARAOGLANIDIS AND BARDAS, 2006) and thiophanate-methyl 5 µg/ml (WEILAND AND HALLOIN, 2001). Primely fungicides were dissolved in sterilized distilled water to adjast appropriate dilution. Autoclaved Petri dishes with PDA cooled to 40 – 50 ºC and amended with fungicide solution were used. Control was PDA medium amended with sterile water. The radial growth (colony diameter) of each isolate was measured with the original mycelial plug diameter (5 mm) subtracted from this measurement, after 7 days of incubation at 25 °C. A relative growth is calculated by dividing an average growth of conidia on PDA treated with a fungicide with an average growth in control. Fungal isolates were classified as resistant if

the colony growth on discriminatory concentration was ≥50% compared to these in control (RUSSEL, 2004). Test for each isolate was carried out in two trials, with three replicates within each trial.

RESULTS

In the current study, sensitivity of the isolates of *C. beticola* to benzimidazole fungicides (carbendazim and thiophanate methyl) was set up. Isolates originated from four different districts in Serbia with different history of fungicide applications. In the district of West Bačka where benzimidazole fungicides has been continually applicated for the past four decades, we determined very high frequency of resistant isolates of *C. beticola* which was 98%. In another region, where benzimidazole fungicides were continually applied, also to control *C. beticola* populations, frequency of resistance was very high reaching up 100% *(Fifure 1.)*. Isolates which originated from beet root crop, which has never been treated with fungicides to control *C. beticola,* from the district of Moravica controversy revealed presence of resistant populations, although selection pressure of fungicides missing. From the 44 analyzed samples, 43 were resistant and determined frequency of resistance to benzimidazole fungicides was 98%. On the other hand in the region of South Serbia, where benzimidazoles likewise has never been used to protect beet root crop, frequency of resistant isolates of *C. beticola* to benzimidazoles was significantly lower with 16% presence of resistant populations *(Figure 1.)*.

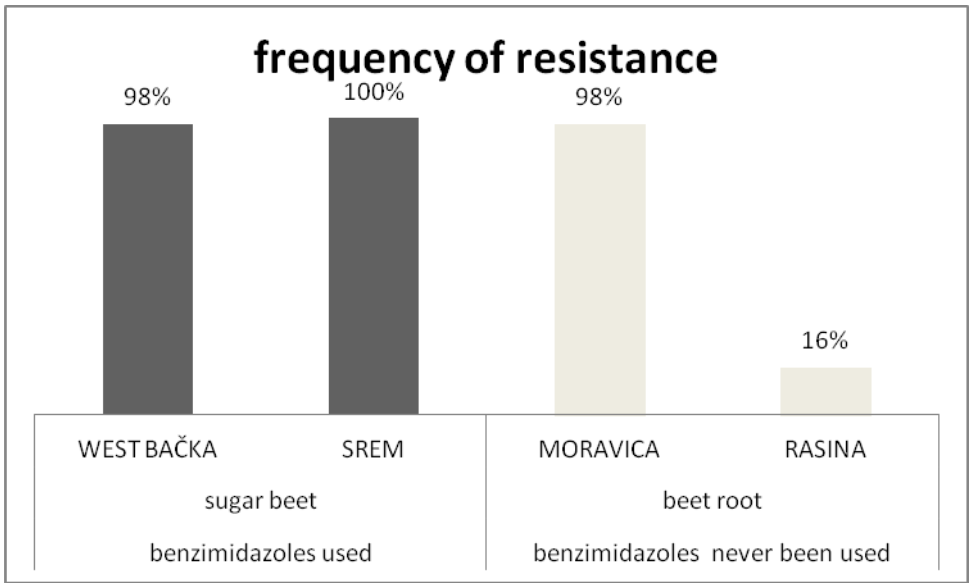

Figure 1. Frequency of resistant isolates of *C. beticola* to benzimidazole fungicides in different regions and history of fungicides applications

CONCLUSIONS

During this study, very high frequency (98 – 100%) of resistance of *C. beticola* isolates to benzimidazole fungicides was determined in two different districts in Serbia (West Bačka and Srem) both with similar history of fungicide applications, which suggested that pressure of fungicides is necessary condition to evolution of fungicides in the field. Due to that, implementation of measures of anti-resistance strategies is essential to stop further development of resistance of *C. beticola* to benzimidazole fungicides, in all regions where significant appearance of resistant populations was detected. Recent studies revealed very high frequency of isolates of *C. beticola* resistant to benzimidazole fungicides in region of the Central, Eastern and Western Serbia where sugar beet production was never established and fungicides to control of *C. beticola* were not used (TRKULJA ET AL., 2011; TRKULJA ET AL., 2012). This study determined very high frequency of resistant isolates of *C. beticola* to benzimidazoles (98%) in the district of Moravica, while in the district of Rasina, which is only 50 – 100 km away from the Moravica, frequency of resistance was dropped to 16%. Discovery of resistance on the locality without presence of selection pressure of fungicides, raise the question of origin of resistant populations in that regions. Based on these studies we can conclude that the usage of benzimidazole fungicides provided ideal conditions for resistant populations of *C. beticola* for their development. However, we can only discuss, that the long time selection pressure of benzimidazoles led to the spread of resistant populations to a regions that surrounds them, or in those regions, environmental conditions were more appropriate for the resistant then for sensitive populations of *C. beticola*, due to which its expansion has occurred. Further research, in terms of genetics of *C. beticola* population could provide an answer to this phenomenon.

ACKNOWLEDGEMENTS

This research was supported by grants of the Ministry of Education, Science and Technological Development of the Republic of Serbia TR-31018.

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