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Studies on *in vitro* activity of picoxystrobin and its mixtures with other fungicides against *Ramulispora herpotrichoides* and *Ramulispora acuformis*

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Abstract: The activity of picoxystrobin and its mixtures with standard fungicides against isolates of eyespot cereal disease agents was estimated in laboratory *in vitro* tests. The tested *Ramulispora herpotrichoides* and *R. acuformis* isolates differed in response to carbendazim. Picoxystrobin at concentration from 0.05 to 50 mg/L not totally inhibited the mycelium growth of tested pathogens on PDA medium. But carbendazim sensitive *R. herpotrichoides* isolates were a little more susceptible to strobilurin derivate than carbendazim resistant *R. herpotrichoides* isolate and *R. acuformis* strains. The effect of picoxystrobin at concentration 0.5 mg/L as partner in mixture with standard fungicide at concentration 0.1 mg/L (carbendazim, prochloraz or cyprodinil) was assessed as the additive. It was relatively most noticeably in tests with *R. acuformis* and picoxystrobin plus cyprodinil mixture that was for mixture recommended as tank-mix or as manufacture product.

Keywords: picoxystrobin, *Ramulispora*, fungicide mixtures

INTRODUCTION

Picoxystrobin is the board spectrum strobilurin fungicide particularly developed for use in cereals [1]. This fungicide was first registered in Poland in 2003 as a one-ingredient product – Acanto 250 SC [2]. In comparison with other commercial strobilurin fungicides picoxystrobin has unique transport properties because it shows both vapour activity and xylem systemicity. After application picoxystrobin is rapidly absorbed into plant tissue. Level of uptake provides

excellent curative properties whilst remaining product on leaf surface retains preventative benefits. Furthermore fungicide remaining on the outside of the leaf can be redistributed around existing leaves and to newly emerging cereal leaves in the vapour phase. Sufficient xylem-mobility of picoxystrobin ensures also disease control towards leaf tip. These attributes of picoxystrobin are an important reason why this fungicide is recommended for early season application in cereals [3, 4]. Other feature which distinguish as picoxystrobin is the fact that application of this compound at early stem extension of cereals provides some reduction of eyespot disease [5]. The eyespot is important stem-base disease, especially for winter wheat, caused by two pathogens: *Ramulispora herpotrichoides* (Fron) von Arx and *R. acuformis* (Nirenberg) Crous (formerly W- and R- growth type of *Pseudocercosporella herpotrichoides* (Fron) Deighton) (teleomorphs: *Tapesia yallundae* Wallwork et Spooner and *T. acuformis* (Boerema, Peters et Hamers) Crous) [6, 7]. In some new reports the fungi are classified to new genus - *Helgardia* (teleomorph - *Oculimacula*) [8, 9].

Possibility of eyespot reduction by picoxystrobin spraying is underlined in practical recommendations [5] and was confirmed to some extent in research studies [10, 11]. For example in the studies carried out by Ray and co-workers application of the mixture including cyprodinil, epoxiconazole and picoxystrobin decreased eyespot incidence and severity especially at disease assessment at late growth stage of winter wheat in comparison to cyprodinil mixed with epoxiconazole and to other treatments including alone cyprodinil or prochloraz. This effects was completed by the DNA decrease of eyespot fungus in plant tissue in comparison with the untreated control [10]. Despite these data we did not come across the studies of actual activity of azoxystrobin or its mixture with other fungicides against eyespot agents. In this place we should remind that manufacturer and plant protection advisory councils put emphasis on the use of picoxystrobin and other strobilurins in mixture with different fungicides as a crucial point of anti-resistance strategy [5, 12].

Taking above into consideration, we aimed, at present studies, to estimate the activity of picoxystrobin and its mixtures with cyprodinil and with systemic fungicides (prochloraz and carbendazim) against eyespot fungi isolates. Cyprodinil, prochloraz and carbendazim are recommended for the protection of winter wheat at early growth stage against eyespot disease [13]. Using these mixtures should assure a simultaneous control of foliar and stem base diseases of wheat crop.

MATERIALS AND METHODS

Isolates. Three *R. herpotrichoides* (code name MZ-159, MA-230 and OPL-16A) and two *R. acuformis* isolates (MA-231 and MZ-166) were used for determination of activity of picoxystrobin and its mixture with other fungicides. The isolates have some differences in carbendazim sensitivity. All isolates came from our collection and were obtained from winter wheat stems. Before tests they were stored at temperature 2-8 (±1)°C on PDA medium slants and subcultured periodically. The characteristic of tested isolates is given in Table 1.

Fungicidal activity tests. The picoxystrobin activity was determined by exposing of all five isolates to different concentrations of the fungicide in laboratory *in vitro* tests. The tests were conducted at the concentrations of fungicide: 0.05; 0.5; 5 and 50 mg/L. For assessment of the fungicide mixtures activity against eyespot fungi the tests were conducted at concentrations: 0.5 mg/L of picoxystrobin and 0.1 mg/L of carbendazim, prochloraz and cyprodinil. The three mixtures were tested at concentration 0.6 mg/L, this is 0.5 mg/L of strobilurin plus 0.1 mg/L of another fungicide. The activity of fungicide mixtures was determined for four isolates (MZ-159, OPL-16A, MA-231 and MZ-166).

In the tests PDA medium (adjusting with 25% lacid acid to pH 4.3-4.8) and suspensions of the commercial formulation of fungicides in sterile distilled water (picoxystrobin as Acanto 250 SC, carbendazim as Sarfun 500 SC, prochloraz as Mirage 450 EC and cyprodinil as Chorus 75 WG) were used. In control treatment PDA was added only with sterile distilled water. Three replicates (Petri plate, 5 cm diameter) were used for each concentration of the compound and isolate. Plates were inoculated centrally with 5-mm diameter discs cut from margin colonies growing actively on PDA. Inoculated plates were incubated in thermostatic chamber at 22 ±1 °C for 14 days and assessed by measurement of the colony diameter. The results (means of the colony diameter) were used to calculate the percentage of growth inhibition of mycelium in relation to the control. The data from measurements were statistically analyzed. The significant differences between treatments were calculated on the basis of the t-Student test (P=0.05) [14]. To estimate the interaction among strobilurin fungicide and the partner standard compound the method suggested by Gisi and quoted by Karaoglanidis and Karadimos was used [15]. With the exception of one isolates and treatments with carbendazim, all tests were conducted twice.

Table 1. Features of *Ramulispora herpotrichoides* (H) and *R. acuformis* (A) tested isolates

Species	Isolate code	Colony morphology on PDA				Sensitivity to fungicides			
		Colour of top mycelium	Margin of colony	Diameter of 14-days colony in mm	Daily growth rate in mm	Carbendazim[1)]	Prochloraz[1)]		Cyprodinil[2)]
						2 mg/L	2 mg/L	0.5 mg/L	0.5 mg/L
H	MZ-159	grey-bright grey	regular	37.0 (35-38)	2.29	100	92.2	68.7	not tested
H	MA-230	grey with different tint	regular	35.3 (34-36)	2.16	100	100	100	not tested
H	OPL-16A	grey	regular	34.5 (33-37)	2.11	1.6*	100	71.6	not tested
A	MA-231	grey or brownish-grey	irregular	16.2 (13-18)	0.80	100	84.1	53.6	100
A	MZ-166	white-grey	irregular, feathery	15.8 (13-17)	0.77	-12.7*	100	not tested	98.7

1) inhibition of linear mycelium growth,
2) inhibition of conidia germination,
* average from two tests

RESULTS

Picoxystrobin at concentrations from 0.05 to 50 mg/L only partially inhibited the mycelium growth of all *Ramulispora* isolates (Table 2).

Independently of concentration the fungicide inhibited significantly the linear growth of all *R. herpotrichoides* isolates. In the case of MZ-159 and MA-230 strains there was no statistical difference in colony growth between treatment with 5 mg/L and with 50 mg/L of strobilurin derivative. The same situation was observed comparing the growth of OPL-16A isolate on plates amended with 0.5 and 5 mg/L of fungicide.

The significant effect of all picoxystrobin concentrations was noted only in one from two conducted tests with *R. acuformis* isolates. In the first test the isolates of this fungus reacted similarly to all fungicide concentrations. In the second test the significant differences were obtained between lowest concentration and treatments with 5 and 50 mg/L of picoxystrobin.

Activity of picoxystrobin against isolates of *Ramulispora* spp. expressed as percent of linear colony growth inhibition varied to some extent (Figure 1). *R. herpotrichoides* isolate MZ-159 was relatively the most sensitive and fungicide restricted colony growth of this strain in 11-63%. Similar effect was obtained for second carbendazim sensitive isolate of this fungus (MA-230). Resistant to carbendazim *R. herpotrichoides* isolate OPL-16A appeared relatively more tolerant to strobilurin derivative.

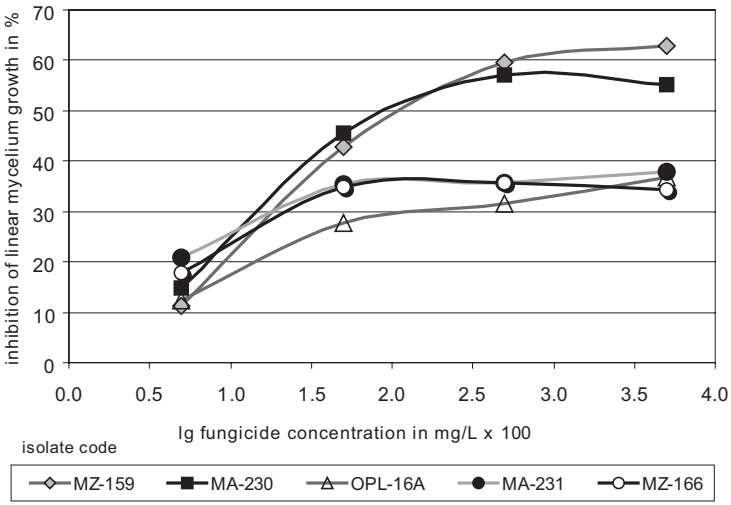

Figure 1. Activity of picoxystrobin against *Ramulispora* spp. isolates (averages from two *in vitro* tests on PDA).

Table 2. Effect of picoxystrobin on linear mycelium growth (in mm) of *Ramulispora* spp. isolates (tests *in vitro* on PDA medium)

Concentration of fungicide in mg/L	*R. herpotrichoides*						*R. acuformis*			
	Isolate code									
	MZ-159		MA-230		OPL-16A		MA-231		MZ-166	
	I. test	II. test	I. test	II. test	I. test	II. test	I. test	II. test	I. test	II. test
50	13.7 d*	13.0 d	18.7 d	14.0 d	24.2 d	22.3 d	10.0 b	9.3 c	11.5 b	9.8 c
5	15.8 d*	13.3 d	17.3 d	14.0 d	26.5 c	24.0 c	10.2 b	9.7 c	11.3 b	9.5 c
0.5	21.8 c	19.2 c	21.0 c	18.7 c	28.2 c	25.0 c	9.3 b	10.7 cb	11.0 b	10.0 cb
0.05	30.8 b	32.7 b	31.5 b	30.7 b	33.5 b	31.0 b	12.2 ab	12.3 b	15.0 ab	11.7 b
0.0 (control)	37.0 a	34.7 a	37.2 a	35.7 a	37.8 a	35.8 a	15.5 a	15.5 a	18.2 a	14.3 a

* Values in columns followed by the same letter do not differ at P=0.05, t-Student test

Activity of picoxystrobin against two *R. acuformis* isolates was comparable and varied in dependence on fungicide concentration from 18 to 38%. Surprisingly, picoxystrobin at lowest concentration was more effective against *R. acuformis* than against *R. herpotrichoides* isolates. At higher fungicide concentration the reaction of *R. acuformis* strains was weaker than carbendazim sensitive isolates of *R. herpotrichoides*.

The weakest reaction to picoxystrobin was noted on plates inoculated with mycelium of carbendazim resistant *R. herpotrichoides* isolate. Activity of fungicide against the mentioned strain was a little worse than against *R. acuformis* isolates and noticeably lower in comparison with two next *R. herpotrichoides* forms at concentration: 0.05; 0.5 mg/L and 0.5; 5 and 50 mg/L, respectively.

The effect of mixtures with picoxystrobin and standard fungicide on linear colony growth of *Ramulispora* spp. only in some cases was significantly stronger than the effect of single antifungal compounds.

In comparison with single benzimidazole and strobilurin treatments the growth of colony on plates amended with carbendazim and picoxystrobin mixture was significantly greater in one test with carbendazim sensitive *R. herpotrichoides* strain (MZ-159) (Table 3). In the case of prochloraz treatments and its mixture with strobilurin the analogues effect was obtained for both *R. herpotrichoides* isolates (in one test with carbendazim sensitive isolate and in both tests with resistant isolate - OPL-16A). The significant poorer colony growth on medium amended with cyprodinil and picoxystrobin in comparison to the medium amended with single fungicide was noted in one from two tests with both *R. acuformis* isolates and resistant to carbendazim *R. herpotrichoides* isolate. It should be added that the effect of 0.5 mg/L picoxystrobin was stronger than the effect of carbendazim and prochloraz (0.1 mg/L) in tests with *R. acuformis* isolates and stronger or comparable with cyprodinil treatment in tests with *R. herpotrichoides*. Moreover carbendazim resistant *R. acuformis* isolate MZ-166 gave more distinct response to cyprodinil than to second standard compound (prochloraz).

Table 3. Effect of picoxystrobin and its mixtures with standard fungicides on linear mycelium growth (in mm) of *Ramulispora* spp. isolates (tests *in vitro* on PDA medium)

Treatments	Concentration in mg/L	*R. herpotrichoides*				*R. acuformis*			
		Isolate code							
		MZ-159		OPL-16A		MA-231		MZ-166	
		I test	II test	I test	II test	I test	II test	I test	II test
Picoxystrobin	0.5	21.2 c	21.3 bc	26.2 b	26.8 b	10.2 c	9.0 bc	9.7 c	9.3 cde
Carbendazim	0.1	11.7 ef *	14.0 d	37.7 a	37.3 a	12.3 b	12.2 b	-	14.7 a
Picoxystrobin + Carbendazim	0.5 + 0.1	10.8 f *	11.5 e	29.7 b	26.5 b	10.5 bc	10.0 bc	-	9.5 cd
Prochloraz	0.1	14.8 d	11.7 de	20.5 d	19.3 c	11.7 bc	10.3 bc	13.3 b	12.2 b
Picoxystrobin + Prochloraz	0.5 + 0.1	12.7 e	12.8 de	17.7 e	17.7 d	11.0 bc	8.7 bc	10.3 bc	8.5 def
Cyprodinil	0.1	24.7 b	23.3 b	26.8 b	26.2 b	12.0 bc	10.7 bc	10.8 bc	10.2 c
Picoxystrobin + Cyprodinil	0.5 + 0.1	20.5 c	20.5 c	24.0 c	25.7 b	7.0 d	7.5 c	8.0 c	7.8 f
Control	-	36.3 a	36.5 a	37.0 a	37.3 a	20.0 a	17.0 a	17.2 a	14.3 a

* Values in columns followed by the same letter do not differ at P=0.05, t-Student test

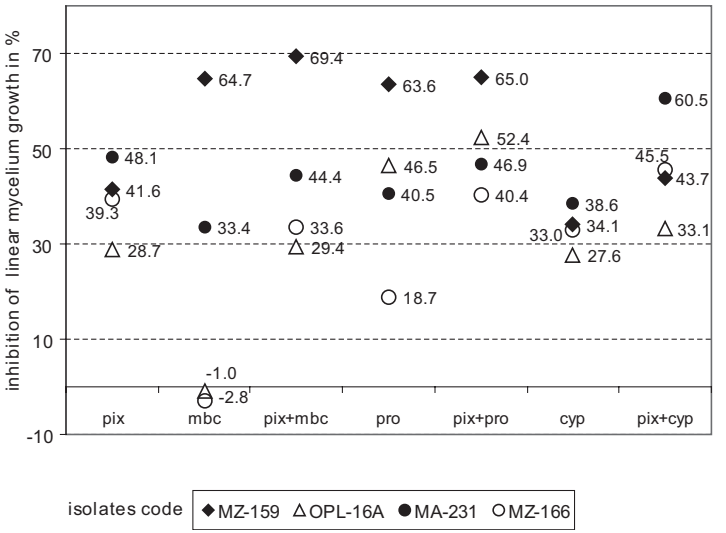

Figure 2. Activity of mixtures with picoxystrobin and standard fungicides against *Ramulispora* spp. isolates (averages from two *in vitro* tests on PDA, pix - picoxystrobin 0.5 mg/L; mbc - carbendazim 0.1 mg/L, pro - prochloraz 0.1 mg/L, cyp - cyprodinil 0.1 mg/L).

Activity of the mixture with picoxystrobin and cyprodinil expressed as percent of linear colony growth inhibition, in comparison with treatments with both alone active ingredient, was higher against all tested strains of eyespot fungi (Figure 2). The same effect was obtained for mixture with prochloraz and both *R. herpotrichoides* isolates and carbendazim resistant *R. acuformis* isolate, and for the mixture with carbendazim and sensitive isolate of first named fungus.

The ratio between the experimentally obtained activity of the tested mixtures and the expected activity were considered to be additive and ranged between: 0.68-0.87 for mixture with carbendazim and sensitive strains; and between 0.68-0.85 and 0.68-0.89 for all strains and mixture with prochloraz and with cyprodinil, respectively.

DISCUSSION AND CONCLUSIONS

Obtained results of laboratory tests indicate some differences in response of *Ramulispora* spp. to picoxystrobin. It seems that *R. herpotrichoides* fungus is more sensitive to this strobilurin derivative than *R. acuformis*. This suggestion

confirms opinions from many studies that generally *R. acuformis* fungus is tolerant or insensitive to many antifungal compounds and other active ingredients used in plant protection in comparison with *R. herpotrichoides* [16]. But it should be underline that tolerant to carbendazim *R. herpotrichoides* strain showed a decreasing sensitivity to picoxystrobin. In the case of eyespot causal agents and to other pathogens the change in sensitivity to one antifungal compound often caused the altering response to other fungicides. In dependence on direction this phenomenon is often named as cross-resistance or cross-negative resistant. For example: in *R. acuformis* fungus the most common prochloraz resistance phenotype is linked with increased susceptibility to triflumizole (another imidazole) and to pyrimidines (fenarimol and nuarimol). Similarly one type of tradimenol resistant isolates of *Septoria tritici* are more susceptible to mentioned fungicides and to fluquinconazole [17]. We could not exclude that response to picoxystrobin of resistant to carbendazim form of *R. herpotrichoides* is a next illustration of this phenomenon. But this idea should be verified in the further, more extensive studies.

It should be added that some studies on the strobilurin fungicides activity *in vitro* against pathogens were carried out in the presence of salicylhydroxamic acid (SHAM), which should suppress fungus resistance due to alternative respiration [18, 19]. In presented studies the agar medium was used without SHAM. But effect of alternative respiration is noted more clearly in laboratory tests with spores than with pathogen mycelium [20, 21]. This way the probability of alternative respiration influence in the presented tests are supposed to be appreciated as very low and without impact to a general trend.

The results of test with fungicide mixtures indicate a positive effect of picoxystrobin as a partner for activity of standard fungicides using for eyespot control. It was particularly noticeable for mixture with cyprodinil (and *R. acuformis* strains) that is for composition which is recommended (as factory product or as tank-mix) for control of eyespot and other cereal diseases [5, 22]. This constructive effect of use some fungicide along with strobilurin derivatives are obtained in some studies carried out in condition similar to plant protection practice (field experiments). And is mainly observed for mixtures with sterols biosynthesis inhibitors e.g. in protection of sugar beet against powdery mildew (*Erysiphe beate*) [15] and in protection of winter wheat against *Septoria tritici* blotch [23].

Taking some interesting results into consideration the studies on activity of picoxystrobin and other strobilurins alone and in mixtures with partner standard fungicides (regarding a ratio of recommended doses) against different forms of eyespot causal agents will be continued.

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