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Crop Protection 21 (2002) 41–47

Crop Protection

www.elsevier.com/locate/cropro

Assessment of interactions between components of fungicide mixtures against *Monilinia fructicola*

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Received 17 October 2000; received in revised form 5 March 2001; accepted 27 April 2001

Abstract

Mixtures of fungicides with different modes of action can exhibit synergism, i.e. an inhibition of pathogen growth above that expected from independent action of the mixture components. Two-way mixtures of commercial formulations of propiconazole with either benomyl, captan, chlorothalonil, cyprodinil or vinclozolin were evaluated in vitro for potential synergism in inhibiting *Monilinia fructicola*, the causal agent of blossom blight and brown rot of stone fruits. Propiconazole was emphasized because of its widespread use and the recent detection of isolates of *M. fructicola* with reduced sensitivity to this fungicide. Experiments included each active ingredient at low, medium and high concentrations in all possible pairwise combinations. Inhibition of radial growth of two isolates of *M. fructicola* was not significantly different ($P > 0.01$) from that predicted by a simple model of independent action for any of the fungicide–concentration combinations, indicating absence of synergism between active ingredients. Results were similar when mixtures of propiconazole with either benomyl, chlorothalonil or cyprodinil were evaluated on peach fruit treated with fungicide. While fungicide mixtures are useful in delaying the development of fungicide resistance, they are unlikely to be used in practice unless synergistic interactions allow for applications at reduced concentrations. The absence of synergism suggests little incentive exists for favoring propiconazole-based fungicide mixtures over a rotating schedule of fungicides for control of and resistance management in *M. fructicola*.

Keywords: Brown rot; Fungicide mixture; *Monilinia fructicola*; Peach; *Prunus persica*

1. Introduction

Control of *Monilinia fructicola* (G. Wint.) Honey is critical in peach (*Prunus persica* (L.) Batsch) production worldwide. Fungicide applications are made during bloom to control the blossom blight phase of the disease and again before harvest to prevent brown rot of the fruit (Byrde and Willetts, 1977; Horton et al., 2000). While control is generally adequate, frequent applications are costly and repeated use of the same active ingredient can lead to the development of fungicide resistance. Indeed, reduced sensitivity toward various fungicides has been well documented in both field and laboratory populations of *M. fructicola* (Ritchie, 1983; Michailides et al., 1987; Zehr et al., 1991; Elmer and Gaunt, 1993; Braithwaite et al., 1995; Sanoamuang and Gaunt, 1995). This includes a recent report from South Carolina of reduced sensitivity to the demethylation-inhibiting fungicide propiconazole (Zehr et al., 1999) which is widely used to control the pathogen on stone fruits in the southeastern USA.

The application of fungicides with different modes of action either on a rotating schedule or in a mixture is a generally recommended resistance management strategy (Staub, 1991; Russell, 1995; Bertrand and Padgett, 1997). Compared with a rotating schedule, fungicide mixtures provide the potential for synergistic interactions, which can increase control to a level above that expected from the sum of the individual components (Gisi, 1996). Because of increased control efficacy with mixtures that act synergistically, concentrations of the mixture components can be reduced, thereby reducing costs (Bertrand and Padgett, 1997). For example, synergistic interactions among cymoxanil, mancozeb and oxadixyl against various *Phytophthora* species in vitro (Gisi et al., 1985) translated into less fungicide needed to control potato late blight, caused by *Phytophthora infestans*, when mixtures containing these active ingredients were used in the field (Samoucha and

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0261-2194/02/$ - see front matter
PII: S0261-2194(01)00062-X

Cohen, 1989). Similarly, the synergistic interaction of pyrazophos and propiconazole against the barley net blotch pathogen *Pyrenophora teres* in an in vitro assay correlated with enhanced disease control when the mixture was applied in the greenhouse (Zeun and Buchenauer, 1991).

Knowledge about the general nature of interactions among active ingredients is important for determining the potential value of fungicide mixtures; however, no such research has been reported in relation to *M. fructicola*. Thus, the objective of this study was to evaluate interactions between fungicides against *M. fructicola* as a first step toward assessing the potential for the development of synergistic mixtures that could provide satisfactory disease control while also aiding in resistance management. Experiments were carried out in vitro (on amended media) and in vivo (on treated peach fruit) with a focus on mixtures containing the fungicide propiconazole. This active ingredient was emphasized because of its widespread use in the southeastern USA and the documented risk of resistance development (Zehr et al., 1999).

2. Materials and methods

2.1. Maintenance of fungal cultures and production of inoculum

Experiments were carried out with two isolates of *M. fructicola*, isolate H-211 from Georgia and isolate ZN-21 from South Carolina (obtained from E. I. Zehr, Clemson University). The effective concentrations of propiconazole that reduced growth by 50% (EC_{50} values) were determined as 0.0027 and 0.0038 μg/ml for H-211 and ZN-21, respectively, using the in vitro assays described below. The isolates were maintained on propiconazole-amended V-8 juice agar slants at 5°C.

Inoculum was produced on canned peach slices (Nevill et al., 1978) on wire racks in sterile tissue culture boxes. Each peach slice was inoculated with an agar plug from a 5- to 7-day-old culture of *M. fructicola* and incubated at room temperature (ca. 25°C) in the dark for 5–7 days. To harvest conidia, peach slices were placed in Erlenmeyer flasks and washed in sterile distilled water for 15 min on a wrist action shaker. The suspension was filtered through two layers of cheesecloth, and conidia were counted with the aid of a hemacytometer. The concentration of the suspension was adjusted to range from 1.2 to 1.8×10^5 conidia/ml.

2.2. Dose–response curves

Dose–response curves were generated to identify concentrations of individual active ingredients that inhibited growth of *M. fructicola* by 10, 50 and 90% (EC_{10}, EC_{50} and EC_{90}, respectively). Serial dilutions of commercial formulations of six fungicides (benomyl, captan, chlorothalonil, cyprodinil, propiconazole and vinclozolin; Table 1) were made in sterile distilled water. Aliquots of the fungicide suspensions were incorporated into Czapek–Dox agar buffered with 11.5 g/l TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Sigma, St. Louis, Missouri) to provide eight different concentrations in the agar medium ranging from 0.0001 to 1000 μg/ml. Czapek–Dox agar, a synthetic medium lacking amino acids, was selected because certain amino acids can interfere with fungicidal action in vitro (Masner et al., 1994). The medium was dispensed into 100-mm plastic petri dishes at a volume of 25–30 ml per dish; five dishes for each fungicide concentration were prepared. The medium was inoculated with 40 μl of a conidial suspension of isolate H-211 dispensed into a 5-mm diameter well cut in the center of each dish with a sterile cork borer. After 7 days at room temperature in the dark, colony diameters were measured across perpendicular axes. For each fungicide and concentration, inhibition of radial growth compared with the untreated check (growth on non-amended medium) was calculated. The experiment was repeated and results were combined for analysis.

Logit-transformed values of growth inhibition were plotted against $\log_{10}$-transformed fungicide concentra-

Table 1
Active ingredients used in the assessment of interactions between components of fungicide mixtures against *Monilinia fructicola*

Common name	Formulation	Manufacturer	EC_{10}[a]	EC_{50}	EC_{90}
Benomyl	Benlate 50WP	Du Pont	0.0091	0.0266	0.0774
Captan	Captan 50WP	Zeneca Ag Products	0.5628	4.662	38.6162
Chlorothalonil	Bravo Weather Stik	Zeneca Ag Products	0.0085	0.0356	0.1485
Cyprodinil	Vangard 75WP	Novartis	0.0064	0.0536	0.4476
Propiconazole	Orbit 3.6EC	Novartis	0.0006	0.0027	0.0113
Vinclozolin	Ronilan DF	BASF	0.0046	0.0668	0.9660

[a] EC_{10}, EC_{50} and EC_{90} are the concentrations (in μg/ml) of active ingredient that reduced radial growth of isolate H-211 on fungicide-amended Czapek Dox agar by 10%, 50% and 90%, respectively.

tion and dose–response curves for each fungicide were generated by fitting linear regression equations. Regressions were statistically significant ($P<0.01$) for all fungicides, with correlation coefficients ranging from 0.825 to 0.965 (data not shown). Based on parameter estimates obtained from the regression equations, EC_{10}, EC_{50} and EC_{90} values were determined (Table 1).

2.3. Evaluation of fungicide mixtures in vitro

Two-way fungicide mixtures consisting of propiconazole with either benomyl, captan, chlorothalonil, cyprodinil or vinclozolin (Table 1) were evaluated with both isolates. Experiments included each component of the mixture at its EC_0 (no fungicide), EC_{10}, EC_{50} and EC_{90} concentration (to simulate interactions at low, medium and high levels) in all possible pairwise combinations, yielding a total of 16 combinations per fungicide pair and isolate. The EC values for each fungicide were as determined with isolate H-211 (Table 1). Fungicide mixtures were made in sterile distilled water and incorporated into Czapek–Dox agar which was prepared, inoculated and assessed for inhibition of radial growth as described above. Relative inhibition compared with the untreated check (non-amended medium) was calculated for each fungicide–concentration–isolate combination. Each combination was tested at least three times.

2.4. Evaluation of fungicide mixtures in vivo

A subset of active ingredients (propiconazole in mixtures with either benomyl, chlorothalonil or cyprodinil) was selected for postharvest testing on firm-ripe peach fruit cv. 'Blake' that received no preharvest fungicide applications. Fruit were surface-sterilized in a solution of 0.5% sodium hypochlorite (NaOCl) for 2 min and allowed to dry overnight. Two-way mixtures of fungicides were prepared in sterile distilled water with each component at 0.5%, 2.5%, and 5% of its standard field application rate (0.13 ml/l, 0.60 g/l, 0.67 ml/l and 0.28 g/l of active ingredient for propiconazole, benomyl, chlorothalonil and cyprodinil, respectively). This range of concentrations resulted in negligible (0.5% rate) to almost complete (5% rate) inhibition of *M. fructicola* in preliminary tests on peach fruit. Fruits were dipped individually in these suspensions for 30 s before placement on plywood racks previously disinfested with 0.5% NaOCl. Each fruit was inoculated with a 30-µl drop of conidial suspension prepared from isolate H-211 or ZN-21 placed on the uninjured cheek surface. The racks holding inoculated fruit were covered with a plastic sheet to maintain high humidity and kept at room temperature. After 5 or 6 days, lesion diameters were measured to the closest mm with a tape measure and expressed as a proportion of the fruit circumference. Relative inhibition compared with the inoculated check (treated with sterile distilled water) was calculated for each fungicide–concentration–isolate combination. Each combination was tested at least three times, each with eight fruit per combination.

2.5. Data analysis

Interactions between the components of the fungicide mixtures were evaluated with the Gowing equation (Gowing, 1960; Levy et al., 1986; Kosman and Cohen, 1996):

$$C_{exp} = C_1 + C_2(1 - C_1), \quad (1)$$

where C_{exp} is the expected level of inhibition with the mixture when the components act independently and C_1 and C_2 are the actual levels of inhibition observed when each component is applied alone. If observed inhibition with the mixture, C_{obs}, is equal to C_{exp}, the components exhibit independent action. If C_{obs} is greater or less than C_{exp}, the mixture components act synergistically or antagonistically, respectively.

For each fungicide–concentration–isolate combination, ΔC, the difference between C_{obs} and C_{exp}, was calculated. Using the repeats of the experiments as replications, *t*-tests were applied to determine whether ΔC deviated significantly from zero. All tests were carried out at $P = 0.01$ because a large number of significance tests was made, thereby increasing the probability of declaring significance by chance alone. The analysis was conducted with the Statistical Analysis System (SAS Institute, Cary, North Carolina).

3. Results

3.1. Evaluation of fungicide mixtures in vitro

The two isolates of *M. fructicola* reacted similarly to increasing concentrations of propiconazole alone or in combination with other fungicides. Isolate ZN-21 showed lower levels of inhibition, particularly at low and medium concentrations of the mixture components. The general response of the two isolates to fungicide mixtures, in terms of relative growth inhibition, is illustrated in Fig. 1 using the combination of propiconazole and benomyl as an example.

ΔC was negative for 65 of 90 fungicide–concentration–isolate combinations evaluated in vitro (Fig. 2), i.e. growth inhibition observed with the mixture was generally less than that expected from Eq. (1). This pattern was similar for both isolates. However, ΔC did

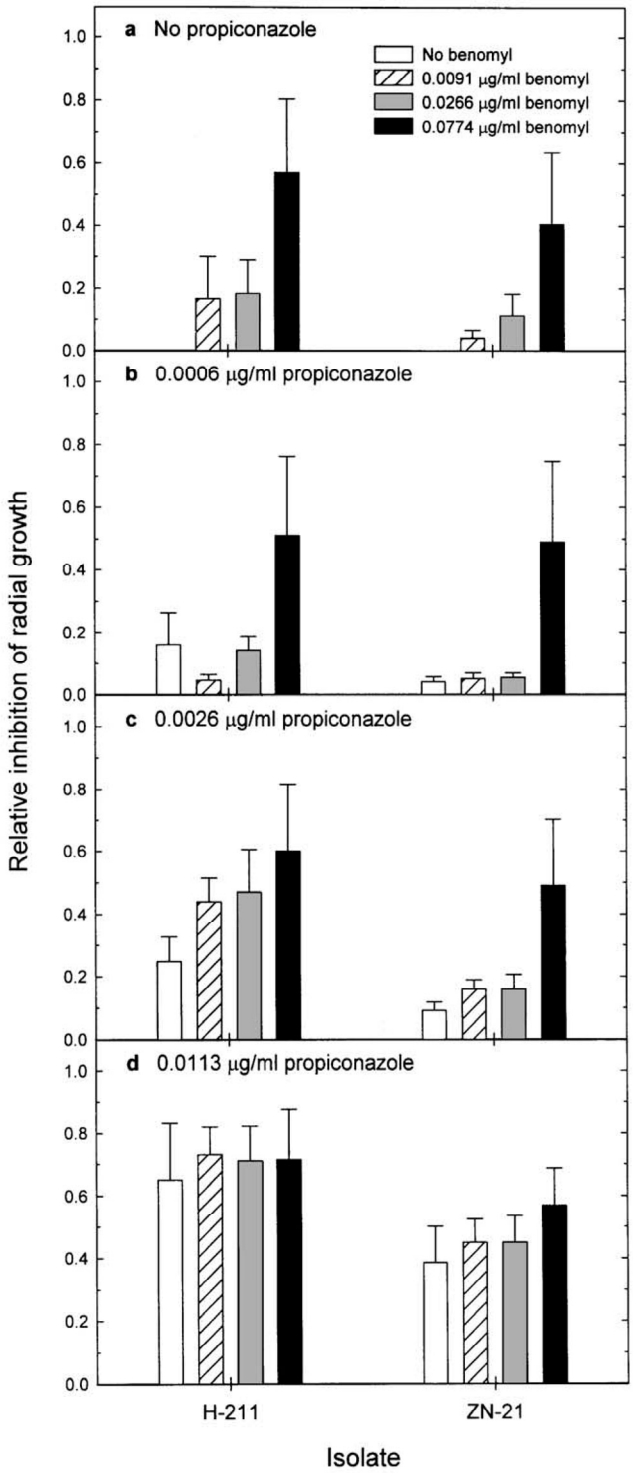

Fig. 1. Effect of two-way mixtures of propiconazole and benomyl at various concentrations on relative inhibition of radial growth of two isolates (H-211 and ZN-21) of *Monilinia fructicola* on fungicide-amended Czapek–Dox medium. Fungicide concentrations correspond to EC_{10}, EC_{50} and EC_{90} values for isolate H-211 determined in vitro. Values are means and standard errors of four experiments.

not differ significantly from zero ($P > 0.01$) for any of the combinations, indicating independent action between the mixture components in all cases.

3.2. Evaluation of fungicide mixtures in vivo

Inhibition of *M. fructicola* on peach fruit ranged from 0% to 100% for the various fungicide–concentration–isolate combinations (data not shown). As in the in vitro experiments, a trend toward negative values of ΔC was apparent (Fig. 3), with 45 of 54 fungicide–concentration–isolate combinations showing less inhibition of *M. fructicola* than that predicted by Eq. (1). For isolate ZN-21, this trend was most pronounced at low concentrations for all fungicides (Fig. 3A); for isolate H-211, it only occurred for mixtures containing cyprodinil at low concentrations. However, no significant antagonism was detected at $P = 0.01$. Similarly, no significant synergism was detected in these experiments.

4. Discussion

Active ingredients in two-way mixtures of propiconazole with other fungicides against *M. fructicola* in culture and on peach fruit generally acted independently, i.e. inhibition achieved with the mixtures was equal to that of the sum of the individual components of the mixture. This result is consistent with the hypothesis that each active ingredient inhibits a fixed proportion of the residual pathogen growth not inhibited by the other. Similar results have been reported in other pathosystems. For example, Couch and Smith (1991) evaluated a range of fungicides for interactions against *Pythium aphanidermatum* on perennial ryegrass and observed mostly independent action. In contrast, other studies that involved screening of various active ingredients either in vitro or in vivo reported a greater incidence of synergistic (Gisi et al., 1985) or antagonistic (Buchenauer, 1980) interactions. It should be noted, however, that most previous studies used predetermined thresholds of "synergy ratios" (calculated as C_{obs}/C_{exp}) to determine whether mixtures acted synergistically or antagonistically and generally did not include formal tests to confirm that observed deviations from independent action were statistically significant.

In the present study, trends toward antagonism were apparent, although not significant at $P = 0.01$, for mixtures of propiconazole with the two contact fungicides captan or chlorothalonil evaluated in vitro at medium and high concentrations (Fig. 2). Similar trends toward antagonism in vitro were reviewed by De Waard and Gisi (1995) and Scardavi (1966) for various fungicide mixtures tested against a range of plant pathogenic fungi. Couch and Smith (1991) observed only one case of significant antagonism when screening a large number of fungicide mixtures for their effect on *P. aphanidermatum* in vivo. Interestingly, this occurred in a mixture used commercially for more than 25 years.

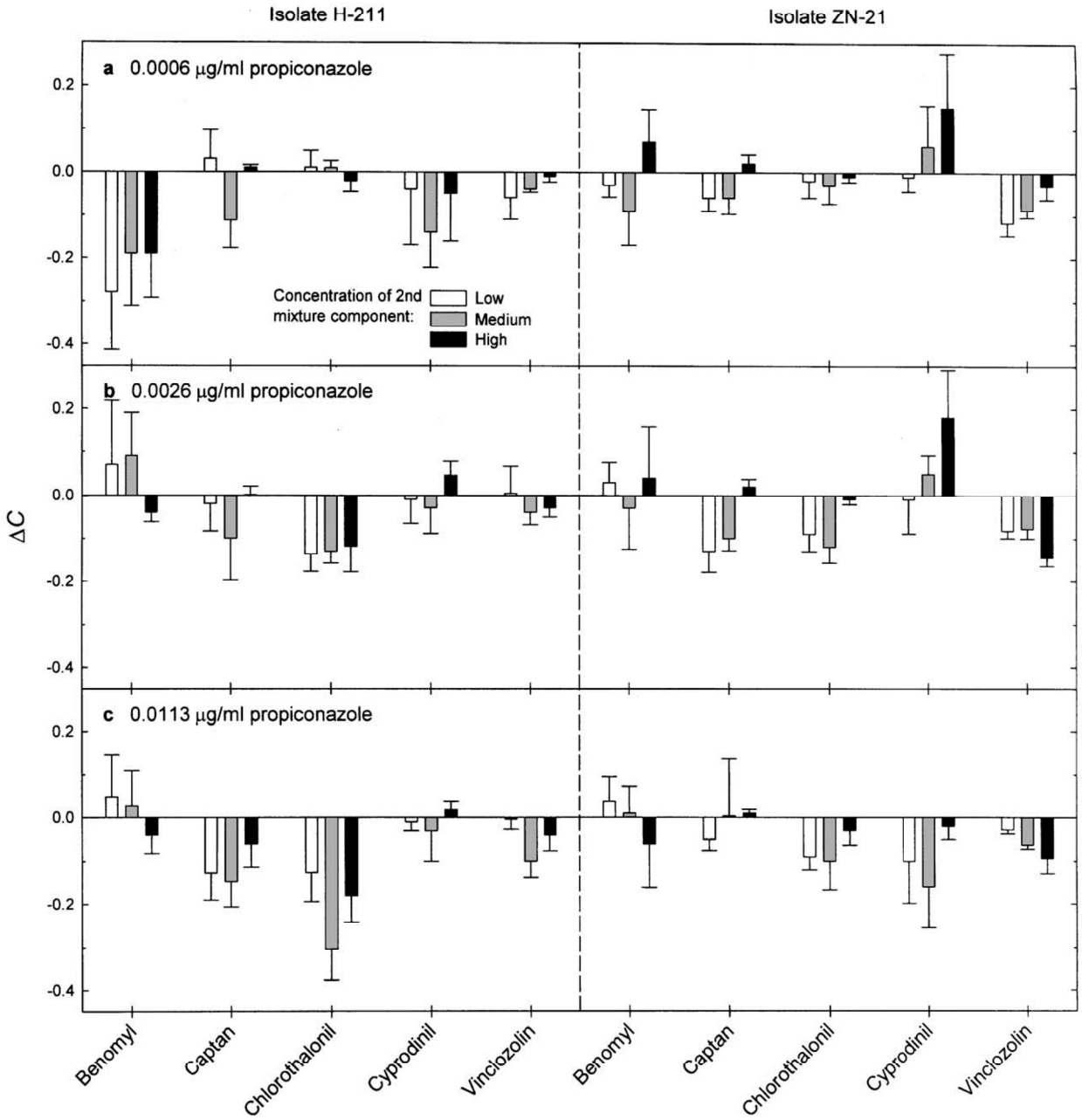

Fig. 2. In vitro interactions between fungicides in two-way mixtures of propiconazole with other active ingredients with respect to inhibition of two isolates (H-211 and ZN-21) of *Monilinia fructicola*. Interactions are expressed as differences (ΔC) between observed inhibition (reduction of radial growth on Czapek–Dox medium amended with the fungicide mixture relative to the non-amended check) and predicted inhibition assuming independent action of the components of the mixture (see Eq. (1)). Low, medium and high fungicide concentrations correspond, respectively, to EC_{10}, EC_{50} and EC_{90} values for isolate H-211 determined in vitro. Values are means and standard errors of at least three experiments.

For the three fungicide mixtures included in both the in vitro and in vivo experiments in the present study, results were similar in that all interactions were of an independent nature. In contrast, Grabski and Gisi (1987) and Zeun and Buchenauer (1991) noted more pronounced activity in vivo than in vitro when fungicide mixtures were evaluated against *P. infestans* or *P. teres*.

Of particular interest in studies with fungicide mixtures are effects on isolates of pathogenic fungi with reduced sensitivity to one of the mixture components (Grabski and Gisi, 1985, 1987; Samoucha and Cohen, 1988; Couch and Smith, 1991; Gisi, 1996). For example, Grabski and Gisi (1987) noted that the degree to which an isolate of *P. infestans* was resistant to a fungicide influenced strongly its reaction to mixtures containing

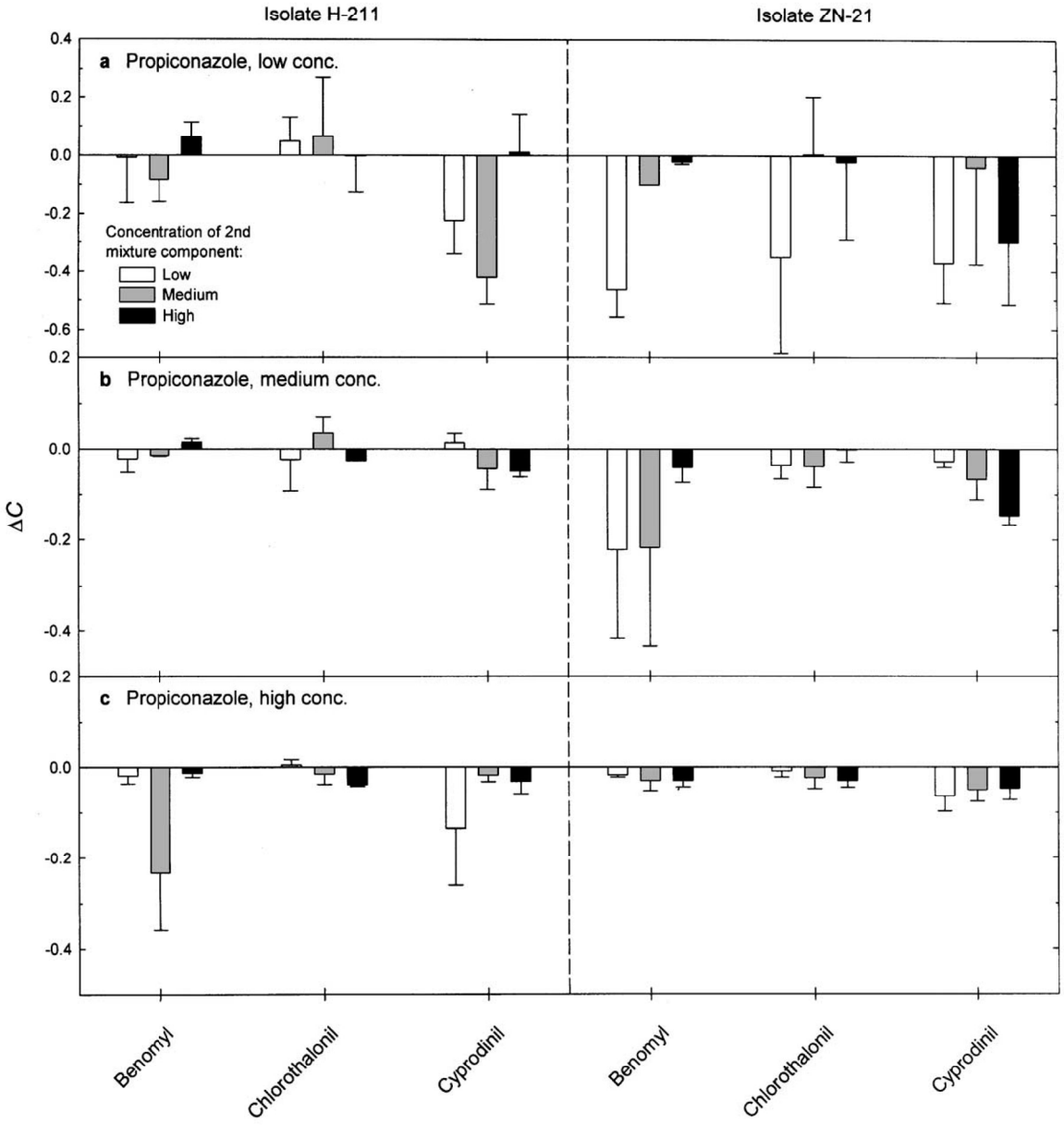

Fig. 3. In vivo interactions between fungicides in two-way mixtures of propiconazole with other active ingredients with respect to inhibition of two isolates (H-211 and ZN-21) of *Monilinia fructicola*. Interactions are expressed as differences (ΔC) between observed inhibition (reduction in lesion diameter caused by *M. fructicola* on peach fruit dipped in the fungicide mixture relative to the untreated check) and predicted inhibition assuming independent action of the components of the mixture (see Eq. (1)). Low, medium and high fungicide concentrations correspond, respectively, to 0.5%, 2.5% and 5% of the compounds' standard field application rates. Values are means and standard errors of at least three experiments.

that fungicide. In contrast, Couch and Smith (1991) determined that mixtures of metalaxyl and mancozeb were equally effective against metalaxyl-sensitive and resistant isolates of *P. aphanidermatum*. Differential responses to fungicide mixtures in relation to fungicide sensitivity were not investigated in the present study because of the similarity in propiconazole-sensitivity of the two isolates of *M. fructicola*.

In the absence of synergism, the control potential of a fungicide mixture at reduced concentrations is less than that of the full concentration of the most efficacious component used alone (Couch and Smith, 1991). Because of the lack of synergistic effects observed in this study, the level of control achieved with propiconazole-based mixtures may not provide sufficient benefit to offset the added costs of using two active ingredients instead of one. Thus, there would be little incentive for favoring fungicide mixtures over a rotating schedule of fungicides for control of and resistance management in *M. fructicola*, assuming both

strategies are equally successful in delaying resistance development.

Acknowledgements

Funded in part by the USDA-CSREES Pest Management Alternatives Program (grant no. 97-34365-5034) and the Georgia Agricultural Commodity Commission for Peaches. We thank J. Lance and L. Chenault for technical assistance.

References

Bertrand, P.F., Padgett, G.B., 1997. Fungicide resistance management. Bulletin 1132, University of Georgia, College of Agricultural and Environmental Sciences, Cooperative Extension Service, Athens.

Braithwaite, M., Elmer, P.A.G., Saville, D.J., Boyd-Wilson, K.S.H., Whelan, H.G., 1995. Reduced sensitivity to DMI fungicides in *Monilinia fructicola* and the efficacy of DMI fungicides for blossom blight control. Proceedings of the 48th New Zealand Plant Protection Conference, pp. 17–21.

Buchenauer, H., 1980. Interaction of different liquid components with various fungicides. Z. Pfl. Krankh. Pfl. Schutz 87, 335–345.

Byrde, R.J.W., Willetts, H.J., 1977. The Brown Rot Fungi of Fruit: their Biology and Control. Pergamon Press, Oxford.

Couch, H.B., Smith, B.D., 1991. Synergistic and antagonistic interactions of fungicides against *Pythium aphanidermatum* on perennial ryegrass. Crop Protection 10, 386–390.

De Waard, M.A., Gisi, U., 1995. Synergism and antagonism in fungicides. In: Lyr, H. (Ed.), Modern Selective Fungicides: Properties, Applications, Mechanisms of Action, 2nd Edition. Gustav Fischer Verlag, Jena, pp. 565–578.

Elmer, P.A.G., Gaunt, R.E., 1993. Effect of frequency of dicarboximide applications on resistant populations of *Monilinia fructicola* and brown rot in New Zealand orchards. Crop Protection 12, 83–88.

Gisi, U., 1996. Synergistic interaction of fungicides in mixtures. Phytopathology 86, 1273–1279.

Gisi, U., Binder, H., Rimbach, E., 1985. Synergistic interactions of fungicides with different modes of action. Trans. Br. Mycol. Soc. 85, 299–306.

Gowing, D.P., 1960. Comments on tests of herbicide mixtures. Weeds 8, 379–391.

Grabski, C., Gisi, U., 1985. Mixtures of fungicides with synergistic interactions for protection against phenylamide resistance in *Phytophthora*. In: Smith, I.M. (Ed.), Fungicides for Crop Protection: 100 Years of Progress. BCPC Publications, Croydon, pp. 315–317.

Grabski, C., Gisi, U., 1987. Quantification of synergistic interactions of fungicides against *Plasmopara* and *Phytophthora*. Crop Protection 6, 64–71.

Horton, D., Gorsuch, C., Ritchie, D. (Eds.), 2000. 2000 Southern Peach, Nectarine and Plum Pest Management and Culture Guide. Bulletin 1171, University of Georgia, College of Agricultural and Environmental Sciences, Cooperative Extension Service, Athens.

Kosman, E., Cohen, Y., 1996. Procedures for calculating and differentiating synergism and antagonism in action of fungicide mixtures. Phytopathology 86, 1263–1272.

Levy, Y., Benderly, M., Cohen, Y., Gisi, U., Bassand, D., 1986. The joint action of fungicides in mixtures: comparison of two methods for synergy calculation. EPPO Bull. 16, 651–657.

Masner, P., Muster, P., Schmidt, J., 1994. Possible methionine biosynthesis inhibition by pyrimidinamine fungicides. Pestic. Sci. 42, 163–166.

Michailides, T.J., Ogawa, J.M., Opgenorth, D.C., 1987. Shift of *Monilinia* spp and distribution of isolates sensitive and resistant to benomyl in California prune and apricot orchards. Plant Dis. 71, 893–896.

Nevill, J.R., Szkolnick, M., Gilpatrick, J.D., Ogawa, J.M., 1978. Mass production of conidia of brown rot fungi on canned peaches. Plant Dis. Rep. 62, 966–969.

Ritchie, D.F., 1983. Mycelial growth, peach fruit-rotting capability, and sporulation of strains of *Monilinia fructicola* resistant to dichloran, iprodione, procymidone, and vinclozolin. Phytopathology 73, 44–47.

Russell, P.E., 1995. Fungicide resistance: occurrence and management. J. Agric. Sci. (Cambridge) 124, 317–323.

Samoucha, Y., Cohen, Y., 1988. Synergistic interactions of cymoxanil mixtures in the control of metalaxyl-resistant *Phytophthora infestans* of potato. Phytopathology 78, 636–640.

Samoucha, Y., Cohen, Y., 1989. Field control of potato late blight by synergistic fungicidal mixtures. Plant Dis. 73, 751–753.

Sanoamuang, N., Gaunt, R.E., 1995. Persistence and fitness of carbendazim- and dicarboximide-resistant isolates of *Monilinia fructicola* (Wint.) Honey in flowers, shoots and fruit of stone fruits. Plant Pathol. 44, 448–457.

Scardavi, A., 1966. Synergism among fungicides. Annu. Rev. Phytopathol. 4, 335–348.

Staub, T., 1991. Fungicide resistance: practical experience with antiresistance strategies and the role of integrated use. Annu. Rev. Phytopathol. 29, 421–442.

Zehr, E.I., Luszcz, L.A., Olien, W.C., Newall, W.C., Toler, J.E., 1999. Reduced sensitivity in *Monilinia fructicola* to propiconazole following prolonged exposure in peach orchards. Plant Dis. 83, 913–916.

Zehr, E.I., Toler, J.E., Luszcz, L.A., 1991. Spread and persistence of benomyl-resistant *Monilinia fructicola* in South Carolina peach orchards. Plant Dis. 75, 590–593.

Zeun, R., Buchenauer, H., 1991. Synergistic effects of pyrazophos and propiconazole against *Pyrenophora teres*. Z. Pfl. Krankh. Pfl. Schutz 98, 661–668.