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Efficacy of Carboxylic Acid Amides Fungicides towards CAA Sensitive and Resistant *Plasmopara viticola* Populations: *in vivo* Tests and Molecular Studies on *PvCesA3* Gene

Nanni IM, Stammler G, Zito R, Collina M

Department of Agricultural Sciences (DipSA), University of Bologna, Viale G. Fanin 46, I-40127 Bologna, Italy.

Email: marina.collina@unibo.it

ABSTRACT

Plasmopara viticola is controlled by fungicides with different modes of action, including carboxylic acid amides (CAAs). The mutations conferring CAA resistance in *P. viticola* located on the *CesA3* gene are G1105S and G1105V. The aim of this work was to evaluate the activity of CAAs on *P. viticola* populations coming from Italian commercial vineyards as well as on CAA sensitive and CAA resistant isolates. In leaf disc assays, CAAs showed different levels of activity and in particular dimethomorph showed lower EC_{95} levels. Detached leaf tests with protective application showed that the CAA-sensitive strains were fully controlled by all CAAs, while the CAA-resistant strains were best controlled by dimethomorph. With curative application all CAAs showed good activity on the sensitive strains, while the resistant strains were not controlled by any CAA. The good activity of dimethomorph on CAA resistant isolates under preventive conditions was also confirmed in grape plants. All CAA resistant strains carried the G1105S/V mutations, which were detected by sequencing and CAPS-PCR. In order to gain a better understanding of the different behaviour among the CAA fungicides, molecular modelling and docking studies are ongoing.

INTRODUCTION

Downy mildew caused by *Plasmopara viticola* may be controlled by utilizing fungicides with different modes of action including the carboxylic acid amides (CAAs). Dimethomorph was the first CAA introduced in 1988, followed by iprovalicarb, flumorph, benthiavalicarb, mandipropamid, valifenalate and latest pyrimorph in 2010 (Gisi *et al*. 2012). The mode of action of CAA fungicides was previously associated with an inhibition of phospholipid biosynthesis, but it has now been confirmed, in studied on mandipropamid, as interference

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with cell wall deposition and cellulose biosynthesis (Blum *et al.*2010b) is linked to the inhibition of cellulose synthesis in the oomycete plant pathogens. Resistance to CAA is based on a single point mutation in the *CesA3* gene, leading to a change of amino acid position 1105 from a conserved glycine to either serine or valine G1105S, G1105V (Blum *et al.* 2010a ; Sierotzki *et al.* 2011). The aim of this work was to evaluate the activity of CAAs on populations of *P. viticola* coming from Italian vineyards and also on CAA sensitive and CAA resistant *P. viticola* isolates. Bioassays on leaf discs, on detached leaves and on grape plants were carried out. Molecular techniques have been applied in order to detect G1105S/V mutations in resistant strains.

MATERIAL AND METHODS

Samples of *P. viticola*

Leaf samples showing downy mildew symptoms collected from 42 commercial vineyards in Northern Italy in 2014 were tested in a leaf disc test. Detached leaf and grape plant tests were conducted with a CAA sensitive strain isolated in Germany in 1999 and a resistant strain (with 100% G1105S mutation) isolated in France in 2002 (Nanni *et al.* 2016b).

Leaf disc test

Assays were carried out utilizing grape (cv. Chardonnay) leaf discs applying 7 concentrations (0, 1, 3, 10, 30, 100, 300 mg/l a.i.) of mandipropamid, MPA (Pergado® SC) and dimethomorph, DMM (Forum® 50 WP) 1 day before inoculation. For each concentration tested, 15 leaf discs were soaked in the fungicide suspensions for fourty-five minutes. The inoculation was conducted by spraying a sporangial suspension ($5x10^4$ spores ml^{-1}) onto the adaxial surface of leaf discs which were then incubated at 23 °C and a photoperiod of 12 hours. The sporulation was assessed 8-10 days after the inoculation by evaluating the percentage sporulating leaf area and the EC_{95} values (mg/l) were calculated by probit analysis.

Detached leaf test

Leaves from 10-week-old greenhouse plants were cut off and placed in Petri dishes with water agar (0.4 % agar + 40 mg/l benzimidazole + 30 mg/l streptomycin) and sprayed with benthiavalicarb (BTN), dimethomorph, iprovalicarb (IPV) and mandipropamid (all tech a.i., from Sigma Aldrich, St. Louis, MO, USA) solved in a standard formulation containing 5% acetone and 0.05% Wettol LF700. Rates of a.i. were chosen based on their use in combination products (Anonymous, 2015), where the maximum registered field rates of the solo active ingredients in combination products are 225 g/ha a.i. dimethomorph, 150 g/ha a.i. mandipropamid, 220 g/ha a.i. iprovalicarb and 35 g/ha a.i. benthiavalicarb, respectively.

Leaves were sprayed to just before run off, which was then calculated with 1000 l/ha, resulting in concentrations of 225, 150, 220 and 35 mg/l, respectively, additionally, half rates were used. For preventive trials, application was 1 day before inoculation, for curative trials 1 day

after inoculation. Inoculation was done with a suspension of $2x10^5$ sporangia ml^{-1}. Four inoculated leaves were used as replicates for each strain (CAA sensitive and resistant). Petri dishes were incubated for 18 to 20 h in darkness in a moist chamber at 18 °C, the Petri dish lids were then removed and the surfaces of the leaves dried in the horizontal laminar flow cabinet. The lids were then replaced and the Petri dishes further incubated at 20 °C with 12 h light/darkness. Seven days after the inoculation the percentage infected area of each leaf was assessed.Efficacy was calculated based on the four replicates (leaves): ([% diseased leaf area untreated - % diseased leaf area treated]/% diseased leaf area untreated) x 100%.

Grape plant test

A greenhouse test with intact grapes plants was performed with 10-week-old cuttings (var. Riesling) with 5 to 6 developed leaves. Plants were treated with DMM and MPA at their full and half of their full registered rates in Germany as previously described, resulting in concentrations of 225 and 112.5 ppm for DMM and 125 and 62.5 ppm for MPA, respectively. As a control treatment with a fungicide not affected by the G1105S mutation, the full and half of the registered rate of metiram (MET) (700 g /kg Polyram® WG) was applied (1600 and 800 ppm). Plants were sprayed just up to run off in a spray chamber. One day after application the plants were inoculated with $2x10^5$ spores ml^{-1} of two CAA-sensitive strains and two CAA-resistant strains, respectively.

Thereafter, the plants were incubated at 90% relative humidity (RH) and 21 °C for one day followed by 4 days at 65% RH and 21 °C and then for additional one day at 90% RH and 21°C. Five plants with each 3 leaves per treatment and strain were used. The diseased leaf area with sporulation was evaluated separately for each leaf resulting in 15 values per strain and treatment.

Mean values were calculated and efficacy was assessed: ([% diseased leaf area untreated - % diseased leaf area treated]/% diseased leaf area untreated) x 100%.

Molecular Analyses

Total genomic DNA extraction from the samples was performed from the same sporangial material (5×10^4 spores ml^{-1}) used for bioassays on leaf disc using the cetyltrimethylammonium bromide (CTAB) method following the protocol of Doyle and Doyle (1987) with some modifications. A *Pv*CesA3 gene fragment including the region coding the G1105S mutation was amplified by using the forward primer *Pv*CesA3F (Blum *et al.* 2010) and a newly designed reverse primer *Pv*CesA3R (Nanni *et al.* 2016a). PCR amplifications were performed, and then the PCR products were digested by 0.25 U of *Pvu*II restriction enzyme (Promega, Madison, WI, USA), which recognises its target site only when the mutation causing G1105S substitution is present, similarly to that used by Aoki *et al.* (2011). The majoriy of the samples were also subjected to Sanger sequencing.

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RESULTS AND DISCUSSION

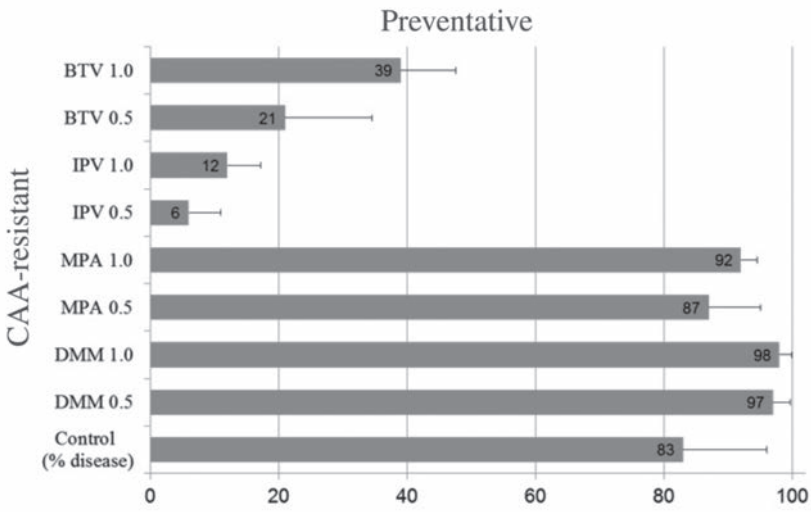

Figure 1a Efficacy of half and full rate of DMM, MPA, IPV, and BTV on a CAA-resistant isolate when applied preventively

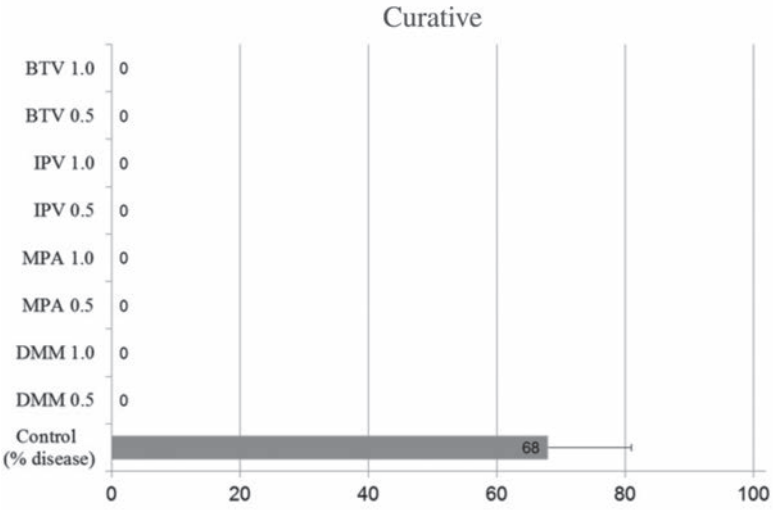

Figure 1b Efficacy of half and full rate of DMM, MPA, IPV, and BTV on a CAA-resistant isolate when applied curatively

Activity of CAAs was evaluated with different methodologies: *in vivo* with a leaf disc assay, detached leaves test in petri dishes and an efficacy test with grape plants; molecular analyses have been also applied in order to evaluate the mutation affecting resistant samples. The CAA compounds tested on 42 samples coming from Italian commercial vineyards on leaf discs showed different levels of efficacy, DMM had lower EC_{95} values when compared to MPA.

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The detached leaf test demonstrated that with protective application the CAA- sensitive strains were fully controlled by CAAs at both rates. The resistant strain was controlled to different degrees by the CAAs under these preventive conditions (Figure 1a), but it was not controlled by any CAAs when applied curatively (Figure 1b). DMM showed the highest acitivy of all tested CAAs followed by MPA, BTN and IPV.

In order to simulate a scenario which is closer to the field grape plant test were performed and inoculated with sensitive and resistant strains, respectively. Mean infection levels of two isolates in the untreated control were about 68% for the CAA-sensitive and and 42% for the CAA-resistant strains. The CAA-sensitive strains were fully controlled by half (0.5) and full field rate (1.0) of all products. Under these conditions the CAA-resistant strain was fully controlled by half and full rate of MET and full rate of DMM, while efficacy of MPA was lower (Figure 2).

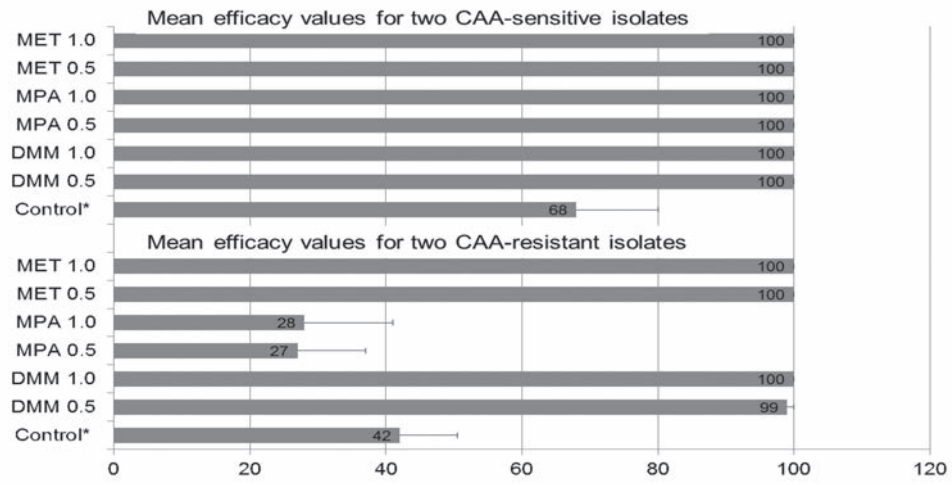

Figure 2 Efficacy of half (0.5) and full (1.0) rates of (DMM), (MPA) and (MET) on two CAA-sensitive and two CAA-resistant strains after 1 day preventative application

All different approaches indicated that there was a significant residual activity, mainly of dimethomorph on CAA-resistant strains. Depending on the test system, there was also activity of MPA and BTN, but lower than DMM. In all tests IPV provided lowest control of CAA-resistant isolates.

PCR-RFLP technique was able to detect the mutation at position 1105 of *CesA3* gene (glycine to serine) in population which have had high EC_{95} values. Sanger sequencing allowed us to detect also the presence of another amino acid change (glycine to valine) in two out of fourty-two samples coming from Italian vineyards. As stated by Sierotzki *et al.,* (2011) there are two different resistance alleles of the *CesA3* gene G1105S and G1105V and each allele can lead to a resistant phenotype but the predominant one is the amino acid change glycine to serine.

Molecular methods are necessary to confirm *in vivo* data in order to minimize the risk of the development of resistance. The effect of this mutation on the different CAAs is different and this may be based on the different structures of the molecules.

Docking studies are still ongoing in order to explain the different behavior on the binding site of CAAs at the CesA3 protein.

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