



DIFFERENTIAL CHANGE PATTERNS FOR FUNGICIDE-EXPOSED CARBOXYLESTERASE AND ACETYLESTERASES IN VINE CLONES OF *VITIS VINIFERA* L. (VITACEAE)

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Abstract: Current analysis shows the effect of different fungicides, usually applied in pest control, on four economically important table-wine grapes cultivars (Italia, Rubi, Benitaka and Brasil) of *Vitis vinifera* on α - and β -esterase patterns. The α - and β -esterase patterns in the bud leaf of vine cultivars in native PAGE analysis have shown that the Cabrio Top[®] compound inhibited EST-2, EST-5, EST-6, EST-7, EST-8, EST-9 and EST-10 carboxylesterases, and EST-4, EST-11, EST-12, EST-13, EST-14 acetylerases and EST-16 carboxylesterase have been detected as weakly stained bands. Carboxylesterases and acetylerases were also detected as weakly stained bands when exposed to Orthocide 500[®], Positron Duo[®], and Folicur PM[®] fungicides. No change for α - and β -esterase patterns were observed when the esterases were exposed to Rovral SC[®], Kumulus DF[®], Curzate M[®], Score[®] and Cuprocarb 500[®] fungicides. Evidences of functional changes for carboxylesterases and acetylerases in the present study have been important to warn grape producers with regard to the danger in the indiscriminate use of potent and modern fungicides extensively used in agriculture.

Key words: esterases; fungicides; grape-vines; isozymes; PAGE system.

1 INTRODUCTION

Esterases are often found in multigene families and sixteen esterase isozymes have been detected in *Vitis vinifera* cultivars (Italia, Rubi, Benitaka and Brasil) (Orasmo et al., 2007). Specific-inhibitor tests for biochemical and functional classification of esterases by PAGE distinguished carboxylesterases (EST-2, EST-3, EST-5, EST-6, EST-7, EST-8, EST-9, EST-10, and EST-16 isozymes) and acetylerases (EST-4, EST-11, EST-12, EST-13, EST-14, EST-15 isozymes) in grapes. Inhibition tests showed that organophosphate compounds (OPCs) and neo-nicotinoid (thiamethoxan) insecticides inhibited vine esterases. Thiamethoxan also acted as an EST-4 and EST-6 inhibitor and induced the appearance of EST-5 and EST-7 isozymes as more intensely stained bands in *Aspidosperma polyneuron* leaves (Carvalho et al., 2003).

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Fungicides are the usual pesticides employed for the control of different pest in grapes and there has recently been a tendency by producers to use synthetic fungicide. Some studies showed that fungicide application significantly affect the *V. vinifera* plant physiology.

The current study verifies the effect of different fungicides usually applied to pest control on esterase pattern in four table-wine grape cultivars of the *Vitis vinifera* species. Economically important cultivars Italia, Rubi, Benitaka and Brasil are traditionally grown in the rural region of Marialva, a town in the north-western region of Paraná, southern Brazil. Exposure to different fungicides may either inhibit or activate the *V. vinifera* esterases action (enzyme-substrate binding).

2 MATERIALS AND METHODS

Young leaves collected from eight stakes of lab-maintained cultivars Italia, Rubi, Benitaka and Brasil, and thus not previously exposed to pesticides, were used for esterase extraction. One young leaf was collected from each stake. Young leaves of each stake were individually homogenized with a glass rod in an Eppendorf microcentrifuge tube, using 40 μ L 0.1 M Tris-HCl buffer, pH 8.5, containing 6% PVP-40, 0.1% ascorbic acid, 0.2% EDTA and 0.5% β -mercaptoethanol. After homogenization, samples were centrifuged at 25,000 rpm, for 30 min, at 4°C, in a Sorval 3K-30 centrifuge; the supernatant (20 μ L) was used for each sample.

Polyacrylamide gels (14%) were prepared with 7.23 mL acrylamide (30%) and bisacrylamide (0.8%), dissolved in 2.66 mL 1.5 M Tris-HCl, pH 7.5, 6.01 mL twice-distilled water, 320 μ L ammonium persulfate (2%), and 16 μ L TEMED. The stack gel was prepared with 3.0 mL acrylamide (10%) and bis-acrylamide (0.5%) dissolved in 3.0 mL 1.5 M Tris-HCl, pH 6.8, 30 μ L twice-distilled water, 250 μ L ammonium persulfate (2%), and 3 μ L TEMED (Orasmo et al., 2007). Electrophoresis was carried out during 10-13 h, at 10 °C, with a constant 200 V. The running buffer was 0.1 M Tris-glycine, pH 8.3.

Fungicide tests for the verification of fungicide effects on esterase pattern in grape clones of *V. vinifera* involved Orthocide 500[®], Positron Duo[®], Cabrio Top[®], Rovral SC[®], Kumulus DF[®], Curzate M[®], Score[®], Folicur PM[®] and Cuprogarb 500[®], whose active principles and concentrations are described at Table 1.

Table 01. Fungicides their active principles and concentrations used to inhibition tests of esterases in cultivars of the *Vitis vinifera*.

Fungicides	Manufacturer	Active principles	Concentration
Orthocide 500 [®]	Du Pont do Brasil S.A.	Captan	0,24%
Kumulus DF [®]		Enxofre	0,4%
Curzate M [®]	Du Pont do Brasil S.A.	cymoxamil + maneb	0,25%
Positron Duo [®]	Bayer CropScience Ltda.	iprovalicarbe + propinebe	0,25%
Cabrio Top [®]	Basf S.A.	metiran + piraclostrobina	0,25%
Score [®]	Syngenta Proteção de Cultivos Ltda.	difenoconazol	0,02%
Folicur PM [®]	Bayer CropScience Ltda.	tebuconazol	0,1%
Rovral SC [®]	Bayer CropScience Ltda.	iproditiona	0,13%
Cuprogarb 500 [®]	Oxiquímica Agrociência Ltda.	oxicloreto de cobre	0,27%

Fungicide solutions were prepared individually in 100 mL of twice-distilled water using recommended concentrations in culture practices. After electrophoresis, separate gels were pre-incubated and stained with each one of the fungicide compounds; fungicide

compound was also added in staining solutions. Staining techniques used for esterase identification were based on protocol described by Orasmo et al, 2007. Gels were soaked for 30 min in 50 mL 0.1 M sodium phosphate, pH 6.2, and 10 mL of fungicide solution, at room temperature. Esterase activity was visualized by placing the gels for 1 h in a staining solution containing 50 mL sodium phosphate solution, 30 mg α -naphthyl acetate, 30 mg β -naphthyl acetate, 60 mg Fast Blue RR salt, 5 mL n-propanol, and 10 mL of fungicide solution. Each leaf bud extract was repeated four times on the same electrophoresis gel, which, after electrophoresis, was vertically divided into four parts for control and for fungicide test.

Polyacrylamide gels were dried at room temperature for 1 h in a mixture of 7.5% acetic acid and 10% glycerol, embedded in 5% gelatin, placed between two sheets of wet cellophane paper stretched on an embroidering hoop, and left to dry for 24-48 h.

3 RESULTS AND DISCUSSION

The α - and β -esterase patterns in bud leaves of grape cultivars in native PAGE analysis showed that Cabrio Top[®] compound inhibited EST-2, EST-5, EST-6, EST-7, EST-8, EST-9 and EST-10 carboxylesterases, whereas EST-4, EST-11, EST-12, EST-13, EST-14 acetylerases and EST-16 carboxylesterase were detected as weakly stained bands (Figure 1).

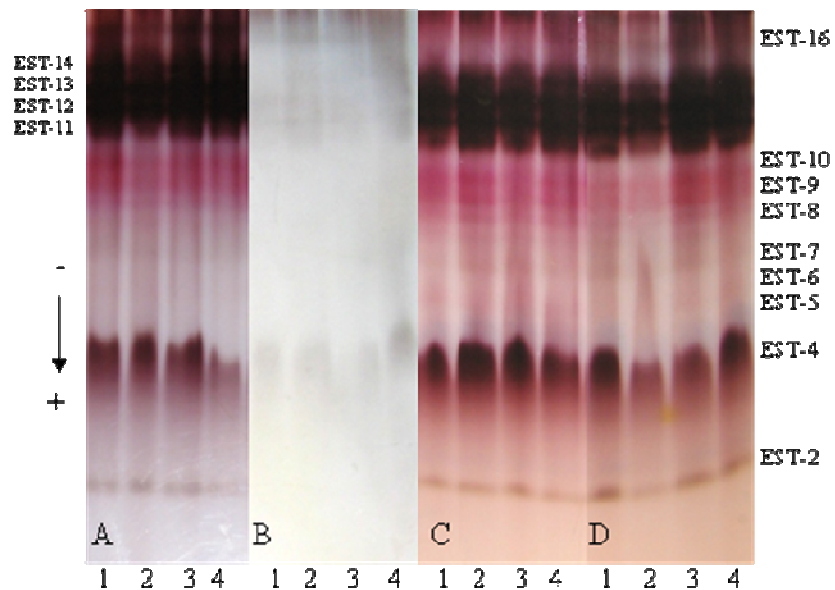


Figure 1. Carboxylesterases and acetylerase in the absence of fungicide (A) and inhibition pattern of carboxylesterases and acetylerases in bud leaf of *Vitis vinifera* cultivars showing EST-2, EST-5, EST-6, EST-7, EST-8, EST-9, EST-10 carboxylesterases were inhibited in the presence of Cabrio Top[®] compound, while EST-2, EST-4, EST-11, EST-12, EST-13, EST-14 acetylerases and EST-16 carboxylesterase were detected as weakly stained bands (B). Carboxylesterases and acetylerases were detected when exposed to Cuprocarb 500[®] (C), and Score[®] (D) fungicides. Lanes 1-4 correspond to bud leaf samples in different vines of the Rubi cultivar.

Carboxylesterases and acetylerases were also detected as weakly stained bands when exposed to Orthocide 500[®], Positron Duo[®] and Folicur PM[®] fungicides (Figure 2).

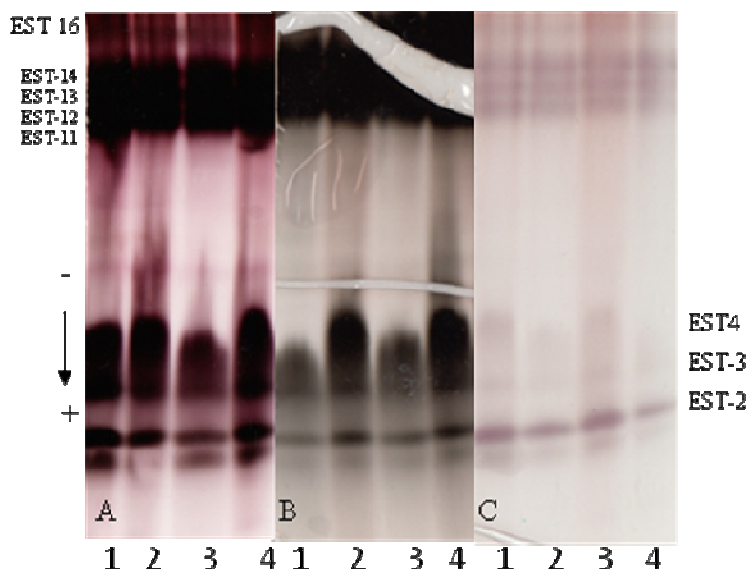


Figure 2. Carboxylesterases and acetylase in the absence of fungicide (A), carboxylesterases and acetylase detected when exposed to Rovral[®] (B), and carboxylesterases and acetylase as more weakly stained bands when exposed to Follicur PM[®] (C). Lanes 1-4 correspond to bud leaf samples in different vines of *Vitis vinifera*.

In current study EST-3 carboxylesterase was not analyzed since high polymorphism for null Est-3 isozyme phenotype (61.7%) has been detected in four *V. vinifera* cultivars (Orasmo, et al., 2007). On the other hand, no change for α - and β -esterase patterns was observed when the bud leaves esterases of grape cultivars were exposed to Rovral SC[®], Kumulus DF[®], Curzate M[®], Score[®], and Cuprocarb 500[®] fungicides. Inhibition effect of fungicides on EST isozyme molecules does not seem to be dependent on the fungicide-chemical group, since Follicur PM[®] and Score[®] compounds belong to the same triazol group. Whereas Follicur PM[®] in solution determined carboxylesterases and acetylase as more weakly stained bands (Figure 2), no change for EST isozymes patterns has been reported when exposed to Score[®] (Figure 1).

The no change for esterases patterns observed were exposed to Kumulus DF[®] (sulphur chemical groups) and Cuprocarb 500[®] (copper chemical groups) fungicides is relevant. Copper-based and sulphur-based fungicides are used in organic production of grapevine in suppressing grapevine diseases. Our study showed that copper-based and sulphur-based fungicides no changed the α - and β -esterase patterns from cultivars of *V. vinifera*.

Changes in esterase isozyme function were first registered as a decrease in carboxylesterase activity of organophosphate-resistant strains. This factor has led to the idea that resistant insects have a reduced ability to hydrolyze carboxylesterase substrates but gained the ability to hydrolyze organophosphate substrates. It may thus be possible that EST isozyme have also the ability to hydrolyze fungicides.

Current study shows that inhibition of EST isozyme molecules for fungicide hydrolysis, is an additional evidence that certain fungicides may affect the plant physiology of *V. vinifera*. Our results have shown that, EST isozymes may be considered as biosensors on methods for checking applications of certain specific fungicides.

Fungicide-induced effects may alter the berry development, the grape growth and the yield. As fungicides are frequently employed for the control of pests continuous and subsequent fungicide applications may lead towards the evolution of resistance processes in vines and/or fungi.

Esterases enzymes may interact directly with pesticides and other pollutants, influence biochemical and molecular polymorphism. High polymorphism for EST-3 α -carboxylesterase (61.7%) (Orasmo et al., 2007) and RAPD markers (65%) (Zequim-Maia et al., 2008) has been detected in four *V. vinifera* cultivars. EST-4 α/β -acetylerase was also absent in one Rubi vine. Somatic mutations have also been used to explain the deep berry skin color polymorphism in the four grape cultivars of *V. vinifera* (Oliveira-Collet et al., 2005).

4 CONCLUSION

Evidences of functional changes (enzyme-substrate binding) for carboxylesterases and acetylerases in the present study have been important to warn vine producers on the danger in the indiscriminate and extensive use (or tissue-plant exposition) of potent and modern fungicides in agriculture. If any esterases are altered to cope with the fungicide's metabolism, other related or non-related enzymes may also be altered to take over their normal metabolic role.

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