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Available in: http://www.redalyc.org/articulo.oa?id=187143301009
Esterase isozymes patterns of grape vine (Vitis vinifera L.) are altered in response to fungicide exposure

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ABSTRACT. Current analysis characterizes the effect of different fungicides often applied for pest control on α– and β-esterase patterns of four economically important table-wine grape cultivars (Italia, Rubi, Benitaka and Brasil) of Vitis vinifera. The α- and β-esterase patterns in bud leaves of the cultivars were assessed by native PAGE analysis. 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 acetyesterases and EST-16 carboxylesterase were detected as weakly stained bands. Carboxylesterases and acetyesterases were also detected as weakly stained bands when exposed to fungicides Orthocide 500®, Positron Duo® and Folicur PM®. No changes in α- and β-esterase patterns were reported when the vines were exposed to the fungicides Rovral SC®, Kumulus DF®, Curzate M®, Score® or Cuprogarb 500®. The evidence of functional changes in carboxylesterase and acetyesterase levels in current study is a warning to grape producers on the dangers inherent in the indiscriminate use of potent and modern fungicides extensively used in agriculture. The inhibition effect of fungicides on esterase isozyme molecules seems to be independent of the fungicide chemical.

Keywords: esterases, isozymes, fungicides, vine clones, differential patterns.

Padrões de isozimas Esterases de videira (Vitis vinifera L.) alterados em resposta à exposição a fungicidas

RESUMO. O presente estudo caracterizou o efeito de diferentes fungicidas comumente aplicados como medidas de controle de pragas sobre padrões de α- e β-esterases de quatro importantes cultivares de uva de mesa (Itália, Rubi, Benitaka e Brasil) de Vitis vinifera. Os padrões de α- e β-esterases de brotos foliares das cultivares foram avaliados por PAGE. O composto Cabrio Top® inibiu as carboxilesterases EST-2, EST-5, EST-6, EST-7, EST-8, EST-9 e EST-10, enquanto as acetilesterases EST-4, EST-11, EST-12, EST-13, EST-14 e a carboxilesterase EST-16 foram detectadas como bandas fracamente coradas. As carboxilesterases e acetilesterases também foram detectadas como bandas fracamente coradas quando expostas aos fungicidas Orthocide 500®, Positron Duo® e Folicur PM®. Não foram observadas alterações nos padrões de α- e β-esterases quando as videiras foram expostas aos fungicidas Rovral SC®, Kumulus DF®, Curzate M®, Score® ou Cuprogarb 500®. A evidência de alterações em nível funcional em carboxilesterases e acetilesterases, apresentada neste estudo, pode servir como um alerta aos produtores de uva dos perigos inerentes ao uso indiscriminado de fungicidas potentes e modernos amplamente utilizados hoje na agricultura. O efeito dos fungicidas sobre as enzimas esterases parece ser independente do grupo químico ao qual pertence o fungicida.

Palavras-chave: esterases, isozimas, fungicidas, clones de videira, padrões diferenciais.

Introduction

Esterases are often found in multigene families (OAKESHOTT et al., 1993; ROBIN et al., 1996). Currently 14-16 esterase isozymes have been detected by polyacrylamide gel electrophoresis (PAGE) in different plant species (PEREIRA et al., 2001; CARVALHO et al., 2003; ORASMO et al., 2007). Sixteen esterase isozymes have been detected in Vitis vinifera cultivars (Italia, Rubi, Benitaka and Brasil), and the biochemical characterization of grape esterases using ester substrates has revealed the existence of α-, β- and α/β-esterases (ORASMO et al., 2007).

Specific inhibitor tests for the biochemical and functional classification of esterases by PAGE have distinguished carboxylesterases (Est-2, Est-3, Est-5, Est-6, Est-7, Est-8, Est-9, Est-10, and Est-16 isozymes) and acetyesterases (Est-4, Est-11, Est-12, Est-13, Est-14, Est-15 isozymes) in grapes (ORASMO et al., 2007). Inhibition tests showed that organophosphate...
compounds (OPCs) and neo-nicotinoid thiamethoxam insecticides inhibit vine esterases (ORASMO et al., 2007). Thiamethoxam also acts as an Est-4 and Est-6 inhibitor and induces the appearance of Est-5 and Est-7, revealed as more intensely stained bands, in Aspisperma polynemon leaves (CARVALHO et al., 2003).

The different esterase activity patterns displayed by vine leaf buds after in vitro incubation with insecticide compounds shows the importance of further investigation on the effects of fungicide compounds on esterase activity. Fungicides are standard pesticides employed for the control of different pests in grapes, and the trend to use synthetic fungicides has recently become more common (M. Collet, personal observations). Moreover, necessary precautions are not always followed during fungicide spray application. Some studies have shown that fungicide application significantly affects the plant physiology of V. vinifera. Fungicides fludioxonil and pyrimethanil stimulate protein accumulation and alter leaf water contents and carbohydrate levels (SALADIN et al., 2003a). Similarly, chlorpyrifos residues and endosulfan sulfate have been registered at higher levels in apple and orange samples, respectively (LATIF et al., 2011).

Enzyme-specific activity in other plant species and in vitro experiments has been shown to either increase or decrease as a response to fungicide treatment. Systemic fungicide (benlate and calixin) application caused a significant decrease in total protein and carbohydrate content of resistant and susceptible varieties of Triticum aestivum (SIDDQUI; AHMED, 2002). The non-systemic fungicide captan inhibited glutathione-S-transferase activity (CHOI et al., 2003). The non-systemic fungicide thiram and captafol affected the activity of cytochrome P450 isoenzymes (RAHDEN-STARON et al., 2001). Enzyme activity in other plant species and in vitro experiments has been shown to either increase or decrease as a response to fungicide treatment. Systemic fungicide (benlate and calixin) application caused a significant decrease in total protein and carbohydrate content of resistant and susceptible varieties of Triticum aestivum (SIDDQUI; AHMED, 2002). The non-systemic fungicide captan inhibited glutathione-S-transferase activity (CHOI et al., 2003). The non-systemic fungicide thiram and captafol affected the activity of cytochrome P450 isoenzymes (RAHDEN-STARON et al., 2001).

The fungicide solutions were prepared individually in 100 mL of twice-distilled water with concentrations recommended in culture practice.

Material and methods

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

Polyacrylamide gels (14%) were prepared with 7.23 mL acrylamide (30%) and bis-acrylamide (0.8%), dissolved in 2.66 mL of 1.5 M Tris-HCl, pH 7.5, 6.01 mL twice-distilled water, 320 μL ammonium persulfate (2%), and 16 μL TEMED. The stacking 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 μL twice-distilled water, 250 μL ammonium persulfate (2%), and 3 μL TEMED. Electrophoresis was carried out for 10-13 h at 4°C at a constant voltage of 200 V. The running buffer used was 0.1 M Tris-glycine, pH 8.3 (ORASMO et al., 2007).

The following fungicides were used to test the effects of fungicides on V. vinifera esterase patterns: Orthocide 500®, Positrion Duo®, Cabrio Top®, Rovral SC®, Kumulus DF®, Curzate M®, Score®, Folicur PM® and Cuprogarb 500®. Their active ingredients and concentrations are described in Table 1.
After electrophoresis, separate gels were preincubated and stained with each compound of the fungicide; the fungicide compound was also added to the 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 in a staining solution containing 50 mL sodium phosphate solution, 30 mg β-naphthyl acetate, 30 mg α-naphthyl acetate, 60 mg Fast Blue RR salt, 5 mL n-propanol, and 10 mL of fungicide solution for 1h. Each leaf bud extract was included four times in the same gel, which was vertically divided into four parts after electrophoresis, for control and fungicide tests.

The polyacrylamide gels were dried at room temperature for 1h 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 24-48h.

Results and discussion

The α- and β-esterase patterns in the bud leaves of grape cultivars analyzed by native PAGE showed that Cabrio Top® compound inhibited the carboxylesterases Est-2, Est-5, Est-6, Est-7, Est-8, Est-9 and Est-10, whereas the acetylesterases Est-4, Est-11, Est-12, Est-13 and Est-14, the carboxylesterase Est-16 were detected as weakly stained bands (Figure 1). Carboxylesterases and acetylesterases were also detected when exposed to Orthocide 500®, Positron Duo® and Folicur PM® fungicides (Figure 2). Est-3 carboxylesterase was not analyzed in this study, as a high level of polymorphism (61.7%) for a null Est-3 isozyme phenotype has been detected in four V. vinifera cultivars (ORASMO et al., 2007).

Contrastingly, no changes in α- and β-esterase patterns were reported when the grape cultivar esterases were exposed to Rovral SC®, Kumulus DF®, Curzate M®, Score® and Cuprogarb 500® fungicides. The inhibition effect of fungicides on EST isozyme molecules seems to be independent of the fungicide chemical group, as the active ingredients of Folicur PM® and Score® compounds belong to the same triazole group.
to appear as more weakly stained bands (Figure 2), no change was observed in EST isozyme patterns in cultivars exposed to Score® (Figure 1).

Particularly relevant was the lack of change in α- and β-esterase patterns observed when the grape cultivar esterases were exposed to Kumulus DF® and Cuprogarb 500® fungicides. Sulfur and copper are the active ingredients of Kumulus DF® and Cuprogarb 500® fungicides, respectively (Table 1), and copper- and sulfur-based fungicides are used in organic grape production to suppress grapevine diseases. Copper has been used as an irreplaceable agent in the suppression of downy mildew of grapevine (GALET, 2002), and sulfur is the primary agent for powdery mildew suppression in organic vineyards. The application of sulfur in organic grape production is the same as in conventional production, but it is applied more carefully to prevent the development of resistance in parasites, which may lead to difficulties in their suppression.

Světlev et al. (2010) showed that research into the protection of grapevines from disease-causing agents has led to a reduction in the amount of copper and sulfur employed. In fact, they are lately being replaced by new products. Studies have shown that copper exposure induces oxidative stress in rice seedlings copper toxicity (MOSTOFIA; FUJITA, 2013). However, current study shows that copper- and sulfur-based fungicides failed to change the α- and β-esterase patterns in the bud leaves of Italia, Rubi, Benitaka, and Brasil cultivars of V. vinifera. These results may have significant implications for organic grape production.

The faint staining intensity of carboxylesterases and/or acetyl esterases observed in PAGE gels is consistent with a lower number of EST isozyme molecules available to associate with α– and β-naphthyl substrates added in the staining solutions. However, the absence of EST isozyme bands in a gel may signify a complete lack of EST isozyme molecules available to associate with substrates. The complete absence or the lower levels of EST isozyme available to react may be caused by EST isozyme inhibition after exposure to Cabrio Top®, Orthocide 500®, Positron Duo® or Folicur PM® fungicides. Wheelock et al. (2008) report that carboxylesterases play a significant role in the metabolism and subsequent detoxification of many agrochemicals. Several different types of carboxylesterase inhibitors have been reported in the literature.

Alternatively, the absence or the lower levels of EST isozymes available may be caused by the ability of carboxylesterase and acetyl esterase to hydrolyze the fungicide compounds (Cabrio Top®, Orthocide 500®, Positron Duo® and Folicur PM®) as substrates instead of their typical α– and β-naphthyl substrates. Indeed, typically esterases are assayed by monitoring their activities with substrates such as nitrophenyl and naphthyl acetates. However, the metabolism of the substrates does not predict the ability of esterases to catalyze the hydrolysis of other specific substrates (HASLAM et al., 2001). Ileperuma et al. (2007) crystallized a carboxylesterase from a kiwifruit species (Actinidia eriantha) and showed that it was significantly inhibited by the insecticide paraoxon, demonstrating that, in plants, carboxylesterase had a similar inhibitor-binding mechanism as mammalian orthologues. Thus, it may be possible that EST isozyme molecules from vine bud leaves also possess the ability to hydrolyze fungicides. Biochemical studies have shown that these enzymes may hydrolyze a wide range of esters that are potentially involved in detoxification processes (ILEPERUMA et al., 2007; MARSHALL et al., 2003).

As demonstrated in current study, the inhibition of EST isozyme molecules for fungicide hydrolysis, alone or in combination, provides additional evidence that certain fungicides may affect the physiology of V. vinifera. Our results are consistent with previous reports by Saladin et al. (2003 a, b) who reported that fungicides exert significant effects on the physiology of V. vinifera, both in vitro and ex vitro, on soil-growth. Similarly, populations of wild potato displayed a significantly reduced time of flowering after exposure to the pesticide Furadant® (DEL RIO et al., 2012).

According to Wheelock et al. (2008), the relation of carboxylesterase activity to agrochemical exposure has been examined in a wide range of species and may therefore be useful for ecosystem-wide environmental monitoring projects. The inhibition of carboxylesterases has been employed as a biosensor for the detection of selenium compounds in Thevetia peruviana seeds (SARITHA; NANDA KUMAR, 2001). In addition, the fungicides metiram and pyraclostrobin (the active principle of Cabrio Top®) were selected to test a new method for the detection of pesticide contact residues on fruit surfaces (ACHARYA et al., 2012).

Carboxylesterase activity has also been investigated for applications in the selective bioactivation of herbicides in crops and weeds (GERSHATER et al., 2006). Proteins from a range of important economically important crops and weeds were assayed for carboxylesterase activity. The crops included maize, rice, sorghum, soybean,
flax and lucerne, and the model plant Arabidopsis thaliana. Significant hydrolysis of the majority of herbicides was observed. Gershater and Edwards (2007) revised the ability of carboxylesterases to control the bioactivity and transport of herbicides, plant signaling agents and secondary metabolites in plants, they covered the roles of carboxylesterases in regulating herbicide bioactivation.

The physiological significance of carboxylesterase and acetyesterase change patterns cannot be fully determined in current study. The physiological role and specific substrates of carboxylesterase and acetyesterase isozymes in vivo is unknown in vines. Carboxylesterases are widely distributed in plants, where they have been implicated in roles that include plant defense, plant development, secondary metabolism, including the processing or degradation of neurotransmitters, hormones and xenobiotics (LePERUMA et al., 2007; HEMINGWAY, 2000). According to Incledon and Hall (1997 apud HASLAM et al., 2001), carboxylesterases are involved in the metabolism of herbicides and several environmental toxicants in plants.

Stuhlfelder et al. (2002) proposed that carboxylesterases might play a role in plant signaling pathways. Other possible substrates for plant carboxylesterases include esters produced by plants to attract pollinators and to deter herbivores (PICHERSKY; GERSHENZON, 2002). Marshall et al. (2003) reviewed the possible role of these enzymes in plant-pathogen interactions and in the suppression of the programmed cell death associated with the hypersensitive response during pathogen attack. Est-7 isozyme in young unexposed leaves of cassava plants was used as a marker of pathogenesis after infection with X. axonopodis pv. Manihotis (PEREIRA et al., 2001).

Acetyesterases seem to be specific for the deacetylation of cell wall polysaccharides (Searle-Van LEEUWEN et al., 1992) by the removal of acetyl groups from different positions of acetylated glycoside. Proteins with acetyesterase activity have been purified from orange peel (WAIDMANN; HEUSER, 1994) and from the cell walls of bean hypocotyls (BORDENAVE et al., 1995). Differential acetyesterase activity during inflorescence development has also been reported in palmarosa (Cymbopogon martini) by Dubey et al. (2003).

The significant and differing physiological roles of carboxylesterase and acetyesterase in plants and the evidence of changes in EST isozyme molecules exposed to certain fungicides (Cabrio Top®, Orthocide 500®, Positron Duo® and Folicur PM®) indicate the importance of further studies on the effects of different types and concentrations of fungicide compounds during field application. Since fungicides are applied during flower or berry development, fungicide-induced effects may alter berry development, growth and yield. Fungicides are frequently employed for the control of different pests, alone or in combination (a mixture of different types), according to changes in the microclimate of regions and the severity of infestations (M. Collet, personal information). Continuous and subsequent fungicide applications may lead to the evolution of resistant properties in vines and/or fungi. A few aryesterases from insect pests displaying resistance against organophosphate insecticides such as paraaxon and chlorpyrifos-oxon have been reported (ZUH; HE, 2000 apud PARK et al., 2008).

Carboxylesterases in the leaf apoplast of wheat seedlings catalyze the hydrolysis of herbicide esters and are substrate specific. The authors hypothesized that esterases contributed to the bioavailability of herbicide in the plant (HASLAM et al., 2001). Further, since certain enzymes (e.g., esterases) may interact directly with pesticides and other pollutants (GERSHATER et al., 2006; GERSHATER; EDWARDS, 2007; WHEELOCK et al., 2008), it may be suggested that fungicides influence biochemical and molecular polymorphism in the four V. vinifera cultivars examined in current study. High polymorphism for the Est-3 α-carboxylesterase (61.7%; ORASMO et al., 2007) and RAPD markers (65%; ZEQUI-MAI, 2009) have been detected in four V. vinifera cultivars. Est-4 α/β-acetyesterase was also absent in one Rubi vine (ORASMO et al., 2007). If a mutation occurs in actively dividing tissue, mutant clones may arise. For instance, if a plant cutting is removed from a stem that includes a mutant somatic sector, the plant that grows from that cutting may also contain the mutant sector. 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).

Conclusion

Evidence of functional changes (enzyme-substrate binding) in carboxylesterases and acetyesterases in current study are important for cautioning vine producers on the dangers inherent in the indiscriminate and extensive use (or tissue-exposure) of potent and modern fungicides in agriculture. If any esterases are altered to cope with

the metabolism of fungicides, other related or unrelated enzymes could be altered to assume their normal metabolic role. The inhibition effect of fungicides on esterase isozyme molecules seems to be independent of the fungicide chemical.

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Received on March 27, 2014.
Accepted on August 19, 2015.

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