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Effect of sterol biosynthesis inhibitors and azole-type inducers on growth and development of *Plasmopara viticola* on grapevine

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Summary

The effect of various azole fungicides (demethylation inhibitors, DMIs), mainly triazoles, that interfere with sterol metabolism in higher fungi, triazole-type inducers and an inhibitor of plant brassinosteroid biosynthesis on growth and spore production of the downy mildew pathogen of grapevine (*Plasmopara viticola*) was tested in a leaf disc-based system. Application of DMIs and azole-type inducers two days before inoculation resulted in a delayed mycelial growth during the first three days pot inoculation, which was not observed at day 5 or 7 post inoculation, and in a weakly reduced sporulation of the pathogen. These effects were not observed when compounds were applied during or post inoculation. Thus, azole treatments do not interfere with infections and epidemics caused by the downy mildew pathogen as observed in the field. However, alteration of brassinosteroid metabolism of the plant induced by the inhibition of a 5α-reductase with finasteride resulted in a markedly reduced sporulation. This indicates that plant derived sterols or those processes modulated by sterols interfere with the reproduction of the pathogen.

K e y w o r d s : downy mildew; finasteride; fungicides; peronosporomycetes; *Vitis vinifera.*

Introduction

Downy mildew caused by *Plasmopara viticola* is one of the major diseases of grapevine worldwide. As other downy mildew pathogens, *P. viticola* belongs to the peronosporomycetes (formerly oomycetes) and represents an organism that is much more related to heterokont algae than to true fungi. Taxonomic separation of downy mildew agents from the fungi is due to several characteristic features that are specific for peronosporomycetes, such as multinuclear, unseptated hyphae, the presence of cellulose in the cell wall, the lack of chitin in most species, and mycolaminarine instead of glycogen as a carbon-based energy source (Webster and Weber, 2007). However, recent research on *P. viticola* revealed that this pathogen exhibits features much more related to true fungi than to other members of stramenopiles. For example, *P. viticola* is able to express at least two different chitin synthases, and chitin is present on the surface of sporangia, sporangiophores, and hyphal cell walls during *in planta* growth (Werner *et al*. 2002). Furthermore, septa are formed at least in stems and branches of sporangiophores (Kortekamp 2005), even though there are species-specific differences.

Developmental stages of *P. viticola* during infection and colonization of grapevine have been well studied cytologically, but little is known about the general biology and physiology at the molecular level concerning changes in gene expression during the life cycle of the pathogen and of the gene products required during these stages. Some gene products are probably similar to those present in saprophytic relatives, others are likely to be important for the rapid establishment of the parasitic relationship. Moreover, the molecular basis of compatibility and disease development in susceptible grapes is poorly understood, even though first results about putative effector genes expressed in zoospores using a candidate gene strategy are recently published (Mestre *et al*. 2012). To visualise the host-pathogen interaction, expression of genes was analysed at early time points post infection (Merz *et al*. 2014, Polesani *et al*. 2010), indicating an alteration of gene activity at one to 24 h post inoculation, and at oil spot stage (Polesani *et al*. 2008). Transcriptional analysis on artificially infected leaves followed by selective amplifications allowed the visualisation of grapevine transcripts belonging to different functional categories. Among them, several transcripts encode enzymes that are involved in lipid and sterol metabolism. Sterols are essential components of cell membranes and sterol-related compounds play a pivotal role in plant development and fruit ripening (Symons *et al*. 2006) but also in growth and propagation of fungi.

In contrast to plants and true fungi, peronosporomycetes seem not to be able to synthesise sterols, even though they require trace amounts for both sexual and asexual reproduction (Elliott 1983, Deacon 2006). Furthermore, sterols seem to be able to stimulate hyphal growth (Elliott 1977). However, since peronosporomycetes are quite different from true fungi regarding their distinct physiology, chemical protection of crops affected by downy mildews requires specific compounds that are effective against peronosporomycetes or, vice versa, fungicides such as azoles used to control other fungal diseases are not effective

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against downy mildews. Azoles, especially triazoles, are widely used to control a large set of pathogens. The main target of triazoles is the biosynthesis of sterols in membranes. The antifungal activity results in an inhibition of a C14-demethylisation step leading to an enrichment of 14-methyl-sterols (Kuck *et al.* 2012) and a reduced level of ergosterol, a major component in fungal membranes that can be used for the estimation of fungal biomass in must and grapes (Porep *et al.* 2014). An incorporation of 14-methyl-sterols into the membranes causes alterations regarding their physico-chemical properties and results in the death of hyphae and germinating spores. On the other side, azoles may also cause side effects such as retarded growth of treated plants due to a reduced synthesis of gibberellins (Buchenauer and Röhner 1981, Burden *et al*. 1989), a delayed senescence, and an increased tolerance to abiotic stress (Buchenauer 1995). Therefore, some azoles are used as fungicides and growth regulators, such as paclobutrazol and probenazole. Both compounds are able to enhance tolerance towards various stresses such as drought and flooding via the production of enzymes, oxygen species, and phenolic compounds and to activate the defence system of the plant (Iwata 2001, Lin 2006). Beside the triazoles, morpholines are also used as inhibitors of sterole biosynthesis in fungi. Among them, fenpropimorph is the most used member of this chemical class and applied as triazoles to inhibit infection of crop plants with pathogens such as rust fungi and powdery mildews.

Genome wide analysis revealed that several sterol biosynthetic enzymes are also encoded in the *Phytophthora sojae* and *Phytopthora ramorum* genome (Tyler *et al*. 2006) and seem to be affected by triazoles. Furthermore, analysis of gene expression in grapevine showed that expression of a 5α-reductase was altered when plants had been inoculated with *P. viticola* (Polesani *et al*. 2008). The 5α-reductase, also known as 3-oxo-5-alpha-steroid 4-dehydrogenase, is a membrane bound enzyme and catalyses the NADPH-dependant reduction of double bonds in a variety of human or animal steroid substrates (Wilson 1975). This enzyme is also involved in the biosynthesis of plant steroid hormones, the brassinosteroids (Li *et al*. 1996). Brassinosteroids are essential for normal plant development and are involved in numerous processes such as cell elongation, cell division, vascular differentiation, reproductive development, and pathogen and stress tolerance (Clouse and Sasse 1998, Clouse 2002). In grapevine, brassinosteroids seem to be important for the ripening of grape berries (Symons *et al*. 2006, Pilati *et al*. 2007) and expression of 5α-reductase is modified during berry growth (Fortes *et al*. 2011).

The role of 5α-reductase in human, animals, and plants has been elucidated through physiological and pharmacological studies. Several different classes of inhibitors have been used to competitively block the enzyme. Among them, finasteride, a 4-azasteroid, was used in most cases (e.g. Thipgen and Russell 1992). Since the expression of genes involved in lipid and sterol metabolism is modified during the infection of leaves with the downy mildew pathogen and azoles as inhibitors of sterol biosynthesis are widely used in viticulture to control e.g. powdery mildew and black rot, the effect of azole-type inhibitors, inducers, and fungicides on growth and development of *P. viticola* on grapevine was investigated in a leaf disc-based test system.

Material and Methods

Plant material and pathogen: Plants of *Vitis vinifera* 'Riesling' were grown in 12 l pots filled with sand and loamy soil (3:1, v/v) in the greenhouse at daylight and 20 °C, and fertilised weekly with 100 mL of a 1 % solution of Hakaphos blau (N/P/K/Mg, 15:10:15:2; Compo, Münster, Germany) supplemented with microelements (Fetrilon Combi, Compo).

Sporangiospores of *P. viticola* (Berk. and Curt.) Berl. et De Toni were collected during summer and autumn from infested vineyards of the Palatinate, mainly from *V. vinifera* 'Riesling' or 'Kerner' and propagated on 'Riesling'. Sporangiospore suspensions (50,000 sporangiospores per ml) were first incubated at 4 °C for 1–2 hours until germination of zoospores occurred, and then used as inoculum (see below).

Pharmacological tests: Leaves of greenhouse plants were treated by spraying aqueous solutions of several fungicides, growth regulators, and inducers at field doses (Table) according to the manufacturers' instructions. All solutions were applied as a fine mist onto the upper side of the leaf to avoid an influence of surfactants and other components of the formulated pesticides on the lower leaf side, placed in plastic boxes, and left therein for 48 h at 20 °C and high humidity prior inoculation to allow an absorption and distribution in the leaf tissues. In addition, all compounds were applied at the same time point of inoculation or two days after an inoculation. In most cases, formulated products had been used except for probenazole and finasteride (Dr. Ehrenstorfer GmbH, Germany) which were first solved in DMSO (dimethyl sulfoxide) and diluted with bidistilled water to a final concentration of 10 % DMSO.

Leaf discs were prepared from these treated leaves using a cork borer (15 mm in diameter). Each experimental unit (treatment or control) consisted of 3 Petri dishes (55 mm in diameter) containing 10 to 12 leaf discs made

Table

List of tested inhibitors and inducers that interfere with fungal or plant sterol metabolism

Active component	Product	Final concentration of active component
Fenpropimorph	Corbel	15 mM
Finasteride	pure	1 and 2 mM
Metconazol	Caramba	5 mM
Paclobutrazol	Bonzi	6,8 mM
Penconazol	Topas	5 mM
Probenazol	pure	4 mM
Propiconazol	Desmel	5 mM
Tebuconazol	Folicur	5 mM

from different leaves. Discs were placed upside down on water and inoculated with 50 µL droplets of a sporangia suspension of *P. viticola* (50,000 sporangia/ml). Controls were performed by applying the same amount of sterile bidistilled water. The finasteride/DMSO-solution was infiltrated into the leaf disc prior inoculation to achieve a homogenous distribution. Infiltration with DMSO dilutions were performed as controls. At least three experimental units were performed (at least 100 leaf discs in total for each treatment or control). Sample preparation for fluorescence microscopy was done following Kortekamp (2005) and sporulation intensity on each leaf disc was determined according to Kortekamp (2006) 7 d post inoculation (dpi) by washing down the spores in 1 ml of 0.1 % Tween 80 in water. The number of spores was estimated in an aliquot with the aid of a haemocytometer. The effect of treatments on intensity of sporulation and symptom development was compared using the Tukey HSD test. Statistical analyses were performed using the SPSS (SPSS Software GmbH, München, Germany) procedure.

Results

The effect of spray applications of different azoles (fungicides, inducers, inhibitors) on growth and spore production in leaves was assessed seven days post inoculation (dpi). Compared to the untreated control, growth of the pathogen was retarded after azole treatment within the first three dpi when azoles were applied two days prior inoculation (Fig. 1). However, this delay in hyphal growth was nearly compensated 5 dpi. No differences in hyphal growth were observed compared to the control 7 dpi. At this time point, affected intercostal fields were completely colonised by the pathogen and a dense net of hyphae was visible after aniline blue staining. Spore production was also only weakly affected, but significantly reduced by tebuconazole, metconazole, propiconazole, fenpropimorph, and probenazole (Fig. 2). Especially tebuconazole treatments reduced spore production when applied two days before inoculation. No effect on growth and spore production was detected when all compounds used were applied simultaneously during or two days after inoculation.

Since the effect of azoles interfering with sterol production in true fungi on growth and spore production of *P. viticola* was very low and only observed when compounds were applied two days before inoculation, finasteride as an inhibitor of brassinosterol biosynthesis in plants was used, in order to investigate the effect on the pathogen when sterol metabolism of the host was modified. To achieve homogenous distribution of this lipophilic compound, finasteride was first solved in DMSO and diluted with water to a final concentration of 10 % DMSO and 1 mM or 2 mM finasteride before infiltration of leaf discs was performed. Infiltration with the solvent DMSO alone did not decrease spore production significantly, but this effect could be enhanced markedly by finasteride at a concentration of 1 mM (Fig. 3). Furthermore, nearly no sporulation was observed using finasteride at a final concentration of 2 mM. Microscopical analysis revealed that

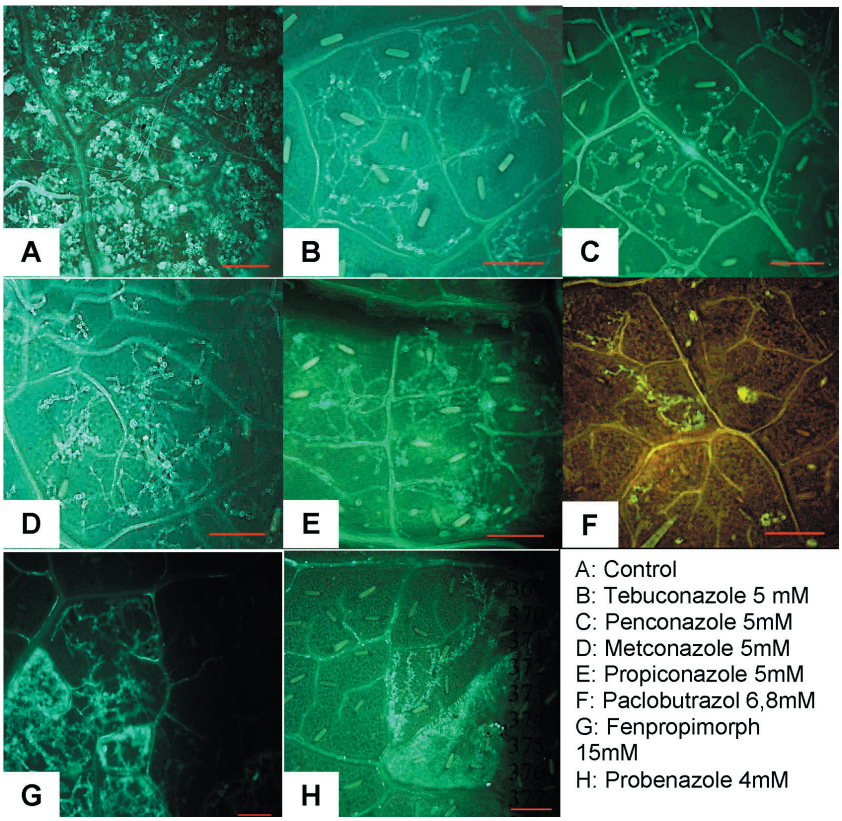

Fig. 1: Effect of sterol biosynthesis inhibitor and plant inducer application two days before inoculation with *Plasmopara viticola* on mycelial growth of the pathogen three days post inoculation. Growth of intercellular hyphae was retarded compared to the untreated control; bar equals 100 µm.

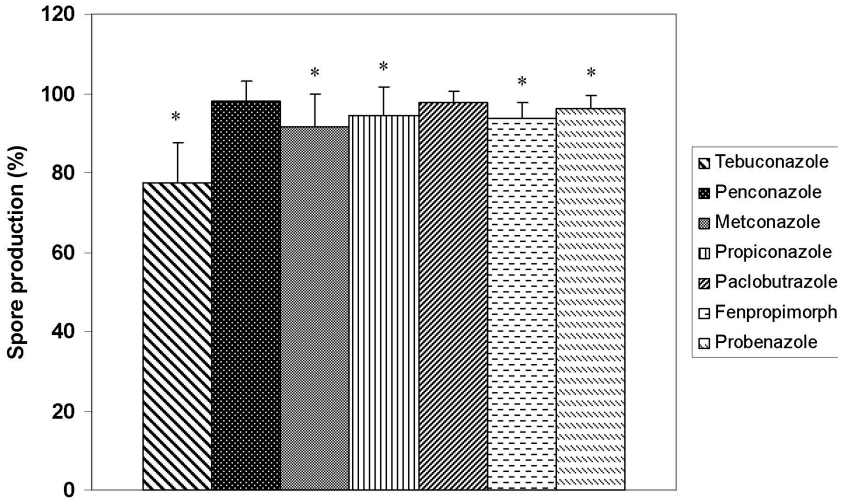

Fig. 2: Effect of sterol biosynthesis inhibitors and inducers on the sporulation of *Plasmopara viticola* on leaf discs compared to an untreated control which was calculated as 100 %. Significant differences according to a Tuckey HSD test are indicated by an asterisk (*).

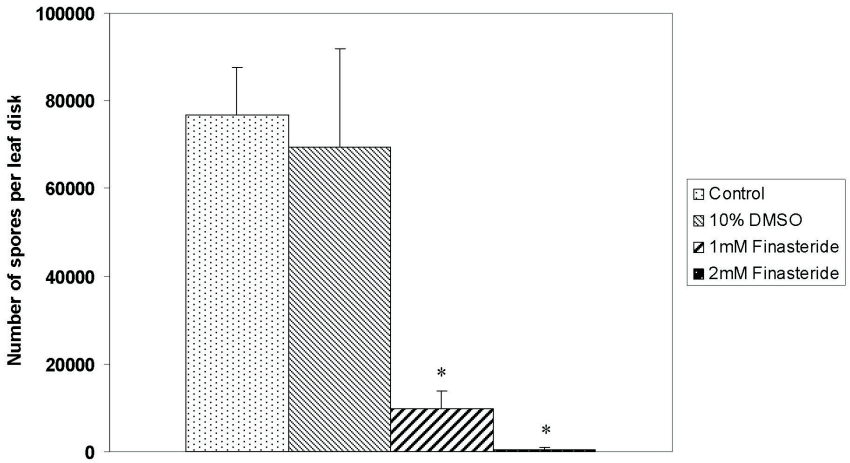

Fig. 3: Effect of finastride as a plant brassinosterol biosynthesis inhibitor on spore production of *Plasmopara viticola*. Significant differences compared to the cotrol according to a Tuckey HSD test are indicated by an asterisk (*).

hyphal growth was restricted to a few intercostal fields that exhibit a poor number of short hyphae deriving from single zoospores (Fig. 4). Interestingly, affected intercostal fields turned brown seven days after an inoculation with *P. viticola* which was not observed in the controls.

Discussion

Triazole fungicides and other fungicidal compounds that interfere with sterol biosynthesis in fungi are widely used to control disease epidemics in many cultivated crops. Such fungicides are applied several times during the season in the vineyard, especially to avoid infections caused by *Erysiphe necator* (powdery mildew) and also *Guignardia bidwellii* (black rot) when needed. It is well known from other crops that azole-type fungicides are able to modify both, fungal and plant metabolism, leading to side effects depending on the plant species and compound used. Even though triazole fungicides are used in grape production for many years, their application seems not to interfere with infections caused by *P. viticola* and the mode of colonisation of vines in the field. Thus, epidemics caused by the downy mildew pathogen are not altered by the application of triazoles. This seems to be obvious from a biochemical or physiological point of view, since peronosporomycetes - to our knowledge - are not equipped with sterol biosynthesis pathways.

However, some (tri)azoles are well known to stimulate defence reactions in the plant. Probenazole activates the phenylpropanoid pathway, the expression of PR genes, and the production of superoxide in rice plants (Iwata 2001) and maybe other crops and is therefore used as a fungicide and an inducer. However, probenazole failed to induce defence responses in parsley cells (Siegrist *et al*. 1998). The triazole paclobutrazol protects plants from stress caused by heat, drought, and herbicides (Kraus and Fletcher 1994, Smith *et al*. 1992) and its molecular structure is quite similar to triadimenol, a worldwide used fungicide. Both compounds, probenazole and paclobutrazol, affected spore production on leaf discs inoculated with *P. viticola* only weakly as it was observed after an application of fun-

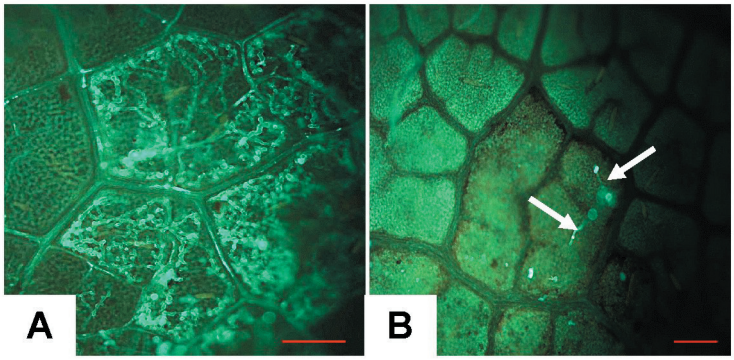

Fig. 4: Analysis of hyphal growth of *Plasmopara viticola* in grapevine leaves using epifluorescence microscopy seven days post inoculation. Extensive growth of the pathogen was observed in several intercostal fields in the control (**A**). Infiltration of leaf samples with 2mM Finasteride inhibited nearly completely growth and propagation of the pathogen (**B**), small hyphae are indicated by arrows); bars represent 100 µm.

gicidal azoles, except tebuconazole. Furthermore, these weak effects on growth and spore production of *P. viticola* were only observed when azole-type fungicides and inducers were applied two days before inoculation, maybe allowing the plant to initiate defence reactions. Further studies revealed that the application of tebuconazole and probenazole slightly increased the content of salicylic acid in leaves which was not significant and not observed after treatments with other azoles (data not shown). This reaction may represent a general stress response to pesticide applications. Stresses caused by agrochemicals, such as herbicides, leading to unexpected side effects during host-fungus interactions (Kortekamp 2008, 2011), may reduce the feeding capability due to a modulated general metabolism and thus to a lower availability of nutrients of the affected tissue. This can result in a reduced growth or spore production, even though the effect on sporulation of *P. viticola* was too weak to reduce the pathogens ability to provoke heavy infestations.

Since peronosporomycetes are not able to produce sterols by their own, but sterols are involved in growth and propagation of these plant pathogens, probably including *P. viticola*, plant derived sterols or appropriate precursors have to be absorbed by the pathogen. Thus, alterations in sterol metabolism and maybe other biochemical and physiological processes linked to the sterol metabolism of the plant may interfere with the development of the pathogen. Finasteride is an inhibitor of 5α-reductase which is involved in the biosynthesis of brassinosteroids. In grapevine, brassinosteroids modify different processes such as accumulation of sugars and anthocyanins in berries (Symons *et al.* 2006). There are no reports on the role of brassinosteroids in grapevine regarding defence responses after infections of fungal pathogens. However, infiltration of finasteride markedly reduced or nearly inhibited growth and spore production of *P. viticola* at millimolar concentrations. In addition, propiconazole as another sterol /brassinosteroid biosynthesis inhibitor (Hartwig *et al.* 2012) also reduced spore production significantly. Since it is unlikely that *P. viticola* produces these kinds of sterols by its own, this indicates that plant derived brassinosteroids or those processes affected by these sterols interfere with the propagation of *P. viticola*. Further investigation is needed to illuminate this biochemical and physiological interaction which might reveal a new Achilles heel of the pathogen.

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