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EVIDENCE FOR FOLIAR UPTAKE OF SULFATED LAMINARIN INTO GRAPEVINE DEPENDING ON SURFACTANT USE AND LEAF SURFACE

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RÉSUMÉ

Certains ß-1,3-glucanes, en particulier la laminarine sulfatée (PS3), sont connus pour être des inducteurs de résistance de la vigne contre l'agent pathogène *Plasmopara viticola*. Bien que leur efficacité soit incontestable, la protection induite reste variable au vignoble. Pour être perçue par la plante, ils nécessitent de franchir les barrières constitutives, et notamment la cuticule. Ainsi, leur faible efficacité au champ pourrait être liée par une pénétration limitée à travers la cuticule des feuilles, suite à leur pulvérisation. Dans cette étude, nous avons mis au point une méthode pour analyser les comportements de diffusion du PS3 dans les feuilles de *Vitis vinifera,* à l'aide d'un microscope biphotonique. Nos résultats ont révélé que ce polysaccharide est vraisemblablement capable de pénétrer à travers la cuticule des feuilles de vigne, lorsqu'il est formulé dans un adjuvant adéquat. Par ailleurs, les stomates, les parois anticlines et les trichomes se sont révélés être des zones de diffusion préférentielle pour cette molécule. Il a été intéressant de constater que son taux de pénétration était plus important sur la face abaxiale des feuilles (stomatique), que la face adaxiale (sans stomate). De plus, nous sommes parvenus à lier le taux de pénétration, avec l'efficacité de PS3 en tant qu'inducteur de résistance contre le mildiou.

Mots-clés : *Vitis vinifera*, induction de résistance, polysaccharides, perméabilité cuticulaire, microscopie.

ABSTRACT

Some ß-1,3-glucans and particularly sulfated laminarin (PS3) are known as resistance inducers (RI) of grapevine against the pathogen *Plasmopara viticola*. Although their efficacy is doubtless in laboratory conditions, the protection they induce still suffer inconsistency, especially in vineyard, which might be caused by a limited penetration through the leaf cuticle following spray. In this study, we developed a method to analyze the diffusion patterns of PS3 in *Vitis vinifera* leaves, with two-photon laser scanning microscopy. Our results first revealed that PS3 is actually able to penetrate the grapevine leaf cuticle when formulated with a suitable adjuvant. Furthermore, stomata, anticlinal cell walls and trichomes were found to constitute preferential diffusion zones for the polysaccharide. Interestingly, we also showed that its penetration rates were much higher on the stomateous abaxial surface of the leaf than on the adaxial surface. Moreover, we were able to link the penetration rates to the efficacy of PS3 as a RI against downy mildew of grapevine when applied on either surface of the leaf.

Keywords: *Vitis vinifera*, induction of resistance, polysaccharides, cuticular permeability, microscopy.

Abbreviations – CLSM, confocal laser scanning microscopy; DAT, day after treatment; DE, Dehscofix CO125; DP, degree of polymerization; DS, degree of substitution; EO, degree of ethoxylation; HAT, hour after treatment; LRB, Lissamine Rhodamine B; MW, molecular weight; RI, resistance inducer; SD, standard deviation; TPSM, two-photon laser scanning microscopy.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an economically important crop which is susceptible to severe fungal diseases. Controlling them requires several fungicide applications, especially in temperate rainy areas. However, for environmental and health concerns, there is a need for reducing antifungal treatments. Inducing resistance by application of elicitors represents an attractive alternative strategy for crop protection. Elicitors are compounds that are perceived by plant receptors as danger signals and that trigger signaling cascades leading to gene expression and, subsequently, to defense responses (Garcia-Brugger et al. 2006). Numerous compounds have been identified as elicitors over the last decades, among which polysaccharides have become a worthwhile subject of studies (Trouvelot et al. 2014). A number of polysaccharides are considered as microbial- or damage-associated molecular patterns (MAMP/DAMP). They are specifically recognized by receptors, known as pattern recognition receptors (PRRs) located in the plant cell plasma membrane (Boller and Felix 2009). Polysaccharides are of special interest for use in crop protection for several reasons. They are (i) extracted from microbial, algae or plant cell walls, which represent renewable sources and (ii) biodegradable and practically deprived of toxicity. Previous studies have shown that laminarin, a ß-1,3-glucan, is an effective resistance inducer (RI) against plant diseases (Aziz et al. 2003, Klarzynski et al. 2000). Furthermore, the sulfated derivative of laminarin (PS3) turned out to be more effective than the native product against the tobacco mosaic virus (Ménard et al. 2004), grape downy and powdery mildews, respectively caused by the oomycete, *Plasmopara viticola*, (Trouvelot et al. 2008) and the ascomycete, *Erysiphe necator*, (Daire, personal communication). Foliar spray application of PS3 primes H_2O_2 production, expression of defense gene and cell wall reinforcement in grapevine challenged with *P. viticola* (Gauthier et al. 2014), and these reactions are associated with the restriction of pathogen colonization and sporulation (Trouvelot et al. 2008).

Application of polysaccharides as RIs in field conditions can be effective against plant diseases (Adrian et al. 2012; Delaunois et al. 2014). For example, in vineyard trials, spray applications of a mixture of oligogalacturonic acid and chitooligosaccharides achieved a significant control of powdery mildew, reducing the disease severity on bunches of grapes by at least 70% (Van Aubel et al. 2014).

However, many data or communications, often unpublished, indicate that polysaccharide elicitor applications either achieve low level of plant protection or suffer inconsistency, and this is the main impediment to their practical use. Unlike the pesticides that directly affect the pathogen, RIs rely on the elicitation of plant defenses. Therefore, various factors can affect the plant responsiveness, e.g. developmental stage (Steimetz et al. 2012), genotype, abiotic stress, or mineral nutrition (Walters et al. 2013). Another factor that received no or only little attention to date is polysaccharide bioavailability. When polysaccharides are sprayed on leaves, they first have to cross the leaf cuticle and cell walls to reach their putative cellular target receptors. The cuticle is a rather thin membrane consisting of cutin, polysaccharides and associated lipids called cuticular waxes. Cuticular waxes are a complex mixture of a homologue series of long chain aliphatic lipids, triterpenoids and minor secondary metabolites, such as sterols and flavonoids (Bernard and Joubès 2013), playing a major role in limiting uncontrolled water loss (Kosma et al. 2009). As cuticle constitutes a hydrophobic barrier to the diffusion of hydrophilic compounds, such as PS3, we hypothesized that low penetration rates into leaves may be responsible for poor efficacy of polysaccharide treatments.

Based on the fact that some surfactants have been shown to enhance the foliar penetration of hydrophilic herbicides (Liu 2004, Ramsey 2005), we used a surfactant that may act in the same way with PS3 and, consequently, could increase the level of induced resistance. In addition, this study aimed at gaining insight into the diffusion of the sulfated laminarin PS3 into grapevine leaf, which is largely unknown as yet. For this purpose, we developed a protocol using two-photon laser scanning microscopy in order to track the penetration of fluorescent-labeled PS3 into grapevine leaves.

MATERIALS AND METHODS

PLANT MATERIAL

The grapevine cultivar *V. vinifera* L. cv. Marselan, susceptible to *P. viticola* (causal agent of downy mildew), was used for this study. The plants were obtained from herbaceous cuttings placed in individual pots (10 cm× 10 cm × 7 cm) containing a mixture of blond peat and perlite (3:2, v/v). They were grown in a glasshouse at a temperature of 24 / 18°C (day / night, respectively) with a photoperiod of 16 h and at a relative humidity (RH) of 70 ± 10% until they developed six leaves. Plants were irrigated with a fertilizer solution (NPK 10-10-10, Plantin, France). Unless otherwise indicated, the second adult leaf under the apex was treated as described below.

SURFACTANT

In order to enhance the foliar uptake of PS3 in grapevine, we used the surfactant Dehscofix CO125® (Huntsmann) at the concentration of 0,5%.

FLUORESCENT LABELLING OF SULFATED LAMINARIN PS3

Lissamine Rhodamine B(LRB)-labelling of native laminarin

Fluorescent labelling of native laminarin was performed as follows. To a suspension of 660mg of laminarin in a mixture of 5 ml of acetic acid and 5 ml of deionized water, were added 150 mg of Lissamine Rhodamine B Ethylenediamine (LRB). The solution was stirred at 60 °C for 1 h. After that time, 42 mg of sodium cyanoborohydride dissolved in 1 ml of acetic acid and 1 ml of deionized water was added and the stirring went on at 60 °C for 4 days. The solvent was removed under strong vacuum and 10 ml of 1 M hydrochloric acid solution was added, followed with continued stirring for 30 min. The acid was removed under reduced pressure and the residue was suspended in 5 ml of deionized water. Free LRBED and the salts were removed by size exclusion chromatography on a PD-MidiTrap G-10 column purchased from GE Healthcare Life Sciences (28-9180-11) with deionized water as eluant. The fractions containing no free LRBED were combined and lyophilized.

Sulfation of labelled laminarin (PS3-LRB synthesis)

Briefly, 1.5 g of labelled laminarin and 3.5 ml of dry pyridine were suspended in 30 ml of dry dimethylformamide under nitrogen. The mixture was heated at 60 °C with vigorous stirring and 8.6 g of SO_3-pyridine complex were added. The solution was stirred at 60 °C for 2 h and then overnight at room temperature. Precipitation of the product was obtained by addition of 20 ml of absolute ethanol. The liquid phase was removed by filtration and the solid residue washed three times with 20 ml of absolute ethanol. The product was suspended in 5 ml of warm deionized water and neutralized with 2 M sodium hydroxide solution. Excess pyridine was removed under strong vacuum and the product was lyophilized and stored at 4 °C until use.

VISUALIZATION OF THE FOLIAR UPTAKE OF FLUORESCENT-SACCHARIDES INTO GRAPEVINE WITH TWO-PHOTON LASER SCANNING MICROSCOPY (TPLSM)

This following section is based on the methodology previously described by Liu and collaborators to study the foliar uptake of xenobiotics (Forster et al. 2006, Liu 2003, Liu et al. 2004). To study the diffusion of PS3 into grapevine leaf tissues with TPLSM, PS3-LRB was either dissolved in water or in 0.5% DE. The first adult leaf under the apex of grapevine plants was cut off with petiole, and put into 15 ml falcon tubes filled with reverse osmosis water. Five droplets (1 µl) per leaf were applied to the intact adaxial or abaxial surface. Leaves were collected, washed and dried 2 DAT, as described above. The samples were mounted in distilled water before examining them on a Nikon A1-MP scanning microscope (Nikon, Japan). Imaging was carried out with a 60x Plan Apo IR objective (NA: 1.27, Water Immersion, Nikon, Japan). An IR laser (Chameleon, Coherent) was used to provide 840 nm excitation. Fluorescence emission was collected on two detection channels: the orange channel FF01-575/25-25 [563-588] nm, and the red channel: FF01-629/56-25 [601-657] nm (Semrock). As the recovered signal tended to decrease with depth, laser intensity was adjusted with the Z-intensity correction function: a linear enhancement from 2 to 5% was set from the surface to a depth of 50 +/- 10 µm, so that the recovered signal could be maintained along the Z-axis. The photomultiplier gain was set at 80V for both orange and red channels. Cross-sections were visualised after setting the depth of the optical section at 50 +/- 10 µm. Images were acquired at 1024 x 1024 pixels resolution, with Z-stacks every 1 µm along the Z-axis.

ASSESSMENT OF PS3-INDUCED RESISTANCE AGAINST *PLASMOPARA VITICOLA* IN GRAPEVINE

Sulfated laminarin (PS3) was dissolved at 5 g l^{-1} either in water or in 0.5% DE and applied to both adaxial and abaxial surfaces of grapevine plants using a hand-held sprayer. Mock-treated plants were used as control and consisted of plants sprayed with water. Five plants were used per condition. The plants were left to dry for 2 h then transferred in the greenhouse. Two days after treatment, the abaxial surfaces of the leaves were inoculated by spraying a freshly prepared suspension of 10^4 spores of *P. viticola* per milliliter of water. Inoculated plants were placed overnight in a humid chamber (100% relative humidity). They were then transferred again in the greenhouse. Six days later, the plants were transferred again in the humid chamber to provoke sporulation of the pathogen. Severity of the disease was visually assessed as percentage of sporulating foliar area.

RESULTS

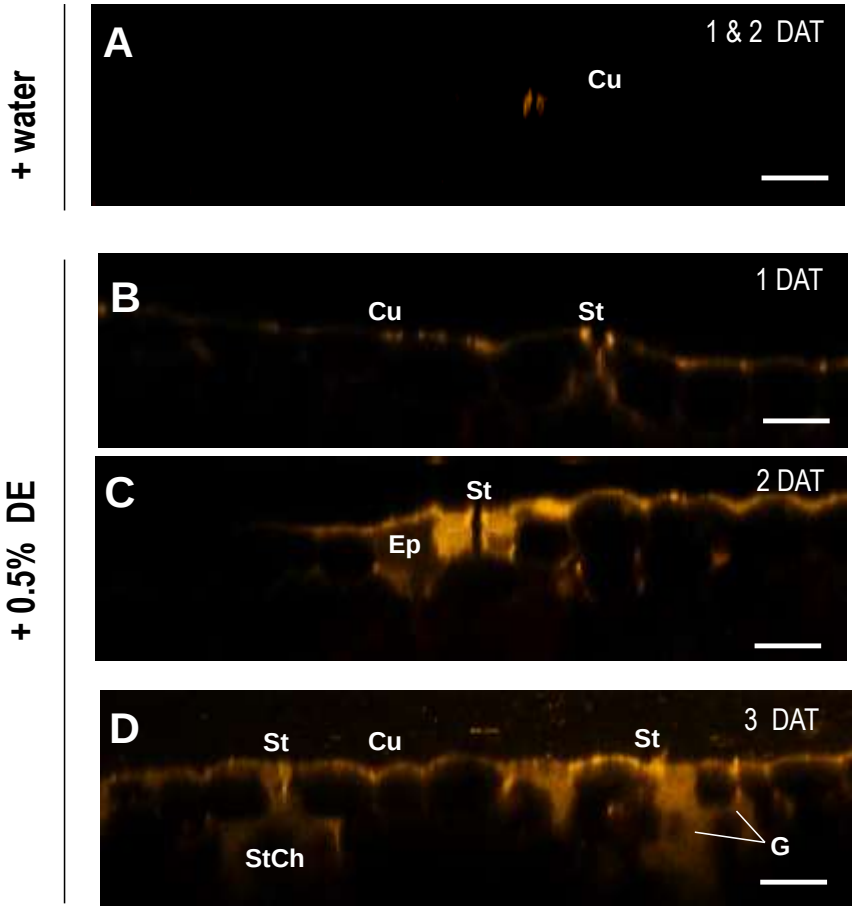
VISUALIZATION OF THE UPTAKE OF FLUORESCENT PS3 INTO THE FOLIAR TISSUES

Visualization of the uptake of LRB-labelled PS3 into grapevine leaf (abaxial surface)

PS3-LRB was prepared in water or in 0.5% DE, and then applied to the abaxial surface of grapevine leaves. The observations were done with TPLSM at 1 and 2 DAT (Fig. 1). The fluorescent signal could barely be detected when PS3 was prepared in water, regardless of the observation time (Fig. 1A). In presence of DE, only the cuticle and the stomatal edges were fluorescent at 1 DAT (Fig. 1B), whereas at 2 DAT, the signal was stronger in the cuticle, and in vacuoles of some epidermal cells, especially those surrounding stomatal guard cells (Fig. 1C). At 3 DAT, the signal was still detected in those zones, but also in substomatal chambers and intercellular gaps near them (Fig. 1D).

Figure 1: Visualization of LRB-PS3 formulated in water (A) or DE 0.5% (B,C,D) in grapevine abaxial leaf tissues, 1, 2 or 3 DAT. Cu= Cuticle; St= Stomata; StCh= Substomatal chamber; Ep= Epidermal cells; G= intercellular gaps. Bar= 20µm

Figure 1: Visualisation du PS3-LRB formulé dans de l'eau (A) ou le DE 0,5% (B, C, D) dans les tissus foliaires abaxiaux de vigne, à 1, 2 ou 3 jours après traitement. Cu= Cuticule; St= Stomate ; StCh= Chambre sous-stomatique ; Ep= Cellule épidermique ; G= Lacune intercellulaire. Echelle= 20µm

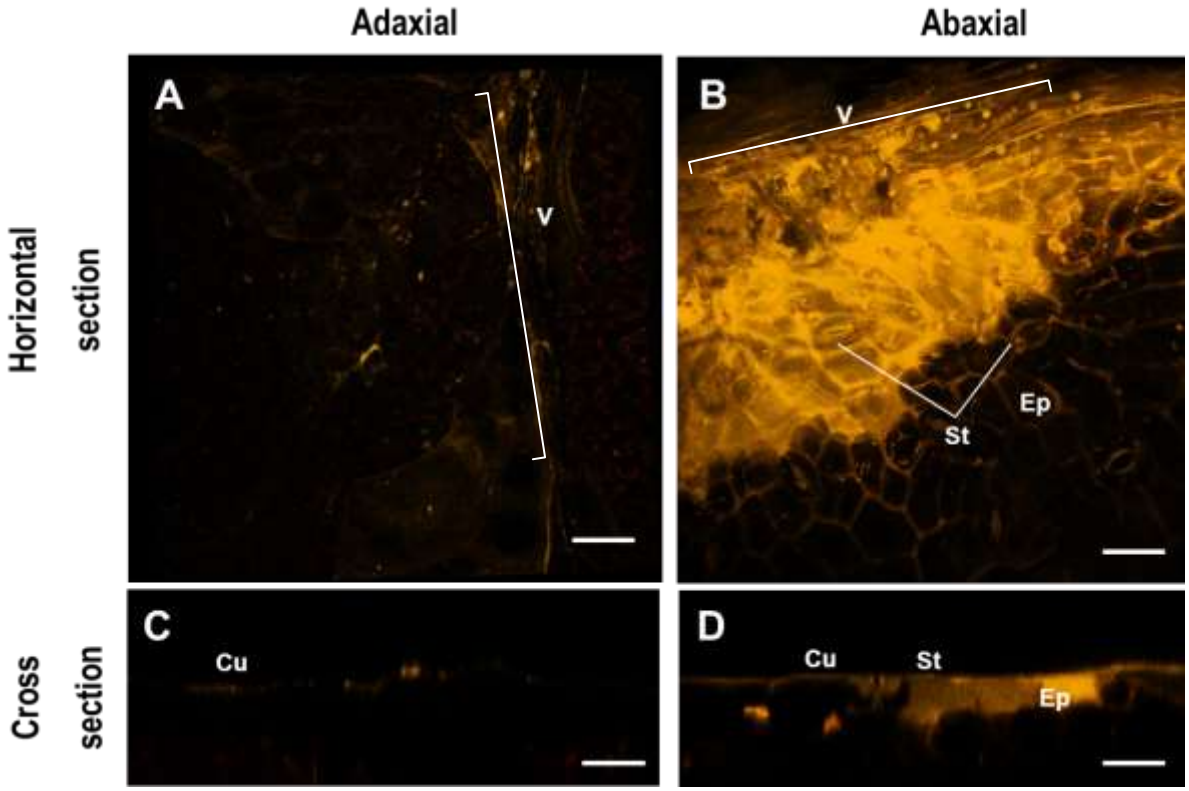


Visualization of the uptake of fluorescently labelled PS3 according to the leaf surface

In grapevine leaf tissues at 2 DAT, a LRB-fluorescent signal was barely detected in samples treated on the adaxial surface (Fig 2A and C). On the contrary, those treated on their abaxial surfaces (Fig. 2B and D) emitted a stronger fluorescent signal, which was found along the depression due to the borders of veins most of the time.

Figure 2: Visualization of LRB-PS3 formulated in DE 0.5% in grapevine abaxial leaf tissues 2 days after treatment, on adaxial (A,C) and abaxial (B,D) leaf surface, by horizontal (A,B) and cross (C,D) sections. Cu= Cuticle; St= Stomata; Ep= Epidermal cells; V= vein. Bar= 20µm

Figure 2: Visualisation du PS3-LRB formulé dans le DE 0,5% dans les tissus foliaires de vigne, 2 jours après traitement, sur face adaxiale (A,C) et abaxiale (B,D), observés en coupe horizontale (A,B) ou transversale (C,D). Cu= Cuticule ; St= Stomate ; Ep= Cellule épidermique ; V= Nervure. Echelle= 20µm.

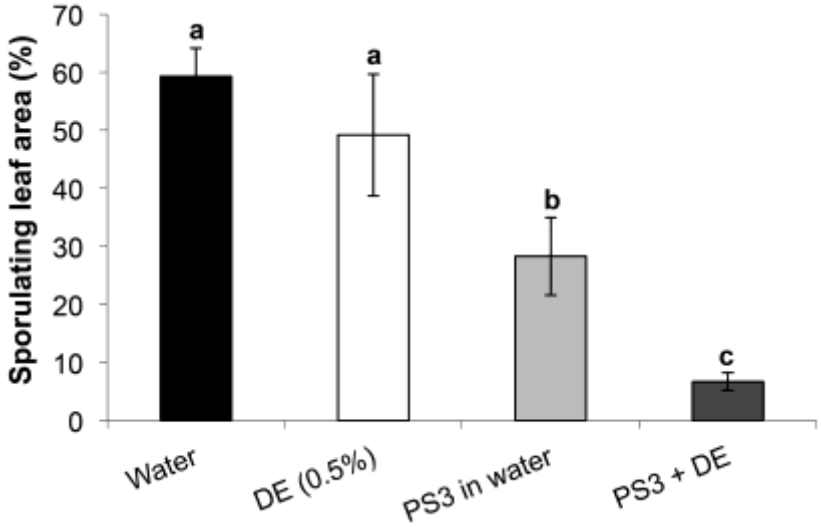


INFLUENCE OF DE ON PS3-INDUCED RESISTANCE TO *PLASMOPARA VITICOLA*

In order to check the hypothesis that increasing RI bioavailability leads to higher induced resistance, experiments were carried out with grapevine challenged by the oomycete *P. viticola*. As shown in Fig. 3, PS3 on its own reduced disease severity (disease reduction rate: 49%) as compared to the water control, however the combination of both polysaccharide and surfactant turned out to be even more effective as compared to the corresponding DE control (disease reduction rate: 86%).

Figure 3: Induced resistance in grapevine to *Plasmopara viticola* following foliar spray application of PS3 (5 $g.l^{-1}$), with or without the surfactant (DE, 0.5%). Both leaf surfaces were treated. Treatment with different letters are statistically different ($p<0.02$).

Figure 3: Résistance induite sur vigne contre *Plasmopara viticola* suite à un traitement foliaire par PS3 (5 $g.l^{-1}$), avec ou sans l'adjuvant (DE 0,5%). Les deux surfaces foliaires ont été traitées. Les lettres différentes indiquent que les modalités sont statistiquement différentes ($p<0.02$).



DISCUSSION

To elicit plant defenses, RIs have to cross the leaf cuticle in order to reach their putative cellular receptor. Our hypothesis is that their bioavailability affects their efficacy and needs optimization to achieve satisfying results.

The development of this microscopic observation method enabled us to show that a polysaccharide RI such as PS3 does not readily enter grapevine leaves upon exogenous application.

In order to increase polysaccharide penetration into grapevine leaves, DE was found effective in enhancing the foliar uptake of PS3, as clearly shown by TPLSM observations. That a surfactant with high EO content and poor spreading properties like DE improved the penetration of PS3 is in agreement with previous findings. Indeed, such surfactants were shown to promote the uptake of a range of hydrophilic compounds such as glucose derivatives (Forster 2005, Tuansheng 2004) and glyphosate (Liu 2004). Surfactants with high EO content are believed to increase the water content of cuticle, and consequently to improve permeability to water-soluble compounds (Ramsey 2005). Such

action may help PS3 to gain access to hydrophilic zones in the cuticle, from which it can reach its cellular targets and be perceived.

Interestingly, the beneficial effect of DE on PS3 uptake into grapevine leaf was further backed by increased PS3-induced resistance in biological tests when both were combined. This supports our hypothesis that insufficient bioavailability of polysaccharide RIs can limit their efficacy.

This study also aimed to gain insight in the way of penetration of these RI into the leaf. It has previously been established that hydrophilic compounds can enter the leaf via polar pathways in the cuticle. Polar paths may likely consist of polysaccharide fibers embedded in the cuticle. These polar zones were shown to occur preferentially in the cuticle above anticlinal cell walls, around the stomata and at the bases of trichomes (Schönherr 2006). Some anticlinal cell walls are stained by fluorescent PS3, which suggests that a transcuticular pathway could not be excluded. However, this is surprising given that PS3 has a MW of approximately 7000 Da and that it is currently established that molecules larger than 800 Da are over the size exclusion limit of the polar path.

Stomata could also constitute a possible entry route for polysaccharides as it is supposed that hydrophilic solutes can diffuse into stomata through either a microbial film or deposited salts and particles present on the wall of guard cells facing the pore, which were already considered as hydrophilic pores with large size exclusion limits, above 10 nm (Eichert et al. 2008). This hypothesis of a stomatal entry path is supported by the TPLSM study with grapevine which clearly revealed that in the presence of DE, the stomata (guard and substomatal cells) were preferentially stained by fluorescent PS3. This penetration pathway could however be limited because less than 10 % of the stomata were shown to participate in the uptake of hydrophilic particles in leaves of different species (Eichert and Burkhardt 2001). We are, furthermore, not able to explain why we needed the use of a specific surfactant in order to observe PS3 uptake through stomata. Eichert and collaborators (2008) for instance could detect the uptake of fluorescent particles in aqueous solution. Also, some PS3 is also probably able to enter the leaf in the absence of DE, via either the stomatal or the cuticular hydrophilic route, since it was shown to induce resistance as such, although to a lesser extent. In this case, we presume that the amount that reached leaf tissues was below the detection thresholds of TPLSM. Elucidating exactly how a polysaccharide such as PS3 can enter the leaf deserves further investigations.

Furthermore, uptake visualization evidenced that the penetration increase due to DE was much higher in the grapevine leaf abaxial surface as compared to the adaxial surface, which remained nearly impervious to polysaccharide penetration. This could be due to the fact that grapevine possesses stomata on the abaxial surface only, and that the difference in cuticular structure between its leaf surfaces could be significant. Therefore, further studies should focus in analyzing differences in the thickness and composition between the two leaf surfaces. Altogether these differences may explain why the penetration rates of hydrophilic compounds are higher in the abaxial surface.

Our findings have two important practical consequences for improvement of RI treatments against grape downy mildew and, likely, other fungal diseases. First, spray application of polysaccharide RI preferentially targeting the abaxial surface of the grapevine leaf may improve the effectiveness of the treatments. Second, one should pay attention to the formulation of RI active ingredient and particularly the nature of the adjuvant. Undoubtedly, the importance of these factors is generally underestimated.

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