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Grapevine downy mildew development as affected by chitosan spray treatments and metabolomics implications

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Abstract. Chitosan has been shown to enhance grapevine tolerance toward downy mildew while reducing the environmental impact of traditional protection products. The *in vitro* study considered potted vines of *Vitis vinifera* L. cv. Merlot grafted on SO4 and K5BB rootstocks. The aim was to assess, at different phenological stages: (1) the role of chitosan chlorydrate denso treatments on the occurrence of downy mildew infection; (2) the change of the plant metabolites elicited by canopy applications of chitosan chlorydrate and subsequent downy mildew inoculation on leaf discs and berries, according to the metabolomics approach; and (3) the role of the different rootstocks on the fungal infection and the metabolomic profile. Four treatments were evaluated, as follows: a) chitosan application followed by downy mildew inoculation (CH-I); b) chitosan application without inoculation (CH-NI); c) untreated control with inoculation (UC-I); d) untreated control without inoculation (UC-NI). Leaves were sampled at the phenological stages of shoots 30 cm long (BBCH 57), fruit set (BBCH 71), and the onset of berry touch (BBCH 77), while berries were sampled at fruit set (BBCH 71).The results demonstrated that chitosan canopy treatments significantly reduced downy mildew infections on both leaves and berries. Metabolomic analysis revealed that chitosan elicited responses in secondary metabolism, with variations depending on phenological stages and rootstock combinations. The induced secondary metabolites were mainly associated with polyphenol, nitrogenous compound, and terpene pathways. These changes suggest that chitosan triggers defense-related metabolic pathways against downy mildew. Additionally, the elicitation of aromatic and antioxidant pathways in berries indicates a potential role in enhancing grape composition. Future studies should be conducted in vineyard conditions, including sensory analysis of both grapes and wines.

1. Introduction

The identification of sustainable plant protection strategies against downy mildew that are both effective and easy to apply represents one of the most important challenges in viticulture.

Natural products such as chitosan offer a promising alternative for sustainable disease management [1].

Chitosan is a linear polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine, linked by β(1-4) bonds. It is one of the most common natural polymers and is primarily obtained as a by-product of the fishing industry, extracted mainly from the exoskeleton of crustaceans such as lobsters, crabs and shrimps. In plants, chitosan has the dual function of growth regulator and an inducer of disease resistance [2].

Its mode of action includes the induction of phytoalexin accumulation, change in the composition of free sterols, activation of glucanases and lipoxygenases, production of reactive oxygen species and stimulation of the lignification in plant tissues [3].

The study aims to assess the efficacy of chitosan chlorydrate denso (chitosan) as a resistance inducer against downy mildew, both at the leaf and berry level.

2. Materials and methods

2.1. 2.1. Experimental layout

The trial was conducted outdoors at the experimental platform of the Department of Viticulture, Università Cattolica del Sacro Cuore, in Piacenza, Italy (45.0374773 N, 9.7283258 E; 46 m asl), in 2023.

The experiment included 4-year-old potted Merlot R18 vines grafted onto SO4 and K5BB rootstocks, vines were trained using the Guyot system (10 buds/vine). Conventional spray treatments were not applied to avoid interference with chitosan activity.

The present study involved chitosan chlorydrate denso (Agrilaete company, Ponte Crepaldo di Eraclea, VE, Italy, patent No. 102020000007237, www.agrilaete.it, agrilaete@agrilaete.it). The formulate was applied at a concentration of 2 g/L to the canopy using a manual sprayer.

Treatments were performed at 3 different phenological phases: a) inflorescence fully developed, flowers separating (5 May); b) fruit set (7 June); c) begin of berry touch (12 July), BBCH 57, 71 and 77, respectively [4]. After each treatment the 4th leaf (from the tip) of every shoot was sampled and downy mildew was inoculated on leaf discs. Sampling and inoculation were performed 48 hours after chitosan treatment applying 4 drops (10 µL each) of inoculum suspension (10^4 sporangia/mL) on each leaf disc (14 mm diameter) placed in petri dishes. All plates were incubated in thermostat at 20°C. After 24 hours, the excess of inoculum was removed from the inoculated discs.

Following treatment at fruit set (second sampling, 7 June), small berries were collected, placed in petri dishes and inoculated 48 hours after the chitosan application by spraying an inoculum suspension (10^4 sporangia/mL). Inoculated dishes were placed in thermostat at 20°C.

After the inoculations, the status of the infections was monitored periodically and after 7 days the incidence and severity of infection were visually assessed for each disc while only the incidence was recorded for berries. Incidence of the infection was assessed as the percentage of infected surface of leaf and berry area, while the sporulation intensity of the infection was assessed on a scale from 0 to 3, where 0 indicated absent sporulation and 3 when sporulation covered the entire surface area.

2.2. Metabolomic analysis

Metabolomic analysis was done on leaf discs and berries at the time of symptom assessment, 7 days after inoculation.

Untargeted metabolomic screening was performed via high-resolution mass spectrometry by using a hybrid Q-TOF spectrometer coupled to an UHPLC chromatographic system, as previously reported [5]. Samples were extracted in 0.1% formic acid in 70% methanol and then MS acquisition was performed in positive mode, in the range 100–1200 m/z and compounds identification carried out using the software Agilent Profinder B.07, against the online database PlantCyc (pmn.plantcyc.org) and according to the whole isotopic patterns ([6]).

Untreated and uninoculated (UC-NI) leaf discs and berries were also included, as control. The treatments under investigation were as follows:

- CH-NI: chitosan application without fungal inoculation;
- UC-NI: untreated control without fungal inoculation;
- CH-I: chitosan application and fungal inoculation;
- UC-I: untreated control and fungal inoculation

2.3. Statistical analysis

The incidence of the disease and sporulation intensity were processed according to an analysis of variance (three-way ANOVA with interactions) with the F test (Fisher test) significant to the two usual probabilities of error ($p<0.05$ and $p<0.01$). Tuckey's post-hoc multiple comparison test was then calculated for $p<0.05$ to identify significant differences between the different leaf discs tested. The values expressed as a percentage were processed after angular transformation of the data. Each sampling (thesis) included three replicates (3 petri dishes/thesis).

3. Results

3.1. Incidence and severity (intensity) of fungal infection in the leaves

The three sources of variations (sampling time, treatment, rootstock) and their interactions affected in a significant way incidence and severity of fungal attack. Incidence of infections and sporulation intensity are shown as the combination of sampling time x treatment x rootstock will be described (Figg.1 and 2). The incidence of fungal attack (Fig. 1) in the first sampling (5 May) was observed only in untreated and inoculated discs, (UC-I) being higher in the vines grafted on SO4 (40%) than in those grafted on K5BB (25%). In the second sampling (June 7th) also chitosan treated and inoculated discs (CH-I) showed incidence, but lower than UC-I, without significant differences between rootstocks (Fig.1).

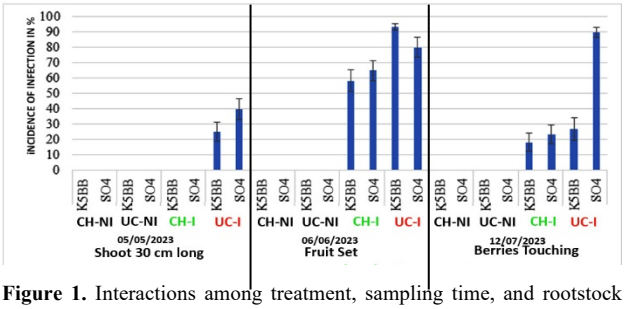

Figure 1. Interactions among treatment, sampling time, and rootstock (K5BB and SO4) on the incidence (%) of downy mildew infections on leaf discs. CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated; UC-NI = untreated control and non-inoculated.

In the third sampling (12 July) chitosan treatments (CH-I) significantly reduced the incidence, as compared to untreated discs (UC-I) in the vines grafted on SO4 (Fig.1). The severity of infection showed the same trend as the incidence (Fig.2).

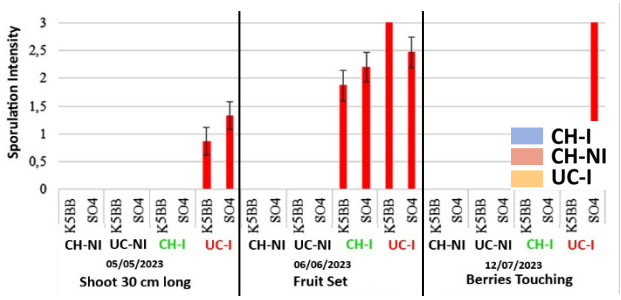

Figure 2. Interactions among treatment, sampling time, and rootstock (K5BB and SO4) on the sporulation intensity of downy mildew on leaf discs. CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated; UC-NI = untreated control and non-inoculated.

3.2. Incidence of fungal infection in the berries

The treatment affected in a significant way the incidence of the fungal attack, while the rootstock did not (Fig. 3). No incidence in uninoculated berries was observed (as expected), while the treatment with chitosan and inoculation with the fungus (CH-I) significantly reduced the incidence, without differences between the rootstocks.

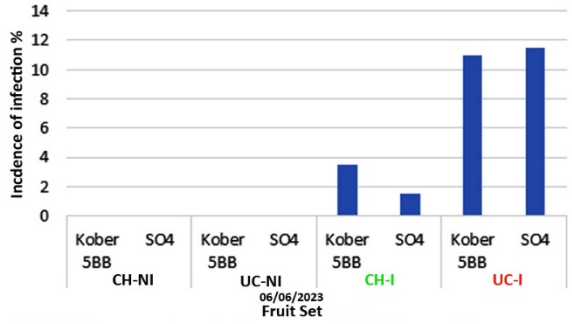

Figure 3. Interactions among treatments and rootstock (K5BB and SO4) on the incidence (%) of downy mildew on grape berries at sampling time 2 (7 June 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated; UC-NI = untreated control and non-inoculated.

3.3. Metabolomic analysis

The variation of components linked to several metabolic pathways altered by the treatments (CH-I, CH-NI and UC-I)[1] have been compared with those of UC-NI, which is taken as a reference. Log fold change[2] (LFC) has been used to express these variations, occurring in main metabolic pathways as well as in the secondary metabolism, hormones and lipids contents.

3.3.1. Leaves

In the first sampling (5 May) (Figg. 4 and 5) especially the secondary metabolites and other substances were affected by the treatment and the rootstock played an important role.

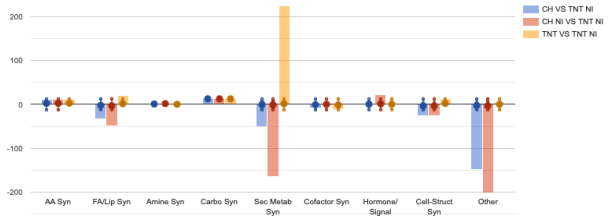

Figure 4. Biosynthesis of compounds induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto K5BB rootstock at the first sampling (5 May 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

Figure 4 shows the treatments in K5BB. Here we can see that in comparison to the control UC-NI, there is a reduction of secondary metabolites in both CH-I and CH-NI, which received the chitosan, while there is an upregulation in UC-I that did not received chitosan but was inoculated.

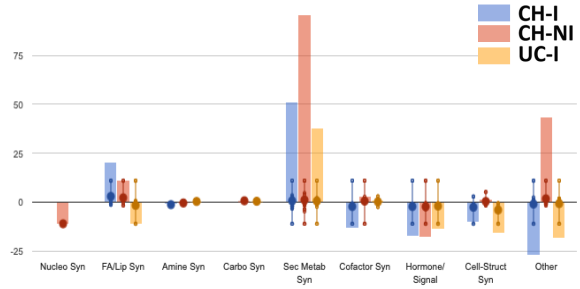

Figure 5. Biosynthesis of compounds induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto SO4 rootstock at the first sampling (5 May 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

On the other hand, figure 5 shows the treatments on SO4 where CH-I, CH-NI and UC-I are all upregulated compared to UC-NI even though in different measures.

[1] CH-I: Chitosan application and subsequent inoculation; UC-I: Untreated control with inoculation; CH-NI: Chitosan application without inoculation; UC-NI: Untreated control without inoculation

[2] The fold change (FC) shows the relative change between two quantities. If for example a gene gets express twice as much compared to control the FC will be 2, while if it is half as much expressed FC will be 0.5. The log fold change is instead the logarithm of the fold change, normally express in base 2. Log fold change (LFC) is often used in bioinformatics and biostatistics in order to exhibit the change in gene expression and discern between two separate conditions such as a thesis and a blank. Indeed a log2(FC) > 0 indicate an up regulation, a log2(FC) < 0 shows down regulation while a log2(FC) = 0 express no variation.

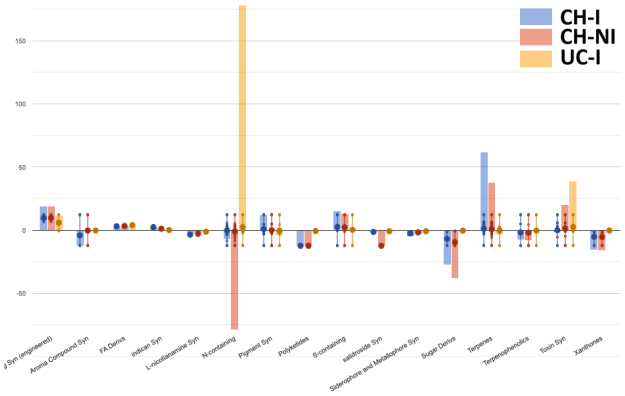

Figure 6. Biosynthesis of secondary metabolites induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto K5BB at the first sampling time (5 May 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

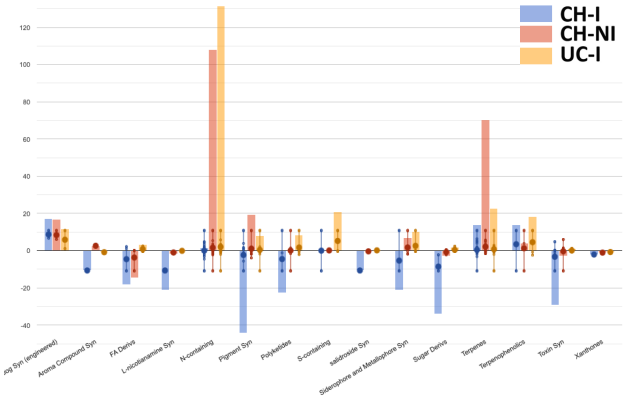

Figure 7. Biosynthesis of secondary metabolites induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto SO4 at the first sampling time (5 May 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

Figure 6 (K5BB) shows an upregulation of terpenes for CH-I and CH-NI compared to UC-NI. This is confirmed in figure 7 in the case of SO4, where also UC-I shows an increase. Figures 6 and 7 shows also that the nitrogenous compounds (N-containing) are abundant in UC-I for K5BB and in CH-NI and UC-I for SO4.

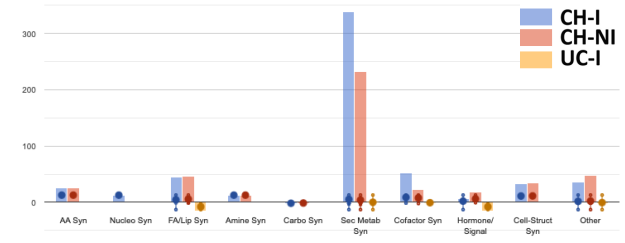

Figure 8. Biosynthesis of compounds induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto K5BB rootstock at the second sampling (7 June 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

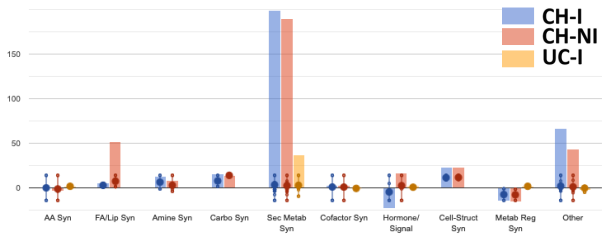

Figure 9. Biosynthesis of compounds induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto SO4 rootstock at the second sampling (7 June 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

In the second sampling (7 June) a higher activity of secondary metabolisms (Figg. 8 and 9) was observed compared to first sampling (5 May).

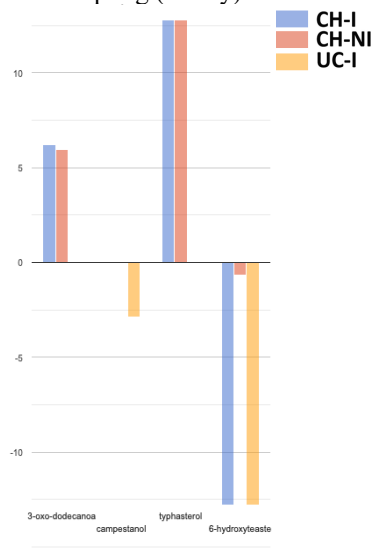

Figure 10. Biosynthesis of phythormones induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto K5BB rootstock at the second sampling (7 June 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

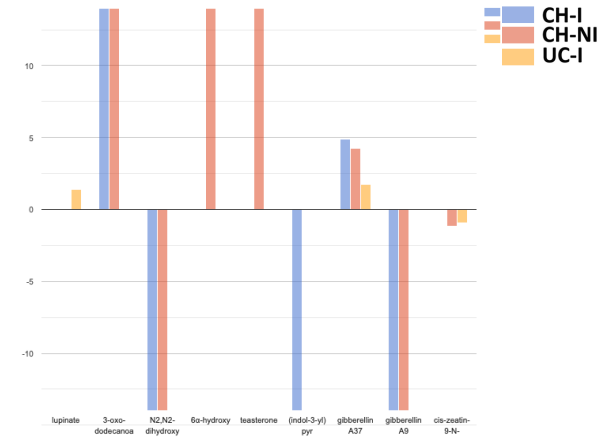

Figure 11. Biosynthesis of phythormones induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto SO4 rootstock at the second sampling (7 June 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

Among the secondary metabolites, sterols, pigments, terpenes and nitrogen containing molecules were enhanced by CH-I treatments, as well as hormones such as 3-oxo-dodecanoate and typhasterol[3]. (Figg. 10 and 11).

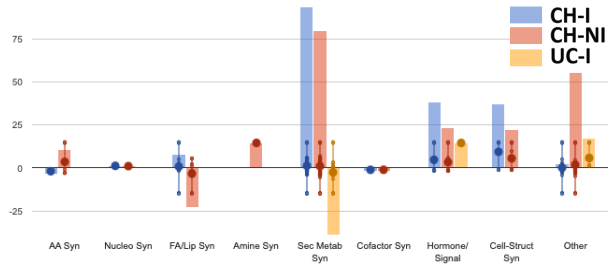

Figure 12. Biosynthesis of compounds induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto K5BB rootstock at the third sampling (12 July 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

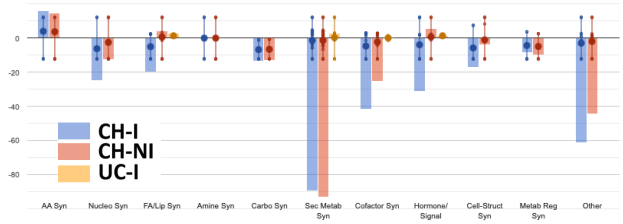

Figure 13. Biosynthesis of compounds induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto SO4 rootstock at the third sampling (12 July 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

In the third sampling (12 July) the data confirm previous observation of chitosan effect on secondary metabolism (Figg. 12 and 13).

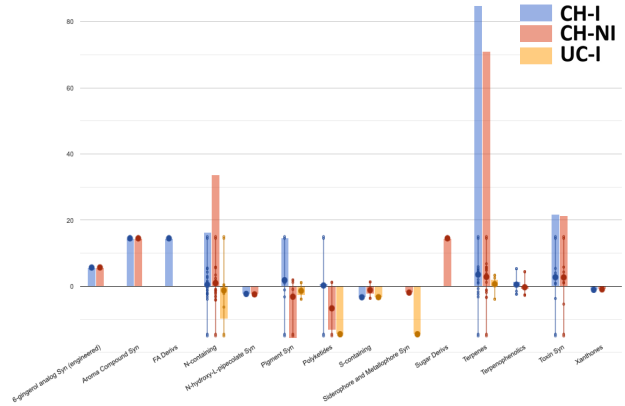

Figure 14. Biosynthesis of secondary metabolites induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto K5BB rootstock at the third sampling (12 July 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

[3] Typhasterol is a molecule belonging to the class of brassinosteroids. It is a plant growth regulator and stress response molecule involved into cell elongation and division, photosynthesis enhancement and managing of abiotic stress, such as in the case of drought.

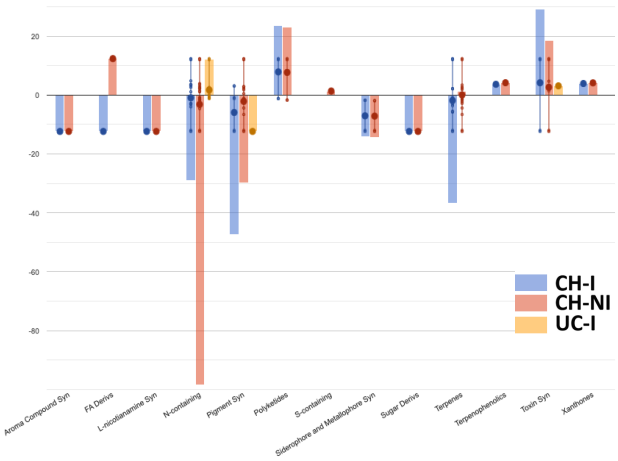

Figure 15. Biosynthesis of secondary metabolites induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in leaves from vines grafted onto SO4 rootstock at the third sampling (12 July 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

Interestingly, terpenes show an increase due to chitosan application in vines of CH-I grafted on K5BB, but not on SO4 (Figg. 14 and 15).

3.3.2. Berries

Focusing on the secondary metabolites, an abundant biosynthesis of pigments, terpenes and N-containing molecules was observed, especially for K5BB (Figg. 16 and 17).

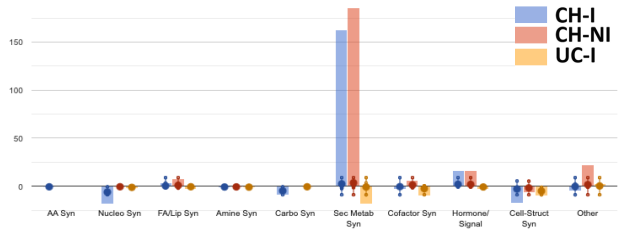

Figure 16. Biosynthesis of compounds induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in grape berries from vines grafted onto K5BB rootstock at the second sampling (7 June 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

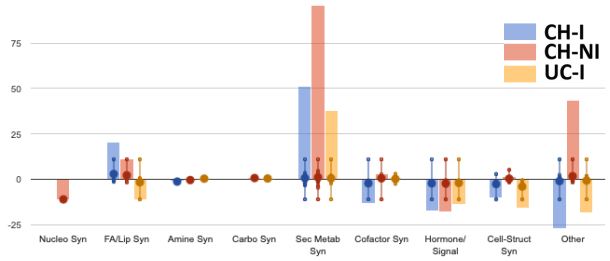

Figure 17. Biosynthesis of compounds induced by chitosan and corresponding Log fold change compared to the untreated, non-inoculated control (UC-NI) in grape berries from vines grafted onto SO4 rootstock at the second sampling (7 June 2023). CH-I = Chitosan treated and downy mildew inoculated; CH-NI = chitosan treated and non-inoculated; UC-I = untreated control and downy mildew inoculated.

4. Conclusions

Chitosan canopy treatments significantly reduced the downy mildew infections on both leaves and berries. As revealed by metabolomic analysis, the response is induced by elicited secondary metabolic pathways with production of polyphenols, nitrogenous compounds and terpenes, acting as defense mechanisms of the vine against diseases. The response of the plant is modulated by the time of application and by the rootstock.

5. Acknowledgements

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