Role of chitosan against grapevine downy mildew

Luigi Bavaresco[1], Edmondo Belisomo[1], Margherita Furiosi[1], and Luigi Lucini[2]

[1]Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy
[2]Department of Sustainable Food Process, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy

Abstract. The study of environmentally friendly active principles against downy mildew represents one of the main challenges in viticulture. In this context, the aim of the present trial was to investigate the efficacy of chitosan as a resistance elicitor against downy mildew and to study the metabolites involved in the interaction plant-chitosan-pathogen by a metabolomics approach. The experiments were carried out on potted vines of Merlot R18 grafted on SO4 rootstock, Guyot trained, during 2022. Leaf disks pre-treated with chitosan were infected by *Plasmopara viticola* sporangia in 3 phenological phases and the development of infection was recorded. The most significant findings were: 1) Chitosan resulted as an effective elicitor of defense mechanism against *Plasmopara viticola*. 2) The elicitor activity of chitosan was explained mainly through the induction of some secondary metabolites (terpenes and resveratrol), fatty acids (involved in the biosynthesis of sterols), and hormones (brassinosteroids and abscisic acid). 3) The best efficacy corresponded to the application of chitosan 48 hours before the infection. The timing of the treatment was, therefore, one of the key factors for the success of chitosan treatment.

1 Introduction

Downy mildew is one of the most serious diseases that affects *Vitis vinifera* and can cause huge production losses, both quantitatively and qualitatively. The causal agent of this disease is *Plasmopara viticola*, a microorganism belonging to the class of *Oomycetes*, order *Peronosporales*, family *Peronosporaceae*.

The identification of sustainable plant protection strategies, which are both effective and easy to apply, represents one of the most important challenges.

Natural products like chitosan could be a good alternative for a sustainable protection plan ([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 polymers in nature present in waste product of the fishing industry, extracted mainly from the exoskeleton of crustaceans such as lobsters, crabs and shrimps. On plants it has the dual function of growth regulator and resistance ([2]).

Its action is carried out through 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 of plant tissues ([3]).

The goal of the paper is to verify the efficacy of chitosan as an inducer of resistance against downy mildew and to verify which metabolites are involved in the resistance reaction.

2 Materials and methods

The trial was carried out outdoor, in the platform of the Department of Viticulture of the Università Cattolica del Sacro Cuore in Piacenza (45.0374773, 9.7283258; 46 m asl), during 2022.

The experiment included 3-year-old potted Merlot R18 vines grafted onto SO4 rootstock and Guyot trained. Spray treatments were not provided to avoid interference with chitosan.

The present study considered chitosan hydrochloride produced by Agrilaete company (Crepaldo, Venezia, Italy), applied at a concentration of 2 g/L on the canopy by using a manual nebulizer.

Treatments were performed at 3 different phenological phases: a) separated flower buttons (May 9th); b) fruit set (June 15th); c) berry touch (July 18th). After each treatment the 4th leaf (from the tip) of every shoot was sampled and fungal inoculation was performed on leaf disks. The samplings and inoculations were done 24, 48, and 72 hours after the chitosan treatment, by placing 4 drops (10 µL each) of inoculum suspension (10^4-10^5 sporangia/mL) on each leaf disk (14 mm diameter) placed in petri dishes. All plates were placed in thermostat at 20°C. After 24 hours the extra inoculum on the inoculated disks was removed.

After the inoculations, the status of the infections was monitored periodically and after a week the incidence and severity of infection were visually assessed for each disk. The incidence of the infection was assessed by counting the number of sporulated drops on each disk, while the severity of the infection was assessed on a scale from 0 to 3, where 0 indicated absent sporulation and 3 very important sporulation.

The incidence of the disease (expressed as % of infected leaf area) and intensity of sporulation (expressed as a class of damage) were processed according to an analysis of variance (one-way ANOVA) 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.

Metabolomic analysis was done on leaf disks of the second treatment (June 15th), inoculated after 48 hours, because the induction of resistance (caused by the

application of chitosan) was the highest. The leaf disks were analyzed at the time of symptom assessment, one week 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 ([4]). Briefly, samples were extracted in 0.1% HCOOH 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 ([5]).

Untreated and uninoculated leaf discs were also included, as control.

3 Results

First treatment (May 9th)

24 hours after the treatment with chitosan, the leaf discs treated with chitosan and not inoculated presented no attack (Fig. 1). Also the untreated and non-inoculated leaf discs presented no attack. The leaf discs treated with chitosan and inoculated presented an average incidence of the attack equal to 68.3%, with an intensity of sporulation equal to 2.6/3. The untreated and inoculated leaf discs presented an average incidence of the attack equal to 98.3%, with an intensity of sporulation equal to 3/3.

All data were statistically different.

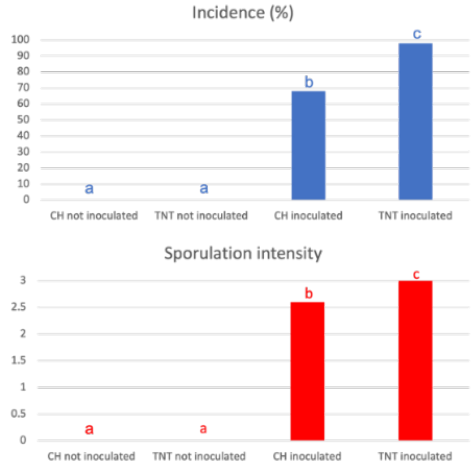

Figure 1. Inoculation 24 hours after the first treatment with chitosan (May 9th, 2022): downy mildew incidence (%) and sporulation intensity, depending on the treatment and the inoculation. TNT: not treated leaf disks. CH: treated leaf disks.

Forty eight hours after treatment with chitosan, the leaf discs treated with chitosan and not inoculated presented no attack (Fig. 2). Also the untreated and non-inoculated leaf discs presented no attack. The leaf discs treated with chitosan and inoculated presented an average incidence of attack equal to 21.6%, with a sporulation intensity of 0.87/3. The untreated and inoculated leaf discs presented an average incidence of the attack equal to 100%, with an intensity of sporulation equal to 3/3.

All data were statistically different.

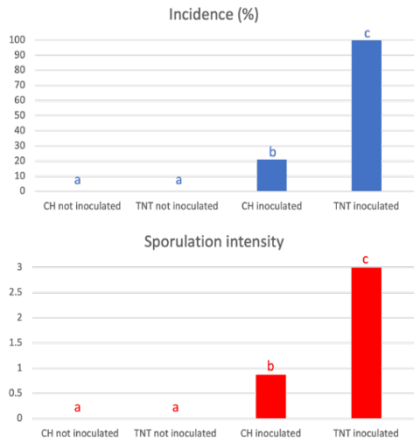

Figure 2. Inoculation 48 hours after the first treatment with chitosan (May 9th, 2022): downy mildew incidence (%) and sporulation intensity, depending on the treatment and the inoculation. TNT: not treated leaf disks. CH: treated leaf disks.

Second treatment (June 15th)

At 24 hours after treatment, the leaf discs treated with chitosan and not inoculated presented no attack (Fig. 3). Also the untreated and non-inoculated leaf discs presented no attack. The leaf discs treated with chitosan and inoculated presented an average incidence of attack equal to 48.3%, with an intensity of sporulation equal to 1.2/3. The untreated and inoculated leaf discs presented an average incidence of attack equal to 95%, with a sporulation intensity equal to 3/3.

All data were statistically different.

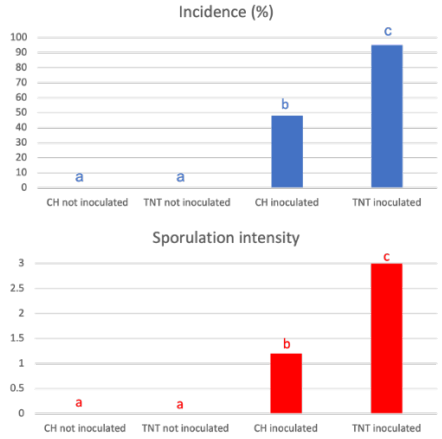

Figure 3. Inoculation 24 hours after the second treatment with chitosan (June 15th, 2022): downy mildew incidence (%) and sporulation intensity, depending on the treatment and the inoculation. TNT: not treated leaf disks. CH: treated leaf disks.

At 48 hours after treatment with chitosan, the leaf discs treated with chitosan and not inoculated presented no attack (Fig. 4). Also the untreated and non-inoculated leaf discs presented no attack. The leaf discs treated with chitosan and inoculated presented an average incidence of the attack equal to 15%, with an intensity of sporulation equal to 0.47/3. The untreated and inoculated leaf discs

presented an average incidence of attack equal to 100%, with a sporulation intensity of 2.87/3.

All data were statistically different.

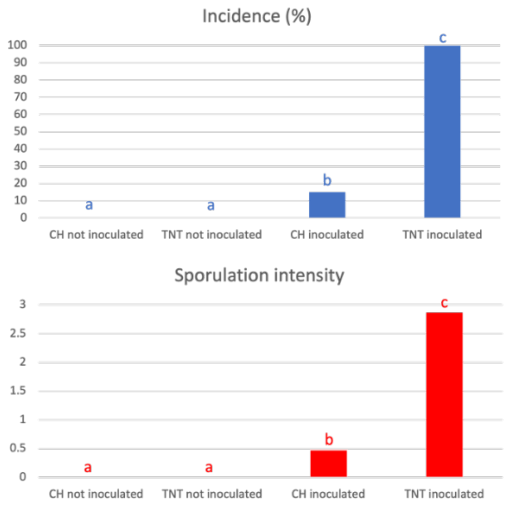

Figure 4. Inoculation 48 hours after the second treatment with chitosan (June 15th, 2022): downy mildew incidence (%) and sporulation intensity, depending on the treatment and the inoculation. TNT: not treated leaf disks. CH: treated leaf disks.

At 72 hours after treatment, the leaf discs treated with chitosan and not inoculated presented no attack (Fig. 5). Also the untreated and non-inoculated leaf discs presented no attack. The leaf discs treated with chitosan and inoculated presented an average incidence of attack equal to 36.6%, with a sporulation intensity of 1.67/3. The untreated and inoculated leaf discs presented an average incidence of attack equal to 98.3%, with a sporulation intensity of 2.87/3.

All data were statistically different.

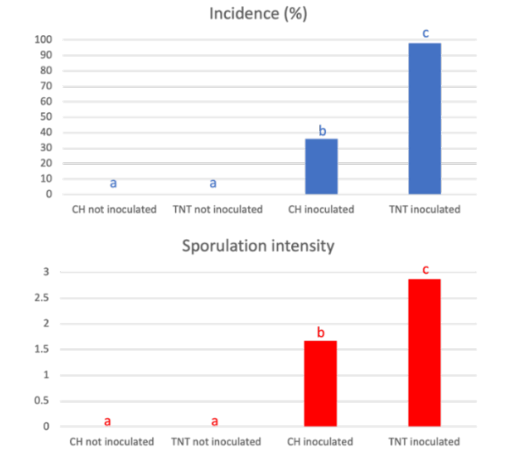

Figure 5. Inoculation 72 hours after the second treatment with chitosan (June 15th, 2022): downy mildew incidence (%) and sporulation intensity, depending on the treatment and the inoculation. TNT: not treated leaf disks. CH: treated leaf disks.

Third treatment (July 18th)

At 24 hours after treatment, the leaf discs treated with chitosan and not inoculated presented no attack (Fig. 6). Also the untreated and non-inoculated leaf discs presented no attack. The leaf discs treated with chitosan and inoculated presented an average incidence of attack equal to 36.6%, with a sporulation intensity of 1.67/3. The untreated and inoculated leaf discs presented an average incidence of attack equal to 98.3%, with a sporulation intensity of 2.87/3.

All data were statistically different.

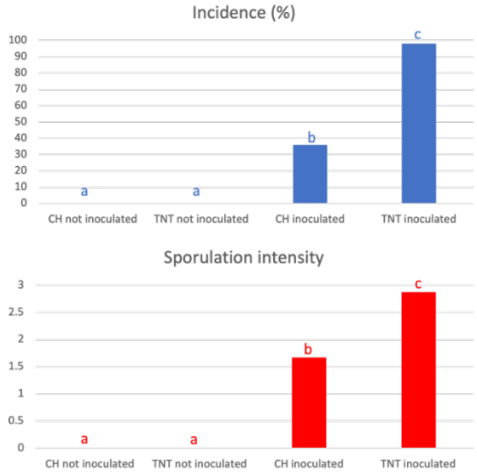

Figure 6. Inoculation 24 hours after the third treatment with chitosan (July 18th, 2022): downy mildew incidence (%) and sporulation intensity, depending on the treatment and the inoculation. TNT: not treated leaf disks. CH: treated leaf disks.

At 48 hours after treatment, the leaf discs treated with chitosan and not inoculated presented no attack (Fig. 7). Also the untreated and non-inoculated leaf discs presented no attack. The leaf discs treated with chitosan and inoculated presented an average incidence of attack equal to 36.6%, with a sporulation intensity of 1.67/3. The untreated and inoculated leaf discs presented an average incidence of attack equal to 98.3%, with a sporulation intensity of 2.87/3.

All data were statistically different.

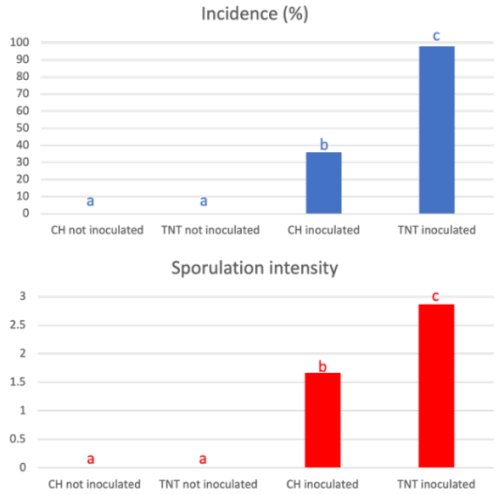

Figure 7. Inoculation 48 hours after the third treatment with chitosan (July 18th, 2022): downy mildew incidence (%) and sporulation intensity, depending on the treatment and the inoculation. TNT: not treated leaf disks. CH: treated leaf disks.

At 72 hours after treatment, the leaf discs treated with chitosan and not inoculated presented no attack (Fig. 8). Also the untreated and non-inoculated leaf discs presented

no attack. The leaf discs treated with chitosan and inoculated presented an average incidence of the attack equal to 55.5%, with an intensity of sporulation equal to 2.2/3. The untreated and inoculated leaf discs presented an average incidence of attack equal to 78.3%, with a sporulation intensity of 2.4/3.

All data were statistically different.

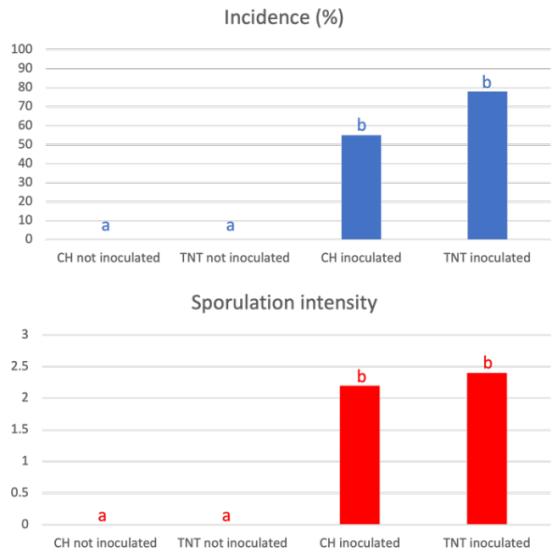

Figure 8. Inoculation 72 hours after the third treatment with chitosan (July 18th, 2022): downy mildew incidence (%) and sporulation intensity, depending on the treatment and the inoculation. TNT: not treated leaf disks. CH: treated leaf disks.

The metabolomic analysis carried out in 2022 on the samples of the second sampling at 48 hours from the treatment identified 1611 compounds produced by the leaves under analysis.

A multivariate analysis was used to investigate the metabolic profiles of each treatment. First, an unsupervised cluster model was carried out considering treatment with chitosan and *Plasmopara viticola inoculation* as factors. The unreviewed model showed that the metabolic profiles of the leaves were significantly affected by the application of chitosan. In fact, this analysis led to the observation of two main clusters, distinguishing the leaf discs not treated from the leaf discs treated with chitosan, and, within each cluster, to the separation of the inoculated leaf discs from the non-inoculated leaf discs.

The heatmap (Fig. 9) shows the abundance of compounds within the dataset. In the graph, different colors indicate a different abundance of compounds in clusters calculated on the fold change with respect to the median. A positive change is indicated in red, no change in yellow and a negative variation of the compound in blue. The metabolites identified in the different samples can therefore be found with a greater abundance, less than or equal to the median of the entire dataset.

Subsequently, OPLS-DA supervised analysis (Fig. 10) allowed to visualize the distribution of samples in space according to the treatment applied. This supervised modelling confirmed the presence of different metabolic profiles in the various leaf discs, as seen with the unsupervised cluster model, and confirmed that chitosan was the main factor in sample separation. The leaf discs treated with chitosan were clearly distinguished from the leaf discs not treated independently of inoculation, demonstrating that treatment with chitosan influenced the metabolomic profiles of the leaf discs such as to make the treated leaf discs distinguishable from the leaf discs not treated.

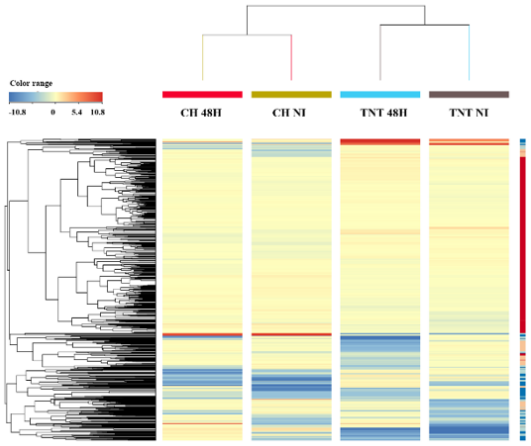

Figure 9. Heatmap: analysis of the unsupervised hierarchical cluster analysis. CH 48H: leaf disks treated with chitosan and inoculated. CH NI: leaf disks treated with chitosan and not inoculated with downy mildew. TNT 48H: untreated leaf disks and inoculated. TNT NI: untreated leaf disks and not inoculated.

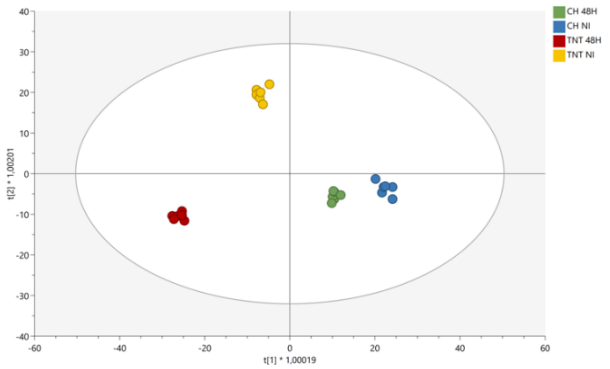

Figure 10. OPLS-DA supervised analysis. CH 48H: leaf disks treated with chitosan and inoculated. CH NI: leaf disks treated with chitosan and not inoculated. TNT 48H: untreated leaf disks and inoculated. TNT NI: untreated leaf disks and not inoculated.

Treatment with chitosan therefore induced a metabolic response by plants. This induction occurred through the regulation of some biosynthetic pathways. For this reason, the significantly induced compounds were subjected to the analysis of the main metabolic pathways.

In Figure 11 we have the abundance of compounds belonging to the above classes for the leaf discs “treated with chitosan and inoculated”, “treated with chitosan and not inoculated” and “not treated with chitosan and inoculated” based on the Log fold changes compared to the leaf discs “not treated with chitosan and not inoculated”. In particular, the biosynthesis of amino acids was stimulated in all leaf discs, in particular in the leaf discs “not treated with chitosan and inoculated” and in the leaf discs “treated with chitosan and not inoculated”. The biosynthesis of nucleic acids was stimulated in all leaf discs, in particular in the leaf discs “treated with chitosan and not inoculated” and slightly in the leaf discs “not

treated with chitosan and inoculated". The biosynthesis of fatty acids and lipids was particularly stimulated in all leaf discs, especially in the leaf discs "treated with chitosan and inoculated" and "treated with chitosan and not inoculated". Regarding amine biosynthesis, this metabolic pathway has not been induced in a major way, except for the leaf discs "not treated with chitosan and inoculated". Carbohydrate biosynthesis was induced only in the leaf discs "treated with chitosan and not inoculated", although not relevant. Secondary metabolites are the class of compounds whose biosynthesis has been most induced in all the leaf discs under analysis, in particular in the leaf discs "treated with chitosan and not inoculated". On the other hand, the biosynthesis of cofactors was poorly induced in all leaf discs, especially in the leaf discs "treated with chitosan and not inoculated". Finally, hormone biosynthesis was also induced in all leaf discs, especially in the "treated with chitosan and inoculated" leaf discs.

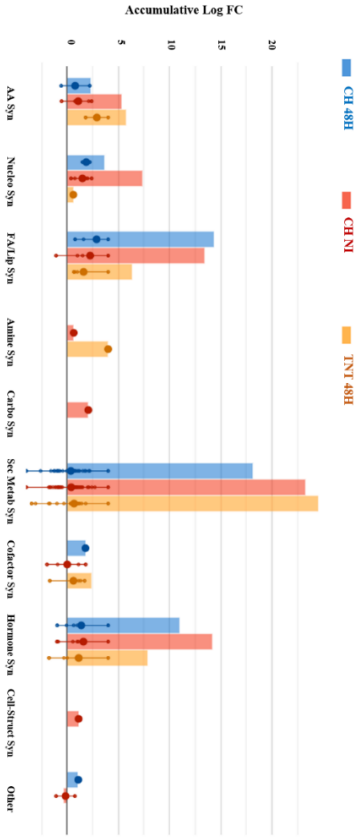

Figure 11. Compounds significantly induced by chitosan and related Log fold change compared to the untreated and uninoculated leaf disks. CH 48H: leaf disks treated with chitosan and inoculated with downy mildew. CH NI: leaf disks treated with chitosan and not inoculated. TNT 48H: untreated leaf disks and inoculated.

As reported in (Figs. 12-14), the biosynthesis of secondary metabolites, fatty acids and lipids and hormones were the most induced by the treatment with chitosan.

As far as secondary metabolites are concerned, the metabolic pathways of the biosynthesis of terpenes, phytoalexins and phenylpropanoid were more stimulated (Fig. 12).

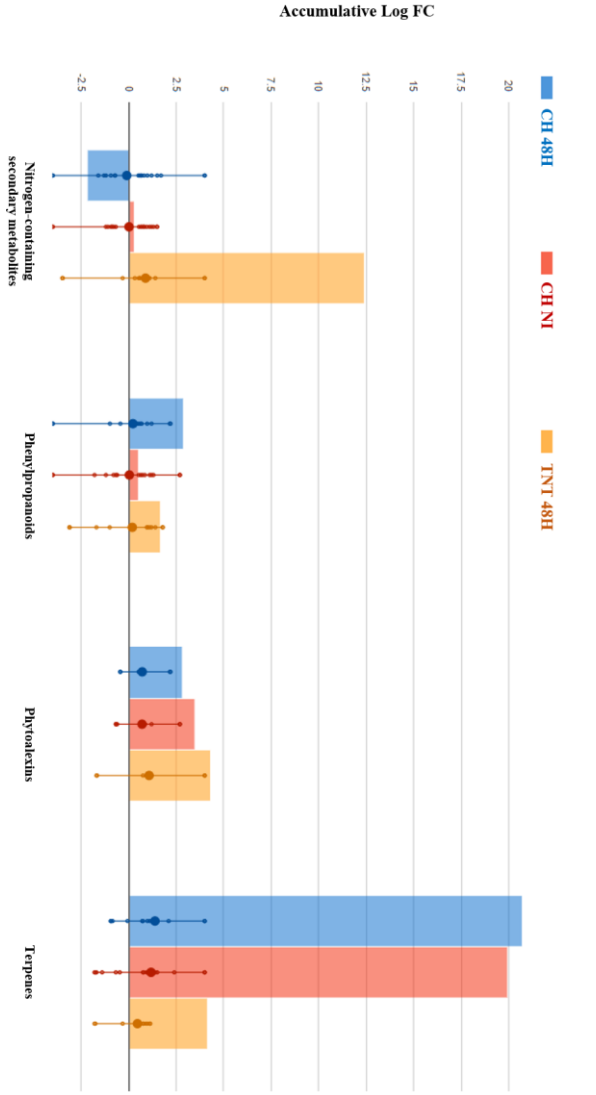

Figure 12. Fold change of some secondary metabolites compared to the untreated and not inoculated leaf disks. CH 48H: leaf disks treated with chitosan and inoculated with downy mildew. CH NI: leaf disks treated with chitosan and not inoculated. TNT 48H: untreated leaf disks and inoculated.

With regard to fatty acids and lipids, an important induction of the biosynthetic pathway of sterols was detected (Fig. 13).

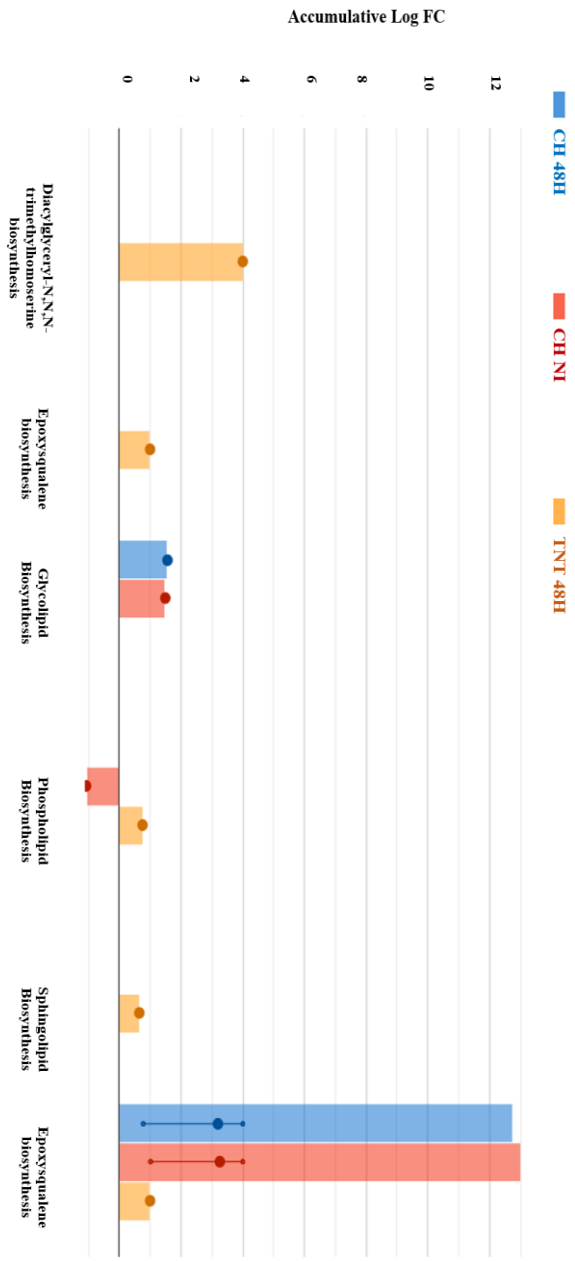

Figure 13. Fold change of some lipids compared to the untreated and not inoculated leaf disks. CH 48H: leaf disks treated with chitosan and inoculated with downy mildew. CH NI: leaf disks treated with chitosan and not inoculated. TNT 48H: untreated leaf disks and inoculated.

Finally, with regard to hormones, the modulation of some important hormones in plant defense, such as brassinoteroids, abscisic acid and giberelline, was detected (Fig. 14).

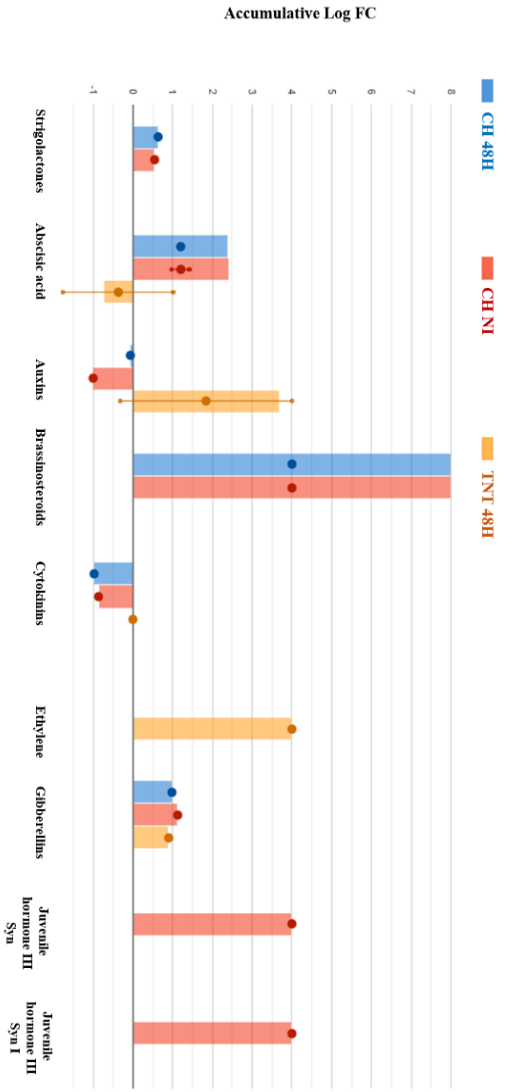

Figure 14. Fold change of some hormones compared to the untreated and not inoculated leaf disks. CH 48H: leaf disks treated with chitosan and inoculated with downy mildew. CH NI: leaf disks treated with chitosan and not inoculated. TNT 48H: untreated leaf disks and inoculated.

4 Discussion

The first treatment was carried out on 9th May when the plants were in the phenological phase of separated flower buttons. In temperate climates, in this phase the vines are particularly susceptible to downy mildew attacks due to the climatic conditions favorable to the germination of the spores and the high receptivity of the tissues in full growth.

The data obtained in this test confirm our hypothesis and highlight an important containment of both the incidence and intensity of infection when chitosan is applied 48 hours before inoculation of the pathogen.

To verify whether the effect of chitosan persists or decays over time, in the second and third trials of 2022 *Plasmopara viticola* was inoculated even 72 hours after chitosan treatment.

The second treatment was carried out on June 15th, when the plants under analysis were in the phenological phase of fruit set. Also in this case, the data obtained confirm the initial hypothesis and highlight an important containment of both the incidence and intensity of the infection when chitosan is applied 48 hours before inoculation of the pathogen. The slight decrease in the incidence and intensity of sporulation found in all the timing of this second test is probably due to the acquisition of ontogenetic resistance by the leaves, which were thus less susceptible to downy mildew attack. In addition, the effectiveness of resistance induction caused by chitosan begins to decrease already at 72 hours after the treatment.

The third treatment was carried out on July 18th when the plants under analysis were in the phenological phase of berry touch. In this case, however, the mean incidences and the sporulation intensity of the untreated leaf disks were not significantly different from those of the treated ones. This probably confirms the hypothesis that the resistance induction effect due to chitosan drops considerably at 72 hours from treatment with the preventive principle. Also in this phenological phase, chitosan was particularly effective at 48 after treatment.

As concerning the metabolomics approach, both the unsupervised statistical analysis and the supervised statistical survey demonstrated the elicitatory effect of chitosan: it was possible to clearly distinguish the metabolic profiles of the treated (with chitosan) leaf disks from the untreated ones. The elicitory effect of chitosan is shown above all by the increase in the abundance of certain metabolites. 82 metabolites were found with greater abundance in the leaf disks treated with chitosan than in the untreated ones. This phenomenon is common in plant organisms subjected to biotic or abiotic stress.

The most induced classes of metabolites were secondary metabolites and fatty acids and lipids. The present experimentation confirms, as already reported in multiple studies, the role of phenylpropanoids, phytoalexins and sterols in the induction of resistance ([3]). In addition, it is interesting to note the considerable increase in the synthesis of terpene compounds resulting from treatment with chitosan, as already observed by ([4]). A modulation of the metabolism of some hormones, such as abscissic acid, brassinosteroids and giberellins, has also been found. These are important bioregulators that trigger cascading effects on the metabolic processes put in place to counteract the impact of stress on the plant.

5 Conclusion

The trial results confirm the efficacy of chitosan as a priming effect against *Plasmopara viticola*. The elicitatory activity of chitosan is expressed through the induction of compounds belonging to different classes, such as secondary metabolites, fatty acids, lipids and hormones.

The reduction in the incidence and intensity of the fungal attack are maximum when the priming principle is applied 48 hours before the fungal attack, while they begin to decrease already 72 hours after treatment. The timing of treatment is therefore one of the key factors for the success of the anti-downy mildew defense with chitosan.

Since the chitosan application has not completely overcome the fungal attack, chitosan is proposed as a biological adjuvant for downy mildew defense. Further studies are needed to verify the efficacy of chitosan in open field conditions.

References

1. Vasyukova N.I., Zinov'eva S.V., Il'inskaya L.I., Perekhod E.A., Chalenko G.I., Gerasimova N.G., Il'ina A.V., Varlamov V.P., Ozeretskovskaya O.L., *Modulation of plant resistance to disease by water-soluble chitosan.* Applied Biochemistry and Microbiology **37**, 103-109 (2001)
2. Romanazzi G., Feliziani E., Sivakumar D., *Chitosan, a biopolymer with triple action on postharvest decay of fruit and vegetables: Eliciting, antimicrobial and film- forming properties*. Frontiers in Microbiology **9**, 2745 (2018)
3. Bavaresco L., Zamboni M., Squeri C., Xu S., Abramowicz A., Lucini L., *Chitosan and grape secondary metabolites: a proteomics and metabolomics approach.* BIO Web of Conferences **9**, 01004 (2017)
4. Pretali L., Bernardo L., Butterfield T.S., Trevisan M., Lucini L. Phytochem. **130**, 56 (2016)
5. Lucini, L., Baccolo, G., Rouphael, Y., Colla, G., Bavaresco, L., & Trevisan, M., *Chitosan treatment elicited defence mechanisms, pentacyclic triterpenoids and stilbene accumulation in grape (Vitis vinifera L.) bunches*. Phytochemistry **156**, 1–8 (2018)