Chemosphere 316 (2023) 137772

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Interaction effect of fungicide and chitosan on non-target lichenized fungi

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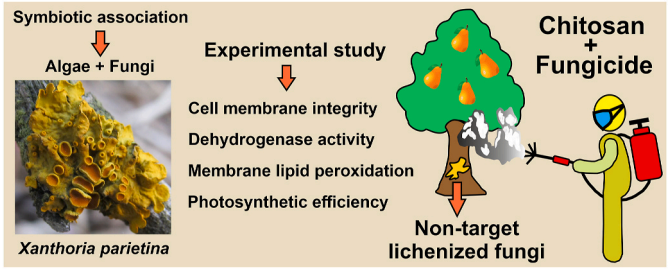
HIGHLIGHTS

- Combined usage of synthetic fungicides and chitosan may impair lichen physiology.
- The disturbances relate to both the algal and fungal partner of symbiotic association.
- Increased membrane lipid peroxidation and cell membrane damage were observed.
- Disturbances in the functioning of mitochondrial respiratory chain was recognized.
- Plant protection agents deserve attention due to their impact on non-target fungi.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Marisa Passos

Keywords:
Lichen
Xanthoria parietina
Chlorophyll fluorescence
Cell membrane damage
Lipid peroxidation
Dehydrogenase activity

ABSTRACT

Excessive use of plant growth stimulants and pesticides is currently a considerable problem, especially in agriculture, horticulture, and arboriculture. Understanding the impacts of these compounds and their combinations on non-target organisms is crucial to minimize unintended consequences, while maintaining their use in plant protection. The aim of this study was to test how long-term spraying with different solutions of natural biostimulator chitosan, synthetic fungicide Switch 62.5 WG, and their combinations affects the physiology of epiphytic lichen *Xanthoria parietina* naturally occurring in fruit orchards and farmlands. We showed that fungicides composed of fludioxonil and cypronidil, as well as the combined use of such fungicides together with chitosan, can cause the considerable impairment of lichen physiology, and these disturbances relate to both algal and fungal partners of the symbiotic association. This negative effect was especially visible in the loss of cell membrane integrity, the high level of membrane lipid peroxidation, and changes in chlorophyll fluorescence parameters on the last day of the experiment. The combined use of these agents also leads to clear disturbances in the functioning of the mitochondrial respiratory chain, which was manifested by increased NADH dehydrogenase activity, while the use of these compounds separately led to a decrease in the activity of this enzyme. We concluded that the regular use of these agents in fruit tree cultivation may cause serious ecological consequences for epiphytic lichen communities as a result of the death of lichen thalli. This study suggests that the impact of some plant protection agents, both individually and in combinations, merits further attention in terms of their impact on non-target fungi.

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https://doi.org/10.1016/j.chemosphere.2023.137772
Received 4 October 2022; Received in revised form 14 December 2022; Accepted 4 January 2023
Available online 6 January 2023

1. Introduction

The excessive use of plant growth stimulants and pesticides is currently a considerable problem, especially in agriculture, horticulture, and arboriculture. Since the use of synthetic fungicides is constantly growing due to the development of the resistance of pathogenic microorganisms, new alternatives that are less harmful to the environment are constantly being sought (Sharma et al., 2019). One such proposal is the use of fungicides in combination with the natural biostimulant and plant protector chitosan to enhance the antifungal action of fungicides. For example, the most recent study showed a positive effect when using low-molecular-weight chitosan in combination with synthetic fungicides against *Botrytis cinerea* (Karpova et al., 2021). Such an approach could significantly reduce the number of synthetic fungicides used, but there is a gap in knowledge on how these affect non-target organisms in interaction with each other.

Chitosan is a natural biopolymer used as a plant stimulant due to its intrinsic properties (Aranaz et al., 2009). Many studies have shown its beneficial effect on plant functional traits, e.g., it induced an increase in chlorophyll content (Chookhongkha et al., 2012), extended the shelf life of fruits (Islam et al., 2018) or improved the drought resistance of seedlings (Dzung et al., 2011). Moreover, chitosan could improve photosynthetic performance and stimulate antioxidant enzyme activity in plants under stress conditions (Zong et al., 2017). Furthermore, chitosan has broad antimicrobial activity against various bacteria and fungi (see Ke et al., 2021a). The mode of action of chitosan against microorganisms involves both intracellular and extracellular effects. There are three main mechanisms proposed as the inhibition mode of chitosan. In the first mechanism, chitosan affects the plasma membrane by causing the release of cellular contents due to the electrostatic interaction between chitosan and phospholipid components of cell membranes (Liu et al., 2004; Xing et al., 2018). Regarding the second mechanism, chitosan acts as a chelating agent that prevents cell growth by binding trace elements and nutrients (Rabea et al., 2003; Roller and Covill, 1999; Skjak-Braek et al., 1989). The third mechanism suggests that chitosan may interact with DNA and, as a result, affect the production of crucial proteins and enzymes (Ing et al., 2012; Kong et al., 2010). Moreover, chitosan can inhibit the mitochondrial function and ATP production (Ke et al., 2021b).

Switch 62.5 WG (Syngenta, Basel, Switzerland) is a commercially available fungicide containing cyprodinil and fludioxonil, having broad-spectrum action against fungi and a wide range of applications. Fludioxonil is a phenylpyrrole fungicide derived from the antibiotic pyrrolnitrin, which was first isolated from bacteria in the genus *Pseudomonas* (Arima et al., 1964; Brandhorst and Klein, 2019). Phenylpyrroles have an inhibitory effect on all stages of fungal development, including spore germination and mycelial growth (Leroux et al., 1992). Fludioxonil is a non-systemic surface fungicide, used for treatments at pre- and post-harvest stages on seeds, leaves, and fruits (Kilani and Fillinger, 2016). Due to its broad-spectrum of antifungal activity against various types of plant pathogens, it is used for their control, but it also has prophylactic activity against a fungal disease provoked by ascomycetes or basidiomycetes (Kilani and Fillinger, 2016; Taiwo et al., 2021). Phenylpyrroles cause an accumulation of metabolites that induces hyphal swelling and burst, membrane hyperpolarization, and changes in carbon metabolism (Kilani and Fillinger, 2016). Cells of plants, fungi and algae could accumulate high levels of osmolytes providing internal hydrostatic pressure regulated mainly by an osmotic MAP kinase cascade, which could operate in two ways: activating glycerol synthesis or uptaking ions from the extracellular medium (Lew, 2010). Fludioxonil, in the absence of hyperosmotic stress, leads to the activation of the osmotic MAP kinase cascade, which leads to cell death (Lew, 2010). The second compound, i.e., cyprodinil, belongs to the chemical class of anilinopyrimidines (Neumann et al., 1992). It is used for the protection of fruits, cereals, and vegetable crops from a wide range of pathogens and is considered safe for consumers and the environment (Heye et al., 1994). The negative effect of this fungicide on phytopathogenic fungi is associated with its ability to inhibit the biosynthesis of methionine, the suppression of cystine and cysteine biosynthesis, and the secretion of hydrolytic enzymes (Hou et al., 2018; Petsikos-Panayotarou et al., 2003). The mode of the action of cyprodinil is not fully understood; however, the primary target is assumed to be cystathionine β-lyase and cystathionine γ-synthase (Fritz et al., 2003; Masner et al., 1994).

Due to the large use of synthetic fungicides in crops all over the world, further studies are very important in the context of their impact on organisms in the surrounding environment. Fungicide sales amounts to 40% of total pesticide sales in the European Union (EUROSTAT database, 2022); moreover, the use of fungicides is likely to increase regionally due to climate changes, and the development of fungicide resistance (Zubrod et al., 2019). Most studies focused on the influence of agrochemical fungicides on aquatic ecosystems and showed a strongly negative effect on many non-target organisms (Zubrod et al., 2019), including fungi. For example, fungicides proved to impoverish aquatic fungal communities, reduce biomass and the sporulation of decomposer fungi (Pimentão et al., 2020), and disrupt host-parasite interactions by decreasing the infectivity of microparasitic yeast in freshwater ecosystems (Machado et al., 2022).

Lichens constitute symbiotic associations central to which are a fungus and a phototroph (alga and/or cyanobacteria). The influence of environmental contaminants on the physiological condition of lichens is particularly challenging to predict, due to the potentially different effect of stressors on the fungus and phototroph, separately, and on lichens as a whole unit. In these organisms both symbiotic partners form a well-defined structural and functional unit; however, they are composed of two main partners of completely different metabolisms and the details of these heterotroph-phototroph metabolic interactions are still poorly understood (Spribille et al., 2022). Lichens grow on a wide range of substrates and habitats, including areas used for agriculture. They are particularly responsive to various pollutants and agents, since due to a lack of root systems, protective cuticles, and filtration mechanisms, they uptake both nutrients and toxic compounds throughout the entire surface of the thallus, directly from the surrounding environment (Nimis et al., 2002). Numerous studies have been carried out on the effects of various types of biocides, e.g., glyphosate (Vannini et al., 2015), MCPA (Sujetovienė et al., 2019) and Koretrel (Tretiach et al., 2007) on lichen physiology. The results showed that these compounds are accumulated and cause serious physiological alterations in both the algal and fungal partner of lichen symbiosis (e.g., Vannini et al., 2016). This predisposes lichens to be important organisms for monitoring the negative biological effects of pesticides and studying their fate in the environment (Vannini et al., 2015).

In the present study, we aimed to determine the effect of fungicide, chitosan and their interaction on the physiological condition on lichenized fungi. We selected a model species - *Xanthoria parietina* (L.) Th. Fr. (Teloschistaceae, Ascomycota), which is a foliose lichen forming regular rosettes with numerous apothecia on the surface of the thallus (Nimis and Martellos, 2022). Representatives of the *Trebouxia* genus (Trebouxiophyceae, Chlorophyta) constitute the photosynthetic partners in *X. parietina* (Nyati et al., 2013). The species was chosen due to its common occurrence on trees in orchards, gardens, plantations, and along roads, i.e., in places where plant protection products are commonly used. Its common presence in farmlands and agricultural areas in many countries is additionally favoured by eutrophication (Gaio-Oliveira et al., 2005).

The main aim was to test how long-term spraying with different solutions of chitosan, Switch 62.5 WG fungicide, and their combinations affects the physiology of *Xanthoria parietina*. The following hypotheses were set: (1) chitosan would not affect the photosynthetic process carried out by the algae, while it would disturb the fungal partner's functioning; (2) Switch 62.5 WG would negatively affect the physiological condition of the mycobiont; (3) combinations of chitosan and Switch

62.5 WG would show a more harmful effect on lichen physiology than their individual effect. Both chitosan and Switch 62.5 WG are widely used in agriculture and horticulture around the world. Nevertheless, to the best of our knowledge, no studies have been conducted on the influence of these compounds and their interaction on symbiotic organisms. The present study may contribute to a more detailed definition of conditions and recommendations for the use of chitosan and fungicides, considering both their individual and combined impact on biotic components.

2. Materials and methods

2.1. Sample collection and handling

Lichen sampling was conducted in the summer season 2022. *Xanthoria parietina* thalli were collected from living trees (*Acer* sp.) in Kraków city (S Poland). A large amount of lichen material required for the entire duration of the experiment was collected, packed into litter boxes, and transported to the laboratory, where lichen thalli were carefully removed from the bark using tweezers, and the surface particles were cleaned with a soft brush.

2.2. Experimental design

Before the experiment, lichen thalli were kept in a chamber with 95% relative humidity at 20 °C and 70 μmol m^{-2} s^{-1} PAR photons for 48 h to reactivate their metabolic activity and maintain cell membrane integrity (Honegger, 2003). Lichen thalli were placed in a climatic chamber in compartments intended for experimental groups; each compartment contained the amount of lichen material required for the entire duration of the experiment.

A total of 9 experimental groups were considered (Fig. 1), which represent various combination doses of chitosan (hereinafter referred to CH) and Switch 62.5 WG fungicide (hereinafter referred to SW; cyprodinil: 375 g in 1 kg of the agent and fludioxonil 250 g in 1 kg of the agent; Syngenta, Basel, Switzerland). Low molecular weight chitosan (50,000–190,000 Da, Sigma-Aldrich) was used to prepare solutions with the following concentrations: 200 (CH200) and 1000 mg L^{-1} (CH1000). Since chitosan is slightly soluble in water, solutions were prepared by dissolving the required amount of chitosan in 100 mL of 0.5% solution of acetic acid, and then its volume was raised to 1000 mL. Then 1 (SW1) and 2 g L^{-1} (SW2) solutions of Switch 62.5 WG were prepared by dissolving the required amount of fungicide in deionized water. During the experiment two solvent controls were considered (with water and with 0.05% acetic acid). Since no significant differences in cell membrane integrity, TBARS concentration and dehydrogenase activity between solvent controls were found, it was decided to only include measurements from the solvent control with water for further statistical analyses, to obtain a fully crossed experimental design (for results see Fig. S1). The design of the experiment and the abbreviations of the experimental groups used thereafter in the text are provided in Fig. 1. All solutions were placed in sprayers. The selection of fungicide concentrations in the experiment was in line with the doses indicated by the manufacturer.

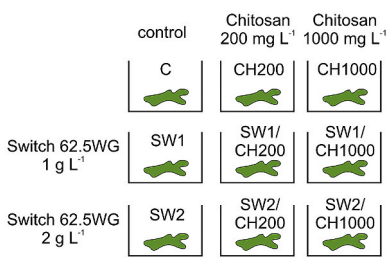

Fig. 1. Experimental design. Abbreviations of experimental groups are provided.

The solutions (ca. 15 mL) were applied every second day (day 1, 3, 5, 7, 9, 11, 13, 15), using hand sprayers to each compartment, with thalli representing a given experimental group. The spraying of lichens was carried out to best reflect the conditions to which the lichens are exposed in the natural environment, since spraying plants in horticulture is also practised. After treating the lichens with the solutions, each compartment was wiped dry. The entire experiment lasted 15 days, and consisted of 8 treatments of lichen thalli with solutions. During the experiment, except for the measurement time, the samples were exposed to photosynthetically active radiation (daytime: 70 μmol m^{-2} s^{-1} PAR photons, night-time: dark) and temperature 20 °C. The measurements of chlorophyll fluorescence were made every second day, 2 h after treatment, while cell membrane integrity, membrane lipid peroxidation, and dehydrogenase activity measurements were made 3 times on the 3rd, 7th, and 15th day of the experiment. In each compartment (representing a certain experimental group), a large amount of lichen material was placed, it was ca. 100 individual lichen thalli (individuals). Each time, the appropriate amount of lichen material required for a given analysis was randomly collected from the compartments. One whole lichen thallus (individual) or its fragment was used to construct each replicate so that the replicates for each analysis constituted separate lichen thalli that were independent. Five replicates were considered for the assessment of cell membranes integrity, the membrane lipid peroxidation level and dehydrogenase activity. For chlorophyll fluorescence measurements, ten replicates were considered.

2.3. Integrity of cell membranes

Prior to analysis, the thalli were gently rinsed in deionized water to remove unbounded particles and ions (Yemets et al., 2015). Procedure followed was as at Paoli et al. (2011), with minor modifications. The lichen samples were then air-dried, and ca. 100 mg DW of material were weighed. Each sample was soaked in 50 mL of distilled water in glass weighting bottles, covered with glass stoppers, and shaken on a vibrating shaker for 1 h (Vibramax 100, Heidolph Instruments, Germany). Next, the initial electrical conductivity (*Ci*) of the distilled water was measured using a conductivity meter (Seven Go Duo SG23-FK5, Mettler Toledo, Switzerland). After that, the conductivity of the samples was measured after soaking the thalli (*Cv*), and the samples were boiled for 10 min at 100 °C to disrupt cell membranes. The samples were then cooled to room temperature, and the conductivity was measured again (*Cf*). Finally, the relative electrical conductivity (*EC*), considered as the level of loss of membrane integrity was calculated according to the formula: $((Cv-Ci)/Cf) \times 100$ (%). Five replicates were measured for each experimental group. For a more detailed description of the measurement procedure see Osyczka and Rola (2019).

2.4. Assessment of membrane lipid peroxidation

The level of membrane lipid peroxidation in lichen samples was estimated using the thiobarbituric acid-reactive substances (TBARS) assay according to Heath and Packer (1968), with modifications of Politycka (1996). After the preceding treatment with the solutions, the thalli were left in the air at 20 °C for 24 h so that the applied agents could be absorbed inside the lichen thalli. First, ca 40 mg of lichen material were weighed. The samples were homogenised in a porcelain mortar using 1.5 mL of ice-cold 0.25% (w/v) thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA). These mixtures were then heated in a water bath (JWE 357, Elpin-Plus, Poland) at 95 °C for 30 min. After that, the samples were cooled to room temperature and were centrifuged at 12, 000×*g* for 15 min (Centrifuge 5424, Eppendorf, Poland). The absorbance of the supernatant was measured at 532 nm (Genesys 180 UV–Vis spectrophotometer, Thermo Fisher Scientific, USA), and corrected for nonspecific absorption at 600 nm. The extinction coefficient specific for thiobarbituric acid-malondialdehyde complex (TBA-MDA; 155

$mM^{-1}cm^{-1}$) was used for the calculation of the concentration of lipid peroxidation products (TBARS). The level of membrane lipid peroxidation was expressed as nmol of TBARS per gram of DW of lichen thalli. Five replicates were measured for each experimental group.

2.5. Dehydrogenase activity

The vitality of the mycobiont, which represents ca. 90% of the lichen biomass, was verified by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to red-coloured triphenylformazan (TPF), which is directly linked to dehydrogenase activity and represents the activity of the mitochondrial respiratory chain (Bačkor and Fahselt, 2005; Ruf and Brunner, 2003). Dehydrogenases are oxidoreductase enzymes involved in cell respiration that oxidize organic compounds by transferring electron pairs from a substrate to NAD + or NADP +, forming NADH or NADPH, respectively (Crane et al., 1991). The application of tetrazolium salt is a widely used method to measure redox reactions in plant and fungal cells (e.g., Lin et al., 2001; Munzi et al., 2017). TTC successfully competes with NAD + for electrons, and thus constitutes an artificial electron acceptor. The TTC accepts electrons directly from the low potential cofactors of the NADH dehydrogenase (complex I) in the respiratory chain (Rich et al., 2001). As TTC accepts electrons, it is reduced to a red formazan. The colorimetric method based on TTC reduction provides an accurate assay of dehydrogenase activity. The lichen material (ca. 40 mg) was incubated in the dark for 20 h at 25 °C in 2 mL of 0.6% TTC (Sigma-Aldrich) and 0.005% Triton X 100 solution (Sigma-Aldrich) in 50 mM sodium phosphate buffer adjusted to pH 6.8. Then the solutions were removed, and samples were rinsed in distilled water until complete removal of Triton X. The samples were then dried on filter membrane. Water-insoluble formazan was extracted with 6 mL of ethanol (95%, Sigma-Aldrich) at 65 °C for 1 h. The test tubes were then centrifuged at 4000×*g* for 10 min and the supernatant absorbance was measured at 485 nm. The results were expressed as absorbance at 485 nm on a dry weight of the thalli. Three replicates (pseudoreplicates) per single lichen sample were measured. These three measurements were then averaged and treated as a single value in further analyses. Five true replicates for each experimental group were analysed.

2.6. Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured using Handy-PEA + fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments Ltd, United Kingdom). Lichen samples were dark-adapted for 20 min and then a saturating pulse of 2400 μmol m^{-2} s^{-1} was provided by 650-nm light-emitting LED diodes. Ten replicates for each experimental group were measured.

The photosynthetic efficiency of the lichen photobiont was assessed by the maximum PSII quantum yield: $F_V/F_M = (F_M - F_0)/F_M$, where F_M is a maximum and F_0 is a minimum chl *a* fluorescence and $F_V = (F_M - F_0)$ variable fluorescence. The F_V/F_M greater than 0.63 were considered to correspond to physiologically healthy thallus (Jensen and Kricke, 2002). Other chlorophyll fluorescence parameters that were analysed are presented in Table S1.

The fast fluorescence kinetic typically outlines a transient curve: when the curve is plotted on a log-time axis, a sequence of steps is called O-J-I-P (Strasser et al., 2000). Transient curves representing particular experimental groups were compared on the first and last day of the experiment (averaged for n = 10).

2.7. Statistical analysis

Two-way analyses of variance (two-way ANOVA; $P < 0.05$) were performed to assess the effect of 'fungicide' and 'chitosan', and their interactions on *EC,* TBARS concentration and dehydrogenase activity in the lichen thalli, separately for each experimental day. The significance of differences between particular experimental groups was then verified with Tukey's HSD post-hoc tests ($P < 0.05$). For testing the significance in multiple comparisons, False Discovery Rate approach was applied to obtain FDR adjusted *P*-values for each test according to two-stage sharpened method (Pike, 2011), and gain sizeable power to identify truly significant comparisons. The significance of differences in terms of photosynthetic parameters that characterize PSII functionality between particular days of the experiment within individual experimental groups was tested with one-way analyses of variance ($P < 0.05$) followed by Tukey's HSD post-hoc tests. If the assumption of variance homogeneity was not met, the Welsh correction was applied. In these cases, the Games-Howell post-hoc tests were applied for multiple comparisons. In a few cases in which distribution was not normal, even after transformation, non-parametric Kruskal-Wallis tests, along with Dunn's post hoc tests, were applied. One-way ANOVAs along with post-hoc Tukey's tests ($P < 0.05$) were also applied to test the significance of differences in F_V/F_M between particular experimental groups on each day of the experiment, separately. Before performing these analyses, the normality distribution within groups was checked using the Kolmogorov-Smirnov test. Brown–Forsythe and Cochran's C tests were used to verify the homogeneity of variances. The box-cox transformation was applied, when necessary. Statistical analyses were performed using STATISTICA 13 (TIBCO Software Inc., Palo Alto, CA, USA) and Statgraphics Centurion 19 (Statgraphics Technologies, Inc., The Plains, VA, USA).

3. Results

3.1. Loss of cell membrane integrity

As regards cell membrane integrity in *X. parietina*, significant interactions between 'fungicide' and 'chitosan' factors have been recorded at each experimental day (Fig. 2, Table S2). The lowest *EC* values (up to 15%) were observed on the 3rd day of the experiment for all experimental groups. On the 7th day of the experiment, groups without SW had the lowest *EC* values, and, as a rule, differ significantly from SW1, SW2, and their combinations with CH200 and CH1000. The highest *EC* values (exceeding 50%) were observed on the last day of the experiment after treatment with SW1, SW2, and their combinations with CH.

3.2. Membrane lipid peroxidation

TBARS concentrations on the 3rd day of the experiment were influenced by both 'fungicide' and 'chitosan' (Fig. 3, Table S3). The treatment with SW1, SW2 and their combinations with CH resulted in significantly higher TBARS concentrations compared to groups without SW. Moreover, all experimental groups without chitosan differed significantly from groups treated with CH1000, which significantly increased TBARS concentrations in the thalli. On the 7th day of the experiment, only the 'fungicide' effect was significant, and groups treated with SW1 and SW2 differed significantly from the groups not treated with SW regardless of chitosan presence. On the 15th day of the experiment, a significant interaction between 'fungicide' and 'chitosan' was found. TBARS reached the lowest concentrations in groups without SW and in SW1/without the CH group (Fig. 3, Table S3). Generally, on each day of the experiment high TBARS concentrations were recorded in SW1 and SW2 groups, and in fungicide-chitosan combinations they reached the highest values on the 15th day of the experiment in SW1/CH200, SW1/CH1000, and SW2/CH200 groups.

3.3. Dehydrogenase activity

Regarding dehydrogenase activity in *X. parietina*, a significant effect of 'fungicide' and 'chitosan' was observed on the 3rd day of the experiment (Fig. 4, Table S4). In detail, dehydrogenase activity was significantly the highest in the groups without SW, and significantly the lowest in groups treated with SW2. Moreover, groups without chitosan had significantly higher dehydrogenase activity than all groups treated with

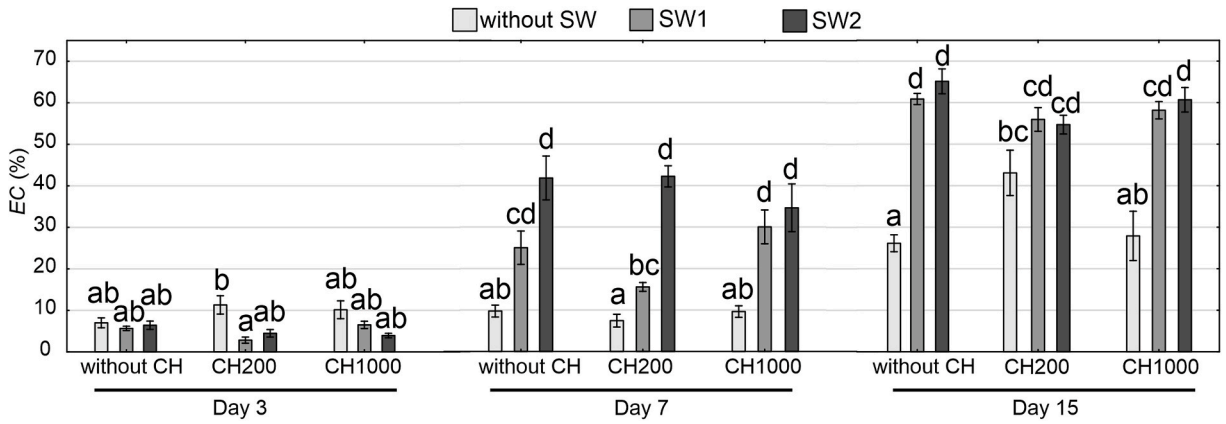

Fig. 2. Effect of chitosan and Switch 62.5WG on the loss of cell membrane integrity in *Xanthoria parietina* thalli expressed by *EC* in particular experimental groups (means ± SE; n = 5) for different concentrations of the agents, alone or in combination, applied on the 3rd, 7th and 15th day of the experiment. Within each experimental day, lowercase letters indicate statistically significant interaction between 'fungicide' and 'chitosan' effects. The different letters above the bars indicate statistically significant differences (FDR adjusted *P*-values <0.05). For explanation of the abbreviations, see Fig. 1. For details on the main effects and interactions see Table S2.

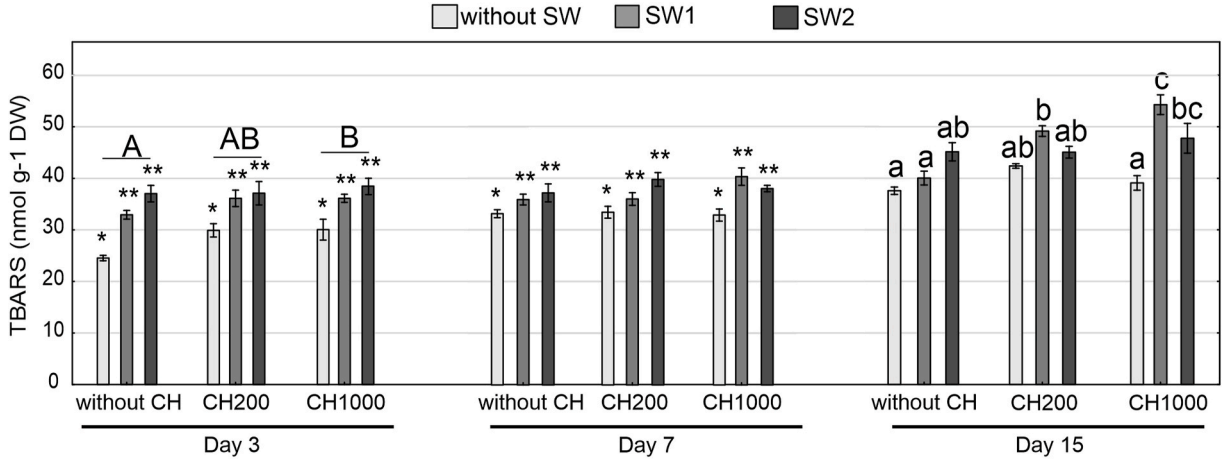

Fig. 3. Effect of chitosan and Switch 62.5WG on the thiobarbituric acid reactive substance (TBARS) concentrations in *Xanthoria parietina* thalli in particular experimental groups (means ± SE; n = 5) for different concentrations of the agents, alone or in combination, applied 3rd, 7th and 15th day of the experiment. Within each experimental day, the lowercase letters above the bars indicate the statistically significant interaction between 'fungicide' and 'chitosan' effects; the capital letters indicate the significant main effect of 'chitosan'; the asterisks (*) indicate the significant main effect of 'fungicide'. The different letters/number of asterisks above the bars indicate statistically significant differences (FDR adjusted *P*-values <0.05). For explanation of the abbreviations, see Fig. 1. For details on the main effects and interactions see Table S3.

CH1000. On the 7th and 15th day of the experiment, a significant interaction between 'fungicide' and 'chitosan' factors was recorded (Fig. 4, Table S4). The highest values on the 7th day of the experiment were observed in the solvent control group without SW and without CH, and in the CH200 group, differing significantly from the remaining groups. On the last day of the experiment, the lowest dehydrogenase activity was only observed in groups treated with fungicide, but without chitosan addition; these groups differed significantly from the remaining groups. As a rule, lichen thalli treated with SW only had lower dehydrogenase activity compared to solvent control groups without SW and without CH, on particular days of the experiment. Interestingly, in the case of CH and SW combinations, dehydrogenase activity increased after repeated doses of agents, reaching the highest values on the 15th day of the experiment.

3.4. Chlorophyll fluorescence parameters

As regards the F_V/F_M parameter, the first significant decrease compared to the control was observed on the 5th day of the experiment, but only in groups SW2 and SW2/CH1000 (Fig. 5, Table S5). The remaining groups did not differ significantly from the control until the 7th day of the experiment, in which a significant decrease in F_V/F_M was also observed for SW1 and SW1/CH1000 groups. On the 9th day of the experiment, most groups differed significantly compared to the control, and from the 11th day of the experiment to the end of the experiment, the greatest decreases in the F_V/F_M parameter were observed. The lowest F_V/F_M values were observed on the last day of the experiment for all groups except CH200 and CH1000 groups.

Most of the studied photosynthetic parameters characterizing PSII functionality underwent significant changes during the experiment (Table S5). Parameters F_V/F_M, F_0, ET_0/RC, Phi (P_0), Psi(E_0), Phi (E_0), Phi (R_0) and PI_{ABS} were the highest at the beginning of the experiment and decreased significantly, over time, with the repeated use of agents in most experimental groups. On the other hand, ABS/RC and DI_0/RC increased significantly after the repeated use of agents.

As regards fluorescence kinetics reflected in the OJIP test, on the first day of the experiment, the curves representing all groups followed a course similar to the control group, with visible characteristic peaks,

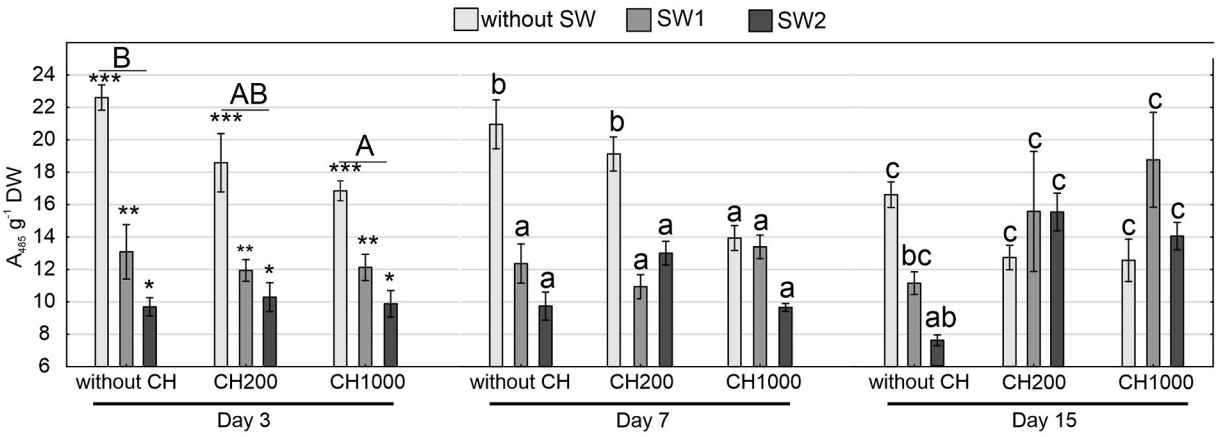

Fig. 4. Effect of chitosan and Switch 62.5WG on the dehydrogenase activity in *Xanthoria parietina* thalli expressed as absorbance at 485 nm on g DW in particular experimental groups (means ± SE; n = 5) for different concentrations of the agents, alone or in combination, applied on the 3rd, 7th and 15th day of the experiment. Within each experimental day, the lowercase letters above the bars indicate the statistically significant interaction between 'fungicide' and 'chitosan' effects; the capital letters indicate the significant main effect of 'chitosan'; the asterisks (*) indicate the significant main effect of 'fungicide'. The different letters/number of asterisks above the bars indicate statistically significant differences (FDR adjusted *P*-values <0.05). For explanation of the abbreviations, see Fig. 1. For details on the main effects and interactions see Table S4.

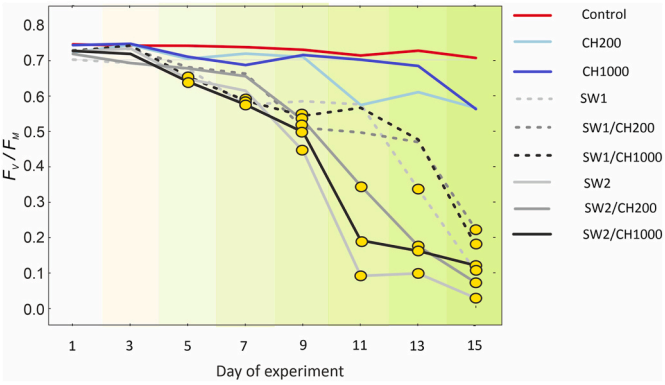

Fig. 5. Effect of chitosan and Switch 62.5WG on the maximum photochemical efficiency of PSII (F_V/F_M) in *Xanthoria parietina* thalli. The agents, alone or in combination, were applied on the indicated days for 15 days and chlorophyll *a* fluorescence measurements (n = 10) were performed 2 h after each application. The dots indicate significant differences from control on particular days of the experiment (one-way ANOVA, post-hoc Tukey's tests; $P < 0.05$). Detailed statistics of the F_V/F_M values are given in Table S5. For explanation of the abbreviations, see Fig. 1.

only the F_M values were slightly lower (Fig. S2). However, on the 15th day of the experiment, the induction curves of most of the experimental groups had a disturbed course. Exposure to SW and in combination with CH had a more apparent negative effect on OJIP shape than exposure to CH alone. A substantial decrease in the chlorophyll fluorescence signal was observed, which was reflected in flattened fluorescence curves without visible characteristic steps and a considerable decrease in the values of F_0 and F_M (Fig. S2).

4. Discussion

4.1. Cell membrane integrity disturbances

Cell membranes have previously been reported to be the main target of the antifungal action of chitosan (García-Rincón et al., 2010; Ing et al., 2012; Singh et al., 2008). Chitosan treatments of *Candida albicans* caused changes in cell structure resulting in swelling and severe cell wall alterations (Tayel et al., 2010). Singh et al. (2008) found increased K^+ leakage in wood-degrading fungi as an early response to chitosan, which may disrupt the ionic balance essential for normal growth. Liu et al. (2004) showed that chitosan increased permeability and ultimately disrupted bacterial cell membranes, causing the release of cellular contents. More evolutionarily advanced fungi also demonstrate similar responses, as shown in the ascomycete *Ceratocystis fimbriata* (Xing et al., 2018). All these reports indicate that chitosan exerts a fungicidal effect through its ability to disturb fungal membranes. Our results do not unequivocally indicate a drastic increase in the degree of damage to cell membranes after treatment with chitosan alone. Perhaps it could be related to the various effectiveness of chitosan antifungal activity that is highly dependent on the type of target organism (Ke et al., 2021a), which determines the interaction of chitosan with cell membranes. The susceptibility of chitosan is associated with the content of unsaturated fatty acids on the cell membranes, and their higher content induces better membrane fluidity leading to a more negative charge on the cell membrane (Kumariya et al., 2015; Palma-Guerrero et al., 2010). Since chitosan binds to negatively charged phospholipids, the alternations could be greater in species containing more polyunsaturated lipids (Palma-Guerrero et al., 2010).

Similar mechanisms were reported in the response of fungi to phenylpyrroles. For example, the treatment of *Neurospora crassa* with fludioxonil caused hyperpolarization of the cell plasma membrane and net H^+ efflux with simultaneous net K^+ uptake, and an increase in turgor (Lew, 2010). This unexpected increase in turgor was the possible cause of the final lysis of fungal cells and death. Moreover, the impaired membrane structure, intracellular plasma leakage, and increased cell membrane permeability was observed after treatment with fludioxonil in both algae and fungi (Duan et al., 2013; Liu et al., 2022). The described phenomena could be responsible for a significant decrease in the integrity of cell membranes on the 7th and 15th day of the experiment in the groups treated with SW and its combinations with CH. The interaction between these two antifungal agents has never been investigated before, but many studies have shown that the simultaneous action of two different fungicides could have stronger antifungal activity than the action of a single agent. For example, Kanetis et al. (2007) found that azoxystrobin and fludioxonil mixtures were significantly more effective when compared to single-fungicide treatments on *Penicillium digitatum*. On the contrary, we did not observe a significantly stronger effect on cell membrane damage in treatments with combinations of SW with CH, compared to treatments with SW alone. However, it should be noted that damage to cell membranes increased with the duration of the experiment, especially after treatment with SW fungicide. This is probably due to the cumulative effect caused by repeated

use of the agents.

4.2. Oxidative degradation of membrane lipids

One of the most crucial biological processes related to the action of ROS is lipid peroxidation. Various biotic and abiotic factors can result in ROS production that leads to oxidative stress and can cause disorders in protein and DNA structure (Kranner et al., 2008). As a result, an increase in thiobarbituric acid reactive substances (TBARS) is often observed as decomposition products of polyunsaturated fatty acids created during the peroxidation of membrane lipids (Mittler, 2002). Many studies have shown that TBARS concentrations in lichens are very responsive to various stress factors, as heavy metals, salinity or nitrogen excess (e.g., Chowaniec et al., 2022; Paoli et al., 2015a; Rola et al., 2022). Our results showed that, as a rule, combinations of SW with CH caused a higher level of TBARS compared to SW1 and SW2 treatments. This trend was especially visible on the 15th day of the experiment in the case of SW1/CH200 and SW1/CH1000 groups. Similarly, the application of chitosan with fludioxonil and difenoconazole resulted in a 2–3-fold more effective antifungal action against *Botrytis cinerea* than the action of each component separately (Karpova et al., 2021). Regarding the effect of biocides on various organisms, many agents are regarded as inducers of oxidative stress (see Jabłońska-Trypuć, 2017). Chitosan treatment also proved to increase the levels of intracellular reactive oxygen species (ROS) that lead to the permeabilization of the plasma membrane in *Neurospora crassa* (Lopez-Moya et al., 2016). Nevertheless, our results showed that significant differences between thalli treated with SW and its combinations with CH in relation to the control were the most pronounced at the beginning, and at the end of the experiment. Such a phenomenon can be related to the efficient operation of defence mechanisms, i.e., enzymatic and non-enzymatic antioxidants scavenging ROS (Kranner et al., 2003). Nevertheless, a much higher level of lipid peroxidation of membranes maintained throughout the experiment in the groups treated with the highest fungicide concentrations compared to the control and their increase after repeated treatments with the agent may indicate that antioxidant mechanisms are not able to eliminate increasing oxidative stress.

4.3. Dehydrogenase activity in the mitochondrial respiratory chain

Mitochondria coordinate many aspects of fungal biology such as cellular energy production or apoptosis (McBride and Neuspiel, 2006). Despite this, detailed mechanisms allowing mitochondria to modify their flexibility to respond to environmental changes are largely unknown. Bačkor and Fahselt (2005) suggested that the level of tetrazolium reduction could be a good indicator of the impact of various stress factors on lichens. The dehydrogenase activity checking test is mainly related to the mycobiont condition, because ca. 90% of lichen thalli consist of fungal hyphae (Hale, 1983). In the following years, over a dozen studies were carried out in which the dehydrogenase activity in lichens exposed to various stress factors was analysed under laboratory (Fačkovcová et al., 2020; Vannini et al., 2015) and field (Paoli et al., 2015b) conditions. The observed negative effect of SW and CH treatments is consistent with results of other studies that tested the effect of pesticides on various organisms. For example, glyphosate treatment resulted in even up to 70% suppression of dehydrogenase activity in *X. parietina* (Vannini et al., 2015), while Mancozeb supplemented with dimethomorph also caused a significant decrease in dehydrogenase activity in soil microorganisms (Cycoń et al., 2010). The observed decrease in dehydrogenase activity after SW treatment may be related to the mechanism of action of pyrrolnitrin (from which fludioxonil was obtained), which involves the disruption of electron transport in mitochondria, predominantly impacting the respiration of mitochondria at complex I (Wong and Airall, 1970; Wong et al., 1971). Pyrrolnitrin was found to uncouple oxidative phosphorylation and inhibit electron transport in the mitochondria of *Neurospora crassa* (Lambowitz and Slayman, 1972). Although later studies indicated other modes of fludioxonil action, as the excessive accumulation of glycerol in cells by triggering the HOG osmoregulation pathway (Pillonel and Meyer, 1997) or the disruption of glutathione homeostasis (Kim et al., 2007), further studies confirmed that both fludioxonil and cyprodinil caused significant reductions in cellular ATP and induced toxic effects on mitochondrial membrane potential in human neuronal and glial cell lines (Coleman et al., 2012). Unexpectedly, we found an increase in dehydrogenase activity in groups treated with SW and CH combinations, which may seemingly suggest that CH somewhat reduces the negative effect of SW. However, the effect of this interaction is an interesting issue that requires the further explanation of its mechanism because the action of chitosan alone only causes a reduction in dehydrogenase activity. Nevertheless, Peña et al. (2013) found that very low concentrations of chitosan stimulated respiration in *Candida albicans*. Similarly, Robles-Martínez et al. (2014) demonstrated that chitosan induces an increase in oxygen consumption and raises NADH dehydrogenase activity in *Rhizopus stolonifer*. This could be interpreted as a stress response in which cells required more energy to repair damages generated by chitosan, which is associated with an increase in mitochondrial respiration to raise ATP synthesis essential for survival. On the other hand, Robles-Martínez et al. (2014) found that, at the same time, cytochorme c oxidase and ATP synthase activities were not affected by the application of chitosan, which indicates that increased oxygen consumption is not coupled with ATP synthesis and could only indicate a dysfunction of the mitochondrial respiratory chain. Since TTC, used in the present study as an artificial electron acceptor, is primarily reduced by complex I in mitochondria (Rich et al., 2001), we cannot clearly verify whether the increased activity of dehydrogenase contributes to increased ATP production in response to the combined application of CH and SW, or is the result of a disorder in the mitochondrial respiratory chain.

4.4. Effect on chlorophyll fluorescence parameters

The measurement of maximum photosystem II efficiency is a widely used method to test the impact of abiotic stress factors on lichens. Chitosan treatment proved to strongly enhance the content of chlorophylls and photosynthetic efficiency and contribute to chloroplast enlargement in vascular plants (Dzung et al., 2011; Limpanavech et al., 2008). Therefore, we assumed that the application of chitosan alone would not adversely affect the photosynthesis efficiency of photobiont cells in the lichen thallus. As expected, a significant reduction in the value of F_V/F_M was observed in the groups treated with SW and its combinations with CH, especially from the 9th day of the experiment until the end, while in the groups treated with CH only, no significant differences compared to the control were observed. To the best of our knowledge, there is no data on the effect of fludioxonil and cyprodinil on symbiotic algae. However, the reduced content of photosynthetic pigments and increased oxidative stress were observed in free-living microalgal taxa *Chlorella vulgaris* after treatment with fludioxonil (Liu et al., 2022). On the other hand, the effect of this fungicide on the photosynthetic parameters of *Scenedesmus obliquus* was negligible (Dewez et al., 2005). Therefore, the effect of fludioxonil depends on algae species, which can differ in their ability to penetrate and distribute this compound among different cellular compartments. Regarding lichens, other pesticides have also been shown to reduce photosynthetic efficiency and in chlorophyll content in the thalli (Sujetovienė et al., 2019; Vannini et al., 2015).

Our results showed that long-term exposure to fungicide and chitosan led to the most considerable decrease in F_V/F_M and PI_{ABS}, as well as a significant increase in ABS/RC and DI_0/RC over the period of the experiment in the lichen algal partner. Nevertheless, this effect was not visible after a single treatment, as significant changes in these parameters were not observed until the 5th day of the experiment, which may be related to the cumulative effect of repeated application. ABS/RC represents the absorption flux per one active reaction centre (RC), a ratio of active to inactive RCs (Strasser et al., 2000), and thus an increase in

ABS/RC could be associated with the inactivation of some PSII RCs. Since the inactive centres could not trap photons, the increase in the rate of energy dissipation of untrapped excitations causes an increase of DI_0/RC. Moreover, the disturbances in the photosynthetic apparatus due to fungicide and chitosan treatments were best reflected in the decline of the PI_{ABS} parameter, which is a global indicator of photosynthetic performance (Strasser et al., 2000). The induction curves also showed disturbance in transient trajectory as decreases in the chlorophyll fluorescence signal were observed, and F_0 and F_M values, especially after treatment with SW and its combination with CH, which could be linked with the disturbances in electron transport on the donor side of PSII and could indicate the accumulation of inactive RCs at PSII (Kalaji, 2011).

5. Conclusions

The use of fungicides and plant growth stimulators/elicitors reduces the population of fungal pathogens and contributes to increased crop productivity and health. Understanding the impacts of these compounds and their combinations on non-target organisms is crucial to minimize unintended consequences, while maintaining their use in plant protection. Our results showed that chitosan itself causes significant disturbances in the functioning of the lichen fungal partner, while the photosynthetic efficiency of the algal partner is not significantly affected. We showed that fungicides composed of fludioxionil and cypronidil, as well as the combined use of such synthetic fungicides together with the natural biopolymer chitosan, can cause the considerable impairment of lichen physiology, and these disturbances are related to both the algal and fungal partner of symbiotic association. The combined use of these agents leads to clear disturbances in the functioning of the mitochondrial respiratory chain, which was manifested by increased NADH dehydrogenase activity. However, the recognition of this mechanism requires further research. We concluded that the regular use of these agents in the cultivation of fruit trees and shrubs may have serious ecological consequences for epiphytic lichen communities, as a consequence of the death of lichen thalli. This study suggests that the impact of some plant protection agents, both individually and in combinations, merits further attention in terms of their impact on non-target fungi.

Credit author statement

Kaja Rola: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Supervision, Visualization, Writing – review & editing. **Emilia Majewska**: Investigation, Writing – review & editing. **Karolina Chowaniec**: Conceptualization, Formal analysis, Investigation, Funding acquisition, Methodology, Resources, Visualization, Writing – original draft.

Funding

The research has been supported by a grant from the Faculty of Biology (grand number U1U/W18/NO/28.03) under the Strategic Programme Excellence Initiative at Jagiellonian University. Partial financial support was received from the Institute of Botany at the Jagiellonian University, project no. N18/DBS/000002. The open-access publication of this article was funded by the programme "Excellence Initiative – Research University" at the Faculty of Biology of the Jagiellonian University in Kraków, Poland. The linguistic proofreading was funded by the Ph.D. Students' Association of Jagiellonian University.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2023.137772.

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