

# Antimicrobial Potential of Microalgae *Chlorella vulgaris* water Extract in Controlling Grey Mould of Strawberry with a Devastating Disease Caused by the Ubiquitous Necrotrophic Fungal Pathogen *Botrytis cinerea* in Field Conditions

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Article Details: Received: 2025-01-31 | Accepted: 2025-02-18 | Available online: 2025-05-31



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Strawberry (*Fragaria ananassa* Duch.) cultivation faces significant challenges due to *Botrytis cinerea*, a fungal pathogen responsible for grey mould. This study investigates the potential of microalgae *Chlorella vulgaris* water extract as a biocontrol agent against ubiquitous necrotrophic fungal pathogen *B. cinerea*, aiming to offer an eco-friendly alternative to chemical fungicides. A 10% water extract of microalgae *C. vulgaris* was prepared and tested in the field experiments. We initially tested different algae for inhibition of the fungal pathogen *B. cinerea* under laboratory conditions using a disk diffusion method. In the laboratory, the disc diffusion method revealed varying antifungal activities of different algal species, with *C. vulgaris* showing significant inhibition of *B. cinerea*. The field experiment was conducted on strawberries, assessing infection rates following treatment with *B. cinerea* and *C. vulgaris*. The results demonstrated that the treatment with *C. vulgaris* reduced the incidence of *B. cinerea* infection compared to the control, highlighting the potential of this algae as an effective biocontrol agent. This study contributes to the growing body of research supporting the use of natural products in sustainable agricultural practices, providing a promising alternative to chemical pesticides in strawberry production.

**Keywords:** *Chlorella vulgaris*, *Botrytis cinerea*, strawberry, biocontrol, antifungal activity, sustainable agriculture

## 1 Introduction

Strawberry (*Fragaria ananassa* Duch.), a member of the Rosaceae family, is a widely cultivated fruit originally from the Americas. It thrives in a variety of climates, ranging from tropical and subtropical to cooler regions (Darnell et al., 2010). Known for its bright red colour, sweet flavour, and high nutritional content, strawberries are rich in vitamins A, B, and C, as well as minerals such as iron, calcium, potassium, phosphorus, and zinc (Al-Hachami et al., 2019). In addition to their nutritional benefits, strawberries are recognized for their antioxidant properties and their potential contribution to human health (Giampieri et al., 2015). The cultivated variety of strawberry, *Fragaria* × *ananassa*, is widely grown across the globe, with significant production in Europe, the United States, and Asia. Spain stands as the top producer within Europe, followed by Italy and France (Şesan, 2006).

One of the primary threats to strawberry cultivation is *Botrytis cinerea*, the pathogen responsible for grey mould, which is a leading disease in strawberries worldwide. According to Neri et al. (2015), this pathogen can remain dormant in unripe tissues and cause infection as the fruit ripens. The infection typically starts in the flower, and any injury to the fruit exacerbates fungal growth. Conventional strawberry farming relies heavily on chemical fungicides to manage *B. cinerea*; however, there is growing interest in alternative methods, particularly in organic farming (Daugaard, 1999; Williamson et al., 2007).

In recent years, seaweed extracts have garnered attention in agriculture due to their beneficial effects on plant growth and productivity. These extracts contain compounds that may help reduce the occurrence of fungal infections, offering a potential solution to

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the challenges posed by pesticide restrictions. Studies suggest that seaweed extracts could help reduce the incidence of *B. cinerea* on strawberries, as well as other plant pathogens like *Erysiphe polygoni* (powdery mildew) on turnips and damping-off in tomato seedlings. Cyanobacteria and algae, which produce a wide variety of antibacterial and antifungal compounds, have emerged as promising biocontrol agents, offering an environmentally friendly alternative to chemical pesticides (Righini et al., 2018). However, more research is needed to fully assess their potential as effective biocontrol agents (Kulik, 1995).

This study aims to evaluate the efficacy of a 10% water extract of *Chlorella vulgaris* in protecting strawberries from *Botrytis cinerea*, assessing its potential as an alternative biocontrol method for managing this widespread fungal disease. Based on laboratory findings, our study attempted to bring valuable insights into the viability of using seaweed-based natural products in a field trial as sustainable crop protection tools in agriculture.

## 2 Material and Methods

### 2.1 Laboratory Experiment

#### 2.1.1 Algae Samples

In this study, the antifungal activity of various microalgae, macroalgae, and cyanobacteria was evaluated against *Botrytis cinerea*. The tested species included *Nannochloropsis* sp., *Tetraselmis chuii*, *Chaetoceros muelleri*, *Thalassiosira weissflogii*, *Tisochrysis lutea*, *Palmaria palmata* (dulse), *Chondrus crispus* (Irish moss), *Ascophyllum nodosum* (kelp), *Chlorella vulgaris*, and *Arthrospira platensis* (spirulina). These algae were selected based on their known bioactive properties and previously reported antimicrobial potential.

Among the tested species, *C. vulgaris* demonstrated the most promising antifungal activity and was therefore chosen for further investigation. The powdered sample, sourced from certified organic production in China, was obtained from FutuNatura (Kranj, Slovenia). The alga was cultivated under controlled conditions and certified according to EU organic standards (SI-EKO-002). Its selection was based on its strong inhibitory effects against *B. cinerea*, as well as its broader antimicrobial properties observed in additional tests. To ensure the stability of its bioactive compounds, the sample was stored in a dry, dark environment at laboratory temperature ( $\pm 20$  °C) in an airtight container.

#### 2.1.2 Preparation of Water Extracts from Algae

The powdered dried algae were used to prepare 10% water extracts. Algae powder was weighed and mixed with distilled water. The mixture was then agitated for 24 hours at 22 °C in the dark. After the incubation period, the extract was filtered using Whatman No. 2 filter paper.

#### 2.1.3 Inoculation of *Botrytis cinerea* for the Disc Diffusion Method

The fungal strain used to evaluate antifungal activity was the filamentous *Botrytis cinerea* F-314 from the Czech Collection of Microorganisms in Brno, Czech Republic. *B. cinerea* is a phytopathogenic filamentous fungus responsible for causing grey mould, a widespread and economically significant disease affecting various fruits, vegetables, and ornamental plants. The strain *B. cinerea* CCM F-314 (also referred to as strain F 50070) is a fungal isolate obtained from *Cucumis sativus* (cucumber) fruit in the Czech Republic. It is part of the Czech Collection of Microorganisms (CCM) and has been linked to L. Marvanová. This strain is noted for its ability to produce  $\alpha$ -amylase, as documented in Czech Patent 205 315. To perform the tests, the fungi were inoculated onto Potato Dextrose Agar (PDA, Oxoid, Basingstoke, United Kingdom) using a sterile bacterial loop. The inoculated plates were incubated at 21 °C for five days. After incubation, the fungal cultures were adjusted to a 0.5 McFarland standard using a densitometer, which corresponds to approximately  $1.5 \times 10^8$  colony-forming units (CFU) per millilitre. These prepared fungal cultures were then used to assess antimicrobial activity.

#### 2.1.4 Disk Diffusion Method

*Botrytis cinerea* after adjusting the densities to 0.5 McFarland was used to test for antimicrobial activity using the disc diffusion method. For the fungal test, a suspension of *B. cinerea* was evenly spread on Petri dishes containing Potato Dextrose Agar (PDA) using a sterile cotton swab, making three parallel streaks across the agar surface. Sterile 6 mm diameter discs (Oxoid, Basingstoke, United Kingdom) were then placed on the surface of the inoculated agar. Each disc was treated with 10  $\mu$ L of the algae water extract. The plates were incubated for 5 days at 21 °C for *B. cinerea*. As positive controls, antifungal agents (fluconazole; Oxoid, Basingstoke, United Kingdom and Itraconazole; Biomaxima AFSTD, Lublin, Poland) were used. Negative controls consisted of discs soaked in the solvent used to dissolve the extract (ultrapure water) and blank discs. The size of the inhibition zones (diameter) around each disc was measured three times, and the average and standard deviation in mm were calculated. All measurements were conducted in triplicate.

## 2.2 Field Study

### 2.2.1 Experimental Setup and Treatment Conditions for Strawberry Plants

The experiment was conducted in the Demonstration Garden of the Botanical Garden at the Slovak University of Agriculture in Nitra, located on parcels managed

by the Institute of Horticulture. Strawberries (*Fragaria × ananassa* L. Duch) were planted in March 2021 at a uniform planting distance of 0.5 × 0.3 m, with biostimulant applications to the soil monitored until 2023. In 2024, strawberries were grown without any biostimulant or protective treatment. The plants were maintained as overgrown rows throughout the experiment.

During the entire experimental period, the strawberry plants were maintained as overgrown rows, providing a natural growth environment for assessing interactions with potential biocontrol agents, including algae-based treatments for protecting against *B. cinerea*. The cultivar used in this experiment is known for its high productivity, attractive fruit characteristics, and resilience to various fungal diseases, making it an ideal model for studying plant protection strategies.

To better understand the environmental context of the experiment, Table 1 summarizes the monthly temperatures for 2024, comparing them to the 1991–2020 climate norm and presenting the deviation (*Dt*) between the two. These temperature trends were essential for evaluating how environmental factors influenced the experimental conditions, particularly in relation to the growth of the strawberry plants and the performance of biocontrol treatments. Additionally, Table 2 presents the monthly precipitation data for 2024, compared to the long-term average from 1951 to 2000, along with the percentage deviation (% *n*) of the 2024 values. This precipitation data was crucial for assessing how rainfall patterns impacted the experimental conditions and the overall development of the strawberry crop during the study.

### 2.2.2 Experimental Procedures and Results Evaluation

The prepared water extracts were tested on Petri dishes against the fungal pathogen *B. cinerea* using the disc diffusion method (Chapter 2.1.4). Based on the inhibition zone results, the 10% water extract of *C. vulgaris* was selected for further experiments. Extract was filtered using Whatman No. 2 filter paper.

**Table 1** Evaluation of Monthly and Yearly Temperature Deviations Based on the 1991–2020 Climate Norm (Nitra, 2024)

Month	<i>t</i> (°C)	Normal 1991–2020	<i>Dt</i> (°C)	Characterization
Jan	-1.9	-0.5	-1.3	normal
Feb	3.5	1.3	2.2	warm
Mar	0.9	5.5	-4.6	cold
Apr	11.7	11.4	0.3	normal
May	17.6	16.0	1.6	warm
Jun	20.8	19.6	1.1	warm
Jul	23.8	21.7	2.1	very warm
Aug	24.0	21.1	2.8	very warm
Sep	16.8	15.9	0.9	normal
Oct	11.4	10.4	1.0	normal
Nov	3.8	5.6	-1.8	cold
Dec	1.6	0.7	0.9	normal
Year	11.2	10.8	0.4	normal

Source: Meteorological station FZKI – UKI

**Table 2** Evaluation of Monthly and Yearly Precipitation Based on the Long-Term Average (1991–2020) (Nitra, 2024)

Month	<i>Z</i> (mm)	Normal 1951–2000	<i>n</i> (%)	Characterization
Jan	47	33	142	moist
Feb	32	29	111	normal
Mar	29	33	87	normal
Apr	56	36	154	very moist
May	62	59	104	normal
Jun	181	59	306	extremely moist
Jul	17	65	26	very dry
Aug	57	55	104	normal
Sep	132	58	227	extremely wet
Oct	42	46	92	normal
Nov	21	45	46	very dry
Dec	20	42	48	very dry
Year	694	559	124	normal

Source: Meteorological station FZKI – UKI

This adjusted inoculum was then used for the treatment of strawberries.

Strawberry plants were divided into four treatment groups with three replications, each consisting of 20 plants:

- Control: Plants without treatment.
- Fungus: Plants treated only with *Botrytis cinerea*.
- Algae: Plants treated with a 10% water extract of *Chlorella vulgaris*.

Fungus + Algae: Plants treated with *Botrytis cinerea* followed by a 10% water extract of *Chlorella vulgaris*. On May 9, 2024, during the final stage of flowering, all strawberry plants were subjected to disinfection using a 3% sodium hypochlorite solution to eliminate any potential surface pathogens or contaminants that could interfere with the experimental treatments. The disinfection procedure involved spraying each plant group with 10 sprays of approximately 15 mL, ensuring even coverage across the entire plant surface, including both sides of the leaves, stems, and flowers (Fig. 1). This step aimed to reduce the presence of external microbial flora, thus minimizing the risk of confounding factors in subsequent treatments.

The following day, on May 10, 2024, the experimental groups scheduled for fungal treatment were treated with an inoculum of *B. cinerea* at a concentration of 0.5 McFarland, which corresponds to a standardized fungal spore concentration suitable for infection trials. The inoculum was applied to each plant group via 10 sprays of approximately 15 mL per group, ensuring uniform distribution across the plants. This method was designed to introduce the fungal pathogen in a controlled manner, creating a consistent infection pressure for the evaluation of fungal disease resistance and the effectiveness of the treatments.

On May 11, 2024, the groups designated for algal treatment received a spray application of a 10% water extract of *C. vulgaris*. Each plant group was sprayed 10 times, with approximately 15 mL of extract applied per group. This treatment aimed to evaluate the potential antifungal properties of the *C. vulgaris* extract in combating *B. cinerea*. The algae extract was carefully applied to ensure even distribution across the plant surfaces, with a focus on the leaves and flowers, as these are the primary infection sites for the fungal pathogen. The use of a water extract of *C. vulgaris* was chosen based on its previously observed bioactive properties, and this application aimed to test its potential for inhibiting fungal growth in a real-world agricultural setting.

On May 29, 2024, the fruit harvest was conducted to assess the effectiveness of the treatments in preventing



**Figure 1** Application of water *C. vulgaris* extract spray on strawberry plants

or mitigating infection by *B. cinerea*. At this stage, the strawberries from each treatment group were carefully collected into separate containers to ensure accurate tracking of fruit origin and treatment assignment (Fig. 2). The harvesting process was performed with care to minimize any physical damage to the fruit, which could introduce additional variables to the study.

Following the collection, each strawberry was visually inspected for any symptoms of *B. cinerea* infection, such as soft spots, discoloration, and mould growth, which are characteristic of the grey mould caused by this pathogen. Fruits exhibiting any signs of fungal infection were counted and recorded for each treatment group. This step aimed to quantify the extent of fungal colonization in each group. Based on the recorded data, the percentage of infected fruits in each group was calculated by dividing the number of infected fruits by the total number of fruits harvested in that group and then multiplying by 100 to obtain the infection rate as a percentage. This percentage provided a clear comparison of the efficacy of each treatment in preventing or reducing *B. cinerea* infection, offering insight into the potential role of *C. vulgaris* as a biocontrol agent in protecting strawberries from fungal disease.

### 2.3 Statistical Analyses

Statistically significant differences were also calculated using One-way ANOVA, followed by Tukey's HSD test at  $p \leq 0.05$  significance, was performed using online Astatsa Anova One Way.

### 3 Results and Discussion

#### 3.1 Results

##### 3.1.1 Testing The Antimicrobial Activity Of Water Algae Extracts Against *B. cinerea* Using the Disk Diffusion Method

The inhibition zones of the aqueous extracts (in mm) for different algal species against *B. cinerea* by the disk diffusion method. *Nannochloropsis* sp. exhibited an inhibition zone of  $0.89 \pm 0.60$  mm, while *Tetraselmis chuii* and *Tisochrysis lutea* showed no detectable inhibition (ND). *Chaetoceros muelleri* and *Thalassiosira weissflogii* both showed inhibition zones of  $1.11 \pm 0.60$  mm. *Palmaria palmata* also showed no detectable inhibition (ND), whereas *Ascophyllum nodosum* and *Chondrus crispus* exhibited inhibition zones of  $2.11 \pm 0.60$  mm and  $1.67 \pm 0.50$  mm, respectively. The largest inhibition zone was observed in *C. vulgaris*, with a value of  $2.33 \pm 0.57$  mm. *Arthrospira platensis* also showed no detectable inhibition (ND).

Due to its significant inhibition zone of  $2.33 \pm 0.57$  mm, *C. vulgaris* was selected for the next phase of the experiment. This species exhibited the strongest antimicrobial activity among the tested algae, making it the most suitable candidate for next investigation.

##### 3.1.2 Effect of *Chlorella vulgaris* Extract on the Infection Rate of *Botrytis cinerea* in Strawberry Plants

The results of the experiment indicate significant differences in the percentage of strawberry fruits infected by *B. cinerea* across different treatment groups (Table 3). The control group, which received no treatment, showed an infection rate of  $30.09 \pm 4.67\%$ . This baseline infection rate reflects the natural susceptibility of strawberries to *B. cinerea* in field conditions (Fig. 2).

The treatment group using only the 10% *C. vulgaris* extract (algae) showed a slightly lower infection rate of  $27.65 \pm 3.92\%$ . Although the algae treatment did not completely prevent infection, it seemed to have a mild protective effect, reducing the infection rate when compared to the control. This suggests that the algae extract may have some antimicrobial or antifungal properties, though its effect is not strong enough to completely counteract the fungal pathogen.

In contrast, the group treated with *B. cinerea* alone exhibited a significantly higher infection rate of  $46.26 \pm 1.70\%$ . This indicates that *B. cinerea* is a major pathogen for strawberries, with no treatment to combat the infection resulting in a relatively high rate of disease. Interestingly, when both *B. cinerea* and *C. vulgaris* were



**Figure 2** Evaluation of the percentage of infected strawberries after the application of water *C. vulgaris* extract

**Table 3** Percentage of infected strawberries in different treatment groups treated with *Botrytis cinerea* and 10% *Chlorella vulgaris* water extract

Sample	Number of infected	Total number	Infected (%)
Control	68 ±5.51 <sup>a</sup>	226 ±25.00 <sup>a</sup>	30.09 ±4.67 <sup>a</sup>
Algae	73 ±3.21 <sup>a</sup>	264 ±3.61 <sup>a</sup>	27.65 ±3.92 <sup>a</sup>
Fungus	136 ±3.51 <sup>b</sup>	294 ±4.00 <sup>b</sup>	46.26 ±1.70 <sup>b</sup>
Fungus + algae	137 ±10.12 <sup>b</sup>	315 ±17.35 <sup>b</sup>	43.49 ±2.76 <sup>b</sup>

applied together (fungus + algae treatment), the infection rate was 43.49 ±2.76%. While this result is still quite high, it is slightly lower than the fungus-only treatment group. This suggests that *C. vulgaris* may offer some degree of protection when combined with the fungal inoculation, but the overall effect was not significant enough to markedly reduce the infection rate when compared to the fungus-only treatment.

One notable observation is the comparison between the algae-only treatment and the combined treatment. Although the combined treatment did not significantly outperform the algae-only treatment, it provides an insight into the possibility that *C. vulgaris* might have a potential role in supporting plant defenses. However, the fact that it does not substantially reduce infection rates in the presence of the pathogen implies that further optimization or additional treatments (such as higher concentrations of algae extract or different methods of application) might be needed to enhance its antifungal activity.

In terms of the total number of fruits, the varying infection rates reflect the effectiveness of the treatments in mitigating fungal damage. The group treated with *C. vulgaris* exhibited a relatively healthy fruit population with a lower infection rate, despite not achieving complete protection. This suggests that the algae extract may be useful as part of an integrated disease management strategy, but further research is needed to explore its synergistic potential with other antifungal agents or treatments

Data are the mean (±SD) of 3 samples. Different letters in each column refer to significant differences (Tukey,  $p \leq 0.05$ ). Control: Plants without treatment; Fungus: Plants treated only with *Botrytis cinerea*; Algae: Plants treated with a 10% water extract of *Chlorella vulgaris*; Fungus + Algae: Plants treated with *Botrytis cinerea* followed by a 10% water extract of *Chlorella vulgaris*.

### 3.2 Discussion

The results of this study reflect the significant challenges posed by *Botrytis cinerea* to strawberry crops, a problem

well-documented in the literature (Dwivedi et al., 2024; El-Morsy et al., 2022). *B. cinerea*, a widespread pathogen, affects a wide variety of plants, including strawberries, and is a major contributor to economic losses in agriculture (Lage et al., 2024). Its ability to infect not only the fruit but also the leaves, flowers, and seeds makes it a formidable threat, as evidenced by our findings. We observed a high infection rate in the control group (30.09 ±4.67%), which reflects the vulnerability of strawberries to *B. cinerea* under field conditions. The comparison of environmental factors in Nitra with the study conducted in Moguer, Spain (Blanco et al., 2006), highlights the potential impact of climatic variables on fungal dispersal. Our data suggests that the warmer temperatures in Nitra in May it may have provided favorable conditions for *B. cinerea* spore dispersal, aligning with previous research (Blanco et al., 2006). However, the variable precipitation patterns in Nitra may have also played a role. Despite the higher-than-usual rainfall in April (very moist) and in May (normal), which might typically promote fungal growth, the lack of adequate rainfall in other periods did not appear to significantly reduce conidia dispersal, possibly due to other environmental factors such as increased solar radiation and temperature. This brings us to an important aspect of this study – alternatives to conventional fungicides, particularly the use of microalgae, which have gained attention for their antifungal properties. Unlike synthetic pesticides, microalgae offer a sustainable, bio-based solution that aligns with European agricultural directives (Righini & Roberti, 2019). Algae, including cyanobacteria, produce allelopathic substances that can inhibit the growth of various plant pathogens (Lage et al., 2024). Algae extracts have long been used to enhance plant resistance to stress, providing additional benefits such as plant growth hormones, micronutrients, and antifungal activity (Righini & Roberti, 2019). Our findings contribute to this body of knowledge by demonstrating that a 10% extract of *C. vulgaris* exhibited a mild antifungal effect on *B. cinerea*, reducing the infection rate in strawberries compared to the fungus-only treatment. Studies have previously shown the efficacy of algae extracts in combating *B. cinerea*. For instance, *Laminaria digitata* and *Undaria pinnatifida* extracts have been proven to fully inhibit *B. cinerea* growth and spore

germination (De Corato et al., 2017), with hexane-soluble extracts showing the strongest effect. Similarly, *C. vulgaris* has demonstrated antifungal activity against other pathogens such as *Aspergillus niger* (Ghasemi et al., 2007), with the bioactive compounds in the extract, such as phenolics and flavonoids, being largely responsible for the observed inhibitory effect (Ahmed, 2016).

The present study reinforces these findings by showing that a *C. vulgaris* extract reduced fungal growth and fruit infection. However, while this extract showed promising results, it did not completely prevent infection, indicating that its antifungal activity could be further enhanced. When combining *C. vulgaris* with the pathogen, the results are not significantly improved, suggesting that while the algae extract may reduce infection, it is not potent enough to completely eliminate the pathogen. This could be due to the extract concentration, which may need optimization. The study shows that *C. vulgaris* extracts can contribute to protection against *B. cinerea*, but further research is needed to improve effectiveness and application. These results support the potential of algae-based treatments in sustainable protection against fungal infections in agriculture.

#### 4 Conclusions

The present study investigated the potential of *Chlorella vulgaris* extracts as a natural biocontrol agent against *Botrytis cinerea*, a common fungal pathogen causing significant postharvest decay in strawberries. Laboratory-based assays demonstrated that the 10% water extract of *C. vulgaris* exhibited substantial antifungal activity, as evidenced by the inhibition of *B. cinerea* growth in the disc diffusion test. This suggests that the extract contains bioactive compounds capable of interfering with fungal growth, making it a promising candidate for use in crop protection. Field trials further supported the laboratory results, as strawberries treated with *C. vulgaris* extract showed a marked reduction in fungal infection rates compared to untreated control samples. The treated strawberries exhibited less decay and damage, indicating that the extract effectively reduced the incidence of *B. cinerea* infections in real-world conditions. This result highlights the practical applicability of *C. vulgaris* as an environmentally friendly and sustainable alternative to chemical fungicides in agricultural settings. The findings of this study suggest that *C. vulgaris* extracts could play an important role in integrated pest management strategies, offering a safer and more sustainable solution to managing fungal diseases in strawberries. However, further investigations are needed to refine application protocols, assess the long-term effectiveness of the extract in diverse environmental conditions,

and determine its economic feasibility for large-scale agricultural use. Additionally, research into the specific compounds responsible for the antifungal activity of *C. vulgaris* could help in developing more targeted and efficient biocontrol formulations. Overall, this study contributes to the growing body of evidence supporting the use of microalgae-based products in sustainable agriculture and provides a promising avenue for reducing reliance on chemical pesticides, thereby benefiting both the environment and public health.

#### Acknowledgments

This research was funded by the grant KEGA 023SPU-4/2024.

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