Microalgae strains of the Mosonmagyaróvár Algal Culture Collection with activity against plant fungal pathogens

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Abstract

Microalgae produce many secondary metabolites that are biologically active, including compounds with antifungal activity. These could potentially function as biofungicides. Selection criteria for potential strains include having good antifungal activity against specific phytopathogenic fungi and high biomass productivity rates to ensure sufficient biomass can be generated. Water extracts were prepared from 280 strains comprising of 33 Cyanophyceae strains (13 genera), 157 Chlorophyceae strains (29 genera), 80 Trebouxiophyceae strains (19 genera), 5 Klebsormidiophyceae strains (1 genus) and 1 Zygnematophyceae strain. These were tested against 9 phytopathogenic fungi. In total, 45% of the species had antifungal activity against at least one fungal pathogen. Cyanobacteria had the highest “hit-rate” (64%), followed by the Chlorophyceae (49%) and Trebouxiophyceae (30%). Water extracts of 19 strains had fungicidal activity – these were predominantly Cyanobacteria. The Cyanobacteria displayed a wider spectrum of activity with five strains being active (either fungicidal or fungistatic) against three or more fungal strains - *Trichormis variabilis* MACC-304 and *Tolypothrix tennis* MACC-205 had antifungal activity against 6 phytopathogens and *Nostoc linckia* MACC-612 inhibited 4 fungi. Each Chlorophyta strain was only active against 1–2 fungal strains. However, the daily productivity rates of Cyanobacteria were significantly lower than Chlorophyta strains. Further investigation of 15 Nostocales species (Families Nostocaceae, Tolypothrichaceae and Calotrichaceae) showed the *Nostoc* species generally had significantly lower biomass generation compared to other Nostocacaeae strains. The most promising strain was *Tolypothrix tenuis* MACC-205 which had the most potent, broad spectrum antifungal activity as well as significantly higher daily biomass productivity rates. Some microalgae strains (8%) had a stimulatory effect, suggesting the potential to screen strains especially from the Klebsormidiophyceae, for stimulating activity of beneficial plant growth promoting fungi. Thus, Cyanobacteria can potentially be developed as effective agricultural tools for environmentally-friendly disease management.

Introduction

Fungal infections are a common problem in agriculture, causing diseases such as leaf blight, vascular wilt, root rot and damping off (Asimakis et al., 2022). Intensive agricultural practices apply pesticides together with synthetic fertilizers for crop protection and to improve yield. However, their long term overuse has led to environmental problems such as soil salinization, eutrophication of water systems and ocean acidification (Wang et al., 2018; Costa et al., 2019). These chemicals are also detrimental to the rhizospheric microbial community which plays an important role in plant health and disease resistance (Yuan et al., 2017). There is now a shift towards more eco-friendly “green” agricultural practices such as the use of natural biostimulants and biopesticides to promote plant growth and improve tolerance to biotic and abiotic stresses (du Jardin, 2015; Costa et al., 2019). Benefits of natural biopesticides over synthetic pesticides include that they are more easily biodegradable than synthetic chemicals and thus have a lower residual; they are more specific and thus safer to non-target organisms such as beneficial soil microorganisms; their diverse mechanisms of action reduce the possibility of microbes developing resistance and thus they have a lower impact on the environment and human health (Costa et al., 2019; Asimakis et al., 2022).

One emerging category of natural biostimulants is microalgae (Colla & Rouphael, 2020). These are a rapidly growing, sustainable resource that can be cultivated on non-arable land using recycled wastewater (Falaise et al., 2016; Costa et al., 2019), thereby further contributing to the reduction of eutrophication. There are numerous studies showing that application of either extracts or living Cyanobacteria and Chlorophyta improve plant growth (reviewed in Chiaiese et al., 2018; Poveda, 2021). For example, hydrolysates of Cyanobacteria strains (*Nostoc* sp., *Tolypothrix* sp. and *Leptolyngbya* sp.) significantly increased root and shoot growth and leaf number in basil plants grown in a hydroponic system (Santini et al., 2022). *Chlorella* sp. and *Chlamydomonas reinhardtii* extracts increased fruit size in tomato. *Chlorella* sp. extracts induced earlier flowering and more flowers and *Chlamydomonas reinhardtii* extracts delayed flowering and reduced flower number. *Chlorella* sp. extracts increased chlorophyll b and carotenoid content and *Chlamydomonas reinhardtii* extracts increased chlorophyll a content (Gitau et al., 2022). This biostimulating activity is attributed to the microalgae being rich in primary metabolites (proteins, lipids and carbohydrates) as well as other beneficial metabolites such as plant hormones, polysaccharides, amino acids, vitamins, polyamines and antioxidant compounds (de Morais et al., 2015; Colla & Rouphael, 2020). The biostimulatory effects are strain specific with the elicited response dependent on the biochemical and structural properties on the microalgae.

Microalgae also produce a large variety of secondary metabolites which are biologically active, including compounds with antifungal activity (reviewed in Stirk & van Staden, 2022). These could potentially function as natural biofungicides. Most antifungal screening has focussed on medicinal applications where microalgal extracts are tested against human pathogens such as *Candida albicans* (Falaise et al., 2016; Poveda, 2021) with fewer studies focusing on plant pathogens. Examples of microalgae with phytopathogenic activity include *Nostoc commune* and *Oscillatoria tenuis* inhibiting *Fusarium oxysporum*, *Phytophthora capsici* and *Alternaria alternata* (Kim, 2006) and *Anabaena subcylindrica*, *Nostoc muscorum* and *Oscillatoria angusta* inhibiting seven pathogenic fungi isolated from infected Faba bean plants (Abo-Shady et al., 2007). There are examples of microalgae extracts reducing fungal diseases in greenhouse and field trials. For example, *Chlorella fusca* extracts (0.4% extract) applied at two-weekly intervals as a foliar application and soil irrigation enhanced strawberry plant growth and increased the chlorophyll content and number of flowers. It also decreased the incidence of *Fusarium* wilt disease in the strawberry crop by supressing the population of *Fusarium oxysporum* (Kim et al., 2020). Dipping wheat grains into the *Oscillatoria agardhii* and *Anabaena sphaerica* extracts and drying them reduced seed-born infection and improved germination when seeds were stored for up to 180 days (Haggag et al., 2014).

The long evolutionary history of microalgae and their diverse ecological habitats confer a vast potential to produce a number of secondary metabolites. However, most research has been conducted on only a few microalgae genera that are commonly cultivated for other biotechnological applications such as biofuel and animal feed (Santini et al., 2022). It is conservatively estimated that there are approximately 70 000 microalgae species of which about 73% have been described (Guiry, 2012). Only a small fraction of these are maintained in culture collections and have been evaluated for their biological activity (de Morais et al., 2015). Thus microalgae provide a rich, untapped reserve of novel compounds which could potentially be developed as biocontrol agents for agriculture.

The commercialization of microalgae-derived biofungicides is still in its infancy and they are only used niche markets such as organic agriculture (Costa et al., 2019). There is a need to screen diverse genera to find suitable strains with good bioactivity (Colla & Rouphael, 2020) coupled with rapid growth rates and a robust morphology so that they can be cultivated in outdoor systems to generate sufficient biomass for commercial applications (Borowitzka & Vonshak, 2017). The aim of the present study was screen taxonomically diverse microalgae for antifungal activity against phytopathogenic fungi in order to identify fast-growing strains with broad-spectrum antifungal activity.

Materials And Methods

Microalgae strains screened for antifungal activity

The Mosonmagyaróvár Algal Culture Collection (MACC) of the Széchenyi István University, Hungary maintains 970 strains in culture. In total 280 strains were selected from the MACC to test for antifungal activity. These comprised of 33 Cyanophyceae strains from 13 genera, 157 Chlorophyceae strains from 29 genera, 80 Trebouxiophyceae strains from 19 genera, 5 Klebsormidiophyceae strains from 1 genus and 1 Zygenematophyceae strain (Table 1; Supplementary Table A). These strains originated from soil and water samples collected in six European countries and Brazil (South America; Supplementary Table A). The main criteria for strain selection was based on their dry weight (DW) and daily biomass production with selected Cyanobacteria strains having over 1.5 $g.L^{-1}$ DW and 0.1 $g.L^{-1}$ daily biomass production and Chlorophyta and Charophyta having over 2.0 $g.L^{-1}$ DW and 0.2 $g.L^{-1}$ daily biomass production. All strains were monoalgal and *Chlorella* and *Chlamydomonas* strains were axenic. Besides microscopic taxonomic determination, *Anabaena*, *Nostoc*, *Chlorella*, *Scenedesmus* and *Chlamydomonas* strains have been identified by molecular sequencing. The global algal database “AlgaeBase” was used in the description of the taxonomic positions of the MACC strains (Guiry & Guiry, 2022).

Table 1
Number of strains and their taxonomic position of the 280 MACC microalgae strains tested for antifungal activity

Class	Order	Family	No. of Genera	No. of strains
Phylum Cyanobacteria				
Cyanophyceae	Nostocales	Nostocaceae	3	12
		Tolypothrichaceae	1	3
		Calotrichaceae	1	4
	Oscillatoriales	Oscillatoriaceae	3	8
		Microcoleaceae	1	1
	Synechococcales	Synechococceae	1	1
		Leptolyngbyaceae	1	1
		Merismopediaceae	1	1
	Chroococcales	Chroococcaceae	1	2
			33 strains	
Phylum Chlorophyta				
Chlorophyceae	Chlamydomonadales	Chlamydomonadaceae	3	31
		Chlorococcaeae	4	30
		Chlorosarcinaceae	2	5
		Haematococcaceae	1	1
		Pleurastraceae	1	1
	Sphaeropleales	Scenedesmaceae	7	41
		Radiococcaceae	3	12
		Neochloridaceae	2	15
		Selenastraceae	2	5
		Mychonastaceae	1	10
		Bracteacoccaceae	1	3
		Hydrodictyaceae	1	1
		Chaetophoraceae	1	2
			157 strains	
Trebouxiophyceae	Chlorellales	Chlorellaceae	4	43
		Oocystaceae	5	13
	Prasinolales	Strichococcaceae	2	11
		Prisinolales incertae sedis	1	1
	Watanabeales	Watanabeaceae	1	8
	Trebouxiophyceae ordo incertae sedis	Coccomyaceae	1	2

Class	Order	Family	No. of Genera	No. of strains
		Trebouxiophyceae incertae sedis	1	1
	Trebouxiales	Trebouxiaceae	1	1
			80 strains	
Ulvophyceae	Ulotrichales	Planophilaceae	1	1
		Ulotrichaceae	2	3
			4 strains	
Phylum Charophyta				
Klebsormidiophyceae	Klebsormidiales	Klebsormidiaceae	1	5
			5 strains	
Zygenematophyceae	Zygenatales	Zygnemataceae	1	1
			1 strain	

The 280 microalgae strains were maintained on solidified medium in dim light at 15 ± 2°C in a stock culture room. Each Cyanobacteria strain was initially inoculated from agar culture into two 500 mL Erlenmeyer flasks containing either 250 mL Zehnder-8 liquid medium (Staub, 1961) or BG-11 medium (Rippka et al., 1979). The Chlorophyta and Charophyta strains were inoculated into Tamiya (Kuznetsov & Vladmirova, 1964) or Bristol (Bold, 1949) liquid medium (Supplementary Table A). The cultures were grown in the apparatus described by Ördög (1982) under controlled laboratory conditions at 25 ± 2°C in a 12:12 h light:dark photoperiod. Cultures were illuminated from below with 130 µmol photon.m^2.s^{-1} light intensity and aerated continuously (1.33 vvm 20 L.h^{-1} per flask) with sterile air enriched with 1.5% CO_2 during the day. After 7 days, the suspension cultures were used to inoculate four 500 mL flasks containing 250 mL culture medium. The starting density of the cultures was 10 mg.L^{-1} DW. The cultures were harvested between 8–21 days when they were in the stationary growth phase.

The dry weight was determined by filtering 5–20 mL cell suspension from each flask through Whatman GF/C glass fibre filters as previously described (Stirk et al., 2020). This was used to calculate the DW of the biomass at harvest and the average daily production of the strain (n = 4). The remainder of the cell suspension from the four flasks was combined and centrifuged (2150 x *g* for 15 min at room temperature). The pellet was freeze-dried (Christ Gamma 1–15) and stored at -18°C until tested for antifungal activity.

Fungal strains

Axenic cultures of nine phytopathogenic fungi isolated from infected plants were obtained from the collection of the Department of Plant Sciences, Széchenyi István University, Mosonmagyaróvár, Hungary (Table 2). Identification of fungal species was carried out based on host plant identification and micro- and macromorphological characteristics. In addition, a fragment of the translation elongation factor 1 alpha (EF-1α) gene was sequenced in *Fusarium graminearum* and the ITS nrDNA region in *Rhizoctonia solani* was sequenced to help with species specific identification. The ITS nrDNA region, fragments of the RNA polymerase 2 gene (*rpb2*) and the glyceraldehyde-3-phosphate dehydrogenase *(gapdh*) genes were also sequenced from *Alternaria alternata* and *Botrytis cinerea* to resolve the species complexes these strains belong to.

Table 2
Fungal phytopathogens used to screen 280 microalgae (Cyanobacteria, Chlorophyta and Charophyta) from the Mosonmagyaróvár Algal Culture Collection for antifungal activity

Fungal pathogens	Diseases	Hosts	Isolated from[a]
Alternaria alternata (Pleosporales)	leaf spot, fruit rot	wide range of host plants including *Allium*, *Beta*, *Brassica*, *Capsicum*, *Lycopersicon*, *Solanum*, *Triticum*	*Vitis vinifera*
Fusarium graminearum (Hypocreales)	ear mold, head blight	wheat, barley, rice, oats, maize	*Triticum aestivum*
Rhizoctonia solani (Cantharellales)	damping off, black scurf, root rot	wide range of host plants including soybean, potato, cereals, sugarbeet	*Solanum tuberosum*
Pythium ultimum (Pythiales)	seed rot, damping off, black-leg of seedlings	wide range of host plants including maize, soybean, potato, wheat	*Zea mays*
Phytophthora infestans (Peronosporales)	late blight	potato, tomato	*Solanum tuberosum*
Plasmopara viticola (Peronosporales)	grape downy mildew	wine grapes	*Vitis vinifera*
Phaeoramularia capsicicola (Mycosphaerellales)	velvet spot, leaf loss	pepper	*Capsicum annuum*
Botrytis cinerea (Helotiales)	grey mold	wide range of host plants including wine grapes, strawberry, tomato, rhubarb	*Fragaria × ananassa*
Sclerotinia sclerotiorum (Helotiales)	white mold	wide range of host plants including herbaceous plants, woody ornamentals	*Helianthus annuus*

[a] Host species from which the fungal strains were isolated for the present study

Fungal cultures were maintained on Potato Dextrose Agar (PDA) medium at 4°C with a monthly passage regime. The exception was the obligate biotrophic parasite *Plasmopara viticola* which was maintained *in vitro* on detached grapevine leaves. Young grapevine leaves were surface sterilized by dipping in 2% sodium hypochlorite (60 s), then 70% ethanol (90 s) and washed in sterile tap water. After drying, leaves were placed with their adaxial surface upwards onto the surface of Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) into Petri dishes (MS medium Mod. No. 1B, Duchefa, solidified with 6.5 $g.L^{-1}$ phyto agar) and the tip of the petiole was inserted into the medium. The dishes were kept at 25 ± 2°C in a 12:12 h light:dark photoperiod and the pathogen was transferred monthly.

Extract preparation

The freeze-dried microalgae biomass was resuspended in distilled water (10 $mg.mL^{-1}$ DW) and sonicated for 3 min (VirSonic 600, Virtis) to disrupt the cells to improve the extraction of secondary metabolites. This extract was used in the agar-diffusion and *Plasmopara* leaf disc assays to test for antifungal activity.

Antifungal assays

The antifungal activity of the 280 microalgae water extracts was determined against the eight fungal pathogens (excluding *P. viticola*) using the *in vitro* agar diffusion method (Aponyiné, 1997). Briefly, 7 day old fungal mycelium cultures were washed and suspended in cooled PDA medium (42°C), gently shaken and then poured into sterile 90 mm petri dishes. Once solidified, four equidistant holes were cut in the agar with a 9 mm cork borer and 150 µL microalgae extract placed in three holes. Sterile tap water was added to the fourth hole as the negative control (C-). The petri dishes were sealed with parafilm and incubated at 25 ± 2°C in the dark. The effect of the microalgae extracts on fungal mycelium growth was evaluated by measuring the diameter of the inhibition ring (including the diameter of the hole) 72 h after incubation. Further observations were made at 96 h and 120 h. The toxicity of the microalgae extract was evaluated by scoring the “clearness” of the inhibition ring. The effect was classified

into one of the following categories: fungicidal activity (F), mild (FS-), medium (FS–) or strong fungistatic (FS—) activity and mild (S+), medium (S++) and strong (S+++) stimulatory activity. Each microalga was tested in four parallels in two repeated experiments (n = 8).

It was necessary to use a different method to test antifungal activity against *P. viticola* as it can only grow on plant tissue. For this, either whole leaves or 10 mm leaf discs cut from grape leaves (*Vitis vinifera* var. Kékfrankos) were placed onto sterile solidified MS medium in a 90 mm Petri dish and sprayed with 500 µL microalgae extract or water (C-). The leaves and leaf discs were dried and then inoculated with *P. viticola* suspension (10^4 zoosporagium cm^{-3}). Petri dishes were incubated at 25 ± 2°C for 7 days. Antifungal activity was evaluated on the 8th day by estimating the leaf area ratio covered with the pathogen and comparing it to the negative control.

Antifungal activity in the Nostocales

Based on the results of the screening study, 15 Cyanobacteria strains from the Nostocales (Families Nostocaceae, Tolypothrichaceae and Calotrichaceae) were selected for further testing. These included 8 strains showing fungicidal (F) or strong fungistatic (FS—) activity in the initial screening (Supplementary Table A). This study was expanded to include an additional seven Nostocales strains that were not previously screened. The microalgae were grown as described above and harvested on day 6. Water extracts were made from the freeze-dried algal pellet as outlined above.

These extracts were tested against four fungal strains. *Alternaria alternata* and *Fusarium graminearum* were tested using the method described above where the fungal mycelium was mixed into the cooled PDA medium (termed mixed-method). As *Botrytis cinerea* and *Rhizoctonia solani* suspensions did not mix homogenously in the cooled PDA medium, it was necessary to modify the assay method. For this, PDA medium was poured into 90 mm Petri dishes and 6 mm discs were cut from growing regions of the fungal colonies and placed upside down in the middle of the PDA plate (termed disc-method). As with the mixed-method, four 9 mm equidistant holes were cut in the solidified PDA plates. Three holes were filled with 150 µL microalgae extract and the fourth hole with sterile tap water (C-). The commercial fungicide Quadris (active ingredient 250 $g.L^{-1}$ azoxystrobin) was included in the assays as a positive control (C+). This was dissolved in sterile tap water to give a final concentration of 1 $\mu g.mL^{-1}$. Each microalgae extract and the positive control were tested in four parallel plates in three independent experiments to give 12 parallel measurements for each Cyanobacteria strain (n = 12).

After 72 h incubation at 25 ± 2°C in the dark, the radius of the inhibition ring (without the radius of the hole) was measured (mm) for the mixed-method assay. For the disc-method, the inhibition zone was measured as the distance (mm) from the fungal colony edge to the treatment holes. The negative control was taken into account in these measurements. The toxicity of the extract was evaluated in two ways depending on the assay method. For the mixed-method, the “clearness” of the inhibition ring as ranked using the scale from 1–4 where 1 = no visible effect, 2 = mild FS, 3 = strong FS and 4 = F effect. The mean of the 12 parallel replicates was calculated and the extract was classed as non-fungistatic (nFS; mean between 1-1.5), weak fungistatic (Fs-; mean between 1.6–2.5), strong fungistatic (Fs–; mean between 2.6–3.5) or fungicidal (F; mean between 3.6-4.0). For the disc-method, the extent of the fungal growth towards the microalgae extracts was measured at 48 h and 72 h. This growth over 24 h (mm) was ranked into four categories, namely nFS (> 8.0 mm), Fs- (5.5-8.0 mm), Fs—(3.0-5.5 mm) and F (0–3.0. mm).

Statistical analysis

Statistical differences in the average growth rates of the microalgae were calculated by One-way ANOVA followed by the post-hoc Tukey test. Data was tested for normality using the Shapiro-Wilk test (SigmaPlot v. 13). To analyse the effect of the Nostocales extracts on mycelium growth, a linear model was used where the treatment (MACC strain, positive and negative controls) was included as a fixed factor. Differences between the microalgae strains and controls were compared by contrasting the emmeans function with the false discovery rate (fdr) p value adjustment method (RStudio Team).

Results

Growth rates of the 280 microalgae strains

Cultures were harvested once they entered the stationary growth phase. This was detected visually by the colour and density of the culture. The average harvest day for strains in each class was 13–14 days. The four Ulvophyceae strains were harvested significantly later at 17-18-days (Table 3). Growth rates measured as dry weight at harvest and daily biomass productivity were significantly similar in the Chlorophyta (Chlorophyceae, Trebouxiophyceae and Ulvophyceae) and Charophyta. The Cyanophyceae had significantly lower growth rates compared to the Chlorophyta strains (Table 3; Supplementary Table A).

Table 3
Growth rates of microalgae strains used in the antifungal screening study harvested in the stationary growth phase. Results are presented as mean ± SEM of the microalgae strains within each family. Different letters in each column represent significant differences ($p < 0.05$)

Class (no. of strains)	Harvest day	Dry weight ($g.L^{-1}$)	Daily biomass productivity ($g.L^{-1}$)
Cyanophyceae (33)	14.3 ± 0.5^{ab}	2.04 ± 0.12^{b}	0.15 ± 0.01^{b}
Chlorophyceae (157)	14.1 ± 0.2^{ab}	2.61 ± 0.06^{a}	0.19 ± 0.04^{a}
Trebouxiophyceae (80)	13.1 ± 0.3^{b}	2.62 ± 0.08^{a}	0.20 ± 0.01^{a}
Ulvophyceae (4)	17.8 ± 1.6^{a}	3.20 ± 0.29^{a}	0.18 ± 0.01^{a}
Klebsormidiophyceae (5)	13.6 ± 2.5^{ab}	2.44 ± 0.68^{ab}	0.18 ± 0.05^{ab}
Zygenematophyceae (1)	14.0	1.68	0.12

Antifungal activity of the 280 microalgae strains

In total, 127 extracts showed some antifungal activity against at least one of the nine fungal pathogens. This was an overall “hit-rate” of 45%. The Cyanophyceae had the highest incidence of strains with antifungal activity with 64% of the strains tested showing some activity. In comparison, less than half the Chlorophyceae (49%) and Trebouxiophyceae (30%) strains tested had antifungal activity. Too few strains were tested from the other taxonomic classes to discern trends (Table 4). A few microalgae strains (8%) had a stimulatory effect on the fungal pathogens. Three of the 5 Klebsormidiophyceae strains (60%) were stimulatory as well as 8% of the Chlorophyceae and Trebouxiophyceae strains. No Cyanophyceae had a stimulatory effect on the fungal pathogens (Table 4). A few strains, namely *Chlorosarcina* sp. MACC-560, *Scenedesmus acutus* var. *globosus* MACC-551, *Scenedesmus* sp. MACC-575, *Gloeocystis* sp. MACC 631 (Chlorophyceae) and *Chlorella* sp. MACC-564 (Trebouxiophyceae) had both inhibitory and stimulatory activity against different pathogen strains (Supplementary Table A). The active microalgae strains were isolated from both soil and water habitats (Table 4).

Table 4
Number of microalgae strains with inhibitory and stimulatory activity against the nine phytopathogens. The "hit-rate" shows the % of strains with activity from the total number of tested strains in each class

Class (no. of strains)	**No. of strains with inhibitory activity**			**No. of strains with stimulatory activity**		
	Strain origin			Strain origin		
	Soil	Water	"Hit-rate"	Soil	Water	"Hit-rate"
Cyanophyceae (33)	13	8	64%	-	-	-
Chlorophyceae (157)	51*	26*	49%	6*	7*	8%
Trebouxiophyceae (80)	19	5*	30%	3	3*	8%
Ulvophyceae (4)	4	-	100%	-	-	-
Klebsormidiophyceae (5)	-	-	-	2	1	60%
Zygenematophyceae (1)	-	1	100%	-	-	-
Total (280)	**87**	**39**	**45%**	**11**	**11**	**8%**
* Some strains with both inhibitory and stimulatory activity						

Water extracts of 19 of the 280 microalgae strains had fungicidal activity against at least one of the nine fungal phytopathogens. Cyanobacteria had a higher hit-rate with 20.5% of the strains (7 strains) having fungicidal activity compared to 4.9% (12 strains) of the Chlorophyta having fungicidal activity. The active Cyanobacteria were all from the Nostocales and the Chlorophyta belonged to the Chlorophyceae and Trebouxiophyceae (Table 5). The Cyanobacteria strains displayed a wider spectrum of activity with five strains being active (either fungicidal or fungistatic) against three or more fungal strains, namely *Trichormus variabilis* MACC-304 and *Tolypothrix tenuis* MACC-205 had antifungal activity against 6 phytopathogens and *Nostoc linckia* MACC-612 inhibited 4 fungi. In contrast, each Chlorophyta strain was only active against 1–2 fungal strains (Table 5).

Table 5

Cyanophyta and Chlorophyta MACC-strains demonstrating fungicide (F) activity against at least one of the nine phytopathogens detected with the agar gel diffusion bioassay. FS shows fungistatic activity. The number indicates the size of the inhibition zone (mm). *Plasmopara viticola* was tested using leaf discs made from grape leaves. Results are the mean of 8 replicates

Microalgae		**Phytopathogen**								
Taxon	MACC strain code	*Alternaria*	*Fusarium*	*Rhizoctonia*	*Pythium*	*Phytophthora*	*Plasmopara*	*Phaeoramularia*	*Botrytis*	*Sclerotinia*
Cyanophyta										
Nostocales										
Trichormus variabilis	304	F-13		F-13	F-13			F-13	F-13	F-12
Nostoc linckia	612	F-9	F-9		F-9			F-9		
Tolypothrix sp.	465				F-15			F-13		
Tolypothrix tenuis	205	F-19		F-17	F-17	FS-25		F-24	F-15	
Nostoc insulare	661							F-13		
Nostoc calcicola	251	F-19							FS-20	
Nostoc sp.	150				FS-15	FS-25		F-23		
Chlorophyta										
Chlorophyceae										
Desmococcus olivaceus	343				FS-22			F-25		
Scenedesmus sp.	540	FS-10						F-14		
Fernandinella alpina	682	FS-21		F-13						
Scenedesmus sp.	9								F-10	
Lobochlamys segnis	10					F-24			F-14	
Desmodesmus dispar	302						F			
Haematococcus lacustris	90					F-25				
Trebouxiophyceae										
Mychonastes homosphaera	339				F-20			F-20		
Scotiellopsis sp.	14						F			
Pseudococcomyxa sp.	76						F			
Stichococcus bacillaris	147							F-21		
Mychonastes homosphaera	151							F-22		

Phaeoramularia capsicicola was the most susceptible fungi with 25% of the microalgae extracts inhibiting its growth with 11 extracts having fungicidal activity. Other susceptible fungi were *Fusarium graminearum*, *Pythium ultimum* and *Botrytis cinerea* where the microalgae extracts mainly had weak fungistatic activity. The most resistant fungi was *Plasmopara viticola* with only 3 microalgae extracts having an inhibitory effect (Fig. 1A). However, this may have been due to the assay used to test the extracts. Four of the fungal strains were stimulated by a few microalgae extracts with *Botrytis cinerea* being the most frequently stimulated fungi (Fig. 1B).

Antifungal activity in the Nostocales

The *Nostoc* strains generally had significantly slower growth (determined as DW and daily biomass productivity on day 6) compared to the other microalgae strains (Table 6).

Table 6
Culture collection details and growth rates of 15 Nostocales strains analyzed for antifungal activity against four phytopathegenic fungi. Cultures were harvested on day 6. Results are presented as mean ± SEM (n = 4)

Microalgae				**Dry weight**	**Daily biomass productivity**
Taxon	MACC	Origin	Habitat	($g.L^{-1}$)	($g.L^{-1}$)
Family Nostocaceae					
Nostoc calcicola	150	Hungary	water	0.371 ± 0.012^{c}	0.062 ± 0.002^{c}
Nostoc calcicola	251	Serbia	soil	0.561 ± 0.026^{ab}	0.093 ± 0.004^{ab}
Nostoc insulare	661	Czech Republic	soil	0.355 ± 0.035^{c}	0.059 ± 0.006^{c}
Nostoc linckia	612	Czech Republic	water	0.405 ± 0.065^{c}	0.067 ± 0.011^{c}
Nostoc muscorum	132	Germany	water	0.388 ± 0.019^{c}	0.065 ± 0.003^{c}
Nostoc muscorum	189	Serbia	soil	0.405 ± 0.066^{c}	0.067 ± 0.011^{c}
Nostoc muscorum	683	Brazil	soil	0.626 ± 0.045^{ab}	0.104 ± 0.007^{ab}
Trichormus variabilis	128	Serbia	soil	0.853 ± 0.030^{a}	0.142 ± 0.005^{a}
Trichormus variabilis	134	Hungary	water	0.708 ± 0.015^{ab}	0.118 ± 0.002^{ab}
Trichormus variabilis	221	Serbia	soil	0.511 ± 0.030^{ab}	0.085 ± 0.005^{ab}
Trichormus variabilis	304	Russia	water	0.675 ± 0.031^{ab}	0.112 ± 0.005^{ab}
Anabaena cylindrica	307	Russia	water	0.617 ± 0.025^{ab}	0.103 ± 0.004^{ab}
Family Tolypothrichaceae					
Tolypothrix tenuis	205	Germany	water	0.647 ± 0.142^{ab}	0.108 ± 0.023^{ab}
Tolypothrix sp.	465	Brazil	soil	0.835 ± 0.049^{ab}	0.139 ± 0.008^{ab}
Family Calotrichaceae					
Calothrix sp.	405	Brazil	soil	0.611 ± 0.051^{ab}	0.102 ± 0.007^{ab}

Overall, *Rhizoctonia solani* was the most susceptible fungi with all the microalgae extracts inhibiting its growth with nine of the extracts having strong fungistatic activity (Fig. 2D). *Alternaria alternata* was the least susceptible fungi with seven microalgae extracts having no inhibitory activity (Fig. 2A). The positive control azoxystrobin (1 $\mu g.mL^{-1}$) had fungicidal activity against the four fungi strains (Fig. 2). The only microalgae extract to have fungicidal activity was *Tolypothrix tenuis* MACC-205 against

Botrytis cinerea (Fig. 2C) as well as strong fungistatic activity against *Alternaria alternata* (Fig. 2A). Other extracts with significantly high antifungal activity were *Tolypothrix* sp. MACC-465 and *Nostoc linckia* MACC-612 against *Fusarium graminearum* (Fig. 2B), *N. muscorum* MACC-189 and *N. calcicola* MACC-150 against *Botrytis cinerea* (Fig. 2C) and *Trichormus variabilis* MACC-304 and *Nostoc insulare* MACC-661 against *Rhizoctonia solani* (Fig. 2D). There was no apparent trend between the growth rates and antifungal activity.

Discussion

The cost of producing microalgae-derived biofungicides is limiting their market potential. Their success will depend on strain selection to encompass rapid growth, a robust morphology suitable for mass cultivation systems and a high level of the target compounds (Righini et al., 2022) It is also necessary keep down-stream production costs (harvesting, drying and biomass processing) to the minimum before microalgae biofungicides can compete with synthetic agrochemicals and become an economically viable agricultural tool (Costa et al., 2019; Gitau et al., 2022; Righini et al., 2022). The least time-consuming and most energy and cost-efficient way to prepare microalgae biomass for use in agriculture is to remove the liquid medium (dewatering) to form a pellet which is then suspended in water and applied as a foliar spray or soil drench (Gitau et al., 2022). This eliminates the need to use expensive solvents or other energy-intensive extraction methods. It is thus best to test for water-soluble compounds when screening for suitable strains to develop as biofungicides (Asimakis et al., 2022) as was done in the present study where water extracts were prepared from the microalgal biomass. While there are many microalgal screening studies testing organic solvent extracts, aqueous extracts are less widely investigated (Righini et al., 2022).

Cell pellets of the 280 strains were freeze-dried and briefly sonicated (3 min) prior to extraction. Freeze-drying damages the cell membrane due to the formation of intracellular ice-crystals, making it more porous so that water soluble, low molecular weight compounds can be released (Lee et al., 2017). The cell wall acts as a barrier for compound extraction and needs to be disrupted to improve compound extraction. For example, electrical conductivity was higher in freeze-dried *Chlorella* and *Scenedesmus* extracts compared to living cultures and electrical conductivity and extract yields further increased when cells were sonicated for 3 min which caused 10–20% disruption to the cell walls (Stirk et al., 2020). The effectiveness of the cell disruption method is dependent on the cell wall structure (Günerken et al., 2015) with sonication being more suitable for species with less resistant cell walls (Alhattab et al., 2019). Highly resistant cell walls require more intense cell disruption methods and this would increase the downstream production costs. Such strains would not be suitable for commercialization of microalgae biostimulants.

In the present study, cultures were harvested once they had reached their stationary growth phase. On average, cultures were harvested between 13–14 days with the four Ulvophyceae strains being harvested later (17–18 days). The dry mass at harvest and the daily biomass productivity were significantly lower for the Cyanophyceae strains compared to the Chlorophyta strains (Table 3). Productivity rates (including inoculum generation time) are a crucial consideration when selecting potential microalgae strains for commercial production of microalgal products (Borowitzka & Vonshak, 2017).

Almost half (45%) the water extracts screened in the present study had some antifungal activity against at least one of the nine phytopathogenic fungi tested. The water extracts of the Cyanobacteria strains had the highest incidence (% hit-rate) of biological activity with 64% of the tested strains showing some antifungal activity, followed by the Chlorophyceae (49% hit-rate) and the Trebouxiophyceae having the lowest incidence of strains with antifungal activity (30% hit-rate; Table 4). Other studies report similar or lower incidences of antifungal activity for both water and organic solvent extracts (Kim, 2006; Prasanna et al., 2008; Najdenski et al., 2013; Mudimu et al., 2014; Cepas et al., 2019). For example, organic solvent (petroleum ether, propanol and methanol) and water extracts of 40 strains from 9 genera of halotolerant Cyanobacteria were tested against five phytopathogenic fungi that had been isolated from diseased plants and seeds. Of these, 20 isolates had antifungal activity (50% hit-rate) with three isolates having “hyper-antifungal activity” (strong antifungal activity). *Synechocystis* sp. had the broadest spectrum of antifungal activity (Pawar & Puranik, 2008).

The Cyanobacteria also had a higher efficacy, having a higher incidence of fungicidal activity (20.5%) compared to the Chlorophyta (4.9%). The Cyanobacteria strains with fungicidal activity were all from the Nostocales. In addition, the Cyanobacteria generally had a broad spectrum of antifungal activity with many strains being active against 3 or more fungal

pathogens while the Chlorophyta were only active against 1–2 fungal pathogens (Table 5). Similarly, in a screening of 225 microalgae from 13 phylum for biofilm inhibitory activity, species from the Crypthophyta, Euglenophyta and Glaucophyta had the best broad-spectrum antimicrobial activity while inhibitory activity in the Chlorophyta was generally limited to *Candida albicans* and *Enterobacter cloaceae* (Cepas et al., 2019). A review of antimicrobial activity based on minimum inhibitory concentrations (MIC) indicated that species from the Nostocales were the most active Cyanobacteria strains while antimicrobial activity was similar in most Chlorophyta orders (Stirk & van Staden, 2022). Comparison of antifungal activity in the 15 Nostocales strains investigated suggest that species from the Family Tolypothrichaceae are the most promising. *Tolypothrix tenuis* MACC-205 had fungicidal activity against *Botrytis cinerea* and strong fungistatic activity against *Alternaria alternata*. These microalgae also had significantly higher growth rates (DW and daily biomass productivity) compared to the majority of the *Nostoc* strains investigated (Table 6).

This suggests that it should be possible to design screening programmes using a taxonomic approach to improve the “hit-rate” with Nostocales being the most promising order to date. It is also important to list microalgae species that had no or weak antifungal activity as a guideline to the taxonomic groups which have a low probability of activity (Stirk & van Staden, 2022) as was done in the present study (Supplementary Table A).

Many antifungal compounds including novel metabolites have been identified in Cyanobacteria with the Nostocaceae being the most widely studied, suggesting that they are a metabolically versatile family (Falaise et al., 2016). Cyanobacteria produce a wide variety of lipoproteins with over 600 identified to date. Many lipoproteins such as nostofungicidine, laxaphycins, lobocyclamides, hassallidins and anabaenolysins have antifungal activity (Shishido et al., 2015). Other biologically active metabolites include polysaccharides and phycobiliproteins (Najdenski et al., 2013; Righini et al., 2022). Polysaccharides precipitated from aqueous extracts of *Anabaena* sp. inhibited growth of *Botrytis cinerea*. Pre-harvest treatments to strawberry fruits reduced infection in a dose-response manner. Post-harvest treatments were not effective, suggesting that the polysaccharides induced plant defence mechanisms in strawberry plants (Righini et al., 2019). Water soluble phycobiliproteins extracted from *Arthrospira platensis* reduced colony growth, colony forming units and spore germination of *Botrytis cinerea* when grown on PDA. Post-harvest treatment of tomato fruits prior to artificially infecting them with *Botrytis cinerea* reduced the disease incidence and its severity in a dose-dependent relationship (Righini et al., 2020). Free (predominately chlorogenic acid) and bound phenolic compounds extracted from *Spirulina* sp. and *Nannochloropsis* sp. (Ochrophyta) had antifungal activity against three *Fusarium* strains while conjugated phenolic compounds and carotenoids had no activity. Natural phenolic extracts from these microalgae were more effective than similar synthetic formulations indicating synergistic effects can alter the potency of the extract (Scaglioni et al., 2019).

In contrast, fewer antimicrobial compounds have been identified in the Chlorophyta. Antimicrobial activity of Chlorophyta extracts is often linked to their fatty acid content (Plaza et al., 2012; Shaima et al., 2022). Polar organic solvents such as methanol are the most effective for fatty acid extraction. This may explain the limited antifungal activity of the water extracts made from Chlorophyta strains in the present study compared to the broad spectrum of activity in the water extracts of the tested Cyanobacteria strains.

However, the diversity of metabolites in Cyanobacteria also has some drawbacks with approximately 26% of Cyanobacteria genera producing toxins that are harmful to humans and animals and elicit toxic effects in plants (Poveda, 2021; Righini et al., 2022). While *Nostoc* sp. was effective at controlling *Sclerotinia sclerotiorum* infections in tomato seedlings, it also elicited some negative effects on the plants. This was probably due to cytotoxic metabolites synthesized by *Nostoc* sp. (Biondi et al., 2004). Similarly, 31 Cyanobacteria strains from 12 genera were tested for biostimulatory activity in the watercress germination assay and for antifungal activity against *Pythium ultimum* in an agar-diffusion assay. One third of the extracts had antifungal activity with five strains having good activity. Most strains improved the germination and seedling growth but eight strains had a phytotoxic effect (Toribio et al., 2021). These negative effects are mainly due to microcystins and cylindrospermopsins which cause structural and physiological changes in the plants (reviewed in Righini et al., 2022). Future screening studies for microalgae biofungicidal agents should also incorporate assays to test for phytotoxicity as this could make application of Cyanobacteria in agriculture problematic.

Excessive use of pesticides is detrimental to the beneficial soil microorganisms (Kim & Kim, 2008). Three of the five tested Klebsormidiophyceae and a few Chlorophyceae and Trebouxiophyceae tested in the present study had a stimulatory effect on the fungi, especially *Botrytis cinerea*. Similarly, *Spirulina* sp. and *Nannochloropsis* sp. promoted *Sclerotium rolfsii* and *Alternaria alternata* growth respectively (Schmid et al., 2022). While microalgae strains must be screened against a range of phytopathogenic strains to ensure they do not promote the growth of some phytopathogenic fungi, there is potential to screen microalgae especially from the Klebsormidiophyceae, for stimulating activity of beneficial plant growth promoting fungi. These strains could potentially be applied as to the soil to promote the rhizosphere microorganism communities and thereby improve soil health.

Phaeoramularia capsicicola was the most susceptible fungal strain tested in the present study with 11 microalgae having fungicidal activity. Other susceptible strains included *Fusarium graminearum*, *Pythium ultimum* and *Botrytis cinerea*. The most resistant strains were *Plasmopara viticola*, *Sclerotinia sclerotiorum* and *Phytophthora infestans* (Fig. 2A). These results are similar to other studies while other results are variable. For example, *Alternaria alternata* and *Botrytis cinerea* were most susceptible and *Pythium ultimum* and *Rhizopus stolonifer* most resistant when tested against 142 cyanobacterial strains (Kim, 2006); *Fusarium moniliforme*, *Fusarium solani*, *Alternaria solani* and *Aspergillus candidus* were the most susceptible when tested against 70 *Anabaena* strains. Other strains including *Pythium debaryanum*, *Fusarium oxysporum*, *Fusarium graminearum*, *Rhizoctonia solani* and *Sclerotium oryzae* were not inhibited. This antifungal activity was linked to the production of hydrolytic enzymes (Prasanna et al., 2008). *Sclerotium rolfsii* was the most susceptible and *Alternaria alternata* was the most resistant when tested against 5 microalgae (Schmid et al., 2022). This variability indicates that there is strong target specificity with activity depending on both the microalgae strain and the phytopathogen.

The mechanisms of action may either be direct action to the fungal mycelium (toxic or inhibitory), may induce defence mechanisms in the plant via elicitor molecules and quorum sensing inhibitors or form biofilms that block sites for fungal infections (Poveda, 2021; Asimakis et al., 2022). Microalgae synthesize secondary metabolites and enzymes that are released into the environment where they can degrade fungal cell walls, damage cell membranes, inactivate enzymes and suppress fungal protein and DNA synthesis. They may also stimulate the defence system in plants by activating various defence-related enzymes (Costa et al., 2019; Poveda, 2021).

Conclusion

Microalgae can potentially provide effective tools in environmentally-friendly disease management. A number of criteria need to be considered when selecting potential strains as natural fungicides in order to be more effective and cheaper than synthetic chemicals. In addition to having good antifungal activity against specific phytopathogenic fungal strains, biomass productivity rates are an important criterion to ensure that sufficient biomass can be generated. Cyanobacteria from the Nostocales showed the most promising antifungal activity with water extracts having broad spectrum activity and nine species having fungicidal activity. However, their daily productivity rates were significantly lower than Chlorophyta strains. The most promising strain was *Tolypothrix tenuis* MACC-205 which had broad spectrum fungicidal activity as well as significantly higher daily biomass productivity rates compared to most of the investigated *Nostoc* strains. Some microalgae strains had a stimulatory effect on the fungi, suggesting the potential to screen strains especially from the Klebsormidiophyceae, for stimulating activity of beneficial plant growth promoting fungi.

Abbreviations

C- negative control; C+ positive control; DW dry weight; F fungicidal activity; FS fungistatic activity; MACC Mosonmagyaróvár Algal Culture Collection; MS Murashige & Skoog medium; PDA potato dextrose agar; S stimulatory activity

Declarations

Ethics approval not applicable

Consent for publication All authors grant consent to publish the manuscript. Lajos Németh passed away since completing the antifungal testing of the 280 microalgae strains.

Availability of data and material All data is available upon reasonable request.

Competing interests The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contribution Áron N. Horváth carried out the molecular identification of the pathogens and tested the 15 Nostocales for antifungal activity, Lajos Németh isolated the fungal strains and tested the 280 microalgae strains for antifungal activity, Lajos Vörös identified the microalgae strains, Wendy A. Stirk was involved in the conceptualization of the project and wrote the manuscript; Johannes van Staden edited the manuscript; Vince Ördög conceptualized the project, collected and produced the microalgae strains.

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Figures

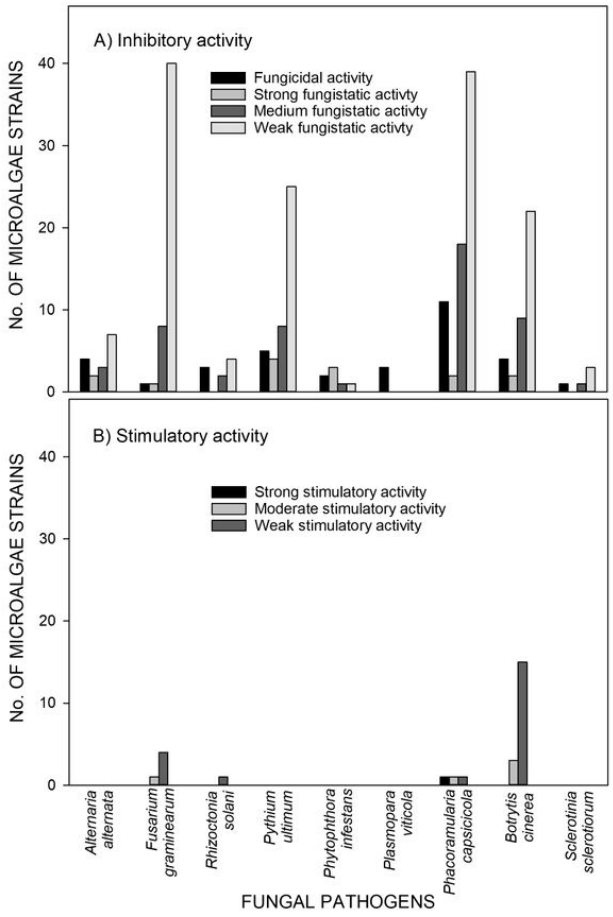

Figure 1

Number of phytopathogenic fungal strains A) inhibited and B) stimulated by the 280 microalgae water extracts

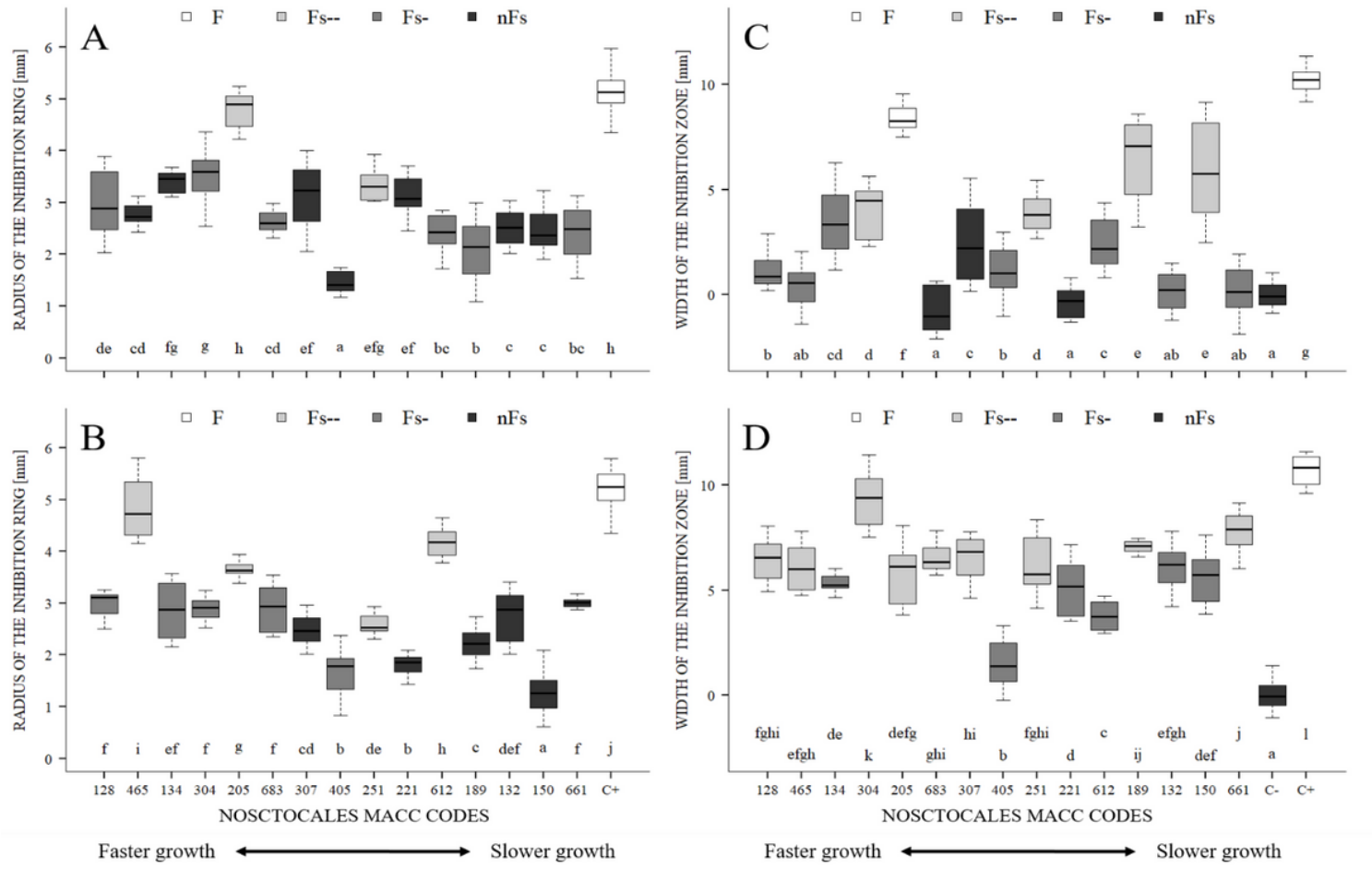

Figure 2

Antifungal activity of the 15 Nostocales strains against A) *Alternaria alternate*, B) *Fusarium graminearum*, C) *Botrytis cinerea* and D) *Rhizoctonia solani* showing the inhibition ring from the mixed-method (A&B) and the width of the inhibition zone from the disc-method (C&D) after 72 h incubation. The microalgae are presented on the X-axis according to their dry weight at day 6 with the highest biomass producing strains nearest the y-axis. Azoxystrobin (1 µg/mL) was included as a postivie control (C+) and sterile tap water as a negative control (C-). Boxes represent four parallel plates each with three technical repeats (n=12) where the thick middle line is the median, the box is the interquartile range and the whiskers extend to the most extreme data points within 1.5 × interquartile range from the box. Lowercase letters below the boxes indicate statistically significant differences in the inhibitory activity of the microalgae water extracts and controls ($p<0.05$). The Degree of Freedom was 176 for the mixed-method (A&B) and 187 for the disc-method (C&D).The shading of the boxes indicates differences in toxicity (fungicidal/fungistatic) effects of the water extracts and control(s).

Supplementary Files

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- SupplementaryTableA280strains.xlsx