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RESEARCH

Cataloguing microalgae and Cyanobacteria strains from the Mosonmagyaróvár Algal Culture Collection with in vitro antagonistic activity against phytopathogenic fungi and oomycetes

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Abstract Microalgae produce many secondary metabolites that are biologically active, including compounds that inhibit microbial growth. These could potentially function as biofungicides. The first selection criteria for potential strains suitable in the phytosanitary market is good in vitro inhibition of growth against specific phytopathogenic fungi and oomycetes and higher biomass productivity rates. In the present study, 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 in vitro against 6 phytopathogenic fungi and 3 phytopathogenic oomycetes. In total, 45% of the species had mycelial growth inhibitory activity against at least one pathogen. Cyanobacteria had the highest "hit-rate" (64%), followed by the *Chlorophyceae* (49%) and *Trebouxiophyceae* (30%). Water extracts of 19 strains had fungicidal and/or oomyceticidal activity – these were predominantly Cyanobacteria. The Cyanobacteria displayed a wider spectrum of inhibition with five strains being active against three or more phytopathogenic strains. *Trichormis variabilis* MACC-304 and *Tolypothrix tenuis* MACC-205 had inhibitory activity against 6 phytopathogens and *Nostoc linckia* MACC-612 inhibited 4 phytopathogenic strains. Each Chlorophyta strain was only active against 1-2 strains. However, the daily productivity rates of Cyanobacteria were significantly lower than Chlorophyta strains. Further investigation of 15 *Nostocales* species (*Nostocaceae*, *Tolypothrichaceae* and *Calotrichacea*e) 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 fungal and oomyceticidal inhibitory activity as well as significantly higher daily biomass productivity rates. Thus, Cyanobacteria

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can potentially be developed as an effective agricultural tool for environmentally-friendly disease management.

Keywords Biofungicides · Chlorophyta · Cyanobacteria · Growth rates · *Nostocales*

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
O	Oomyceticidal activity
OS	Oomycetistatic activity
PDA	Potato dextrose agar
S	Stimulatory activity

Introduction

Fungal infections cause diseases such as leaf blight, vascular wilt, root rot and damping off (Agrios, 2005). Intensive agricultural practices apply pesticides together with synthetic fertilizers for crop protection and to improve yield. However, their long term use 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 sustainable resource that can be cultivated on non-arable land using recycled wastewater and are characterized by high growth rates (Falaise et al., 2016; Costa et al., 2019). There are studies showing that application of either extracts or living Cyanobacteria and Chlorophyta improve plant growth (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 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 *C. reinhardtii* extracts delayed flowering and reduced flower number. *Chlorella* sp. extracts increased chlorophyll b and carotenoid content and *C. reinhardtii* extracts increased chlorophyll a content (Gitau et al., 2022). This biostimulating activity is attributed to the microalgae having a high primary metabolite (proteins, lipids and carbohydrates) content 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 of the microalgae.

Microalgae also produce a large variety of secondary metabolites which are biologically active, including compounds that inhibit fungal growth (reviewed in Stirk & van Staden, 2022). These could potentially be utilized as biofungicides. Antifungal screening has mainly focussed on medicinal applications where microalgal extracts were 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 also examples of microalgae extracts that reduced fungal diseases in

greenhouse experiments and field trials. For example, *Chlorella fusca* extracts applied at two-weekly intervals enhanced strawberry plant growth and decreased the incidence of *Fusarium* wilt disease by supressing the population of *Fusarium oxysporum* (Kim et al., 2020).

Most research has been conducted on 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 and untapped reserve of unexplored compounds which could potentially be developed as biocontrol agents for sustainable agriculture.

The commercialization of microalgae-derived biofungicides is still in the early stage and are only used in niche markets as plant biostimulants (Costa et al., 2019). 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 bioactive compounds (Righini et al., 2022). It is also necessary keep downstream 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). While there are many microalgal screening studies testing organic solvent extracts, aqueous extracts are less widely investigated (Righini et al., 2022).

There is a need to screen more genera to find suitable strains with good bioactivity (Colla & Rouphael, 2020) coupled with rapid growth rates and a robust morphology that can be cultivated in outdoor systems to generate sufficient biomass for commercial applications (Borowitzka & Vonshak, 2017). The aim of the present study was to screen taxonomically diverse microalgae for in vitro inhibitory activity against phytopathogenic fungi and oomycetes to identify fast-growing strains with broad-spectrum inhibitory activity.

Materials and methods

Microalgae strains

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 in vitro mycelia inhibitory 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 *Zygnematophyceae* 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).

All 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 & Vladimirova, 1964) or Bristol (Bold, 1949) liquid medium (Supplementary

Table 1 Number of strains and their taxonomic position of the 280 MACC microalgae strains tested for in vitro mycelial inhibitory activity against nine phytopathogens

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	*Synechococcaceae*	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	*Stichococcaceae*	2	11
		Prasinolales incertae sedis	1	1
	Watanabeales	*Watanabeaceae*	1	8
	Trebouxiophyceae ordo incertae sedis	*Coccomyaceae*	1	2
		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	*Zygnematales*	*Zygnemataceae*	1	1
			1 strain	

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 and 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 remaining 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 inhibitory activity.

Fungal and oomycete strains

Axenic cultures of six phytopathogenic fungi and three oomycetes 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 and oomycete species was carried out based on host plant identification, micromorphological and macromorphological characteristics. In addition, a fragment of the translation elongation factor 1 alpha (*EF-1α*) gene was sequenced to identify *Fusarium graminearum*. The ITS nrDNA region was sequenced in *Rhizoctonia solani* for species specific identification. The ITS nrDNA region, fragments of the RNA polymerase 2 gene (*rpb2*) and the glyceraldehyde-3-phosphate dehydrogenase *(gapdh*) genes were sequenced for *A. alternata* and *Botrytis cinerea* identification.

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

Fungal pathogens	Diseases	Hosts	Isolated from[a]
Kingdom Fungi			
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*
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*
Kingdom Chromista			
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*

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

Fungal and oomycete cultures were maintained on Potato Dextrose Agar (PDA) medium at 4 °C with a monthly passage regime with the only exception being *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.

Crude microalgae extract preparation

The freeze-dried microalgae biomass was re-suspended 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 in vitro inhibitory activity.

Assay to determine mycelial inhibitory activity

In vitro mycelial inhibitory activity of the 280 microalgae water extracts was determined against eight fungal and oomycete pathogens (excluding *P. viticola*) using the in vitro agar diffusion method (Aponyiné, 1997). Briefly, 7 day old 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-). Petri dishes were sealed with Parafilm and incubated at 25 ± 2 °C in the dark. The effect of the aqueous microalgae extracts on fungal and oomycete mycelium growth was evaluated by measuring the diameter of the inhibition ring (including the diameter of the hole) after 72 h 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 (F) and oomyceticidal (O) activity, mild (FS-, OS-), medium (FS--, OS--) or strong (FS---; OS---) fungistatic or oomycetistatic activity and mild (S+), medium (S++) and strong (S+++) stimulatory activity. Each microalgal strain was tested in four parallels in two repeated experiments ($n = 8$).

It was necessary to use a different method to test inhibitory activity against *P. viticola* (obligate parasite) 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. Inhibitory activity was evaluated on the 8th day by estimating the leaf area ratio covered with the pathogen and comparing it to the negative control.

In vitro fungal inhibitory 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 eight strains showing in vitro 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. *A. 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). Since *Botrytis cinerea* and *Rhizoctonia solani* suspensions did not mix homogenously in the cooled PDA medium, the assay method was modified. 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 µg.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 according to the assay method. For the mixed-method, the "clearness" of the inhibition ring was ranked using the scale from 1 to 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 and 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 rate of the 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 *Cyanophycea*e had significantly lower growth rates compared to the Chlorophyta strains (Table 3; Supplementary Table A).

In vitro mycelia inhibitory activity of the microalgae strains

In total, 127 extracts showed some inhibitory activity against at least one of the nine phytopathogens. This

Table 3 Growth rates of microalgae strains 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}
Zygnematophyceae (1)	14.0	1.68	0.12

The significance of the superscirpt letters is included in the figure caption ($p < 0.05$)

was an overall "hit-rate" of 45%. The *Cyanophyceae* had the highest incidence of strains with inhibitory activity with 64% of the strains tested showing some activity. In comparison, less than half the *Chlorophyceae* (49%) and *Trebouxiophyceae* (30%) strains tested had inhibitory 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 phytopathogens. 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 phytopathogens (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).

Water extracts of 19 of the 280 microalgae strains had in vitro fungicidal and/or oomyceticidal activity against at least one of the nine phytopathogens. Cyanobacteria had a higher hit-rate with 20.5% of the strains (7 strains) having fungicidal and/or oomyceticidal activity compared to 4.9% (12 strains) of the Chlorophyta. 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 inhibitory against three or more phytopathogen strains, namely *Trichormus variabilis* MACC-304 and *Tolypothrix tenuis* MACC-205 inhibited the mycelial growth of six phytopathogens and *Nostoc linckia* MACC-612 inhibited four phytopathogen strains. In contrast, each Chlorophyta strain was only active against 1-2 strains (Table 5).

Phaeoramularia capsicicola was the most susceptible fungus with 25% of the microalgae extracts inhibiting its growth with 11 extracts having fungicidal activity. Other susceptible fungi and oomycetes were *Fusarium graminearum*, *Botrytis cinerea* and *Pythium ultimum* where the microalgae extracts mainly had weak fungistatic and oomycetistatic activity. The most resistant strain 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 phytopathogen strains were stimulated by a few microalgae extracts with *Botrytis cinerea* being the most frequently stimulated phytopathogen strain (Fig. 1B).

In vitro inhibitory 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).

Overall, *Rhizoctonia solani* was the most susceptible fungus with all the microalgae extracts inhibiting its growth with nine of the extracts having

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%
Zygnematophyceae (1)	–	1	100%	–	–	–
Total (280)	87	39	45%	11	11	8%

*Some strains with both inhibitory and stimulatory activity when tested against *Alternaria alternata*, *Fusarium graminearum*, *Rhizoctonia solani*, *Phaeoramularia capsicicola*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Pythium ultimum*, *Phytophthora infestans* and *Plasmopara viticola*

Table 5 Cyanophyta and Chlorophyta MACC-strains demonstrating in vitro fungicidal (F) and oomyceticidal (O) activity against at least one of the nine phytopathogens detected with the agar gel diffusion bioassay. FS shows fungistatic activity and OS indicates oomycetistatic 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*	*Phaeoramularia*	*Botrytis*	*Sclerotinia*	*Pythium*	*Phytophthora*	*Plasmopara*
Cyanophyta										
Nostocales										
Trichormus variabilis	304	F-13		F-13	F-13	F-13	F-12	O-13		
Nostoc linckia	612	F-9	F-9		F-9			O-9		
Tolypothrix sp.	465				F-13			O-15		
Tolypothrix tenuis	205	F-19		F-17	F-24	F-15		O-17	OS-25	
Nostoc insulare	661				F-13					
Nostoc calcicola	251	F-19				FS-20				
Nostoc sp.	150				F-23			OS-15	OS-25	
Chlorophyta										
Chlorophyceae										
Desmococcus olivaceus	343				F-25			OS-22		
Scenedesmus sp.	540	FS-10			F-14					
Fernandinella alpina	682	FS-21		F-13						
Scenedesmus sp.	9					F-10				
Lobochlamys segnis	10					F-14			O-24	
Desmodesmus dispar	302									O
Haematococcus lacustris	90								O-25	
Trebouxiophyceae										
Mychonastes homosphaera	339				F-20			O-20		
Scotiellopsis sp.	14									O
Pseudococcomyxa sp.	76									O
Stichococcus bacillaris	147				F-21					
Mychonastes homosphaera	151				F-22					

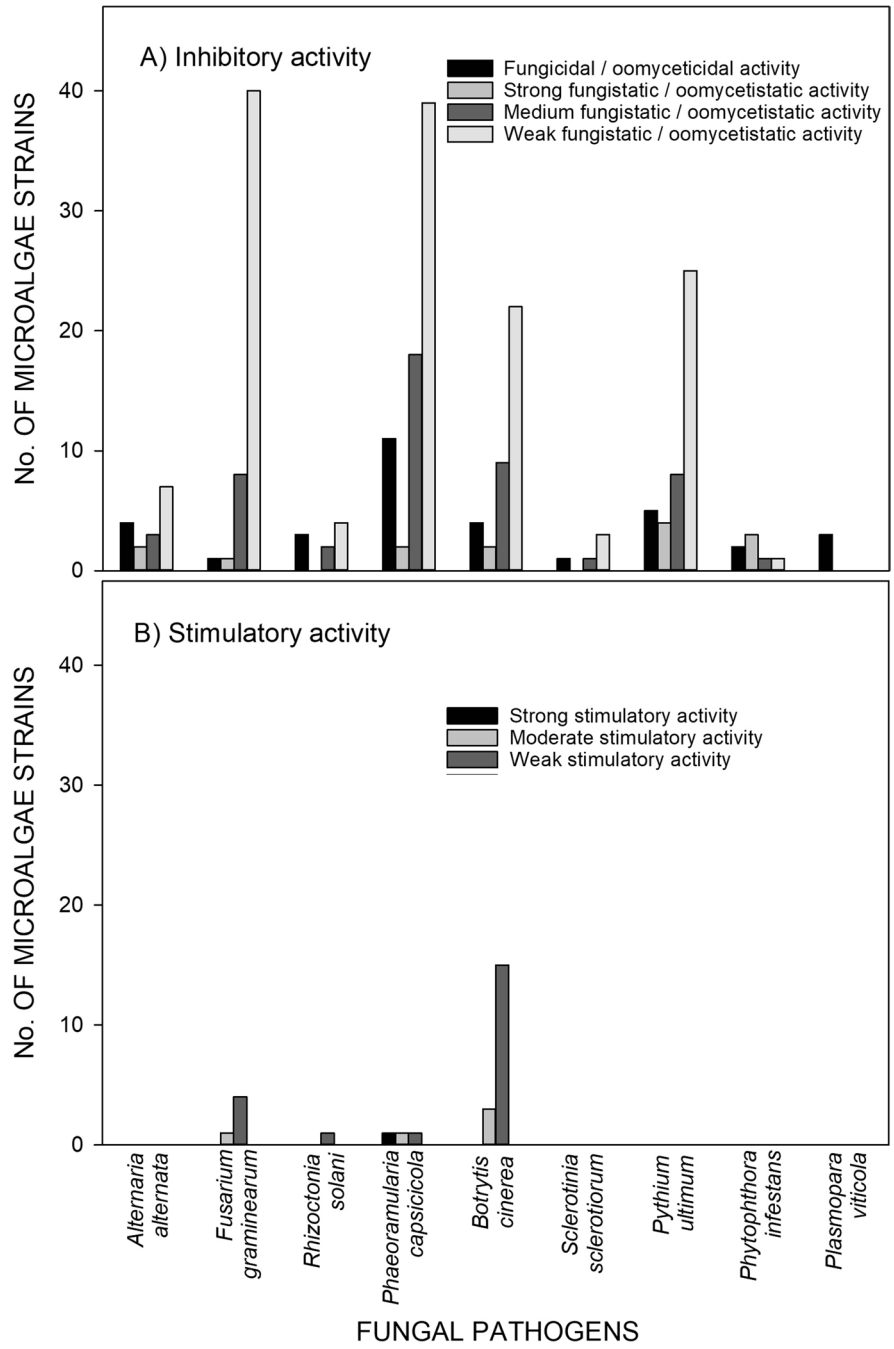

Fig. 1 Number of **A**) inhibiting and **B**) stimulating water extracts prepared from 280 microalgae and Cyanobacteria strains tested on phytopathogenic fungal and oomycete strains

strong fungistatic activity (Fig. 2D). *A. alternata* was the least susceptible fungus with seven microalgae extracts having no inhibitory activity (Fig. 2A). The positive control azoxystrobin (1 µg.mL^{-1}) had fungicidal activity against the four fungi strains (Fig. 2). The only microalgae extract to have fungicidal activity was *T. tenuis* MACC-205 against *Botrytis cinerea* (Fig. 2C) as well as strong fungistatic activity against *A. alternata* (Fig. 2A). Other extracts with significantly high mycelial inhibitory activity were *Tolypothrix* sp. MACC-465 and *N. linckia* MACC-612 against *Fusarium graminearum* (Fig. 2B), *N. muscorum* MACC-189 and *N. calcicola* MACC-150 against *Botrytis cinerea* (Fig. 2C)

Table 6 Culture collection details and growth rates of 15 *Nostocales* strains analyzed for in vitro fungal inhibitory activity against four phytopathogenic 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}

Phytopathogenic strains tested were *Alternaria alternata, Fusarium graminearum, Botrytis cinerea and Rhizoctonia solani*

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 inhibitory activity.

Discussion

In the present study, 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). Compound extraction is further improved by cell wall disruption. 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 highly resistant cell walls requiring more intense cell disruption methods. This would increase the downstream production costs. Such strains would not be suitable for commercialization of microalgae biostimulants and thus more energy-intensive cell disruption methods were not used in the present study.

In the present study, cultures were harvested once they had reached their stationary growth phase. On average, cultures were harvested between 13 and 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 in vitro mycelial inhibitory activity against at least one of the nine phytopathogenic fungi and oomycetes tested. The water

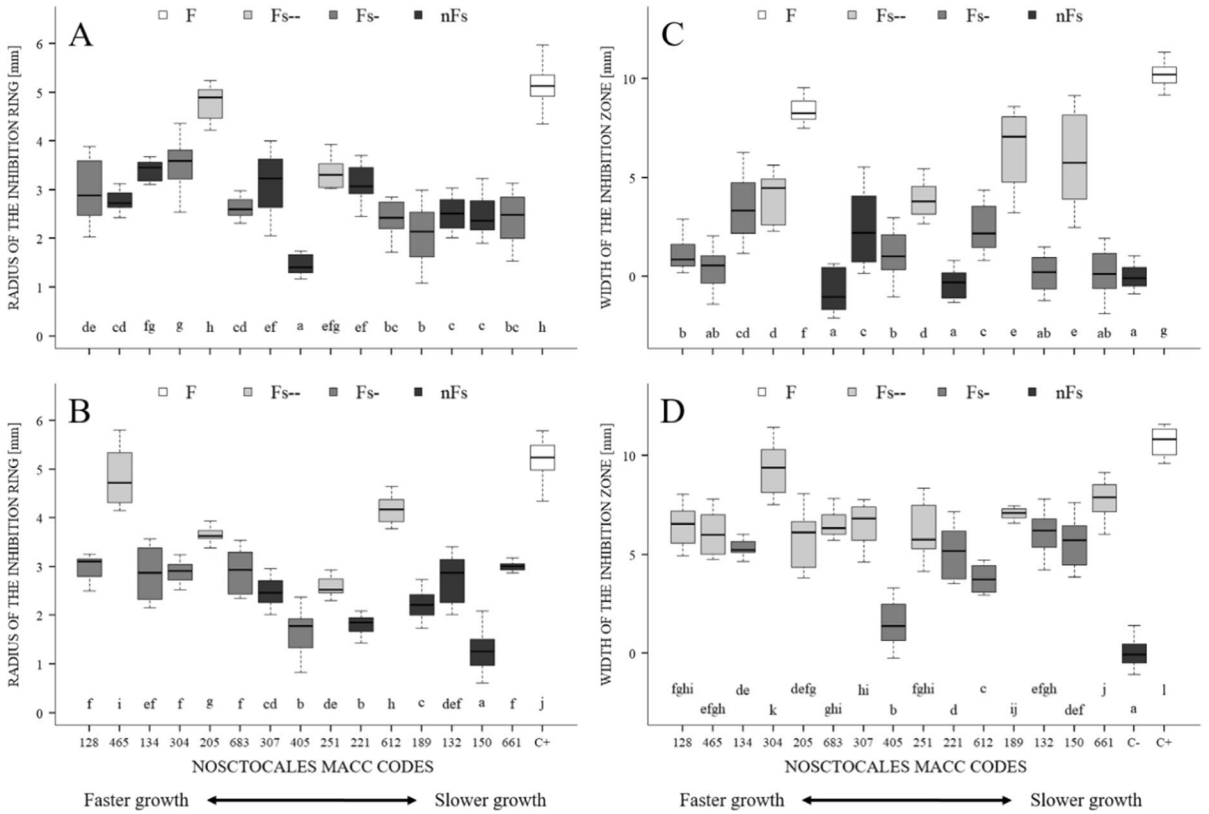

Fig. 2 In vitro fungal inhibitory activity of the 15 *Nostocales* strains against **A**) *Alternaria alternata*, **B**) *Fusarium graminearum*, **C**) *Botrytis cinerea* and **D**) *Rhizoctonia solani* showing the inhibition ring from the mixed-method (A&B) and the width of the inhibition zone from the 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^{-1}) was included as a positive 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)

extracts of the Cyanobacteria strains had the highest incidence (% hit-rate) of biological activity with 64% of the tested strains showing some inhibitory activity, followed by the *Chlorophyceae* (49% hit-rate) and the *Trebouxiophyceae* having the lowest incidence of strains with mycelial inhibitory activity (30% hit-rate; Table 4). Other studies report similar or lower incidences of in vitro fungal inhibitory 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 inhibitory activity (50% hit-rate) with three isolates having "hyper-antifungal activity" (strong activity). *Synechocystis* sp. had the broadest spectrum of inhibitory activity (Pawar & Puranik, 2008).

The Cyanobacteria also had stronger in vitro inhibitory activity with a higher incidence of fungicidal and oomyceticidal activity (20.5% of the tested strains had activity) compared to the Chlorophyta

(4.9% of the tested strains had activity). The Cyanobacteria strains with fungicidal and oomyceticidal activity were all from the *Nostocales*. In addition, the Cyanobacteria generally had a broad spectrum of inhibitory activity with many strains being active against 3 or more phytopathogens while the Chlorophyta were only active against 1-2 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 *C. 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 inhibitory activity in the 15 *Nostocales* strains investigated in the present study suggest that species from the Family *Tolypothrichaceae* are the most promising. *T. tenuis* MACC-205 had in vitro fungicidal activity against *Botrytis cinerea* and strong fungistatic activity against *A. 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). It is also important to list microalgae species that had no or weak inhibitory 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).

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. 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). In contrast, fewer antimicrobial compounds have been identified in the Chlorophyta with activity often linked to their fatty acid content (Plaza et al., 2012; Shaima et al., 2022).

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 (Biondi et al., 2004). Similarly, 31 Cyanobacteria strains from 12 genera were tested for biostimulatory activity in the watercress germination assay and for inhibitory activity against *P. ultimum* in an agar-diffusion assay. One third of the extracts had inhibitory 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). The microalgae and Cyanobacteria strains identified in the present study as having in vitro inhibitory activity against phytopathogens need to be further screened to ensure that they are not phytotoxic towards plants.

The strain of *Phaeoramularia capsicicola* was the most susceptible phytopathogenic strain tested in the present study with 11 microalgae having fungicidal activity. Other susceptible species included strain of *Fusarium graminearum*, *P. ultimum* and *Botrytis cinerea*. The most resistant species were strains of *Plasmopara viticola*, *Sclerotinia sclerotiorum* and *Phytophthora infestans* (Fig. 2A). These results are similar to other studies while other results are variable. For example, *A. alternata* and *Botrytis cinerea* were most susceptible and *P. 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 species including strains of *Pythium debaryanum*, *Fusarium oxysporum*, *Fusarium graminearum*, *Rhizoctonia solani* and *Sclerotium oryzae* were not inhibited (Prasanna et al., 2008). *Sclerotium rolfsii* was the most susceptible and *A. 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.

In the present study, three of the five tested *Klebsormidiophyceae* and a few *Chlorophyceae* and *Trebouxiophyceae* tested in the present study had a stimulatory effect on the fungi and oomycete strains, especially *Botrytis cinerea*. A few strains

(*Chlorosarcina* sp. MACC-560, *S. acutus* var. *globosus* MACC-551, *Scenedesmus* sp. MACC-575, *Gloeocystis* sp. MACC 631 and *Chlorella* sp. MACC-564) had both inhibitory and stimulatory activity against different pathogen strains (Supplementary Table A). Similarly, *Spirulina* sp. and *Nannochloropsis* sp. promoted *Sclerotium rolfsii* and *A. alternata* growth respectively (Schmid et al., 2022). Thus, once promising strains have been selected as potential biopesticides, they should be screened against a larger range of phytopathogenic strains to ensure they do not promote the growth of some phytopathogenic species.

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 inhibitory activity against specific phytopathogenic strains, biomass productivity rates are an important criterion to ensure that sufficient biomass can be generated. Cyanobacteria from the *Nostocales* showed the most promising in vitro mycelial inhibitory activity with water extracts having broad spectrum activity and nine species having fungicidal and oomyceticidal activity. However, their daily productivity rates were significantly lower than Chlorophyta strains. The most promising strain was *T. tenuis* MACC-205 which had broad spectrum phytopathogenic activity as well as significantly higher daily biomass productivity rates compared to most of the investigated *Nostoc* strains. Additional research on these promising strains is required to test their activity against a broader spectrum of pathogens to ensure no species specific stimulatory effects and that they have no phytotoxic effects. In vivo activity and their mechanisms of action also need to be elucidated.

Authors' contribution Áron N. Horváth carried out the molecular identification of the pathogens and tested the 15 *Nostocales* for fungal inhibitory activity, Lajos Németh isolated the fungal and oomycete strains and tested the 280 microalgae strains for inhibitory 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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Data availability All data is available upon reasonable request.

Declarations

Ethics approval Not applicable.

Consent to participate 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.

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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