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New Strain of *Cyphellophora olivacea* Exhibits Striking Tolerance to Sodium Bicarbonate

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Abstract: The cyanobacterium strain *Synechococcus cedrorum* SAG 88.79 stock culture has fungal contamination stated by the *Sammlung von Algenkulturen der Universität Göttingen* itself. In this recent work, this particular fungal strain was isolated, identified, and morphologically characterised. The fungal strain AGSC12 belongs to the species *Cyphellophora olivacea*, with respect to the sequence similarity, phylogeny, and morphology of the strain. Colony morphology and growth capability were examined on SMA, EMMA, PDA, MEA, YEA, and YPA plates. Growth of the colonies was the most successful on YPA plates, followed by PDA and MEA containing plates. Surprisingly, the AGSC12 strain showed extreme tolerance to $NaHCO_3$, albeit it, is is considered a general fungistatic compound. Moreover, positive association between the AGSC12 and SAG 88.79 strains was revealed, as the SAG 88.79 strain always attained higher cell density in co-cultures with the fungus than in mono-cultures. Besides, a taxonomic note on the SAG 88.79 strain itself was also stated.

Keywords: *Cyphellophora*; *Cyanobacterium*; *Synechococcus*; fungal phylogeny; sodium bicarbonate; microbial interaction

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1. Introduction

The genus *Cyanobacterium* (family: *Cyanobacteriaceae*, order: *Chroococcales*) originally proposed by Rippka and Cohen-Bazire in 1983 comprises more than 13 species [1–4]. The type species of this genus is *Cyanobacterium stanieri* Rippka and Cohen-Bazire, a free-living unicellular photosynthetic bacterium [1]. Its homotypic synonym is *Synechococcus cedrorum* Sauvageau according to the NCBI Taxonomy [5,6]. The strain *S. cedrorum* SAG 88.79 was originally isolated in Chad by M. Lefevre in 1963. This strain was originally described as *Synechococcus* on the basis of morphology, then transferred to the genus *Cyanothece*, and later to *Cyanobacterium*, based on the results of more thorough morphological examinations, the type of cell division, and the ultrastructural patterns [7,8]. Phylogenetic analyses of the 16S rDNA and ITS rDNA sequences suggested that the strains *C. stanieri* PCC 7202 and *S. cedrorum* SAG 88.79 are identical, but there were some observable differences in the carotenoid compositions between the two strains [3].

It is broadly known that algae and cyanobacteria exhibit a wide range of relationships with fungi, from mutualistic relations to parasitic characteristics. Symbiotic relationships reach their highest levels in the form of lichens or mycophycobiosis [9–11]. However, free living cells also could be beneficial to each other, even if they do not have strong physical or metabolic interdependences [12,13].

The SAG 88.79 culture, which is maintained in SAG (The Culture Collection of Algae at Goettingen University), has fungal contaminants (https://sagdb.uni-goettingen.de/detailedList.php?str_number=88.79, accessed on 15 October 2022). Though the origin of the fungal contamination is not known, its presence is fascinating on its own. It is widely known that $NaHCO_3$ and Na_2CO_3 both have a strong statin effect on most fungi, as the compounds are able to inhibit fungal growth even at relatively low concentrations [14–20]. In contrast, the Spirulina medium (which is used for the maintenance of the SAG 88.79 strain) contains

approximately 160 mmol $NaHCO_3$ and 38 mmol Na_2CO_3, whose amounts are more than enough to hinder fungal growth. It would be interesting to know whether the fungal companion is less sensitive to $NaHCO_3$ and Na_2CO_3, or whether the metabolism of the cyanobacterial strain makes fungal propagation possible in that hostile medium.

We strongly believe it would be beneficial to the scientific community if we identified the fungal companion. Thus, the aim of this study was to isolate and identify that certain fungal strain and to provide taxonomical and morphological characterisation. We assessed the sensitivity of the strain to different compounds and compared it to other fungal strains. Furthermore, we established the nature of the relationship between the cyanobacterial and fungal strains.

2. Materials and Methods

2.1. Strains and Culturing Media

The strains used in this study are listed in Table 1. The cyanobacterial strain was maintained and cultivated in Spirulina and ES medium (in liquid or on slant agar) at room temperature (25 °C) with atmospheric CO_2 under natural light. Fungal strains were maintained in PDA medium at room temperature.

Table 1. List of strains used in this study.

Species	Strain	Origin	Maintaining Medium
Cyanobacterium stanierei	SAG 88.79	SAG	Spirulina
Aspergillus niger	CBS 554.65	CBS-KNAW	PDA
Penicillium expansum	SZMC 2175	SZMC	PDA
Fusarium graminearum	FGSC 9075	ATCC	PDA
unkown fungus	AGSC12	This study	PDA

Recipes of the Spirulina and ES media are available at the website of SAG (https://www.uni-goettingen.de/en/186449.html, accessed on 15 October 2022) [21]. YEL, YEA, YPL, YPA, SML, and SMA were described by Sipiczki and Ferenczy [22], while EMML and EMMA minimal media were made according to Mitchinson [23]. PDA and MEA were purchased from VWR (VWR, Debrecen, Hungary) [24,25].

PDA plates containing $NaHCO_3$ and NaCl were prepared as follows: a twofold concentration of PDA medium was sterilised in an autoclave and mixed with sterile $NaHCO_3$ or NaCl aqueous solution after cooling to 35–40 °C. $NaHCO_3$ containing media were prepared in two ways: $NaHCO_3$ version I was sterilised by autoclaving, $NaHCO_3$ version II was filter sterilised (VWR, Debrecen, Hungary). The reason for that is the heat instability of the $NaHCO_3$. According to PubChem, $NaHCO_3$ begins to decompose around 50 °C, releasing CO_2, H_2O and Na_2CO_3. As the recipe of the Spirulina medium does not mention filter sterilisation in the case of the $NaHCO_3$, both $NaHCO_3$ solutions were tested. Consequently, the effect of Na_2CO_3 tested only indirectly.

2.2. Isolation of the Unknown Fungal Strain

The unknown fungal strain was isolated from the culture broth of *S. cedrorum* by transferring 10 µL of liquid drops to Petri dishes (VWR, Debrecen, Hungary) containing EMMA medium. The Petri dishes were stored at room temperature. The obtained fungal isolate was transferred to PDA medium and was maintained at room temperature.

2.3. Morphological Observations and Microscopy

Fungal colony morphologies and growth ability were investigated on YEA, YPA, SMA, EMMA, MEA, and PDA containing plates using quadruplicate samples in the case of each medium. Fungal hyphae were observed with the sandwich method under an Olympus BX-40 light microscope (Olympus, Tokyo, Japan) and were photographed with an Olympus DP-70 camera (Olympus, Tokyo, Japan) [26,27]. Aerial mycelia were investigated under a Carl Zeiss Jena stereo microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.4. Tolerance Tests

The $NaHCO_3$ and NaCl tolerance of the fungal strains were tested with the drop plate method using PDA medium. A loopful amount of fungal net and conidia were washed into 1 mL of sterile water and mixed thoroughly, then droplets of 20 µL volume from the supernatants were placed onto the surface of the PDA plates. The plates were incubated at ambient temperature, and the growths of the fungal colonies were checked and measured occasionally. Since the growth rates of the fungal strains listed in Table 1 are substantially different, a custom scoring system, ranging from 0 to 3, was used for the evaluation of fungal growth. Unit 3 indicates normal growth in comparison to the untreated control, whereas unit 2 represents slower growth and/or altered phenotypes (e.g., less pigmented colonies). Unit 1 denotes retarded growth, which generally means small and unusual phenotypes of the fungal colonies. Consequently, unit 0 means no fungal growth at all. Categorisations were made by statistical significance or thorough investigation by eye.

2.5. Interaction Tests

S. cedrorum and *C. olivacea* were separated by consecutive dilutions then mono- and co-cultivated in liquid media and on agar plates. Solid Spirulina media were flooded with either a cell mixture or sequentially (first with cyanobacteria, then with fungi), and the growth of the cultures was monitored by eye and photographed on occasion.

At the same time, exponentially growing cyanobacteria were inoculated to fresh Spirulina medium with or without the fungal strain. In this case, approximately 7 × 7 mm samples from the fungal net growing in liquid media (YEL or YPL) were cut with a sterile scalpel (VWR, Debrecen, Hungary). The remaining liquid from the surface of the hyphal net was dried with a sterile filter paper (VWR, Debrecen, Hungary) to avoid adding extra nutrients to the fresh media. Then, the hyphal net was added to the cyanobacterial culture with sterile forceps (VWR, Debrecen, Hungary). The optical density (OD) at λ = 730 nm was measured using an UV-1601 Shimadzu spectrophotometer (Shimadzu Europa GmbH, Duisburg, Germany) to monitor the cyanobacterial growth. Individually cultivated cyanobacteria were used as a control. We performed the experiments with 2 replicates twice.

2.6. DNA Analysis Methods

Genomic DNA was isolated from exponential-phase cyanobacterium cultures grown in 50 mL of Spirulina medium for 7 days with the glass bead method [28]. DNA isolation from fungal hyphae was performed as follows: the fungal strain was grown in a 300 mL Erlenmeyer flask (VWR, Debrecen, Hungary) in 100 mL YEL medium at room temperature for 10 days. Then, the hyphal batch was ground to powder under liquid nitrogen with a pestle in a mortar. Genomic DNA was isolated with the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Hungary Ltd., Budapest, Hungary), according to the manufacturer's protocol.

The isolated genomic DNAs were used as templates for the PCR reactions. Bacterial 23S rDNA was amplified with the primers 23SU1 and 23SU2 in the case of *S. cedrorum* [29]. PCR was conducted under the following cycling conditions: one cycle at 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 2 min. These cycles were followed by a final extension step at 72 °C for 10 min.

The fungal ITS1-5.8S-ITS2 region was amplified with the primer pair of ITS1-ITS4 in the case of the fungal strain [30]. PCR cycling conditions were the following: one cycle at 95 °C for 2 min; 30 cycles of 95 °C for 60 s, 55 °C for 60 s, and 72 °C for 90 s, followed by a final extension step at 72 °C for 10 min. DreamTaq polymerase KIT (Life Technologies Hungary Ltd., Budapest, Hungary) was used in both cases.

To check the efficiency of the DNA amplifications, PCR products were subjected to electrophoresis through 1.0% agarose gels in 1xTBE buffer. Gels were stained with ethidium-bromide. Qualities and sizes of the bands were checked by comparing them to 1 kb DNA ladder (Thermo Scientific SM0332, Life Technologies Hungary Ltd., Budapest, Hungary)

under UV-transilluminator (UVP Bio-Doc-It Imaging System, Ultra-Violet Products Ltd., Cambridge, UK).

Quality-checked PCR products were purified with the ethanol precipitation method [31]. Concentrations of the purified DNAs were measured with a Qubit fluorometer (Life Technologies Hungary Ltd., Budapest, Hungary). Sanger sequencing reactions were performed by GeneArt or Microsynth AG using the primer pairs mentioned earlier.

2.7. Bioinformatics

The DNA sequences resulting from the Sanger sequencing were curated manually by creating pairwise alignments with a Needleman-Wunsch algorithm available at the website of EMBL-EBI (https://www.ebi.ac.uk/Tools/psa/emboss_needle/, accessed on 15 October 2022). The refined consensus sequences were used further. BLASTn searches were performed at the website of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&PAGE_TYPE=BlastSearch&PROGRAM=blastn, accessed on 15 October 2022) using megablast with standard parameters either in the standard nucleotide collection database or in the rRNA/ITS database of fungi type and reference material. Fungal ITS sequence was also checked in the UNITE database (https://unite.ut.ee/analysis.php, accessed on 15 October 2022) [32].

The concerning DNA sequences extracted from the database of NCBI are listed in Table S2 [33–52]. Phylogenetic trees were created on the website of Phylogeny.fr (http://www.phylogeny.fr/, accessed on 15 October 2022) [53]. DNA sequences were aligned with MUSCLE (full mode, maximum iteration: 16) (http://www.phylogeny.fr/one_task.cgi?task_type=muscle, accessed on 15 October 2022) [54], and the ambiguous regions were removed with GBLOCKS v0.91b (http://www.phylogeny.fr/one_task.cgi?task_type=gblocks, accessed on 15 October 2022) [55]. For the phylogeny, BioNJ and PhyML algorithms were used. In the case of BioNJ (http://www.phylogeny.fr/one_task.cgi?task_type=bionj, accessed on 15 October 2022), the K2P substitution model was applied [56]. The PhyML v3.0 algorithm (http://www.phylogeny.fr/one_task.cgi?task_type=phyml, accessed on 15 October 2022) was used with the HKY85 substitution model [57]. The number of substitution rate categories was adjusted to 4. Gamma distribution parameters, proportions of invariable sites, and transition/transversion ratio were all estimated. Branch supports were estimated from bootstrap analysis (100 replicates—BioNJ) and the approximate likelihood ratio test (aLRT—PhyML). The trees were displayed with FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 15 October 2022).

2.8. Statistical Analyses

Normal distribution of the data was tested by the Shapiro-Wilk test. In the case of normally distributed data, one-way ANOVA and repeated-measures ANOVA tests were performed, followed by Tukey's pairwise as post hoc tests. In other cases, the Kruskal-Wallis test was used, followed by Dunn's post hoc test with Bonferroni correction. *P* values were considered significant below the alpha level of 0.05. All statistical analyses were performed in PAST v.4.03 software [58].

2.9. Data Availability

The isolated fungal strain AGSC12 is available upon request. The sequences generated during the current study are available in the GenBank repository with the following accession numbers: MW363512 and MT860449.

3. Results

3.1. Taxonomic Note on the SAG 88.79 Strain

The analyses of the 16S rDNA and ITS rDNA sequences suggested that the strains *C. stanieri* PCC 7202 and *S. cedrorum* SAG 88.79 were identical, but minor differences could be observed in the carotenoid compositions between the two strains [3]. We amplified the 23S rDNA sequence of the SAG 88.79 strain and sequenced it from both directions,

then we performed a similarity search on the website of NCBI with the 769 nucleotide length consensus sequence (GenBank accession: MW363512). According to our findings, the most similar sequence was the 23S rDNA of the *C. stanieri* PCC 7202 strain with a 99.22% sequence identity.

3.2. Isolation of the Unknown Fungal Strain

As was mentioned earlier, the culture collection SAG itself stated that the SAG 88.79 strain has a fungal contaminant. At the very first cultivation of the cyanobacterium strain upon arrival, no fungal strain could be spotted by the naked eye. However, when we transferred 10 µL of liquid drops from the original cyanobacterial culture broth to different media (EMMA and YEA) suitable for fungi, a single greyish mould-like colony appeared. We scraped away some fungal material with a sterile loop from the surface of the colony and streaked it onto Petri dishes containing YEA medium to obtain single colonies. Single colonies were then transferred to fresh PDA media and maintained at room temperature. As the single colonies were identical in their morphology, we presumed that the isolates belonged to the same strain. We gave the name AGSC12 to the new strain (Figure 1A).

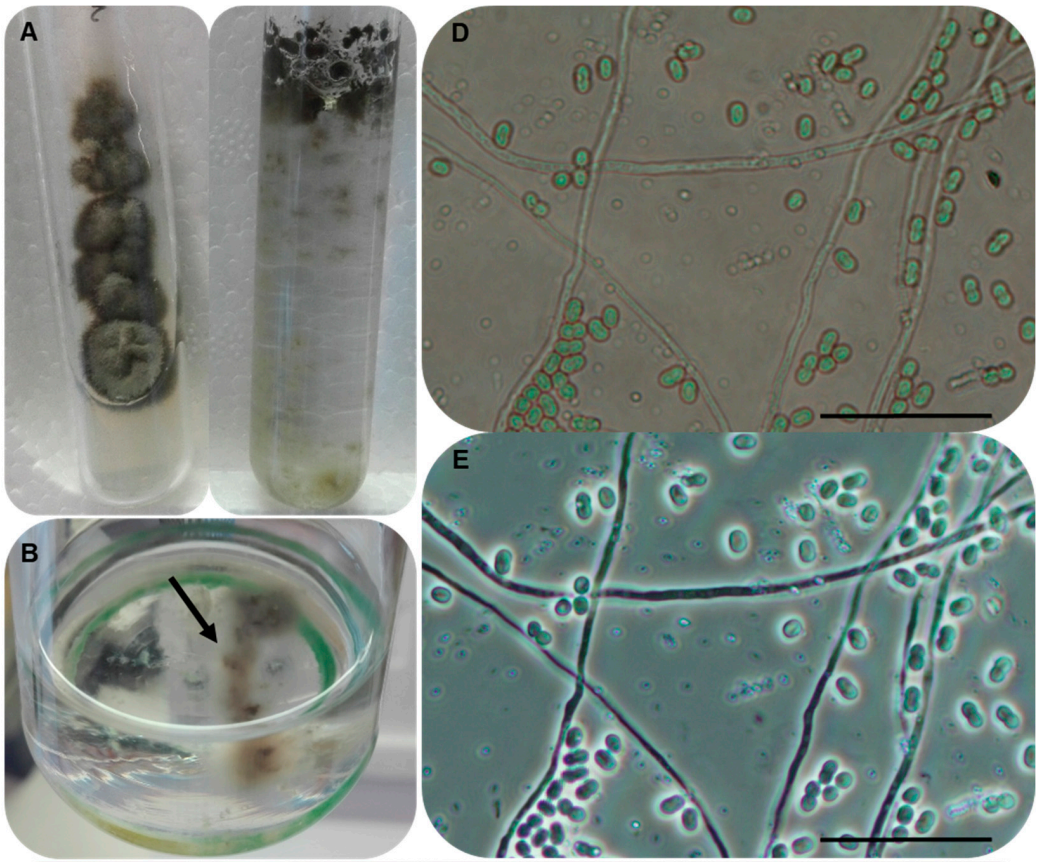

Figure 1. *Cont.*

Figure 1. The fungal strain AGSC12 from the culture broth of the cyanobacterium SAG 88.79. (**A**) The isolated strain AGSC12 on EMMA slant agar and in EMML liquid medium. (**B**) The presence of the AGSC12 strain in SAG 88.79 culture broth after photobleaching the cyanobacterial cells in Spirulina medium. (**C**) Fungal hyphae among the cyanobacterial colonies and at the edges of the colonies on ES and Spirulina media containing plates. On ES medium, the fungal growth was stronger, and on Spirulina medium, the fungal presence was indicated by the brownish tone on the top of the cyanobacterial colony. (**D**,**E**) Microscopic images of the fungal hyphae among the cyanobacterial cells. (**D**): Nomarski microscopy. (**E**): phase-contrast microscopy. Scale bars = 20 μm.

After a long cultivation period, we observed that the fungal strain appeared in both the liquid and solid cultures of the cyanobacterial strain, as well (Figure 1B,C). A stock culture of the SAG 88.79 strain has been maintained on Spirulina slant agar in our lab for years, and the fungal strain AGSC12 is still observable among the cyanobacterial cells (Figure 1D,E).

3.3. Molecular Identification of the AGSC12 Strain

For taxonomic identification, the ITS1-5.8S-ITS2 region of the new strain was amplified and sequenced from both directions. After that, a consensus sequence of 612 nucleotides (GenBank accession: MT860449) was subjected to similarity searches at the websites of NCBI and UNITE. We searched for type/reference strains whose sequences were not different from the AGSC12 sequence by more than three substitutions or indels. The *Cyphellophora olivacea* CBS 122.74 type strain proved to be the most similar to our isolate, as they shared a 99.64% sequence identity. The next best search hit was *C. pluriseptata* CBS 286.85, with a 91.13% pairwise identity. Accordingly, the inclusion of additional barcode sequences was not necessary. Based on multiple alignments of the isolate AGSC12, *C. olivacea* CBS 122.74 and CBS 123.74, we assigned our strain to the species *Cyphellophora olivacea* (Figure 2A).

A

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AGSC12          CCCGGGCCCGCGCCTGTCGACGGCCCCAACCACAAAAACTCTTGTTAAAAAACGTGTCGT
CBS123.74       CCCGGGCCCGCGCCTGTCGACGGCCCCAACCAC-AAAACTCTTGTTAAAAAACGTGTCGT
CBS122.74t      CCCGGGCCCGCGCCTGTCGACGGCCCCAACCAC-AAAACTCTTGTTAAAAAACGTGTCGT
                ********************************* ************************

AGSC12          CTGAGTACCACATTTTAATCAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
CBS123.74       CTGAGTACCACATTTTAA-CAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
CBS122.74t      CTGAGTACCACATTTTAA-CAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
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B

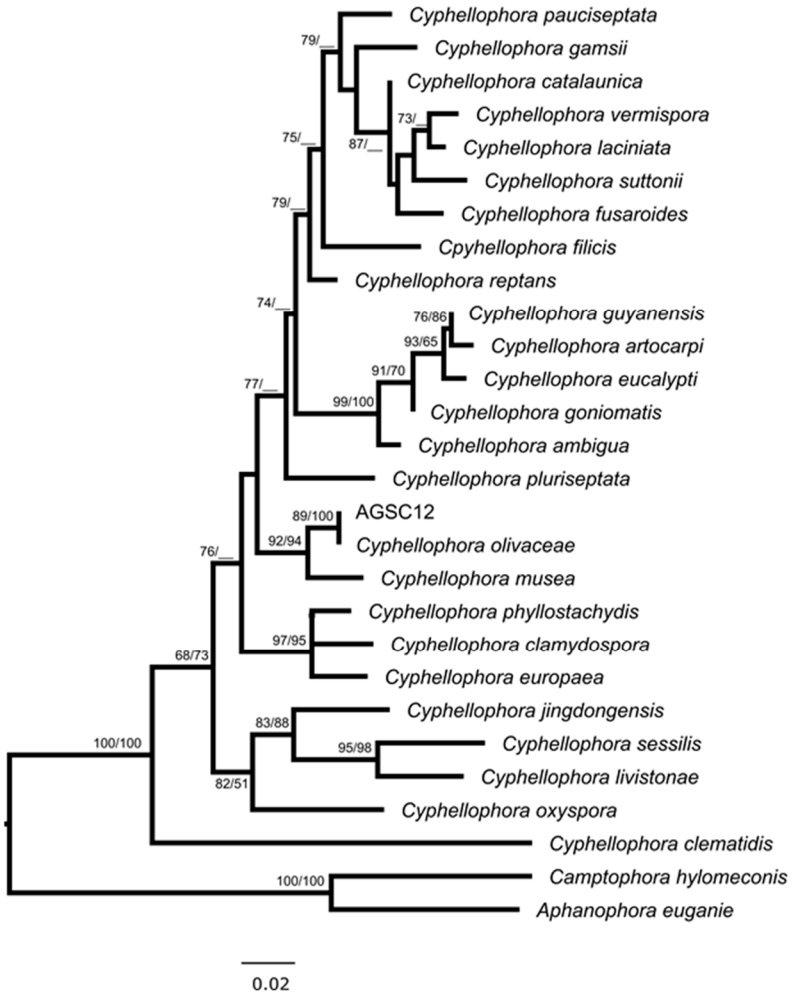


Figure 2. (**A**) Multiple sequence alignment of the ITS1-5.8SrDNA-ITS2 regions of the strain AGSC12 and *C. olivacea* strains CBS122.74 (ex-type strain) and CBS123.74. Only those regions are depicted from the alignment, which exhibited discrepancies (highlighted with green). Otherwise, the alignments were identical. (**B**) Phylogenetic analyses of the strain AGSC12 and other *Cyphellophora* species. The numbers at the branches indicate aLRT of Maximum Likelihood (ML) analysis, the other values come from bootstrapping of neighbour joining (NE) analysis (aLRT/bootstrap). Only aLRT and bootstrap values larger than 50 are shown. The current tree layout represents the ML analysis and comparison of ML. NJ can be seen in Supplementary Figure S1. Although some minor differences can be seen at the branching points, the strain AGSC12 clustered to the same branch with the *C. olivacea* ex-type strain in both the ML and NJ analyses.

3.4. Phylogenetic Analyses of the AGSC12 Strain and Its Relatives

For further conviction on the taxonomic position of the strain AGSC12, we searched for all the publicly available *Cyphellophora* sequences of type strains that were assigned to a species. According to the MycoBank database, there are 35 identified species, which belong to the genus *Cyphellophora* to date (https://www.mycobank.org/, accessed on 6 October 2022). We managed to find most of the ITS sequences of ex-type strains in that genus. *C. indica* and *C. taiwanensis* neither have cultures nor have any sequences available, so we were not able to include them in any phylogenetic analyses [33]. *C. eugeniae* and *C. hylomeconis* were transferred to new genera, as *Aphanophora eugeniae* and *Camptophora hylomeconis* [34]. According to Crous et al., *C. vermispora* is identical to *C. aestival* [59]. Finally, we used the sequences of 27 ex-type strains to build phylogenetic trees (Supplementary Table S1). We created character-based (PhyML) and distance-based (BioNJ) phylogenetic trees using 401 well-aligned sites (broadly 44% of the concerning ITS sequences) (Figure 2B and Supplementary Figure S1). Although the two trees exhibited some minor discrepancies in the topologies, our strain AGSC12 was situated in a common branch with *C. olivacea* at both of the trees. Thus, we were convinced that AGSC12 was likely a new strain of *C. olivacea*.

3.5. Morphological Characterisation and Growth Ability of the Strain AGSC12

Species: *Cyphellophora olivacea* (W. Gams), Réblová & Unter [34].

Basionym. *Phialophora olivacea* W. Gams [49].

Teleomorph: unknown.

Material examined: fungal contaminant of the cyanobacterial strain SAG 88.79 obtained from the culture collection of *Sammlung von Algenkulturen der Universität Göttingen*.

Colony morphology and growth capability were examined on SMA, EMMA, PDA, MEA, YEA, and YPA plates (Figure 3A–F). Young colonies are greyish with a pale olivaceous margin. The surface is covered with aerial mycelia. Colonies attain 12–19 mm in diameter after nine days of cultivation at room temperature, depending on the type of media. Mature colonies exhibit the same look as the young colonies but have darker colours and more definitive margins.

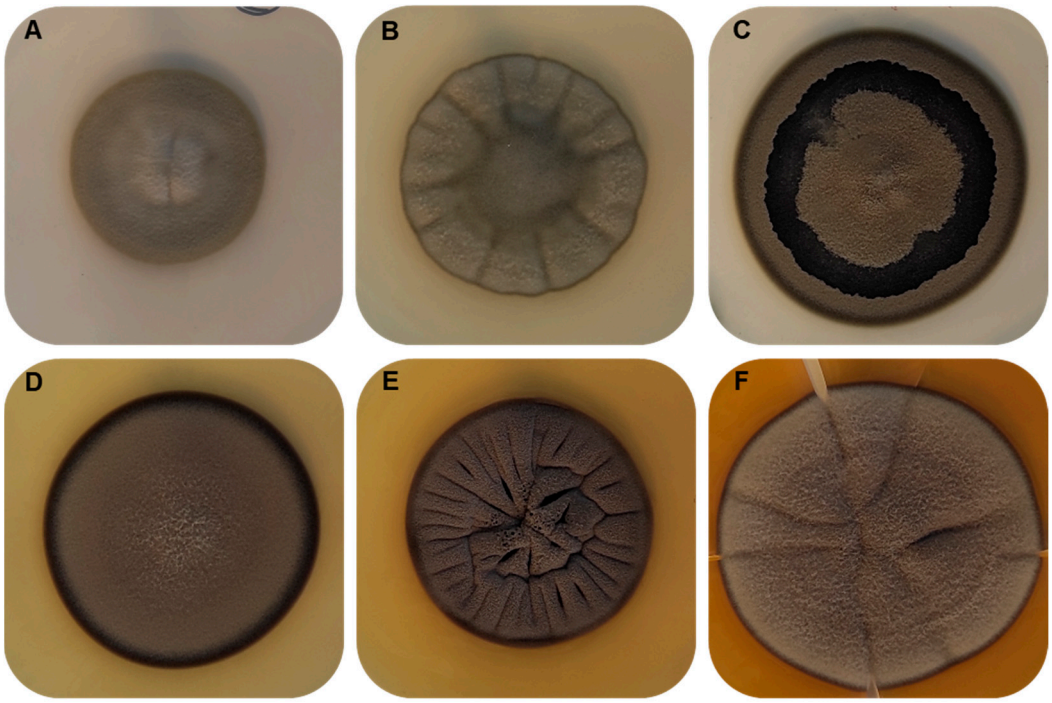


Figure 3. *Cont.*

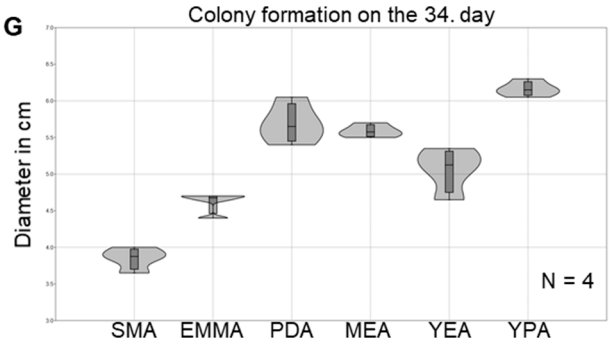


Figure 3. Morphology and growth capability of the *C. olivacea* strain AGSC12. (**A**–**F**) Colony formation of SMA, EMMA, PDA, MEA, YEA, and YPA plates in the order shown. Photos were taken after 34 days of cultivation. (**G**) Distribution of the measured diameters of the fungal colonies growing on different media at the 34th day. Violin plots show the kernel density for each sample. The box plots in the violin plots indicate the 25–75 percent quartiles. Horizontal lines within the boxes show the medians of the samples. Minimal and maximal values are depicted by the whiskers. N = sample size. Colony expansion was the most efficient on YPA plates (one-way ANOVA test, $P = 2.162 \times 10^{-11}$).

Since the strain AGSC12 does not belong to a new species, only a short description is provided here. A thorough description can be seen in [34]. Young vegetative hyphae are smooth, thin, rarely septate, and hyaline, with an approximate width of 1–2 µm. Intercalary branching of the vegetative hyphae is observable. Old hyphae are a bit melanised, and they are sometimes widened and bulky (Supplementary Figure S2A). Phialides are slightly pigmented and are formed terminally, laterally, or intercalary (Supplementary Figure S2B). Conidia are nonseptate at maturity with a shape from clavate to fusiform but mainly ovoidal (Supplementary Figure S2C,D).

The strain AGSC12 can grow in both aerobic and anaerobic conditions in liquid media, but it prefers the aerobic condition. On the plates of complete media, colony expansion proved to be the most successful on YPA (one-way ANOVA test, $P = 2.162 \times 10^{-11}$), followed by PDA and MEA (Figure 3G). The SMA minimal medium was the least preferred by the strain (Figure 3G).

3.6. Salt Tolerance of the Strain AGSC12 Compared to Other Fungal Strains

As the *S. cedrorum* strain is maintained in Spirulina medium, which contains a notable amount of $NaHCO_3$, we wanted to test the salt tolerance of the fungal companion compared to other fungi. First, we performed a general NaCl tolerance test on PDA plates to exclude potentially sensitive strains. All the used fungal strains were able to grow successfully even at 0.9 M NaCl concentration (Figure 4A and Table 2). *F. graminearum* responded to the emerging concentration of NaCl with less pigment production (Figure 4A). However, it seemed that the strain AGSC12 showed a weak but definite sensitivity to NaCl as the strain produced significantly smaller colonies (Kruskal-Wallis test, $P = 3.96 \times 10^{-6}$) (Figure 5A).

In contrast to that, on $NaHCO_3$ version I and version II containing media, colony formation of *A. niger* and *P. expansum* was completely inhibited above 0.1 M and 0.2 M concentrations, respectively (Table 2 and Supplementary Figures S3 and S5). Although *F. graminearum* was able to initiate colony formation even at 0.7 M concentrations, their colonies showed pigmentless and altered phenotypes compared to the untreated controls (Table 2 and Supplementary Figures S3, S5 and S6). The colony morphology seemed normal only at the 0.1 M concentration (Supplementary Figures S3 and S5). Strikingly, *C. olivacea* AGSC12 was able to produce viable colonies, even at ~1.1 M concentrations, but the colonies were smaller in diameter (Kruskal-Wallis test, $P = 4.66 \times 10^{-7}$) (Figures 4B and 5B, Table 2 and Supplementary Figures S4–S6). However, the AGSC12 strain did not show any substantial differences in the colony forming ability on the two $NaHCO_3$-containing media but had a darker colour on the version II medium (Figure 4B). According to the

stereo microscopic examinations, the aerial hyphae of *C. olivacea* AGSC12 turned melanised from hyaline and bulky from thin in response to the elevated $NaHCO_3$ concentrations.

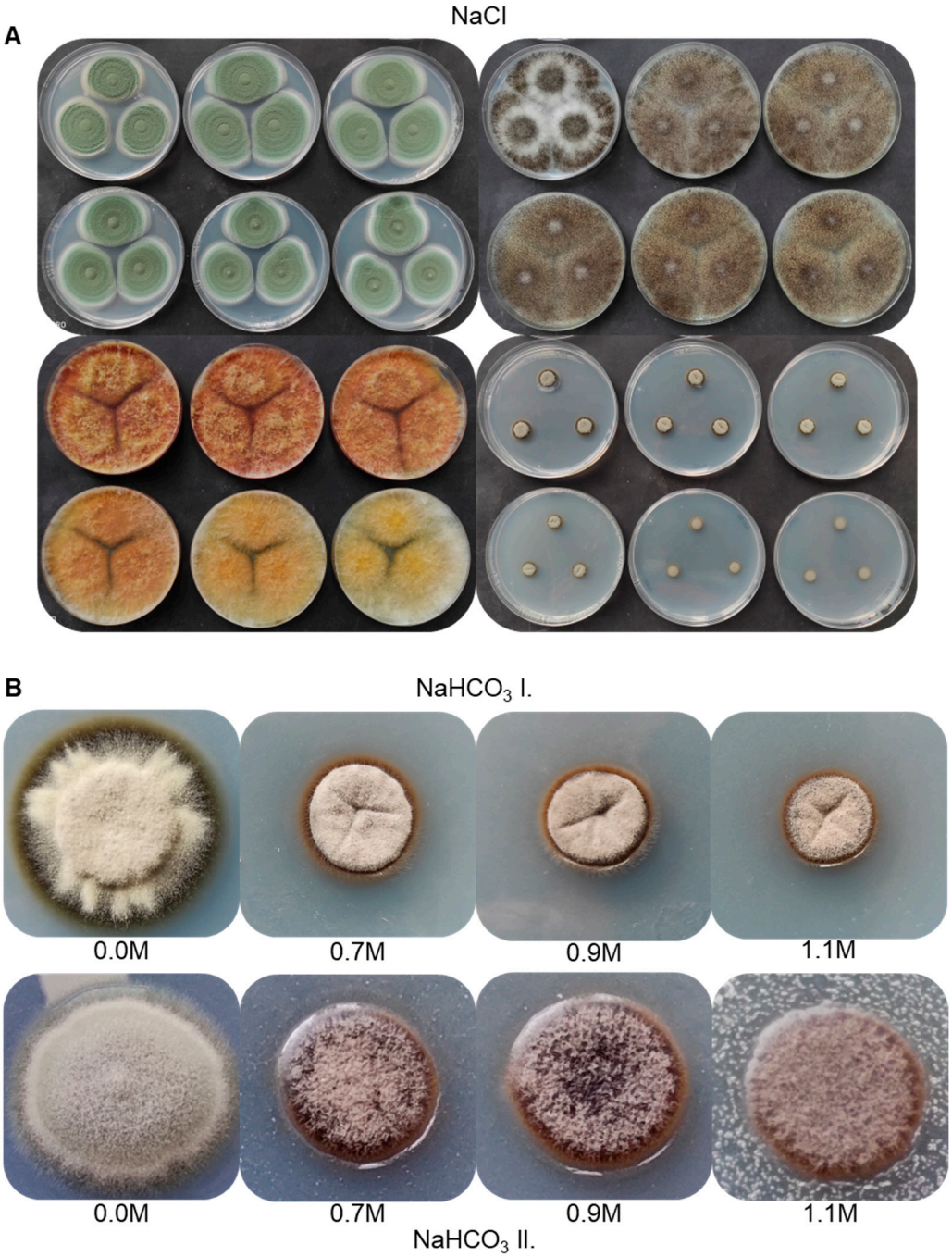
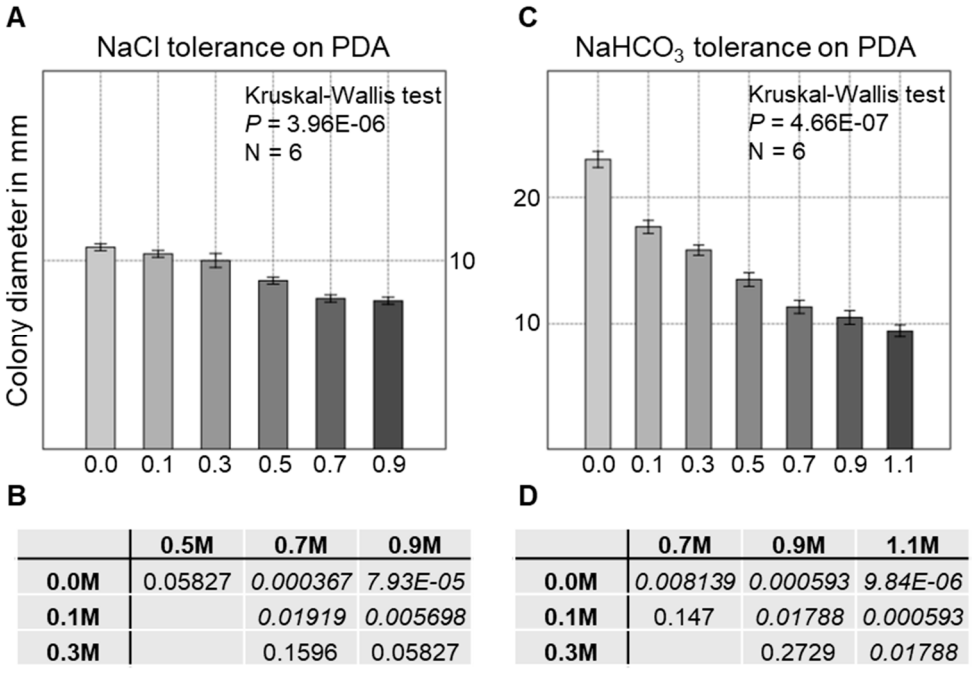


Figure 4. Colony forming ability of the examined fungal strains on NaCl- and on $NaHCO_3$-containing PDA plates. (**A**) Tolerance to NaCl. Green mold: *Penicillium expansum* SZMC 2175; blackish mold: *Aspergillus niger* CBS 554.65; orange-yellowish mold: *Fusarium graminearum* FGSC 9075; small grey colonies: *Cyphellophora olivacea* AGSC12. The NaCl concentrations are the following in the top line from left to right: 0.0 M (control), 0.1 M, 0.3 M and in the bottom line from left to right: 0.5 M, 0.7 M, 0.9 M. All the tested strains were able to produce colonies at the different concentrations. Photos were taken after 7 days of cultivation. (**B**) The strain *C. olivacea* AGSC12 tolerance to $NaHCO_3$ I and II contained PDA plates (see methods). This strain showed a striking tolerance to $NaHCO_3$. Photos were taken after 10 days of cultivation. The diameters of the colonies are not to scale; these photos concentrate on the colony morphologies.

Table 2. Evaluation of the tolerance of the fungal strains to different concentrations of salts (see methods).

Species	NaCl Tolerance on PDA						
	0.0 M	0.1 M	0.3 M	0.5 M	0.7 M	0.9 M	
A. niger	3	3	3	3	3	3	
P. expansum	3	3	3	3	3	3	
F. graminearum	3	3	3	3	3	3	
C. olivacea	3	3	3	3	2	2	
	$NaHCO_3$ I. Tolerance on PDA						
	0.0 M	0.1 M	0.3 M	0.5 M	0.7 M	0.9 M	1.1 M
A. niger	3	0	0	0	0	0	ND
P. expansum	3	2	0	0	0	0	ND
F. graminearum	3	3	1	1	1	0	0
C. olivacea	3	3	3	3	2	2	2
	$NaHCO_3$ II. Tolerance on PDA						
	0.0 M	0.1 M	0.3 M	0.5 M	0.7 M	0.9 M	1.1 M
A. niger	3	0	0	0	0	0	ND
P. expansum	3	2	0	0	0	0	ND
F. graminearum	3	3	1	1	1	0	ND
C. olivacea	3	3	3	3	2	2	2



	0.5M	0.7M	0.9M
0.0M	0.05827	*0.000367*	*7.93E-05*
0.1M		*0.01919*	*0.005698*
0.3M		0.1596	0.05827

	0.7M	0.9M	1.1M
0.0M	*0.008139*	*0.000593*	*9.84E-06*
0.1M	0.147	*0.01788*	*0.000593*
0.3M		0.2729	*0.01788*

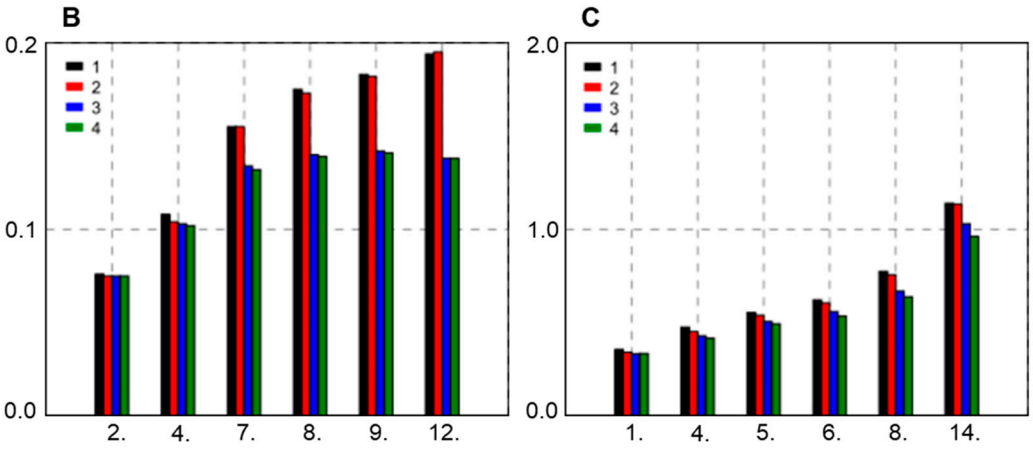
Figure 5. Colony formation of the AGSC12 strain on NaCl- and $NaHCO_3$-containing PDA plates. (**A**) Colony diameters after 7 days of cultivation at different NaCl concentrations. There were significant differences among the values (Kruskal-Wallis test, $P = 3.96 \times 10^{-6}$). (**B**) The data indicate those concentrations when the measured colony diameters showed significant discrepancies according to the Dunn's post hoc test. The values are the relevant *P* values. Only those cases are shown where the *P* values were less than 0.5. Significant values are in italics. (**C**) Colony diameters at various $NaHCO_3$ (II) concentrations after 14 days of cultivation. There were significant differences among the values (Kruskal-Wallis test, $P = 4.66 \times 10^{-7}$). (**D**) Same as (**B**) for the $NaHCO_3$ concentrations. Whiskers on the bar charts represent standard deviations. N stands for sample size.

3.7. Establishing Interaction between the Strains SAG 88.79 and AGSC12

Since we had no information about the interactions (if any) between the strains AGSC12 and SAG 88.79, we wanted to examine the possibility of that. Thus, we separated the two strains with serial dilutions. After this, we obtained fungal-free cyanobacterial

cultures, and we cultivated them in mono- and co-cultures in Spirulina medium. As the AGSC12 strain was not able to increase its biomass substantially in the Spirulina medium, afterwards we concentrated only on the growth of the SAG 88.79 strain.

We inoculated an equal amount of cyanobacterial cells into liquid Spirulina medium with or without the AGSC12 strain. We monitored the changes in the cell density with an OD measurement (λ = 730 nm). We performed the experiments at two different times with 2–2 samples (initial OD = 0.03 and 0.3). In both experiments, co-cultures gained significantly higher OD per unit time than mono-cultures (repeated-measures ANOVA, $P = 0.001427$ and $P = 0.000232$, respectively) (Figure 6A–E).



D

	1	2	3	4
1	1.0	0.9985	*0.01196*	*0.00956*
2		1.0	*0.01635*	*0.01308*
3			1.0	0.9994
4				1.0

E

	1	2	3	4
1	1.0	0.7857	*0.00684*	*0.00042*
2		1.0	*0.04243*	*0.00254*
3			1.0	0.4952
4				1.0

Figure 6. Cultivation of the cyanobacterial strain SAG 88.79 with or without the fungal strain AGSC12. (**A**) Co-cultures (**left**) and mono-cultures (**right**) of the SAG 88.79 strain in liquid Spirulina medium. A black arrow shows the hyphal batch in the medium. (**B**,**C**) Bar charts indicate the differences between the co- (sample 1 and 2) and mono-cultures (sample 3 and 4). The y-axles depict the OD values (λ = 730 nm) and the x-axles show the days of sampling. The differences were significant in both cases (repeated-measures ANOVA, $P = 0.001427$ and $P = 0.000232$, respectively). (**D**,**E**) Tukey's pairwise post-hoc test demonstrated that all the co-cultures exhibited significantly higher cyanobacterial growth than the mono-cultures did. As in (**B**) and (**C**), samples 1 and 2 are for the co-cultures, and samples 3 and 4 are for the mono-cultures. The values in the tables are the corresponding pairwise *P*-values. Italics show the significant values. The cyanobacterial strain SAG 88.79 attained higher cell density in the presence of the fungal strain AGSC12.

On the plates of Spirulina medium, we used two different approaches. At one point, we flooded the plates with a mixture of the SAG 88.79 and AGSC12 strains. At another time,

we flooded the plates with the fungus-free cyanobacterial strain first, let them dry, then flooded them with fungus-containing sterile water. We observed that the cyanobacterial strain became denser around the fungal strain in both cases (Figure 7A–C).

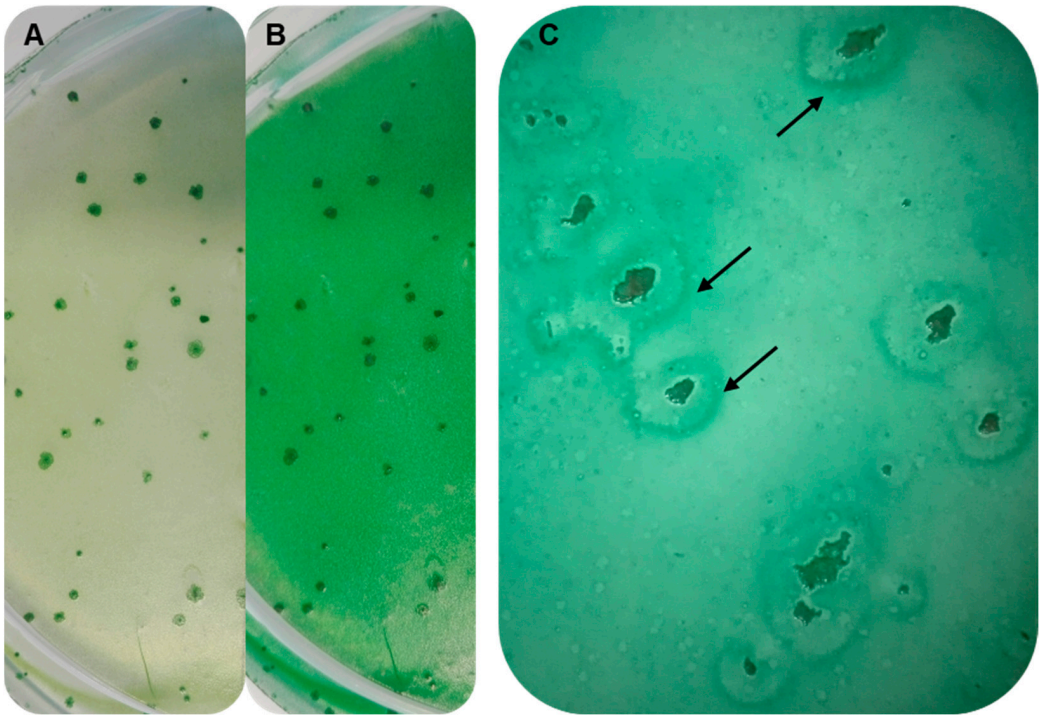


Figure 7. Cultivation of the cyanobacterial strain SAG 88.79 with or without the fungal strain AGSC12 on agar plates. (**A**,**B**) Spirulina plate flooded by the mixture of SAG 88.79 and AGSC12 cells. The cyanobacterial cells became denser near the fungi within a few days of cultivation (**A**) and retained the advantage over a long cultivation period, too (**B**). (**C**) Sequential flooding of the cells. The SAG 88.79 cell-mass was thicker around the fungal colonies as the black arrows indicate. The cyanobacterial strain SAG 88.79 attained higher cell density in the presence of the fungal strain AGSC12 on plates, as well.

4. Discussion

Here, we provided a brief report on the fungal contaminant of the *S. cedrorum* (*C. stanieri*) SAG 88.79 strain, besides a taxonomic note on the cyanobacterium itself.

The 23S rDNA sequence of the SAG 88.79 strain was almost identical with the *C. stanieri* PCC 7202 strain. Thus, our results are in accordance with Komárek et al. [2] and with Moro et al. [3] that the strain SAG 88.79 could be a strain of *C. stanieri*, rather than a strain of *S. cedrorum*. However, it is important to note that the original *S. cedrorum* strain described by Sauvageau is not available for comparison to gain more evidence, so the exact taxonomic evaluation of the strain is cumbersome.

The fungal strain AGSC12 isolated from the culture broth of the SAG 88.79 strain belongs to the *Cyphellophora olivacea* species according to the sequence, phylogenetic, and morphological analyses. The genus *Cyphellophora* (G.A. de Vries emend. Réblová & Unter) consists of mould-like species from widespread ecological niches. Some of the species can be hazardous to health: for example, the type species of the genus *C. laciniata*, along with *C. europaea* and *C. pluriseptata* were isolated from human skin and nails [41,48,50]. Other species, such as *C. guyanensis*, *C. oxyspora*, *C. sessilis*, *C. jingdongensis* and *C. olivacea* were isolated from plant materials or non-biological substrates [43,47,49,60]. The genus is continuously expanding with the introduction of new species and with the occasional transfer from other genera [59,61,62].

Cyphellophora olivacea (W. Gams) and Réblová & Unter (Basionym. *Phialophora olivacea* W. Gams) ex-type strain CBS 122.74 was originally isolated from wallpaper in Kiel-Kitzeberg (Germany) in 1966 [34,49]. Other strains originated from various substrates, such as stalactites, dead petioles of *Chamaerops humilis*, surface of *Inonotus obliquus*, or the surface of treated pine poles and washing machine soap dispensers [49,63,64]. The variety of occurrences of this fungus predicts that the species has a wide tolerance range for environmental factors. Although we have not tested the AGSC12 strain in many different circumstances, we showed that the strain has a striking tolerance for the $NaHCO_3$ on its own. In general, $NaHCO_3$ is considered as a harmless compound, but it could be dangerously harmful to aquatic life (from planktonic organisms to fishes and amphibians) [65]. Besides, this compound has a strong statin effect on most fungi even at low concentrations [14–20]. For instance, *Penicillium digitatum* is completely inhibited by 14.1 mmol of $NaHCO_3$, while *Botrytis cinerea* could be inhibited by 50 mmol [15,20]. Letscher-Bru et al. tested the effect of $NaHCO_3$ on 70 different yeasts, moulds, and dermatophytes, and they found that a concentration of 10 g/L $NaHCO_3$ inhibited the growth of 80% of all the fungal isolates [19]. According to our findings, the isolated strain AGSC12 can colonize even a medium containing 1.1 M (~92.4 g) $NaHCO_3$. Consequently, this strain may be a good candidate in cooperation with certain cyanobacteria and algae for the bioremediation of $NaHCO_3$-containing wastewater (e.g., natural coalbed water and used thermal water).

Although the species inhabits various niches, to our best knowledge, this is the first report of a strain co-existing with cyanobacteria. However, it is unclear whether SAG 88.79 was already accompanied by the fungal strain at its isolation in 1963 or if its culture became contaminated by the fungus afterwards, during its maintenance in the culture collection. According to Göttlich et al., the fungal flora of groundwater-derived public drinking water in certain regions of Germany was dominated by *Phialophora* species [66]. We should bear in mind that the genus *Cyphellophora* was proposed first in 2013 by Réblová et al. and that *C. olivacea* was considered as *Phialophora olivacea* earlier [34]. Thus, there is a chance that the AGSC12 strain appeared in the culture broth of the SAG 88.79 strain during its maintenance. Nevertheless, it seemed that the presence of the fungal strain was beneficial to the cyanobacterium, as we observed that the SAG 88.79 strain always attained a higher cell density in co-cultures with the fungus than in mono-cultures.

To reveal the exact mode of interaction between the strains is outside the scope of the current study, but we are planning to further investigate this phenomenon. There is emerging knowledge to prove that "artificial" symbiosis can be beneficial to the participant species even if the strains do not have common evolutionary history [67,68].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14121023/s1, Figure S1: Comparison of different phylogenetic tress; Figure S2: Microscopic examination of the fungal strain AGSC12; Figure S3: $NaHCO_3$ (II) tolerance of the comparator species; Figure S4: $NaHCO_3$ (II) tolerance of the AGSC12 strain; Figure S5: $NaHCO_3$ (I) tolerance of the examined fungal species; Figure S6: $NaHCO_3$ (I) tolerance of the FGSC 9075 and AGSC12 strains; Table S1: List of species used for the phylogeny.

Author Contributions: Conceptualization: L.A.-S. and M.S.; Methodology: L.A.-S., H.C. and M.S.; Validation: L.A.-S.; Formal analysis: L.A.-S. and M.S.; Investigation: L.A.-S. and L.A.P.; Resources: I.M. and M.S.; Writing—original draft preparation: L.A.-S.; Writing—review and editing: L.A.-S., L.A.P., H.C., I.M. and M.S.; Visualization: L.A.-S.; Supervision. M.S.; Funding acquisition: M.S. and I.M. All authors have read and agreed to the published version of the manuscript.

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