Developing Effective ManagementOptions for Nostoc spp. in Florida Nurseries

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ABSTRACT

Nostoc is a genus of cyanobacteria (blue-green algae) that is commonly found on gravel, ground cloths, aisles, and countless other production areas of nurseries and greenhouses. It is considered a noxious weed and is often referred to as “star jelly” and even “ground boogers”. *Nostoc* colonies are composed of chains of cells that grow on the surface of soil, gravel, and cement producing macroscopic mats. During dry periods, these mats desiccate and become flaky, but when water on any moisture is present, they swell to form thick, dark green, gelatinous masses that can completely cover container production areas. While very unsightly, the primary concern with this species is that it is extremely slippery and wet, which poses serious health hazards for nursery employees. Other concerns are their ability to produce cyanotoxins and allelopathic compounds, which can affect plant growth and physiology.

Another caveat of this species is the unique ability to reestablish growth following algaecide applications due to their ability to cyst when stressed and/or application of chemicals incapable of killing all single cells. Additionally, large *Nostoc* growths usually contain several species of cyanobacteria, each with a unique threshold for algaecide applications. In order to find truly effective and long-term management options for this cyanobacterium, a multi-disciplinary approach was adopted. For more efficacious management of nurseries, a combined understanding of *Nostoc* ecology, taxonomy, systematics, and chemical susceptibility is necessary. The objective of this research project is to address the *Nostoc* dilemma using a combination of laboratory and field methodology to provide growers with more data on effective *Nostoc* management.

OBJECTIVES

1. ***Isolate and culture Nostoc strains from nurseries.***
2. ***Resolve taxonomy and systematics of Nostoc strains.***
3. ***In situ and in vitro percent growth of Nostoc post-treatment.***
4. ***Cell viability of Nostoc strains post-treatment.***

METHODS

Isolation and culturing
Fresh algal and soil material were collected from sites at the Ft. Lauderdale Research and Education Center (FLREC) (Davie, FL) and the Mid-Florida Research and Education Center (MREC) (Apopka, FL) and stored in sterile 50ml falcon tubes. Samples were then processed by both inoculating in sterile BG-110 media (nitrogen free media) and inoculating on BG-110 agar plates. Plates and flasks were incubated under constant fluorescent lighting at 25°C for three weeks. Single colonies that formed were restreaked and then transferred into liquid media and given an ID number based on the Berthold-Laughinghouse Culture Collection (BLCC) of terrestrial algae. All strains were cultured in 75ml BG110 media. Images were captured using a compound microscope (LEICA CTR5500).

Molecular and phylogenetic analyses
Fresh unialgal biomass was placed into an Eppendorf tube and submerged into liquid nitrogen for initial cell lysis. After lysis, a DNeasy UltraClean Microbial Kit (Qiagen, USA) was used to extract and purify DNA. The 16S rRNA gene was amplified using polymerase chain reaction (PCR) and the primers 359F and 1487R (Wilmotte et al. 1993; Nübel et al. 1997). PCR reactions were carried out on a ProFlex thermal cycler (Applied Biosystems) using the PCR profile as indicated by Wilmotte et al. (1993). PCR products were purified using a Qiaquick PCR purification kit (Qiagen, USA). Purified amplified DNA was then submitted to Eurofins for Sanger sequencing (Eurofins, Kentucky, USA). BLAST (Basic Local Alignment Search Tool - NCBI) was used to locate strains and sequences similar to the sequences of our taxa. We retrieved 22 sequences from Genbank. Sequences were aligned using Muscle and then manually edited considering conserved regions on MEGA (7.0.26) (Kumar et al. 2015). To construct the phylogenetic tree, a maximum likelihood (ML) analysis was run using GTR+I+G model over 1000 bootstrap replicates SeaView (v4.6.3) (Gouy et al 2010).

In situ and in vitro percent growth of Nostoc post-treatment
0.5m2 of greenhouse tarp and gravel substrate were sectioned off using laboratory tape and fluorescent spray paint, respectively, and labelled with waterproof markers. Images of plots were taken every seven days for 28 days.

For the laboratory studies, sterilized ceramic tiles were placed within petri dishes and with BG110 and inoculated with 1 ml of seed culture of *Nostoc commune* (UTEX 1621). Plates with ceramic tiles were cultured at 25°C under constant lighting (50µmol) for two weeks until enough algae covered the surfaces and then analyzed every 7 days after application. Chemicals for testing were mixed to the desired label rate concentrations and placed into dark spray bottles. For application of the chemical, each greenhouse plot was sprayed 40 times and each petri plate with the ceramic tile was sprayed twice.

Images were taken on an iPhone (v8) and uploaded onto Adobe Photoshop (2017.1.1) in order to crop each picture within the marked plot areas of both field and laboratory images. These images were then uploaded onto iLastik (1.3.0) for image analysis. Live and dead algae material were labeled with two colors including green (live) and red (dead). Material not considered algae were also labeled red. Converted images with the pixel analysis software were uploaded onto a pixel analysis software (coolphptools.com) using the color extract feature to calculate color pixel percentages. Percent coverage of live and dead algae were then calculated based on the initial coverage on Day 0 prior to chemical applications. A total of 5 chemicals with one control were used in this study. No replicates were established in this preliminary trial.

Cell viability post-treatment
In order to analyze the degradation (presence or absence) of algal DNA, ±100 mg of material from each greenhouse plot (gravel and tarp) and ceramic tiles were removed and placed in 1.5ml Eppendorf tubes. Tubes underwent a freeze-thaw cycle three times for cell lysis. Samples were processed for DNA using the UltraClean Microbial DNA isolation kit (Qiagen, USA). DNA extracts were then amplified using PCR primers for cyanobacteria and chlorophytes using the 16S and 18S rRNA genes, respectively. For the 16S rRNA gene, the primers used were the forward CYA359F and two reverse primers CYA 781R (a) and CYA 781R (b) (Nübel et al. 1997). For 18S rRNA gene, the primers used were the forward SSU1 and reverse primer SSU2 (Saunders and McDevit 2012).

RESULTS

Isolation and cultivation of Nostoc strains
Two nurseries/greenhouses were sampled for cyanobacteria at the Ft. Lauderdale REC (Davie, FL) and Mid-Florida REC (Apopka, FL) greenhouses. The *Nostoc* strains were isolated from several surfaces including floor, gravel, tarp, pots, plastic sheets, walls, and soil crusts adjacent to greenhouses. A total of 65 unialgal strains were isolated from the samples. Of the 65 strains, 22 strains macroscopically resembled the ‘*Nostoc*’ genus and were therefore chosen for imaging; 15 isolates are shown in Figure 1 (on page 44). Of the 22 isolates, the five strains that most resembled *Nostoc* were chosen for algaecide screening, and a total of ten strains were used for molecular phylogenetic analyses.

Molecular and phylogenetic analyses
Of the 22 *Nostoc* strains isolated, an initial group of ten strains were chosen for phylogenetic analyses. From the phylogenetic tree based on the 16S rRNA gene sequence, only two strains from this research grouped together with *Nostoc sensus stricto* (Figure 2, on page 45). The remaining eight strains formed a novel clade sister to the genus *Tolypothrix*. These strains will be further studied, since they most likely represent a new genus and several new species to science.

In situ percent growth of Nostoc post-treatment
The field experiments on cyanobacterial crusts *in situ* varied among treatments. From the results of Table 1a, the highest percent coverage decrease on tarps on day 28 was observed with sodium carbonate peroxyhydrate with a total decrease of 67%. The second and third most effective algaecides were germicidal bleach and pelargonic acid with a 60% and 53% decrease, respectively. In terms of percent coverage of algae growth on gravel (Table 1b), the greatest percent decrease on day 28 was observed with Zerotol 2.0 with a total decrease of 71%, while the second and third most effective formulations were sodium carbonate peroxyhydrate and germicidal bleach with 46% and 10% decrease of algae coverage, respectively.

Table 1. Results of algaecides on plots containing algae expressed as a percent (%) coverage of live algae on a) tarp and b) gravel for every week over 28 days. Red boxes indicate death and reduction of algae growth, and blue boxes indicate percent increase of algae growth/coverage.

a)

%Change Tarp	Day 7	Day 14	Day 21	Day 28
Control	-39.66	7.18	52.30	70.20
Copper Sulfate	19.40	15.55	21.52	51.00
Zerotol 2.0	-4.25	25.23	0.92	-11.83
Pelargonic Acid	-40.48	29.97	36.35	53.18
Germicidal Bleach	31.01	9.02	60.20	60.08
$NaCO_3$ Peroxyhydrate	-41.87	28.63	80.72	67.41

b)

%Change Gravel	Day 7	Day 14	Day 21	Day 28
Control	18.50	59.93	81.73	78.20
Copper Sulfate	7.99	-29.68	-2.73	-0.61
Zerotol 2.0	44.57	43.96	25.10	70.90
Pelargonic Acid	-67.25	0.08	-66.38	-11.32
Germicidal Bleach	-37.09	-20.58	-18.54	9.79
$NaCO_3$ Peroxyhydrate	8.66	-5.35	9.95	45.57

These data indicate that chemicals have different effects depending on the surface in which they are applied, such as with Zerotol 2.0 and pelargonic acid with different results on gravel and tarp. Our data indicate that sodium carbonate peroxyhydrate is the most effective algaecide on both surface types, while Zerotol is effective on gravel surfaces. Results may vary as the amount in grams (g) of algae on tarp and gravel was not measured and standardized with the amounts of chemicals added. In other words, all plots received the same amount of chemicals regardless of the amount of algae present. Environmental factors, such as seasonality, rain and temperature, can affect algal growth and species composition.

In vitro percent growth of Nostoc post-treatment
When *Nostoc commune* UTEX 1621 was used in a preliminary experiment to test the efficacy of different algaecides on ceramic tiles, a week of application indicated that the most effective algaecides were pelargonic acid, sodium carbonate peroxyhydrate, and germicidal bleach with a growth reduction of 98%, 80%, and 30%, respectively (Table 2). Conversely, copper sulfate increased the growth of *Nostoc commune* by 41%, which was higher than that of the control, which increased increase of 16%). After two weeks post-application, all treatments except copper sulfate witnessed a loss of algal coverage on the tile. Three weeks post-application demonstrated that the most efficient algaecides against *Nostoc commune* were sodium carbonate peroxyhydrate, pelargonic acid, and germicidal bleach with a reduction of growth around 98%, 96%, and 26%, respectively, as shown in Figure 3 (on page 45).

Table 2. Percent area growth increase or decrease of *Nostoc commune* inoculated onto ceramic tiles, treated with various algaecides and analyzed every week over three weeks. Negative values and blue indicates growth of *Nostoc*, while red and positive values indicated reduction/death of algal area cover.

Treatment	Day 7	Day 14	Day 21
Control	-15.53	16.56	-5.49
Copper Sulfate	-41.17	-55.68	-62.91
Zerotol 2.0	-3.77	0.66	0.55
Pelargonic Acid	98.03	95.84	95.89
Germicidal Bleach	29.91	31.43	26.12
$NaCO_3$ Peroxyhydrate	79.49	95.57	98.03

Cell viability post-treatment
Verifying that algaecides effectively remove and degrade algal DNA is vital in suppressing the re-establishment of algae after application. From Table 3, DNA extraction revealed that from the laboratory plates inoculated with *Nostoc commune*, when observing the raw extracted DNA, all of the plates contained DNA. After amplifying the extracted raw DNA with primers that are specific to coccoid cyanobacteria (16S) like *Nostoc*, we found that the plates treated with sodium carbonate peroxyhydrate o did not contain any cyanobacterial DNA that could stem from *Nostoc commune*. The remaining plates treated with copper sulfate, germicidal bleach, and pelargonic acid were not effective in removing the DNA of *Nostoc commune* and therefore could allow the cyanobacteria to establish growth again after application.

Table 3. Results from molecular studies of in vitro ceramic tiles inoculated with *Nostoc commune* indication presence (+) or absence (-) of either cyanobacterial 16S rRNA gene.

Treatment	Total DNA	16S
Control	+	+
Copper Sulfate	+	+
Zerotol 2.0	+	+
Pelargonic acid	+	+
Germicidal Bleach	+	+
Sodium carbonate peroxyhydrate	+	-

CONCLUSION

In this study, five common algaecides used in greenhouses and nurseries for the control of algae were assessed for their efficacy in treating algal contamination. We analyzed the effects of various algaecides on three different surfaces including field gravel and tarp *in situ* and ceramic tiles *in vitro*. The main factors that influence the actions of chemicals on combating greenhouse algae include 1) the type of chemicals used and 2) the material in which the algae are growing. From the field studies, sodium carbonate peroxyhydrate was the most efficient at reducing algae growth on tarp and gravel. On the tarp material, sodium hypochlorite, pelargonic acid, and copper sulfate were also moderately effective in removing algae growth (Table 1a). Hydrogen dioxide was not an effective agent when applied to algal mats found on tarp possibly because it is easily oxidized in the exposed tarp. Alternatively, hydrogen dioxide was the most effective in reducing algal cover on gravel. Hydrogen dioxide is a dioxide-based product that penetrates into the gravel creating a high success rate when targeting algae bound onto gravel. Besides hydrogen dioxide, sodium carbonate peroxyhydrate was also moderately efficient at removing algae from the gravel surface. On the other hand, copper sulfate, pelargonic acid, and sodium hypochlorite were not successful at removing algae (Table 1b). Copper sulfate applied onto gravel and ceramic along with pelargonic acid applied on gravel were especially not efficient at removing algae, but instead stimulated growth in our study.

From the *in situ* studies, results show that a few chemicals effectively targeted *Nostoc*. By inoculating *Nostoc* onto ceramic plates and removing the uncontrollable environmental variables, we were able to observe that hydrogen dioxide, sodium hypochlorite, pelargonic acid, and sodium carbonate peroxyhydrate were increasingly effective against *Nostoc commune* grown on ceramic tiles. However, copper sulfate resulted in increasing growth coverage on the ceramic surface over 21 days. Copper sulfate indicated signs of hormesis, where growth was actually stimulated rather than reduced (Figure 3, on page 45). Other herbicidal compounds, when applied in low concentrations, have also indicated hormesis with cyanobacteria (Shen et al. 2009).

When observing the raw extracted DNA, all of the plates contained DNA whether fungal, bacterial, or algal in nature. Alternatively, once the DNA from the ceramic plates were amplified using the specific filamentous cyanobacteria primer, only sodium carbonate peroxyhydrate resulted in the complete destruction of *Nostoc commune* genetic material. The complete degradation of *Nostoc commune* genetic material is essential in preventing cyanobacteria from reestablishing growth after chemical application.

This study provides an understanding for horticulturists to target the various forms of growth on gravel, tarp, and ceramic surfaces. It seems that chemicals have different results depending on the surface applied, such as the case of hydrogen dioxide and sodium hypochlorite, with different results on gravel and tarp. We found sodium carbonate peroxyhydrate to be the most effective formulation on all surfaces examined here, specifically for the removal of *Nostoc*, while sodium hypochlorite is moderately effective on both tarp and gravel surfaces. Sodium carbonate peroxyhydrate is effective at penetrating the surfaces assessed here and renders the surfaces free of contamination by degrading the genetic material. Our results indicate that a better understanding of algal community structure found on varying surfaces is a requisite for efficacious management practices in curbing algae contamination in greenhouses and nurseries.

Since algal mats and crusts in greenhouses are composed of several species, it is especially vital to account for the complexity of the community. Since different strains may have different properties, such as varying degrees of mucilage and pigmentation, their susceptibility to any specific algaecide will vary. The next phase of our work will focus on using the '*Nostoc*' strains isolated in this research to screen the individual strains with the algaecides *in vitro*. This will allow us to grasp a better understanding of the effects of algaecides on individuals commonly found in the algae community mats in nurseries.

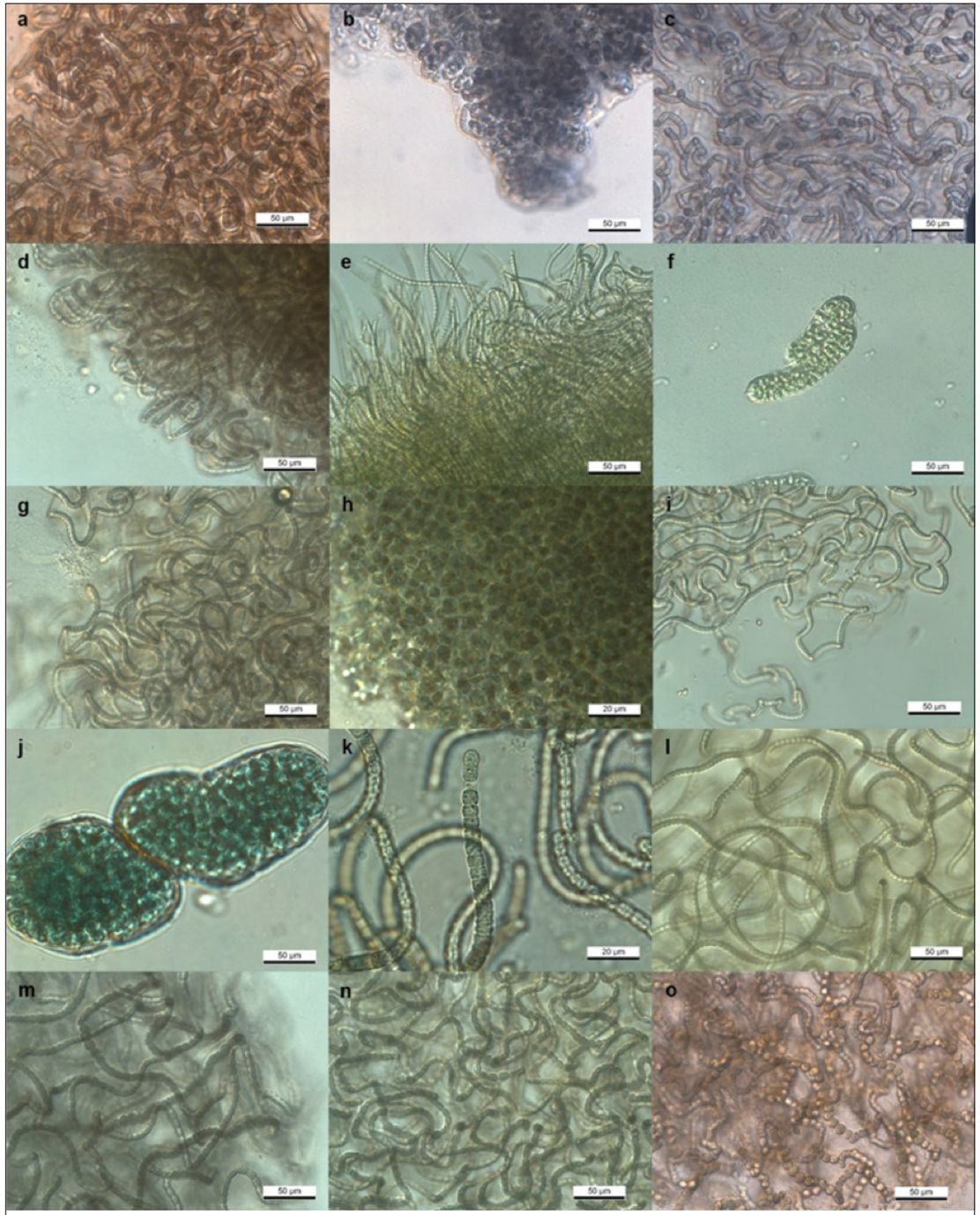

Fig. 1. Strains of *Nostoc*-like cyanobacteria isolated from various nurseries in Florida. Strains include '*Nostoc*' BLCC a) T3, b) T5, c) T6, d) T9, e) T10, f) T11, g) T12, h) T13, I) T14, j) T15, k) T16, l) T17, m) T18, n) T19, and o) T20. Scale bars are as indicated.

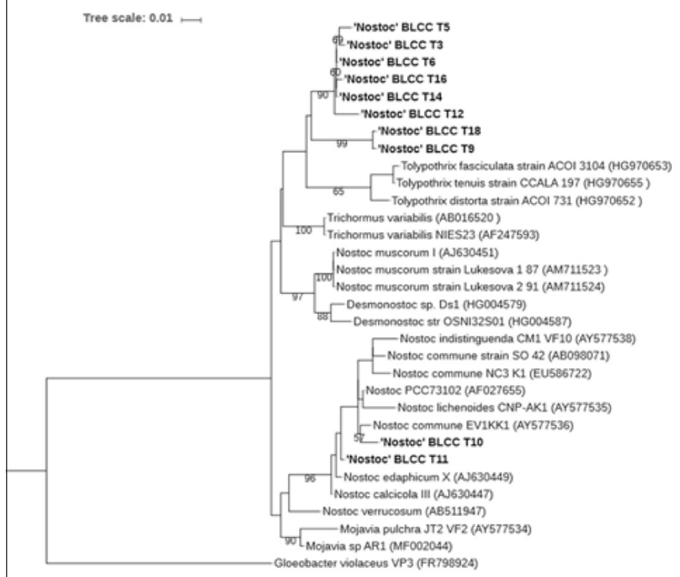

Fig. 2. Phylogenetic analysis (ML tree) based on 16S rRNA gene sequences. Bootstrap values above 50 are shown. '*Nostoc*' strains isolated in this research are bolded.

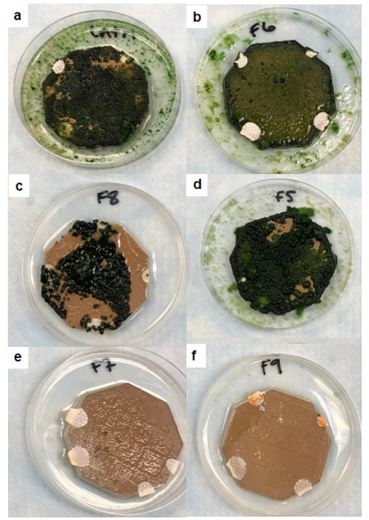

Fig. 3. *In vitro* experiment of *Nostoc commune* inoculated on ceramic tiles and treated with varying algaecides over three weeks. The experiment involved a) control, and the cyanobacterium treated with b) Zerotol 2.0, c) germicidal bleach, d) copper sulfate, e) pelargonic acid, f) sodium carbonate peroxyhydrate.

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