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Foliar Application of *Chlorella* Supernatant Protects Turfgrass against *Clarireedia jacksonii* by Eliciting Induced Resistance and Modulating the Rhizosphere Microbiota

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Large-scale culture of the microalga *Chlorella* produces valuable products. Cultivation also generates tons of supernatant waste that require detoxification and disposal. Recent research has focused on recycling waste supernatant as a plant protectant and biofertilizer, although, to date, most studies have considered its use as a biological control of pathogens infecting dicot plants. By contrast, the current study evaluated whether *Chlorella* supernatant could protect turfgrass (*Agrostis stolonifera*), a monocot plant widely used as a turfgrass, against dollar spot disease caused by the fungal pathogen *Clarireedia jacksonii* (formerly *Sclerotinia homoeocarpa*) under greenhouse and field conditions. Foliar application of supernatants from *Chlorella* sp. ABC001 and HS2 cultures reduced the incidence of dollar spot disease in turfgrass under both greenhouse and field conditions without directly inhibiting growth. The effects of supernatant application on the rhizosphere microbiome were investigated using 16S rRNA amplicon sequencing. Application of ABC001 and HS2 supernatants modulated the structure of the rhizosphere microbiome and enriched specific microbial taxa that improved turfgrass health in the presence of *C. jacksonii*. The application of waste *Chlorella* supernatant therefore offers an alternative method for protecting monocot plants against fungal pathogens, while also enhancing the composition of soil microbes in the rhizosphere.

Keywords **:** *Chlorella*, biological control, dollar spot disease, rhizosphere microbiome, turfgrass

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Dollar spot disease, caused by *Clarireedia jacksonii* (formerly *Sclerotinia homoeocarpa*), is the most common and serious disease affecting turfgrass species worldwide. Dollar spot disease is prevalent in all areas in which turf is grown, including golf courses, domestic lawns, and sports fields and stadia (Mitkowski and Colucci, 2006; Sapkota

et al., 2022). Its symptoms include white to straw-colored lesions that spread across the leaf blades to form circular, sunken patches of varying diameters; these resemble silver dollars, hence its name (Bennett, 1937). The severity of dollar spot disease increases at temperatures between 15°C and 27°C, as well as during high humidity (>85%) or nutrient deficiency (Allen et al., 2005; Sapkota et al., 2022; Walsh et al., 1999). Disease management is difficult due to *C. jacksonii*'s wide host range, ability to survive across a wide range of temperatures and overwinter in dead tissue, and the lack of resistant grass cultivars (Bishop et al., 2008; Jo et al., 2008; Ok et al., 2011; Ostrander et al., 2014; Popko et al., 2018; Rioux et al., 2021; Walsh et al., 1999).

Synthetic fungicides, such as demethylation-inhibiting (DMI) fungicides, benzimidazoles, and succinate dehydrogenase inhibitors, are commonly used to manage dollar spot disease. The continuous use of chemical fungicides is, however, a cause of increasing concern due to their ecotoxicological risks, the emergence of fungicide-resistant pathogens, and dysbiosis caused to the phyllosphere and beneficial soil microbiota (Akinnifesi et al., 2006; Geiger et al., 2010; Jo et al., 2008; Ok et al., 2011; Popko et al., 2018). An effective, safe and environmentally sustainable alternative method for controlling grass diseases is required. Biological control, defined as the reduction of inoculum density or disease-producing activity of plant pathogens through the action of one or more organisms, is an alternative control strategy that addresses the issues associated with synthetic agrochemicals (Cook and Baker, 1983; Pal and Gardener, 2006). Biological control agents may suppress phytopathogen growth directly by producing antimicrobial compounds, competing for resources or space, or by hyperparasitism, but they may also protect plants indirectly by activating plant immunity through induced resistance (IR) (Cook and Baker, 1983; Kloepper et al., 1992; Pal and Gardener, 2006; Pieterse et al., 1996). IR is a state of systemically activated plant immunity triggered by an inducing agent that provides protection against a broad spectrum of phytopathogens (Alström, 1991; Kuć, 1982; Pieterse et al., 2014; Ross, 1961; Van Peer et al., 1991; Wei et al., 1991; Zimmerli et al., 2000). Crop protection through IR is an attractive biological control strategy, as it provides long-lasting effects and is effective against a wide range of pathogens (Pieterse et al., 2014). Although associations between IR in crops and beneficial microbes, including bacteria, fungi, and archaea, have been widely studied (Pieterse et al., 2014; Song et al., 2019; Wang et al., 2021), few biocontrol agents are known to activate IR in turfgrass species, enabling the control of dollar spot disease.

Chlorella is a genus of eukaryotic photosynthetic microalgae that are cultivated on an industrial scale because they produce a variety of nutrients and secondary metabolites used in health supplements, cosmetics, and biofuels (Cho et al., 2015; Lee and Ryu, 2021; Liu et al., 2016). *Chlorella* species were originally identified in aquatic habitats and their role among the beneficial plant-associated microbiota has been the subject of debate (Lee and Ryu, 2021). A recent paradigm shift, however, has led to microalgae such as *Chlorella* being recognized as ubiquitous microorganisms that are present in plant tissues as well as soil environments. *Chlorella* treatment has beneficial effects on various plants as a bioprotectant or biofertilizer (Lee and Ryu, 2021). Foliar application of *Chlorella fusca* elicits IR against *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis* (Lee et al., 2020a). The industrial cultivation of *Chlorella* uses only the microalgal cells, however, and various studies have demonstrated the beneficial activities of the waste cell-free supernatant, leading to the suggestion it could be recycled for crop protection. D-lactic acid in the cell-free supernatant of *C. fusca* acts as an inducing agent and primes innate immune responses in Arabidopsis (*Arabidopsis thaliana*), including reactive oxygen species burst and callose deposition (Lee et al., 2020a). In addition, cell-free supernatants from *C. fusca* and *Chlorella* sp. ABC001 and HS2, two high biofuel-producing strains, delay senescence in the ornamental plant *Erinus alpinus* (Lee et al., 2020b).

This study evaluated the potential use of *Chlorella* supernatant as a biological control agent against dollar spot disease, a fungal disease caused by *C. jacksonii*, in turfgrass (*Agrostis stolonifera*), a monocot plant widely used as a turfgrass. Spraying turfgrass with *Chlorella* sp. ABC001 and HS2 supernatants reduced the severity of both manually inoculated and naturally occurring dollar spot disease in the greenhouse and on a golf course. Additionally, treatment with either supernatant modulated the rhizosphere microbiome by enriching the abundance of protective microbial taxa that enhance grass health. These findings suggested that *Chlorella* supernatant could protect turfgrass from fungal pathogen attacks by eliciting IR, while also improving the health of the soil microbiome in the rhizosphere.

Materials and Methods

Cultivation of turfgrass plants. Turfgrass samples were collected from a field of turfgrass using an 18 cm diameter hole cutter and transplanted to pots, which were placed in an environmentally controlled growth room at 26°C under

fluorescent lights (light intensity approximately 7,000 lux) and 12 h light/12 h dark cycles. Grass leaves grew continuously under these conditions and were cut using an automatic mower 1 day before pathogen challenge.

***Chlorella* cultivation and preparation of cell-free supernatant.** *Chlorella* sp. ABC001 and HS2 were cultivated under mixotrophic conditions, as described previously (Lee et al., 2020a). To avoid bacterial contamination, culture media were filtered prior to *Chlorella* cultivation using a 0.45 μm syringe filter (Lee et al., 2020a). A hemocytometer (INCYTO, Cheonan, Korea) was used to measure *Chlorella* cell concentration (Lee et al., 2020a). When cultures reached the exponential phase (10^7 cells/ml), cells were harvested by centrifugation at 4,000 ×*g* for 10 min to separate the supernatant from the pellet.

To evaluate the biological control activity of *Chlorella* sp. ABC001 and HS2 supernatants on turfgrass under both indoor and field conditions, supernatants from both cultures was sprayed onto grass leaves. In greenhouse experiments, turfgrass was sprayed with 20 ml of *Chlorella* sp. ABC001 and HS2 supernatant, 20 ml of 0.5 mM benzothiadiazole (BTH), an inducer of disease resistance, or 20 ml of BG11 (Blue-Green medium) broth containing 1 g of glucose, a control treatment, three times with 7 days intervals. Outdoor experiments were conducted at a site in Incheon (37°26′41.82″N, 126°28′47.28′E). Turfgrass growing in the field was sprayed with 1 liter per 30 m^2 of *Chlorella* sp. ABC001 and HS2 supernatant, with a mixture of 15% azoxystrobin (a DMI fungicide) and 10% thiophanate-methyl (a benzimidazole fungicide), or with tap water (control treatment) four times at 2-week intervals.

Pathogen challenge and disease measurement. *Clarireedia jacksonii* (formerly *Sclerotinia homoeocarpa*) was cultivated in oatmeal agar medium for 7 days at 30°C. In greenhouse experiments, turfgrass leaves were inoculated with 2 g of oatmeal medium containing *C. jacksonii*. Inoculated grass was covered with aluminum foil at 26°C. Symptoms of dollar spot disease were observed at 2 weeks post-inoculation. In field experiments, the incidence of naturally occurring dollar spot disease was measured 0, 2, 4, 6, and 8 weeks after treatments were applied.

Soil sampling and analysis of the rhizosphere microbiome. Samples of rhizosphere soil were collected in the field for microbiome analysis. Replicate samples were collected at 8 weeks post-treatment from three grass plants per treatment. Each rhizosphere soil sample was passed through a 2 mm mesh and suspended by mixing with sterile distilled water and centrifuging at 200 rpm for 30 min to eliminate larger particles and plant material, leaving only the rhizosphere soil. The soil suspension was centrifuged at 8,000 rpm for 10 min to obtain a microbiome-enriched soil pellet, which was stored at −80°C until further analysis.

Genomic DNA was extracted from rhizosphere soil samples using the FastDNA Spin Kit (MP Biomedicals, Irvine, CA, USA) and quantified using an Epoch Spectrophotometer (Biotek, Winooski, VT, USA). PCR amplification was performed using primers targeting the V3 and V4 regions of the 16S rRNA gene. The primer pair 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3') and 805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATC-3') was used in the initial round of amplification with the following conditions: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 5 min. A secondary amplification step was performed to attach the Illumina NexTera barcodes using the primer pair i5-F (5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTC-3'; X indicates the barcode region) and i7-R (5'-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-GTCTCGTGGGCTCGG-3'; X indicates the barcode region) under the same conditions as before, but with eight amplification cycles. PCR products were separated by electrophoresis on 1% agarose gels and visualized using a Gel-Doc system (Bio-Rad, Hercules, CA, USA). PCR products were purified using the CleanPCR Kit (CleanNA, Waddinxveen, the Netherlands). Equal quantities of purified products were pooled. Non-target short fragments were removed using the CleanPCR Kit. The quality and size of PCR products were checked using the DNA 7500 chip on an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). The pooled amplicons were sequenced at ChunLab, Inc. (Seoul, Korea) using the Illumina MiSeq platform and following the manufacturer's guidelines.

***In silico* analysis of microbiota structure.** The quality of the raw sequencing data was assessed using FastQC to determine the appropriate quality thresholds for the forward and reverse reads. These reads were imported into QIIME2 (v. 2020.2) (Bolyen et al., 2019) for quality filtering, diversity analysis, and sequence classification. The DADA2 quality control function (Callahan et al., 2016) was applied to trim the forward and reverse reads, remove noise, and detect and eliminate chimeras. Alpha and beta diversity metrics were analyzed using a rarefied operational taxonomic units (OTU) table, generated with the rarefy_even_

depth function in the PhyloSeq package to standardize read depth. Alpha diversity indices, such as the Shannon index, were used to estimate community abundance, while OTUs and community evenness were assessed using Pielou's evenness index. To analyze beta diversity, phylogenetic trees were generated in QIIME2 and pairwise beta diversity calculations were performed using the weighted Unifrac matrix. Sequence classifications were assigned at the species level based on the SILVA (v. 138.1) Reference Taxonomy Database (Quast et al., 2013, https://doi.org/10.1093/nar/gks1219). Relative abundance at the class level was calculated as the change in proportion, at this level may reflect soil conditions.

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) using JMP 4.0 software (SAS Institute Inc., Cary, NC, USA). Significant treatment effects were determined using the magnitude of the F-value ($P < 0.05$). When significant F-values were obtained, the separation of means was analyzed by determining Fisher's protected least significant difference at $P < 0.05$. Microbiome data were analyzed using the R software and QIIME2. Alpha diversity was assessed through the Shapiro-Wilk normality test, followed by a Student's t-test using stats R package. Beta diversity was evaluated using PERMANOVA (permutational multivariate analysis of variance) with 999 permutations, performed via the function adonis2 in the vegan R package (Oksanen et al., 2022). The randomForest function from the randomForest package in R was applied for predictive modeling to identify OTUs that distinguish between groups.

Data availability. Raw data were deposited in the Sequence Read Archive National Center for Biotechnology Information (SRA-NCBI) under the accession number PRJNA1185631.

Results

***Chlorella* supernatant suppresses dollar spot disease in turfgrass under greenhouse conditions.** To determine whether application of cell-free supernatants from *Chlorella* sp. ABC001 and HS2 cultures reduced the occurrence of dollar spot disease in turfgrass, we tested the biological activity of supernatants against the pathogenic fungus *C. jacksonii* under greenhouse conditions (Fig. 1). *C. jacksonii* was inoculated onto grass leaves, which were sprayed with each supernatant three times at 7 days intervals (Fig. 1A). Although both the supernatants and the fungal pathogen were applied to the same leaves, the growth of *C. jacksonii* hyphae on the leaves was similar across all treatments (data not shown). We therefore used BTH, a chemical trigger of plant innate immunity, as a positive control. Compared with the control, BTH treatment reduced the symptoms of dollar spot disease on grass leaves, including chlorosis and necrosis, at 7 days post-inoculation (Fig. 1B). Foliar application of either ABC001 or HS2 supernatant also significantly reduced the symptoms of dollar spot disease on grass leaves compared with control plants (Fig. 1B). Treatment with the two *Chlorella* supernatants or BTH did not affect turfgrass growth under greenhouse conditions (data not shown). These results indicated that *Chlorella* supernatant controlled dollar spot disease in turfgrass without antifungal activity.

Application of *Chlorella* supernatant suppresses naturally occurring dollar spot disease. To test the biological activity of supernatants against naturally occurring dollar spot disease under field conditions, cell-free ABC001 and HS2 supernatants were sprayed on turfgrass growing outdoors at Incheon, South Korea, that had experienced frequent occurrences of dollar spot disease (dollar spot-conducive soil). A mixture of fungicides (15% azoxystrobin and 10% thiophanate-methyl) was applied as a positive control. We measured the incidence of naturally occurring dollar spot disease at 0, 2, 4, 6, and 8 weeks post-treatment (Fig. 2). Disease symptoms were not observed in all groups in the first 4 weeks post-treatment (Fig. 2B). At 6 weeks post-treatment, fungicide application reduced the disease incidence by 95.6% compared with the control treatment (Fig. 2A and B). Application of supernatants from ABC001 and HS2 cultures reduced the disease incidence by 60.8% and 52.2%, respectively, at 6 weeks post-treatment, compared with the control treatment (Fig. 2A and B). At 8 weeks post-treatment, fungicide application reduced the incidence of disease by 95.3% compared with the control treatment, but neither of the *Chlorella* supernatant treatments suppressed disease in turfgrass (Fig. 2B); this may have been because of the high humidity. These results suggested that cell-free supernatants from ABC001 and HS2 cultures could act as a bioprotectant under field conditions, reducing the occurrence of naturally occurring dollar spot disease.

Assessment of turfgrass growth under field conditions. To investigate whether application of *Chlorella* supernatants affected plant growth, shoot and root growth, as well as the visual quality of grass plants, were examined at 8 weeks post-treatment under field conditions (Supplementary Fig. 1). Neither growth nor visual quality was altered in plants treated with either of the supernatants. Turfgrass

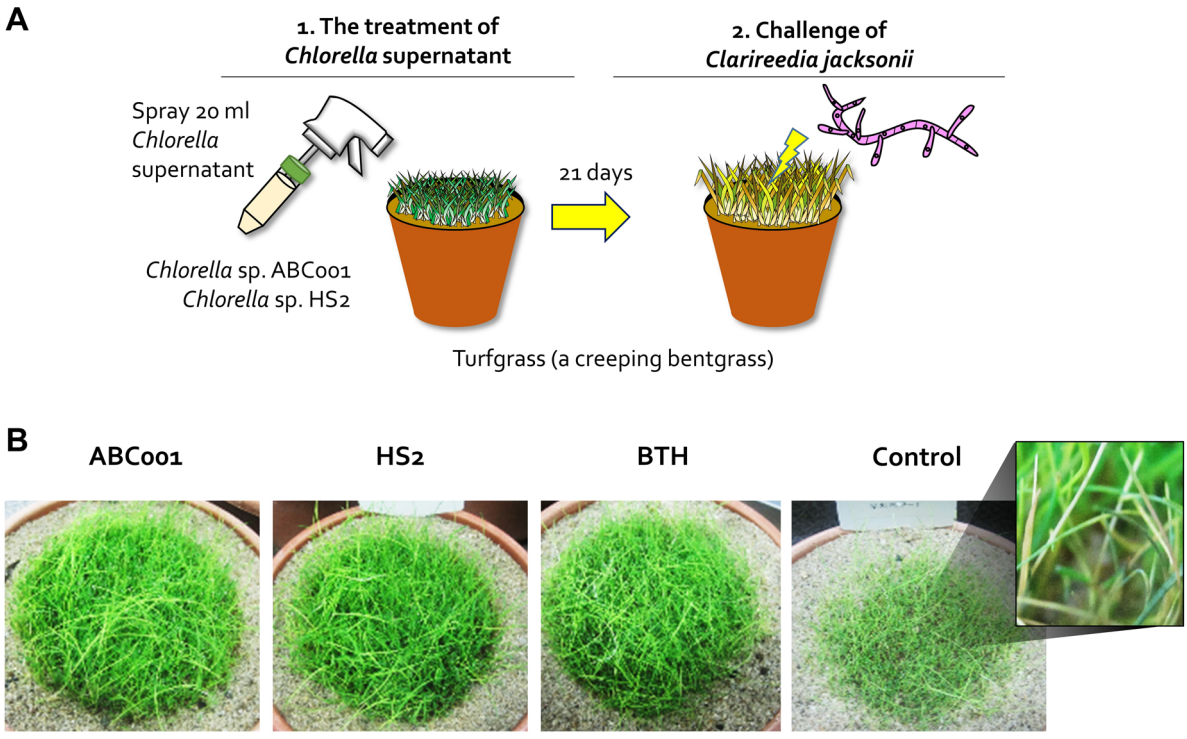

Fig. 1. *Chlorella* supernatant reduces the incidence of dollar spot disease in turfgrass. (A) Experimental diagram for testing the biological control effect of *Chlorella* supernatant on dollar spot disease. Leaves of 2-week-old turfgrass plants were sprayed with *Chlorella* sp. HS2 or ABC001 supernatant, benzothiadiazole (BTH), or BG11 broth (Blue-Green medium) three times at 7-day intervals. At 21 days post-treatment, grass leaves were inoculated with 2 g of oatmeal medium containing *Clarireedia jacksonii*. (B) Symptoms of dollar spot disease 2 weeks post-inoculation in turfgrass plants sprayed with *Chlorella* supernatants, fungicide, or BG11 broth (control). ABC001, *Chlorella* sp. ABC001 supernatant treatment; HS2, *Chlorella* sp. HS2 supernatant treatment; BTH, 0.5 mM BTH treatment; control, BG11 broth treatment.

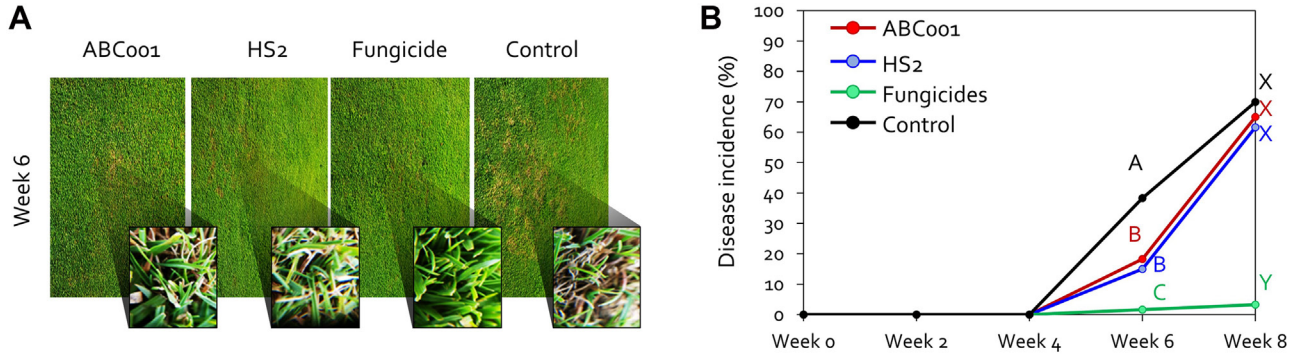

Fig. 2. Foliar application of *Chlorella* supernatant suppresses naturally occurring dollar spot disease in turfgrass. (A) Symptoms of dollar spot disease in turfgrass 6 weeks post-treatment with *Chlorella* supernatant, fungicide, or tap water. (B) Incidence of dollar spot disease in turfgrass grown in the field at Incheon. ABC001, *Chlorella* sp. ABC001 supernatant treatment; HS2, *Chlorella* sp. HS2 supernatant treatment; fungicide, azoxystrobin 15% + thiophanate-methyl 10% treatment; control, tap water treatment. Different letters indicate significant differences between treatments at $P < 0.05$ (least significant difference).

plants treated with the two *Chlorella* supernatants showed similar root and shoot growth, as well as visual quality, compared to the control-treated and fungicide-treated plants (Supplementary Fig. 1). Thus, both the ABC001 and HS2 supernatants acted as bioprotectants and controlled dollar spot disease without imposing a growth penalty.

***Chlorella* supernatant-induced modulation of the rhizosphere microbiota.** Treatment with *Chlorella* culture solutions modulates the structure of the bacterial community in plant root microbiomes (Cho et al., 2022; Lee et al., 2022, 2023). To investigate whether treatment with cell-free supernatant also affected the bacterial community in the turfgrass rhizosphere upon pathogen challenge, we sequenced the 16s rRNA gene in samples of rhizosphere soil from plants treated with the *Chlorella* supernatants (Fig. 3). In total, 273,460 sequences were obtained from all samples after the chimeric and chloroplast genome sequences were removed. The reads were clustered into OTUs with sequence similarity >97%, resulting in 1,907 OTUs being considered. Rarefaction curves were constructed for all samples showing the number of observed OTUs; these appeared close to saturation (Supplementary Fig. 2).

A total of 1,907 OTUs were obtained from samples of turfgrass rhizosphere treated with *Chlorella* supernatants. An alpha diversity analysis was performed based on Observed_features and the Shannon index (Fig. 3). No differences in the bacterial uniformity and abundance indices were found across all the rhizosphere samples (Fig. 3A). In addition, a principal coordinate analysis based on Bray-Curtis dissimilarity showed that the two sets of cell-free supernatant-treated samples clustered separately from the control samples (Fig. 3B). In the Bray-Curtis dissimilarity index, axis 1 and axis 2 accounted for 27.2% and 14.8% of the variance, respectively (Fig. 3B).

To investigate the differentially abundant bacterial communities in turfgrass rhizospheres treated with *Chlorella* supernatants, we performed a relative abundance analysis at the class level. Relative abundance data indicated that the classes Alphaproteobacteria, Gammaproteobacteria, Bacteroidia, and Desulfuromonadia were enriched in rhizosphere soil samples treated with ABC001 and HS2 supernatants. By contrast, the abundances of the classes Acidobacteriae, Blastocatellia, Actinobacteria, and Acidimicrobiia were reduced in soil samples treated with either supernatant (Fig. 3C). These results suggested that treatment with *Chlorella* supernatant modified the structure of the bacterial community in rhizosphere soil.

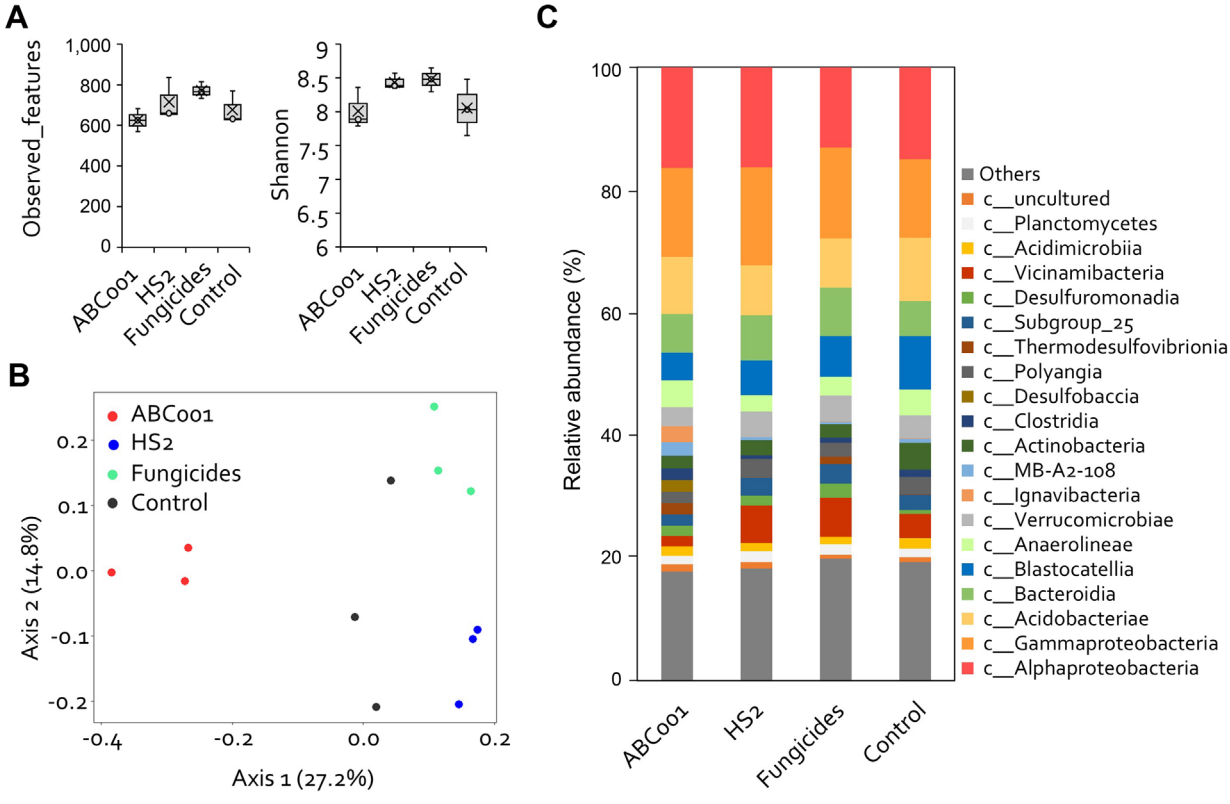

Fig. 3. Bacterial community structure in the turfgrass rhizosphere. (A) Changes in alpha diversity of the turfgrass rhizosphere microbiome. (B) Two-dimensional principal coordinate analysis based on the Bray-Curtis distance metric. (C) Relative abundance of taxa in the turfgrass rhizosphere at the class level. The relative abundance is the mean value, calculated from the abundances in three samples of rhizosphere soil per treatment. ABC001, *Chlorella* sp. ABC001 supernatant treatment; HS2, *Chlorella* sp. HS2 supernatant treatment; fungicide, azoxystrobin 15% + thiophanate-methyl 10% treatment; control, tap water treatment.

Table 1. List of the top 10 OTUs identified by the random forest model as differing most greatly in abundance between the ABC001 supernatant and control treatments

# OTUs	Mean Decrease Gini (ABC001 vs. control)	Average relative abundance (%)				Taxonomy
		ABC001	HS2	Fungicides	Control	
OTU1550[a]	0.030	0.043	0.000	0.006	0.000	Patescibacteria; Parcubacteria; Candidatus_Jorgensenbacteria; Candidatus_Jorgensenbacteria; Candidatus_Jorgensenbacteria; Candidatus_Adlerbacteria
OTU0114[a,b]	0.030	0.024	0.026	0.000	0.019	Patescibacteria; Saccharimonadia; Saccharimonadales; LWQ8; LWQ8; uncultured_bacterium
OTU0717	0.043	0.000	0.024	0.014	0.052	Verrucomicrobiota; Verrucomicrobiae; Chthoniobacterales; Chthoniobacteraceae; Chthoniobacter; uncultured_bacterium
OTU0831[a]	0.047	0.157	0.000	0.000	0.000	Desulfobacterota; Desulfobaccia; Desulfobaccales; Desulfobaccaceae; Desulfobacca; uncultured_bacterium
OTU1746	0.053	0.000	0.038	0.050	0.063	Gemmatimonadota; Gemmatimonadetes; Gemmatimonadales; Gemmatimonadaceae; Gemmatimonas
OTU1843	0.053	0.000	0.000	0.000	0.015	Proteobacteria; Gammaproteobacteria; uncultured; uncultured; uncultured; uncultured_bacterium
OTU1599[a,b]	0.060	0.225	0.131	0.020	0.029	Desulfobacterota; Desulfuromonadia; Geobacterales; Geobacteraceae
OTU1239[a]	0.060	0.122	0.000	0.000	0.000	Proteobacteria; Gammaproteobacteria; Burkholderiales; Rhodocyclaceae; Candidatus_Accumulibacter; uncultured_bacterium
OTU1237[a]	0.060	0.513	0.267	0.265	0.385	Proteobacteria; Alphaproteobacteria; Rhizobiales; Xanthobacteraceae
OTU0580[a]	0.060	0.080	0.000	0.075	0.012	Bacteroidota; Bacteroidia; Chitinophagales; Chitinophagaceae; uncultured; uncultured_Chitinophaga

[a]The key operational taxonomic units (OTUs) enriched in the ABC001 supernatant treatment compared with control and fungicide treatments.
[b]OTUs enriched in both the ABC001 and HS2 supernatant treatments.

Specific bacterial taxa affected by treatment with *Chlorella* supernatant. We conducted a random forest analysis, which uses a machine learning method (Breiman, 2001), to explore the discriminant bacterial OTUs in rhizosphere soil treated with ABC001 or HS2 supernatants (Tables 1 and 2, Fig. 4). This identified the top 10 differential OTUs in the ABC001 and HS2 supernatant treatments (Fig. 4A and C). We found that seven OTUs were enriched in rhizosphere soil treated with ABC001 supernatant, compared with both the control and fungicide treatments; these were the Patescibacteria (OTU1550 and OTU0114), Desulfobacterota (OTU0831 and OTU1599), Proteobacteria (OTU1239 and OTU1237), and Bacteroidota (OTU0508) phylum groups (Table 1, Fig. 4A). In particular, OTU1599 (class Desulfuromonadia), OTU1239 (class Gammaproteobacteria), and OTU0580 (class Bacteroidia) increased in abundance by 7.9-fold, 12.2-fold, and 6.87-fold, respectively, in soil treated with ABC001 supernatant compared with their abundance in control-treated soil (Fig. 4B).

Treatment with HS2 supernatant enriched the abundance of eight OTUs in rhizosphere soil compared with control-treated samples; these OTUs were from the class Proteobacteria (OTU1750 and OTU1137) and the phyla Verrucomicrobiota (OTU1735), Planctomycetota (OTU1863), NB1-J (OTU0919), Acidobacteriota (OTU1779), and Bacteroidota (OTU1343) (Table 2, Fig. 4C). In soil samples treated with HS2 supernatant, OTU1343 (class Bacteroidia), OTU1750 (class Alphaproteobacteria), and OTU1863 (phylum Planctomycetes) showed 9.1-fold, 6.3-fold, and 5.8-fold higher abundance, respectively, than in the control samples (Fig. 4D). Additionally, the abundance of seven OTUs (OTU1750, OTU1736, OTU1863, OTU0919, OTU1137, OTU1779, and OTU1343) also increased in rhizosphere soil treated with HS2 supernatant relative to their abundance in fungicide-treated soil (Table 2). Treatment with ABC001 or HS2 supernatant also enriched the abundance of diverse species of uncultured bacteria in rhizosphere soil; these included OTU0114, OTU0831, OTU1239, OTU0580, OTU1736, OTU1863, OTU0919, OTU1137, OTU1343, OTU0025, OTU1742, OTU0209,

Table 2. List of the top 10 OTUs identified by the random forest method as differing most greatly in abundance between the HS2 supernatant and control treatments

# OTUs	Mean Decrease Gini (HS2 vs. control)	Average relative abundance (%)				Taxonomy
		ABC001	HS2	Fungicides	Control	
OTU0514	0.047	0.724	0.006	0.345	0.634	Chloroflexi; Anaerolineae; Anaerolineales; Anaerolineaceae
OTU0119	0.053	0.027	0.000	0.039	0.027	Patescibacteria; Saccharimonadia; Saccharimonadales; Saccharimonadales; Saccharimonadales; uncultured_bacterium
OTU1750[a,b]	0.057	0.175	0.089	0.000	0.014	Proteobacteria; Alphaproteobacteria; Tistrellales; Geminicoccaceae; Candidatus_Alysiosphaera; metagenome
OTU1736[a]	0.057	0.009	0.012	0.000	0.030	Verrucomicrobiota; Verrucomicrobiae; Pedosphaerales; Pedosphaeraceae; Pedosphaeraceae; uncultured_bacterium
OTU1863[a,b]	0.057	0.012	0.058	0.052	0.000	Planctomycetota; Planctomycetes; Pirellulales; Pirellulaceae; uncultured
OTU0919[a]	0.057	0.045	0.076	0.028	0.045	NB1-j; NB1-j; NB1-j; NB1-j; NB1-j
OTU1137[a]	0.057	0.034	0.189	0.014	0.072	Proteobacteria; Gammaproteobacteria; Xanthomonadales; Rhodanobacteraceae; Dokdonella; uncultured_Dokdonella
OTU1779[a]	0.057	0.000	0.080	0.000	0.000	Acidobacteriota; Acidobacteriae; Solibacterales; Solibacteraceae; Candidatus_Solibacter
OTU0025	0.060	0.139	0.000	0.000	0.100	Bacillota; Limnochordia; Limnochordia; Limnochordia; Hydrogenispora; uncultured_bacterium
OTU1343[a]	0.090	0.000	0.175	0.079	0.019	Bacteroidota; Bacteroidia; Chitinophagales; Chitinophagaceae; Terrimonas; uncultured_bacterium

[a]The key operational taxonomic units (OTUs) enriched in the HS2 supernatant treatment compared with the control and fungicide treatments.
[b]OTUs enriched in both the ABC001 and HS2 supernatant treatments.

OTU0463, and OTU0554, which, by contrast, were reduced in soil treated with fungicide and in the control treatment (Tables 1 and 2).

Both the ABC001 and HS2 supernatant treatments enhanced the abundance of four of the top 10 OTUs (OTU0114, OTU1599, OTU1750, and OTU1863) present in rhizosphere soil. We investigated these commonly enriched key OTUs further (Fig. 5, Supplementary Table 1). Of the top 50 differential OTUs identified using the random forest method, 20 were keystone OTUs that were enriched in the soil rhizosphere by both supernatant treatments (Fig. 5, Supplementary Table 1). These included OTUs from the classes Alphaproteobacteria (OTU1171, OTU1750), Gammaproteobacteria (OTU1430, OTU1606, OTU0209), and the phyla Chloroflexi (OTU1742), Desulfobacterota (OTU1599), and Myxococcota (OTU1253) (Fig. 5, Supplementary Table 1). Taken together, these results indicated that treatment with cell-free supernatant enriched specific microbial taxa in turfgrass rhizosphere soil in the presence of a fungal pathogen.

Discussion

Industrial cultivation of *Chlorella* sp. ABC001 and HS2 produces a lipid-rich biomass suitable for biofuel production but also generates a large volume of waste supernatant that requires detoxification (Jin et al., 2017; Sung et al., 1999; Yun et al., 2019, 2021). Previous studies have focused on using waste supernatant as a biocontrol agent, although such research has mostly focused on dicot plants, including Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), and cucumber (*Cucumis sativus*). We recently showed that *Chlorella* supernatant has considerable potential for the biological control and biofertilization of diverse crop and ornamental plant species (Lee and Ryu, 2021; Lee et al., 2020a, 2020b). The current study extends this line of research to monocot grasses by showing that spraying turfgrass with *Chlorella* supernatant was an effective method of controlling dollar spot disease, caused by *C. jacksonii*, under greenhouse and field conditions. An analy-

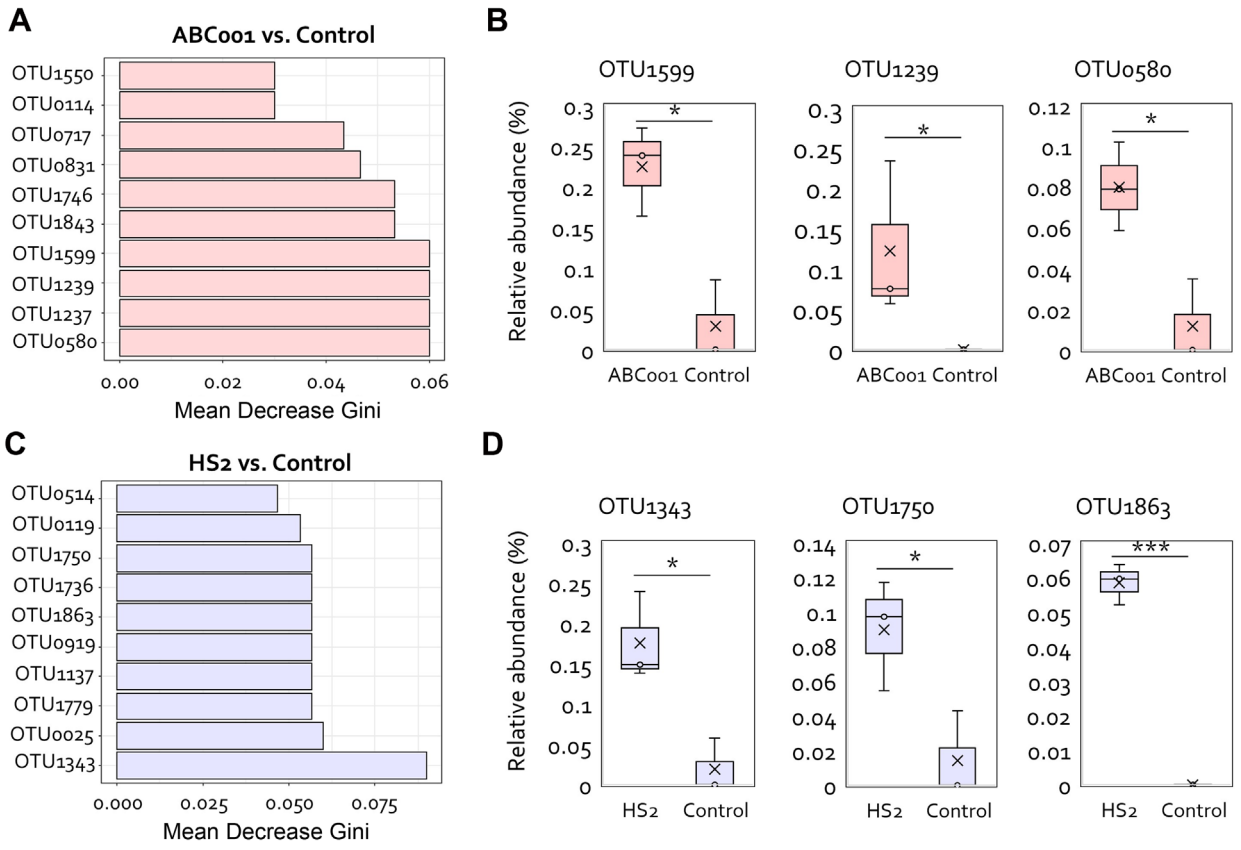

Fig. 4. The operational taxonomic units (OTUs) that showed the greatest responses to treatment with *Chlorella* sp. ABC001 and HS2 supernatants in turfgrass rhizosphere soil. (A, C) The top 10 OTUs responding to ABC001 supernatant (A) or HS2 supernatant (C), identified using random forest analysis. (B, D) The relative abundance of three signature OTUs enriched in ABC001 (B) or HS2 supernatant treatments (D), respectively. The mean values ± SEs are shown (n=3). Significant differences were determined using Student's t-test; *P< 0.05, ***P<0.001. The diamonds and bolded lines in the boxplot are the mean and median of the relative abundance of OTUs, respectively. ABC001, *Chlorella* sp. ABC001 supernatant treatment; HS2, *Chlorella* sp. HS2 supernatant treatment; control, tap water treatment.

sis of the rhizosphere microbiome revealed that supernatant application altered the structure of the soil microbial community, enriching beneficial taxa that may enhance plant health. These findings underscored the dual role played by *Chlorella* supernatant. Its application both reduced the occurrence of fungal disease and enhanced the abundance of specific key bacteria, thus offering a sustainable solution for managing supernatant waste while controlling disease in an important species of turfgrass.

The beneficial effects of Cyanobacteria, which are prokaryotic microalgae, on plant health are increasingly well understood; by contrast, few studies have investigated the use of eukaryotic microalgae, such as *Chlorella* species, as biocontrol agents against fungal pathogens (Lee and Ryu, 2021). Foliar application of *C. fusca* cultures protects cucumber and Chinese chive (*Allium tuberosum*) plants against the pathogenic fungi *Colletotrichum orbiculare* and *Botrytis squamosa* (Kim et al., 2018a, 2018b; Lee et al., 2016, 2017), as well as inducing cell wall modification, which is part of the defense response to pathogen attack, in Arabidopsis and cucumber plants (Kim et al., 2018a, 2018b; Lee et al., 2016, 2017, 2020a). Furthermore, *C. fusca* reduces levels of anthracnose caused by *C. orbiculare* on cucumber leaves by decreasing the number of conidia and suppressing appressorium formation (Kim et al., 2018a, 2018b; Lee et al., 2016, 2017). This antifungal activity depends, however, on direct cell contact between microalgal and fungal cells (Kim et al., 2018a, 2018b; Lee et al., 2016, 2017). By contrast, cell-free supernatants from ABC001 and HS2 cultures did not directly inhibit growth of *C. jacksonii* hyphae on grass leaves in the greenhouse, which contradicted previous findings (data not shown).

These results led us to consider whether *Chlorella* supernatant activated IR in grass prior to *C. jacksonii* infec-

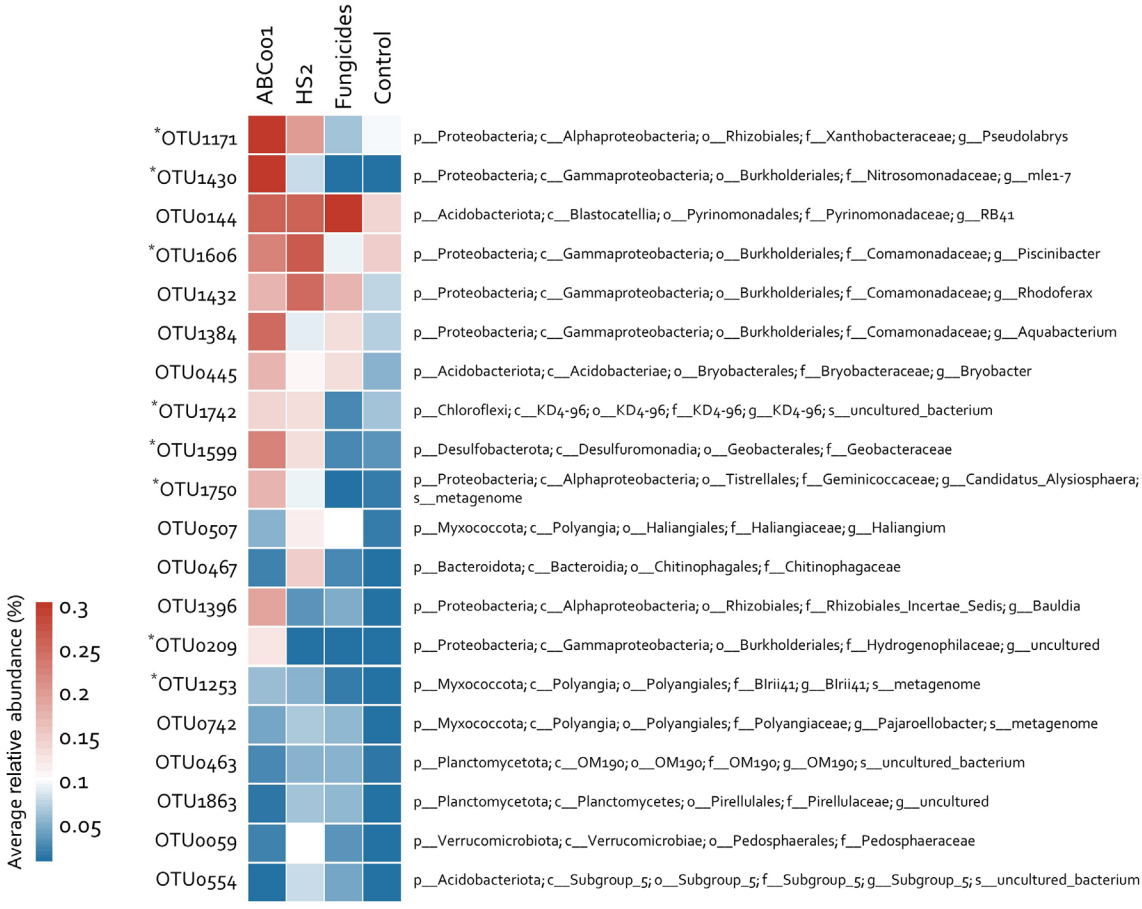

Fig. 5. Relative abundance (%) of keystone taxa in turfgrass rhizosphere soil that responded to *Chlorella* sp. ABC001 and HS2 supernatant treatments. The 20 operational taxonomic units (OTUs) were enriched in rhizosphere soil from both *Chlorella* supernatant treatments. The heat map shows the average relative abundance values of the 20 OTUs calculated from three replicate soil samples. Asterisks indicate OTUs that increased in both supernatant treatments relative not only to the control treatment but also the fungicide treatment. ABC001, *Chlorella* sp. ABC001 supernatant treatment; HS2, *Chlorella* sp. HS2 supernatant treatment; fungicide, azoxystrobin 15% + thiophanate-methyl 10% treatment; control, tap water treatment.

tion. Plants in which IR has been activated trigger faster and stronger responses upon encountering a pathogen, a phenomenon known as defense priming (Balmer et al., 2015; Heil and Baldwin, 2002; Karasov et al., 2017; Lämke and Bäurle, 2017; van Hulten et al., 2006). Maintaining a primed state is less costly than mounting a full defense response, benefiting both disease control and stable plant growth (Balmer et al., 2015; Heil and Baldwin, 2002; Karasov et al., 2017; Lämke and Bäurle, 2017; van Hulten et al., 2006). ABC001 and HS2 supernatants did not affect the growth or visual quality of the turfgrass under field conditions (Supplementary Fig. 1). Thus, spraying with HS2 and ABC001 supernatants may control dollar spot disease by eliciting IR, thus enhancing the defense response to *C. jacksonii*. To understand more fully how IR is mediated in grass species by *Chlorella* supernatant, further mechanistic studies are required.

Supernatant from *Chlorella* cultures can control crop disease, increase yield, and enhance soil microbiome structure, but previous studies of its effects on the root microbiome have focused solely on the dicot plants (Cho et al., 2022; Kublanovskaya et al., 2019; Lee et al., 2022, 2023). *Chlorella* supernatant also induces alterations in the soil microbiota of turfgrass species, potentially supporting disease suppression in the host plants, both currently and continuously (Berendsen et al., 2018; Kong et al., 2019; Lee et al., 2021). In the current study, the keystone taxa that were enriched following ABC001 or HS2 supernatant treatment can help restore soil nitrogen levels through nitrification, nitrogen fixation or nitrogen mineralization; these taxa included *Pseudolabrys* (OTU1171), a member of the orders Nitrosomonadaceae (OTU1430) and Rhizo-

biales (OTU1237), Patescibacteria from the candidate phyla radiation (OTU1550, OTU0114), and a member of the Comamonadaceae family (OTU1606). The alterated soil nitrogen levels caused by these key taxa may have reduced the virulence of *C. jacksonii* and affected host plant immunity to dollar spot disease (Tables 1 and 2, Fig. 5, Supplementary Table 1) (Garrido-Oter et al., 2018; Hu et al., 2020; Li et al., 2022; Prosser et al., 2014; Sapkota et al., 2022; Townsend et al., 2021; Xiao et al., 2022). In golf course, infected turfgrass is removed and replaced, but dead tissues can serve as a primary inoculum of *C. jacksonii*. Meanwhile, the abundance of myxobacteria (OTU1253, OTU0507, and OTU0742) increased by supernatant treatment (Supplementary Table 1). Thus, myxobacteria enriched by the supernatant may prevent infection through direct antagonism of pathogens (Fudou et al., 2001; Li et al., 2019; Xia et al., 2023). Moreover, sulfate-reducing bacteria from the phylum Desulfobacterota (OTU0831 and OTU1599) and the Hydrogenophilaceae family (OTU209) are essential for sulfur cycling and energy processes within soil microbial environments (Jeanthon et al., 2002; Orlygsson and Kristjansson, 2014). Treatment with ABC001 or HS2 supernatant also enriched unique uncultured bacterial taxa in the rhizosphere; the abundance of these taxa decreased in the fungicide treatment (Tables 1 and 2), however, implying that *Chlorella* supernatant could act as a prebiotic to reverse microbial dysbiosis (Lee et al., 2021). Future use of supernatant as a treatment to enhance soil microbiomes should be optimized to ensure its efficacy in turfgrass protection.

Following treatment with *Chlorella* culture solutions, Gram-positive bacteria are less likely to increase in abundance in the root microbiomes of Arabidopsis, bean (*Phaseolus vulgaris*), and strawberry (*Fragaria* × *ananassa*) plants than Gram-negative bacteria, such as *Stenotrophomonas*, members of the Beijerinckiaceae and Xanthobacteraceae families, *Rhizobium*, *Massilia*, *Flavisolibacter*, and *Sphingomonas* (Cho et al., 2022; Kublanovskaya et al., 2019; Lee et al., 2022, 2023). In our experiments, treatment with ABC001 or HS2 supernatant enriched the abundance of Gram-negative bacteria in the turfgrass rhizosphere. We did not, however, detect most of the Gram-negative taxa previously reported as increasing in abundance, with the exceptions of *Pseudolabrys* (OTU1171), *Pirellula* (OTU1863), and a member of the Xanthobacteraceae family (OTU1237) (Tables 1 and 2, Supplementary Table 1) (Cho et al., 2022; Kublanovskaya et al., 2019; Lee et al., 2022, 2023). Such differences in the microbial community structure may result from the genetic differences between monocot and dicot hosts (Chan et al., 2024; Naylor et al., 2017). Furthermore, as the aboveground parts of plants were sprayed with *Chlorella* supernatants, the systemic changes observed in the rhizosphere microbiome were more likely to be caused by IR in the host plant than by direct effects of the supernatant on the rhizosphere microbiota. Signaling pathways involving the defense hormones, salicylic acid (SA) and jasmonic acid (JA), along with changes in root exudates, may also influence microbiome structure (Carvalhais et al., 2015; Lebeis et al., 2015). *Chlorella* supernatant contains lactic acid and thus foliar application may have activated SA and JA signaling pathways in turfgrass (Lee et al., 2020a; Yan et al., 2024), thereby increasing the abundance of various keystone bacterial taxa (Tables 1 and 2).

At present, synthetic fungicides are the most commonly used method of disease control in turfgrass. Use of such chemicals is increasingly restricted, however, because of rising environmental and health concerns. In addition, the long-term use of fungicides such as azoxystrobin and thiophanate-methyl has generated resistant pathogens (Ok et al., 2011). Continuous fungicide treatments on many golf courses in South Korea have been insufficiently effective at controlling dollar spot disease in turfgrass. Although the application of *Chlorella* supernatant was not an effective means of disease control over an extended period (Fig. 2), our results demonstrate its potential as a novel method of reducing disease occurrence in turfgrass when applied weekly. Recycling waste supernatant from the industrial cultivation of *Chlorella* as a turfgrass disease control agent has several advantages. Unlike fungicides, *Chlorella* supernatant activates innate plant immunity responses. Preventing disease without applying synthetic fungicides minimizes the likelihood of resistant fungal isolates developing. The use of waste supernatant in combination with fungicides reduces the quantity of chemicals used and, thus, decreases environmental pollution while lowering production costs. Application of the supernatant increases the abundance of beneficial uncultured microbes in the soil, thus it acts as a prebiotic and reversing dysbiosis in the soil microbiota caused by repeated fungicide use. Finally, filtered supernatant can be easily applied to turfgrass in the field using automatic systems, like sprinklers, that are already in place for watering and disease management.

Although this study offers strong evidence that *Chlorella* supernatant can be used in the biological control of turfgrass diseases, it has several limitations. We do not yet understand the driving force that modulates the rhizosphere microbiota. Elucidating this mechanism requires a detailed investigation of both the indirect influences of turfgrass root exudates and the direct effects of supernatant on the

rhizosphere microbiota. Coupling between the *Chlorella* supernatant-mediated immune activation and the modulation of the turfgrass microbiota also needs to be assessed. Finally, the protection offered by the observed changes in the keystone taxa must be validated to determine the level of biological control offered by treatments involving *Chlorella* supernatant, either alone or in combination with fungicides, if disease control is to be managed sustainably on golf courses.

To conclude, we propose a novel use for recycled industrial *Chlorella* supernatant as a means of controlling fungal disease in turfgrass. Our results provide the first report of the protection provided by *Chlorella* supernatant to a monocot plant and, more importantly, offer new insights into the development of environmentally friendly and sustainable agricultural technologies that will reduce the need for the agrochemicals commonly used in modern agriculture.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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